The role of Human papillomaviruses in the aetiopathogenesis of head and neck cancer in South Asia, and approaches to treatment

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Submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy

January 2017
To my Parents

Professor Md. Ali Akbar and Mrs. Minnatun Ara,

the reason of what I become today

Thank you for all your enormous support and care
STATEMENT OF ORIGINALITY

I declare that the work presented in this thesis is, to the best of my knowledge and belief, original and performed by me within Griffith University under the supervision of Professor Newell W Johnson and Professor Nigel A J McMillan, except where acknowledged in the text.

The research presented in this thesis is for Doctor of Philosophy (PhD).

This work either as a whole or in part, has not been submitted for a degree or diploma at this or any other institution. To the best of my knowledge, the thesis contains no materials that were published or written by others except where due reference is made here.

Signatures

(Signed) ____________
Candidate: Mushfiq H Shaikh

(Signed) ____________
Principal Supervisor: Professor Newell W Johnson

(Signed) ____________
Associate Supervisor: Professor Nigel AJ McMillan
STATEMENT OF CONTRIBUTIONS

In chapter 3, I developed the protocol and performed the electronic search of the published literature. All authors agreed on selected papers. I also extracted data and prepared the meta-analyses, tables and diagrams. I wrote the first draft, and corrected versions and agreed the final manuscript. Prof. McMillan advised and contributed on molecular aspects. Prof. Johnson contributed to data interpretation, critical correction and manuscript revision and agreed the final manuscript.

In chapter 4, the designs of the experimental protocols were performed by me. I have carried out all the laboratory experiments by myself. I also extracted, interpreted the data, prepared tables & diagrams, performed statistical analysis and prepared the manuscript. The head and neck cancer tissue samples were kindly provided by Prof. A.I Khan (Dhaka, Bangladesh). The p16 immunohistochemical staining was performed by Mr. Chris Philippa (Gold Coast University Hospital, Australia). I performed all the other immunochemistry work. The immunohistochemistry slide scoring was performed by Prof. Alfred Lam. Prof. McMillan advised with experimental design and revised the manuscript. Dr. Daniel Clarke advised with experimental design and revised the manuscript. Prof. Johnson contributed to experimental design, data interpretation, critical correction, manuscript revision and agreed the final manuscript.

In chapter 5, I designed the experimental protocols and performed all the related laboratory experiments by myself. I also extracted, interpreted the data, prepared tables & diagrams and performed statistical analysis. The HPV-positive HNSCC cell lines were kindly provided by Prof. Tom Carey, Prof. Hoffmann and Prof. Paul Lambert. The contributions of these individuals are stated in chapter 2. Prof. McMillan contributed in experimental design and data interpretation. Dr. Daniel Clarke advised with experimental design.

In chapter 6, I designed the experimental protocols and performed all the related laboratory experiments by myself. I interpreted the data, prepared tables & diagrams and performed statistical analysis. Prof. McMillan contributed in experimental design and data interpretation. The aurora kinase drugs (AMG900, ZM447439 and
CCT244941) were kindly provided by Prof. Gabrelli’s Lab. The Western blots for MLN8237 in HNSCC cells were performed by Mr. David Martin from the University of Queensland.

Appendix-1, I performed the electronic search of the published literature. I also extracted data and prepared the tables, wrote the first draft of the manuscript, corrected versions and agreed the final manuscript. Prof. McMillan advised on and performed some critical corrections, manuscript revision and agreed the final manuscript. Dr. Daniel Clarke advised and revised the manuscript. Prof. Johnson contributed to manuscript revision and agreed the final manuscript.
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I am grateful to all the staff members of School of Medicine and School of Dentistry and Oral Health and the School of Medical Science for their sincere co-operation. I would like to thank Griffith University for offering me the GUIPRS and GUPRS scholarships.

Finally, I would like to thank my friends and family for their enormous support. I owe much to my parents, whose prayers are always with me: this has helped me to go through thick and thin even while I am studying in a different place far away from my home. At last but not the least, the special thanks goes to my lovely wife Fahima Islam who has always been here with me, sacrificing her own career and constantly supporting me throughout my PhD. I will not forget those days when I was so tired, stressed and returning home from the Lab in the middle of the night and found nicely cooked food waiting for me on the dinner table. Thank you so much for all your gracious support and love during this strenuous time.
Publications arising from the thesis and concomitant work


Conference presentations

- **Shaikh MH**, Daniel Clarke, McMillan NA, Johnson NW. *The role of HPV E7 and Aurora A kinase in the growth and survival of HPV-positive head and neck cancer*. Oral (podium) presentation at Gold Coast Health and Medical Research Conference, Gold Coast, QLD, Australia, December, 2016.


- **Shaikh MH**, Daniel Clarke, McMillan NA, Johnson NW. *The role of human papillomaviruses in the aetio-pathogenesis of head and neck cancer and approaches to gene therapy*. Poster presentation delivered at Gold Coast Health and Medical Research Conference, Gold Coast, QLD, Australia, December, 2014.

Abstract

Malignancies of the upper aero-digestive tract are a major public health problem, especially in South Asia. The major risk factors in South Asia remain smoked/smokeless tobacco, areca nut, alcohol abuse and poor diet, with limited evidence for human papillomavirus (HPV). Although HPV-associated head and neck squamous cell carcinoma (HNSCC) is well documented in the western world, studies on South Asian populations are few and inconsistent. However, the incidence of HPV-associated head and neck cancer (HNC) has increased in recent years. Certain high-risk types of HPV infection are regarded as well-established risk factors for cervical cancer and a subset of HNSCC; however, their true role and importance in the progression of HNSCC remain unclear. Although HPV-associated HNC patients generally have a better prognosis than those with HPV-negative disease, current chemo- and radio-therapies are largely non-specific and have considerable toxicities. RNA interference (RNAi), which has shown great promise as a highly specific therapy for other diseases, has potential for treating HPV-associated HNC, especially if disease progression is dependent on the continual expression of HPV oncogenes.

The aims of our project are to investigate the prevalence of HPV in HNSCC in Bangladesh, this being the most common cancer in males in the country, and to overview the role of HPV oncogenes in the development of HNSCC with the use of short interfering RNA (siRNA) technology as a novel therapeutic approach against HPV-positive head and neck cancer. Further, we also investigate the role of the Aurora A kinase inhibitor Alisertib on the inhibition of HPV-associated HNSCC derived cells.

For our first aim, we have included 195 patients with histologically confirmed primary SCC of different anatomical subsites of the head and neck region. Involvement of HPV DNA was investigated on formalin-fixed paraffin-embedded (FFPE) tumour tissues by p16\(^{INK4A}\) immunohistochemistry, and by nested polymerase chain reaction (PCR) followed by sequencing for HPV types. For the second aim, four different HPV type 16 - positive HNC cell lines, and one HPV-negative HNC cell line were investigated. Cell lines were transfected with siRNA E6/E7 before examining
HPV E7 gene knockdown (by real time qPCR), cellular metabolism, cell viability and siRNA efficacy. Further, these cells were treated with Alisertib and cell death measured via cell viability assays.

We found approximately 21% of head and neck cancer cases from Bangladesh to be HPV positive by PCR. p16\textsuperscript{INK4A} immunohistochemistry was positive in a slightly lower percentage of lesions and HPV type 16 was the most common type detected.

Our analyses on HPV-positive HNC cells showed significant knockdown of the HPV E7 gene and cell death when treated with siRNA E6/E7, suggesting a reliance on this oncogene for survival. Further, the HPV positive HNC cells with high E7 expression level were shown to be highly sensitive to Alisertib (IC50 value of less than 1µmol/L).

Our study on the Bangladeshi population is important in light of the very different mix of risk factors for HNSCC in different parts of the world. This study will also be a valuable addition towards the understanding of the HPV-associated HNSCC prevalence in South Asian populations. Further, our findings with HPV-positive HNC cells suggest siRNA-targeting E6/E7 could be a potential therapeutic strategy in combination with conventional treatment modalities for effectively treating HPV-positive HNC patients. Further, sensitivity to Alisertib makes it a possible candidate for HPV-positive HNC treatment in the near future.
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# LISTS OF ABBREVIATIONS

## General Abbreviations

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<tr>
<td>AURKA</td>
<td>Aurora kinase A</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CONSORT</td>
<td>Consolidated Standards Of Reporting Trials</td>
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<td>COX-2</td>
<td>Cyclooxygenase-2</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>G2/M boundary</td>
<td>Point of entry into mitosis</td>
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<td>Gy</td>
<td>Gray unit</td>
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<td>HNC</td>
<td>Head and Neck Cancer</td>
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<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
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<td>HPV</td>
<td>Human papillomavirus</td>
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<tr>
<td>HR</td>
<td>High Risk</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
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<td>ICD -10</td>
<td>The International Classification of Disease tenth Revision</td>
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<tr>
<td>kDa</td>
<td>Kilodaltons</td>
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<td>LCR</td>
<td>Long control region</td>
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<td>M phase</td>
<td>Mitosis</td>
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<td>Micro RNA</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>NNK</td>
<td>4-(methylNitrosamino)-1-(3-pyridyl)-1-Butanone</td>
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<td>N-nitrosonornicotine</td>
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<td>PRISMA</td>
<td>Preferred Reporting Items for Systematic Reviews and Meta-Analysis</td>
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<td>rt-qPCR</td>
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<td>shRNA</td>
<td>Short hairpin RNA</td>
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<td>siRNA</td>
<td>Short interfering RNA</td>
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<td>TKI</td>
<td>Tyrosine-kinase inhibitor</td>
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<td>TROLS</td>
<td>Trans-oral robotic laser surgery</td>
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<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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<td>VLP</td>
<td>Virus like particle</td>
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<td>Western Blot</td>
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## Abbreviations of compounds and chemical substances:

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<tr>
<td>APS</td>
<td>Ammonium per-sulphate</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline with Tween 20</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodiumdodecylsulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction and Literature review
Introduction

Head and Neck Cancers (HNCs) are a biologically diverse group of malignancies arising from the moist mucous membranes lining the internal spaces of the head and neck. This includes the lining of the lips, oral cavity (cheeks, gingiva, floor of the mouth, anterior two-thirds of the tongue and the hard palate); salivary glands; nasal cavity; nasopharynx and accessory sinuses, the oropharynx (base of the tongue, soft palate, tonsils & the lateral and posterior walls of the oropharynx), the hypopharynx, the vallecula and the larynx (Syrjanen, 2005, Argiris et al., 2008). Approximately 85% of malignancies in this region (excluding nasopharyngeal carcinomas) display histological features of squamous cell carcinoma and are morphologically characterized as Head and Neck Squamous Cell Carcinomas (HNSCC) (Mehanna et al., 2010). HNSCC of the lip, oral cavity, oropharynx, larynx and hypopharynx (not nasopharynx) together represent the sixth most common malignancy diagnosed in the world, with an estimated incidence of ~ 634,766 new cases (~ 4.5% of all cancer cases) and a mortality rate of ~ 330,000 per annum. (Globocan 2012 ref.) (Parkin et al., 2005, Ferlay et al., 2015). It is also one of the most common malignancies throughout South Asia, comprising of approximately one third of the total HNSCC incidence in the world, with an annual incidence rate of 198,152 p.a (D’Costa et al., 1998). Although tobacco (both smoked and unsmoked), areca nut and alcohol abuse are well-established risk factors, recent data from meta-analysis and case-control studies suggest “high risk” types of human papillomavirus as an independent risk factor for HNSCC (Parkin and Bray, 2006). Recent studies have revealed a rising incidence of oropharyngeal squamous cell carcinoma (OPSCC), despite the reduction of tobacco use in several countries of the western world, which has been thought to be attributed to an increase in HPV infection (Ryerson et al., 2008, Doobaree et al., 2009, Hammarstedt et al., 2007, Ariyawardana and Johnson, 2013, Herrero et al., 2003, Ramqvist and Dalianis, 2011). Although HPV-associated HNSCC is well documented in the western world, few studies have looked at the prevalence of this cancer in South Asian populations as the majority of the HNSCCs from this region are correlated with extensive use of tobacco in smoked or smokeless forms, and with consumption of areca nut. Therefore, it is necessary to understand the true association
of HPV in HNSCC in this region as the worldwide incidence of this particular type of HNC has increased in recent years.

Although HPV-associated HNC patients generally have a better prognosis than those with HPV-negative disease, current chemotherapy and radiation therapies are largely non-specific and have considerable toxicities. RNA interference (RNAi), which has shown great promise as a highly specific therapy for other diseases, has potential for treating HPV-associated HNC, especially if disease progression is dependent on the continual expression of HPV oncogenes.

In this thesis, we demonstrate the prevalence of HPV in HNSCC in a Bangladeshi population utilizing sensitive and specific HPV detection methods and overview the role of HPV oncogenes in the development of HNSCC with the use of short interfering RNA (siRNA) technology as a novel therapeutic approach against HPV-positive head and neck cancer. Further, we demonstrate the role of the Aurora A kinase inhibitor Alisertib on the inhibition of HPV-associated HNSCC derived cells.

1.1. Literature review:

Cancer is one of the five major causes of morbidity and mortality worldwide. The incidence of cancer is on the rise around the world and poses a particular threat in many low- and middle-income countries. Death tolls globally far exceed those from devastating infectious diseases like AIDS, tuberculosis and malaria. The International Agency for Research on Cancer, part of the World Health Organization at the United Nations, estimates that 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred worldwide in 2012 (IARC: GLOBOCAN 2012 website, www. data, accessed February, 2016). Much research has been carried out to combat this deadly disease and still continues today. Although surgery and radiotherapy remain the mainstay of treatment, two-thirds of cancer deaths can be prevented through lifestyle modification and early detection followed by effective treatment.
1.1.1. Head and Neck Cancer (HNC):

Head and neck cancers are malignant neoplasms affecting the structures or tissues of the head and neck region including oral cavity, oro-pharynx, naso-pharynx, hypo-pharynx, larynx, paranasal sinuses and salivary glands. They may be a primary lesion originating at the site, metastasising from a distant site or extending from adjoining sites. Squamous cell carcinoma (SCC) accounts for more than 90% of all head and neck malignancies (Johnson et al., 2011, Syrjanen, 2005). The majority of squamous cell carcinomas in the head and neck region arise in the mucous membrane of the mouth and pharynx (Johnson, 1991).

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide and eighth most common cause of cancer-associated death (Parkin et al., 2005, Warnakulasuriya, 2009). Its relative position varies depending on geographical location. In South Asia, it accounts for more than 40% of all malignancies and is regarded as the second most frequent cancer seen in males and the fourth most in females (D'Costa et al., 1998). Overall, HNSCC of the oral cavity and of the oropharynx are the two most common. HNC has much in common with squamous cell carcinomas arising elsewhere in the upper aero-digestive tract and shares common risk factors.
**Figure 1.1**: Anatomical sites for the head and neck cancer. The cartoon shows that cancer involving these anatomical sites in the head and neck region are designated as head and neck cancer. (The picture is adapted from National Institute of health (NIH) website, accessed in November, 2016.)

**Figure 1.2.** Histologic sections of HNSCC showing mild, moderate and poorly differentiated lesions (H&E staining) (from left to right). These tissues are head and neck cancer samples of three different Bangladeshi patients. The histologic grading was performed following the WHO. *IARC WHO Classification of Tumours*, (3rd ed., vol. 9, 2005)
1.1.2. Aetiology & Risk factors of HNSCC:

The key risk factors for head and neck squamous cell carcinomas are tobacco in various forms: smoking and smokeless or chewed tobacco; areca nut/betel quid chewing, and heavy alcohol intake. These frequently act in a background of poor diet, poor oral hygiene and there is a small genetic predisposition. In recent times, there is rising evidence of a strong association of ‘high-risk’ genotypes of human papillomavirus (HPV) noticed in HNSCC, especially in the oropharyngeal regions. The major risk factors for the HNSCC are briefly described below.

Tobacco:

Tobacco is the single most common risk factor for HNSCC development. A recent comprehensive study has estimated more than 967 million smokers existed in 187 countries in 2012 (Ng et al., 2014). If the total number of countries in the world were 196, then the estimation of smoker would have easily passed a billion by now. Surprisingly, 5-6 million people die every year from smoking related diseases and the majority of these deaths (~ 80%) occur in less developed countries (Li and Guindon, 2013). South and Southeast Asia are the home of approximately half of the total worldwide smokers (~ 400 million) and experience ~ 1.2 million tobacco related death each year (Sreeramareddy et al., 2014). Tobacco is consumed in the form of smoking (cigarettes, hookah and beedi) or smokeless (eg: zarda, gul, oral snuff and toombak). It contains a large number of carcinogenic components, among which nitrosamines [N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butaneone (NNK)] and polycyclic aromatic hydrocarbons are commonly found. It has been suggested that these components are causally correlated to the development of cancers in the upper aero-digestive tract and in more remote body sites, including bladder. The consumption of tobacco in its different forms generates a large amount of reactive oxygen species, which increases oxidative stress in many tissues, and produces free radicals that directly damage DNA, ultimately leading to malignant transformation if critical oncogenes, onco-suppressor genes or DNA-repair genes are affected. (Haddock et al., 2001, Hecht, 2003, Zain, 2001).
Betel quid & Areca nut:

The betel quid is commonly consumed by middle to older aged males and females in South Asia, although there is recent concern about rising rates of oral submucous fibrosis amongst children who have become habitués (Gupta and Ray, 2004). It is composed of betel leaf (leaf of the *Piperaceae* vine), slaked lime (calcium hydroxide), areca nut (seed of *Areca catechu*), clove, and sweetener and sometimes may contain tobacco (often the product known as Zarda in much of South Asia). Betel quid is consumed by approximately 600 million people worldwide (2004). The areca nut (Secretan et al., 2009, 2004) and slake lime, function synergistically, reducing the pH and releasing the pharmacologically active agents present in the nut. The slaked lime erodes the oral mucous membrane, thus allow the carcinogens of the betel quid to penetrate into the mucosa and ultimately lead to the development of cancer at the site where the quid is habitually held. Apart from South Asia, the use of areca nut is also common in Southeast Asia, Melanesia and a few areas in China, notably Taiwan. It is used as a masticatory substance, alone or as a component of betel quid. Betel quid and areca nut alone have now been designated as Class I carcinogens by the IARC (International Agency for Research on Cancer) (2004). Areca nut contains fibers, minerals, alkaloids and polyphenols. The alkaloids and polyphenols are carcinogenic components that produce nitrosamines and reactive oxygen, respectively, which ultimately facilitate malignant transformation, principally in the head and neck, where these agents, dissolved in saliva, contact the mucosae. Areca does, importantly, also have distant [eg carcinoma of the pancreas] and systemic effects on brain, heart, lungs and gastrointestinal tract. It also aggravates some pre-existing conditions like asthma; type 2 diabetes, neuronal injury, cardiac arrhythmia and myocardial infarction. , (Garg et al., 2014, Nair et al., 1996).

Alcohol Abuse:

Alcohol is one of the most common risk factors for HNSCC. Approximately, 2 billion people in the world consume alcohol regularly (Secretan et al., 2009). However, the
consumption of alcohol varies across geographic regions and in different populations. In South Asia, alcohol-related HNSCC is less common compared to tobacco related HNSCC. Nevertheless, tobacco intake in different forms and the consumption of alcohol demonstrate a synergistic effect, which at least doubles the risk for head and neck cancer (OR=2.15; 1.53 – 3.04) (Goldstein et al., 2010, Hashibe et al., 2009). Commercial alcoholic beverages contain mainly ethanol and water. The alcohol dehydrogenase enzyme from alcohol oxidizes ethanol to acetaldehyde, which adducts DNA and is directly carcinogenic. For religious reasons data on alcohol use is often unreliable in South Asia, and its true contribution to the high rates of malignant disease difficult to quantify accurately.

**Human papillomavirus :**

The so-called “high-risk” types of human papillomavirus, (HPVs: particularly, genotypes 16 & 18) are now regarded as an independent risk factors for the development of head and neck cancers, especially in the oropharynx) (Andrews et al., 2009, D'Souza et al., 2007). Details on the correlation of HPV and HNSCC are discussed later in this chapter.

**Herpes viruses:**

**a) Epstein Bar virus:**

Epstein Bar virus (EBV) has shown to be strongly associated with nasopharyngeal carcinoma (NPC), gastric carcinoma and Burkitt’s lymphoma (Young and Rickinson, 2004). EBV infection is perhaps the most studied aetiological factor for NPC, which is the major head and neck cancer seen in the East Asian countries (Alemany, 2013). Although EBV is invariably associated with NPC, the association of EBV with HNSCC was less commonly understood. However, recently, A comprehensive study from Sweden examining FFPE samples from 8 different countries, has shown that
western European countries (70-80% cases) and African nations (75%) have higher EBV-association with OSCC compared to South Asian populations (35-45%) (Jalouli et al., 2012). This suggests the possible potential role of EBV in HNSCC, which needs more investigation to confirm.

b) Herpes simplex virus:

The serum IgA antibody to Herpes simplex virus (HSV), type 1 has been reported to be higher in smoking-related oral cancer patients compared to the healthy normal control, suggesting HSV may act as a co-carcinogen with tobacco (Shillitoe et al., 1983). Further, the co-carcinogenic activity of HSV with tobacco and other chemical carcinogens was also demonstrated in an animal study (Hirsch et al., 1984). However, in recent years a little progress has been seen in the study of HSV and its association with HNSCC.

Poor nutrition:

Dietary factors are linked with approximately 20 - 30% of all cancers worldwide. Poor nutrition refers to poor eating habits, which includes over- or under-eating, consuming unhealthy food and drinks everyday (such as foods which are low in fibre and high in fat, salt and sugar) and not having enough healthy food. Thus poor diet is also shown to be an important predisposing factor for head and neck cancers (Garavello et al., 2009, Levi et al., 1998, Pavia et al., 2006). A recent study reported that consumption of green leafy vegetables or citrus fruits every day reduces the risk of oral cancer development by as much as 50% as they contain antioxidants (Pavia et al., 2006). Further, a recent comprehensive study from Sri Lanka suggested that people living in the rural area and having low daily consumption of fruits and vegetables, typically have less cancer preventive properties. This property makes them vulnerable to other carcinogenic products like tobacco and alcohol takeover the are limited and are swamped by the known carcinogenic products (Amarasinghe et al., 2013)
Solar Radiation:

The most common risk factor for the development of SCC of the lip is prolonged exposure to sunlight. Such cancers are common in people with fair complexion and outdoor workers (Baker and Krause, 1980). Typically, people from those countries that have clear air (Scandinavia, Australia, New-Zealand) or close to the equator (Greece, India) are more likely to suffer from lip cancer due to high level of UV ray exposure, which induces DNA damage (Pukkala and Notkola, 1997, Wiklund and Dich, 1995, Moore et al., 1999, Antoniades et al., 1995).

Genetic predisposition:

A component of inherited or genetic predisposition is also regarded as a risk factor for HNSCC (Tai et al., 2010). The hazard of head and neck cancer is higher in people who have affected siblings rather than parents, indicating that a common environment needs to be segregated from a genetic contribution (Negri et al., 2009). It is likely that environmental and genetic interaction, with key epigenetic changes, will also be shown to have an effect. These effects are distinct from the well-known inherited genetic defects, such as Fanconi anaemia and dyskeratosis congenita, which have a high risk of HNSCC development (especially hypopharyngeal cancer) (Baez, 2008).

Common oral diseases:

It has been suggested that poor oral hygiene and associated periodontal diseases may contribute to the development of HNSCC (Guha et al., 2007, Mehanna et al., 2009). Studies suggest that Candida albicans, an oral commensal, frequently associated with leukoplakia, has a higher risk of facilitating the carcinogenic process of HNSCC (De Pasquale and Sataloff, 2003, Holmstrup et al., 2006). Indeed, a systematic review has reported that approximately 12.1% of oral dysplasia with associated fungal infection, has the potential for malignant transformation after 4.3 years follow up (Mehanna et al., 2009). Further, mechanical trauma arising from ill-fitting denture, a denture clasps
and sharp or fractured teeth may cause chronic ulceration, which in turns, if untreated, may facilitate cancer progression (Velly et al., 1998)

There is currently considerable interest in the microbiome associated with oral cancer: the mycome and the virome, as discussed, but also the bacteriome. A recent review has highlighted the relationship between oral perio-pathogenic bacteria, such as Porphyromonas gingivalis and Fusobacterium nucleatum, and oral cancer, suggesting the potential of these bacteriome to develop oral cancer (Perera et al., 2016). However, the carcinogenic potential of these bacteriome has yet been demonstrated in preclinical studies, which requires further investigation in clinical settings. Moreover, a comprehensive review has also suggested a strong relationship between periodontal disease (PD) and the oral cancer development showing that patients with PD had 2-5 fold increased risk of oral cancer development compared to those without PD (Javed and Warnakulasuriya, 2016). However, the availability of next generation sequencing is providing new data on the association of microorganisms with many cancers, including of the head and neck: in considering these it is important to remember that so far the studies mostly show associations, from which cause and effect cannot be deduced.

**Air pollution:**

Air pollution is considered as a risk factor for HNSCC. The indoor air pollution occurring from the burning of wood, animal dung or coal used as fuel for cooking, especially in the villages of many developing countries, poses a significant health hazard (Dietz et al., 1995, Sapkota et al., 2008). Continuous inhalation and exposure to this polluted air may induce HNSCC development. Therefore, IARC has recognized indoor air pollution as a human carcinogen (2010). Recently, the IARC also classified outdoor air pollution and particulate matter (PM) as a Group1 carcinogen, indicating that environmental factor is also related to HNSCC development. A recent epidemiological study has demonstrated that outdoor air pollution, similar to smoking, is linked with the upper aerodigestive tract cancers (Wong et al., 2014). An earlier study from US, suggested that the carbonaceous
aerosols and polycyclic aromatic hydrocarbons from outdoor air pollution is associated with the increased risk of upper aerodigestive tract cancer including lung cancers and head and neck cancer development in the urban populations (Grant, 2009).

1.1.3. Use of ICD-10 coding system in HNC:

The International Classification of Disease tenth revision (ICD-10) is the latest coding system developed and approved by the World Health Organization (WHO). It is an international standard for defining and reporting diseases. The relevant codes for HNC are shown in Table 1.1.

Table 1.1: ICD-10 classification of malignant neoplasms of head and neck cancers. (http://apps.who.int/classifications/icd10/browse/2010/en#/C00-C14) (www. Data) (adopted from WHO website)

<table>
<thead>
<tr>
<th>Codes</th>
<th>Specific sites</th>
</tr>
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<tbody>
<tr>
<td>C00</td>
<td>Malignant neoplasm of lips (external upper &amp; lower lips, internal upper and lower lips, unspecified external and inner aspect of lips, commissure of lip)</td>
</tr>
<tr>
<td>C01</td>
<td>Malignant neoplasm of base of the tongue (dorsal surface of base of tongue, fixed and posterior part of the tongue)</td>
</tr>
<tr>
<td>C02</td>
<td>Malignant neoplasm of other and unspecified parts of the tongue (dorsal and ventral surface of the tongue, ventral surface and anterior-two third of the tongue, border of the tongue and lingual tonsil)</td>
</tr>
<tr>
<td>C03</td>
<td>Malignant neoplasm of gum (upper and lower gingiva/ alveolar mucosa)</td>
</tr>
<tr>
<td>C04</td>
<td>Malignant neoplasm of floor of the mouth (Anterior and lateral floor of the mouth)</td>
</tr>
<tr>
<td>C05</td>
<td>Malignant neoplasm of palate (Hard palate soft palate and uvula)</td>
</tr>
<tr>
<td>C06</td>
<td>Malignant neoplasm of other and unspecified parts of the mouth (cheek mucosa, vestibule of mouth and retro-molar area)</td>
</tr>
<tr>
<td>C07</td>
<td>Malignant neoplasm of parotid glands</td>
</tr>
<tr>
<td>C08</td>
<td>Malignant neoplasm of other and unspecified major salivary glands (Submandibular glands &amp; sublingual glands)</td>
</tr>
<tr>
<td>C09</td>
<td>Malignant neoplasm of tonsils (tonsillar fossa and tonsillar pillar)</td>
</tr>
<tr>
<td>C10</td>
<td>Malignant neoplasm of oro-pharynx (vallecula, anterior...</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>C11</td>
<td>Malignant neoplasm of naso-pharynx superior, posterior and lateral walls of the naso-pharynx</td>
</tr>
<tr>
<td>C12</td>
<td>Malignant neoplasm of piriform sinus</td>
</tr>
<tr>
<td>C13</td>
<td>Malignant neoplasm of hypo-pharynx (posterior, aryepiglottic fold, posterior wall of hypopharynx)</td>
</tr>
<tr>
<td>C14</td>
<td>Malignant neoplasm of other ill-defined sites of the lip, oral cavity and pharynx. (pharynx, Waldeyer’s ring)</td>
</tr>
<tr>
<td>C33</td>
<td>Malignant neoplasm of larynx (glottis, supraglottis, subglottis and laryngeal cartilage)</td>
</tr>
</tbody>
</table>

While this coding system has been adopted and practiced by most developed countries, many developing countries still lack such a scheme. The implementation of this coding system bears great importance in defining and classifying head and neck cancer accurately as different parts of the head and neck region are susceptible to different types of risk factors. Therefore, it is necessary to employ this system all over the world while categorizing site specific HNCs, especially in developing countries (like those of South Asia).

### 1.2.1. Human papillomavirus (HPV):

Papillomaviruses are a group of small circular, non-enveloped and icosahedral capsid coated double stranded DNA viruses. They belong to a large family of viruses, the ‘papovaviridae’ (Rowson and Mahy, 1967). Richard Shope first described Papillomaviruses in 1933 from cutaneous papillomatosis in a cottontail rabbit (Christensen, 2005). These viruses are widespread in nature and induce warts (papilloma) in a variety of higher vertebrates, including humans. Some of them have the potential to progress cells into malignancy (McMurray et al., 2001, Longworth and Laimins, 2004). Usually, they are highly species specific and preferentially infect
cutaneous or mucocutaneous epithelia (Longworth and Laimins, 2004). A number of human papillomaviruses (HPVs) have shown frequent association with cervical cancer and other epithelial neoplasms, notably anal carcinoma, penile cancer and oropharyngeal carcinoma.

HPVs are a large heterogenous group of viruses, epitheliotropic in nature, that are small in diameter (55nm), with a genome of 7200 – 8000 base pairs in length (McCance, 1986). All the papillomavirus genomes contain similar characteristics. The viral genes (ORFs) are located on one strand, which serves as a template for transcription. This transcriptional strand consists of 3 regions: (1) an upstream regulatory region (URR) or Long Control region (LCR) or Non-coding region, which has no open reading frames (ORFs) but incorporates sequences that regulate viral transcription and replication; (2) an early region, ORFs E1, E2, E4, E5, E6 and E7, that are engaged in various functions including trans-activation of transcription, transformation, replication and viral adaptation and (3) a late region coding for L1 and L2 capsid proteins that are involved in the formation of virion structure and facilitate selective encapsidation and maturation of viral DNA (Burk, 1999, zur Hausen, 1977).
Due to the insufficiency of an ideal in vitro culturing system, the serological classification of HPVs could not be established. Thus, their classification is based on the origin of the species, the extent of the homology of the DNA genomes and referred to as “Genotypes” (Burk et al., 2009, Schiffman et al., 2005). A distinct ‘type’ of HPV is established by comparing the complete nucleotide sequence of the L1 region of the desired type to that of any other characterised type of HPV with at least 10% difference (Bernard et al., 2010, de Villiers et al., 2004). More than 189 types of fully characterized HPVs are currently recognised; among which approximately 60 are predominantly located in mucosal epithelia. Based on the L1 nucleotide sequences, phylogenetic analysis has grouped all the mucosal epithelial HPV types into alpha-papillomavirus genus (α-PV genus) and species (α1 – α15) (de Villiers et al., 2004, Bernard et al., 2010). A phylogenetic tree was generated
depending on the nucleotide and amino acid sequences of ORFs (E6,E7,E1,E2,L2,L1) to characterize all the available α-papillomavirus sequences according to their probable malignant transformation potential (Schiffman et al., 2005). HPV types that are allocated in a species tend to share common characteristics, such as tissue tropism and oncogenic potential. The phylogenetic tree separates the α-HPVs into 3 groups: (1) Low-risk 1 (LR-1) or non-oncogenic type-1 (NOT1) group (α-1, α-8, α-10, α-13); (2) LR-2 or NOT2 group (α-2, α-3, α-4, α-15) and (3) High-risk (HR) or OT (oncogenic type) group (α-5, α-6, α-7, α-9, α-11) (Schiffman et al., 2005).
Figure 1.4: Phylogenetic tree of the mucosal/genital α-HPVs. Showing different HPVs categorized according to their oncogenic potential. (taken from Burk RD et al., 2009) (with permission)(Burk et al., 2009).
HPVs are pervasively present in humans, but only small subsets develop cancer. The majority of HPV types are NOTs that induce benign lesions or may not cause any detectable lesion. In contrast, most of the OT or HR types of HPV are preferentially associated with cervical, ano-genital and oro-pharyngeal cancers. Epidemiological studies from a wide range of countries have identified at least 15 oncogenic types of HPV (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) with 2 probable high risk types (HPVs 26 & 66) and 13 commonly observed low risk types (HPVs 6, 11, 40, 42, 43, 44, 53, 54, 61, 70, 72, 81 & 89) (Munoz et al., 2003, Munoz et al., 2006, Bosch and de Sanjose, 2007). However, among all the high-risk types, HPV-16 (α-9 species) and HPV-18 (α-7 species) are associated with more than 70% of cervical cancers cases worldwide (Smith et al., 2007). HPV-16 is most frequently involved in squamous cell carcinoma of the cervix (>50%). Interestingly, more than 90% of the cases of HPV-related HNSCCs are suggested to be associated with HPV 16 (Marur et al., 2010). Recent studies reported approximately 25 HPV types that are associated with different oral lesions, not necessarily malignant (Syrjanen, 2003, Thorland et al., 2003).

1.2.2. HPV-associated Head and neck cancer:

It is widely accepted that HPV-16 and HPV-18 are responsible for approximately 70% of cancers of the uterine cervix (zur Hausen, 2002) and are strongly associated with anal, vulvar, and penile cancers (Kayes et al., 2007, Crum et al., 1997). In 1983, the association of HPV with a subset of HNCs was first documented (Syrjanen et al., 1983). Recent reports show high-risk HPV infections are a strong risk factor for approximately one third of the total HNC cases in the world (Ang et al., 2010, Shaikh et al., 2015). Further, a growing body of evidence suggests that approximately 20% of oral cancers and 60-80% of oropharyngeal cancers are associated with HPVs (Ang et al., 2010, Syrjanen et al., 2011, Mehanna et al., 2013). Recently, the cancer research agency of WHO, the International Agency for Research on Cancer (IARC) has declared HPV as an etiological factor of oropharyngeal squamous cell carcinoma (OPSCC) (Dalianis, 2014). Approximately 25 HPV types are associated with oral
1.2.3. Epidemiology of HPV-associated HNC:

Head and neck cancer has long been a serious problem in many parts of the world, with increases in recent time. According to GLOBOCAN 2012 data, the estimated burden of HNC is approximately 634,766 new cases per annum worldwide, including approx. 263,020 for oral cavity, 150,677 for larynx, 136,622 for oropharynx, excluding the naso-pharynx because of its distinctive biological characteristics (Ferlay et al., 2010). While the majority of these are observed in developing countries, there is considerable geographical variation in incidence. South Asia falls into the high incidence category for head and neck cancer. The annual incidence is 143,152 new cases p.a. (Gupta et al.) (Ferlay et al., 2010). The age standardized incidence rate of HNC in South Asian countries [approximately 17.27 per 100,000 persons p.a.] is higher than other parts of the world, because of extensive use of tobacco, areca nut and poor oral health (Ferlay et al., 2010).

Although HPV-related cancers are mostly seen in the uterine cervix, as more than 99% of these harbor HPV (Walboomers et al., 1999), a significant association of HPV with HNSCC is now well recognized, with an overall prevalence of approximately 25 – 35 % with a substantial variability (McKaig et al., 1998, Gillison and Shah, 2001, Kreimer et al., 2005). The HPV association with HNSCC was first reported in 1983 (Syrjanen et al., 1983) and her hypothesis was validated by Loning et al. and de Villers et al. on oral cavity (OSCC) and oro-pharyngeal squamous cell carcinoma (OPSCC) in 1985 (Loning et al., 1985, de Villiers et al., 1985). However, recent rising rates of OPSCC, especially of the tonsil and base of the tongue, have been reported in Europe and in the USA, many of which showed clear association with HPV (Hammarstedt et al., 2006, Chaturvedi et al., 2011, Shiboski et al., 2005,
Braakhuis et al., 2009). Similar trends have been described in Australia where the incidence of HPV-related oro-pharyngeal cancer increased from approximately 19% in 1982 to ~29% in 2005 (Hocking et al., 2011). Although a high incidence of oral cavity cancer in South Asia has been recognized for decades due to pervasive use of tobacco (smoking and smokeless form) and areca nut, some recent data show significant association of HPV with the HNSCC in the South Asian region. Studies from South Asia, present a striking variability of the prevalence of HPV-associated HNSCC ranging from 3% to 74% (Shaikh et al., 2015). Studies from India also reported variability in the prevalence of HPV in OPSCC, ranging from 22.8 - 74% (Balaram et al., 1995, Bahl et al., 2013, Nagpal et al., 2002). A study from the western part of India showed approximately 28.56% prevalence of HPV in OSCC (Koppikar et al., 2005). However, the prevalence of HPV in laryngeal squamous cell carcinoma (LSCC) varies widely across the world. The reported prevalence of HPV in LSCC has ranged from 9.4 - 27% in USA, 4.4 – 52% in Europe, and slightly higher in South America, ranging from 32 - 37.3% (Shen et al., 1996, Paz et al., 1997, Baumann et al., 2009, Stephen et al., 2012, de Oliveira et al., 2006, Torrente et al., 2005, Salam et al., 1995, Venuti et al., 2000, Snijders et al., 1996, Koskinen et al., 2007, Hoffmann et al., 2009, Morshed et al., 2010). There are a few studies from South Asia, reporting the prevalence of HPV in LSCC ranging from 34% - 47% (Jacob et al., 2002, Talukdar et al., 2015).

1.2.4. Risk factors of HPV-associated HNSCC:

Current evidence across the world indicates that patients with HPV-related HNSCC are generally younger, male, of comparatively high socio-economic status, are non-smokers, non- or light-drinkers and are of Caucasian ethnicity (Benard et al., 2008, Gillison et al., 2008, Llewellyn et al., 2001, Ryerson et al., 2008, Gillison, 2006, Klozar et al., 2008, Lindel et al., 2001). Several studies indicate that sexual behavior is the most important risk factor for HPV-positive HNSCC. The prevalence of HPV in
HNSCC is 5-10 fold lower than that of cervical cancers and the transmission of high-risk HPVs in both these cancers is highly correlated with sexual behaviour (Satterwhite et al., 2013, D'Souza et al., 2009). These includes sexual debut at early age, high number of lifetime vaginal or oral sex partners, open-mouth kissing, oral sex in both oral-oral and oral-genital forms (D'Souza et al., 2009) (Rosenquist et al., 2007, Sanders et al., 2012). Case-control studies indicate a 2-fold increase in those individuals who have had more than one lifetime oral sexual partner and five-fold increase in individuals who had more than six (D'Souza et al., 2007, Rajkumar et al., 2003, Smith et al., 2004). Further, homosexuals are more commonly infected than heterosexuals, as they tend to have greater number of sexual partners (Esquenazi et al., 2010). Another study shows a significant association of HPV with head and neck cancers in men having sex with men who have regular oral-genital contact (Zou et al., 2015).

HPV prevalence in HNSCC tends to be higher in Caucasians, 34%, compared to the black population (4%) in a recent comprehensive study in the U.S.A. (Settle et al., 2009). Although it seems that HPV-positive HNSCC is more likely to develop in Caucasians, a significant prevalence has also been noted in South Asian populations, probably due to the changing of traditional sexual behaviors and the rapid infiltration of western culture (Rajkumar et al., 2003).

The actual mechanism of HPV transmission remains unknown. However, unprotected sexual intercourse could be a leading cause, especially with oro-genital sex where the genital mucosa could be infected by HPVs and during the intercourse it would transmit from genital region to oral cavity (Feller et al., 2009, Sanchez-Vargas et al., 2010). Other possibilities could be direct skin-skin contact, considering that anogenital warts (caused by low risk HPVs), are often spread by self-inoculation from other skin sites by contaminated objects (fomites) and/or by hands to the genital region (Durzynska et al., 2011, Esquenazi et al., 2010, Sinclair et al., 2005). HPVs can also be transmitted by other non-sexual routes, most commonly mother to child transmission during birth. Vertical transmission of HPV-DNA may occur while the foetus passes through the infected cervix and birth canal of the mother, which can lead to the development of laryngeal papillomatosis in the newborn (Rombaldi et al., 2009, Sinclair et al., 2005).
Current understanding suggests that tobacco use, marijuana use, and alcohol abuse could be regarded as additional risk factors as they might contribute in the transmission of HPV virus and cancer progression (Pickard et al., 2012, Sanders et al., 2012). Recent studies have demonstrated a decrease in the progress free survival rate of HPV-associated HNC patients who have used tobacco for more than 10 pack-years (Gillison et al., 2012, Maxwell et al., 2010). This indicates a potential interplay between chemical carcinogens and the HPV. Several studies have reported that men are at 2 - 3-fold greater risk of developing HPV-associated head and neck cancer compared to women (D'Souza et al., 2009, Pickard et al., 2012, Sanders et al., 2012). HPVs have a high predilection for the squamous mucosal epithelium of the oropharynx compared to the other parts of head and neck region (Chaturvedi et al., 2011, Chaturvedi et al., 2008). The oropharynx, especially the tonsils and base of the tongue provide a particularly suitable microenvironment for HPV infection. These sites contain lymphoid tissues and are part of Waldeyer’s ring (Syrianen, 2004, Paz et al., 1997). Lymphoid aggregations are lined by non-keratinized epithelium containing crypts (Syrianen, 2004). The crypts, lined by basal keratinocytes, function as traps for antigens but may also be a target for nuclear integration of HPV DNA (Syrianen, 2004, Gillison et al., 2000). Another possibility could be that this region of the oropharynx acts as an embryonic transformation zone for which HPVs may have a predilection (Gillison et al., 2000). Consistent with such origins, HPV positive HNSCCs exhibit poorly differentiated, non-keratinized morphology with many basaloid tumour cells (Wilczynski et al., 1998, Barnes et al., 1996, Andl et al., 1998).

1.2.5. HPV entry and replication in the basal epithelial cells of head and neck mucosae:

HPVs are commonly present in the anatomical crypts of normal genital epithelium. They are also located in the oropharynx, especially in the tonsillar crypts (Syrianen, 2004, Begum et al., 2005). It has been suggested that the epithelium of the crypts is a specialized tissue, which contains non-keratinised, partially stratified epithelium, and patches of reticulated or sponge-like epithelium, the spaces of which are filled by other specialised cells, in particular M cells, which function to trap antigens and
present them to the immune system) (Kim et al., 2007, Surjan, 1988). The reticulated epithelium lining of the tonsillar crypt functions as the first line immune defence by providing a favourable environment for effector cells of the immune response. As stated, the crypts facilitate direct transport of antigens and contain a pool of immunoglobulins as effectors of the humoral immune response (Surjan, 1988).

HPVs critically depend on the cellular machinery of the host for replication of their genome. A portion of the virus reaches the nucleus to undergo replication (Thierry, 2009), by a similar mechanism to that of eukaryotic chromosomes. The E2 gene acts as the initiating factor for replication, which facilitates the recruitment of E1 gene product, by binding to the LCR region of the viral genome. The E1 gene then exploits replication machinery proteins of the host: replication protein A, topoisomerase 1 and polymerase alpha-primers, and interacts further with cellular genes to coordinate viral replication with the host cell during the S phase of the cell cycle (Doorbar, 2005, Thierry, 2009).

HPV can only initiate infection once it successfully binds and integrates into host epithelial cells. Typically, non-enveloped DNA viruses are internalised by the clathrin-mediated endocytosis pathway (Day et al., 2003). However, HPVs might enter cells either via clathrin-mediated or via caveola-mediated endocytosis (Bousarghin et al., 2003). In squamous epithelia, HPVs infect undifferentiated basal cells after trauma or erosion and exist as a long-term latent infection. However, during an active viral DNA replication phase (productive phase), HPVs can amplify/generate more than 1000 copies/cell (Wang et al., 2009). This leads to the expression of L1 and the production of mature viral particles in the cells of the uppermost layers of differentiated epithelia. The L2 gene is important for viral infectivity and the endosomal escape of the viral genome, which ultimately allows the virus to enter into the host nucleus (Kamper et al., 2006, Sapp and Bienkowska-Haba, 2009). A recent concept regarding cellular receptor binding suggests the presence of HPV receptors for α6β4 integrins and sulphated proteoglycans (HSPGs) (eg: syndecan-1) (Shafti-Keramat et al., 2003). Over-expression of α6β4 integrins and syndecan-1, respectively, is seen strongly in migrating epithelial cells and in basal cells following trauma (Shafti-Keramat et al., 2003, Letian and Tianyu, 2010). HPVs probably bind to α6β4 integrins and syndecan-1 during cellular entry. Once cell entry has been
achieved, HPV undergoes endosomal acidification for viral uncoating, resulting in effective infection (Yang et al., 2003, Letian and Tianyu, 2010).

1.2.6. Molecular Pathogenesis of HPV-associated HNSCC:

The exact biological mechanisms of HPV-associated HNSCC are not completely understood. The carcinogenic process is thought to commence by integration of HPV DNA into the host cell (keratinocyte) genome. Two oncogenes of HPV, E6 and E7, are thought to be responsible for malignant transformation (Figure 1.5). Suppression by transcriptional repressor (E2) of HPV allows continuous expression of viral oncogenes E6 and E7 that ultimately lead to malignancies (Munger et al., 2004). It has been suggested that E6 & E7 oncoproteins of high-risk HPVs bind to the p53 protein and Rb (retinoblastoma) proteins respectively, with approximately 10 fold higher affinity than those of the low-risk types (Farthing and Vousden, 1994, Brachman et al., 1992), and this alone has been shown to be sufficient to immortalise primary human genital and oral keratinocytes in vitro (Munger et al., 1989, Barbosa and Schlegel, 1989). However, additional alterations including activated ras gene mutations or deletions of DCC gene are also required in vivo (Crook et al., 1988, Klingelhutz et al., 1995, Matlashewski et al., 1987).

The E6 oncogene, localized in the nuclear matrix, targets and degrades p53 tumour suppressor gene, thus inhibiting p53-dependent cell cycle arrest & inducing apoptosis (Scheffner et al., 1990, Werness et al., 1990). The E6 oncogene also enhances telomerase activity, favoring immortalisation of affected keratinocytes (Klingelhutz et al., 1996). The other viral oncogene E7 promotes cellular proliferation by down-regulating the cyclin-dependent kinase (CDK) inhibitors p21 & p27, and by inactivating Rb (retinoblastoma) protein by binding with the hypophosphorylated forms of Rb: pRb, p130 and p107 (Dyson et al., 1989, van der Riet et al., 1994, Zhu et al., 1993, Claudio et al., 1994). Inactivation of Rb protein mediates the release of E2F transcription factor that trans-activates cell cycle regulatory genes that eventually lead to abnormal cellular growth spurts (Davies et al., 1993). Of the two viral oncogene
products, E7 protein plays the more critical role in promoting transformation (Shindoh et al., 1995, Barbosa and Schlegel, 1989, Halbert et al., 1991). The E7 oncogene also down-regulates another CDK inhibitor, i.e: inhibits p16 gene transcription: this then up-regulates CDKN2A, which ultimately mediates the over-expression of p16^{INK4A} gene in HPV-positive cancers (Benevolo et al., 2006, Li et al., 1994).

Figure 1.5: A schematic diagram of the process of HPV carcinogenesis.

The diagram presents two HPV oncoproteins E6 and E7 targeting cell cycle regulatory protein (pRb - retinoblastoma protein) and tumour suppressor gene (p53) respectively. E7 binds with pRb, which inactivates pRb leading to the release of E2F transcription factor that drives the expression of S-phase genes and ultimately progresses to uncontrolled cell proliferation. In addition, a reciprocal relationship has been observed between pRb and p16 protein where inactivation of pRb leads to over expression of the p16 gene [p16(INK4)]. P16 protein binds to CDK4 & CDK6 (cyclin-dependent kinases) and inhibits their ability to interact with cyclin D and stimulates passage through the G1 phase to S phase. E7 also degrades p21 protein function, which is thought to play a major role in cellular response to DNA damage. On the other hand, oncoprotein E6 binds to p53 protein and leads to its degradation, allowing the cancer cell to escape apoptosis. The E6 gene also increases telomerase activity. All of these changes ultimately promote cancer progression.
This expression of p16 gene has been recognized as a surrogate marker for diagnosing that a given HNSCC contains an oncogenic HPV (Kuo et al., 2008).

Since the mucosae of the upper aero-digestive tract are exposed to a wider range and higher concentrations of chemical carcinogens compared to genitalia, it is likely that different carcinogenic mechanisms could be implicated in HPV-associated HNSCC than in cervical or anal cancer. Recent studies have suggested that apart from p53 and pRb pathways, HPV onco-proteins E5, E6 and E7 coordinate and target multiple pathways for the development of HPV-induced HNSCC. These include the EGFR pathway, the TGFβ pathway, PI3-PTEN-AKT pathway (evading apoptosis, thus providing a survival advantage for HPV-positive cancer cells), and the angiogenesis pathway, involving up-regulation of hypoxia-inducible factor (HIF) (Munger et al., 2004, Moody and Laimins, 2010, Maufort et al., 2010). Activation of WNT pathways by E6 and E7 in head and neck cancer has also been reported (Rampias et al., 2010). Interference in these pathways by HPVs lead to mutations of cellular genes, which increase genomic instability: ultimately full transformation takes place (Leemans et al., 2011). Understanding these molecular mechanisms is essential for effective bioprevention and individualised biotherapies.

1.2.7. Detection methods for HPV in Head and Neck Cancers:

In recent times, it has been recommended to perform HPV-testing for all head and neck cancer patients, especially those with OPSCC, as standard practice. HPV-associated HNSCCs, especially OPSCC, constitute a distinct tumour entity with distinct clinical features and better prognosis. Nevertheless, heterogeneity in both clinical and biological behaviours has been noticed (Braakhuis et al., 2004, Lindquist et al., 2007, Reimers et al., 2007). Histopathological study of tissue (using Haematoxylin and Eosin- H&E staining) is the most commonly practiced and one of the oldest methods for diagnosing and understanding the tissue morphology of any
cancerous lesion. Histologically, HPV-associated HNSCC and HPV-negative HNSCC differ. The typical HPV-associated HNSCC presents with epithelial invasion in the stroma, which appears as islands, cords, sheets or isolated epithelial malignant cells. The cytological hallmark of HPV infection is the presence of koilocytic atypia of the prickle cell (intermediate) layers of stratified squamous epithelia. This is manifested as ballooning of keratinocytes with dense staining of irregular nuclei, often pressed to the side of the cell, surrounded by a perinuclear halo with condensed, amphophilic cytoplasm at its margins. Distinction should be made between true koilocytosis and vacuolization of cells, such as that seen in white sponge naevus of the oral mucosa, which is not HPV related. In HPV infection there may also be parakeratinized (dyskeratotic) cells individually or in clusters below the surface layers. Both koilocytic and dyskeratotic cells may be bi- or multinucleate, and show enlarged nuclei. Where HPV-associated HNSCC arises from lymphoid mucosae, strands and islands of infiltrating malignant epithelial cells have basaloid morphology and are surrounded by lymphoid stroma.

A range of biomarkers has been used for detection of biologically active HPV infection in SCC. Among these, p16\textsuperscript{INK4A} immunohistochemistry is used frequently as a surrogate marker (Ang et al., 2010). However, studies have shown that approximately 10 – 20% of p16 positive tumours did not show evidence of HPV when screened by polymerase chain reaction (PCR) (Shah et al., 2009, Weinberger et al., 2006, Harris et al., 2011). Ideally, p16 immunohistochemistry should not be used as a diagnostic tool alone but could be used in conjunction with nucleic acid based assay PCR to accurately detect an HPV infection (Smeets et al., 2009, Jordan et al., 2012). The accurate detection, or exclusion, of HPV in tumour tissue is critical when planning personalised therapy for patients with HN or genital cancers: de-escalation of therapy is possible for HPV-positive cases.

Diagnostic assays available for the detection of HPV-DNA in cancerous tissue vary considerably in sensitivity and specificity. It is important to select a technique that is highly sensitive, subject to standardisation, easily accessible, economically viable and has short turn-around time. Southern blot is one of the oldest and reliable techniques for detecting HPV-DNA, highly sensitive and has the ability to identify specific HPV
types (Singhi and Westra, 2010). Unfortunately, it is not suitable for routine clinical use as it is very time consuming and requires a large quantity of cellular DNA. In situ hybridization (ISH), has high specificity but is less sensitive than Southern Blotting (SB); SB with 76% sensitivity and 87% specificity, while ISH has shown 50% sensitivity and 93% specificity (Caussy et al., 1988). Furthermore, sensitivity may vary in different disease categories: ISH is more sensitive when applied to condylomatous tissue but less sensitive in invasive neoplasms (Caussy et al., 1988). PCR can be used when only a small amount of sample is available and HPV loads are low. However, false positive results may be an issue if strict contamination control is not maintained (Kim et al., 2003, Seo et al., 2006). Studies have shown that combination of two sets of degenerate primers (MY09/11 & GP5+/6+) (de Roda Husman et al., 1995), amplifying fragments of the widely conserved HPV L1 gene in “Nested PCR”, is more efficient for detection of HPV in oropharyngeal cancer tissues with low viral load compared to conventional PCR (Haws et al., 2005, Winder et al., 2009). Generally, PCR followed by direct sequencing is performed to detect HPV types. Another advanced PCR method, real time quantitative PCR (qPCR) has been shown to permit high-throughput analysis and to quantitatively detect & genotype HPV-DNA (Seaman et al., 2010). qPCR can discriminate between HPV-positive and HPV-negative cancers by measuring viral load, but is very sensitive (with greater chance of errors from contamination) and is expensive (Yoshida et al., 2008). Both Nested PCR and qPCR are very sensitive and frequently used methods, but are prone to cross contamination and require a strict, de-contaminated working facility. Multiplex tandem-PCR (MT-PCR) assays, which are capable of detecting HPV-DNA very efficiently have also been described, (Stanley and Szewczuk, 2005). Ideally, the gold standard method for identifying clinically relevant HPV would be detecting transcriptionally active viral oncoproteins E6 and E7 mRNA in fresh or frozen tissue (Westra, 2014). Two commercial assays are available: PreTect® Proofer (PPT) assay and APTIMA® assay (Cuschieri and Wentzensen, 2008, Dockter et al., 2009). These function by the transcription-mediated amplification of the full length of E6 and E7 oncogenes. However, they cannot be used in retrospective molecular studies on formalin fixed paraffin embedded (FFPE) tissues, and are unlikely to be used for routine diagnosis (Marur et al., 2010). They require good quality mRNA, which can generally be extracted from fresh materials or even tissues stored at -80°C for a short
period, which is not possible in case of FFPE tissue as mRNA degrades during the fixation process.

Genotyping is important for clinicians to identify oncogenic HPV types in patient samples and monitor the patient accordingly. For HPV type determination, direct bi-directional sequencing of an amplicon of L1 sufficiently detects any HPV type present. However, to detect multiple HPV types in a mixed infection the current Food and Drug Administration (FDA) approved method- the Hybrid Capture II system (HC-II; Digene Corp) - is available. However, this method can only detect HR-HPV and LR-HPV types as a group, and not specific genotypes (Garcia-Sierra et al., 2009). Very recently, a method claiming to be superior to HC-II, known as the Array-based HPV genotyping method or CAPH (Clinical Arrays Papillomavirus Humano) (Garcia-Sierra et al., 2009), has been shown to be capable of detecting single or mixed infections with up to 35 HPV types (20 HR-HPVs and 15 LR-HPVs). In the future, next-generation sequencing could play a greater role in HPV genotyping as it examines the entire HPV genome and can detect any HPV type present, even in a mixed infection (Barzon et al., 2011, Conway et al., 2012). Nevertheless, being costly and time consuming, it is unlikely to be used routinely.

1.2.8. Prevention and Management of HPV-associated Head and Neck Cancer:

Treatment of HNSCC still relies largely on surgery with or without adjunctive radiotherapy and/or chemotherapy. Several immunotherapies are in early clinical trial.

HPV-related HNSCCs are regarded as a distinct entity as they have differences in demography of patients, clinical presentations and prognostic outcomes (Ang et al., 2010). Research suggests that HPV certainly plays a significant role in the alteration of the epidemiology and survival outcome of a subset of head and neck cancer patients (Chaturvedi et al., 2011, Friedman et al., 2014). HPV-positive HNSCC
patients have better survival rates and better therapeutic responses to chemotherapy and radiotherapy (RT) compared to HPV-negative HNSCCs (Fakhry et al., 2008, Lassen, 2010, O'Rorke et al., 2012). Recent clinical trials have also reported a higher response rate towards chemo-radiation therapy with superior progression-free survival of HPV-positive patients (Fakhry et al., 2008, Posner et al., 2011). Possible reasons for this could be that affected patients tend to be sexually active younger individuals with a better immune system (Ang et al., 2010, Weinberger et al., 2006). However, younger age with improved prognosis requires reduction in treatment-related toxicity. Hence, the focus of current treatment strategies is to overcome such harmful effects.

Current standard treatment option for HPV-positive HNSCC is a combination of platinum-based and fractionated radiation therapy (Coppock et al., 2013, Spanos et al., 2009). Three phase III trials (RTOG 1016, De-ESCALaTE and TROG 12.01) are underway assessing the possibility of de-intensifying the existing standard combination treatment of cisplatin & RT by reducing RT dosage and replacing platinum-based agents, with Cetuximab (Masterson et al., 2014, Nguyen-Tan et al., 2014). Early studies suggested that 10% enhancement of RT dosage would increase tumour control rates by 5% - 30% (Bentzen, 1994, Begg et al., 2011). However, this increased RT dosage results in high levels of normal tissue toxicity. Unfortunately, recent studies have shown that approximately 10% of HPV-positive HNSCC patients with de-escalation strategies are likely to develop distant metastasis (O'Sullivan et al., 2013, Kimple and Harari, 2014). While these dose reductions have shown positive outcomes to a certain extent, a less invasive surgical approach is now being employed. This is trans-oral robotic laser surgery (TROLS), which seems to be equally effective and oncologically safe primary treatment (Leonhardt et al., 2012, Cohen et al., 2011). Traditionally, HPV driven HNSCC populations tend to be younger, wealthier and socially better-networked TROLS might be a good option for them. Considering the developing parts of the world, where HPV-positive HNSCC also seem to be on the rise, TROLS may prove too expensive. The recurrence rate is high in HNCs. Surgeons and oncologists often face difficulties in controlling the disease. Considering the entire above, there is clearly a need for novel therapeutic approaches, such as molecular targeted therapy, researched later in this thesis. And of course primary prevention remains essential.
Since HPV-positive HNSCC is biologically different from HPV-negative cases, the molecular targeted therapeutic approaches also differ. Although much of the genome of HPV-positive and HPV-negative HNSCC share amplification of 1q, 3q, 5p and 8q regions and deletions, including 3p, 5q and 11q regions, some striking genetic aberrations apply only to the HPV-associated HNSCC patients. One such is located at 14q32, which has been reported to undergo deep deletion in HPV-positive tumours while very few occurrences are detected in HPV-negative tumours (Keck et al., 2015). EGFR is commonly over-expressed in HPV-negative HNSCC with focal amplification of chromosome 7, while it is less or absent in HPV-positive cancer (Keck et al., 2015). HPV-negative tumours have shown over-expression of transcription factor TP63, of the oncogene PIK3CA and of the CDKN2A gene (Gymnopoulos et al., 2007, Keck et al., 2015). In contrast, as discussed above, HPV-associated cancers overexpress viral oncogenes E6 and E7 that critically deregulate cell cycle and initiate genomic instability (Keck et al., 2015). The design of targeted molecular therapies will have to vary accordingly.

Since HPV-positive tumours are induced by a viral infection, the virus can produce a range of foreign antigens that might enhance the immune system’s response towards the tumour. Humoral immune responses against viral antigens E6 and E7 are frequently found in HPV-positive cancer patients and can be correlated with increased survival, making immunotherapy a potentially effective approach. This can involve vaccines against HPV. The concept behind the existing prophylactic vaccines is to stimulate the immune system to elicit an adequate neutralising antibody response prior to exposure to high-risk HPVs, so that any infection is transient. It is also expected that this effect would subsequently abolish the development of invasive cancers. Currently, two prophylactic HPV vaccines are available with FDA (Food and Drug Administration) approval. The first is the quadrivalent vaccine (Gardasil by Merck & Co., Inc.), which showed 98% of efficacy in protection against HPV types 6, 11, 16 & 18 in anal, cervical and vaginal pre-cancers (Garland and Smith, 2010, Lu et al., 2011). The second HPV vaccine is Cervarix (HPV2), a bivalent vaccine that has a protective effect against high-risk types HPV16 and HPV18-associated pre-cancers with a high efficacy (97%). In fact lower efficacy is demonstrated when individuals already have an HPV16 and/or HPV18-associated cancer (Lu et al., 2011, D'Souza and Dempsey, 2011). The impact of the present prophylactic vaccines and vaccination
programmes around the world on the incidence of head and neck cancer is just beginning to emerge: early data are encouraging. Other issues might be the price of prophylactic vaccines, which are very expensive to afford by the developing parts of the world and may have a less protective effect against HPV-associated HNSCCs. Therapeutic vaccines aim to treat HPV-infected cells, and this might be achieved by developing a T cell immune response, which can recognize and eliminate HPV-infected cells. While the HPV 16 E6 and E7 genes are uniquely expressed by virus infected cells, a HPV specific therapeutic vaccine would be a promising approach for treating HPV-associated HNSCC patients. High frequencies of T regulatory cells that inhibit cellular immune response are often found in HPV-positive HNSCC tissues. Therefore, using low dose of cyclophosphamid (200 mg/m$^2$) along with the therapeutic vaccine for HPV-associated HNSCC can effectively reduce T regulatory cells activity as this has been effectively demonstrated to increase the efficiency of GM-CSF tumor vaccines and thereby enhanced the vaccine-induced immunity in breast cancer patients (Emens et al., 2009).

From current knowledge, although the vaccines are very effective in the prevention of HPV infection, there is limited evidence of an effect on reducing the incidence of HNSCC. Recently, some countries have started vaccinating young boys. The true efficacy of prophylactic vaccines against HNC will take several decades to become clear. Further, these vaccines are not likely to be effective in individuals who already have an HPV-associated cancer, although work is proceeding on therapeutic vaccines. New approaches are required. Could this be siRNA therapy? siRNAs can act as direct anti-virals by targeting the mRNA sequences of viral oncogenes. It is a highly specific therapy with few side effects, and overcomes the non-specific toxicity to surrounding cells. siRNAs targeting HPV infected cells, lead to induction of TLRs (toll like receptors), which in turn upregulate IFNs (interferons), with consequent enhancement of innate immunity. Further the activation of T cell immunity and an increased response to cytotoxic T cells, ultimately induces apoptosis (Kanzler et al., 2007). The role of siRNA in treating cervical cancer, effectively by knocking down E6 and E7 oncogenes, has already been demonstrated in in vitro and in vivo models (Gu et al., 2006, Niu et al., 2006). Such therapy could be effective for HPV-associated head and neck cancer.
An additional targeted therapy comes from a novel drug, a small molecular inhibitor, (Trade name Alisertib), which blocks the activity of the cell cycle regulatory protein, aurora kinase A. It has also shown promising therapeutic advantage, reducing tumour growth in different cancers, including HNSCC (Manfredi et al., 2011, Tanaka et al., 2013). Several studies have shown that the Aurora kinase A is over-expressed in HNSCC, suggesting a possible role in carcinogenesis, such that Alisertib could be used as a potential therapeutic agent for HPV-positive head and neck cancers.

1.3.1. RNAi

Ribonucleic acid has always been considered as the intermediate carrier of genetic instructions to produce proteins essential for normal cellular functions. Other important roles are now emerging. One is its gene silencing competence, which directly regulates gene expression levels. This is termed RNA interference (RNAi) (Ghildiyal and Zamore, 2009). While the functionality of RNAi was first reported in the plant kingdom, the efficiency of its gene suppression in mammalian cells, shown in 2001, was the actual gateway towards its potential use as a therapeutic (Napoli et al., 1990, Elbashir et al., 2001a).

The RNAi machinery is arranged in cells by means of short double stranded non-coding RNAs that are 20-30 nucleotides long, include microRNA (miRNA), small interfering RNA (siRNA) and short hairpin RNA (shRNA). The recognized function of siRNA became more appealing when it was shown successfully to block the expression of the specific mRNA by triggering specific degradation of the targeted gene (Fire et al., 1998). Structure wise, these siRNAs are 21 nucleotides (base pairs) in length and have a characteristic structure of symmetric nucleotides (19 nucleotides) that form a duplex and the remaining 2 nucleotides incorporates 3’ end overhangs (Elbashir et al., 2001b).
1.3.2. Fundamental mechanisms of RNAi:

RNAi describes the mechanism of sequence-specific silencing of the expression of a targeted gene by a class of small noncoding RNAs such as siRNA, miRNA and shRNA. Approximately 2% of the human genome is transcribed into mRNA and translated into protein (the protein coding transcript), so that approximately 98% of all transcriptional genomic output in humans is noncoding RNA (Mattick, 2001, Mattick and Makunin, 2006, Carninci et al., 2005). The RNAi machinery is arranged in eukaryotic cells by means of short, double-stranded, non-coding RNAs that are 20-30 nucleotides long. RNAi is thought to knock down protein expression by three different mechanisms. Firstly, it can inhibit transcription from a genomic DNA by targeting complementary promoter regions through epigenetic silencing (Kawasaki and Taira, 2004, Morris et al., 2004). Secondly, it can inhibit the ribosomal translation of mRNA into protein (Lee et al., 2004). The third and well-understood mechanism is to form a cytoplasmic RNA-induced silencing complex (RISC) that causes destruction of the targeted mRNA (Elbashir et al., 2001b, Hammond et al., 2001) as seen in Figure 1. In this mechanism, the single strand of RNAi (siRNA) has to be exactly complementary to the target mRNA and it is this which allows it to be implemented as a potential therapeutic tool for treating numerous diseases including viral infections, cancer and inherited genetic disorders.

1.3.3. Gene silencing mechanism of siRNA:

In structure, the short-interfering, double-stranded RNAs (siRNAs) are 21 nucleotides (base pairs) in length and have a characteristic symmetric nucleotide (19 nucleotides) that forms a duplex with two nucleotide 3’ overhangs (Elbashir et al., 2001b). siRNAs can be introduced into the cytoplasm of malignant cells in three different forms: (a) as short, synthetic, double-stranded siRNA transfected directly into the cell (Ketting et al., 2001); (b) as longer dsRNA, transfected into cells, cut by the cytoplasmic enzyme Dicer (RNase III endonuclease) into siRNAs (Matzke and Birchler, 2005) and (c) as
endogenously produced short-hairpin RNA (shRNA), which are exported from the nucleus to cytoplasm and cut into small RNAs (siRNAs) by Dicer (Yi et al., 2003).

The sense strand of the activated siRNA is separated by endogenous endonucleases, while the antisense strand (complimentary to the target mRNA) is assembled into the RISC (Hammond et al., 2000). The helicase efficiently unwinds the duplexes and permits it’s binding with the homologous-targeted mRNAs through base-pair interaction, while the Argonaute-2 (AGO2) protein plays a major role in assembling of the siRNA. Further, these mechanisms lead to endonucleolytic cleavage and subsequent degradation of targeted mRNA in the RISC by the action of the cellular exoribonucleases (Takeshita and Ochiya, 2006) (Figure 1). Thus the targeted gene expression is suppressed, which is termed as post-transcriptional gene silencing (PTGS). The catalytic RISC may be recycled to induce multiple rounds of silencing. Importantly, as the guide-strand of siRNA is protected by the RISC from degradation, multiple mRNAs can be cleaved from a single siRNA (Takeshita and Ochiya, 2006). Since siRNA induces transient gene silencing, it is modified in the form of an expression vector (shRNA) if extended gene silencing effect is required. A newly developed bi-functional shRNA (bishRNA) has shown to be more efficient and durable in terms of silencing gene expression compared to shRNA (Rao et al., 2009).
Figure 1.6.: The RNAi pathway. Long double-stranded RNA (dsRNA) or small hairpin RNA (shRNA) is processed by Dicer to form a siRNA, which associates with RNA-inducing protein complex (RISC) and mediates target sequence specificity for subsequent mRNA cleavage.

1.3.4. The concept of siRNA therapy targeting HPV in HPV-positive HNSCC:

The HPV genome is composed of approximately 8,000 base pairs and encodes two groups of viral proteins; the non-structural early genes (\(E1, E2, E4, E5, E6 & E7\)) that are associated with viral replication and the structural late genes (\(L1\) and \(L2\)) that regulate viral packaging (McLaughlin-Drubin and Munger, 2009, Blitzer et al., 2014). E6 and E7 oncoproteins have the ability to promote the transformation of the host’s
epithelium that ultimately leads to tumourigenesis (Adams et al., 2014, Blitzer et al., 2014). Since E6 and E7 oncoproteins are constitutively expressed throughout cancer progression, they have become attractive targets for antiviral or virus-related cancer therapy (Stern et al., 2012). Given the specificity of RNAi-based therapy, any viral gene including genes of HPV could potentially be targeted by siRNAs. Jiang and Milner were the first to use siRNA against E6 and E7 oncogenes and successfully silenced HPV in cervical cancer with a significant reduction in tumour growth (Jiang and Milner, 2002). Since then, several studies have examined the knockdown of E6 and E7 oncogenes in the cervical cancer setting by inducing cellular senescence using siRNA in vitro (Putral et al., 2005, Yamato et al., 2006). Interestingly, using a single siRNA sequence can silence both E6 and E7 genes (Figure 1.8) as these genes are produced by the same transcriptional unit and exist as a bicistronic open reading frame (Figure 1.7) (Liu et al., 2009).

**Figure 1.7 :** Locations within the HPV16 bicistronic E6/E7 transcript targeted by siRNAs (upward pointing arrows) in recent studies and previous studies (downward pointing arrows). The E6 and E7 open reading frames run from nucleotide 83-559 and 562-858, respectively. Splicing events within the pre-mRNA lead to transcription termed E6 I, II, III.
Several reports have suggested that knockdown of both E6 and E7 oncoproteins, either using E6 or E7 directed siRNA treatment produces a better outcome (Lea et al., 2007, Bousarghin et al., 2009). To complement in vitro studies, the effect of siRNA has also been investigated on a pre-clinical animal models (in vivo) with positive outcomes (Gu et al., 2006, Niu et al., 2006). Although the fact that siRNA targeting E6/E7 can effectively inhibit tumour growth is well documented in cervical cancer settings, the efficacy of siRNA in HPV-positive HNSCC settings remains unclear. Few studies have looked at the possible role of E6/E7-targeted siRNA in HPV-positive HNSCCs. One of the primary reasons could be the paucity of well-established HPV-positive HNSCC cell lines. However, a recent study has shown a promising potential of siRNA targeting E6/E7 reduced cancer cell viability in an HPV-positive HNC cell line in vitro (Adhim et al., 2013). Further, siRNA targeted against wild-type Tp53 in HPV-positive HNC cells resulted in increase sensitivity towards radiotherapy has also been demonstrated (Kimple et al., 2013).
In comparison with other therapies, siRNA has the advantage of being highly specific and therapeutic with limited side effects. Both *in vitro* and *in vivo* experiment results have supported the effectiveness of siRNA directed against HPVE6 and E7. These promising results have urged for the development and progression towards clinical trial, which might lead the successful use of siRNA therapy in treating HPV-positive cancers in the foreseeable future.

1.4.1. **Aurora Kinases:**

Mammalian cells undergo an organised cycle of events that allow the cells to divide and generate into two daughter cells. These events include a DNA replication or synthesis phase (S phase), a mitosis or cell division phase (M phase) and two gap phases (G1 and G2 phases). These gap phases play an important role in cell division by ensuring correct DNA replication and genomic integrity (Harper and Brooks, 2005). Further, the genomic integrity is also maintained by the activity of three major checkpoints in cell cycle; the G1/S, G2/M and spindle assembly checkpoints. In addition, a family of serine/threonine kinases governs the well-ordered progression at critical phases of the cell cycle. These are known as cyclin dependent kinases (CDKs): CDK1, CDK2, CDK4 and CDK6 (Malumbres and Barbacid, 2006). In addition to CDKs, checkpoint kinases (Chk1 and Chk2), Polo-like kinases (Plk) and Aurora kinases also play important regulatory roles in the coordinated cell cycle (Bartek and Lukas, 2003, Petronczki et al., 2008, Carmen and Earnshaw, 2003).

The Aurora kinases are a well conserved family of serine/threonine protein kinases that play a crucial role in the mitosis of the cell cycle, starting from mitotic entry to cytokinesis (Carmena, 2009). Hence, they are also called as mitotic kinases. Chan and Botstein et al, discovered and described the first member of the Aurora kinase family in *Saccharomyces cerevisiae* (yeast) while screening for mutations that led to abnormal
increase in chromosome numbers (Chan and Botstein, 1993). Later, a new family of Aurora kinases was discovered in *Drosophila melanogaster* (Glover et al., 1995). Subsequently, three homologous Aurora kinases were postulated in mammals, categorised as Aurora A, Aurora B and Aurora C (Bischoff and Plowman, 1999, Nigg, 2001). Aurora kinases have similar structural features to other protein kinases, consisting of a C-terminal catalytic domain and a short N-terminal domain. In terms of structure, Aurora A, B and C share 71% homology in their C-terminal catalytic domains, but differ in their N-terminal domains, and have distinct localisation and functions (Giet and Prigent, 1999, Carmena and Earnshaw, 2003).

Although Aurora kinases are nuclear proteins they are localised in different intra-cellular locations during different steps of mitosis (Figure 1.9). For example, Aurora A is localised at the centrosome in prophase (early step of mitosis but late G2-phase), but during metaphase to anaphase, it relocates to spindle microtubules (Katayama et al., 2003, Shindo et al., 1998). Whereas, Aurora B, a chromosomal passenger protein (CPP), is located at peri-centromeric chromatins in prophase but in metaphase they are found at the kinetochores. Thereafter, Aurora B relocates to spindle midzone and finally concentrates in the midbody before cytokinesis (late step of mitosis but the early step of G1-phase) (Terada et al., 1998, Adams et al., 2000). Aurora C, also a CPP, with similar sub-cellular location to Aurora B, is found in centromeres through prophase to metaphase, and later relocates to midzone microtubules (Sasai et al., 2004). The distribution of Aurora kinases in different steps of mitosis strongly relates to their function. The activity of the Aurora kinases A and B, thought to increase in S and G2 phases, and persist at high levels throughout mitosis, degrades when the cells exit from mitotic phase (Crane et al., 2004, Stewart and Fang, 2005). Aurora A plays an important role in the timing of mitotic entry, maturation of centrosomes, separation of centrioles, bipolar spindle assembly and cytokinesis (Hirota et al., 2003). Although Aurora B has no influence on mitotic entry of cells, it is involved in chromosomal condensation, chromosomal bi-orientation, modification of kinetochore-microtubule attachment, spindle-assembly checkpoint activation and cytokinesis (Carmena et al., 2012). Aurora C has been shown to have similar functions as Aurora B (Yan et al., 2005).
Figure 1.9. Location and function of aurora kinase A and B. Aurora A is first located at the centrosome during S phase. Activity of Aurora A increases in centrosome and it gets translocated into nucleus. After the nuclear envelope breakdown (NEBD), the activated aurora A is localized in the spindle poles during prometaphase and metaphase. In cytokinesis the aurora A becomes undetectable. The aurora B is located on chromosome during prophase and at the centrosome during prometaphase and metaphase. At the late stages of mitosis it is localized into spindle and during cytokinesis it is relocated into midbody. (taken from Muramoto T. et al. Nat. Rev. Can., 2005) (with permission).
1.4.2. Role of Aurora kinases in tumour development:

As Aurora kinases play critical roles in mitosis, any alteration in their signalling pathway may result in abnormal mitotic cell division that eventually could lead to chromosomal abnormality, genetic instability or aneuploidy, and cancer (Dar et al., 2010). In tumour cells, Aurora A may either be diffusely detected or overexpressed as both inhibition and overexpression lead to cancer progression. The disruption of Aurora A activity halts the mitotic progression of cells by mitotic checkpoint activation. The lack or absence of Aurora A has shown to trigger multiple events including: disruption in normal cell cycle progression, defects in spindle pole organization, misalignment of chromosomes and excess phosphorylation of substrates during G1/S phase, which ultimately leads to incomplete cytokinesis and neoplastic transformation (Hirota et al., 2003, Liu and Ruderman, 2006). On the contrary, the overexpression of Aurora A has been shown to provoke centrosome amplification, chromosomal instability that leads to failures in correct cytokinesis, formation of multinucleated daughter cells and, finally, progression to cancer (Zhou et al., 1998, Meraldi et al., 2002). This suggests that any alteration in the normal function of Aurora A may lead to cancer progression.

Aurora A is located in chromosome 20q13.2, a region which is frequently altered in different cancers. The amplification of Aurora A is oncogenic and has been reported in many human cancers including breast, colon, ovarian, cervical, prostate and neuroblastoma (Sen et al., 1997, Bischoff et al., 1998, Zhou et al., 1998). Moreover, centrosome amplification leading to multipolar mitotic spindle formation has been reported in head and neck cancers (Tatsuka et al., 2005). Aurora A has been shown to phosphorylate p53, a tumour suppressor protein, and facilitate MDM2-mediated p53 degradation, which causes the cells to undergo several rounds of failed cell division, leading to centrosome instability and high level of aneuploidy (Katayama et al., 2004). In contrast, the activity of Aurora kinase A can be suppressed by p53 gene product, which in turn may lead to overexpression or increased activation of Aurora kinase A in p53 mutant neoplasms (Chen et al., 2002). The overexpression of Aurora A also influences Ras-oncogene induced transformation, indicating its possible role in oncogenesis and tumour growth (Tatsuka et al., 2005).
Ectopic expressions of Aurora B may also play an important role in cancer progression. The inhibition of Aurora B kinase also leads to the failure of mitotic checkpoint activation, causing the cells to bypass checkpoint, generating polyploidy and resulting in failure of cytokinesis, genomic instability and finally cancer (Ditchfield et al., 2003). Aurora B is located in chromosome 17p13.1, a region that lies close to the location of the p53 gene. The overexpression of Aurora B has also been shown to phosphorylate p53 protein, resulting in functional loss of p53, which then leads to carcinogenesis (Lacroix et al., 2006). Although the exact function of Aurora B is not clearly understood, it is seen to be overexpressed in several types of cancers include; ovarian carcinoma, hepatocellular carcinoma, glioblastoma multiforme and gastric carcinoma (Chen et al., 2009, Aihara et al., 2010, Zeng et al., 2007, Honma et al., 2014). The high-level of Aurora B expression has been shown to cause its mislocalisation, which led to tetraploidy and tumour formation in a nude mouse model (Nguyen et al., 2009). Further, the overexpression of Aurora B was exhibited to mediate chromosomal instability in gastric cancer, which in turn lead to DNA aneuploidy and ultimately to cancer progression (Honma et al., 2014).

The role of Aurora C in carcinogenesis has been least explored. An earlier study suggested overexpression of Aurora C was seen in colorectal and prostate cancers, although it is not clear that whether the overexpression is the cause or an effect of carcinogenesis (Sasai et al., 2004).

1.4.3. Aurora Kinases in Head and Neck Cancer:

Raised expression of Aurora kinase A has also been reported in HNSCC, like many other cancers, which indicates its likely role in the pathogenesis of head and neck cancer. It is thought that the over expression may occur due to the spread of Aurora A from the chromosome to cytoplasm or nucleus, which eventually leads to carcinogenesis (Tatsuka et al., 2009). A comprehensive study compared the Aurora kinase A expression in 6 different HNSCC cells (Tu138, UMSCC1, Tu167, OSC19,
Tu177 and JMAR) with the normal human epithelial keratinocyte (NHEK) (from the oral cavity), and showed approximately 10 - 20 fold higher Aurora kinase A mRNA expression level in different HNSCC cells than in NHEKs (Mazumdar et al., 2009), indicating that Aurora kinase A may have been regulated at the transcriptional level. Further, the study has also suggested a possible posttranscriptional regulatory role of Aurora kinase A in HNSCC. Another study has demonstrated that suppressing the endogenous Aurora kinase A using target specific shRNA led to 50% reduction in the invasive capability and also a reduced chromosome segregation abnormality in the laryngeal cancer cell line, HEp-2, suggesting a potential role of overexpressed Aurora kinase A in the carcinogenesis of laryngeal squamous cell carcinoma (Zhang et al., 2012). A recent study has demonstrated a strong correlation between Aurora kinase A and mitotic phosphoprotein, FLJ10540 or matrix metalloproteinase (MMP) family proteins (MMP-7 and MMP10), indicating that Aurora kinase A, by regulating the function of FLJ10540 protein may contribute in cancer cell proliferation and metastasis in HNSCC as the high FLJ10540 protein expression promotes cell proliferation and metastasis in human cancer (Chen et al., 2015). Further, the study also demonstrated the regulatory role of Aurora kinase A in modulating the activation of MMP7 and MMP10 in HNSCC cell lines, suggesting potential role of Aurora kinase A in cell migration and invasion (Chen et al., 2015).

Apart from in vitro study, some earlier studies have observed the overexpression of Aurora A kinase in clinical samples of human head and neck cancer tissue biopsies. Reither et al. systematically measured the Aurora A kinase mRNA expression level and Aurora kinase A protein expression via qPCR and immunohistochemistry, respectively, in 66 HNSCC tissues, showing that 22 out of 34 (~65%) head and neck cancer samples expressed Aurora kinase A protein (Reiter et al., 2006). They have shown that the over expression or the up-regulation of the AURKA mRNA was frequently observed in HNSCC cases and was strongly correlated with tumour stage, distant metastasis, poor prognosis and shortened disease free survival (Reiter et al., 2006). Another study has assessed the Aurora kinase protein expression comparing between healthy oral tissue and the HNSCC tissue, in which most of the of HNSCC tissue samples - approximately 85% (53/63) - showed high Aurora kinase A expression with 65% (41/63) strong and 19% (12/63) moderate expression, whereas the healthy normal tissue exhibited significantly low to minimal nuclear staining for
Aurora kinase A in immunohistochemistry (Mazumdar et al., 2009). Another study has also shown overexpression of Aurora kinase A in laryngeal cancer human tissue samples, approximately 64% (16 out of 25) (Zhang et al., 2012). A recent study on a small number of HNSCC tissue samples also suggested overexpression of Aurora kinase A in 50% (7/14) of cases compared to adjacent non-tumour healthy tissue and this overexpression was correlated with aggressive disease phenotype (patients with high TNM stages - TNM stages III/IV) and poor prognosis, while patients with low Aurora kinase A expression showed better 5 year survival (Chien et al., 2014).

However, few studies have investigated the relationship between the overexpression of Aurora kinase B and HNSCC. One study on 40 clinical samples from HNSCC patients has demonstrated that Aurora kinase B expression level was significantly higher (16.5 ± 8.5%) in HNSCC samples compared to non-cancerous healthy epithelial tissue (4.4 ± 1.9%) (p < 0.01) via immunohistochemical analysis (Qi et al., 2007). The study also suggested the correlation between Aurora kinase B overexpression and proliferative marker, Ki-67 expression, indicating that the Aurora kinase B overexpression is related with cellular proliferation in HNSCC (Qi et al., 2007). However, more studies are required to understand the true role of Aurora kinase B in the genesis of HNSCC.

The above studies have demonstrated the overexpression of Aurora kinase A in HNSCC cell lines and on clinical samples, suggesting Aurora kinase A as a potential prognostic factor and a promising therapeutic target for HNSCC.

1.4.4. Aurora kinase inhibitors:

Since Aurora kinases, especially Aurora A and Aurora B, have been shown to be mechanistically involved in cancer progression, they were considered as potential targets for anticancer strategies. Based on this principle, a number of small molecule Aurora kinase inhibitors (AKIs) had been developed. The innovations of AKIs were driven by the application of diverse experimental approaches, including: structure based drug design; structure based virtual screening and rational design, followed by combinational expansion (Howard et al., 2009, Mortlock et al., 2005, Cancilla et al.,
2008, Oslob et al., 2008). Approximately, 30 AKIs have been developed, the majority of which are in various stages of preclinical and clinical trials. Initial outcomes have not been promising.

The core of the small molecule inhibitors, AKIs, is designed to bind to the hinge region of the Aurora kinase via hydrogen bonds and prevent access of ATP to the catalytic domain (Zhao et al., 2008). The remaining portions of the AKIs are thought to interact with different active sites via various types of non-covalent bonds. Since the hinge region of the Aurora kinase is practically similar for both Aurora A and Aurora B, most of the available AKIs can target both kinases and are termed Pan-Aurora inhibitor, such as VX-680 (Aliagas-Martin et al., 2009, Zhao et al., 2008). Recent efforts have been made to develop selective Aurora kinase inhibitors, such as Alisertib and Barasertib, which selectively target Aurora A kinase and Aurora B kinase, respectively.

Among Aurora kinase inhibitors, Alisertib (MLN8237) has been broadly characterized investigating different cancers both \textit{in vitro} and in preclinical models \textit{in vivo}. A comprehensive study has demonstrated that Alisertib effectively inhibited cellular proliferation in a range of human cancer cell lines, including lung, prostate, ovarian, colon, breast, pancreatic and lymphoma cells with an IC\textsubscript{50} ranging from 15 nmol/L to 469 nmol/L (viz: all less than 1umol/L) Lymphoma cells showed the greatest sensitivity (IC\textsubscript{50} value 15 to 86 nmol/L) to Alisertib compared to cell lines from solid tumours (Manfredi et al., 2011). Moreover, the same study showed anti-tumour activity of Alisertib in xenograft models of cancers of colon, lung, prostate, breast and lymphoma, with dose-dependent tumour growth inhibition ranging from 43.3\% to 94.7\%. Greater than 75\% inhibition was seen at 30mg/kg (the maximum tolerated dose) in all models (Manfredi et al., 2011). Further, Alisertib was shown to reduce viability of acute meyoloblastic leukemia (AML) cell lines and primary AML cells \textit{in vitro} (Kelly et al., 2012). A broad study on nine different human oral squamous cell carcinoma cell lines (Ca9-22, HSC2, HSC3, HSC4, SCC111, SCC66, SCC9, SCC25 and SAS) tested the efficacy of Alisertib, showing that the viability of these cell lines was reduced by more than 50\% with the doses between 50nM to 100nM: approximately 40\% reduction in tumour size was reported \textit{in vivo} with a treatment dose of 20mg/kg (Tanaka et al., 2013). A recent study has also
demonstrated the antitumour activity of Alisertib in a human tongue squamous cell carcinoma cell line (HSC-3), showing that Alisertib effectively inhibited the growth of HSC-3 cells at a concentration of ~ 0.60uM (IC$_{50}$ value of 0.60 uM/ 600nM) via apoptosis (in vitro) and significant tumour growth inhibition in animal xenografts (Qi and Zhang, 2015). A phase one clinical trial of Alisertib on HNSCC reported the stability of the disease after more than six cycles of treatment in three out of six patients, and the drug is claimed to have been well tolerated (Dees et al., 2012). A recent large phase 2 clinical trial of Alisertib on 5 different types of cancers that included a cohort with HNSCC reported four out of forty-five (9%) patients showing objective responses (Melichar et al., 2015). Approximately 2.7 months of progression-free survival after the recommended trial dose (50mg twice daily for 7 days with 14-day treatment free period, in 21 cycles for 2 years). Frequent drug-related adverse events were noticed in the majority of patients: these include neutropenia, leukopenia and anaemia. However, the HPV status of the HNSCC patients included in this trial was unknown.

Given the broad spectrum of activity of Alisertib in preclinical models, it has been moved forward for further evaluations in the Phase I and Phase II clinical trials for different types of cancers. Since Alisertib is a well-established anti-cancer agent and its efficacy has been already demonstrated in treating cancers, including head and neck cancer, this paves the pathway for the potential use of Alisertib as a therapeutic strategy in HPV-positive HNSCC treatment.

1.5.1. Significance of the project:

Cancer is a major health problem. One in eight deaths occur due to cancer worldwide. Cancer is the leading cause of death in developed countries and 2$^{nd}$ leading cause of death in developing countries (following heart disease) (de Martel et al., 2012). Throughout the world, malignant neoplasms of the mouth and pharynx rate as the 5$^{th}$ most common cancer in men and seventh in women (overall sixth), although there are marked geographical variations. The incidence of HNSCC is rising worldwide and
continues to be the most prevalent cancer related to the consumption of tobacco, alcohol and areca nut. According to GLOBOCAN 2012, deaths from HNSCCs were approximately 324,794 worldwide (7/100,000 in men and 2/100,000 in women). While the incidence of HNSCC is highest in South and South-East Asia due to the use of traditional carcinogenic products, there is now evidence of a significant association with HPV infections. Much variation has been observed in the prevalence of HPV in HNSCC in this region. Although HPV-associated HNSCC is well documented in the western world, studies on South Asian populations are few and inconsistent. However, the reported prevalence of oropharyngeal carcinomas attributable to HPV varies strikingly by geographical region, with the highest rates in North America and Japan (over 50%) and Europe (approximately 40%), according to the most recent data. However, these figures may be low. The national population-based database maintained by the Danish Head and Neck Cancer Group (DAHANCA), reported a striking 12-fold increase in oropharyngeal cancer between 1977 and 2012, with HPV-positive disease increasing from 37% to 74% of cases. This mirrors what has been seen elsewhere in Europe, in the United States and in Australia. This increasing incidence prompts questions about management approaches to this evolving epidemic. HPV-positive HNSCC has better prognosis compared to HPV-negative HNSCC, with chemo-radiotherapy, and treatment de-intensification is now widely practiced. Such patients are relatively young, however, so side effects in later life are probable. A new effective treatment approach is clearly required.

1. **6.1. Research Hypotheses:**

1. The prevalence of HPV in HNSCC in Bangladesh is the same as the prevalence of the total South Asian populations.
2. HNSCC relies on HPVE7 for their HPV-associated cancer progression.
3. The loss of HPVE7 will lead to significant loss of HNSCC cell viability.
4. HNSCC are dependent on the expression of Aurora Kinases for their malignant transformation.
5. HNSCC are sensitized to the loss of Aurora Kinase activity by the level of HPVE7.
1.6.2. Research Aims:

While we know that the overall worldwide prevalence of HPV in HNSCC is ~35 %, and that this group of patients has a comparatively favourable treatment outcome, this prevalence may not be true in South Asia, where tobacco dominates as a risk factor. Few published studies on HPV-associated HNSCC have appeared from this region. A comprehensive search of the literature is required to ascertain the current state of understanding. Therefore, our first aim is:

- To determine the prevalence of HPV in HNSCC in Bangladesh. From this we might infer the situation in other South Asian countries.

In cervical cancer it is well known that the malignant cells are oncogene addicted – i.e. the cells are completely reliant on the ongoing expression of E6 and E7 for their survival. This might be the same for HPV-positive HNSCC. If so, this type of cancer would be a target for siRNA therapy: this powerful gene-silencing tool could be targeted to the E6 and E7 genes, which are of viral origin.

Thus the 2nd aim –

- To investigate the role of the HPVE7 gene in driving HNSCC and to examine the efficacy of siRNA in vitro and in vivo to downregulate expression of this oncogene, as a potential therapeutic agent in HPV-positive HNSCC.

Since Aurora kinases play vital roles in the co-ordination of cell division and are overexpressed in a variety of cancers, it makes them attractive targets for anticancer therapy. The Aurora kinase A inhibitor, Alisertib (MLN8237) is a novel anticancer drug, which is in phase 2 and phase 3 clinical trials (Melichar et al., 2015). Our previous study on cervical cancer cell lines suggested a potential link between the Aurora kinase A and the expression of the HPVE7 oncogene, where the loss of aurora A resulted in selective cell death in HPV-positive cells (with high E7 expression) while normal cells remained unaffected (Appendix 2). Based on this, we believe that E7 is involved in making Alisertib more effective in its action as the high E7
expression make cells more sensitive to Alisertib. The third aim of this thesis is, therefore:

- To investigate the role of Aurora kinase A in the growth and survival of HPV-associated HNSCC and assess the therapeutic implication of Alisertib in this type of cancer treatment.

References:


CHAPTER 2

Materials and Methods
2.1 Cell culture protocols

2.1.1. Cell lines:

Numerous oral and oro-pharyngeal squamous cell carcinoma cell lines are preserved in cell banks in laboratories around the world. While there are approximately 300 HNSCC cell lines from different sites of the oral and oropharyngeal cavities,\(^1\), HPV positive oral and/or oropharyngeal cancer cell lines are very few. Four established HPV16-positive HNSCC cell lines, one HPV-negative HNSCC cell line and an HPV-16-positive cervical cancer cell line have been used (Table 2.1).

Table 2.1: Information about the HNSCC cell lines and a cervical cancer cell line included in the project.

<table>
<thead>
<tr>
<th>Name of cell line</th>
<th>Origin</th>
<th>Anatomical site</th>
<th>HPV status (type)</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDSCC 2</td>
<td>A squamous cell carcinoma of the hypopharynx obtained from a 58 year-old man.</td>
<td>Hypo-pharynx</td>
<td>HPV-positive (type 16)</td>
<td>A gift from Prof. Hoffmann and Dr. Silke Schulz, University of Ulm, Germany</td>
</tr>
<tr>
<td>UMSCC 104</td>
<td>A moderately to poorly differentiated recurrent squamous cell carcinoma of floor of the mouth (fom) from a 56 year-old man.</td>
<td>Oral cavity (floor of the mouth)</td>
<td>HPV-positive (type 16)</td>
<td>Kindly provided by Prof. T. Carey, University of Michigan, USA.</td>
</tr>
<tr>
<td>UPCI:SCC90</td>
<td>A poorly differentiate squamous cell carcinoma of the base of the tongue obtained from a 46 year-old man.</td>
<td>Oro-pharynx (base of the tongue)</td>
<td>HPV-positive (type 16)</td>
<td>A gift from Prof. Paul Lambert, University of Wisconsin, Madison, USA and from Prof. Susanne M Golin, University of Pittsburgh, USA</td>
</tr>
<tr>
<td>Lines</td>
<td>Description</td>
<td></td>
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<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>93VU147T</td>
<td>A moderately differentiated squamous cell carcinoma of the floor of the mouth (fom) obtained from a 58 year-old man.</td>
<td>Oral cavity (floor of the mouth)</td>
<td>HPV-positive (type 16)</td>
<td>A gift from Prof. Paul Lambert, University of Wisconsin, Madison, USA and from VU university, Netherlands</td>
</tr>
<tr>
<td>UMSCC1</td>
<td>A moderately to poorly differentiated recurrent squamous cell carcinoma of the floor of the mouth (fom) obtained from a 56 year-old man.</td>
<td>Oral cavity (floor of the mouth)</td>
<td>HPV-negative</td>
<td>A gift from Prof. Paul Lambert, University of Wisconsin, Madison, USA.</td>
</tr>
<tr>
<td>CaSki</td>
<td>An epidermoid carcinoma of the cervix obtained from a 40 year old female.</td>
<td>Cervix</td>
<td>HPV-positive (type 16)</td>
<td>American type culture collection (ATCC)</td>
</tr>
</tbody>
</table>

### 2.1.2. Cell Culture:

All cells were maintained in complete DMEM (Dulbecco’s Modified Eagle’s Medium: Gibco-Invitrogen, Mount Waverly, Australia) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Gibco-Invitrogen) and 1% of antibiotic/glutamine preparation (100u/ml penicillin G, 100unit/ml streptomycin sulphate, and 2.9mg/ml of L-glutamine: Gibco-Invitrogen). However, for UMSCC104 cells 1% MEM (Gibco-Invitrogen) was added. For the UPCI: SCC90, UMSCC1 and 93VU147T cell lines; 0.4µg/ml of hydrocortisone (Invitrogen,) was added.
Cells were cultured in T75 flasks, were trypsinised by first washing with 10 ml of sterile 1XPBS (Appendix 2), adding 2 ml of 0.1% Trypsin + Ethylene diamine tetra-acetic acid (EDTA) (Gibco-Invitrogen) and incubating for 8 minutes at 37°C and 5% CO₂ / 95% air for the detachment of cells. Later 8ml of fresh culture medium was added to inactivate the trypsin. The UDSCC2, 93VU147T, UMSCC1 and UPCISSC90 cells were split 1 in 5, UMSCC104 cells 1 in 3 and the CaSki cells 1 in 10. Prior to subculture, 70-80% cellular confluency was ensured. Cells were routinely examined for mycoplasma using polymerase chain reaction (PCR).

2.1.3. Storage of Cells:

Cells were stored in liquid nitrogen for further use. Before freezing down, the cells were detached by trypsinisation for 8 minutes and pelleted by centrifuging at 400 X g for 5 minutes and further washed twice with 1xPBS and centrifuged. Pellets were then re-suspended in freezing media [93%v/v FBS, 7% dimethylsulfoxide (DMSO)] and transferred into cryo-vial (Greiner Bio-one, USA). Vials were cooled at 1 °C per minute in -80 °C freezer using a Nalgene freezing container and transferred into liquid nitrogen after 2 days.

2.1.4. Defrosting and thawing of cells:

Aliquots of cellsin cryo-vials were taken from liquid nitrogen and immediately thawed in a pre-heated 37 °C water bath. After 2 minutes cellswere transferred into a 15ml tube and 10 ml of fresh media gently added, mixed and centrifuged at 400 g for 5 minutes at 4°C to get rid of DMSO present in the freezing media. After removing the supernatant 2 ml of fresh media was added to the cell pellet and the cell suspension transferred into a T25 flask containing 3 ml of pre-warmed medium and incubated in the tissue culture incubator at 37°C and 5% CO₂ / 95% air.
2.1.5. Tissue culturing solutions and reagents

10X PBS - NaCl 80g, KCl 2g, Na₂HPO₄ 14.4g, KH₂PO₄ 2.4g per 2 L (used as 1x PBS)
Trypsin-EDTA - 0.25% Trypsin, 1mM EDTA and 1X PBS (without Ca²⁺ and Mg²⁺).

2.2 RNAi protocols

2.2.1. Small interfering RNAs (siRNAs):

The siRNAs, siRNA targeting HPV16E6/E7 (sequence 10) termed si-E6/E7, the control siRNA targeting green fluorescent protein (GFP) named siGFP, and another control siRNA targeting polo-like kinase 1 (PLK1) termed as siPLK1, were purchased from IDT technology (IDT, Boronia, Vic, Australia). The sequence of the oligonucleotide templates are given below:

HPV16Si-E6/E7 (si-E6/E7) (sequence 10):
Sense: 5’ - GCAACAGUUACUGCGACGUUU – 3’
Antisense: 5’- ACGUCGCAGUAACUGUUGCUU – 3’

siGFP sequence (control siRNA seq.):
Sense: 5’- GCACGACUUCUUCUCAAGUCCUU – 3’
Antisense: 5’- GGACUUGAAGAAGUCGUGCUU – 3’

siPLK1 sequence (control siRNA seq.):
Sense: 5’- CAACACGCCUCCUCUAAU - 3’
Antisense: 5’- UAGAGGAUGAGGCGUUGUU – 3’
2.2.2. siRNA Transfections (in vitro):

2.2.2.1 Fast forward transfection (6 well plate):

Cells were plated out at a density of 150,000 cells/well for CaSki, 250,000 cells/well for all the HNSCC cell lines and incubated for at least 24 hours prior to transfection to allow the cells to grow up to 40-50% confluence in a six well plate. The plate was designed in the format of untreated cells, mock control (lipid/oligofectamine only), non-specific/internal control (siGFP), si-E6/E7 treated cells and a positive control (siPLK1).

For each desired siRNA treated sample, Oligofectamine (Gibco-Invitrogen) was used as per manufacturer’s protocol. Briefly, oligofectamine was added with OptiMEM and si-E6/E7 was added at a final concentration of 40nM to that mixture and incubated at room temperature (RT) for 20 minutes to allow the siRNA to merge in to the lipid vehicle (oligofectamine). The wells containing adherent cells were washed and the mixture complex of siRNA was added to the desired siRNA treated wells. For non-specific control siRNA (siGFP) was also used at a final concentration of 40nM. Further, for positive control siRNA (siPLK1) was used at final concentration of 40nM. After transfecting the cancer cells with respective siRNAs, they were incubated in the tissue culture incubator (37°C and 5% CO₂ / 95% air) for 6 hours. Following incubation, transfection complexes were removed and replaced with fresh complete DMEM media (1ml in each well) and incubated again up to 48 hours.

2.2.2.2 Reverse transfection:

For reverse transfection, first the cells were seeded in T75 flasks and allowed to become 80-90% confluent. Then they were washed with 1XPBS and trypsinised by adding 0.1% Trypsin + Ethylene diamine tetra-acetic acid (EDTA) (Gibco-Invitrogen) and incubated for 8 minutes at in the tissue culture incubator. Later, fresh culture media was added to inactivate the trypsin. The cell suspension was then centrifuged at 400 g for 5 minutes to acquire cell pellets. The supernatant was removed and replaced with fresh complete DMEM media. Cells were counted using a haemocytometer. In the meantime, the OptiMEM, oligofectamine and the siRNA complex mixture were prepared in a similar way (fast forward transfection) and
incubated for 20 minutes. The transfection complex is added to desired wells in the 6 well plate first, before adding the cells. Then the cell suspension at 300,000 cells/well for CaSki and 500,000 cells/well for all HNSCC cells were directly added to the transfection complex in 6 well plate and incubated for 6 hours. Following incubation, transfection complexes were removed and replaced with fresh complete DMEM media and incubated again up to 48 hours before the gene expression analysis was performed.

2.3 Gene expression protocols

2.3.1. RNA isolation:
For RNA isolation, TRIzol (Gibco-Invitrogen) reagent was used. Prior to TRIzol treatment, the cells from each well of six well plates were washed with 1XPBS. TRIzol (Gibco-Invitrogen) was added at 500µl per well in 6 well plates and mixed by rocking the plate for 5 minutes. The mixture was transferred to a micro-centrifuge tube and 100µl of chloroform was added. The tube was incubated for 15 minute at RT and vigorously vortexed prior to spinning down samples at 12000g at 4°C for 15 minutes. Only the top aqueous layer (containing RNA) was collected from each sample tube without touching the middle DNA and the bottom pink protein layers and transferred to a sterile microcentrifuge tube. Equal volume of 100% isopropanol was added to the collected aqueous layer to precipitate the RNA. Samples were then incubated for a further 30 minutes at -30°C with intermittent vortexing. RNA was pelleted from the samples by centrifuging at 12000g at 4°C for 15 minutes and the acquired RNA pellets were re-suspended in 1ml of 75% ethanol solution to remove isopropanol. Further incubation of the samples was carried out at -80°C overnight. The next morning, RNA was re-pelleted again by vortexing and then centrifuging at 12000xg at 4°C for 15 minutes. After the supernatant was carefully removed from each tube, the pellets were allowed to dry at RT for 10 -15 minutes to evaporate the remaining ethanol. Once the RNA pellets were air-dried 50µL of (diethylpyrocarbonate) DEPC (Gibco-Invitrogen) water added to each sample in the microcentrifuge tube and incubated at 60°C for 10 minutes. RNA quantification was
performed using the Nanodrop 1000 (Thermo-Fischer) to assess the RNA purity at 260 and 280 nm wavelength absorbance ratio.

2.3.4. DNase Treatment:

The purity of RNA samples showed readings below 1.8 at 260/280 absorbance in the Nanodrop 1000 machine. DNase treatment was therefore carried out for further purification. The RQ1-RNase free DNase (Promega, Madison, WI) and 10xRQ1 DNase buffer (Promega) were added to each sample of RNA suspension and incubated for one hour at 37°C. An equal volume of cold phenol/chloroform solution was added to the reaction and incubated at room temperature for 10 minutes. The aqueous phase was separated by centrifugation at 13200g at 4°C for 15 minutes prior to transfer to a fresh and sterile micro-centrifuge tube. An equal volume of chloroform was added and incubated at RT for a further 5 minutes. Once again, the mixtures were centrifuged at 13200g at 4°C for 15 minutes and transferred to another new pre-chilled tube. 2.5 times and 0.1 times the sample volume of 100% ethanol and 3.2M sodium acetate at pH 5.2, respectively, were added to pre-chilled tube containing samples. Further incubation was carried out at -20°C for 30 minutes. RNAs were pelleted by spinning at 13200g at 4°C for 20 minutes and re-suspended in 75% ethanol solution followed by 30 minutes incubation at -20°C. Once again, the RNAs were pelleted by centrifuging at 13200g at 4°C for 20 minutes and allowing the pellet to air-dry for 10-15 minutes. 50 µl of DEPC water was added and incubated at 60°C for 20 minutes. Finally, the purity of the RNA was checked using the Nano-drop 1000.

2.3.5. cDNA synthesis:

To synthesize the cDNA from the extracted RNA samples, Quantitech reverse transcription kit (Qiagen, Hilden, Germany) was used. Desired RNA samples and the
kit were thawed on ice prior to mixing the RNA template (~2µg or 2000ng/µl) to 2µl of genomic (gDNA) wipe out buffer (Qiagen, Hilden, Germany). The total volume of the reaction was prepared up to 14µl adding RNase free water in a micro-centrifuge tube and subsequently incubated at 42°C for 2 minutes. The further steps of the reverse transcription reactions were performed on ice. 1µl of Quantuscript reverse transcriptase, 4µl of RT buffer and 1µl of RT (reverse transcription) primer mix were added to previously prepared 14µl of template RNA mixture, vortexed and short spinned prior incubating at 42°C for 15 minutes. Further, the reactions were heat-inactivated at 92°C for 3 minutes and the cDNA concentration measured using the Nano-drop 1000 at 260 and 280 nm wavelength absorbance ratio.

### 2.3.6. Polymerase Chain Reaction (PCR):

For conducting PCR, all the reaction mixtures were prepared on ice. The reaction mix for PCR was prepared by the mixture of 2.5µl of 10x ThermoPol II (mg-free) reaction buffer (New England Biolab, Ipswich, MA), 0.5µl of 100mM MgSO4 (New England Biolab, Ipswich, MA), 0.5µl of 10mM dNTPs, 0.5µl of 10µM forward primer (beta-actin/E7) and 0.5µl reverse primer (beta-actin/E7) and 0.125µl of Taq DNA polymerase with final concentrations of 1X, 2.0mM, 200µM, 0.2 µM, 0.2µM and 1.25units/50µl of PCR reaction respectively. Less than 200 ng of desired cDNA template was added to the reaction mix and finally, the remaining volume of 25µl was made up by adding nuclease-free water in the PCR tubes. Once all components were mixed, the tubes were kept on ice and immediately transferred to the thermocycler (Kyratec, Mansfield, QLD, Australia), which was calibrated to 2 steps thermo-cyclic condition with the initial denaturation phase 95°C for 30 seconds, 40 cycles including denaturation stage 95°C for 30 sec, annealing stage 60°C and extension stage 68°C for 1 min. Final extension was at 68 °C for 5 minutes and infinite hold for 4°C for overnight.
2.3.7. Gel electrophoresis:

Once the PCR products were generated, they were analysed using agarose gel electrophoresis. 2% agarose gel was prepared in 1xTAE (Tris-acetate EDTA) (Biorad, Hercules, CA) buffer, placing a comb to create wells in the gel. Prior to gel setting, 0.2-0.5µl/ml of Ethidium Bromide (EtBr) (Invitrogen,) was added. The PCR product samples were mixed with loading dye at 5:1 ratio (Sample: loading dye) and loaded in the well chambers of the agarose gel, which was immerged into the 1xTAE buffer containing 0.2µl/ml of EtBr. A PCR marker of 100 -1000 bp DNA ladder (New England Biolab, Ipswich, MA) was loaded in a well for comparison. The power unit was set at 80 volts for 75 minutes. The DNA would migrate from negatively charged to positively charged electrodes. Once a run was completed the gel was de-stained in water for 5 minutes and bands were visualized under UV light using the Chemi-Doc (BioRad, Hercules, CA).

2.3.8. Quantitative Real Time Polymerase Chain Reaction:

To analyse the expression of the HPV16E7 genes in all the HPV-positive HNSCC cell lines and CaSki cells and to determine the E7 mRNA knockdown efficiency of siRNA, Rotor-Gene SYBR Green real time quantitative-PCR kit (Qiagen, Hilden, Germany) was used. Reactions from each sample was set up in 100µl rotor-gene strip tubes (Qiagen) with a mixture of 6.25 µl 2X Rotor-Gene SYBR Green RT-PCR master mix, forward and reverse primers (of both HPV16E7 gene and house keeping gene (β-actin) (Table 2.1)) to a final concentration of 1µM, 0.125 µl of Rotor – Gene RT mix, 100ng of template RNA and RNase free water to make each reaction up to 12.5µl. The samples were labelled and transferred to the Rotor-gene Q machine and analysed with the Rotor-gene software.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ – 3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV16 E7 forward</td>
<td>GAACCGGACAGAGCCCATTA</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>HPV16 E7 reverse</td>
<td>CGAATGTCTACGTGTGTGCTTTTGG</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Human β-actin forward</td>
<td>AGCCTCGCCTTTGCCGA</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Human β-actin forward</td>
<td>CTGGTGCTGCTGGGCG</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
</tbody>
</table>

### 2.4. Protein expression studies:

#### 2.4.1. Preparation of protein extraction:

Cell lysates from all treated and untreated cells were extracted by collecting the plated cells from the flask/well by washing with 1xPBS, trypsinising, adding fresh culture media to inactivate trypsin and the cell suspension was then transferred in to a 15ml tube. Cells were pelleted by centrifuging at 400g for 5 minutes at 4°C. Pellets were washed in 1xPBS and mixed vigorously and centrifuged once again at 400g for 5 minutes at 4°C. This step was repeated once, and the pellets re-suspended in 500µl of RIPA buffer (Pierce-Thermo Scientific, Waltham, MA) and 5µl of Halt Protease Inhibitor (Thermo Scientific, Waltham, MA). Samples were kept on ice for 20 minutes and centrifuged at 13000g for 5 minutes at 4°C to get rid of cell residue. The supernatant was carefully collected (containing protein) and the lysates were immediately transferred and stored at -30°C for further analysis.
To determine the protein concentration of the samples, Pierce BCA Protein assay kit (Thermo scientific, Waltham, MA) was used. Prior to detect the concentration of desired protein sample, a standard curve was generated by the nanodrop spectrometer 1000 using 5 different concentrations of BSA (bovine serum albumin) with working buffer (both supplied with the kit) according to the kit protocol. Once the standard curve was achieved, the concentration of each sample was tested and compared against the standard curve to acquire the actual concentration of protein in the desired sample.

2.4.2. Western blotting solutions

Solution Composition

- PBS (pH 7.4) - 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.47mM KH₂PO₄
- TBS (pH 7.4) - 50mM Tris, 150mM NaCl
- Running Buffer - 25mM Tris, 192mM glycine, 0.1% SDS
- Transfer Buffer - 25mM Tris, 192mM Glycine, 0.1% SDS, 20% methanol
- Stripping Buffer - 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris (pH6.7)
- Sample Buffer (5X) - 62.5mM Tris, 30% glycerol, 2% SDS, 0.04% bromophenol blue, 1% β-mercaptoethanol
- Blocking solution - 5% skim powder milk in 1x PBS containing 0.05% Tween 20 (PBST) or TBS containing 0.1% Tween 20 (TBST)

2.4.3. SDS polyacrylamide gel electrophoresis:

The proteins in sample were separated by tris-glycine polyacrylamide gel electrophoresis (PAGE). In resolving gel, the acrylamide percentage ranged from 10 to 15% (Appendix 2) depending upon the molecular weight of the protein of interest. The resolving gel was cast by mixing 7 ml of 37% polyacrylamide (BioRad), 3.9ml of deionized water (dH₂O), 3.8 ml of 1.5M of Tris-Cl (pH8.8), 0.150 ml of 10% Sodium dodecyl sulphate (SDS), 0.150 ml of 10% ammonium persulfate (APS) and 0.009 ml of tetramethylethylenediamine (TEMED) (BioRad, Hercules, CA). 5% stacking gel was prepared by mixing 1 ml of 37% polyacrylamide, 4.1ml of dH₂O, 0.75 ml of
1.5M of Tris-Cl (pH8.8), 0.060 ml of 10% Sodium dodecyl sulphate (SDS), 0.060 ml of 10% ammonium per-sulphate (APS) and 0.006 ml of TEMED and layered on the top of the resolving gel.

Once the resolving gel was prepared and the concentration of the protein was determined, the protein lysates from the samples were loaded into the wells of the stacking gel at a concentration of ~ 30µg/ well. Prior to loading, equal volume of 2x loading buffer (100mM Tris-Cl, pH 6.8, 4% SDS; 0.2% bromophenol blue; 20% glycerol; 10% 2-mercaptopethanol (2-ME)) and dithiothreitol (DTT) (0.1 volume of the sample) were added to the samples and boiled at 100°C for 5 minutes followed by a short spin. A molecular weight marker (Biorad, USA) was also loaded into a well. SDS-PAGE was performed using Mini-protein II Western blot apparatus (Biorad) for 20 minutes at 80 V in the SDS-PAGE buffer to allow the samples to leave from the stacking gel and enter into the resolving gel. The machine was then set at 160 V for 1 hour to allow the samples to run through the resolving gel until the dye front reach the bottom level.

2.4.4. Blot Transfer and Enhanced Chemiluminescence:

After the SDS-PAGE, the polyacrylamide gel containing separated proteins was soaked in transfer buffer (Appendix 2) for approximately 15 minutes to remove SDS-PAGE buffer. Prior to the preparation of the transfer cassette, all the components added in the cassette were briefly soaked in the transfer buffer. The transfer cassette was prepared in the following order: Black plastic of the cassette, a supporting pad, two Whatman’s filter papers, the polyacrylamide gel containing proteins, polyvinylidene difluoride (PVDF) membrane (Millipore, Australia) (pre-wetted with methanol for its activation and laid down on top of the gel), two Whatman’s papers, a supporting pad and the white plastic of the cassette. Wet transfer was performed using polyvinylidene difluoride (PVDF) membrane (Millipore, Australia) at 100V for 1 hour in 1X transfer buffer using a mini Transblot® electrophoretic transfer cell (BioRad). An ice pack was used in the apparatus while the transfer was performed to prevent overheating. Following the transfer, the PVDF membrane was blocked overnight at 4°C with 5% skimmed dried milk in Tris buffered saline with 0.05%
tween 20 (TBS-T). Membrane was washed 3 times for 5 minutes in TBS-T prior applying antibodies. The primary antibodies were used at 1:1000 ratio in 5% skimmed dried milk in TBS-T and incubated overnight at 4°C. The membrane was washed once again with the TBS-T solution 3 times for 5 minutes and HRP conjugated secondary antibody (anti-mouse or anti-rabbit antibody) applied at 1:1000 ratio in 5% skimmed milk in TBS-T, to the membrane and incubated for 1-2 hours at RT. Finally, the blot was washed with TBS-T three times for 5 minutes prior to enhanced chemiluminescence (ECL) detection.

ECL reagent was prepared by combining 4.5 ml of dH2O with 0.5 ml of 1.5M (Tris) pH 8.8 into each of the two 15ml tubes. In addition, in one tube 50µl of 250mM luminol and 22µl of 90mM coumaric acid was added- taking care to protect luminol from light. Hydrogen peroxide (3µl) was added to another tube. The mixture of two tubes was prepared and also poured on the membrane in a darkroom and incubated for 2 minutes. Cling film was used to wrap the membrane and imaging was acquired and analysed by using the Chemi-Doc scanner.

2.4.5. Antibodies used for Western blotting:

The antibodies used are listed below in Table 2.3.

**Table 2.3. List of antibodies.**

<table>
<thead>
<tr>
<th>Name of the antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PARP1 antibody (against PARP-1 protein)</td>
<td>Cat. No. #9542, Cell Signalling Technology, Danvers, MA, USA</td>
</tr>
<tr>
<td>Anti-Bcl-2 antibody</td>
<td>Cat no. #2872, Cell Signalling Technology, Danvers, MA, USA</td>
</tr>
<tr>
<td>Anti-Mcl-1 antibody</td>
<td>Cat no. #4572, Cell Signalling Technology, Danvers, MA, USA</td>
</tr>
<tr>
<td>Anti-Bim antibody</td>
<td>Cat no. 2819, Cell Signalling</td>
</tr>
</tbody>
</table>

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2.5. Viability assay protocols

2.5.1. Metabolic activity Assay, Cell Titre Glo assay:

Cellular metabolic activity (presence of active ATP) of the samples was assessed by luminescence based Cell Titer-glo viability assay (Promega, Madison, WI). Minimal light condition is required for this. Following the transfection of 10000 cells/well in a 96 well plate, plates were incubated at RT for 30 minutes prior to adding Cell-Titer-Glo reagent to each well, equal to the amount of media present (for 100ul of media, 100ul of cell titre glo reagent added). Plates were shaken gently in a shaker for two minutes in the dark room at RT to aid cell lysis. The plate was then covered with foil paper for 10 minutes to allow the luminescence to stabilize. Plates were then analysed by the BMG FLUOstar OPTIMA (Offenburg, Germany) plate reader machine to record the luminescence, measured by setting the photomultiplier (PMT) sensitivity at 135 with an integration time of 0.25-1 second per well in the plate reader.

2.5.2. Metabolic activity Assay, Real time Glo™ MT assay:

Cell viability was assessed in real time using a luminescence based Real –time Glo™ MT assay (Promega, Madison, WI), a non-lytic, homogeneous, bioluminescent cell viability assay. The Real –time Glo™ MT kit contains MT cell viability substrate and NanoLuc Enzyme. The enzyme produces a luminescence signal, which correlates with the number of viable cells. Following the transfection of 10000 cells/well in a 96 well plate with a total OptiMEM media volume of 100ul/well, the culture medium
was replaced by fresh complete DMEM after 6 hours and subsequently, the plate and the Real –time Glo™ MT reagent were incubated at 37°C for 30 minutes to equilibrate the temperature. The 2X Real –time Glo™ MT reagent was prepared from stock by diluting 2ul of MT cell viability substrate and 2ul of NanoLuc Enzyme in 996ul of cell culture media. Equal volume of 2X Real –time Glo™ MT reagent was added to the amount of culture media present in each well (for 100ul of media, 100ul of Real –time Glo™ MT reagent) to produce the final concentration of 1X Real –time Glo™ MT reagent. Following the addition of Real –time Glo™ MT reagent, the plate was incubated for 1 hour and the first reading was taken using a microplate reader with an integration time of 0.25-1 second per well, keeping the plate reader temperature at 37°C. The consecutive readings were taken every 12 hours.

2.5.3 Colony Forming Assay:

To observe the siRNA efficiency, the siRNA treated cells, positive control and also untreated cells from the six well plates were trypsinized, centrifuged and counted using a haemocytometer. About 150 cells from each of these samples were plated once again in a 6 well plate and in a 12 well plate with 2ml and 1ml of complete DMEM media in each well of 6 and 12 well plates, respectively, and incubated at 37°C and 5%CO₂. After 2 days, the DMEM in each well was replaced by conditioned media of the respected cell lines by filtering with 0.2um filter (PALL, port Washington, NY) and incubating for 2-3 weeks. After this time, the media were discarded and cells were fixed with formaldehyde, stained by 1% crystal violet, and proceeded for colony counting under the compound light microscope.

2.6. Annexin V apoptotic assay/ Flow cytometry:

Following the transfection, cells were trypsinised and transferred into a 15ml tube and pelleted by centrifuging at 400g for 5 minutes. Cell pellets were re-suspended into
fresh DMEM culture media and cells were and counted using haemocytometer and 300,000 to 500,000 cells containing cell suspension was collected from each sample and transferred to a fresh 10ml tube. Cells were washed with 1XPBS and centrifuged at 400g for 5 minutes, twice. Cells were then re-suspended in 500ul of binding buffer from the Annexin V apoptosis assay kit (BD bioscience, San Jose, CA) and transferred into flow cytometer tube. Five ul of annexin V (FITC) and 5 ul of propidium iodide (PI) from the kit were added to the binding buffer containing cells for each sample, except the unstained control. These were incubated in a dark room at RT for 15 minutes and then the sample was analysed using BD LSR FORTESSA™ cell analyser (a flow cytometry machine) (BD bioscience, San Jose, CA) to detect the fluorescent-labeled cells. The excitation wavelength was 640 for PE channel (Red LASER) and the emission was 488 for FITC channel (Blue LASER).

### 2.7. Study of Aurora Kinase Inhibitors on HNSCC Cells:

#### 2.7.1. Chemotherapeutic drugs:

MLN8237 (Alisertib) was purchased from Jomar Life Research (Caribbean park, VIC, Australia) dissolved in DMSO to make an aliquot of 25mM in a 1.5 ml microcentrifuge tube. The other aurora kinase inhibitors were kindly provided by Prof. Brian Gabrelli’s lab, University of Queensland (Australia). The list of Aurora kinase inhibitors used in this project are given in the following Table 2.4.

**Table 2.4. Aurora kinase inhibitors.**

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Inhibiting action against</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLN8237</td>
<td>Aurora kinase A</td>
<td>Jomar Life research</td>
</tr>
<tr>
<td>AMG900</td>
<td>Aurora kinase A and B</td>
<td>Prof. Brian Gabrelli</td>
</tr>
<tr>
<td>CCT244941</td>
<td>Aurora kinase A</td>
<td>Prof. Brian Gabrelli</td>
</tr>
<tr>
<td>ZM447439</td>
<td>Aurora kinase B</td>
<td>Prof. Brian Gabrelli</td>
</tr>
</tbody>
</table>
2.7.2. Drug treatment:

Cells were plated at 10000 cells/well with a volume of 100ul/well of complete DMEM media in a 96 well plate for 24 hours. After 24 hours, the media were removed and replaced with drug and fresh complete DMEM. The Aurora kinase inhibitors were added at a serial dilution from 40μM to 40 nM of dosage in each desired well with a final volume of 100ul/well. The DMSO was added in a well as vehicle only control in appropriate wells. Following the drug addition, cells were incubated for 72 hours. After the incubation, cells were analysed for viability.

2.8. Protocol for HPV DNA detection in HNSCC sample

2.8.1. Head and neck cancer tissue sample collection:

Archival head and neck cancer tissue blocks of 196 patients were kindly provided by Professor Aminul Islam Khan from Al Khan Laboratory, Dhanmondi, and Department of Pathology, Dhaka Medical College Hospital, Dhaka, Bangladesh, following the approval of Ethics Committees of Griffith University, Australia and Dhaka Medical College Hospital, Dhaka, Bangladesh. The ethics related documents are presented in appendix 3.

2.8.2. DNA isolation and testing of sample integrity:

The FFPE blocks were sectioned at 5um whilst maintaining utmost precautions to avoid inter-block contamination of DNA. This was achieved by pre-chilling and moistening each block in a separate ice container before sectioning, thorough cleaning of the microtome, single use of brush and forceps, changing gloves between each block, changing the water bath for each block and using a new blade for each block. Genomic DNA was extracted from a 10um thick section; using Gene Read FFPE kit (Qiagen, Germany). A section from a blank paraffin block was cut and processed along with the sections from the cases to check for any contamination. DNA concentrations were determined with a NanoDrop 1000 spectrophotometer (Thermo
Scientific). DNA integrity was assessed for 120 bp fragments of the β-actin (Table 2.3) housekeeping gene by PCR using the GoTaq Green PCR kit (Promega, Madison, WI) with the following PCR condition (Table 2.5).

### Table 2.5. Standard PCR condition for HPV16 DNA amplification using β-actin primers.

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95°C (denaturation)</td>
<td></td>
<td>30 seconds</td>
</tr>
<tr>
<td>55°C (annealing)</td>
<td></td>
<td>30 seconds</td>
</tr>
<tr>
<td>72°C (extension)</td>
<td></td>
<td>30 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

Nuclease free water was used as negative control, while genomic DNA from the HPV16 positive head and neck cancer cell line, UDSCC-2 (kindly provided by Dr. Schuwarz and Prof. Thomas Hoffman, University of Ulm, Germany) was used as a positive control in the first PCR run. From the 2nd PCR run onwards, samples that had shown positivity in the first PCR run were used as positive controls.

PCR product was analysed by gel electrophoresis.
2.8.3. Nested PCR:

Nested PCR, consisting of two sets of degenerative/consensus primer pairs, MY09/11 and GP5+/GP6+ (Sigma-Aldrich, St. Louis, MO, USA) was used (Table 2.6). A gradient PCR was performed to optimise the annealing temperature for each primer set. The HPV \textit{L1} gene was amplified using primers MY09/11 in the first round, followed by GP5+/GP6+ in the second round. The PCR reaction mix contained forward and reversed primers (0.5 µM of each), 1\times PCR buffer (containing 1.5mM MgCl\textsubscript{2}) (Phusion High-Fidelity 5\times PCR Buffer, New England Biolabs, MA, USA), 200 µM of dNTPs (10mM dNTP Mix, New England Biolabs, MA, USA), 1.0 unit of Phusion DNA polymerase (Taq polymerase 1unit/50 µl, New England Biolabs, MA, USA) and nuclease-free water, up to a final volume of 20 µl. Positive and negative reaction controls were included in each PCR run. DNA amplification was carried out in an automated thermal cycler (Kyratec, Mansfield, QLD, Australia). Reactions were brought to 98°C for 30 sec (initial denaturation), followed by forty cycles consisting of a denaturing step for 10 sec at 98°C, an annealing step for 30 sec at 55.5°C (MY09/11 in first round) or 55°C (GP5+/GP6+ second round), and an extension step for 20 sec at 72°C. A final extension step at 72°C was carried out for 5 mins. A total of 2 µl of the first-round PCR product was used in the second round of amplifications.

The PCR products of the samples from both rounds were electrophoresed in 2% agarose gel prepared with 1x TAE (Tris-acetate-EDTA) buffer (DNA Agar, Marine Bio Products Inc., Quincy, MA, USA), stained with 0.5 g/mol of ethidium bromide (Merck, KGaA, Darmstadt, Germany) and visualized under ultraviolet light using the Chemi-Doc machine (BioRad, USA). The size of the amplified product was determined by comparing with a base-pair ladder size marker for HPV-L1, (Quick Load, 100bp DNA Ladder, New England Biolab, MA, USA). Any sample that showed a positive band in the gel for both first (band size 450bp) and second (band size 150bp) round of PCR was taken as an HPV-positive sample and purified for sequencing.
Table 2.6. Nested PCR primers and sequences.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ – 3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP5+</td>
<td>TTTGTTACTGTGGTAGATACTAC</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>GP6+</td>
<td>GAAAAATAAAACTGTAAATCATATTC</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>MY09</td>
<td>CGTCCMARRGGAWACTGATC</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>MY11</td>
<td>GCMCAGGGWCATAAYAATGG</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Humanβ-actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>forward</td>
<td>CAGCAAGCAGGAGTATGACG</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Humanβ-actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reverse</td>
<td>TGGTTTCTGCGCAAGTTAGG</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
</tbody>
</table>

2.9. Cell analysis protocols

2.9.1. Haematoxylin and eosin staining (H&E staining):

The head and neck paraffin tissue block was sectioned at 5um using a microtome (Leica biosystem, Wetzlar, Germany) and fixed on charged microscopic slides (Superfrost™ Plus, Thermofisher Scientific, Waltham, MA) and incubated for 48 hours at 37°C. Prior to H&E staining, the protocol was optimized for the tissue sample. After the optimisation, the slide containing tissue was dewaxed in Xylene (2X3 mins), and rehydrated with 100%, 90% and 70% ethanol respectively for 3
minutes each. Slide was rinsed in running tap water and stained with Mayers haematoxylin for 4 minutes, differentiated with acid-alcohol solution (1% HCl in 70% ethanol) for 10-15 seconds, and further washed in running tap water for 1 minute. The slide was then dipped in Scott’s bluing solution for 1 minute and washed in running tap water for 2 minutes. Afterwards, the section was stained with alcoholic eosin solution for 5 minutes, washed in running tap water for 1 minute, followed by dehydration with 70%, 90% and 100% ethanol solution respectively for 3 minutes each, and cleared with Xylene (2X5 mins). The slide containing tissue section was finally mounted with mounting media and cover slip.

2.9.2. Immunohistochemistry:

Immunohistochemistry (IHC) for p16, p53, pRb and Cyclin D1 was performed in the laboratories of MHIQ and/or Gold Coast University Hospital, using DAKO histology kits (DAKO, Agilent, Santa Clara, CA), ~800 slides manually and 200 in an Intellipath (Biocare Medical, Concord, CA) autostainer. Briefly, from each FFPE block, a 4um thick section was cut and affixed to Menzel-Glazer super-frost plus slides (Thermo Fisher Scientific) and air-dried at 37°C for 48 hours. Slides were preheated at 60°C, followed by de-waxing and re-hydration using Xylene, ethanol and water. Antigen retrieval was performed with the DAKO EnVision Kit (DAKO, Agilent, Santa Clara, CA) followed by TBS wash. Staining with primary antibodies was according to the protocol recommended by DAKO, using an Intellipath autostainer, where staining steps and incubation times were programmed according to the DAKO EnVision FLEX Mini Kit protocol. A similar procedure was followed for manual staining. The primary antibodies mouse anti-p16INK4a (#2D9A12; DAKO, Agilent, Santa Clara, CA), mouse anti-human p53 (#DO-7; DAKO, Agilent, Santa Clara, CA), rabbit anti-human Cyclin D1 (#EP12, DAKO, Agilent, Santa Clara, CA), and rabbit anti-human pRB (#9308; Cell Signaling, Danvers, MA), were optimized for antibody concentration and incubation time according to their respective company protocols. Positive and negative IHC controls were used in every run. A tonsillar SCC with high p16 expression was taken as positive control for p16 IHC; for p53 a positive colon cancer; for Cyclin D1 and pRb, a known positive tonsillar carcinoma.
2.10. Liposome protocols

2.10.1. Lipids used for Liposome:

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids, Alabama, USA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti Polar Lipids), cholesterol (Avanti Polar Lipids) and succinyl (methoxy-polyethylene glycol) 2000 Ceramide (PEG2000C16CER) were stored at -20°C.

2.10.2. Liposome preparation:

Prior to Liposome preparation, the tertiary butanol was warmed up at 37°C in a water bath for at least 2 hours to allow any crystalline structure to dissolve. In the meantime, the following amount of lipids per dose (Table 2.7) were measured out in a sterile serum vial/ round-bottomed flask with an overall molecular ratio of lipids of 50:35:5:10. Lipids were then dissolved in tertiary butanol in separate serum vials and the required volume of butanol was calculated by multiplying actual amount of lipid (mg) weighed out by 0.125mL of butanol, and divided by mg of lipid per dose. The serum vial containing lipid mixed with butanol was placed in a 37°C incubator for at least 2 hours (with gentle swirl every 10 mins) and the lipid dissolved fully in butanol. Further, the sterile sucrose solution (using a sterile filter) containing si-E6/E7 or siGFP was prepared by adding requisite amount of sucrose to dH2O to get a final concentration of 55.5mg/ml. For preparing one dose of siRNA in liposome for injecting one mouse, 40ug of si-E6/E7 or 40ug of siGFP was added to 500 ul of sterile sucrose solution, mixed well and left at room temperature for 10 minutes. Afterwards, the sucrose solution containing siRNA/siGFP mixture (500 ml) was added to 500 ml of 4 lipids/butanol mixture (125ml of each lipid/butanol solution) to make lipoplex and mixed well with gentle shaking to bring up a total of 1ml of solution for one dose. This lipoplex mixture was then snap-frozen by swirling the serum vial on dry ice/ethanol to form a thin frozen layer of this mixture. Before the samples were transferred to a lyophiliser (ALPHA 1–2 LDplus, Martin Christ, Germany), the serum vials, containing samples, were uncapped, covered with parafilm with holes pierced
into it to allow lyophilisation. Samples were loaded onto the lyophiliser (ALPHA 1–2 LDplus, Martin Christ, Germany) and maintained at -110°C with 0.1mbar pressure for 48 hours. A nitrogen and phosphate ratio of 4:1 was used for all formulations. After lyophilisation, the dry si-E6/E7 containing lipid cake inside the serum vial was capped and stored at -20°C to protect from moisture, for up to 4-6 weeks.

Table 2.7. Required amount of lipids for per dose.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>1 Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTAP</td>
<td>0.352 mg</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.136 mg</td>
</tr>
<tr>
<td>DOPE</td>
<td>0.037 mg</td>
</tr>
<tr>
<td>PEG Ceramide</td>
<td>0.263 mg</td>
</tr>
</tbody>
</table>

These liposome dry cakes were rehydrated in 300ul of sterile filtered dH₂O for each dose and left on a gentle shaker at room temperature for at least 2 hours to allow the particles to dissolve. Liposome particles were then analysed.

2.10.3. Characterization of Liposome particles:

The size, zeta potential and polydispersity of the resultant liposome particles were analysed using a Zetasizer 3000™ (Malvern Instruments, UK) machine. The sample was diluted at a 1:10 dilution in distilled water prior to measurement. All the measurements were carried out at room temperature.
2.11. Animal experiment protocols

2.11.1. Tumour generation in mice:

UMSCC104 and UDSCC2 cells were separately cultured in T175 tissue culture flasks with complete DMEM media, allowing the cells to become 80% confluent. Cells were then trypsinised and pelleted by centrifuging at 400g for 5 minutes. Further, they were washed twice with sterile 1XPBS, centrifuged and counted using haemocytometer. 1X10^7 cells were collected, centrifuged and re-suspended in 100 ul of sterile 1XPBS and mixed gently to allow the cells to homogenously mix with the PBS. Cells (1X10^7 cells) were immediately injected subcutaneously into the flank of 8 weeks old female nude strain immune-deficient BALB/c mice (BALB/c-Foxn1nu/Arc mice) (ARC, Perth, Australia). After 1 week, tumours were detectable in the mice.

2.11.2. Administration of siRNA in mice and analysis of tumour:

The PEGylated lipoplex formulation (Liposome + Si-E6/E7/siGFP) made as described above was tested and five mice were used for per treatment group (untreated control; vehicle only; non-specific control and Si-E6/E7 treated group): a total of fifteen mice. Once the tumour was detected and the size had reached ~200mm³, the lipoplex containing 40 ug of Si-E6/E7 or siGFP was injected with a total volume of 200ul, intravenously (IV) via tail vein. Twenty-four hours after IV administration, each mouse was anaesthetised using isoflurane (Abbott, Kurnell, Australia) and the tumour volume measured using a high resolution digital ultrasound scanner, VEVO® 3000 system (Fujifilm, Visual sonic, Toronto, Canada). This measurement was carried out every day up to 21 days. The results were analysed by VEVO® LAB software.

All experiments were approved by the Griffith University Animal Ethics Committee under the animal ethics number, GU Ref No: MSC/09/14/AEC.
2.12. Statistical analysis:

Statistical analyses of the acquired data were performed using GraphPad Prism™ software, version 7.0.1. Error bars in the relevant figures indicated the standard deviation of the observed values. Unpaired t-test was used to analyse statistical difference. One-way ANOVA was also used to analyse the statistical difference among 3 or more groups. For meta-analysis, STATA version 10, software was used to analyse the odds ratio and the forest plot was performed to observe the effect size and standardised mean difference of selected studies. The chi-square test was performed in SPSS version 22 to analyse the difference between the categorical variables of two groups.
CHAPTER 3

HPV-associated head and neck cancers in the Asia Pacific: A critical literature review & meta-analysis
Introduction:

Despite the reduction of tobacco and alcohol use, human papillomavirus (HPV)-associated head and neck squamous cell carcinoma (HNSCC) incidence is on the rise, especially in the western world. However, recently it has become a global problem. Since tobacco and alcohol are the major risk factors in developing countries, less importance has been given to search for the role of HPV in HNSCC in these nations. We attempted better to understand the role of HPV in the Asia Pacific region, encompassing countries from South Asia, South East Asia and East Asia and, if data were available, Pacific islands). This region has the highest burden of HNSCC cases in the world. We extended our search to all electronic databases and collected approximately 67 published studies from this region and performed a systematic review and meta-analysis. Our results demonstrate that approximately ~35% of total HNSCC cases are HPV-associated in the Asia Pacific region with the highest association seen in the oropharynx (~ 40%). We segregated information on prevalence of HPV by region, country and anatomical site. Our data show that the prevalence of HPV in HNSCC is ~36% in South Asia, ~39% in South East Asia, ~43% in East Asia and ~42% in Oceania. Moreover, the highest prevalence of HPV-positive oropharyngeal cancer was seen in Oceania (~49%), HPV-positive oral cavity cancer from the East Asia (~48%) and the laryngeal cancer from the South Asia (~46%). Our study was in accord with the current literature that indicates approximately one third of total HNSCC in the world are HPV associated. Further, our meta-analysis showed higher association of HPV with oropharyngeal cancer compared to oral cavity and laryngeal cancer. Our study stretches a brief but important understanding of the involvement of HPV in HNSCC in the Asia Pacific region, where less attention has hitherto been paid to the matter. More importance should be given to detecting the presence or absence of high risk HPVs (HRHPVs) while diagnosing HNSCC patients from this part of the world.

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HPV-associated head and neck cancers in the Asia Pacific: A critical literature review & meta-analysis

Mushfiq Hassan Shaili, Nigel A.J. McMillan, Newell W. Johnson

ABSTRACT

Background: Malignancies of the upper aero-digestive tract are a major public health problem, especially in the Asia Pacific. Certain Human papillomaviruses (HPVs) are well-established risk factors for carcinoma of the oropharynx and for a subset of head and neck carcinomas; however, their true importance in different populations and anatomical subsites remains unclear. The major risk factors in Asia Pacific remain smoking/smokeless tobacco, alcohol and poor diet, with limited evidence for HPVs. We reviewed published studies of association of HPV with anatomical site-specific Head & Neck Squamous Cell Carcinoma (HNSCC) in these populations and attempted a meta-analysis.

Methods and materials: From MEDLINE/PubMed/WEB OF SCIENCE/EMBASE/Scopus databases we found 67 relevant studies with a total of 72800 cases. 15 case-control studies met our inclusion criteria for meta-analysis, totaling 106 cases & 838 controls. HPV detection rates, sample site and size, and methods of tissue preservation and HPV detection were tabulated for each study.

Results: Studies were heterogeneous in terms of sample selection and method of detection of HPVs. Most were of limited quality. Averaging data from 67 studies of HNSCC, the prevalence of HPV of any subtype is approximately 34%. PCR (polymerase chain reaction) was the most used detection method and HPV 16 the most common genotype reported. Meta-analyses of case-control studies from this region reveal significant heterogeneity but suggest higher HPV prevalence in oropharyngeal cancer (OR: 14.66; 95%CI: 6.09-35.26) compared to oral cavity cancer and laryngeal cancer (OR: 4.06; 95%CI: 3.05-5.39 & OR: 3.23; 95%CI: 1.37-7.61) respectively.

Conclusions: In view of the significant association of HPV with HNSCC, studies with accurate subsite classification and more sensitive detection methods are necessary. Accurate data from this geographical region are essential to inform public health policies and treatment decisions, especially as studies from Europe and North America reveal HPV-driven cancers to be less aggressive, permitting treatment de-intensification.

Introduction

Epidemiology & risk factors

Molecular pathogenesis of HPV-associated HNSCC

Materials and methods

Search strategy
1. Introduction

Head and neck cancers (HNCs) represent a biologically heterogeneous group of neoplasms involving numerous distinct anatomical sites within the head and neck. Use of the term usually excludes the skin, central nervous system, eyes, lymphatic system, and endocrine glands. Most malignancies of the head and neck arise from the mucous membranes lining (International Classification of Disease, Tenth revision (ICD-10)); lips (C00); base of the tongue (C01); oral cavity including the anterior two-thirds of the tongue (C02–C05); tonsils (C06); the oropharynx (C10); nasopharynx (C11); the hypopharynx (C13); and pterygoid sinuses (C12); nasal cavity/middle ear (C30); and accessory sinuses (C31); and the larynx (C32) [1]. Most, approximately 85%, are morphologically characterized as Head & Neck Squamous Cell Carcinomas (HNSCC) and share common risk factors [2]. Nasopharyngeal carcinomas are often recorded separately because they are biologically distinct, having a strong association with Epstein-Barr virus [3,4]. Here, we do not include salivary gland neoplasms. HNSCC is the fifth most common cancer diagnosed in the world [5]. It is second among the most common malignancies throughout the Asia-Pacific region (South East Asia, Southeast Asia, East Asia and Oceania) accounting for 40–50% of all cancers [6]. Apart from the influence of ultraviolet light on lip cancer, tobacco, both smoked and unsmoked, areca nut (in these populations often consumed as a condiment with betel quid, which contains areca nut and tobacco) and alcohol abuse, often in the context of diets poor in antioxidants and minerals, are well-established major risk factors. It is no surprise, therefore, that HNSCC are most common in those countries where the consumption of tobacco, areca nut and alcohol is high (including Australia, Brazil, China, France, India, Japan, Netherlands, Papua New Guinea, South Africa, and Switzerland) [7]. Recent studies have shown a rising incidence of Squamous Cell Carcinoma (SCC) of the oropharynx, despite the reduction of tobacco use in several of these countries [8–11]. This suggests increasing prevalence of another risk factor, now clearly identified as persistent infection with so-called “high risk” types of human papillomavirus (HPV) [12,13].

Historically, HPV-associated cancers are best described in the uterine cervix where >99% of cases harbor an oncogenic type [14]. However, a significant association of HPV with HNSCC was reported around the turn of the century, with overall worldwide proportion of 25–35% of cases harboring the virus [15–17]. This association is now well documented in the western world, where it has been established that HPV is particularly related to SCC of the oropharynx. The Asia-Pacific region, on the other hand, has the highest rates of oral cancer in the world and, whereas this is traditionally related to tobacco, areca nut/betel quid, poor nutrition and alcohol abuse, there is a need for a detailed understanding of the part played by HPV in all head and neck cancers across this region.

We present here, for the first time, a critical review of published studies from this region and attempt a meta-analysis of case-control studies. We assess the proportion of HPV-associated HNSCC by cancer sub-site & by country, finding that variation is independent of the viral detection methods used.

2. Epidemiology & risk factors

HNC is a serious problem in much of the world. The burden is approximately 599,677 new cases per annum worldwide: 300,373 for lip plus oral cavity; 156,877 for larynx; 142,387 for other pharynx (oro-hypopharynx) excluding the nasopharynx [18,19]. While there is considerable geographical variation, the major burden is in developing countries. This includes South Asia where the annual incidence is 194,742 new cases and mortality 136,199 p.a. (an age-standardized incidence rate of approximately 20.70 per 100,000 persons p.a.), South East Asia with 35,228 incident cases and East Asia with 78,477 new cases per annum [19]. This is largely because of extensive use of tobacco and areca nut, alcohol abuse, poverty, poor diet and poor oral health [18]. The overall incidence of HNSCC in the whole of the Asia-Pacific is 314,718 new cases per year, which is more than half of the total HNSC cases worldwide. The mortality rate is also very high, approximately 199,537 deaths p.a. [19].

Since the association of HPV with HNSCC was first reported in the mid 1980s, rising rates of oropharyngeal cancer (includes cancers of C01, C09 and C10) especially of the tonsil (C01) and base of the tongue (C01) have been shown in Europe and in the USA, [20–23]. In the USA, between 1964 and 2004, the incidence rates of HPV-associated oropharyngeal cancer increased from 0.8/100,000 to 26/100,000 persons p.a. from 16.3% of cases carrying HPV to 71.7% over this period [21]. In Sweden and Finland the prevalence of HPV in cancers of the tonsil (C01) rose 3-fold between 1970 (29%) and 2007 (93%) and 2-fold between 1956 and 2000, respectively [22,23]; in England also, there was a 3-fold increase between 1995 and 2010 [24]. Significant trends have been described in Australia, where HPV involvement in oropharyngeal cancer (C01, C09, C10 & C13) increased from 20.2% in 1987–1995 to 63.5% in the 2006–2010 period [25]. A recent meta-analysis of contemporary studies on oropharyngeal cancer estimates 69.7% prevalence of HPV association in North America and 73.1% in Europe [26]. Such studies from the Asia-Pacific region as have been reported show striking differences from the West: reports only from India have given prevalence rates of the association of HPV with oropharyngeal cancer (C01, C09, C10 & C13) averaging much lower proportions, 27.28% (22.85–31.78) [29,30].

Head and neck cancer at sites with high prevalence of HPV [31] have a distinctly different structure than the more common alcohol- and tobacco-related neoplasms, and are associated with the lymphoid mucosa of Waldeyer's ring [25,32]. These areas are lined by folded, non-keratinized stratified squamous epithelium, the crypts of which contain basal keratinocytes, which function as a protective barrier to microorganisms, and are susceptible to invasion by HPV DNA [25,31]. Histologically, HPV-positive HNSCCs exhibit poorly differentiated, non-keratinized basaloid morphology [33–35].

In the western world, patients with HPV-related HNSCC are generally Caucasian, are younger, male, have a comparatively high socio-economic status, are non-smokers and non- or light drinkers [36–41]. There are important associations with sexual behavior, especially sexual debut at early age and a high number of lifetime vaginal and especially oral sex partners, these contributing to a 2–5
fold increase in the incidence rate [8,42–50]. Although it seems that HPV-positive HNSCC is more likely to develop in Europeans (including most Australians), a recent prevalence has also been noted in Asia Pacific populations (except Oceania), perhaps associated with changing sexual mores and the infiltration of Western culture [48].

2.1. Molecular pathogenesis of HPV-associated HNSCC

HPVs are a large family of viruses (the Papillomaviridae) that are small in diameter (55 nm), epitheliotropic, with a genome 7200–8000 base pairs in length [51] (Fig. 1). Depending on their nucleotide sequence, HPVs are classified into more than 100 different genotypes, about 15 of which are regarded as “high risk” due to their oncogenic potential [52,53]. HPV16 and HPV18 are especially closely linked with malignant transformation [54,55]. Approximately 25% of HPV are associated with oral lesions [54], and more than 50% of cases of HPV-related HNSCC are associated with HPV type 16 [31,56]. It is widely accepted that HPV16 and HPV18 are responsible for approximately 70% of cancers of the uterine cervix [57] and are strongly associated with anal, vulvar, and penile cancers [31,58,59]. However, the exact biological mechanisms are not completely understood. The process is thought to commence with integration of HPV DNA into the host keratinocyte genome. Two of the early viral genes, E6 and E7, are thought to be responsible for malignant transformation (Fig. 1). Suppression of the transcriptional repressor gene E2 of HPV allows continuous expression of E6 and E7, which then function as oncogenes throughout the life of what becomes malignant keratinocytes [60].

The E6 oncoprotein localized in the nuclear matrix targets and degrades the product of the cell’s p53 tumor suppressor gene, thus inhibiting p53-dependent cell cycle arrest & induced apoptosis [61,62]. E6 oncoprotein also enhances telomerase activity, favoring immortalization of affected keratinocytes. In addition, viral E7 promotes cellular proliferation by down-regulating the cyclin-dependent kinase (CDK) inhibitors p21 & p27, and by inactivating Rb (retinoblastoma) protein [63,64] by binding with its hypophosphorylated forms (pRb, p110, p107) [65–67]. Thus, loss of both p53 and Rb function ultimately leads to malignancy.

HPV-positive and HPV-negative HNSCC are distinctly different diseases. Understanding their molecular mechanisms is essential for effective bio-prevention and individualized biotherapies.

3. Materials and methods

3.1. Search strategy

Japan', 'Taiwan', 'Hong Kong', 'South Korea', 'North Korea', 'Australia', 'New Zealand' and 'Papua New Guinea'). A secondary search was also performed on the references cited in the selected articles.

3.2. Study selection and data extraction

Data extraction was performed by all three authors, focusing on: country of origin, demographics of participants, number of cancer cases, proportion of HPV positive cancers in relation to all cancer cases at HNC sub-sites, HPV detection methods, and HPV genotypes. Based on the CONSORT statement 2010 (Consolidated Standards of Reporting Trials), papers with Asia Pacific data were identified by reading the abstracts and then studied in full. In order to explore the possibility of a meta-analysis, we applied the additional criterion of case control study design. PRISMA 2009 (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines were followed while selecting studies for meta-analysis (Fig. 2).

3.2.1. Statistical analysis

STATA x12 (Stata Corp LP, College Station, Texas, USA) software was used. Firstly, to evaluate any significant difference, two-sided 95% confidence intervals (95% CI) were calculated by the 'Wilson Score' method in all 67 selected studies (Tables 2 and 3). For meta-analysis, dichotomous data of the numbers of HPV-positive and -negative head and neck cancers were analyzed by Forest plot. Then the odds ratios (OR) and 95% confidence intervals (CI) were calculated by the 'Wilson Score' method [68] for individual studies. The overall proportions were examined by meta-analysis to estimate the pooled prevalence of HPV in non-site specific HNCCC using the Mantel-Haenszel method. The Cochrane Chi-squared or I-squared statistic ($I^2$) -based Q statistic and a random effects model (REM) with the DerSimonian-Laird method were used to assess the variation between study results [69,70].

4. Results

We identified 67 studies from the Asia Pacific region that have reported prevalence of one or more HPV types, patients' demographics, methods of tissue preservation, HPV detection methods, and have provided information about anatomical subites (Table 1). A total of 7284 cases include: 3153 from the oral cavity (CO0-CO6); 2768 from the tonsil (CO9) plus oropharynx.

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Fig. 2. PRISMA flowchart of the selection process of studies on HPV-associated head and neck squamous cell carcinomas from the Asia Pacific.
Table 1
Details of published studies from the Asia-Pacific reporting HPV association with Head and Neck Cancer.

<table>
<thead>
<tr>
<th>Authors/year of publication</th>
<th>Country</th>
<th>Habit described</th>
<th>Age groups (mean age years)</th>
<th>Tissues collected</th>
<th>Tumor site(s) (ICD-10 for head and neck cancer)</th>
<th>No. of cases</th>
<th>HPV positive/ total cases (%)</th>
<th>Overall HPV positivity (%)</th>
<th>HPV positive (control group) (%)</th>
<th>HPV genotypes</th>
<th>DNA (HPV) detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balaram et al. [71]</td>
<td>India</td>
<td>Smokers, no tobacco users (majority)</td>
<td>30–70 (55)</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>60</td>
<td>42/60 (70%)</td>
<td>72%</td>
<td>16/18 specified</td>
<td>11</td>
<td>PCR (MY09/11 &amp; GP5/ 6 primers)</td>
</tr>
<tr>
<td>D’Souza et al. [6]</td>
<td>India</td>
<td>All are smokers tobacco users (for 10–30 yrs)</td>
<td>22–70 (50)</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>57</td>
<td>5/57 (9%)</td>
<td>5%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11) &amp; Southern Blot</td>
</tr>
<tr>
<td>Pihlai et al. [72]</td>
<td>India</td>
<td>not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C02/04)</td>
<td>60</td>
<td>19/60 (31.7%)</td>
<td>33.6%</td>
<td>7/16 specified</td>
<td>11</td>
<td>ISH &amp; p53, cyclin D1, Ki67, KiIC</td>
</tr>
<tr>
<td>Nispel et al. [73]</td>
<td>India</td>
<td>All are smokers tobacco users (for 10 yrs)</td>
<td>≤ 50 &amp; &gt; 50 (50)</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>56</td>
<td>21/56 (37.5%)</td>
<td>31.6%</td>
<td>7/16 specified</td>
<td>11</td>
<td>PCR (MY09/11 &amp; 16)</td>
</tr>
<tr>
<td>Jacob et al. [74]</td>
<td>India</td>
<td>not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>Larynx (C42)</td>
<td>44</td>
<td>15/44 (34%)</td>
<td>34%</td>
<td>0/10 specified</td>
<td>16</td>
<td>PCR (GP5/52) &amp; 18-35</td>
</tr>
<tr>
<td>Kumar et al. [75]</td>
<td>India</td>
<td>Smokers &amp; non-smokers tobacco users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>Larynx (C42)</td>
<td>22</td>
<td>7/22 (31.8%)</td>
<td>31.8%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (GP5/52) &amp; 16-35</td>
</tr>
<tr>
<td>Herrozo et al. [76]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>Fresh Frozen specimen</td>
<td>OC (C01-06)</td>
<td>200</td>
<td>12/200 (6%)</td>
<td>6%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (18-35 &amp; 16) &amp; 35</td>
</tr>
<tr>
<td>Kopkar et al. [77]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>Larynx (C42)</td>
<td>23</td>
<td>4/23 (17.4%)</td>
<td>17.4%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (18-35, 16) &amp; 35</td>
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<tr>
<td>Mithra et al. [78]</td>
<td>India</td>
<td>not specified</td>
<td>not specified</td>
<td>Fresh Frozen specimen</td>
<td>Lip (C03)</td>
<td>11</td>
<td>4/11 (36.4%)</td>
<td>36.4%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11 &amp; 16)</td>
</tr>
<tr>
<td>Mitra et al. [79]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>29</td>
<td>9/29 (31.0%)</td>
<td>31.0%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11 &amp; 16)</td>
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<tr>
<td>Bhattacharya et al. [80]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>Fresh Frozen specimen</td>
<td>BM (C06)</td>
<td>200</td>
<td>120/200 (60%)</td>
<td>60%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11 &amp; 16)</td>
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<tr>
<td>Chaudhary et al. [81]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>Fresh Frozen specimen</td>
<td>Larynx (C42)</td>
<td>32</td>
<td>10/32 (31.2%)</td>
<td>31.2%</td>
<td>16 &amp; 35</td>
<td>16 &amp; 35</td>
<td>PCR (GP5/52) &amp; 18-35</td>
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<tr>
<td>&quot;Tokai et al. [82]</td>
<td>India</td>
<td>not specified</td>
<td>not specified</td>
<td>Saliva samples</td>
<td>OC (C01-04)</td>
<td>34</td>
<td>24/34 (70.6%)</td>
<td>70.6%</td>
<td>25/25 specified</td>
<td>25/25</td>
<td>PCR (Unspecified)</td>
</tr>
<tr>
<td>Elango et al. [83]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>Fresh Frozen specimen</td>
<td>BM (C06)</td>
<td>60</td>
<td>23/60 (38.3%)</td>
<td>38.3%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11 &amp; GP5/6 primers)</td>
</tr>
<tr>
<td>Jalewali et al. [84]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>10</td>
<td>5/10 (50%)</td>
<td>50%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11 &amp; 16)</td>
</tr>
<tr>
<td>Bahlo et al. [85]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>55</td>
<td>22/55 (40%)</td>
<td>40%</td>
<td>16 specified</td>
<td>16</td>
<td>PCR (MY09/11 &amp; GP5/6 primers)</td>
</tr>
<tr>
<td>Talukdar et al. [86]</td>
<td>India</td>
<td>Smokers, not specified</td>
<td>not specified</td>
<td>Fresh Frozen specimen</td>
<td>BM (C06)</td>
<td>60</td>
<td>23/60 (38.3%)</td>
<td>38.3%</td>
<td>16 &amp; 35</td>
<td>16 &amp; 35</td>
<td>PCR (MY09/11 &amp; GP5/6 primers)</td>
</tr>
<tr>
<td>Authors/year of publication/Ref. no.</td>
<td>Country</td>
<td>Habits described</td>
<td>Age groups (mean age years)</td>
<td>Tumors collected</td>
<td>Tumor site(s) (ICD-10 for head and neck cancer)</td>
<td>No. of cases</td>
<td>HPV type(s)</td>
<td>Overall HPV positivity (%)</td>
<td>HPV positive (control group)</td>
<td>HPV genotypes</td>
<td>DNA (HPV) detection methods</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>---------</td>
<td>-----------------</td>
<td>-----------------------------</td>
<td>------------------</td>
<td>---------------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-----------------------------</td>
<td>-------------------------------</td>
<td>------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Castillo et al. [84]</td>
<td>Pakistan</td>
<td>Smokers &amp; non-smokers tobacco users &amp; alcohol users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
<td>48</td>
<td>27/48</td>
<td>56%</td>
<td>not specified</td>
<td>10, 18, 6 &amp; 11</td>
<td>PCR (SP1/SP2 &amp; ISH)</td>
</tr>
<tr>
<td>Akhtar et al. [85]</td>
<td>Bangladesh</td>
<td>Smokers &amp; non-smokers tobacco users (smoking) &amp; smokers</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>Unspec. cavity</td>
<td>11</td>
<td>1/11</td>
<td>9%</td>
<td>not specified</td>
<td>10, 18, 6 &amp; 11</td>
<td>PCR (MY09/MY11 primers)</td>
</tr>
<tr>
<td>Jeyasree et al. [86]</td>
<td>Sri Lanka</td>
<td>Non-smokers tobacco users</td>
<td>≤50 &amp; 60+ (69)</td>
<td>FF &amp; PE</td>
<td>BM (C06) T (D1, C02) Lyp (C00)</td>
<td>62</td>
<td>23/62</td>
<td>37.2%</td>
<td>not specified</td>
<td>15, 16,45,66</td>
<td>PCR (G5/G6/G8 primers)</td>
</tr>
<tr>
<td>Jahan et al. [82]</td>
<td>Sri Lanka</td>
<td>Smokers</td>
<td>40-79 (59)</td>
<td>FF &amp; PE</td>
<td>FuM (C04) Unspec. oral</td>
<td>20</td>
<td>8/20</td>
<td>40%</td>
<td>not specified</td>
<td>10</td>
<td>PCR (MY09/MY11 primers)</td>
</tr>
<tr>
<td>Saini et al. [87]</td>
<td>Malaysia</td>
<td>Smokers, non-smokers tobacco users</td>
<td>50-59 (59)</td>
<td>FF &amp; PE</td>
<td>Lyp (C00) BM (C06)</td>
<td>11</td>
<td>4/11</td>
<td>36%</td>
<td>not specified</td>
<td>10</td>
<td>PCR (MY09/MY11 &amp; G5/G6/G8 primers)</td>
</tr>
<tr>
<td>Lim et al. [88]</td>
<td>Malaysia</td>
<td>Smokers, non-smokers tobacco users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>67</td>
<td>20/67</td>
<td>30%</td>
<td>not specified</td>
<td>10, 18 &amp; 11</td>
<td>PCR (SP1/SP2 &amp; ISH)</td>
</tr>
<tr>
<td>Khandelwal et al. [89]</td>
<td>Thailand</td>
<td>Smokers, non-smokers tobacco users</td>
<td>30-88 (59)</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
<td>45</td>
<td>10/45</td>
<td>22%</td>
<td>not specified</td>
<td>10, 18 &amp; 11</td>
<td>PCR (MY09/MY11 &amp; G5/G6/G8 primers)</td>
</tr>
<tr>
<td>Wen et al. [90]</td>
<td>China</td>
<td>Smokers, non-smokers tobacco users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06) Larynx (C32)</td>
<td>100</td>
<td>20/100</td>
<td>20%</td>
<td>not specified</td>
<td>10, 18, 33, 6 &amp; 11</td>
<td>PCR (p16/18-16-28) &amp; Southern blot</td>
</tr>
<tr>
<td>Chen et al. [92]</td>
<td>China</td>
<td>Smokers, non-smokers tobacco users</td>
<td>40-79 (60)</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
<td>40</td>
<td>20/40</td>
<td>50%</td>
<td>not specified</td>
<td>10 &amp; 18</td>
<td>PCR &amp; Southern blot</td>
</tr>
<tr>
<td>Li et al. [93]</td>
<td>China</td>
<td>Smokers, non-smokers tobacco users</td>
<td>37-72 (55)</td>
<td>FF &amp; PE</td>
<td>Tonsil (C09)</td>
<td>100</td>
<td>10/100</td>
<td>10%</td>
<td>not specified</td>
<td>10</td>
<td>PCR (G5/G6/G8)</td>
</tr>
<tr>
<td>Zhang et al. [94]</td>
<td>China</td>
<td>Smokers, non-smokers alcohol users</td>
<td>20-54 (37)</td>
<td>FF &amp; PE</td>
<td>BM (C06) UCP (C13)</td>
<td>35</td>
<td>10/35</td>
<td>29%</td>
<td>not specified</td>
<td>10, 18</td>
<td>PCR (primers for HPV types 16 &amp; 18)</td>
</tr>
<tr>
<td>Lin et al. [95]</td>
<td>China</td>
<td>Smokers, non-smokers alcohol users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>Larynx (C32)</td>
<td>11</td>
<td>2/11</td>
<td>18%</td>
<td>not specified</td>
<td>10, 18</td>
<td>PCR (type-specific HPV16 &amp; 18 &amp; 18 37,69 &amp; 89-18 &amp; 18-28 primers)</td>
</tr>
<tr>
<td>Wei et al. [96]</td>
<td>China</td>
<td>All are smokers tobacco users (for 30yrs)</td>
<td>40-75 (53)</td>
<td>FF &amp; PE</td>
<td>UCP (C13)</td>
<td>108</td>
<td>2/108</td>
<td>1%</td>
<td>not specified</td>
<td>10, 18 &amp; 52</td>
<td>PCR (G5/G6/G8-1/PGV-1/PHLV-1 primers used)</td>
</tr>
<tr>
<td>Xu et al. [97]</td>
<td>China</td>
<td>Smokers, non-smokers alcohol users</td>
<td>21-93 (57)</td>
<td>FF &amp; PE</td>
<td>LCM (C13)</td>
<td>21</td>
<td>2/21</td>
<td>10%</td>
<td>not specified</td>
<td>10, 18</td>
<td>PCR (G5/G6/G8) followed by hybridization</td>
</tr>
<tr>
<td>Li et al. [98]</td>
<td>Hong Kong</td>
<td>Smokers, non-smokers tobacco users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>Tonsil (C09)</td>
<td>100</td>
<td>30/100</td>
<td>30%</td>
<td>not specified</td>
<td>10, 18 &amp; 11</td>
<td>PCR</td>
</tr>
<tr>
<td>Chen et al. [99]</td>
<td>Taiwan</td>
<td>Smokers, non-smokers tobacco users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
<td>40</td>
<td>10/40</td>
<td>25%</td>
<td>not specified</td>
<td>10, 18 &amp; 11</td>
<td>PCR &amp; ISH</td>
</tr>
<tr>
<td>Chang et al. [100]</td>
<td>Taiwan</td>
<td>Smokers, non-smokers tobacco users</td>
<td>25-78 (51)</td>
<td>FF &amp; PE</td>
<td>UCP (C13) BM (C06)</td>
<td>10</td>
<td>5/10</td>
<td>50%</td>
<td>not specified</td>
<td>10, 18, 6 &amp; 11</td>
<td>PCR (MY09/MY11 &amp; G5/G6/G8 primers)</td>
</tr>
<tr>
<td>Yang et al. [101]</td>
<td>Taiwan</td>
<td>Smokers, non-smokers tobacco users</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
<td>10</td>
<td>3/10</td>
<td>30%</td>
<td>not specified</td>
<td>10, 18 &amp; 11</td>
<td>PCR (MY09/MY11 primers)</td>
</tr>
<tr>
<td>Kuo et al. [102]</td>
<td>Taiwan</td>
<td>Smokers, non-smokers tobacco users</td>
<td>29-79 (51)</td>
<td>FF &amp; PE</td>
<td>Tonsil (C09)</td>
<td>20</td>
<td>3/20</td>
<td>15%</td>
<td>not specified</td>
<td>10, 18, 33, 6 &amp; 11</td>
<td>PCR (MY09/MY11 &amp; G5/G6/G8 primers)</td>
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<tr>
<td>Al-Sweihh et al. [103]</td>
<td>Taiwan</td>
<td>Smokers, non-smokers tobacco users</td>
<td>20-69 (59)</td>
<td>FF &amp; PE</td>
<td>Soft palate (C05)</td>
<td>20</td>
<td>20/20</td>
<td>100%</td>
<td>not specified</td>
<td>10, 18 &amp; 11</td>
<td>PCR (MY09/MY11 &amp; G5/G6/G8 primers)</td>
</tr>
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</table>

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<table>
<thead>
<tr>
<th>Authors/Year of publication/</th>
<th>Country</th>
<th>Habit described/</th>
<th>Age groups (mean years)</th>
<th>Tissues collected</th>
<th>Tumor sites (IBD-10 for head and neck cancer)</th>
<th>No. of cases</th>
<th>HPV positive/no. of cases</th>
<th>Overall HPV positivity (%)</th>
<th>HPV positive (control group)</th>
<th>HPV genotypes</th>
<th>DNA (HPV) detection methods</th>
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</thead>
<tbody>
<tr>
<td>Gh et al. [104]</td>
<td>South Korea</td>
<td>not specified</td>
<td>32-77 (58.2)</td>
<td>FF &amp; PE</td>
<td>Tornil (C09)</td>
<td>39</td>
<td>25/39</td>
<td>64.1%</td>
<td>3 (3)</td>
<td>10, 33, 58 &amp; 6</td>
<td>PCR &amp; DNA microarray</td>
</tr>
<tr>
<td>Shin et al. [105]</td>
<td>South Korea</td>
<td>not specified</td>
<td>not specified</td>
<td>not specified</td>
<td>not specified</td>
<td>10</td>
<td>9/16</td>
<td>70.0%</td>
<td>not specified</td>
<td>10, 18 &amp; 32</td>
<td>Real time PCR (primers 152, 226, 1698, and probes 152 &amp; 1698 &amp; RBC)</td>
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<tr>
<td>Kim et al. [106]</td>
<td>South Korea</td>
<td>not specified</td>
<td>not specified</td>
<td>not specified</td>
<td>not specified</td>
<td>52</td>
<td>18/52</td>
<td>73%</td>
<td>not specified</td>
<td>10, 18, 32</td>
<td>PCR (type specific)</td>
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<tr>
<td>Egawa et al. [107]</td>
<td>Japan</td>
<td>not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OP (C10)</td>
<td>11</td>
<td>0/11</td>
<td>0.0%</td>
<td>not specified</td>
<td>10 &amp; 18</td>
<td>PCR (Primers for HJV types 10 &amp; 18)</td>
</tr>
<tr>
<td>Amare et al. [108]</td>
<td>Japan</td>
<td>not specified</td>
<td>42-90 (66)</td>
<td>FF &amp; PE</td>
<td>Larynx (C32)</td>
<td>43</td>
<td>16/43</td>
<td>37.2%</td>
<td>2 (11)</td>
<td>10, 18 &amp; 33</td>
<td>PCR (type specific primers)</td>
</tr>
<tr>
<td>Sashita et al. [109]</td>
<td>Japan</td>
<td>not specified</td>
<td>42-90 (56)</td>
<td>FF &amp; PE</td>
<td>Larynx (C32)</td>
<td>45</td>
<td>11/45</td>
<td>24.4%</td>
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<td>10 &amp; 18</td>
<td>PCR (type specific primers)</td>
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<tr>
<td>Shindo et al. [110]</td>
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<td>not specified</td>
<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
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<td>0/11</td>
<td>0.0%</td>
<td>not specified</td>
<td>10 &amp; 18</td>
<td>PCR (type specific agents)</td>
</tr>
<tr>
<td>Chiba et al. [111]</td>
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<td>not specified</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
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<td>21.1%</td>
<td>not specified</td>
<td>10 &amp; 18</td>
<td>PCR (type specific primers)</td>
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<tr>
<td>Mineta et al. [112]</td>
<td>Japan</td>
<td>not specified</td>
<td>not specified</td>
<td>Fresh frozen</td>
<td>OC (C01-C06)</td>
<td>14</td>
<td>3/14</td>
<td>23.6%</td>
<td>not specified</td>
<td>10 &amp; 18</td>
<td>PCR (type specific primers)</td>
</tr>
<tr>
<td>Nishikawa et al. [113]</td>
<td>Japan</td>
<td>Smokers</td>
<td>50-55 (52.6)</td>
<td>FF &amp; PE</td>
<td>OC (C01-C06)</td>
<td>15</td>
<td>5/10</td>
<td>33.3%</td>
<td>not specified</td>
<td>10 &amp; 18</td>
<td>PCR &amp; Slot blot</td>
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<tr>
<td>Tsukada et al. [114]</td>
<td>Japan</td>
<td>Smokers or alcohol users</td>
<td>28-94 (64)</td>
<td>FF &amp; PE</td>
<td>Lip (C05)</td>
<td>3</td>
<td>1/3</td>
<td>33.3%</td>
<td>not specified</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR &amp; Southern blot</td>
</tr>
<tr>
<td>Shima et al. [115]</td>
<td>Japan</td>
<td>not specified</td>
<td>47-91 (69)</td>
<td>Fresh frozen</td>
<td>Lip (C05)</td>
<td>3</td>
<td>0/3</td>
<td>0%</td>
<td>not specified</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Kojima et al. [116]</td>
<td>Japan</td>
<td>not specified</td>
<td>30-80 (51)</td>
<td>FF &amp; PE</td>
<td>Lip (C05)</td>
<td>3</td>
<td>0/3</td>
<td>0%</td>
<td>not specified</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Higa et al. [117]</td>
<td>Japan</td>
<td>Smokers or alcohol users</td>
<td>59 (59)</td>
<td>Fresh frozen</td>
<td>Lip (C05)</td>
<td>3</td>
<td>1/3</td>
<td>33.3%</td>
<td>not specified</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
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<tr>
<td>Sugiyama et al. [118]</td>
<td>Japan</td>
<td>not specified</td>
<td>39-90 (58.5)</td>
<td>FF &amp; PE</td>
<td>Lip (C05)</td>
<td>7</td>
<td>5/27</td>
<td>69.6%</td>
<td>10 &amp; 18, 6 &amp; 14</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Shimizu et al. [119]</td>
<td>Japan</td>
<td>not specified</td>
<td>59-94 (64)</td>
<td>Lip (C05)</td>
<td>BM (C06)</td>
<td>7</td>
<td>0/7</td>
<td>0%</td>
<td>10 &amp; 18, 6 &amp; 14</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Sugiyama et al. [120]</td>
<td>Japan</td>
<td>Smokers or alcohol users</td>
<td>40-91 (67.5)</td>
<td>FF &amp; PE</td>
<td>Lip (C05)</td>
<td>4</td>
<td>0/4</td>
<td>0%</td>
<td>not specified</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Kayama et al. [121]</td>
<td>Japan</td>
<td>not specified</td>
<td>37-79 (58)</td>
<td>FF &amp; PE</td>
<td>BM (C06)</td>
<td>11</td>
<td>11/11</td>
<td>100%</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Bhawal et al. [122]</td>
<td>Japan</td>
<td>not specified</td>
<td>48-90 (67)</td>
<td>Fresh frozen</td>
<td>OC (C01-C06)</td>
<td>19</td>
<td>11/29</td>
<td>58.1%</td>
<td>not specified</td>
<td>10, 18, 32, 6 &amp; 14</td>
<td>PCR (type specific primers: 15a, 15b, 16, 17)</td>
</tr>
<tr>
<td>Author(s)</td>
<td>Year of publication</td>
<td>Country</td>
<td>Smoking status</td>
<td>Age groups (mean age in years)</td>
<td>Tumor site(s) (ICD-10 &amp; ICD) (number of cases)</td>
<td>Overall HPV positivity (%)</td>
<td>HPV positive cases</td>
<td>HPV types</td>
<td>DNA HPV detection methods</td>
<td></td>
<td></td>
</tr>
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<td>----------</td>
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<td>--------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ishihashi et al.</td>
<td>2013</td>
<td>Japan</td>
<td>Not specified</td>
<td>Not specified (mean age 52.2)</td>
<td>OC, C00 (C00) (50)</td>
<td>50/60</td>
<td>12%</td>
<td>Not specified (50, 51, 52)</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deng et al.</td>
<td>2014</td>
<td>Japan</td>
<td>Smokers or alcohol users</td>
<td>28-90 (64.8)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moriyama et al.</td>
<td>2015</td>
<td>Japan</td>
<td>Smokers or alcohol users</td>
<td>&lt;40</td>
<td>OC, C00 (C00)</td>
<td>50/60</td>
<td>12%</td>
<td>Not specified (50, 51, 52)</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deng et al.</td>
<td>2016</td>
<td>Japan</td>
<td>Smokers or alcohol users</td>
<td>28-95 (65)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hongo et al.</td>
<td>2017</td>
<td>Australia</td>
<td>Not specified</td>
<td>Not specified (mean age 50.8)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hongo et al.</td>
<td>2018</td>
<td>Australia</td>
<td>Smokers or alcohol users</td>
<td>31-89 (55)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hongo et al.</td>
<td>2019</td>
<td>Australia</td>
<td>Smoking history</td>
<td>54-83 (50.2)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hongo et al.</td>
<td>2020</td>
<td>Australia</td>
<td>Smoking history</td>
<td>30-90 (50)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Armstrong et al.</td>
<td>2021</td>
<td>Australia</td>
<td>Smokers or alcohol users</td>
<td>50-80 (56)</td>
<td>OC, C00 (C00)</td>
<td>28/50</td>
<td>56/100</td>
<td>20%</td>
<td>PCR (MY09/MY11 and GP5+/6+ genotyping)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Studies of head and neck cancer in the Asia-Pacific by major cancer sites and overall HPV prevalence.

<table>
<thead>
<tr>
<th>Site</th>
<th>Geographic location</th>
<th>Number of studies</th>
<th>Number of cases</th>
<th>Overall HPV prevalence (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>South Asia, East Asia, Australia, and Oceania</td>
<td>47</td>
<td>3553</td>
<td>57.5% (35.8% - 59.3%)</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>South Asia, East Asia, Australia, and Oceania</td>
<td>26</td>
<td>2768</td>
<td>40.5% (38.7% - 42.3%)</td>
</tr>
<tr>
<td>Larynx</td>
<td>South Asia, East Asia, Australia, and Oceania</td>
<td>19</td>
<td>826</td>
<td>23.9% (21.4% - 26.4%)</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
<td>57.5% (35.8% - 59.3%)</td>
</tr>
</tbody>
</table>

Note: The highest HPV prevalence in oral cavity squamous cell carcinoma is reported from South Asia (48.6%), followed by East Asia (42.8%), and Australia and Oceania (5.0%). In oropharyngeal cancer, reports show HPV prevalence to be higher in Oceania (49.3%) compared to East Asia (34.7%). The lowest HPV prevalence in oropharyngeal cancer was noted in South Asia (29.8%), but this region reported the highest HPV prevalence in laryngeal cancer (49.5%). The prevalence of HPV-associated laryngeal cancer reported was the lowest in Oceania (6%) and North America. No data on HPV-associated oropharyngeal or laryngeal cancer were available from South Asia. Considerable differences were present between countries.
HPV association in Oral Cavity Cancer

HPV Association in Oropharyngeal cancer

HPV association in Laryngeal cancer

Fig. 3. Prevalence of HPV in head and neck squamous cell carcinoma by anatomical sites and countries of Asia Pacific.

[74,91]. HPV18 was the second most common oncogenic type detected [73,87,91,126]. Other less common oncogenic types detected were HPV31, 33, 35, 39, 45, 51, 52, 53, 58, 66, 68 & 70 (Table 1). In addition, some low risk and non-oncogenic types were also detected, including HPV6, 11, 38, 44, 54, 61, 69 and 22 (Table 1). Multiple HPV infections were detected in only 5–10% of cases and in most instances HPV16 was also found [73,78,91,144].

There were no significant differences in the average age of patients by country: 56.4 years overall; 58.6 years in East Asia [113–115,120]; 59 years in South East Asia [87–89], 55.2 years in Oceania [128,131,132] and 52.8 years in South Asia [50,77–79]. Most studies were separated by sex, the prevalence being slightly higher in males [30,72,78,94,104,114,131,132]. The majority of cases in all populations presented with stage III and IV disease. Cases of moderately to poorly differentiated squamous cell carcinoma showed higher HPV prevalence than did well-differentiated cancers [30,72,77,94,106,118,128,131]. All except 2 studies used PCR as HPV DNA detection method; these two used ISH [29,72].

Meta-analysis stratified by anatomical site also indicates that HPV association, compared to non-cancerous patients, is highest for oropharyngeal cancer (OR: 14.66; 95%CI: 6.08–35.26) (Fig. 4).

Assocation of HPV with Oropharyngeal cancer in South Asia

Study ID | OR (95% CI) | Weight
---|---|---
Nishikawa B. et al. (1992) | 8.60 (1.09, 24.11) | 23.31
Oh TA. et al. (2004) | 18.60 (4.77, 71.44) | 34.87
Koppikar P. et al. (2009) | 7.70 (1.20, 30.35) | 13.90
Deng Z. et al. (2015) | 20.70 (2.73, 158.30) | 27.42
Overall (I-squared = 0.00%, p = 0.644) | 14.06 (6.09, 26.26) | 190.00

Fig. 4. Forrest plot of 4 case-control studies from the Asia Pacific (HPV association with oropharyngeal cancer).
less for oral cavity cancer (OR: 4.06; 95% CI: 3.05-5.39) (Fig. 5) and least for laryngeal cancer (OR: 3.23; 95% CI: 1.37-7.61) (Fig. 6). Although there was heterogeneity between these effects for oral cavity cancer ($I^2=71.0\%, p=0.003$), no heterogeneity was noted for laryngeal cancer ($I^2=0\%, p=0.553$) and oropharyngeal cancer ($I^2=0\%, p=0.644$). However, it should be noted that the study effect size and number of samples were small in both oropharyngeal cancer and laryngeal cancer studies so the results should be interpreted with caution.

5. Discussion

Since tobacco and areca nut/quid-related HNSCC has long been the major concern, few studies on the role of HPV have been conducted on Asia-Pacific populations. The overall pooled prevalence in the 62 studies we located was 36.45% (95% CI: 35.36-37.54), concordant with the recent meta-analysis by Termine et al. but slightly higher than that by Nalaye et al., which indicated a worldwide prevalence of 34.5% (95% CI: 28.4-40.6) and 29.5% (95% CI: 25.3-33.6) respectively [133,134]. The prevalence of HPV in oropharyngeal cancer from our study of the Asia Pacific is similar to...
the worldwide average prevalence reported by Ndiaye et al. (45.8%), whereas the average association is much higher in North America (59.9%), but similar in Europe (59.7%) [28,134]. Significant differences of HPV-association with OPC were noted when analyzed by separate parts of the Asia Pacific region: 25.8%, 38.7% & 40.2% in South Asia, East Asia and Oceania respectively (Fig. 3). For OCC, HPV prevalence appears to be higher in the Asia Pacific (38.9%) compared to other parts of the world: South America (33.1%); Europe (17.3%), and North America (13.8%) [134]. The prevalence is higher if we exclude Oceania from the Asia Pacific, namely 42.9%. This is consistent with the reviews from Kreimer et al. and Ndiaye et al. who reported higher proportions in Asian populations: 33% and 43.4% respectively [17,134]. Estimates for an association with cancer of the larynx are much lower, averaging ~2% in the Asia Pacific; with a wide range of 6-48.05%. Similar variability has been suggested from other parts of the world, ranging from 9.4-27% in the USA, 4.4-52% in Europe and 52-37.3% in South America [32,135-146]. These differences could be explained by the number of cases assessed, types of methods, tissue preservation, techniques used for DNA detection, geographical location and/or differences in risk factors.

The existing literature suggests that the prevalence varies considerably among anatomical sites and subtypes; we have not, therefore, pooled data across all head and neck cancer sites for meta-analysis. Our meta-analyses, segregated by anatomical sites, support the fact that oropharyngeal cancer has stronger association with HPV compared to oral cavity and laryngeal cancer in the Asia Pacific. However, studies on HPV-associated oropharyngeal cancer from the Asia Pacific region are few compared to oral cavity cancer.

Although the demography of HPV-associated HNC patients around the world emphasizes young adult males with no or limited history of smoking or drinking, the majority of the cases from the Asia Pacific reviewed here were habitual users of tobacco in smoked or smokeless forms and/or alcohol users/abusers and in whom a high incidence of HPV infection has often been reported [6,7,13,75,89,94,99,113]. It has also been suggested that former/ex smokers are more prone to have HPV-associated HNSCC compared to non-smokers and current smokers [30,134]. Although most studies from the Asia Pacific have shown slight male predilection, there are issues with small study groups and unequal sex distribution. Majority of HPV-associated HNC patients from the South Asia, South East Asia, East Asia and Oceania are relatively young and from lower socio-economic backgrounds than in the West [8,14,147]. One recent extensive study from north India has shown a strong association of higher number of lifetime sexual partners and high-risk sexual behaviors (oral and anal sex) with HPV-positive oropharyngeal cancer group as compared to patients who are HPV negative [88 vs. 21%] [30]. Another study from Australia shows a significant association of HPV with head and neck cancers in men having sex with men who have frequent oral-genital contact [148].

There are several limitations in our study. Although we have stratified by cancer sub-site and by geographical region, heterogeneity still persists because of the limited literature base and absence of relevant data in many publications. Moreover, only fifteen studies out of sixty-six used control subjects. Misclassification of anatomical sub-site is also a concern. Nevertheless, it seems that carcinomas of the tonsil appear to have the highest association with HPV in East Asia and in Oceania [102,104,108,128,129,131], whereas for "tongue cancer" it is South Asia and South East Asia which are reported to have the highest prevalence [6,7,37,81,82,87]. Unfortunately, most studies do not separate the oral tongue from the posterior third/base of tongue (CD1). The latter is partly part of the oropharynx and, worldwide, has a higher prevalence of HPV in carcinomas arising from these than those arising in the mobile tongue [149]. Generally, much less frequent associations of HPV are noted with the mobile tongue (CD2), floor of the mouth (CD4), buccal mucosa (CD3) and palate (CD5) [21,150]. With ambiguity in definitions, we were forced to consider tumors described as "tongue" as oral cavity cancers, which may have resulted in overestimation of HPV association with the mouth (oral cavity). Such misclassification could also contribute to the heterogeneity in the meta-analysis found for oral cavity cancer. Further categorization of other anatomical sub-sites was also imprecise with terms like 'oral cavity', 'Mandible', 'Maxilla', 'others' or even 'Unspecified/Unknown site' being used (Table 1). We strongly recommend the systematic use of detailed ICD 10 (International Classification of Diseases – Revision 10) codes for all studies of HNC.

Information about age and sexual behaviors of subjects were not properly addressed in most studies across the Asia-Pacific Region, nor are there adequate data on tobacco, alcohol or consumption of participants.

Selection of the type of specimen is important. Tissue biopsy is better than exfoliative cytology or saliva because it includes basal layer epithelial cells where HPV is likely to be present in latent state [151]. Transmission of HPV is primarily through skin-to-skin contact and integration of an oncogenic strain of the virus into the tissue of the host genome is essential for cancer to supervene [152,153]. A large number of saliva samples from the general public, which would include exfoliated epithelial cells, have been screened across USA, and 3.1% carriage of high-risk HPV genotypes reported [154]. In interpreting the public health implications of this finding, it would be important to know how many individuals cleared or retained this infection, and the proportion of such individuals in whom integration of the virus might have occurred [155].

Other limitations include variation in HPV DNA detection method and lack of standardised histo-pathological assessment. Techniques used should be reproducible, subject to standardisation, free from contamination and be economically viable. PCR assay could be the technique of choice as it is sensitive, time-efficient, and requires minimal tissue, but is moderately expensive [156] and there is a risk of false positives. Inappropriate length of the consensus primer pair or over-amplification of the HPV DNA type-specific primers may result in excessive sensitivity with false positives from contamination [157]: it is always important to ensure a clean and sterile environment while performing PCR. Further, recent studies have shown that combination of two sets of primers such as "CMV (CP1) + MY09/11 + G-P5R" in nested PCR is more efficient for detection of HPV in oropharyngeal cancer tissues, which may have very low viral load [158-160]. More recently, detection of mRNA for E6/E7 proteins has been proposed as the gold-standard test. However, the sensitivity of this test will be limited in formalin fixed paraffin embedded (FFPE) tissues because of nucleic acid degradation during the fixation process. Real-time quantitative PCR (RT-qPCR) permits high-throughput analysis, but is expensive. Conventional PCR remains the preferred method, especially in resource-poor settings. Recently, a commercial "Diagene 2 kit (Hybrid capture 2) has been promoted to screen large numbers of samples in a short time frame. This, however, is currently very expensive, 100 tests costing approximately 2000 USD (www.eigen.com, accessed on 12 February, 2015). In situ hybridisation (ISH) for virus and immunohistochemistry for p16 protein are highly sensitive, but lack specificity: a recent study by Boy et al. showed 12% HPV positivity in oral squamous cell carcinoma with quantitative real-time PCR, but zero with ISH on the same samples [161].

Management protocols for HNC patients differ from person to person depending on anatomical location, age, co-morbidities and tumour stage [55,162-163]. Recent reports indicate better...
survival and treatment outcomes for HIV-associated HNSCC patients compared to HIV-negative cancer patients, permitting de-

6. Conclusion

In the past 30 years, there has been growing evidence of a significant involvement of HPV in head and neck squamous cell carcinoma. This review covers available data from the Asia Pacific as a whole, a region where tobacco-related HNSCC is a major concern, but suggests that HPV-related cancers are also significant. The prevalence of high risk HPVs appears similar to other countries. Further studies using the best techniques available with clear site-specific (preferably using ICD10 codes) and larger & better-defined populations with more appropriate control subjects & tissues are clearly needed. It would be of value to screen populations at risk for carriage and persistence of high-risk genotypes, especially in subjects with existing oral potentially malignant disorders.

Conflict of interest

None.

Authors contribution

MHS devised the protocol and performed the electronic search of the published literature. All authors agreed on selected papers. He also extracted data and prepared the meta-analyses, tables and diagrams. He wrote the 1st full draft, corrected versions and agreed the final manuscript. NMJ advised and contributed on molecular aspects and agreed the final manuscript. NWJ contributed to data interpretation, critical correction, manuscript revision and agreed the final manuscript.

Acknowledgement

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References


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CHAPTER 4

Prevalence and types of high-risk HPVs in head and neck cancers from Bangladesh
Introduction:

Head and neck cancer (HNCs) is one of the commonest cancers in South Asia, mostly affecting males and commonly related with extensive use of tobacco both in smoking and smokeless forms. Although the rising incidence of Human papillomavirus (HPV) – associated head and neck squamous cell carcinoma (HNSCC) is well documented in the western world, studies from South Asia are limited and inconsistent. Little is actually known about the HPV-positive HNSCC prevalence in Bangladesh. Since Bangladesh has a large number of HNSCC patients and the HPV-associated HNSCC is increasing in the world, it has become necessary to understand the actual association of HPV with HNSCC in these populations. Therefore, in this chapter we attempted to investigate the prevalence of HPV in HNSCC in Bangladesh and explored the possible value of cell cycle markers in clinical diagnostic settings.

The work in this chapter has been constituted as an article and submitted for publication.
Prevalence and types of high-risk HPVs in head and neck cancers from Bangladesh

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Novelty and Impact: Most head and neck squamous cell carcinomas in South Asia are regarded as tobacco-related and treated accordingly. We show that up to 21% are associated with high-risk HPV types, known to be amenable to treatment deintensification. Screening for HPV should become routine, worldwide, as part of treatment planning for such cases.

Abstract:

There is a dramatic rise in the incidence of Human papillomavirus (HPV) – associated head and neck squamous cell carcinoma (HNSCC) in the world, with considerable variation by geography, gender and ethnicity. Little is known about the situation in Bangladesh, where tobacco-related head and neck cancers (HNCs) are the most common cancers in men. We aimed to determine the prevalence of HPV in HNSCC in Bangladesh and to explore the possible value of cell cycle markers in clinical diagnostic settings. A total of 196 archival HNSCC tissue samples were analysed for the presence of HPV DNA. DNA quality was assured, then amplified using a nested PCR approach. Typing was by automated DNA sequencing. Cellular markers, p53, Cyclin D1 and pRb were tested on all samples by immunohistochemistry (IHC), as well as p16 as a surrogate for PCR detection of HPV. HPV DNA was detected in 36/174 (~21%): ~37% of cancers in the oropharynx; 20% or oral cancer, 14.1% for the larynx, and 15% for hypopharynx. HPV-16 was most common, being present in 33 samples, followed by HPV-31 (2 samples) and HPV-33 (1 sample). Twenty-eight out of 174 samples were positive for p16, predominantly in HPV-positive tissues (p<0.001). No statistically significant association was observed between the cellular markers and HPV DNA positive cases. However p16 positivity had excellent predictive value for the presence of HPV by PCR. In conclusion, there is a significant burden of HPV-associated HNSCC in Bangladesh, particularly in the oropharynx but
also oral and laryngeal cancers. Whilst a combination of PCR-based DNA detection and p16 IHC is useful, the latter has excellent specificity, acceptable sensitivity and good predictive value for carriage of HPV and should be used for prognostic evaluation and treatment planning of all HNSCC patients in South Asia, as in the Western world.

**Keywords:** Head and neck squamous cell carcinoma (HNSCC), Human papillomavirus (HPV), Nested Polymerase chain reaction (PCR), Immunohistochemistry (IHC), South Asia, Bangladesh.

**Introduction:**

Head and neck cancer (HNC) is a major health problem worldwide, with an annual incidence of approximately 600,000 cases and close to 300,000 deaths, mostly in less developed countries (Globocan 2012)(Ferlay et al., 2015). Whilst most – 80-90% in some countries - are squamous cell carcinomas and variants thereof, neoplasms in this region are diverse at clinical and biological levels, which make them difficult to manage. Although tobacco, areca nut and alcohol are the major risk factors for HNSCC, infection with high-risk types of Human papillomavirus (HPV) has been shown to be strongly associated with a significant proportion of cases(Gillison and Shah, 2001). This association varies by anatomical site/subsite, with a predilection for
mucosa associated with the lymphoid aggregations of Waldeyer’s ring, and are thus seen in the oropharynx, especially base of tongue and palatine tonsils, compared to the oral cavity, larynx and hypo-pharynx (Rautava and Syrjanen, 2012). Recently, the International Agency for Research on Cancer (IARC) has acknowledged HPV as an aetiological factor for oropharyngeal squamous cell carcinoma (OPSCC) (Dalianis, 2014; 2012). HPV-associated HNSCC represents a distinct entity with increase in incidence over the last three decades, mostly in developed countries and commonly affecting young adult males who tend to be non-smokers, non- or light- drinkers and many have relatively high socioeconomic status (Gillison et al., 2015). It is suggested that this is related to changing sexual behaviour, with an increase in oral-genital contact (D’Souza et al., 2009), sexual debut at early age and a high number of lifetime sex partners (Schwartz et al., 1998). Because HPV-related HNC patients have significantly better treatment response and 3-year overall survival rates (82.4% vs 57.1%) irrespective of age, gender or tumour stage (Ang et al., 2010, Bonilla-Velez et al., 2013), knowledge of HPV status is mandatory in most Tumour Boards for the planning of treatment.

Approximately one-third of the total HNC cases in the world have been shown to be associated with high-risk HPV infection, but wide geographic variation exists (Ang et al., 2010, Shaikh et al., 2015).

The prevalence of HPV in HNC, especially, OPSCC is much higher in North America (~70%) and Europe (~50%) compared to rest of the world (Stein et al., 2015). An increasing trend is noticed in Australia, where HPV-positive OPSCC rose from 20% to 63% of cases over the last two decades (Hong et al., 2016). South Asia (including the Indian subcontinent) has the highest incidence rates and disease burden of
HNSCC in the world with approximately, 200,000 new cases each year and more than 100,000 deaths (GLOBOCAN 2012) (Ferlay et al., 2015, Shaikh et al., 2015). However, relevant studies in South Asian populations are few and inconsistent. HNSCC is the leading cancer in males and 3rd most common in females in India (Badwe et al., 2014). Bangladesh shares similar cultural & social norms as India. Likewise, HNSCC is also the most common cancer in males in Bangladesh, surpassing lung cancer (Hussain, 2013). As extensive use of tobacco (in smoking and smokeless forms) and chewing of areca nut dominate the risks for head and neck cancer across South Asia, less attention has been given to the role of HPV. There are no comprehensive data from Bangladesh. Because of the high burden of this disease in S and SE Asia it is essential to have accurate data on the role of HPV across the region.

The primary objective of our study was to investigate the prevalence of high risk HPV in HNSCC in a Bangladeshi cohort of patients; assembling tumours from different sites of the head and neck region. We also determined the concordance between commonly used HPV detection methods, namely; polymerase chain reaction (detects the presence of HPV DNA in tumour tissue) and p16 immunohistochemistry (IHC), a commonly used surrogate marker for HPV-associated cancers. Increased p16 expression is a direct consequence of E7 (HPV oncoprotein) -induced retinoblastoma (pRb) protein inactivation (no/low expression) (Boyer et al., 1996), and Cyclin D1 protein expression is dependent on intact pRb expression (Bates et al., 1994) Thus, analyzing the expression of both pRb and Cyclin D1 could provide useful prognostic information about the biological activity of HPV in HNSCC. Further, HPV-positive HNSCC is associated with low level of p53 expression due to the suppressing action
of another viral oncoprotein, HPV E6 (Scheffner et al., 1990). Based on these considerations, here we address the correlation of the potential prognostic markers (p16, p53, pRb and Cyclin D1) with HPV status. Our data provide insights into the relative burden and aetiology of HNCs in Bangladesh, likely generalisable to South Asia as a whole.

Materials and Methods:

Study population and data collection:

A total of 196 de-identified HNC cases are included. Patients were from Dhaka Medical College Hospital (DMCH) and from private clinics in the city. All were over 20 years of age, clinically and pathologically diagnosed with head and neck cancer between December 2014 and May 2016. The cancers were classified into different subsites of the head and neck following the ICD-10 classification: oral cavity (C02-C06), oropharynx (C01, C09 & C10), hypopharynx (C13) and larynx (C32). Cancers of the salivary glands and nasopharynx were excluded. Diagnostic biopsy specimens were preserved as formalin fixed paraffin embedded (FFPE) blocks in the Department of Pathology, DMCH and in A. I. Khan Pathology Laboratory, Dhanmondi, and Dhaka. Clinico-pathological data of tumour sites, tumour differentiation, and demography of patients were retrieved from the pathological records. The study was approved by the Griffith University Human Research Ethics Committee in Australia (GU Ref No: DOH/13/14/HREC) and DMCH Human Ethics Committee (Memo No. DMC/ECC/2016/32) in Bangladesh.
**HPV DNA detection & type determination:**

**DNA isolation and testing of sample integrity:**

The FFPE blocks were sectioned whilst maintaining utmost precautions to avoid inter-block contamination of DNA. This was achieved by pre-chilling and moistening each block in a separate ice container before sectioning, thorough cleaning of the microtome, single use of brush and forceps, changing gloves in between each block, changing the water bath for each block and using a new blade for each block. Genomic DNA was extracted from a 10μm thick section using Gene read FFPE kit (Qiagen, Germany). A section from a blank paraffin block was cut and processed along with the sections from the cases to check for any contamination. DNA concentrations were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). DNA integrity was assessed for 150 bp fragments of the Beta-actin housekeeping gene by PCR using the GoTaq Green PCR kit (Promega, Madison, WI). Nuclease free water was used as negative control, while genomic DNA from the HPV16 positive head and neck cancer cell line, UDSCC-2 (kindly provided by Dr. Schuwarz and Prof. Thomas Hoffman, University of Ulm, Germany) was used as a positive control in the first PCR run. From the 2nd PCR run onwards, samples that had shown positivity in the first PCR run were used as positive controls.

**Detection of HPV DNA by PCR:**
Nested PCR, consisting of two sets of degenerative/consensus primer pairs, MY09/11 and GP5+/GP6+ (Sigma-Aldrich, St. Louis, MO, USA) was used (de Roda Husman et al., 1995, Gravitt et al., 2000). A gradient PCR was performed to optimise the annealing temperature for each primer set. The HPV L1 gene was amplified using primers MY09/11 in the first round, followed by GP5+/GP6+ in the second round. The PCR reaction mix contained forward and reversed primers (0.5 µM of each), 1×PCR buffer (containing 1.5mM MgCl₂) (Phusion High-Fidelity 5×PCR Buffer, New England Biolabs, MA, USA), 200 µM of dNTPs (10mM dNTP Mix, New England Biolabs, MA, USA), 1.0 unit of Phusion DNA polymerase (Taq polymerase 1unit/50 µl, New England Biolabs, MA, USA) and nuclease-free water, up to a final volume of 20 µl. Positive and negative reaction controls were included in each PCR run. DNA amplification was carried out in an automated thermal cycler (Takara Bio Inc, Japan). Reactions were brought to 98°C for 30 sec (initial denaturation), followed by forty cycles consisting of a denaturing step for 10 sec at 98°C, an annealing step for 30 sec at 55.5°C (MY09/11 in first round) or 55°C (GP5+/GP6+ second round), and an extension step for 20 sec at 72°C. A final extension step at 72°C was carried out for 5 mins. A total of 2 µl of the first-round PCR product was used in the second round of amplifications.

The PCR products of the samples from both rounds were electrophoresed in 2% agarose gel prepared with 1x TAE (Tris-acetate-EDTA) buffer (DNA Agar, Marine Bio Products Inc., Quincy, MA, USA), stained with 0.5 g/mol of ethidium bromide (Merck, KGaA, Darmstadt, Germany) and visualized under ultraviolet light using the Chemi-Doc machine (BioRad, USA). The size of the amplified product was determined by comparing with a base-pair ladder size marker for HPV-L1, (Quick Load, 100bp DNA Ladder, New England Biolab, MA, USA). Any sample that
showed a positive band in the gel for both first (band size 450bp) and second (band size 150bp) round of PCR was taken as an HPV-positive sample and purified for sequencing.

*Sequencing for HPV type:*

A PCR DNA purification kit (Qiagen, Germany) was used to purify the PCR product of the HPV-DNA positive samples detected in gel electrophoresis. These were submitted to the Australian Genome Research Facility (AGRF) for automated sequencing. Sequences were compared with available HPV genome sequences in Genebank using the NCBI (National Center for Biotechnology Information) Blast programme.

*Histological diagnosis:*

Adjacent H&E sections were used to confirm diagnoses and to grade. Grades 1, 2 and 3 are referred as well, moderately and poorly differentiated, respectively (WHO, 2005).

*Immunohistochemistry Analysis:*
Immunohistochemistry (IHC) for p16, p53, pRb and Cyclin D1 was performed in the laboratories of MHIQ and/or Gold Coast University Hospital, using DAKO histology kits (DAKO, Agilent, Santa Clara, CA), some manually, others in an Intellipath (Biocare Medical, Concord, CA) autostainer. Briefly, from each FFPE block, a 4um thick section was cut and affixed to Menzel-Glazer super-frost plus slides (Thermo Fisher Scientific, Waltham, MA) and air-dried at 37°C for 48 hours. Slides were preheated at 60°C, followed by de-waxing and re-hydration using Xylene, ethanol and water. Antigen retrieval was performed with the DAKO EnVision Kit (DAKO, Agilent, Santa Clara, CA) followed by TBS wash. Staining with primary antibodies was according to the protocol recommended by DAKO, using an Intellipath autostainer, where staining steps and incubation times were programmed according to the DAKO EnVision FLEX Mini Kit protocol. A similar procedure was followed for manual staining. The primary antibodies mouse anti-p16^{INK4a} (#2D9A12; DAKO, Agilent, Santa Clara, CA), mouse anti-human p53 (#DO-7; DAKO, Agilent, Santa Clara, CA), rabbit anti-human Cyclin D1 (#EP12, DAKO, Agilent, Santa Clara, CA), and rabbit anti-human pRB (#9308; Cell Signaling, Danvers, MA), were optimized for antibody concentration and incubation time according to their respective company protocols. Positive and negative IHC controls were used in every run. A tonsillar SCC with high p16 expression was taken as positive control for p16 IHC; for p53 a positive colon cancer; for Cyclin D1 and pRb, a known positive tonsillar carcinoma.

IHC slides were scored independently by three head and neck pathologists (AL, MS & VG). Discordant cases were few and agreed by discussion. Cases with moderate to strong staining of all neoplastic areas were recorded as positive, while sections with weak and focal staining were regarded as negative. To be regarded as p16 positive, sections had to show both nuclear and cytoplasmic staining in 50% or more of
neoplastic cells. (Lewis, 2012, Liu et al., 2015). For p53, Cyclin D1 and pRb proteins, in which reaction product is present in nuclei only, slides were scored dichotomously, negative being <10% of cells staining, positive being >10%. (Shiraki et al., 2005, Rodriguez-Pinilla et al., 2004) (Shin et al., 1996, Shiraki et al., 2005). Typical staining patterns of each cellular marker are presented in Figures 1 (a) to 1(d).

Statistical Analyses:

These were carried out using SPSS version 22. To compare the characteristics of HPV-positive and -negative patients, $\chi^2$ or Fischer’s exact tests were used. However, to determine the mean age differences between HPV-positive and -negative groups, an independent t-test were used. Spearman’s rank coefficient was used to analyse possible correlations among and between p16, p53, pRb and cyclin D1 expression levels. All analyses were two-sided and p-values below 0.05 were regarded as significant. In addition, the predictive values of p16 for pcr-determined HPV status were examined by standard 2X2 table analyses, and the sensitivity and specificity calculated. (Gardner and Greiner, 2006, Altman DG, 2000).

Results:

Patient demography:

A total of 174 of the 196 blocks were analysed: 22 did not contain PCR-amplifiable DNA as determined by B-actin. The mean age of patients was 54.2 (Table 1): 138
(~80%) were men and 36 (~20%) women. Primary tumour sites were: oral cavity 55 (31.6%), oropharynx 35 (20.1%), larynx 64 (36.8%) and hypopharynx 20 (11.5%). The majority, 92 (52.9%), were moderately differentiated; followed by well differentiated 48 (27.6%) and poorly differentiated 34 (19.5) (Table 1) (Figure 1).

![Figure 1: (a) Well differentiated SCC showing keratin pearls, (b) Moderately differentiated SCC with nuclear pleomorphism and limited keratinization, (c) Poorly differentiated SCC having cells with high nucleus:cytoplasm ratio. (10x magnification).](image)

**Presence of HPV DNA and HPV type:**

Overall, 36/174 (21%) of blocks were positive for HPV DNA (Figure 2). HPV prevalence was significantly higher for tumours in the oropharynx (37.1%), followed by oral cavity (20.0%), larynx (14.1%) and hypopharynx (15.0%). Sequencing showed that HPV-16 was most common, 33/36 (94%) of the HPV-positive tumours, followed by HPV-31 (2 cases) and HPV-33 (1 case).
Figure 2: Gel electrophoresis of the 2nd round Nested PCR of 11 samples. The product size is 150 base pairs. Seven of 11 samples are showing positive for HPV DNA.

HPV-positive patients were younger (mean ~54 years) than HPV-negative cases (57.2 years) and HPV prevalence was inversely correlated with age, \( (p=0.014) \). A higher proportion of male cases were HPV-positive (30/138, 21.7% cf 6/36, 16.7% for women) but this was not statistically significant (Table 1) than women. Among the HPV-positive cases, a significantly higher proportion fell into the moderately differentiated group \( (p = 0.011) \) (Figure 3).
Figure 3: (a) Poorly differentiated HPV-associated SCC of tonsil (H&E), in this area beneath an intact surface, (b) Intense staining of nuclei and cytoplasm for p16 in the same tumour (20X magnification of original image).

Correlation between presence of HPV DNA and p16 expression:

Only 28 of 174 samples (~16%) exhibited p16 overexpression, mostly in HPV positive cases (p <0.0001) (Table 1). Of the 36 cases positive for HPV DNA, 26 (72%) showed p16 overexpression (true positive), whereas 138 (94%) of the 146 cases were negative for both HPV DNA and p16 (true negative). The number of discordant cases was 12 (7%): 10 being positive for PCR but negative for p16, 2 being positive for p16 but negative for PCR (Table 2). Taking PCR data as standard, p16 as a surrogate marker for presence of HPV showed excellent specificity of almost 99%, an acceptable sensitivity of 72%, with both Positive Predictive Value and Negative PVs of 93% (Table 3).
<table>
<thead>
<tr>
<th>Molecular Markers</th>
<th>Low expression (Score 1)</th>
<th>High expression (Score 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) p16</td>
<td><img src="image1.png" alt="p16 Low Expression" /></td>
<td><img src="image2.png" alt="p16 High Expression" /></td>
</tr>
<tr>
<td>(b) p53</td>
<td><img src="image3.png" alt="p53 Low Expression" /></td>
<td><img src="image4.png" alt="p53 High Expression" /></td>
</tr>
<tr>
<td>(c) pRb</td>
<td><img src="image5.png" alt="pRb Low Expression" /></td>
<td><img src="image6.png" alt="pRb High Expression" /></td>
</tr>
<tr>
<td>(d) Cyclin D1</td>
<td><img src="image7.png" alt="Cyclin D1 Low Expression" /></td>
<td><img src="image8.png" alt="Cyclin D1 High Expression" /></td>
</tr>
</tbody>
</table>

**Figure 4:** (a) Typical p16 staining of both nucleus and cytoplasm of tumour cells (20X magnification). (b) Nuclear staining pattern of tumour cells by p53 (20X magnification). (c) Nuclear staining pattern of pRb (20X magnification). (d) Low to strong Cyclin D1 staining of nuclei (20X magnification).
Expression of cell cycle proteins: (Figure 4).

Overexpression of p53 was seen in overall half of cases, 86/174 (~49%), a higher proportion in the HPV-positive cases 23/36 (63.9%) compared to HPV negative cases (63/138 (45.7%) of positivity rates for Cyclin D1 and pRb were 62/174 (35.6%) and 66/174 (37.9%) respectively, predominantly in HPV-negative cases: 49/62 (79%) and 51/66 (77.3%), respectively. However, none of these differences were statistically significant (Table 1).

Correlations between over-expressions of cell cycle proteins and presence of high-risk HPV infection are presented in Table 4. There was a highly significant positive correlation between HPV and p16 status (p < 0.01) and a low level of association between HPV status and p53 expression. There was a slight inverse (negative) relationship observed between HPV status and pRb and Cyclin D1 expression. These negative correlations indicate malignancy may not need both Cyclin D1 and pRb pathways. However, there was no significant correlation between the HPV status and expressions of p53, pRb and Cyclin D1 markers.

Among the cell cycle proteins, a significant positive association was observed between p16 and p53 expression (p value < 0.05). However, no significant interrelationship has been identified among p16, Cyclin D1 and pRb expression. There were significant correlations between p53 & Cyclin D1 (p < 0.05), and between p53 & pRb (p <0.01) but at a low to moderate levels (r value 0.177 and 0.222, respectively) (Table 4). A significant association is also seen between Cyclin D1 and pRb proteins but at low level.
Table 1. Demographic and clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients (n=174) (%)</th>
<th>HPV DNA+ve (n=36) (%)</th>
<th>HPV DNA-ve (n=138) (%)</th>
<th>X² (Chi-square)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age</td>
<td>56.6</td>
<td>54.2</td>
<td>57.2</td>
<td>0.143</td>
<td></td>
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<tr>
<td>Age groups (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 – 59</td>
<td>94 (54.1)</td>
<td>26 (72.2)</td>
<td>68 (49.3)</td>
<td>6.053*</td>
<td>0.014</td>
</tr>
<tr>
<td>60 and above</td>
<td>80 (45.9)</td>
<td>10 (27.8)</td>
<td>70 (50.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>138 (79.3)</td>
<td>30 (83.3)</td>
<td>108 (78.3)</td>
<td>0.448</td>
<td>0.503</td>
</tr>
<tr>
<td>Female</td>
<td>36 (20.7)</td>
<td>6 (16.7)</td>
<td>30 (21.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary Tumour sites</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oral Cavity (C02-C06)</td>
<td>55 (31.6)</td>
<td>11 (20.0)</td>
<td>44 (80.0)</td>
<td>8.412*</td>
<td>0.038</td>
</tr>
<tr>
<td>Oropharynx (C01, C09 &amp; C10)</td>
<td>35 (20.1)</td>
<td>13 (37.1)</td>
<td>22 (62.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx (C32)</td>
<td>64 (36.8)</td>
<td>9 (14.1)</td>
<td>55 (85.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypopharynx (C13)</td>
<td>20 (11.5)</td>
<td>3 (15.0)</td>
<td>17 (85.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histopathological Grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Well differentiated</td>
<td>48 (27.6)</td>
<td>4 (11.1)</td>
<td>44 (31.9)</td>
<td>8.947*</td>
<td>0.011</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>92 (52.9)</td>
<td>20 (55.6)</td>
<td>72 (52.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>34 (19.5)</td>
<td>12 (33.3)</td>
<td>22 (15.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunohistochemistry (IHC) Analysis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>p16 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>28 (16.1)</td>
<td>26 (72.2)</td>
<td>2 (1.4)</td>
<td>105.914*</td>
<td>0.0001</td>
</tr>
<tr>
<td>Negative</td>
<td>146 (83.9)</td>
<td>10 (27.8)</td>
<td>136 (98.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>86 (49.4)</td>
<td>23 (63.9)</td>
<td>63 (45.7)</td>
<td>3.799</td>
<td>0.051</td>
</tr>
<tr>
<td>Negative</td>
<td>88 (51.6)</td>
<td>13 (36.1)</td>
<td>75 (54.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>62 (35.6)</td>
<td>13 (36.1)</td>
<td>49 (35.5)</td>
<td>0.05</td>
<td>0.946</td>
</tr>
<tr>
<td>Negative</td>
<td>112 (64.4)</td>
<td>23 (63.9)</td>
<td>89 (64.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRb expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>66 (37.9)</td>
<td>15 (41.7)</td>
<td>51 (37.0)</td>
<td>0.269</td>
<td>0.604</td>
</tr>
<tr>
<td>Negative</td>
<td>108 (62.1)</td>
<td>21 (58.3)</td>
<td>87 (63.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Concordance between HPV positive nested PCR and p16 IHC results.

<table>
<thead>
<tr>
<th>P16 by IHC</th>
<th>HPV DNA detection by PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV positive</td>
<td>HPV negative</td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>136</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>138</td>
</tr>
</tbody>
</table>

Table 3. Sensitivity and specificity between HPV DNA detection by PCR (“the disease”) and p16 by IHC.

<table>
<thead>
<tr>
<th>Point estimate</th>
<th>Value</th>
<th>Confidence limits (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>72.22%</td>
<td>54.81% to 85.80%</td>
</tr>
<tr>
<td>Specificity</td>
<td>98.55%</td>
<td>94.86% to 99.82%</td>
</tr>
<tr>
<td>Positive Likelihood Ratio</td>
<td>49.83</td>
<td>12.40 to 200.21</td>
</tr>
<tr>
<td>Negative Likelihood Ratio</td>
<td>0.28</td>
<td>0.17 to 0.48</td>
</tr>
<tr>
<td>“Disease” prevalence</td>
<td>20.69% (*)</td>
<td>14.93% to 27.47%</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>92.86% (*)</td>
<td>76.50% to 99.12%</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>93.15 % (*)</td>
<td>87.76% to 96.67%</td>
</tr>
</tbody>
</table>

* Does not reflect real prevalence of the “disease”.

Table 4. Agreement between HPV status and cell-cycle proteins in HNSCC tumours.

<table>
<thead>
<tr>
<th></th>
<th>HPV</th>
<th>p16</th>
<th>p53</th>
<th>Cyclin D1</th>
<th>pRb</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>0.766**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>0.132</td>
<td>0.224**</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>- 0.005</td>
<td>0.01</td>
<td>0.177*</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>pRb</td>
<td>- 0.001</td>
<td>0.077</td>
<td>0.222**</td>
<td>0.160*</td>
<td>1.00</td>
</tr>
</tbody>
</table>

** p <0.01, **correlation is significant at 0.01 level (2-tailed)
* p <0.05, *correlation is significant at 0.05 level (2-tailed)
Discussion:

Epidemiological, clinical & molecular studies indicate that high-risk HPV plays a pivotal role in the aetiopathogenesis of some HNSCCs. It is well documented that HPV-associated HNSCC are mostly seen in the oropharyngeal region due to the presence of lymphoid tissue, which makes it vulnerable to HPV infection (Rautava and Syrjanen, 2012). An increasing trend of HPV-associated HNSCC, especially oropharyngeal cancer, is seen in developed countries, where tobacco and alcohol related HNSCC cases are decreasing (Chaturvedi et al., 2013). Similar trends are not (yet) seen in South Asia, South East Asia or East Asia, perhaps partly because there are so far few data from these regions. Such studies as have been published have small sample sizes and have examined mostly cancers of the oral cavity, possibly because this is the dominant site for HNSCC, most of which are tobacco and alcohol related (Shaikh et al., 2015).

Bangladesh, being the 3rd most populous country in South Asia and 8th in the world (~160 million), has the highest HNSCC incidence in the region, 21/100,000 per annum (approximately 25,000 p.a. new cases), mostly affecting males (Ferlay J, 2013). The mortality rate is very high, approximately 16,500 deaths annually (15/100,000 p.a.), about half of which are from cancers of the oropharynx (~ 8,500 p.a.) (Ferlay J, 2013). A survey by WHO in 2004 estimated that ~130,000 head and neck cancer patients existed in Bangladesh (Hussain and Sullivan, 2013). Although tobacco (smokeless or smoking) is the major risk factor, a recent study gives a reduction of tobacco use from 42.4% to 36.3% between years 2009 and 2012, (Nargis et al., 2015) among those aged 15 and above: in the 40 – 54 year age group, this fell
from 64% to 54%. Bangladesh is a rapidly “progressing” country, embracing much of the good and bad characteristics of western culture. This is associated with reductions in the use of smokeless tobacco, and increasing smoking rates. There is no published evidence to assess changes in sexual behaviours, and it has become necessary to investigate the true prevalence of HPV-associated HNSCC in the country and the wider region.

The reported prevalence of HPV-associated HNSCC varies widely across the world (Ndiaye et al., 2014). Possible reasons for this include geographical location, sexual behaviours and other lifestyle factors, inclusion of mixed ethnicities and, importantly, differences in detection methods. Sensitivities of the latter are critical, so that lesions with low copy numbers of HPV may fail to be correctly ascribed. Although PCR-based assay, ISH and p16 IHC are widely available, there is no consensus on the optimum technique for routine screening. Recent studies have largely used PCR which is sensitive and cost effective (Venuti and Paolini, 2012, Ndiaye et al., 2014). However, these can be too sensitive and may amplify contaminant HPV from the laboratory environment if appropriate measures are not taken. ISH offers specificity, but it is time consuming, has background staining and is less sensitive (Smeets et al., 2007). It is necessary to use a highly sensitive but controlled detection system, a well-characterised study population and site-specific tissue samples for accurate estimation of HPV prevalence in HNSCC.

We applied a combination of nested PCR- for detection of HPV DNA and IHC for expression of p16 (Braakhuis et al., 2009). Combination of two sets of primers in nested PCR has been shown in previous studies to be efficient and accurate with oropharyngeal tissue (Winder et al., 2009, Fuessel Haws et al., 2004, Syrjanen, 2010).
The p16 protein has recently emerged as an important biomarker for HPV in HNSCC. In healthy cells, pRb (a cell cycle check point protein) normally suppresses the transcription of p16 protein. However, in HPV-related cancers, pRb protein is functionally inactivated by HPV E7 protein, leading to overexpression of p16 (Klussmann et al., 2003, Adelstein et al., 2009). While consensus PCR is highly sensitive, without p16 IHC, the clinical relevance of HPV infection might be falsely interpreted as the presence of HPV DNA does not necessarily indicate that the virus is biologically active in the tumour (Smeets et al., 2007). On the other hand, simplicity, cost effectiveness and high sensitivity make p16 IHC attractive as a surrogate marker, even in the absence of a direct mechanistic association between HPV integration and p16 overexpression. Moreover, SCC of the oral cavity and of the larynx typically bear low numbers of transcriptionally active HPVs, suggesting that the expression of p16 at these sites may be elevated via a non-viral mechanism, leading to a false positive interpretation (Bishop et al., 2012). In spite of this, the specificity of p16, and its acceptable sensitivity, makes it a valuable tool. In an ideal world, both methods would be used.

A recent study from Australia showed higher discordance between the detection of HPV DNA in HNSCC tissue samples [50/248 (20%)] and p16 IHC [61/248 (28%)] (Antonsson et al., 2015). A study from the USA had the opposite result, with 54/79 (61%) of cases HPV DNA positive but only 19/79 (24%) p16 positive (Weinberger et al., 2006). The reasons for these discordances could be either the high sensitivity of nested PCR or the low sensitivity of p16 IHC (especially in the oral cavity and larynx) due to somatic alterations in chromosome 9 (Combes and Franceschi, 2014). Moreover, subjective evaluation/lack of standardised p16 scoring criteria make
comparision of different studies dangerous. Although a high cut-off point for p16 staining (> 50% tumour cells moderately or strongly stained) has been used for cervical cancer, a low cut-off point, used for cases with limited p16 staining, has the potential for over-diagnosing the involvement of HPV (Sano et al., 1998). It is not clearly understood whether HPV DNA positive cancers with limited p16 positivity are HPV-driven or whether the p16 is silenced by mutations or by DNA methylation of promoter regions, as has been reported in cervical cancers (Nuovo et al., 1999).

The overall worldwide prevalence of HPV in HNSCC averages approximately 30% with wide variation depending on geographical location and tumour sites (Ndiaye et al., 2014). Few studies from South Asia have been published; the majority from India, and these suggest an overall prevalence of approximately 37% (Shaikh et al., 2015). We found 21% of total HNSCC cases positive for HPV DNA in this Bangladeshi series, highest in the oropharynx (~ 36%), lowest in the hypopharynx (~11%). High prevalence in oral cavity SCC (OSCC) has been reported from South East Asia (~48%), Eastern Asia (~43%) and South Asia (~38%) (Shaikh et al., 2015); whereas our study shows lower HPV frequencies in OSCC (20%). In oropharyngeal cancer, our study shows the prevalence of HPV to be higher (37.1%) compared to a recent Indian study, where the proportion was approximately 23% (Bahl et al., 2014). Our findings for laryngeal cancer (~14.1%) are closed to the overall HPV-positive laryngeal cancer prevalence in the Asia-Pacific region (23.6%) (Shaikh et al., 2015).

To our knowledge, there is only one published study from Bangladesh describing the prevalence of HPV in HNSCC, using conventional PCR methods. This had a small sample size (n=34), included samples only from the oral cavity and found just 3% of
HPV positive cases (Akhter et al., 2013). Possible reasons for this discordance could be less sensitive detection method or samples having poor DNA quality.

HPV16 is the most commonly detected HPV type, being present in 90% of cases of HPV-positive HNSCCs worldwide (Kreimer et al., 2010). Our data are similar: 34/36 (~94%) and all were single HPV infections. Similar findings are reported from North America, Europe and the Asia-Pacific. (Ndiaye et al., 2014, Shaikh et al., 2015) This is likely to be related to social norms, as a relatively high proportion of men in western countries tend to have multiple sexual – including the practice of oral sex – partners. Male behaviour could be a contributing factor in the USA, as men tend to have more lifetime sexual partners (59.7% of cases seen in men vs 41.0% in women) and oral sex partners (32.4% of cases in men vs 17.6% in women) compared to women (D'Souza et al., 2014).

Our data show the prevalence of HPV to be inversely correlated with age, a significantly higher prevalence being seen in those less than 60 yrs old (mean age 54.2), compared with older patients (p = 0.025). This accords with the mean age reported from Australia (55.2 years), North America (58 years) and Europe (< 60 years), although the mean age of our Bangladeshi HPV-positive HNSCC patients was slightly higher than reported from other South Asian countries (52.8 years), especially India (Shaikh et al., 2015, Gillison et al., 2015).

Several recent studies have demonstrated the prognostic value of cell cycle markers in HPV-positive HNSCC patients. A recent cohort study suggests that high p16 expression is correlated with better survival: p16 positive and HPV-positive cases had a 2 year disease free survival of 86.2%, (95% CI 79 – 91.1) compared to p16 negative and HPV –negative cases of only 44.2%, (CI 30.2 – 58.1) (Lewis et al.,
Some studies have suggested that high expressions of p53, Cyclin D1 or pRb also relate to poor prognosis. For example, a study from Sweden suggests that patients with HPV+ve DNA and low/absent p53 had a 5-year survival of 88.2% compared to 33.3% for HPV+ve cases with high p53 expression. Patients with HPV-negative and low/absent p53 had a better survival than HPV-negative but high p53 cases (52.6% vs 9.1%) (Sivars et al., 2014).

A comprehensive study of 226 patients from Australia suggests a strong association between HPV positivity and downregulation or absent expression of Cyclin D1. In an HPV-positive tumour group, Cyclin D1-positive cancers had 8 fold-increased risk of poor prognosis compared to Cyclin D1-negative cancers with 3.3 years overall survival. However, the effect of Cyclin D1 was small in HPV-negative HNSCC (Hong et al., 2011). A strong inverse relationship between pRb and p16 expressions has also been reported: cases with low pRb and high p16 expression had better survival (Holzinger et al., 2013).

In our study, HPV-positive cases tended to have high expression of p16, low expression of Cyclin D1 and of pRb protein and moderate p53 expression levels, but none of the associations are statistically significant, consistent with previously reported studies from China, India and Australia (Ma et al., 1998, Mitra et al., 2007, Antonsson et al., 2015).

There are several limitations to the present study. We have a modest cohort size, with small numbers in some anatomical subsites. Use of FFPE archival samples resulted in poor quality of DNA in some samples, which had to be excluded. Further, the E6/E7 mRNA expression analysis was not possible due to the lack of fresh tissue sample. We also have limited information on tobacco, areca nut and alcohol habits, and on the
sexual lives of our cases. These may lead to differences in identifying and categorising HPV-positive tumours accurately. Most importantly no treatment and patient outcome information was available to us.

Patients are from a single hospital and single pathology laboratory, which may limit generalizability. However, this is the largest public hospital in the nation and receives a wide range of patients and tissue samples from all corners of Bangladesh. This is the first comprehensive study from Bangladesh and one of the first studies from South Asia to use a combination of detection methods for HPV and their interrelationship with cell cycle markers of putative prognostic value.

**Conclusion:**

Our data show that HPV is associated with, and probably responsible for, ~21% of HNSCC in Bangladesh. Because it is now well known that such cases respond comparatively well to treatment, routine assessment of HPV status in HNSCC should be mandated. We strongly recommend the use of ICD-10 for proper site-specific classification of cases. We urge the Bangladesh Government to mandate tumour boards and perform longitudinal studies on HNC to confirm the importance of HPV in treatment planning. We recommend the use of both p16 IHC and PCR-based detection of virus, though p16 alone is a satisfactory surrogate marker. Routine use of IHC for the status of p53, pRb and cyclin D1, does not seem to be indicated.
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CHAPTER  5

HNSCC relies on HPVE7 for their HPV-associated cancer progression
Introduction

To establish a causal relationship between a risk factor and cancer requires epidemiological information, molecular pathological studies and experimental evidence (Carbone, Klein, Gruber, & Wong, 2004). Almost all (more than 99%) of cervical cancer cases are caused by HPVs. A number of epidemiological and molecular pathological studies have also suggested the aetiological association of HPV, especially type 16, with a subset of head and neck squamous cell carcinoma (HNSCC). However, the actual role of HPV in HNC initiation and progression is not as well understood as it is in cervical cancer.

It is well documented that the HPV-driven malignant transformation in cervical cancer is correlated with certain molecular events, which are mediated by the functions of HPV E6 and E7 onco-proteins. Numerous experimental studies have indicated that continuous expression of the $E_6$ and $E_7$ oncogenes is essential for maintaining the transformed phenotype in cervical cancer (Klaes et al., 1999). Further, the continued combined effects of $E_6$ and $E_7$ oncogenes targeting and inactivating the p53 and pRb proteins leads to cell cycle disruption. Therefore, several studies have focused on developing approaches, such as antisense RNAi strategies, against these viral oncogenic products, repressing the expression of $E_6$ and $E_7$ (Goodwin & DiMaio, 2000; Hall & Alexander, 2003). However, the effects of repressing HPV16 E6 and E7 on head and neck cancer cells are less studied.

Although the molecular pathological studies have shown the presence of high-risk HPV types (HPV16, HPV18, HPV31 and HPV33) in HNSCC tissues, this does not prove a causal relationship (Gillison et al., 2000). Recent studies on HNSCC
(especially oropharyngeal cancer) have suggested HPV-positive HNSCCs that express transcriptionally active HPV share similar molecular features as cervical cancer, including maintenance of a wild type $p53$ gene and a high level of $p16$ expression (Gillison & Shah, 2001; Weinberger et al., 2006). However, a lack of HPV-positive HNSCC cell lines has made these studies difficult. Nevertheless these data suggest that HPV-positive HNSCC cells may also rely on oncogene expression for cancer maintenance and progression. This is a key question in HNSCC biology and, therefore, in this study we investigated the role of HPV $E6/E7$ genes on HPV-positive HNSCC maintenance on four transcriptionally active HPV16-positive HNSCC cell lines and a HPV negative cell line (UMSCC1). Further, we assessed the possibility of employing $E6/E7$ targeted siRNA as a therapeutic model for HPV-positive HNSCC.

5.1. Results:

5.1.1. siRNA targeting of the $E6/E7$ genes of HPV:

The first approach was to select an siRNA that targets HPV oncogenes. We and others have published a number of studies demonstrating the use of potent $E6/E7$ siRNAs that led to the inhibition of cancer cell growth in cervical cancer settings (both $\textit{in vitro}$ and $\textit{in vivo}$) (Putral L et al. 2005, Gu W et al. 2006, Wu S. et al. 2011 and Liu W-I et al. 2009). $E6$ and $E7$ transforming genes are transcribed from the same promoter (P97 in HPV16) and are expressed from a bicistronic open reading frame with 3 splice variants, all of which express $E7$ and a single variant encoding the full length of $E6$ onco-gene. Careful selection of targets for these siRNAs allows for the silencing of
both genes. For this study, E6/E7 siRNA (sequence 10, denoted here as si-E6/E7) (shown in section 2.2.2) was chosen as it has previously been shown to be highly effective both \textit{in vitro} and \textit{in vivo} in silencing E6 and E7 in cervical cancer cell lines and highly specific to HPV type 16 (Purtal L. et al. 2005). A dose of 40nM was chosen as this concentration was previously described as the highest most effective dose out of a pool of various concentrations of siRNA that ensured effective silencing of E6 and E7 transforming genes with minimal off target effects in cervical cancer settings (Putral L et al. 2005). However, siRNA doses higher than 40nM are more likely to induce off target effects.

We firstly set out to show that si-E6/E7 was effective in the cervical cancer cell line, Caski, as previously shown. This would show that the method of transfection was effective and establish the basis for further work on HNSCC. Cells were treated with 40nM of siRNA (control and siE6E7) and 48 hours later the level of E7 was determined by qPCR (as described in section 2.3.8). We found a knockdown of E7 mRNA of approximately 80%, while controls showed no non-specific reductions had occurred (Figure 5.1 a). We next examined whether this loss of E7 expression would result in a loss of cell viability. Transfections were carried out as before and at 72 hours cell viability was determined using Cell-Titre-Glo, which measures the level of ATP in cells indicating the presence of live cells. It can be seen that si-E6/E7 resulted in a 70% loss of viability (Figure 5.1. b). The negative control for siRNA, siGFP, did not significantly reduce viability while the positive control, siPLK reduced viability but not as significantly as si-E6/E7. Overall these data show that si-E6/E7 works as expected and confirms that the methodology to examine E7 siRNA treatment is appropriate.
Figure 5.1. Evaluation of the capability of E6/E7 siRNA to reduce E7 expression level and inhibit cancer cell growth in cervical cancer cell line (CaSki).
(a) CaSki cells were transfected with 40nM of E6/E7 siRNA (red bars), siGFP (non-specific control siRNA) and a ‘mock’ transfection using transfection reagents only (oligofectamine) (control). Forty-eight hours after the treatment, the downregulation of oncogenic E7 mRNA was determined by quantitative real-time PCR (qPCR). (b) Following the treatment, the cellular viability was ascertained using a Cell Titre Glo assay at 72-hour time point. In this experiment siPLK1 was used as positive control. siGFP was used to observe non-specific cytotoxicity and siPLK1 was used to determine the mechanistic efficiency of siRNA in CaSki cells. The E7 mRNA expression knockdown analysis was performed by quantitative real time PCR (qPCR) using β-actin as housekeeping gene. The E7 mRNA expression levels of HPV+ve HNSCC cells were analysed using Rotor-Gene Q software and the graph was prepared using GraphPad Prism V.7. Each bar represents the mean +/- standard error of mean (SEM) (N=3).

5.1.2. Confirming HPV type 16 positive HNSCC cell lines:

As we have validated the effectiveness of an E6/E7 siRNA approach targeting HPV E6/E7 oncogenes in HPV16–positive cervical cancer cell line leading to cancer cell death, we next examined whether si-E6/E7 would work in the same way in a HPV16-positive HNSCC cell lines. Prior to this, we searched for HPV-positive HNSCC cell lines and imported all those available from various laboratories around the world, obtaining four lines (UM-SCC 104, UDSCC-2, UPCI:SCC 90 and 93 VU-147T – see
table 2.1 in chapter 2 section 2.1). We firstly set out for confirming the HPV type present in these cell lines using published primer pairs of HPV types 16 and 18 analysed the output by conventional PCR method as described in section 2.3.6. For type 16-positivity determination, the primer pair would amplify a 460bp band and CaSki cells were used as positive control. For HPV18, the primer pair would amplify a 150bp band and HeLa cells were used as positive control. Our findings show that all four HPV-positive HNSCC cell lines were HPV16 positive (Figure 5.2a). Both positive and negative controls behaved as expected with amplification from Caski but not HeLa cells. No amplification was observed in any of the HNSCC cells (Figure 5.2b). These results confirm that all four HPV-positive HNC cell lines were HPV type 16 positive.

Figure 5.2. Assessment of HPV16-positive HNC cell lines.
(a) All HPV-positive cell lines were examined for HPV type16 positivity using HPV16 E6/E7 primer pair (product size 460 bp) and visualized in 2% agarose gel stained with ethidium bromide. HeLa cells (HPV18 positive) and CaSki (HPV16 positive) were taken as negative and positive controls, respectively. (b) All HPV positive cell lines were analysed using HPV18 E6/E7 primer pair (product size 150 bp) in ethidium bromide stained agarose gel. HeLa cells were taken as positive control. NTC = No template control.
5.1.3. Examining the presence of target sequence in HPV+ve HNSCC cell lines:

Once we had confirmed that all our selected HPV-positive HNSCC cell lines are HPV16-positive, next we wanted to confirm whether the E6/E7 siRNA targeting sequence was present or had been mutated. Presence of the target sequence would give us confidence that si-E6/E7 would be able to effectively knockdown E7 mRNA and act as an effective molecular targeted strategy for these cells. To confirm the presence of the si-E6/E7 target sequence we purified total RNA and generated cDNAs using random hexamers and reverse transcription following the protocol described in section 2.3.5. Following HPV16 E7 PCR amplification of the whole HPVE6 and E7 sequence (881 bp) and gel electrophoresis, the band was excised and the DNA purified. The purified PCR product was sent for sequencing at the Australian Genome Research Facility (AGRF). The resulting sequence was analysed using NCBI Blast search, (www.ncbi.nlm.nih.gov/BLAST), which indicated a match with HPV 16 E6 and E7. Moreover, the entire E6/E7siRNA target sequence was present within the UMSCC104 cell line, a representative example of HPV16 +ve HNC cell lines (Figure 5.3). Our result suggests that the Si-E6/E7 has the ability to effectively target and silence E6 and E7 genes in HPV-positive HNSCC cells.
Figure 5.3. Authentication of the presence of E6/E7 siRNA sequence in HPV+ve HNC cell line, UMSCC104 (a representative example).
The HPV16 E6/E7 primers were used to amplify portion of E6 and E7 sequences in UMSCC104 cell line by conventional PCR and visualized in 2% agarose gel stained with ethidium bromide. The product size was 731bp. The targeted amplified band was then purified and sequenced. NCBI nucleotide blast programme was used to locate the antisense sequence of our desired E6/E7 siRNA (seq. 10). The red highlighted nucleotides indicate the presence of E6/E7 siRNA target sequence in HPV16-positive HNSCC cell line (UMSCC104).

5.1.4. Scrutinizing the transfection condition of siRNA uptake in vitro:
As we have confirmed the presence of the target sequence of Si-E6/E7 in HPV-positive HNSCC cell line, the next important issue to address was whether these head and neck cancer cells could be efficiently transfected with siRNA. Many cell lines are resistant to transfection for a range of reasons. Therefore, to determine the efficient uptake of siRNA in HPV-positive HNSCC cells, a qualitative transfection indicator, siGlo red (Dharmacon), was used to transfect all four HPV-positive HNSCC cells via oligofectamine (a lipid vector) following the transfection protocol
(refer to section 2.10) and incubated for 24 hours at 37°C in a 5% CO₂ incubator. siGlo red is a fluorescent oligonucleotide duplex that accumulates in the cytoplasm of cells and allows visualisation of uptake in mammalian cells. It is used to determine the optimal siRNA transfection conditions of siRNA. The analysis was performed by flow cytometry using phycoerythrin (PE) fluorescent dye channel (Ex488/Em570 nm). The uptake of siGlo was highest in UMSCC104 cells (97%) compared to UPCI:SCC90 (92%) and UDSCC2 (91%) (Fig 5.4). However, the 93VU147T cells showed a lower transfection efficiency of 82%. Our data indicate that all cell lines were highly efficient for transfection (>80%) and we therefore concluded that oligofectamine was able to efficiently deliver siRNA into these cells.
Figure 5.4. Intracellular uptake of siGlo in siRNA transfected HPV+ve HNC cell lines (histogram).

All four head and neck cancer cell lines were individually transfected with 40nM of siRNA (siGlo) indicator and compared with their respective untreated cells. Twenty-four hours after the treatment, the transfection efficiency was detected by FACS analysis (using phycoethrin (PE) fluorescent dye). A shift of the cell population to the right side on the figures of the right panel represents transfection of sufficient cells. The figures on the left panel represent the untreated cells of the respective cell lines. (a) & (b) UDSCC2 cells untreated and siGlo treated, respectively; (c) & (d) UMSCC104 cells untreated and siGlo treated respectively; (e) & (f) UPCI:SCC90 cells untreated and siGlo treated, respectively; (g) & (h) 93VU147T cells untreated and siGlo treated, respectively.
5.1.5. Determination of effective silencing time point:

As we have previously shown in this chapter that our selected siRNA sequence is present in HPV-positive HNSCC cells and the head and neck cancer cells are highly transfection efficient, next we determined the optimal time point for most effective silencing of E6/E7 in the HPV16-positive HNSCC cell (UMSCC104). To test this, the UMSCC104 cells were transfected with 40nM Si-E6/E7 for 24, 48 and 72 hours. Following this total RNA was isolated from treated and untreated cells as per previously described protocol (Section 2.3.2) and subsequently, cDNA was reverse transcribed from total RNA (section 2.3.4) and the E7 knockdown was analysed by qPCR. The result shows that the most effective silencing of E7 occurred at 48 hours (~75%, Fig 5.5) while minimal silencing was observed at 72 hours (~25%). The reasons for such findings could be due to the finite duration of the siRNA effect, dilution of siRNA during cell division or a grow-through effect from untransfected cells over 72 hours. Nevertheless excellent silencing at 48 hours was observed and this time was chosen for further studies as a standard post transfection incubation time for further experiments in this chapter.
5.1.6. Silencing of HPV E7 gene in vitro / E7 gene silencing in HPV+ve HNSCC cell lines:

Now we had confirmed the efficiency of si-E6/E7 targeting HPVE6/E7 oncogenes in cervical cancer cell line (CaSki) and high transfection efficiency of HPV16-positive HNSCC cells. We next addressed whether the HPV16-positive HNSCC cells show similar molecular characteristics as cervical cancer cells when treated with siRNA. All four HPV16-positive HNSCC cell lines were transfected with si-E6/E7. After 24 hours of transfection, the expression level of E7 was determined by real time qPCR as before (section 2.3.8). The expression of the gene of interest, E7, was normalized by comparing with the house keeping/normalised gene, β-actin, then these normalised
values were further normalised relative to a calibrator or an untreated control. The acquired data were analysed using the Delta $C_t$, relative quantitation method described by Livak et al. (2001).

Our results showed that siRNA significantly knocked down E7 mRNA in all four HPV16-positive HNSCC cell lines compared to negative control siRNA (siGFP) (Fig 5.6). An 80% reduction in E7 mRNA was seen in UMSCC104 cell line followed by 76% in UPCI:SCC90 cells, with lower reductions observed in UDSCC2 (60%) and 93VU147T (55%) cell lines (Figure 5.6). Although the siGFP has been chosen as a non-specific control, in UDSCC2 and 93VU147T cells, the siGFP seems to be showing non-specific response possibly via binding to non-specific genes and causing off target effects. Our results suggest that Si-E6/E7 can effectively silence the HPVE7 gene in HPV16-positive HNSCC cells.
5.1.7. Assessment of cell viability in vitro:

As the E6/E7 siRNA was shown to effectively knockdown E7 expression level in all four HPV-positive HNSCC cell lines, the next question we wished to answer was whether this reduction of E7 might have a biological effect on the growth of HNSCC cells. In other words, does the loss of E7 lead to cancer cell death in HPV-positive HNSCC cell lines, as has previously been shown in cervical cancer cell lines. To examine this, we carried out Cell Titre Glo assay (a luminescence-based cell viability assay) (described in section 2.5.1) using 12,000 cells/well in 96 well plates transfected with 40nM of E6/E7-siRNA, siGFP (non-specific control siRNA), siPLK1 (positive control) or a ‘mock’ transfection using transfection reagents only (oligofectamine) (control). At 72 hours post-transfection cells were analysed in a fluorescent plate reader. We found a significant reduction of cell viability with siE6E7-treated cells in all four HPV16-positive HNSCC cell lines compared to 3 control treatments (untreated, mock transfection and siGFP) (Fig 5.7).

Morphologically, the siRNA-treated cells were found to be shrivelled, slow growing, contained vacuoles in the cytoplasm, and appeared mostly dead, under phase contrast
compound light microscope. The highest cellular growth reduction was seen in UMSCC104 cells (~55%), followed by ~52% in UDSCC2, ~43% in UPCI:SCC90, and the lowest in 93VU-147T cell lines (~38%) (Figure 5.7). The siGFP has been selected as a non-specific control. However, in UPCI:SCC90 and 93VU147T cells, the siGFP showing non-specific response possibly via binding to non-specific genes and leading to cancer cell death. Further, the cytokine release of these cells may also make them sensitive to siGFP. However, no significant difference between siGFP and oligofectamine control was seen. Our data show statistically significant reduction in all four HPV-positive HNSCC cells when treated with E6/E7 –siRNA indicating that siRNA could be an effective molecular targeted technology to induce cancer cell death in these cells in vitro.

![Figure 5.7](image_url)

**Figure 5.7. Growth inhibition effect in HPV16-positive HNSCC cell lines by siRNA.**
The UMSCC104, UPCI:SCC90, UDSCC2 and 93VU147T cells were individually transfected with 40nM of E6/E7 siRNA (purple bars), siGFP (non-specific control siRNA) (green bars), siPLK1...
(positive control) (orange bars) and a ‘mock’ transfection using transfection reagents only (oligofectamine) (control) (red bars). Following the treatment, the cellular viability was determined using a Real time Glo assay at 72-hour time point. The siGFP was used to observe non-specific cytotoxicity and siPLK1 was used to determine the mechanistic efficiency of siRNA in these cells. The percentage of the loss of cell viability was analysed using micro-plate reader software and the graph was prepared using GraphPad Prism V. 7. Each bar represents the mean +/- standard error of mean (SEM) (N=3).

5.1.8. Evaluation of the mechanism of cancer cell death in vitro:

As we found significant growth reduction by siRNA in HPV-16 positive HNSCC cells, we then sought to determine the biological mechanism of cell death in these cells. To investigate the mechanism, Annexin V/PI apoptosis flow cytometry assays were carried out (described in section 2.6). The assay is based on binding of the calcium dependant phospholipid, Annexin V to the cell membrane phospholipid phosphatidylserine (PS), which is translocated from the inner to outer surface of cells if they undergo apoptosis. PI detects the loss of nuclear membrane integrity and the combination allows the detection of early and late apoptosis or necrosis. The HNSCC cells were transfected with 40nM of E6/E7siRNA prior to testing the mechanism of cell death. Forty-eight hours after transfection, cells were analysed for apoptosis using FACS for annexin V and propidium iodide (PI) staining. Our results show that transfection with siE6E7 resulted in cells undergoing apoptosis compared to untreated cells in all four HPV16-positive cells (Figure 5.8). The highest apoptotic cell death was seen in UMSCC104 cells (~45%) followed by the UDSCC2, UPCI: SCC90 and the lowest in 93V-147T cells: ~ 40%, ~33% and ~ 28%, respectively (Figure 5.8).
Further, to validate our findings with the flow cytometry (FACS) on HPV16-positive HNSCC cells, we performed western blot analysis (Section 2.4) to determine the cleaved PARP protein, as the cleavage of PARP by caspase-3 is a classic indicator of apoptosis. All four HNSCC cells were transfected with 40nM of si-E6/E7, 40nM of siGFP and untreated cells were taken as control. The protein extracts were prepared from both treated and untreated groups of cells after 72 hours of treatment. The PARP protein was detected at 116 KDa and the cleaved PARP at 89KDa. Following the Western blot using PARP antibody, the cleaved PARP (at 89KDa) was visible in cell extracts of UMSCC104, UDSCC2 and UPCI:SCC90 cells treated with siRNA [Figure 5.9 (a)]. However, for the 93VU147T cells, siE6E7-treated cell extracts showed cleaved PARP band, but at a lower intensity, indicating less apoptotic cell death, consistent with the flow data above. Further, the quantitative analysis of the western blot results clearly suggest that the UMSCC104 cells treated with siE6E7, showed the highest signal intensity of cleaved PARP, indicating higher apoptotic cell death [Figure 5.9(b)]. Overall, our data indicate that the loss of E7 expression results in substantial apoptotic death of HPV16-positive HNSCC cell lines.
Figure 5.8. Apoptosis of the all four HPV16 – positive HNSCC cells following transfection with E6/E7siRNA.

All four head and neck cancer cell lines were individually transfected with 40nM of E6/E7siRNA and compared with their respective untreated cells. Cells were analysed for apoptosis using FACS for annexin V and propidium iodide (PI) staining 48 hours after the treatment. The logarithms of PI and annexin V – FITC (Fluorescein isothiocyanate fluorescence) were plotted in X and Y-axis in the cytogram, respectively. The lower left quadrant (Q3) shows live cells, the lower right quadrant (Q4) represents the cells that are undergoing early apoptosis; the upper right quadrant (Q2) shows cells in late apoptotic phase and the upper left quadrant (Q1) displays dead cells. The figures on the left panel represent the untreated cells of the respective cell lines and the right panel figures demonstrate siRNA
treated cells. (a) & (b) UMSCC104 cells untreated and siRNA treated, respectively; (c) & (d) UDSCC2 cells untreated and siRNA treated respectively; (e) & (f) UPCI:SCC90 cells untreated and siRNA treated, respectively; (g) & (h) 93VU147T cells untreated and siRNA treated, respectively.
Figure 5.9. Analysis of apoptotic cell death in HPV16-positive HNSCC cell lines by E6/E7 siRNA using western blot.
(a) The protein extracts were prepared from the untreated, siGFP transfected and E6/E7 siRNA transfected cells of all four HPV16-positive HNSCC cell lines (UMSCC104, UDSCC2, UPCI:SCC90 and 93VU147T) individually after 72 hours of treatment. The extracted proteins were then subjected to electrophoresis and immunoblotting with PARP (poly ADP-ribose polymerase) antibody to determine apoptotic cell death. The cleaved PARP indicates the cells undergoing apoptosis. The expression of alpha-tubulin was used as control to demonstrate equal amount of protein loading and protein integrity in all samples. (b) The quantitative analysis of the band intensity of western blot results were analysed using Image J software. The graph was prepared using GraphPad Prism V. 7. Each point represents the mean +/- standard error mean (SEM) of (N=3).

5.1.9. Correlation between E7 knockdown and cell death:

We have found significant knockdown of E7 mRNA expression by Si-E6/E7in all HPV-positive head and neck cancer cells in vitro, however, differences in the percentage of knockdown exist. Here we assess the correlation between the E7 expression level and the loss of cell viability in HPV16-positive HNSCC cells when treated with siRNA, with an aim to find whether these cells are dependent on E7 expression level for their survival. A statistical correlation analysis was performed to determine the association. The result shows a linear correlation between the level of E7 expression and the percentage of the loss among the HPV16-positive cells. Although no significant differences in loss of cell viability were noticed between the
high E7 and low E7 expressing cells, the $R^2$ value indicates that the results of all four HPV-positive cells are closely related to linear regression line, which indicates strong association between E7 expression level and loss of cell viability. These data show that the higher the E7 expression level, the more the loss of cell viability when treated with si-E6/E7 (Figure 5.10). The higher the E7 expression level, the higher the percentage of loss of cell viability, indicating that these cells are reliant on E7 expression.

![Figure 5.10. Correlation between the siRNA related E7 expression level and loss of cell viability in HPV16-positive HNSCC cells.](image)

The E7 knockdown percentages of all four HPV+ve HNSCC cells were plotted in Y-axis and the cell death percentage in X axis. A Linear regression analysis was performed to assess the correlation. The $R^2 = 0.8565$ value indicate that the results of HPV-positive HNSCC cells are closely related to the linear regression line, indicating a relationship between the E7 expression knockdown and siRNA mediated cell death. The graph was prepared using GraphPad Prism V. 7. Each point represents the mean +/- standard error mean (SEM) of (N=3).

**5.1.10. Evaluation of long-term efficacy of siRNA:**

We have already demonstrated in this chapter that siE6E7 can effectively down-regulate E7 mRNA levels, which leads to a cell death in HPV16-positive HNSCC cells. While our assays measure cell viability over 3 days, and reveal that apoptosis is
occurring, they do not tell us if these effects are long-term or if cells may recover from this treatment. To address this we examined clonogenic survival of HPV-positive HNSCC cells following siRNA treatment via colony forming assays. Cells were treated with siRNA for 48 hours before 100 – 200 cells were plated out and incubated for 3 weeks (section 2.5.3). Following this the cells were fixed and stained with crystal violet to visualize the colonies.

Our data show a significant reduction in colony formation of cells treated with siE6E7 compared to control treatments [Fig 5.11(a) and (b)]. We observed that UMSCC104 (~55%) and UDSCC2 (~53%) cell lines had more significant colony loss compared UPCI:SCC90 (~45%) while 93VU147T showed no significant change. Once again these data are consistent with the level of E7 in each of these cell lines.
Figure 5.11. Analysis of siRNA efficacy in HPV16-positive HNSCC cells by colony forming assay.
(a) All four HPV16-positive HNSCC cells were individually transfected with 40nM of E6/E7 siRNA, siGFP (non-specific control siRNA) and a ‘mock’ transfection using transfection reagents only (oligofectamine) (control). After 48 hours of treatment, 150 cells from each treated and untreated group were seeded out in each well of 12 well plate. Cells were kept in the cell culture incubator at 37°C in 5% CO2 up to 3 weeks to allow the cells to form colonies. After 3 weeks, the colonies were visualised by fixing with formaldehyde and staining with crystal violet. Here are examples of colony forming assay of two out of four HPV16-positive HNSCC cells. (b) After 21 days the quantitative measurement
of colonies in each treatment group in all four HNSCC cells were counted by compound light microscope. The graph was prepared using GraphPad Prism V. 7. Each point represents the mean +/- standard error mean (SEM) of (N=3).

5.1.11. Tumour growth inhibition by si-E6/E7 with nanoparticle based delivery system (Xenograft model):

As the use of siRNA to down regulate E6/E7 gene expression led to growth inhibition in HPV-positive HNSCC cells in vitro, we then tested the efficacy of siRNA in an animal model. For this we selected two HPV-positive HNSCC cell lines (UDSCC2 and UMSCC104) as treated with si-E6/E7 they have shown high knockdown of E7 mRNA and significant reduction of cell growth in vitro. Prior to the siRNA treatment, we tested these two cell lines to find out which cell line has the ability to grow subcutaneous tumours in mice. To grow tumours, we injected 10 million cells of each cell line in 100ul of 1XPBS, subcutaneously, in two separate immuno-deficient nude mice. Mice injected with UMSCC104 cells showed macroscopically tumours after 8 days, whereas the mice injected with UDSCC2 did not show any sign of tumour growth after 14 days of observation. We thus selected the UMSCC104 cell line for further siRNA experiments in the animal model. For the siRNA experiment, we accommodated 15 nude mice and split them into 3 groups of 5 mice: untreated control group; control group (siGFP), and siRNA-treated group. First, all fifteen mice were subcutaneously injected with 10 million UMSCC104 cells in 100ul of 1XPBS. Once the tumour grew up to 100mm³ in volume, after 8 days, on day 2, the treatments were administered via the tail vein. Control group mice were injected with 200ul of 1XPBS, the mock control with ~ 2.5mg/kg (40ug/dose) of lipid particle loaded-siGFP and the siRNA treatment group was treated with one dose of 40ug of si-E6/E7 loaded
into lipoplex. We observed a significant post-treatment delay in tumour growth rate in the group of mice treated with si-E6/E7 compared to control groups (Figure. 5.12), demonstrating the specificity and efficacy of our siRNA. The tumour growth was significantly slowed in si-E6/E7 treated mice from day 5 till day 10 with a reduction in tumour size of ~35% compared to the control group observed. Statistical analyses among the groups were performed by one way ANOVA using GraphPad prism software.
Figure 5.12: The *in vivo* identification and tumour growth inhibition analysis in nude mice using si-E6/E7.

(a) & (b) Detection of subcutaneous tumour volume induced by injecting UMSCC104 cells using high resolution ultrasound imaging (Vevo 3100 Fujifilm Visual sonic). (a) 2D ultrasound imaging of the flank of mouse bearing subcutaneous tumour [(5.3mm (green line) x 4.6mm (yellow line)], vertical red arrows indicating margin of the tumour. (b) The red outlined area represents the total circumference of the tumour. The tumour volume was measured by Vevo Lab software (v 1.7). (c) All the treatments were administered once tumours had grown to approximately 100 – 150 mm³ following inoculation of 10 million UMSCC104 cells. The mice were treated on day 2 with a single dose of siRNA at a concentration of 40 ug. The mice treated with si-E6/E7 formulated in lipoplex showed significant delay in tumour growth compared to mice treated with PBS or control siRNA (siGFP). Error bars represent the SEM of tumour size in 5 mice. * represent p value < 0.05. Graph pad prism was used for statistical analysis.

5.2.1. Discussion:

The reliance of cervical cancer cells on the continuous expression of *E6* and *E7* oncogenes leads to the development of therapeutic strategies targeting *E6* and *E7* mRNAs, reducing tumour cell growth and restoring normal functions of *p53* and *pRb*
genes. A rational approach to restore the functions of these genes could be via siRNA technology (Bradford et al., 2003; Butz et al., 2003; Hall & Alexander, 2003; Jiang & Milner, 2002). Jiang and Milner were the first to demonstrate the effect of siRNA by downregulating E6 and E7 genes in cervical cancer cells (Jiang & Milner, 2002). Most studies have suggested that HPV type 16-positive HNSCC cells having transcriptionally active HPV16, and share similar characteristic molecular features of HPV type 16-positive cervical cancer cells (Gillison & Shah, 2001; Weinberger et al., 2006). However, the dependency of HPV16-positive HNSCC cells on the continuous expression of E6 and E7 oncogenes has been less documented and was further investigated here.

In this study, we have demonstrated the effectiveness of si-E6/E7 to suppress mRNA of E7 oncogene in a number of HPV16-positive HNSCC cell lines, which resulted in cancer cell death. Given the understanding that E6 and E7 proteins are expressed on the same mRNA transcript, here we used the similar siRNA sequence (sequence 10) against E6 oncogene that was shown in a previous study to effectively reduce E7 protein level by 81% in HPV-16-positive cervical cancer cells (CaSki) (Putral et al., 2005). In our experimental system, the delivery of siRNA targeting E6/E7 in HPV-positive HNSCC cells led to ~60 to 80% reduction of E7 mRNA expression level. Moreover, our results show similarity with the previous study on HPV-positive HNSCC, which had shown 54% and 85% reduction of E7 mRNA expression level in 93VU147T and UPCI:SCC90 cells, respectively (Rampias, Sasaki, Weinberger, & Psyrri, 2009). A possible explanation for the differences in knockdown of E7 mRNA expression level in different HNSCC cell lines could be related to the viral copy number and the E7 expression levels of those cells. Another reason could be the
possible ability of HPV-positive HNSCC cells to maintain cell growth partially via
different unknown pathways other than E7. Further, variations in the expression levels
of E7 gene in all of these HPV-positive HNSCC cell lines may play another role in
these differences. Previous studies on HPV-positive cervical cancer cells (CaSki)
have shown approximately 60% growth reduction but ~80% of $E6/E7$ mRNA
knockdown (Gu et al., 2006; Liu, Green, Seymour, & Stevenson, 2009; Putral et al.,
2005). We found similar results when we treated HPV16 cervical cancer cell (CaSki)
with E6/E7-siRNA. However, our data on HPV-positive HNSCC cells show
approximately 40-50% reduction in cell viability with siRNA treated cells after 72
hours, with the highest suppression seen in UMSCC104 cells (54%) and the lowest in
93VU147T cells (35%). This can be compared with another published study on head
and neck cancer cells which showed reduction in cell viability of UPCISCC90 and
93VU147T cell lines, of only ~35% and ~23%, respectively (Rampias et al., 2009).

Although we found significant growth inhibition in HNSCC cells, the actual
mechanism of cell death remains controversial. Earlier studies in HPV-positive
cervical cancer cells have reported that the mechanism of cancer cell death via siRNA
could be either by apoptosis or by inducing senescence (Butz et al., 2003; Jiang &
Milner, 2002; Yoshinouchi et al., 2003). Horner et al. using BPV E2 (Bovine
papillomavirus) protein to supress $E6$ and $E7$ oncogenes in HeLa cells had shown
impairment of cell growth via senescence (Horner, DeFilippis, Manuelidis, &
DiMaio, 2004). Likewise, another group of scientists also suggested the loss of $E6$
and $E7$ expression via siRNA resulted in cellular senescence in a number of cervical
cancer cells (CaSki and HeLa), which is thought to be initiated by the reactivation of
dormant p53 tumour suppressor pathway (Putral et al., 2005). On the contrary, a
previous study suggested that selectively targeting E6 mRNA in HeLa cells without affecting the E7 mRNA expression level down regulated E6 mRNA expression but up regulated p53 and eventually led to apoptotic cell death (Butz et al., 2003). DeFillipe et al. had shown that repression of the E6 protein activated the p53 pathway, without activating the pRb pathway, in HeLa, promoted both apoptosis and senescence, but the repression of E7 protein activated the pRb pathway and triggered only senescence (DeFilippis, Goodwin, Wu, & DiMaio, 2003). Nevertheless, Gu W et al. had indicated that shRNA designed against HPV18 E6 reduced E7 protein level and led to apoptotic cell death in HeLa cells and not senescence (Gu et al., 2006). The same study showed that shRNA targeting both E6 and E7 proteins led to senescence at low-dose infection, where as a high multiplicity infection resulted in early apoptosis in HeLa cells, suggesting that the level of E6 and E7 protein expression might be related to host cellular response and high dose infection, which may permit longer lasting suppression of these proteins (E6 and E7) and cause additional stress to cells and eventually trigger apoptotic cell death (Gu et al., 2006).

Our study is in concordance with a recent study on HPV16-positive HNSCC cells (UMSCC-47), which suggested ~ 32% of apoptotic cell death, in spite of showing 55 to 70% loss of cell viability via Alamar Blue assay (Adhim et al., 2013). Similar percentage of apoptotic cell death (~35%) was also shown in HPV16-positive cervical cancer cells (CaSki) when treated with E6/E7 siRNA (Liu et al., 2009). However, an earlier study on HNSCC cells showed much higher percentage of apoptotic cell death in two oropharyngeal cancer cell lines (93V-147T and UPCI:SCC90), namely approximately 60% (Rampias et al., 2009). In our study we found ~32% and ~40% apoptotic cell deaths in 93V-147T and UPCI: SCC90 cells, respectively, while the
highest apoptotic cell death was seen in UMSCC104 cells (45%). These data indicate
the reduction of E7 expression level by siRNA led to early apoptosis in HPV16
positive HNSCC cells. Further, we demonstrated the long-term efficacy of si-E6/E7 in
HPV-positive HNSCC cells via colony forming assay. Our data suggest effective
reduction in the colony formation in UMSCC104, UDSCC2 and UPCISSC90 cells
when treated with si-E6/E7. Once we had found the long-term efficacy of si-E6/E7 in
vitro, it ultimately led us to investigate our findings in in vivo models to observe
whether the Si-E6/E7 is effective in a living being.

Our in vitro study results presented here suggest that HNSCC cells treated with siRNA
targeting E6 and E7 genes of HPV have highly potent antiviral response. However,
we further characterised the effective functionality of siRNA targeting HPV E6/E7 in
vivo for its potential benefits as a therapeutic strategy to treat HPV-positive HNSCC.
For siRNA to be successfully used as a therapeutic agent, an effective delivery agent
is necessary that would allow longer existence of siRNA in the circulation and silence
target gene efficiently. The major challenge to delivery of siRNA is to find an
effective formulation and procedure that would be suitable for in vivo application. In
this experiment, we used the HFDM method to formulate stable siRNA-loaded
liposomes, a similar delivery vehicle that has been previously described and
implemented in a mouse xenograft model by Wu et al. (Wu et al., 2011).

It is challenging to grow tumours in an animal model with HPV-positive head and
neck cancer cells. Unlike cervical cancer cells, HNSCC cells take time and require
more cells to establish tumours in animal models. A recent study has suggested HPV-
positive HNSCC cells may require as high as 10 million cells to grow a tumour in a
mouse model (Pogorzelski et al., 2014). We chose two particular HNSCC cell lines (UMSCC104 and UDSCC2) of the four, based on our in vitro results, because the E6/E7 siRNA was effective in these lines, significantly reducing E7 mRNA expression level, reducing tumour cell growth and have a long term effect.

We first tested the growth ability of UMSCC104 and UDSCC2 in a nude mouse model, injecting 10 million cells. UMSCC104 cells showed tumour growth after 8 days but no tumour growth were seen in nude mice injected with UDSCC2 cells. UMSCC104 cells were therefore chosen for further in vivo experiments. We segregated 15 mice in three groups for this experiment; untreated control group, non-specific control (siGFP) group and Si-E6/E7 treated group. Once the tumour was established, the siRNA targeting E6/E7 loaded in liposome complex (Lipoplex) was injected intravenously in mice bearing subcutaneous tumours. We showed a significant delay in tumour growth when the mice were treated with si-E6/E7 compared to the control groups, demonstrating the specificity and efficacy of the si-E6/E7. These data suggest the potential effectiveness of E6/E7 siRNA as a therapeutic strategy in HPV-positive HNSCC. Further, our result suggests that, therapeutically, multiple doses are required to achieve a consistent tumour growth inhibition effect. Using multiple doses, we will acquire three situations; a) Firstly, stasis, that is killing = active cell division, it is about the balance between how much transfected cells are dying and how much are not surviving. This also suggests how efficient the delivery of the siRNA is to those cancer cells. b) Tumour shrinkage or elimination, and c) finally, there will be stasis but the killing of cancer cells will attract the immune response, which in turn lead to the accumulation of mast cells, macrophages and lymphocytes. Therefore, from the third situation perspective, we might think about using both siE6/E7 and anti PDL-1 siRNAs. Since the tumour microenvironment is
immune-suppressive, this combination may actively knock off the HPV E6/E7 genes and thereby reduce tumour growth.

In conclusion, we have demonstrated the efficacy of siRNA targeting E6/E7 in a number of well-established HPV-positive HNSCC cell lines. Further, the intravenous liposomal delivery of siRNA appears to be safe, stable in the circulation and ensures sequence-specific inhibition of HPV-positive HNSCC tumour growth. Our in vitro and in vivo studies demonstrate growth suppression of HPV16-positive HNSCC cells by siRNA, encouraging speculation of possible future application in treatment of HPV-associated head and neck cancers. Since combination therapy has shown to be more effective than the single mode of therapy in cancer treatment, our result indicates the possibility of using siRNA therapy in combination with the current standard of therapy for HNSCC, including surgery, chemotherapy (cetuximab) and radiotherapy. However, further experiments are necessary to assess possible synergisms when siRNAs are used as adjuncts to surgery, radiotherapy and both cytotoxic and small molecular drug chemotherapies.

References:


particles and its application with cisplatin in cervical cancer mouse models. *Gene Ther, 18*(1), 14-22. doi:10.1038/gt.2010.113

CHAPTER  6

Investigation of MLN8237, an Aurora kinase inhibitor
selective for E7, in HNSCC cells
**Introduction**

Aurora kinases play important roles in regulation of the cell cycle, including entry of cells into mitosis, microtubule spindle assembly, chromosomal condensation, chromosomal bi-orientation, modification of kinetochore-microtubule attachments and cytokinesis. Therefore, any alteration in the signaling pathways of Aurora kinases may result in abnormal cell division that can cause chromosomal abnormality and cancer (Dar et al., 2010). In fact, the overexpression of Aurora kinases has been reported to induce aneuploidy and genomic instability, which led to the pathogenesis of a range of human malignancies (Dar et al., 2008, Hoque et al., 2003, Mazumdar et al., 2009). To date, three types of Aurora kinase have been discovered: Aurora A, Aurora B and Aurora C kinases (Glover et al., 1995). Among these, Aurora A has been studied extensively.

Amplification or overexpression of Aurora kinase A has been described in various cancers with poor prognosis: these include breast (Sen et al., 1997), colon(Bischoff et al., 1998), ovarian, cervical, prostate, head and neck cancers and neuroblastoma (Zhou et al., 1998). A recent study has shown approximately 64% (16 out of 25) of human laryngeal cancer tissue samples had overexpression of Aurora kinase A (Zhang et al., 2012). The same study also suggested that suppressing the Aurora kinase A using siRNA led to reduced invasive ability and chromosomal abnormality in a laryngeal cancer cell line, HEp-2, indicating potential role of overexpressed Aurora kinase A in laryngeal carcinogenesis (Zhang et al., 2012). Another study reported that ~65% (22/34) of head and neck cancer samples examined had high expression of Aurora kinase A mRNA and protein, compared to that of healthy tissue (Reiter et al., 2006).

Since Aurora kinase A plays a critical role in mitosis and its overexpression has been shown to be linked with carcinogenesis (Nikonova et al., 2013), there is a clear rationale for Aurora kinase A inhibition as an anticancer, therapeutic agent. This has led to the development of small molecular inhibitors that could specifically target and inhibit the over-expressed Aurora kinases. Alisertib, or MLN8237, is such an inhibitor. It currently has an investigational drug licence, is bioavailable by mouth,
and selectively targets Aurora kinase A. It has exhibited high efficacy and potent anti-tumour effects against a range of malignancies (colon, lung, breast, prostate, ovarian cancers and lymphoma) in preclinical experimental models (Manfredi et al., 2011), and is currently progressed to phase 1 and phase 2 clinical trials (Dees et al., 2012, Melichar et al., 2015). Experimental and clinical data concerning MLN8237 and HNSCC are few.

Although an earlier study has observed knockdown of Aurora kinase A mRNA leading to apoptotic cell death in HNSCC (Zhang et al., 2011a), no study has systematically researched the effect of MLN8237 in HPV-transformed HNSCC cells. We have demonstrated in our recently published article that targeting of Aurora kinase A using MLN8237 in a panel of HPV-transformed cervical cancer cell lines resulted in growth inhibition both in vitro and in vivo (Appendix 2). Our results showed the sensitivity of MLN8237 was more specific to HPV-driven cancer and this selectivity was due to the expression of E7. However, the exact mechanism or the link between the Aurora kinase A over expression and the HPVE7 expression status is not well understood.

Thus, in this chapter, we explore the effect of the Aurora kinase A inhibitor, MLN8237 (MLN8237), in a number of HPV-positive and a HPV-negative HNSCC, in light of its therapeutic potential for the treatment of HPV-associated HNSCC.
6.1. Results:

6.1. Analysis of the relative HPV16 E7 mRNA expression levels of HPV-transformed HNSCC cell lines:

As we have shown in our published study (Appendix 2) that the sensitivity of HPV-transformed cancer cells to MLN8237 depends on their E7 mRNA expression levels, we first set out to investigate the E7 mRNA expression levels of our selected HPV16-positive HNSCC cell lines. To screen for the HPV16 E7 mRNA expression level, total RNA was extracted (section 2.3.1), cDNA was reverse-transcribed from the total RNA (section 2.3.3). Then the real time qPCR (denaturation 95°C for 30 sec., annealing temp. 60°C for 30 sec., extension temp. 72°C for 30 sec. and 40 cycles) was carried out using HPV16E7 primers (95 base pairs product size) (Chapter 2, section 2.3.6). In this experiment, the expression of the gene of interest, E7, was normalised with the house keeping gene, β-actin, then these normalised values were further compared to the relative E7 expression level of CaSki. The E7 expression level of CaSki was taken as a positive control or a reference standard and compared with the E7 expression level of HPV-positive HNSCC cells using the Delta delta Ct (ΔΔCt) method (a relative quantitation method) described by Livak et al. (2011). The relative E7 expression levels of HNSCC cell lines were shown in Figure 6.1. The results showed that based on the CaSki HPV16E7 mRNA expression, two HPV16-positive HNSCC cell lines showed higher E7 mRNA expression levels (UDSCC2 and UMSCC104) compared to Caski, while UPCI:SCC90 and 93VU147T had less than 50% the Caski level.
Figure 6.1. Screening of relative E7 mRNA expression levels in HPV+ve HNSCC cells. The UMSCC104, UPCiSCC90, UDSCC2, 93VU147T and CaSki cells were seeded in a 6 well plate, and once they were 70-80% confluent, total RNA was extracted using Trizol (Gibco, Invitrogen), further the cDNA was reverse transcribed from total RNA and the oncogenic E7 mRNA expression was determined by quantitative real-time PCR (rtPCR). The relative E7 mRNA expression levels of HPV+ve HNSCC cells to CaSki were analysed using Rotor-Gene Q software and the graph was prepared using GraphPad Prism V. 7. Each point represents the mean +/- standard error mean (SEM) of (N=3).

6.2. HPV-transformed and non-HPV HNSCC cells showed sensitivity to MLN8237:

As we had earlier determined the E7 levels of the HPV16-positive HNSCC cells, we next investigated whether the HPV E7 expression level has any effect on the sensitivity of HPV-transformed HNSCC cells to MLN8237. To test this, our panel of HPV16-positive HNSCC cells lines plus controls were subjected to various doses of MLN8237 and cell viability determined using a luminescence-based assay (Cell Titre Glo assay). All the HNSCC cells were seeded at 10,000 cells/well in 96 well-plates and incubated for 24 hours before being treated with MLN8237 (40µM to 40nM) for 72 hours. After 72 hours of incubation, cell viability was determined. The results showed that UDSCC2 and UMSCC104 were highly sensitive to MLN8237 at optimal concentrations of 82nM and 92nM, respectively (Figure 6.2).
Figure 6.2 (A). Dose response experiment of HNSCC cell lines showing the sensitivity to MLN8237. Summary of the IC\textsubscript{50} values generated from a dose-response experiment showing the effect of the MLN8237 on HPV and non-HPV HNSCC cell lines and also a HPV-positive cervical cancer cell line, CaSki as positive control. Cells were subjected to a viability assay using the luminescence based Cell Titre Glo assay after 72 h of drug treatment (dose range 40 µM to 40 nM). Each point is the mean and SEM of quadruplicate determinations. IC\textsubscript{50} values were calculated via log(inhibitor) vs. response curve using GraphPad Prism V.7. The HPV-transformed HNSCC cell lines UMSCC104 and UDSCC2 and the CaSki, together with UMSCC1 (non-HPV) had IC\textsubscript{50} values within the nanomolar range. The two other HPV-positive HNSCC cell lines, UPCI SCC90 and 93VU147T showed very high IC\textsubscript{50} values. Each point represents the mean +/- SEM of three observations.
HNSCC cells were therefore more sensitive to MLN8237 than Caski cells (217nM) (Fig 6.3A). However, the UPCI:SCC90 and 93VU147T cells were more resistant (IC\textsubscript{50} values of 49uM and 202uM, respectively) while the HPV-negative HNSCC cells (UMSCC1) showed IC\textsubscript{50} of 0.113uM. These results indicate that high E7 expression in HNSCC cells also correlated with sensitivity to MLN8237, as previously shown by us for cervical cancers cells (Appendix 2). However, an alternative explanation may be that variation in sensitivity could relate to cell doubling time given the role Aurora A plays in cell division. To address this we performed a cell growth experiment on all HNSCC cells seeded in 96 well plates. The cell growth was analysed every 12 hours using luminescence based Real Time Glo (Chapter – 2, section 2.5.2) assay for 3 consecutive days. Results showed that the cell doubling time of HPV-positive HNSCC cells was more or less similar except 93VU147T and UMSCC1 cells (Figure 6.3). ANOVA was used to see if there is a significant difference between these cell lines. UMSCC1 cells grew the fastest and 93VU147T was the slowest compared to the others (Table 6.1). The cell doubling time was calculated using cell doubling time equation,

\[
\text{Doubling time} = \frac{\text{duration} \times \log(2)}{\log(\text{Final con.}) - \log(\text{Initial con.})}.
\]

This suggests the MLN8237 sensitivity may not be a consequence of proliferative differences among HPV-positive HNSCC cells.

![Cell doubling time graph](image)

**Figure 6.3. The doubling time of HNSCC cell lines.**
All HNSCC cells were seeded at 15,000 cells in 96-well plates in their standard growth medium. After 6 hours of incubation, the Real Time Glo reagent was added to each well. Cell viability was measured every 12 hours using a Plate reader. The values are pooled data from three independent experiments.
The obtained data were analysed using GraphPad Prism v.7 software. Each point represents the mean +/- standard error mean (SEM) of the three observations.

Table 6.1: Cell doubling time of HNSCC cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Doubling time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  UDSCC 2</td>
<td>33.7</td>
</tr>
<tr>
<td>2  UMSCC 104</td>
<td>36.9</td>
</tr>
<tr>
<td>3  UPCI:SCC90</td>
<td>32.1</td>
</tr>
<tr>
<td>4  93VU147T</td>
<td>41.2</td>
</tr>
<tr>
<td>5  UMSCC 1</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Taken together, our data show that both HPV-positive and HPV-negative HNSCC cell lines are sensitive to MLN8237, although HPV-positive HNSCC cells showed more sensitivity. Further, for the HPV-transformed HNSCC cells, the sensitivity to MLN8237 may vary depending on their HPV E7 mRNA expression level.

6.3. Correlation between E7 mRNA expression level and MLN8237 sensitivity:

As our previous experiments showed differences in the level of E7 mRNA expression and variations in MLN8237 sensitivity for the HPV-transformed HNSCC cell lines, the question arises as to whether there is a relationship between the E7 mRNA expression level and MLN8237 sensitivity in these cells. To investigate this, a statistical correlation analysis was carried out and the data obtained from the E7 mRNA expression levels and the IC_{50} of HPV-positive HNSCC cells were plotted (Figure 6.4). This shows a significant correlation between the levels of E7 mRNA expression and sensitivity of MLN8237 in HPV-transformed HNSCC cells, that is, the higher the E7 mRNA expression level, the more sensitive cells are to MLN8237 and less amount of MLN8237 drug (low IC_{50}) is required for cell death in those cells. This experiment suggests that HPV E7 expression level plays a role in the sensitivity of the HPV-transformed HNSCC cells to MLN8237.
Figure 6.4. Correlation between HPVE7 expression and MLN sensitivity.
The E7 expression level of four HPV+ve HNSCC cell lines and the Log of MLN concentration (the IC50 value) were plotted in Y-axis and X-axis, respectively. Based on the E7 expression level of CaSki cells and taken as standard control (100%), E7 expression levels of the four HPV-positive HNSCC cells were determined. The relationship between HPV E7 expression levels and MLN concentration (IC50 values) performed by linear regression analysis using GraphPad Prism V.7. The coefficient determinant, R2 value indicate a strong linear correlation between E7 expression and IC50 value.

6.4. Assessing the mechanism of MLN8237 induced apoptosis in HNSCC cell lines:

As we have demonstrated that MLN8237 effectively induces loss of viability in HNSCC cells, we next want to examine whether the mechanism behind this biological loss was associated with apoptosis. To determine the precise pathway, a number of apoptotic proteins were tested by immunoblotting of three HPV-transformed and one non-HPV HNSCC cell lines treated with 5μM of MLN8237 for 24, 48 and 72 hours, and with 5μM of DMSO (vehicle only) as control. Here, we have chosen one HPV-positive HNSCC cell line (93VU147T) between the two (93VU147T and UPCI:SCC90) that have shown low E7 expression level and low sensitivity to MLN8237. The dose was kept within the clinical range (5μM) as the tolerable plasma...
concentration of 1 to 5µmol/L has been reported in patients (Cervantes et al., 2013). After the treatment, the cell lysates were extracted and subjected to proapoptotic and anti-apoptotic protein analysis. The cleaved poly-ADP ribose polymerase (PARP) was observed in all the cell lines treated with MLN8237 in a time-dependent manner. The overall levels of cleaved PARP were higher in UDSCC2 and UMSCC104 cells and were maximal at 72 hours of treatment, consistent with the increased loss of viability previously observed. However, 93V107T and UMSCC1 cells showed modest levels and no maxima at 72 hours (Figure 6.5).

Figure 6.5. Analysis of cell lysate from HPV and non-HPV HNSCC cell lines for apoptotic components.
Cells were seeded and cell lysate was collected after treatment with MLN8237 for 24, 48, and 72 h. Western blotting was then performed using PARP, the pro-apoptotic BIM, and the anti-apoptotic Mcl-1 and Bcl-2. α-tubulin was used as a loading control. Western blot imaging was acquired using a ChemiDoc system.

Further, we measured the concentrations of the anti-apoptotic proteins myeloid leukaemia cell differentiation protein (Mcl-1) and B-cell lymphoma 2 protein (Bcl2). Bcl2 did not show differences in expression levels in any of the HNSCC cell lines, indicating MLN8237 treatment has little effect on the level of Bcl2 in these cells. On the contrary, Mcl-1 showed differences in expression levels at 3 different time points.
in UMSCC104, UDSCC2 and UMSCC1 cells, with the highest expression seen at 0 hours of MLN8237 treatment and lowest at 72 hours, suggesting a loss of anti-apoptotic effect over time and consistent with findings in cervical cancer cells. However, lower Mcl-1 expression is noticed in 93VU147T cells. For the proapoptotic BIM (Bcl-2-like protein11), the level of protein expression showed differences in all of the HNSCC cell lines except 93VU147T cells. In 93VU147T cell, the BIM protein showed an increasing trend of expression with the highest observed at 72 hours of MLN8237 treatment indicating high pro-apoptotic activity. These data indicate, depending on the results of PARP cleavage, higher apoptotic cell death occurred in high E7 expressing HPV-transformed HNSCC cells compared to non HPV-HNSCC cells treated with MLN8237.

6.5. Assessing the sensitivity of HPV-positive HNSCC cells to Aurora kinase A and B inhibitors:

As we found, from earlier experiments described in this chapter, that the UDSCC2 and UMSCC104 cell lines, which have high E7 expression, showed high sensitivity to MLN8237, we tested these two cell lines for sensitivity to other Aurora kinase inhibitors, including the pan Aurora kinase inhibitor, AMG900, an Aurora kinase B inhibitor, ZM447439, and a different Aurora kinase A inhibitor, CCT244941. Further, by testing this range of inhibitors, we wanted to examine whether inhibiting both Aurora kinases A and B, or only Aurora kinase B, in HPV-positive HNSCC cells can reduce their growth. Our results show that UDSCC2 and UMSCC104 cells are highly sensitive to AMG900 with an IC₅₀ of 330 nM and 16 nM, respectively. However, cells did not show great sensitivity to another commercially available Aurora kinase A inhibitor (CCT244941) with IC₅₀ values of 26μM for UMSCC104 cells and 20μM for UDSCC2 cells.

The results with Aurora kinase B inhibitor, ZM447439, showed distinctly different sensitivities of cell lines UMSCC104 and UDSCC2, with IC₅₀s of 3.3 μM and 35.3
µM, respectively. It can therefore be speculated that AMG900 would be a better choice than selective inhibitors like ZM447739 or even MLN8237 for this particular cell line (UMSC104) as the AMG900 has the ability to inhibit both Aurora kinases A and B and these cells are very sensitive to it. Our overall results suggest that AMG900 is more effective and sensitive compared to Aurora A and Aurora B selective inhibitors, CCT244941 and ZM447439, in HPV-positive HNSCC cell lines.
AMG900 (Aurora A & B inhibitor)

I\(C_{50}\) = 0.016\ mM/16\ nM

CCT244941 (Aurora B inhibitor)

I\(C_{50}\) = 26\ mM

ZM447439 (Aurora A inhibitor)

I\(C_{50}\) = 3.3\ mM

I\(C_{50}\) = 0.33\ mM/330\ nM

I\(C_{50}\) = 35.3\ mM
6.2. Discussion:

Since Aurora kinases play key roles in mitosis and are overexpressed in many malignancies, they have been considered as promising targets for cancer treatment. Among several Aurora kinase inhibitors that have been identified, MLN8237 (Aurora kinase A inhibitor) has been tested in over 30 clinical trials for different types of cancer, showing modest responses (Friedberg et al., 2014, Dees et al., 2012, Melichar et al., 2015). Although Aurora kinase A has been shown to be overexpressed in head and neck cancer, and the novel drug MLN8237 (an Aurora kinase A inhibitor) has proceeded to a few clinical trials with head and neck cancer patients, the responses have been limited (Melichar et al., 2015). Few studies have observed the effect of this particular inhibitor in HPV-transformed HNSCCs.

With the goal to investigate the effect of specifically targeting Aurora kinase A in HPV-transformed HNSCC cells, we assessed the possible impact of MLN8237 (MLN8237), in vitro. We demonstrated effective reduction in cell viability in HPV-positive HNSCC cells that resulted apoptotic cell death. However, the sensitivity of HPV-positive HNSCC cells to MLN8237 varied due to a possible link with the E7 mRNA expression levels of these cells. Our screening for determining the relative HPV E7 mRNA expression levels on a panel of four HPV-positive HNSCC cells showed that UDSCC2 and UMSCC104 cells had high E7 expression levels while the other two, UPCI:SCC90 and 93VU147T cells, had moderate expression. The cell
viability dose response experiment on HPV-positive HNSCC cells treated with MLN8237 suggested that the cells with high HPV E7 expression levels (UDSCC2 and UMSCC104) were highly sensitive to MLN8237 and had lower IC$_{50}$ s, ~ 82 nM and ~92nM, indicating a lower dose of drug was required for their loss of viability and this sensitivity could translate into lower cytotoxic side effects in the clinic. Therefore we conclude that sensitivity of HPV-transformed HNSCC cells to MLN8237 depends on E7 mRNA expression levels. Our study is in agreement with our previous published work on HPV-transformed cervical cell lines, where we have shown that MLN8237 selectively inhibited growth of HPV-transformed cervical cancer cells and that this was dependent on HPV E7 expression levels (Appendix 2). In that particular study, we have also shown that the MLN8237 sensitivity was a direct consequence of the E7 mRNA expression levels in HPV E7 expressing cells, by transfecting HPV-negative cells (C33A) and HPV-negative oral squamous cell carcinoma cells (SCC25) with high, medium and low levels of HPV E7 oncogene. We found a significant difference in the MLN8237 sensitivity between the high and the low E7 expressing cells, where the high E7 expressing cells were more sensitive to MLN8237, further showing that HPV E7 expression induces sensitivity to MLN8237. This was further supported by showing that the low E7 expressing cervical cancer cell line, SiHa (de Boer et al., 2007) was less sensitive to MLN8237.

Our results on HPV-negative HNSCC cells (UMSCC1) revealed that despite of the absence of E7 oncogene in these cells, they were also sensitive to MLN8237 (less than 1µmol/L), indicating that regardless of HPV status HNSCC cells may be sensitive to MLN8237. This is consistent with our previous published study where we have shown that MLN8237 was selectively sensitive for HPV-transformed cervical cancer cells, but also showed sensitivity for non-HPV HNSCC cells (HaCaT). We had also found differences in MLN8237 sensitivity in 3 HPV-negative HNSCC cell lines (FaDu, Detroit 562 and SCC25), which we had chosen for the above published study. Interestingly, we found HPV-negative HNSCC cells, FaDu and Detroit 562, which were derived from pharynx (subsite was unknown) showed low sensitivity to MLN8237 with IC$_{50}$ 2 µM and 8 µM, respectively (Appendix 2). By contrast, SCC25 cell line derived from oral cavity (tongue), an HPV-negative HNSCC cells, did not show sensitivity to MLN8237 with IC$_{50}$ > 30µM (Appendix 2). This observation is similar to the findings presented in the current chapter, where we have shown that the
HPV-negative HNSCC cell line, UMSCC1 that was derived from the oropharynx, was sensitive to MLN8237 with IC_{50} of 1.13 µM. Therefore, from our findings on HNSCC cells, we could speculate that the anatomical site might be another factor to be considered for the variation in sensitivity of these cell lines to MLN8237. However, the exact mechanism is unknown and this requires further investigation.

We found differences in the mechanism of cell death in HSNCC cells when treated with MLN8237. Our data on PARP immunoblotting suggest that the HNSCC cells treated with MLN8237 are undergoing apoptosis. However, the exact mechanism remains unclear as our findings with the anti-apoptotic (Bcl-2) and pro-apoptotic (BIM) proteins showed inconsistent results on the panel of HNSCC cells. In contrast, the Mcl-1, another anti-apoptotic protein, which was overexpressed in UMSCC104, UDSCC2 and UMSCC1 at 0 hours in control cells, indicating that these cells are protected by Mcl-1 from apoptosis, that is, MLN8237 would be less effective at 24 hour time point. This is in concordance with the previous studies, which also showed that the over-expression of Mcl-1 protects cells from undergoing apoptosis (Zhang et al., 2011b). Further, a gradual decrease of the expression of Mcl-1 was noticed from 24 hour to 72 hours, indicating that at 72-hour time point the effect of Mcl-1 would decrease, the cells would become sensitive to MLN8237 and might undergo apoptosis. However, the low E7 expressing HPV-positive HNSCC cell line, 93VU147T did not show expression of Mcl-1 protein but BIM was highly expressed when treated with MLN9237. These data suggest that the both the HPV-positive and HPV-negative HNSCC cells treated with MLN8237 are undergoing apoptosis, although E7 expression might play an important role in sensitising the HPV-positive HNSCC cells to apoptosis. A recent report suggest that HPV E7 may increase the level of Aurora A kinase (Spardy et al., 2009).

A possible mechanism could be that HPVE7 might indirectly upregulate the p53-related p73 expression level by phosphorylating the retinoblastoma protein (pRb) and releasing E2F1 transcription factor, which in turn upregulates p73 gene. The p73 gene may upregulate the pro-apoptotic proteins PUMA and NOXA, and induce apoptosis by that route. In contrast, Aurora A has been reported to phosphorylate the E7-regulated p73 protein which ultimately leads to the inhibition of apoptosis (Brooks et al., 2002, Dar et al., 2008) (Figure 6.7).
Since the HPV E7 oncogene inhibits the function of the retinoblastoma protein (pRb), a negative cell cycle regulator that binds and inactivates the function of the E2F family of transcription factors (regulates cell proliferation and apoptosis), E7 might have an indirect link with apoptosis. Further, the E2F proteins were shown to bind with PUMA and Noxa (pro-apoptotic proteins) and inhibit their expression that leads to reduction in apoptosis (Hershko and Ginsberg, 2004). Moreover, our data on Mcl-1 suggest a link with E7 expression level. Therefore, there is a possibility that E7 may sensitise cells to apoptosis via sequestering Mcl-1 function and indirectly inducing Noxa or PUMA expression. However, the HPV-negative HNSCC cells are also shown to undergo apoptosis even though E7 is absent in these cells. More work is needed to fully understand these pathways in HPV-negative HNSCC cells.

In conclusion, this chapter demonstrates novel findings using MLN8237 in HPV-positive HNSCC cell lines. The inhibition of Aurora kinase A using MLN8237 induced apoptotic cell death in both HPV-positive and HPV negative HNSCC cells.
Further, a relationship has been described between the level of E7 expression and the sensitivity to MLN8237 of HPV-positive HNSCC cells. These results suggest that MLN8237 could be a potential therapeutic agent for HPV-associated HNSCC, especially for those with high expression of the E7 oncogene. Further, our study on a number of commercially available inhibitors, AMG900, ZM447439 and CCT244941, on HNSCC cells, show that the pan Aurora inhibitor (inhibits both Aurora A and B), AMG900 could be also potential therapeutic agent for HPV-positive HNSCC cases. Moreover, as the use of these Aurora kinase inhibitors take time to go through mitosis and Mcl-1 starts to reduce and eventually the cell die, we could think of using the combination of Aurora kinase A and Mcl-1 inhibitor. This might improve the outcome in HPV-positive HNSCC cells with low E7 expression level. Although we have demonstrated promising findings with Aurora kinase inhibitors on HPV-positive HNSCC cells in vitro, the validation of our results require further investigation in an animal model. If encouraging, only then can human trials be contemplated.

References:


CHAPTER 7

General discussion, Conclusions and Future directions
General discussion, Conclusions and Future directions:

Over the last decade, it has become increasingly clear that high-risk HPV is aetiologically linked to HNSCC, especially those arising from the oropharynx. The worldwide prevalence of HPV-associated HNSCC is approximately 35%, although the percentage may vary from 10% to 70% depending on the tumour sites, the geographical location and the method of detection used (Kreimer et al., 2005, Termine et al., 2008, Ndiaye et al., 2014, Gillison et al., 2015). Although the incidence of tobacco and alcohol-related HNSCC are decreasing, high-risk HPV-associated HNSCCs, especially oropharyngeal cancers, is on the rise (Chaturvedi et al., 2011, Ariyawardana and Johnson, 2013, Dalianis, 2014). This may be attributed to increased sexual activities including multiple partners, oral sex, and transmission of HPV from the ano-genital region to the oral cavity (Schache et al., 2011, Dalianis, 2014). The structural characteristics shared by head and neck mucous membranes may play an important role in creating an appropriate environment for HPV infection, including thin epithelial linings, high probability of inflammation from trauma or infection and micro-abrasions, which may facilitate viral passage to, and integration into, the basal epithelial layers. Although HPV-associated HNSCC is well studied in the western world, few studies have been performed in developing countries, such as those of South Asia, in order to understand the true prevalence of HPV-driven HNSCC. Because tobacco- and alcohol-related HNSCCs are so common in these countries, less focus has been given to assess the role of HPV in the HNSCC in these parts of the world (South Asia, South East Asia, East Asia, Africa and South America), until recently.

In Chapter 3, we performed a systematic study and meta-analysis of published studies on HPV-associated HNSCC prevalence from the Asia Pacific region. Our data showed that the overall prevalence of HPV-associated HNSCC in the region was 36.45%, which was concordant with the recent meta-analysis by Nadiya et al. indicating a worldwide prevalence of ~34.5% (Ndiaye et al., 2014, Termine et al., 2008). The highest HPV-associated HNSCC prevalence we found was in the oropharynx, approximately 40%, which was also supported by our results of the site-specific meta-analyses showing that HPV has stronger association with the
oropharyngeal cancer (OPC) (OR: 14.66; 95% CI: 6.09 – 35.26) compared to oral cavity and laryngeal cancer in the Asia Pacific. However, significant geographic differences in the prevalence of HPV-associated OPC were seen with 25.8%, 38.7% and 49% in South Asia, East Asia and Oceania respectively. Although HPV-positive HNSCC patients are typically younger males with limited history of smoking or drinking, interestingly the majority of HPV-positive HNSCC patients from the Asia Pacific region were habitual tobacco users with an often unknown contribution from alcohol drinking. This suggests a possible relation might exist between the HPV infection and the tobacco or/and alcohol. Indeed, a recent study from India suggested that former habitual smokers are more prone to develop HPV-associated HNSCC compared to the non-smoker or current smokers (Kumar et al., 2015). However, the exact mechanism behind this relationship is not clear.

Although we analysed data in the literature by anatomical sites/subsites and by geographical locations, heterogeneity still existed, which could be explained by the small number of cases described, issues with methods of tissue preservation or variations in methods used for detection of HPV DNA. Further, misclassification, or lack of information about the anatomical sites at which the carcinomas arose, was a major concern. Therefore, we urge systemic classification of the sites and subsites of the head and neck when preforming any study related to HNC and the use of the ICD-10 classification, and any subsequent revisions of this (Section 1.9). As to standardisation of methods for detection of HRHPVs, PCR would be our method of choice as it is sensitive, requires minimal tissue and is but moderately expensive (Smeets et al., 2009, Jordan et al., 2012). The existence of kits for in situ hybridisation and other methods under development by industry may facilitate better uptake of this essential information in the future. Further studies using standardised HPV DNA detection methods, clear site-specific classification of the origin of the cancers, and well-defined larger populations are necessary for better understanding of the relevant epidemiology. Most importantly the planning of patient management requires this information. In the treatment centres of developed countries with well established multidisciplinary Tumour Boards, such information is mandatory, albeit that most seem to rely on the surrogate maker, p16 positivity by immunocytochemistry.
Since one of the aims of this thesis is to understand the role of the HPV in South Asian populations, we have extensively highlighted the site-specific prevalence of HPV-associated HNSCC in the South Asia in our published article (chapter 2). Our data showed that the overall HPV-associated HNSCC prevalence is ~ 36%, with the highest seen in the larynx (~48%) followed by oral cavity (~37%) and oropharynx (~26%). It is understood that the sample size was small and the number of studies were also limited, which makes it difficult to estimate the actual prevalence; moreover variations in the detection method may play an important role. Consequently, the range of prevalence widely varied among the countries and also with the anatomical sites in South Asia. Further, majority of these studies (17/21) appeared to be only from India while very few studies were from Bangladesh, Pakistan and Sri-Lanka, which may limit the generalizability of the overall HPV-positive HNSCC prevalence in this region. Therefore, more studies are needed from Bangladesh, Pakistan and Sri-Lanka to estimate the actual prevalence of HPV in HNSCC in South Asia.

In Chapter 4, we have undertaken a comprehensive study to determine the prevalence of HPV in a modest Bangladeshi cohort of 196 patients who were diagnosed with HNSCC between December 2014 and May 2016 in Dhaka Medical College hospital, in Bangladesh. Bangladesh, being the 3rd most populous country in South Asia with the highest HNSCC incidence in the region, 21/100,000 per annum (Ferlay J, 2013), smoking and smokeless tobacco are the major risk factors. However, there was a lack of published evidence to understand the true prevalence of HPV-associated HNSCC in this country, which necessitated further investigation. To investigate the presence of HPV in the HNSCC, FFPE tissue samples were used and assessed by nested PCR method using two sets of consensus L1 primers of HPV DNA, and the IHC for the expression of p16. Using these test algorithms, among the HNSCC cases approximately 20% (36/174) was positive for HPV DNA by nested PCR, while approximately 16% positive for p16 IHC with the highest prevalence was seen in the oropharynx (36.1%) and HPV-16 was the most common type detected. Our result suggests a significant burden of HPV-associated HNSCC in Bangladesh. When the HPV-detection methods were compared, PCR data was taken as standard and p16 as a surrogate marker, the presence of HPV showed remarkable specificity of almost 99% with both positive predictive values. The correlation between HPV positivity by PCR and p16 positive cases suggest that the HPV is transcriptionally
active and may contribute to the carcinogenesis process in HNSCC in these populations.

Although nested PCR followed by sequencing is time consuming, it does accurately identify the presence and type of HPV in tissue samples (Winder et al., 2009). L1 consensus primers have the ability to detect any known HPV type present. While our study supports the fact that PCR is accurate and most informative test, it would be more effective if p16 IHC were included. The clinical consequence may be severe if p16 positive cases were in fact false positive and thus (over) treated as HPV-positive cases. A standardised, accurate and economical HPV-detection method is required for tissues, which could be used all over the world. Fortunately, the WHO through its HPV Laboratory Network (http://www.who.int/biologicals/vaccines/hpv/en/), has the matter in hand. Training in laboratory methods and in interpretation of results is provided. This work is especially important in monitoring the outcomes of HPV vaccination programmes around the world. Meantime, we propose a combination of nested PCR and p16 IHC for diagnosing the presence of HPV in HNSCC cases.

The current treatment guidelines for HNSCC mostly following the National Comprehensive Cancer Network (NCCN) and United Kingdom National Multidisciplinary Guidelines (UKNMG) are largely based on surgery with ongoing clinical trials of chemotherapy and radiotherapy, and meta-analyses of all relevant data (Homer and Fardy, 2016, Kelly, 2016, Nutting, 2016, Robson et al., 2016, Adelstein et al., 2012, Jordan et al., 2012, Budach et al., 2006, Chau et al., 2012, Chen et al., 2012, Cooper et al., 2012, Duarte et al., 2014, Hitt et al., 2014). However, recently, there has been a significant advancement in the development of new therapeutic agents for HNSCC. Since HPV-positive HNSCC patients have favourable prognosis and better treatment response compared to HPV-negative HNSCC patients, an avenue of opportunity exists for investigating less intensive treatment strategies for these patients. The prime objective of these treatment strategies should be lowering the risk of potential post-treatment side effects without compromising the survival outcome. Currently, clinical trials are investigating the de-escalation of treatment in HPV-positive HNSCC patients (Chapman et al., 2016, Marur et al., 2016).
Therefore, the development of effective treatments for HPV-positive HNSCC is very important, as the chemotherapy and the radiotherapy are associated with serious side effects. In Chapter 5, we have demonstrated the potential use of siRNA as therapeutic strategy for HPV-positive HNSCC by targeting against the E6 and E7 oncogenes and showing that effective knocking down of mRNA of the E7 gene lead to the inhibition of cancer cell growth in culture, \textit{in vitro} and \textit{in vivo} experiments. In addition, we also suggested the reliance of HPV-positive HNSCC cells on the expression of E7 gene for cancer progression. Our results suggested that a single transfection of Si-E6/E7 in a number of HPV-positive HNSCC cell lines, showed growth inhibition via silencing of E6 and E7 oncogenes. However, this silencing effect of siRNA was transient, lasting for only 48 hours, but the molecular events initiated from this were long lasting. Although apoptosis is a more desirable outcome for cancer therapy compared to senescence, this assumption may not be true in all cases. Xue et al. have shown that the up regulation of p53 in liver cancer led to cellular senescence and resulted in tumour clearance via the innate immune system (Xue et al., 2007). Further, a group of scientists have suggested that siRNA targeting against E6 and E7 resulted in suppression of these genes and led to senescence in cervical cancer cell lines (CaSki and HeLa) (Putral et al., 2005). In contrast, an earlier study suggested that selective silencing of the E6 gene, up-regulated p53 gene and led to apoptotic cell death in HeLa cells (Horner et al., 2004). Further, Gu W et al. have also shown an effective reduction of E7 protein by shRNA that led to apoptotic cell death in HPV-positive HeLa cells (Gu et al., 2006). We have shown 30 - 45% apoptotic cell death in all four HPV-positive HNSCC cells when treated with E6/E7-siRNA. Our result was in concordance with a recent study on HPV16-positive HNSCC cells (UMSCC-47) suggesting ~ 32% of apoptotic cell death. However, an earlier study on HPV16- positive HNSCC cells suggested even higher percentage of apoptotic cell death, approximately 60%, (Rampias et al., 2009). All these studies including ours, suggest that HPV-positive HNSCC cells when treated with E6/E7 targeted siRNA are more likely to undergo apoptotic cell death. Taken together our results suggest reliance of HPV-positive HNSCC cells on HPVE7 expression for carcinogenesis.
Since the reliance of E7 was confirmed by \textit{in vitro} study, we tried to understand the pathway through which the expression level of E7 might affect the loss of cell viability in four different of HPV-positive HNSCC cells. Our results in chapter 5 suggest that cell with high level of HPV E7 showed higher percentage of loss of cell viability compared to those that have low HPV7 expression level. Further, the next answer we want to sought was to measure the functional E7 as we have demonstrated in chapter six that there is difference in the level of E7 expression in HNSCC cell lines. To find this, here we propose to investigate the pRb pathway by measuring the pRb and E2F transcriptional factors expression levels. We are assuming the loss of E2F gene, which modulates other cell cycle regulatory genes including p16, will also show reduction in its expression level (Nevins, 2001). In turns, this will up regulate the expression level of pRb. Theoretically and biochemically, the high E7 expression level down regulates pRb, and pRb is up regulated due to loss of E7 (Giarre et al., 2001). Here we could think of two explanations to find out the functional E7 level in all these HPV-positive HNSCC cell lines. First explanation could be, cells with low E7 may have low pRb expression level, and if the level of pRb were the same in all cells, the level of E7 would vary. This could be confirmed by looking at the level of pRb. The other explanation is that cells may have high E7 expression level and high pRb expression level, where the threshold might change. But if the level of pRb were the same in all cells, then by knocking down E7 mRNA expression level would suggest the dependency of cells on E7. Therefore, we would like to measure the level of pRb expression in these HPV-positive HNSCC cells to find out the functional E7 expression levels in these cells.

In \textbf{Chapter 6}, we have demonstrated the significance of Aurora kinase A as a potential therapeutic target and the potential use of Aurora kinase inhibitors as therapeutic strategies in HPV-positive HNSCC. Due to the important roles of aurora kinases in mitosis, their implication in cancers, and the availability of commercial small molecular inhibitors, they have been the investigative subjects in this thesis.

Our results suggest that HPV-positive HNSCC cells are sensitive to MLN8237. Interestingly, there is a variation in the sensitivity of HPV-positive HNSCC cells to MLN8237 depending on their E7 oncogene expression level. The cells with high E7 expression level (UMSCC104 and UDSCC-2) showed great sensitivity to MLN8237,
whereas HPV-positive HNSCC cells (93VU-147T and UPCI: SCC90) with low E7 expression level showed resistance to MLN8237 resulted with high IC50 value (more than 1mmol/L). In fact, cells with no E7 expression level, that is, HPV-negative HNSCC cells showed high sensitivity to MLN8237. However, this might not be the case with cervical cancer as our previous study (appendix 2) suggested selectivity of MLN8237 to HPV-positive cervical cancer cells, whereas the HPV-negative cervical cancer cells were less sensitive to MLN8237.

However, the logical explanation for HPV negative HNSCC cells being sensitive to MLN8237 is that, the over-expression of aurora kinase A in HNSCC, both in clinical samples and in cell lines, regardless of HPV status, had been demonstrated by several earlier studies (Mazumdar et al., 2009, Zhang et al., 2012, Reiter et al., 2006). This indicates that HPV-negative HNSCC cells directly become sensitive to MLN8237 due to the over-expression of aurora kinase A. Our findings on HPV-negative HNSCC cell line sensitivity to MLN8237 was justified as the previous studies have also suggested the effectiveness of MLN8237 in reducing cancer cell growth in HPV-negative HNSCCs both in vitro and in vivo (Dees et al., 2012, Tanaka et al., 2013, Qi and Zhang, 2015). Further, this novel MLN8237 drug is currently under phase-2 clinical trial on a number of cancers, including HNSCC (HPV-negative) (Melichar et al., 2015). Since the major focus of our study is HPV-positive HNSCC, our results showed that MLN8237 could effectively inhibit the cancer cell growth via apoptosis; therefore this could be an effective therapeutic agent for HPV-positive HNSCC with high E7 expression level, but not for those with low E7 expression.

Although we have shown that there is a correlation between the E7 expression level of HPV-positive HNSCC cell lines and the sensitivity of MLN8237, the mechanism behind this relationship remains unclear. One of the possible mechanisms could be increase in the length of mitosis in HPV-transformed HNSCC cells by high E7 expression level. In our previous study (Appendix 2) we have shown that the HPV-transformed cancer cells had approximately 5 times longer mitotic delay compared to HPV-negative cells, this delay is thought to contribute to the selective killing of Alisertib.
Since a number of apoptotic components were reported to be regulated by mitosis (Tunquist et al., 2010, Chen et al., 2005), Aurora A was thought to regulate the stability of Mcl-1 that might be related to the increased delay in mitosis. Further, the level of E7 expression may influence the expression of CDC20 (a mitotic regulatory protein) (Harley et al., 2010), which could also stabilize Mcl-1 in mitosis, thus increase the length of mitotic delay (Figure 7.1). The high level of E7 expressing cells, increase mitotic delay and aids Alisertib to destroy Mcl-1, thus reducing the expression of Mcl-1 in Alisertib treated cells. Where as, low level of E7 may have little effect on mitosis. This statement is reflected in our current experiment in this chapter, as we found that HPV-positive HNSCC cells with high E7 expression level (UDSCC2 and UMSCC104) showed low expression of Mcl-1 apoptotic protein after 72 hours of MLN8237 treatment while the low E7 expressing HNSCC cells, 93Vu147T showed very little effect. Therefore, this increased delay in mitosis, allow the Alisertib to effectively destroy the Mcl-1 and reduce the antiapoptic signal, ultimately leading to apoptosis.

From our experiment we could speculate that both HPV-positive and HPV-negative HNSCC cells are sensitive to MLN8237, suggesting the efficacy of MLN8237 in both HNSCCs. However, this sensitivity to MLN will vary in HPV-positive cells depending on their E7 expression level. Therefore, MLN8237 could be a potential
therapeutic approach in the future for treating patients with HPV-positive HNSCC having high E7 expressing HPV infection.

Conclusions:

The works presented in this thesis have revealed the strong association of HPV with HNSCC in South Asian populations, suggesting a significant burden of this cancer type. The results from our in vitro and in vivo study showed that the E6/E7-siRNA targeting E7 gene could effectively reduce tumour cell growth, suggesting a possible application of siRNA as an effective therapeutic strategy in HPV-positive HNSCC treatment. As the combination therapy is more effective than the single treatment regimen, we could think about the possibility of using E6/E7-siRNA combined with one of the traditional treatment regimen, either surgery or chemotherapy. This would allow us to reduce the traditional treatment related toxicity. Moreover, our studies on HPV-positive HNSCC cell lines using MLN8237 drug, also showed promising outcome, especially for those cells expressing high viral oncogene, HPV E7. Since MLN8237 has already demonstrated its efficacy to reduce tumour growth in a various type of cancers, including HNSCC (HPV-negative), and currently under phase 2 and 3 clinical trials, we could think about the use of this drug as another potential therapeutic modality for treating HPV-positive HNSCC. Despite significant advancement in the field of HNSCC and HPV, more research is required to establish E6/E7-siRNA and/or MLN8237 as effective treatment strategies for HPV-positive HNSCC cases and justify the routine individualised patient treatment in HNSCC cases.
**Future directions:**

More longitudinal research is needed to understand the actual role of HPV in HNSCC development in Bangladesh as well as other countries of South Asia. It is very important to establish a standardised method for accurately screening HNSCC for HPV, which would be fast, cost effective and easily available. Further, we recommend the use of ICD-10 for proper site-specific classification of all cases. It is very important for developing countries such as those in South Asia, to mandate Tumour Boards and perform longitudinal studies on HNC, including a focus on the HPV-associated cases.

Since HPV-positive HNSCC is a distinct subset of head and neck cancer having relatively favourable outcome, de-intensification of traditional treatment modalities are already in place. However, it is important to focus on the development of cancer therapy that would be more effective and have fewer side effects. Our data on HPV-positive HNSCC cell lines demonstrated the efficiency of siRNA targeting E6/E7 genes, leading to significant loss of cell growth, indicating the dependency of these cells on E7. However, the exact mechanism behind this dependency is unclear, although we intend to explore the pRb and p16 pathways to validate our findings. Irrespective of the above, our results from both *in vitro* and *in vivo* studies suggest that siRNA is a potential therapeutic agent for HPV-positive HNSCC cases, perhaps in combination with traditional therapies until its independent effects become manifest.

The rationale for testing MLN8237 on HPV-positive HNSCC cell lines is not only because this drug has been shown to be effective in killing cells in other types of cancer but is under phase 2 and phase 3 clinical trials for several cancers (NCT01045421). Our data demonstrated a significant lethal selectivity of MLN8237 in HPV-positive HNSCC cells, which express E7 at high levels. However, the mechanistic link between the Aurora A kinase and the E7 expression level in HPV-transformed HNSCC cell lines would be an interesting future study. It would be also important to investigate whether Aurora A signalling pathway activation is necessary to induce apoptosis in HNSCC cells. However, based on our findings in the present *in vitro* study, we propose first to validate our findings in animal experiments.
Since combination therapies are becoming commonplace for many cancers, combining siRNA therapy and MLN8237, initially in preclinical models, could be attractive. In fact, we have already tested this combination on an HPV-transformed cervical cancer cell line (unpublished data): unfortunately without a promising outcome so far. The reason for the combination didn’t work well because, we targeted same gene $E7$ using both siRNA and MLN8237. The siRNA effectively reduced the $E7$ mRNA expression level in HPV-transformed cells, so the cells became less sensitive to MLN8237. Further treating those cells with MLN8237 did not show significant loss of cell viability. Based on our findings, one might consider treating HPV-positive cancers that have high HPVE7 expression levels with MLN8237, and cancers, which may show resistant to MLN8237, could be treated with siRNA therapy. However, these approaches require further investigation.

Finally, to increase our understanding of the aetiology, prevalence and incidence, and treatment of HPV-associated HNSCC, extensive future research is necessary. Longitudinal studies of the natural history of oral/oropharyngeal HPV infections are important for determining optimal strategies for preventative immunisation and for evaluating vaccines given as part of therapy. Multicentre clinical trials of screening technologies and of the associated logistics for determining carriage, clearance and persistence of HPV in the upper aero-digestive tract are necessary, with long term follow-up, to determine rates of malignancy, and any association with continued or renewed HPV infection.

References:


APPENDIX -1
**Introduction**

The discovery of RNAi by Fire and Mello in the early twenty first century amazed the scientific world by displaying that a simple small double stranded RNA could silence any complementary genetic sequences and were soon found to be able to inhibit specific genes in human cells. Afterwards, it became the method of choice for understanding gene function. Since RNAi yields temporary loss of gene function, scientists became interested in this new powerful tool that could be designed to produce complete loss of function of a critical mutated gene. Recently, an immensely powerful gene editing toolbox has been discovered, which is known as the CRISPR/Cas system. This is now widely used for genome editing in humans. These two powerful tools offer great promise in cancer treatment. We have discussed the possibility of the application of these tools for treating head and neck cancer in the form of a review article.

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Can gene editing and silencing technologies play a role in the treatment of head and neck cancer?

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ABSTRACT

Conventional treatment strategies have done little to improve the prognosis or disease-free survival in head and neck cancer (HNC) patients. Recent progress in our understanding of molecular aspects of head and neck squamous cell carcinoma (HNSCC) has provided insights into the potential use of cellular targets in combination with current treatment strategies. Here we review the current understanding of treatment modalities for both HPV-positive and HPV-negative HNSCCs with the potential to use gene editing and silencing technologies therapeutically. The development of sequence-specific RNA interference (RNA) with its strong gene-specific silencing ability, high target specificity, greater potency, and reduced side effects, has shown it to be a promising therapeutic candidate for treating cancers. CRISPR/Cas gene editing is the newest technology with the ability to delete, mutate or replace genes of interest and has great potential for treating HNSCCs. We discuss the major challenges in using these approaches in HNSCC; that being the choice of target and the ability to deliver the payload. Finally, we highlight the potential combination of RNAi or CRISPR/Cas with current treatment strategies and outline the possible path to the clinic.

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Introduction

Head and neck cancer (HNC) is a major health problem and a leading cause of morbidity and mortality worldwide. It is regarded as the sixth most common cancer in the world. More than 90% of these malignancies are head and neck squamous cell carcinoma (HNSCC), which mostly comprise of oral squamous cell carcinoma (OSCC), oropharyngeal squamous cell carcinoma (OPSCC) and laryngeal squamous cell carcinoma (LSCC) [1,2]. Tobacco use in different forms (including smoking and smokeless tobacco), areca nut and alcohol consumption are the most common risk factors for the development of HNSCC [3,4]. However, there is now irrefutable evidence of strong association of human papillomavirus (HPV) with HNSCC, especially for OPSCC in younger adult males [5,6]. Current treatment of HNSCC still involves conventional surgery, chemotherapy, and radiotherapy, or a combination of these.

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Radical surgical excision followed by radiotherapy, with or without adjunct chemotherapy, is now commonplace. Due to the complex and delicate anatomical structure of the face and neck, the extent of surgery may be limited, as wide excision results in visible and functional facial deformities: radiotherapy and chemotherapy have low specificity for individual neoplasms and have toxicities affecting the whole body [7]. HNSCC show a wide arrangement of genetic aberrations, increasing with tumour progression and these contribute to a therapeutic failure with conventional therapies [8]. Although in Western countries the average 5-year survival rate for HNSCC patients is now ~60%, one-third of patients develop local or/and regional metastases which reduce survival to less than a year [9,10]. It is clear that there is a need for the development of new treatment approaches, which would better preserve the patient’s physical functions and their quality of life. Targeted molecular therapeutics are promising alternatives for treating cancer patients more efficiently and with limited side effects. One approach is blocking or silencing the expression of a specific gene (or genes) that is vital for continued growth of the neoplasm. Gene silencing or editing, via RNA interference (RNAi) and clustered, regularly interspaced, short palindromic repeats (CRISPR/Cas) respectively, offer new possibilities for HNSCC treatment. The ability of
RNAi to suppress the expression of target genes with high efficiency and specificity is well known. CRISPR/Cas is a more recent discovery that allows the in situ manipulation of target genes but its clinical efficacy is yet to be proven [11]. The issue is which gene does one target and how will the therapy be delivered? Here we discuss gene editing and silencing mechanisms, potential candidate genes and review the various delivery technologies that could be used for HNSCC treatment. We highlight the potential path from bench to bedside for the improved treatment of HNSCC.

Gene editing and silencing

Both RNAi and CRISPR/Cas are programmable systems that allow one to specifically target a mRNA or gene, respectively. By providing each with sequence-specific RNA templates to their target, one is able to precisely change the output of that gene. RNAi achieves this via small non-coding RNAs such as short interfering RNA (siRNA), micro RNA (miRNA) or short hairpin RNAs (shRNAs) [12-14] and takes advantage of a normal cell regulatory system built into every eukaryotic cell. Structure-wise, siRNAs are 21 base pairs in length and are comprised of a 19 nucleotide duplex with a two nucleotide overhang at each 3' end [15]. They can be introduced into cell cytoplasm in three different forms: (a) as synthetic siRNA or miRNA transfected directly into the cell [16]; (b) as longer dsRNA, transfected into cells, that are subsequently processed into siRNAs by the cytoplasmic enzyme Dicer (an RNase III endonuclease) [17]; or (c) as plasmid DNA that expresses an siRNA, which is exported from the nucleus to cytoplasm and also processed by Dicer into an siRNA [18]. RNAi knocks down protein expression by functioning through three different cellular mechanisms. Firstly, it can inhibit transcription from genomic DNA by targeting complementary promoter regions through epigenetic silencing [19,20]. Secondly, it can inhibit the ribosomal translation of mRNA into protein [21]. The third and most well understood mechanism of RNAi is to form a cytoplasmic RNA-induced silencing complex (RISC) that causes destruction of the targeted mRNA [15,22], as seen in Fig. 1. In this mechanism, the single strand of RNAi (siRNA) has to be exactly complementary to the target mRNA.

The siRNA is loaded into the RNA-induced silencing complex (RISC) and the strands separated by the helicase activity with the antisense strand (complementary to the target mRNA) staying associated with the RISC. This then permits its binding with the homologous-targeted mRNAs through base-pair interactions, leading to endonucleolytic cleavage and subsequent degradation of targeted mRNA in the RISC by the action of the cellular exoribonucleases [23] (Fig. 1). Importantly, as the antisense-strand of siRNA is protected by the RISC from degradation, multiple mRNAs can be cleaved from a single siRNA [23]. This, therefore, allows RNAi to be used as a potential therapeutic tool for treating different diseases including viral infections, cancer and inherited genetic disorders.

Until recently it was though that adaptive immunity was a distinct feature only seen in eukaryotes. However, the recent discovery of CRISPR-Cas system suggest that the prokaryotes and archaea also possess a naturally occurring, complex and adaptive defence system [24]. CRISPR-Cas has gained much attention due to its powerful genome editing ability and possible therapeutic potential. The system is composed of a CRISPR — RNA sequence (crRNA), a

Fig. 1. The gene silencing mechanism of siRNA. The synthetic siRNA directly and long double-stranded RNA (dsRNA) processed by dicer to form siRNA into the cytoplasm. The activated siRNA associates with RNA-inducing protein complex (RISC) and mediates target sequence specificity for subsequent mRNA cleavage. This RISC complex is recycled and induces multiple rounds of silencing.
leader sequence plus an array of identical repeats interspersed with non-repetitive spacer sequence targeting foreign DNA, plus the associated Cas endonuclease protein [25]. Typically, the length of identical repeats in a CRISPR array is 28–40 base pairs but may vary in different organisms [25].

The CRISPR-Cas system is classified into two main classes and six types based upon the effector Cas proteins [26]. The class 1 CRISPR-Cas system (includes types I, III and IV) consists of a multi-protein complex, which uses a large complex of Cas proteins to guide crRNA to the targeted site. Whereas, the class 2 CRISPR-Cas system (including types II, V and VI) requires only one effector protein to mediate RNA-guided nucleic acid cleavage [26]. This makes it a useful tool in genome engineering and the type II CRISPR-Cas system containing Cas9 protein, was the first to be adapted to target, edit or modify genome in eukaryotic cells [27]. This ability of the CRISPR-Cas9 system to recognise and cleave any genome has enabled it to be used in a broad range of applications, including genetic modification of cells, genome screening and in animal models [11,28]. Recently, the CRISPR-Cas9 system has also shown to be applied successfully for genome editing in human cells [29].

Despite the rapid progress in the development of CRISPR-Cas9 technology, the exact mechanism of recognition and cleavage of the targeted DNA remains unclear. However, it is believed that, the CRISPR/Cas9 system allows for RNA-guided, DNA-cleavage [30] and can be utilized as a programmable platform to generate precise insertions or deletions (indels) in living cells [11,28,31]. It requires basically two elements; a guide RNA of 17–24 bp in length that programs the Cas9 endonuclease to its target site, and the Cas9 protein itself, which induces double-stranded breaks (DSB) at specific sites of targeted DNA via the use of HNH and RuvC-like domains. These breaks are usually repaired by either non-homologous end-joining (NHEJ), often resulting in variable-length indels (as this process is error-prone), or by homology-directed repair (HDR), which results in replacing the target sequence with a more desirable one via a user provided donor DNA template (Fig. 2) [32,33]. Alternatively, you can have two targets close to each other, which can result in deletion of the sequence in between the sites following repair. This ability to alter genes in vivo is a remarkable step forward in the gene therapy field and follows on from a number of previous systems such as Transcription activator-like effector nucleases (TALENs) and Zinc-finger nucleases (ZFNs). CRISPR/Cas9 has several advantages over these previous systems including the ability to edit multiple genes simultaneously, it is relatively small, no need for target site modification and is significantly cheaper and easier to manufacture. Overall, RNAi is able to silence a target mRNA but the gene is still present and functional so the interference needs to always be present whereas CRISPR/Cas only needs to alter the target gene and is no longer required. Moreover, the change is permanent. A comparison of the features between RNAi and CRISPR/Cas is outlined in Table 1.

**CRISPR/Cas (gene editing) in cancer treatment**

The recent advent of genome editing technologies based on programmable nucleases have improve the ability to delete or modify specific nucleic acid sequences in the genome of cells or in the animal models that aided us to explore the role of particular genes in
<table>
<thead>
<tr>
<th><strong>Mechanism</strong></th>
<th>Knockdown (siRNA)</th>
<th>Knockout (CRISPR/Cas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location of activation</td>
<td>RNA machinery appears to be mostly active in the cytoplasm, which makes transgenic mice more difficult to target.</td>
<td>CRISPR/Cas system is active on nuclear chromosome and allows the gene expression from their endogenous genomic locus.</td>
</tr>
<tr>
<td>Loss-of-function</td>
<td>Post-transcriptional RNA degradation</td>
<td>Frame shift, deletion or insertion</td>
</tr>
<tr>
<td>End result</td>
<td>Reversible knockdown, some functional RNA remains and transcribed into lower levels</td>
<td>Permanent knockout</td>
</tr>
<tr>
<td>Guiding sequence</td>
<td>siRNA</td>
<td>sgRNA</td>
</tr>
<tr>
<td>Off-target space</td>
<td>Transposon</td>
<td>Genome</td>
</tr>
<tr>
<td>Off-target effect</td>
<td>Significant off target effects</td>
<td>Binds to a large number of off target sites, but the specific knockdown efficiency is superior to RNA</td>
</tr>
<tr>
<td>Delivery</td>
<td>Similar issues to CRISPR/Cas</td>
<td>Similar to RNA</td>
</tr>
<tr>
<td>Components</td>
<td>Synthetic siRNA</td>
<td>Cas protein (as plasmid DNA, mRNA or protein) plus guide RNA</td>
</tr>
<tr>
<td>Application</td>
<td>When transient loss of genetic function is required</td>
<td>Introduction of point mutation, correction of pre-existing mutation and producing true gene null allele (deletion)</td>
</tr>
</tbody>
</table>

**Advantages**

a. The silencing machinery is present in almost all mammalian cells
b. Genetic manipulation is not required for the target cell line prior to use
c. RNA is the simplest system compared to CRISPR/Cas
d. As most mammalian cells contain RNA silencing machinery, the use of RNA technology is rapid and saves time and money

**Limitations**

a. The artificial introduction of siRNAs or shRNAs into a target cell line can cause non-sequence-specific off-target effects
b. Delivery to a specific site is still limited

c. CRISPR/Cas has superior knockdown efficiencies compared to RNA

- a. CRISPR/Cas may have few of target effects however, it still requires more research for its validation
b. CRISPR/Cas produces a more consistent and permanent knockdown

c. Significantly stronger loss-of-function phenotypes

CRISPR/Cas is rapidly moving towards the application of it in the clinic with five clinical trials recently approved. Most propose using CRISPR/Cas to improve the performance of cell-based immune therapy by inactivating the PD-1 protein as this approach has proved successful using monoclonal antibodies with drugs such as pembrolizumab (Keytruda\textsuperscript{*}). The first clinical trial started in late 2016 at Sichuan University in China, delivering CRISPR/Cas modified cells in a lung cancer patients [37]. The other four trials are not yet recruiting at this time but include; a trial led by Edward Stadmayer of the University of Pennsylvania which was approved by the NIH in mid 2016 and will undertake three separate CRISPR edits in a range of different cancer types [38]; The other three recently register trials from Peaking University to study the efficacy of CRISPR/Cas targeting PD-1 in bladder, prostate and renal cell cancers.

**Currently available delivery technologies**

The ultimate success of these therapies relies on the adequate delivery of the siRNA or CRISPR/Cas system into the cell. While siRNA delivery is either via synthetic RNA or a DNA plasmid, CRISPR/Cas can be delivered in a variety of forms including guide RNA (gRNA)/DNA (expressing Cas protein), as gRNA/mRNA for Cas, or RNA/Cas protein. Nevertheless, the challenges for delivery are the same for both systems as delivery of RNA or DNA encounters a range of barriers that must be overcome. These include avoiding degradation by serum components, rapid renal clearance, immunostimulation, achieving uptake into cells, subsequent escape into the endosomal compartment and, most importantly, having maximal uptake into target cells – be specific for the neoplasm itself. Conventional siRNAs on their own (m 化 siRNA) where found to be not stable in body fluids due to their size, charge, short half-life and susceptibility to nuclease attack, although a range of clever chemical modifications have alleviated this issue [39]. To overcome these hurdles nanoparticles (NPs) or delivery vectors (such as viral vectors) were developed (Table 2).

Due to their high efficiency of gene delivery and ability to hold large payloads, viruses have been used extensively [40]. Adenovirus, Adeno-associated virus and Lentiviruses are commonly...
used viral vectors for RNAi delivery [40–42]. However, viral vectors have some limitations, include high immunogenicity, short duration of shRNA expression, increased cellular toxicity, insertional mutagenesis and low level of silencing of gene expression [43].

Another popular method for delivery of shRNA is the use of nanoparticles (NPs) (Table 1). NPs are 15–100 nm in diameter and are manufactured from a range of different materials that act as carriers or transporters of drugs, small molecules (nucleic acids), and ligands [44]. Due to their size and large surface area to volume ratio, they can be highly efficient at loading and delivering their cargo [45]. More importantly, they protect RNA and DNA from attack and degradation from nucleases and other proteins found in bodily fluids. As RNA and DNA are highly negatively charged, NPs are designed to incorporate positively-charged components to aid in encapsulation and packaging. These include cationic lipids (1,2-dioleoyl-3-trimethylammonium propane = DOTAP and N-[1-[2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride = DOTMA), ionising lipids (1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-[bis(2-aminoethyl)iminooxy]ethane-N,N,N,N-tetraacetic acid (EGTA), and liposomes (polylethylene–amine – PEI) [46]. Further, NPs can be coated with hydrophilic polymers, such as polyethylene glycol (PEG), to prevent opsonisation by the mononuclear phagocytic system (MPS) and increase circulation time [47,48]. The RNA/DNA-NP complex is generally taken up by cells via endocytosis but must then escape from the endosomes before they mature to lysosomes and are degraded (Fig. 1). To achieve this, fusogenic lipids such as Ectosmitt-DMA or dioleylphosphatidylethanolamine (DOPE) can be added, which destabilize the endosomal membrane and allow RNA/DNA release into the cytosol [49]. Alternatively, one can incorporate ammonium groups to take advantage of the proton-sponge effect, whereby endosomes swell and break open to release their contents [50,51]. The PEGylated liposomes are one of the most commonly used NPs for effective delivery of shRNA into cancer cells. We will discuss the practical use of delivery systems for HNSCC below.

**Target selection**

The treatment-related toxicities, complications and recurrence rates, especially in advanced disease stages, with current conventional therapies have necessitated the focus on molecular targeted therapy. Currently, we are using the same treatment strategies to treat HPV-positive and HPV-negative HNSCC but they are clearly two different types of malignancy. Moreover, the key drivers of cancer formation and maintenance are also different in each type (and likely there are many more sub-types within these groups). The key question therefore is what are the appropriate targets for gene silencing or editing. EGRF is commonly over-expressed in HPV-negative HNSCC while it is rare or absent in HPV-positive cancer [52]. HPV-negative tumours also exhibit an over-expression of transcription factor (TP53, oncopogene p53 and the CDKN2A gene [53]. Alternatively, the key genes to target in HPV-positive HNSCC are the viral oncoproteins E6 and E7 as they critically deregulate cell cycle and initiate genomic instability [8].

**Application of shRNA in HPV-negative HNSCCs**

A number of molecular targets that are over expressed in HPV-negative HNSCC have been investigated for shRNA therapy (Table 3). For example, the most commonly overexpressed gene detected in HPV-negative HNSCC is epithelial growth factor receptor (EGFR), with more than 95% of cases being associated with poor prognosis [55]. Currently, cetuximab, a monoclonal antibody produced by Merck KGaA & Bristol-Myers Squibb that specifically targets the extracellular domain of EGFR, is used as a therapeutic agent in combination with either radiotherapy or chemotherapy for treating locally advanced HNSCC cases [56–59]. A recent comprehensive study on a phase 3 clinical trial has shown that HNSCC patients treated with cetuximab and radiotherapy have a higher percentage of 5 year survival (45.6%) compared to those treated with radiotherapy alone (36.4%) [59]. However, the recent study also reported that majority of the patients (~95%) treated with cetuximab suffered from skin toxicity (acneiform rash with grade 2 or more severity) within 35 days of the treatment [58]. A study...
on siRNA targeting EGFR has shown efficient tumour growth inhibition (~37%) in a tumour xenograft mouse model [60].

Other example targets in HPV-negative HNSCCs are critical transcription factors (TFs). As TFs have flexible structures, they have proven very difficult to target via small molecular drugs. This is where siRNA can play an effective therapeutic role as siRNA has the ability to target such undruggable proteins [61,62]. The nuclear factor-κB (NF-κB) protein/p65 protein (essential for growth and survival of the neoplasm) and hypoxia inducible factor 1α (HIF-1α) (TF of oxygen regulated genes) are commonly overexpressed in HNSCC cell lines [63,64]. Studies on siRNA targeting NF-κB have shown a significant knockdown of NF-κB gene (~50%) that effected ~25% growth inhibition in HNSCC cancer cell lines but sensitized the cells to proteasome inhibitor, Bortezomib. The combination of NF-κB-siRNA and Bortezomib has shown approximately ~65% cell death in HNSCC cells [65,66]. Another study has shown 80–90% reduction of HIF-1α activity with approximately 35% growth inhibition in HNSCC cells and significant reduction in tumour size in an animal model [67]. This study also showed that combination of photodynamic therapy and HIF-1α-siRNA was a more effective approach, resulting in approximately 85% reduction in tumour volume [67].

Application of siRNA in HPV-positive HNSCCs

HPV-positive HNSCC is regarded as an entity discrete from HPV-negative HNSCC, with different molecular mechanisms and different molecular targets. Although the discovery of preventive and therapeutic vaccines has been well demonstrated in cervical cancer, the effectiveness of these vaccines in HPV-positive HNSCC is still under investigation. Since HPV-driven cancers mostly rely on the virus for cancer progression [68], selectively targeting and inactivation of HPV using RNAi could be a viable approach to treating these cases.

As the E6 and E7 oncoproteins are constitutively expressed throughout cancer progression, they have become attractive targets for antiviral or virus-related cancer therapy [69]. This has been demonstrated clearly on other HPV-positive cancers, such as cervical cancer, where siRNA targeting E6, E7, or both genes results in growth inhibition, apoptosis, senescence, and efficient growth inhibition in animal models [70–73]. However, the efficacy of siRNA in HPV-positive head and neck cancer settings remains unclear; few studies have been reported. A major reason could be the paucity of well-established HPV-positive HNSCC cell lines.

One recent study has, however, shown promising results: siRNA targeting of E6 and E7 reduced cancer cell viability by ~40%, with approximately 60% knockdown of E7 gene and significant reduction in tumour volume in an animal model [74]. Another study, using HPV-positive HNSCC cells in vitro shows that radiation sensitivity of HPV-positive HNSCC is related to HPV E6 gene expression level, because down regulation of wild-type p53 gene by siRNA produced radio-resistance [75].

Since RNAi and CRISPR/Cas function more or less in similar fashion, the CRISPR/Cas system could also be used as an alternative and more advanced approach to treat HNSCC cancer efficiently. However, studies in cervical cancer have shown promising outcomes. For example, CRISPR/Cas9-mediated deletion of E6/E7 was shown to produce cell death in cultured cells and a significant reduction tumour growth in an animal model in vivo [80,81]. This looks to be a promising approach for
Table 3
An overview of potential molecular targets in HPV-negative HNSCC targeted by RNAi (Pre-clinical studies).

<table>
<thead>
<tr>
<th>Molecular Targets</th>
<th>Technique (targeting agents)</th>
<th>Nanoparticle based delivery platform (vectors)</th>
<th>Effects on HNSCC cells (in vitro and in vivo)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth factors/Receptors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>siRNA</td>
<td>Liposome (cationic)</td>
<td>(1) Reduction in cellular proliferation</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2) Increased cell viability</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>siRNA</td>
<td>Lipid calcium phosphate nanoparticles</td>
<td>(3) Increase in cellular proliferation</td>
<td>[104,105]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4) Reduction in angiogenesis and tumourigenes in vivo</td>
<td></td>
</tr>
<tr>
<td>CD74</td>
<td>siRNA</td>
<td>Lentiviral particles</td>
<td>(5) Reduction in tumour growth both in vitro and in vivo</td>
<td>[106]</td>
</tr>
<tr>
<td>SET</td>
<td>siRNA</td>
<td>Cationic Polymer</td>
<td>(6) Reduction in tumour growth both in vitro and in vivo</td>
<td>[107]</td>
</tr>
<tr>
<td><strong>Transcription factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-kB</td>
<td>siRNA</td>
<td>Liposome (cationic)</td>
<td>(1) Reduction in tumour growth both in vitro and in vivo</td>
<td>[65,66]</td>
</tr>
<tr>
<td>HIF-1x</td>
<td>siRNA</td>
<td>Lipid nanoparticles</td>
<td>(2) Reduction in tumour growth both in vitro and in vivo</td>
<td>[67]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(3) Induction of apoptosis</td>
<td></td>
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<tr>
<td><strong>Protein kinases/Cell cycle regulatory proteins</strong></td>
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<tr>
<td>PKCc</td>
<td>siRNA</td>
<td>Plasmid vectors (siRNA expression vectors)</td>
<td>(1) Reduction in invasion and migration of tumour cells</td>
<td>[108]</td>
</tr>
<tr>
<td>AURKA</td>
<td>siRNA</td>
<td>Lipid nanoparticles</td>
<td>(2) Induction of apoptosis</td>
<td>[109]</td>
</tr>
<tr>
<td>B-RMM2</td>
<td>siRNA</td>
<td>CALAA-01 nanoparticle (cyclodextrin based polymer)</td>
<td>(3) Increased proliferation</td>
<td>[110]</td>
</tr>
<tr>
<td>CK2</td>
<td>siRNA</td>
<td>Liposome</td>
<td>(4) Reduction in tumour growth both in vitro and in vivo</td>
<td>[111]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(5) Inhibiting cellular migration</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(6) Increased cell sensitivity</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>siRNA</td>
<td>Liposome</td>
<td>(7) Reduction in cellular proliferation</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8) Increased cell sensitivity</td>
<td></td>
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<tr>
<td><strong>EML components</strong></td>
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<tr>
<td>MMP-9 (MMP-9 and MMP-11)</td>
<td>siRNA</td>
<td>Lentiviral particles</td>
<td>(1) Reduction in invasion and migration metastasis of tumour cells both in vitro and in vivo</td>
<td>[113]</td>
</tr>
<tr>
<td><strong>Apoptotic proteins</strong></td>
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</tr>
<tr>
<td>BCL-XL</td>
<td>siRNA</td>
<td>Lipid nanoparticles</td>
<td>(1) Induction in apoptosis</td>
<td>[114]</td>
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EGFR = epithelial growth factor receptor, VEGF = Vascular endothelial growth factor, NF-kB = Nuclear factor kappa B, HIF-1x = Hypoxia inducible factor 1x. PKCc = Protein kinase C, AURKA = Aurora kinase A, B-RMM2 = Ribonucleotide-diphosphate reductase M2, CK2 = Casein kinase 2, MMP-9 = Matrix metalloproteinases, BCL-XL = B-cell lymphoma extra large, TERT = Telomerase reverse transcriptase, Mcl-1 = Myeloid leukemia cell protein.

HPV-positive HNSCC and has the advantage of permanently removing the driver oncogenes rather than just silencing the EGFR output, as RNAi would achieve. However, this remains to be explored.

**Delivery in the HNSCC setting**

Here we will consider delivery in the context of siRNA but as mentioned above, the challenges apply equally to CRISPR/Cas. In the HNSCC setting, the ability to deliver siRNA in the tumour microenvironment for effective gene silencing is a critical issue. Here we suggest several approaches that offer a potential way forward (Fig. 4). The preferred approach would be localised delivery directly to the tumour site as this avoids a number of issues found with systemic delivery, such as poor targeting of tumours, damage to normal cells, rapid clearance, and inflammatory responses. Local delivery requires less material and can potentially achieve higher delivery of RNA or DNA.

Our first suggestion is to use a biodegradable PEG (polyethylene glycol)-entrapped Lipoplex-entrapped Alginate Scaffold (PLAS) system, which is a sustained release system that has the ability to release RNAi continuously in the tumour over at least 24 h [82]. It allows the siRNA to reside longer at the site where NP-only delivery, or even naked siRNA would be quickly removed by flow of body fluids. Indeed this has been shown in the vaginal setting where PLAS dramatically improved siRNA uptake and gene silencing in vivo [83]. The PLAS system works by breaking down in the presence of sodium ions within body fluids, which displace the calcium used to crosslink the scaffold and triggers the release of RNAi slowly over time [82]. This has been demonstrated with vaginal tumour settings in mouse models, where a 9-fold increase in siRNA delivery compared to cationic lipid NPs was seen and a remarkable 85% knockdown of the targeted Lamin A/C gene was achieved [82]. A second approach is use of micro- or nano-needle arrays that penetrate the skin barrier and efficiently deliver RNA/DNA to provide local gene silencing (or, potentially, editing) [84,85]. For example, Nanopatch™ micro-projection arrays have been developed that allow consistent and controlled delivery of NP containing siRNA to the targeted area, where they diffuse through skin and effective silence the target gene [86-88]. These have the advantage of requiring fewer doses and a lower amount of siRNA. As Nanopatch™ works on skin there is no reason to think these systems will not be effective on mucosal surfaces such as the oral cavity and elsewhere in the upper aero-digestive tract.

Other potential methods include the use of medicated chewing gum, muco-adhesive hydrogels, or other versions of alginate patches such as ALG (alginate acid and sodium and potassium alginate) oral patches [89-91] (Fig. 4). These can provide prolonged retention with prolonged drug release, and would clearly be more acceptable to patients [90]. ALG mucoadhesive oral patches have
good sol-gel transition properties and have been shown to have acceptable biocompatibility, with appropriate biodegradation times [92]. These could be used to deliver EBV7-targeted siRNA encapsulated within lipoplex for local treatment of HPV-positive oral cavity cancers, although we are not aware of any publications to date in this area. It is understood that a visible resectable mass may not need local treatment as the patient would undergo surgery, therefore, such patches could be used in combination with traditional therapies: the siRNA containing patches could be placed on the tumour site followed by observation of response before considering surgery or chemo/radiotherapy. Alternatively the patches could be placed after surgery, before considering chemo- or radiotherapy, in the hope of avoiding their harmful side effects, or on precancerous lesions as a means to avoid surgery. In fact, these local delivery approaches could be very useful for oral potentially malignant disorders. The siRNA therapy could also play a role in recurrent cancer cases.

While the oral cavity itself is easily accessible, most HPV-positive head and neck carcinomas are in the oropharynx, a more difficult site to access; this could be helped using modern transoral robotic technology. Alternatively one could consider systemic delivery of EBV7-targeted siRNA encapsulated in PEGylated lipoplex. This has been demonstrated in cervical cancer settings in mouse models with approximately 50% reduction in tumour size achieved[93].

Challenges and gaps

Both the RNAi and CRISPR/Cas system have the potential to play a major role in HNSCC treatment. RNAi-based therapies for HNSCC are still at the preclinical stage but show considerable promise. The CRISPR/Cas approach has the added advantages of being able to repress multiple target genes simultaneously and to permanently change genetic codes at genome level, but it is still too early to predict their clinical use. To date, only 10 early-phase clinical trials (phase I or phase I/II) of siRNA and miRNA therapeutics for cancer have been reported, and none of them have reached routine clinical use [94]. The reasons for this low success rate of the RNAi approach relate to of delivery challenges, targeting and off-target effects, although we now understand in detail the reasons for these off-target effects and can design accordingly [95]. Similar challenges would apply in the HNSCC setting. We already have a list of molecular targets for HPV-positive and HPV-negative HNSCC, although more work is required to refine and expand this list. A major bottleneck is the paucity of cell lines, animal models and sequencing data from primary tissues, and improved local delivery. There are other general challenges in the HNSCC setting including tumour heterogeneity and a lack of deep bioinformatics that would allow an understanding of the driver pathways for survival, growth and drug resistance. However, we believe that combination therapy of siRNA and surgery with or without chemotherapy will offer the most promising way forward clinically. Finally, in terms of delivery systems there are a number of challenges to address including a lack of mechanistic insight into how these systems might be effective in the oral cavity to achieve local delivery, limit systemic spread, and offer effect treatment. While there are many nanoparticle systems, few studies have investigated this in the oral setting.

Conclusion

Even with the rapid progress of science and technology and advancement of treatment strategies, head and neck cancer remains a significant cause of morbidity and mortality worldwide.
While RNAi therapies against cancer have not yet been tested in human trials, they have been shown to reduce the expression of targeted proteins with very limited side effects, both efficiently and specifically. This suggests their potential for treating cancers in humans efficiently. We believe that gene silencing and editing holds great promise to break our current treatment impasse; however, appropriate target selection and optimal delivery mechanisms remain major hurdles. As we rapidly generate more genetic knowledge about HNSCC, via whole genome sequencing, RNASeq and related approaches, the information reviewed here will allow us to develop novel therapeutic approaches to improve survival and quality of life of HNSCC patients in the future.

Conflict of Interest
None declared.

Acknowledgements
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References


Appendix 2
Aurora kinase A is critical for survival in HPV Transformed cervical cancer

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Aurora A Is Critical for Survival in HPV-Transformed Cervical Cancer

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Abstract

Human papillomavirus (HPV) is the causative agent in cervical cancer. HPV oncoproteins are major drivers of the transformed phenotype, and the cancers remain addicted to these oncogenes. A screen of the human kinome has identified inhibition of Aurora kinase A (AURKA) as being synthetically lethal on the background of HPV E7 expression. The investigational AURKA inhibitor MSNR257/Alisertib selectively apoptotic in the HPV cancers. The apoptosis was driven by an extended mitotic delay in the Alisertib-treated HPV E7-expressing cells. This had the effect of reducing Akt-1 levels, which is destabilized in mitosis, and increasing Bim levels, normally destabilized by Aurora A in mitosis. Overexpression of Mcl-1 reduced sensitivity to the drug, the level of HPV E7 expression influenced the extent of Alisertib-induced mitotic delay and Mcl-1 reduction. Xenograft experiments with three cervical cancer cell lines showed Alisertib inhibited growth of HPV and non-HPV xenografts during treatment. Growth of non-HPV tumors was delayed, but in two separate HPV cancer cell lines, regression with no resumption of growth was detected, even at 50 days after treatment. A transgenic model of premalignant disease driven solely by HPV E7 also demonstrated sensitivity to drug treatment. Here, we show for the first time that targeting of the Aurora A kinase in mice using drugs such as Alisertib results in a cure, suggesting a new treatment strategy that may be useful in treating HPV-driven cancers.

Introduction

Human papillomaviruses (HPV) have been identified as a definitive agent in cancers of the cervix, penis, vulva, vagina, anus, skin, eye, and head and neck, and is responsible for more than 6.1 million deaths, 5% of the total cancer burden worldwide (1). The papillomaviruses are small, double-stranded DNA viruses belonging to the family Papillomaviridae. High-risk HPV have been identified as the causative agent in 99.7% of cervical cancers (2), have been detected in more than 50% of other anogenital cancers, and in more than 70% of cancers of the oropharynx (3, 4). The most prevalent high-risk HPV types are HPV-16 and HPV-18, which account for approximately 70% of HPV-related cervical cancers, with another 10 high-risk types making up the other 30% (5).

Although HPV vaccines are available and highly effective (reviewed in ref. 6), they are clearly most useful if given before viral exposure. Even with the advent of vaccines, cervical cancer will remain a serious health issue in unvaccinated and under-vaccinated women (7). The use of more targeted approaches is now beginning to improve outcomes in other cancers but no such therapy for HPV-driven cancers is in the clinic. Indeed, chemoradiotherapy is still the primary treatment modality and there has been little improvement in 5-year outcomes (8).

High-risk HPV promotes cancer via the action of the E6 and E7 oncoproteins. The E6 gene product binds to the p53 tumor-suppressor protein and targets it for ubiquitin-mediated degradation (9). E6 also blocks senescence by stimulating inositol phosphatase activity as well as an increasing number of other proteins (10). The E7 protein also has a range of targets, including the retinoblastoma protein (pRb) family, the stb family, and directly drives genomic instability (10, 11). Therefore, the overexpression of E6 and E7 allows uncontrolled cell growth and increased genomic instability, which promotes transformation and carcinogenesis. This process occurs over an extended timeframe (up to 20 years) due to the fact that E6/E7 alone are not sufficient to drive cancer secondary mutational events also contribute (12), thus making specific treatment difficult as each HPV cancer has a different spectrum of mutations.

The one consistent feature of cervical cancers is the continued dependence on HPV E6 and E7 expression. Depletion of E6/E7 is sufficient to drive even long established cell lines into either
Materials and Methods

Cell culture

All cervical cancer cell lines were originally obtained from the ATCC except for C33A. Cervical cancer cell lines (HeLa, CaSki, ME-180, SiHa, C33A, HTS, and C33A HPV16/E7) were maintained in complete DMEM (GIBCO; Invitrogen) supplemented with 10% serum supplement (BioWhittaker, Lonza), 1 mM sodium pyruvate (GIBCO) and 2 mM L-glutamine (GIBCO) at 37°C and 5% CO2. Squamous cell carcinoma cell lines were kindly given by Associate Professor Nicholas Saunders (The University of Queensland Diamantina Institute, Brisbane, Australia) and were cultured in DMEM/F12 (1:1; GIBCO) containing 10% serum supplement (Lonza) at 37°C and 5% CO2. All cell lines were tested and free of Mycoplasma and authenticated with short tandem repeat fingerprinting at the time of use. SCC25 cells were transduced with lentivirus expressing HPV18 E7 or empty vector as described previously (14). The vector places the HPV18 E7 5' of an IRIS GFP resulting in GFP expression at a level which is an indicator of the level of E7 expression. qPCR analysis of relative E7 gene expression was undertaken using ΔΔCt analysis and β-actin as the housekeeper.

siRNA screening

Detailed methods for the siRNA screening are provided in Supplementary Material.

How cytometry

All tested cells were exposed to final concentrations of 5 μM Alisertib or DMSO (vehicle) for 24, 48 or 72 hours. Cells were analyzed for DNA content by flow cytometry using BD FACs Canto II (BD Biosciences) and data analyzed with FlowJo software (FlowJo Co.) as described previously (15).

Time-lapse microscopy

Cells were either treated with 5 μM Alisertib or DMSO (vehicle) followed by time lapse microscopy using a Zeiss Axiovert 200M Cell Observer microscope equipped with an incubation chamber at 37°C and 5% CO2. Images were captured at 20 minute intervals with a minimum of 150 cells per condition per cell line as described previously (13). Time in mitosis and exit from mitosis was monitored and assessed for successful cellular division, failure of cytokinesis, or cell death.

Immunoblotting

Cells were lysed and immunoblotted as described previously using chemiluminescence detection imaged with a CCD camera (15). Band intensities were quantified using ImageJ software. Antibodies to β-actin, Bcl-2, PARP, cleaved caspase-3, Cell Signalling Technology, Aurora A and Aurora B (Becton-Dickinson), Bcl-XL (AbCam), and α-tubulin (Sigma-Aldrich) were purchased from the indicated suppliers.

Mouse xenograft models

Mice (6-weeks-old female nude: ARC) were inoculated s.c. in the right flank with 1 x 10^6 cells in Matrigel. For each cell line, 6 mice were used for the treatment with Alisertib and 6 for the vehicle control only. When tumors were palpable (3-4 weeks following injection), 100 μl of a 20 mg/ml solution of Alisertib was administered daily for 10 consecutive days. Mice were then dosed daily by scoring any tumor regrowth or until culled. All animal studies were approved by the University of Queensland Animal Ethics.

K14E7 transgenic mouse grafting experiments

Groups of 7 mice with well-healed (up to 5 months) grafts of either wild-type or K14E7 skin were treated with or without two cycles of Alisertib as for the xenograft experiments. At between 2 and 10 days after the final cycle, mice were sacrificed and grafts harvested for immunohistochemical staining. Formalin-fixed, paraffin-embedded samples were either immunostained for cleaved caspase-3 (Cell Signalling Technology) or stained using toluidine blue, pH 1. The number of cells stained was visually assessed by microscopy.

Immunofluorescence

Cells were cultured on poly-L-lysine coated glass cover slips with vehicle (DMSO) or 5 μM Alisertib for 24 or 48 hours. Coverslips were fixed with 20% methanol and stored at -20°C until processing. Coverslips were stained for microtubules and DNA as described previously (16).

qPCR

Extract of RNA, cDNA generation, and qPCR were carried out as described previously described (17).

Results

siRNA library screen and target selection

To discover synthetic lethal interactions with the HPV oncogenes E6 and E7 in cervical cancers, we undertook an siRNA library screen using the Dharmacon human siGENOME siRNA Library for Protein Molecules (targeting 778 genes). The primary screen used CaSki (cervical cancer HPV16), and C33A (cervical cancer non HPV) cell lines. Data were normalized using Z-score transformation for each assay and cell line, and genes sorted by Z-score on CaSki viability. Using the parameters of viability, cytotoxicity, and cell number, we identified genes that when depleted were selectively lethal to the HPV-positive CaSki cell line compared with the HPV-negative C33A cells (Fig. 1A; full screening data presented in Supplementary Table S1). This was visualized using hierarchical clustering and Principle Components Analysis (details of the analysis are provided in Supplementary Fig. S1). We identified a group of genes whose knockdown resulted in reduced viability in all cells, including PLK1, WEE1, and CDK1, which were excluded from further analysis. From this primary screen, we identified a set of 54 genes for secondary screening using the OnTarget Plus siRNA Smart Pool that has reduced off-target effects due to their modified passenger strand and >90% have different target sites
Figure 1.
Kinome siRNA screen of cervical cancer cells. A, cell viability (resazurin) analysis following treatment of CaSki and C33A cells with the Dharmacon Human siGENOME SMARTpool siRNA Library. Each data point represents a mean Z-score of three replicates. B, summary of the top 54 selected hits from the primary screen reviewed using OnTarget Plus siRNA in an expanded panel of cell lines. Only the cytotoxicity data are shown. The top two hits from this validation set are indicated (box). C, cells transfected with either Aurora A kinase (AURKA) or Aurora B kinase (AURKB) ON-Target Plus siRNA and harvested 24 hours after transfection. Non-targeting siRNA (siNT) was used as a control. The levels of Aurora A or B were assayed for the appropriate siRNA, with α-tubulin as a loading control.

(Supplementary Table S2). The secondary screen siRNAs were applied to a larger panel of cell lines including HeLa (HPV18), SiHa (HPV16), and HaCaT (HPV negative) cells using the viability, cytotoxicity, and cell count assays parameters.

From the secondary screen, we identified the genes AURKA and AURKB as the strongest hits (those with the highest Z-scores) in all three assays (Fig. 1B and Supplementary Fig. S2). Other genes such as CSG2, STK, MAPK13, PRR5AR2B, and STK23C showed activity in one or two assays, but not in all three. Using Western analysis, we confirmed siRNA depletion of the respective target proteins of our two top hits, Aurora A and Aurora B kinase. Moreover, we observed no obvious differential expression of these proteins in HPV and non-HPV cancer lines (Fig. 1C). With three of the top seven targets (AURKA, AURKB, and CSG2) acting as regulators of mitosis it suggested that mitosis may be the common target in the HPV cancers. However, two mitotic inhibitors, the Plk1 inhibitor BI2536 and paclitaxel showed no selectivity between the HPV-positive and -negative cell lines (Supplementary Fig. S3), suggesting a more specific mechanism may be responsible for the HPV-mediated sensitivity.

HPV cancer cell lines are highly sensitive to inhibition of Aurora A kinase in vitro and in vivo.

To validate the Aurora kinases as selective targets in HPV-driven cervical cancer, we assessed the activity of well-characterized inhibitors of Aurora A and B. The Aurora B Inhibitor 2M4447 [18] was not selective for the HPV lines (Supplementary Fig. S4). The potent, orally active inhibitor of Aurora A kinase MLN8237
Alietorib (19) was investigated in a panel of HPV-transformed cervical cancer cell lines. Hela, CaSk, and ME180 (HPV16/18) were highly sensitive to Alietorib with IC50 values of less than 1 μM/L, whereas SiHa were less sensitive with an IC50 of 1.2 μM/L (Fig. 2A and Supplementary Table S3). The non-HPV cervical cancer cell lines HT3 and C33A were less sensitive with an IC50 value of 2 and 16 μM/L, respectively (Fig. 2B and Supplementary Table S3). We also tested a panel of squamous cell carcinoma (SCC) cell lines to increase the number of non-HPV cancer cell lines from a keratinocyte origin. These were significantly less sensitive to Alietorib with IC50 values above 5 μM/L in all cases (Fig. 2C). The difference in drug sensitivity was not a consequence of different proliferative rates, as all cell lines tested had similar doubling times. The sensitivity of the HPV cervical cancer cell lines are clinically relevant as plasma concentrations of 1 to 5 μM/L of Alietorib have been reported in patients (20).

To assess the ability of Alietorib to inhibit tumor growth in vivo, nude mice were injected s.c. with either Hela (HPV16), CaSk (HPV16), or C33A (non-HPV) lines. When tumors had formed and were palpable, Alietorib treatment (orally, 30 mg/kg daily for 10 days) was initiated. The non-HPV C33A tumors showed an initial inhibition of growth that continued to 10 days after the fatal treatment, but tumor growth recovered to control levels thereafter. By contrast, Hela and CaSk tumors reached approximately 20 to 35 mm3 during the treatment phase then regressed with Alietorib treatment, with no signs of tumor at day 50 after treatment (Fig. 3A), and excision of the original site of inoculation showed no residual tumor.

We also assessed a transgenic model of HPV16 E7-dependent precancer using a skin graft model. In this model, donor mice have HPV16 E7 expression controlled by the keratin 14 promoter (K14-E7) resulting in E7 expression in squamous epithelial keratinocytes, driving hyperplasia of the keratinocytes (21). Grafting of skin from either wild-type or K14-E7 mice onto a syngeneic host results in well-healed grafts (22). Mice with well-healed grafts underwent two cycles of 10 day Alietorib treatment, and the mice were then sacrificed and the grafts harvested for immunohistochemical analysis. This treatment resulted in swelling and redening of the K14-E7 grafts only. In Alietorib-treated E7 grafts, we observed a significant increase in apoptotic cells compared with untreated E7 grafts (Fig. 3B). No apoptotic cells were detected in the adjacent wild-type grafts. There was also an increase in the number of mast cells immediately adjacent to the epidermis in the Alietorib-treated E7 grafts compared with both the adjacent wild-type grafts and untreated E7 grafts (Fig. 3C and Supplementary Fig. S5), likely to be in part responsible for the increased swelling of the Alietorib-treated E7 grafts.

Treatment with Alietorib induces polyplody and cell death in HPV-transformed cervical cancer cell lines.

Cell-cycle progression was analyzed in cells after Alietorib treatment by flow cytometry. Treatment of cells with 5 μM/L Alietorib for 24 hours resulted in increased cells with 4N and >4N DNA and a reduction in the 2N and S phase population in all cell lines (Figs. 4A; Supplementary Figs. S6 and S7A). There was an increase in the sub-diploid population (<2N) in a time-dependent manner in all cell lines, which was more pronounced in HPV-transformed cells. After 72 hours of treatment, 3 of the 4 HPV-transformed showed high sub-diploid population (Hela, SiHa, and ME180, 70%, 69%, and 57%, respectively) indicating cell death. The exception was SiHa, where the sub-diploid population was 86%. By contrast, the two non-HPV cancer cell lines C33A and HT3, demonstrated lower sub-diploid populations of 14% and 31%, respectively, but accumulated cells with high polyplody (>4N) suggesting a failed cytokinesis, but this did not result in cell death.

To confirm that the induction of 4N and >4N DNA content was a consequence of failure of cytokinesis, all cell lines were subjected to immunofluorescence staining of the microtubule cytoskeleton with anti-α-tubulin and DAPI for DNA (Supplementary Fig. S7A). The percentage of binuclear and multinuclear cells 1 and 2 days after Alietorib treatment increased to a similar level in all cell lines (Supplementary Fig. S7C). Together, these data suggest that the accumulation of cells with 4N or greater DNA content was indeed a consequence of failure of cytokinesis, but only in HPV-transformed cells did this result in a significant loss of viability.

The effects of Alietorib on cell viability were confirmed using time lapse microscopy. In all cell lines, drug treatment caused cells to arrest in mitosis (rounded mitotic morphology), and undergo failed cytokinesis (producing single daughter cells). However, apoptosis was prominent in the HPV-transformed cell lines (Supplementary Fig. S8). Analysis of the timing of cell death in
the HPV-transformed cell lines showed that 50% to 60% of cells underwent two rounds of mitosis before triggering cell death quickly, whereas a further 20% acquired a single mitosis, but then death was delayed for >20 hours (Supplementary Fig. S9). Surprisingly, the length of mitotic arrest induced by the drug was up to 5 times longer in the HPV-transformed lines (Fig. 4B). Aurora A inhibition normally results in a relatively short mitotic delay then exit into failed cytokinesis (21-25). Our data suggest a unique sensitivity in cells where HPV is present that results in a highly extended mitotic arrest.

**Mechanism of Alisertib-induced death in HPV cancer cell lines**

To examine the apoptosis induced by Alisertib treatment, Hela cells overexpressing either Bcl-2 or Mcl-1 were assessed for their sensitivity to Alisertib. Etoposide and taxol were used as respective positive controls. The Mcl-1-Hela cells were highly resistant to Alisertib compared with the parental Hela, with a >5-fold increase in EC50 (90 nM vs. 1.47 μM, respectively). Bcl-2 overexpression had a more modest effect on sensitivity to Alisertib (Fig. 5A). Mcl-1 and Bcl-2 overexpression was protective against taxol and etoposide, respectively (Supplementary Fig. S10), suggesting Alisertib functions via an Mcl-1-sensitive apoptotic mechanism.

A panel of apoptotic components was examined by immunoblotting of the HPV-transformed cervical cancer cell lines after 24, 48, and 72 hours of Alisertib treatment. The level of full-length PARP decreased with a concomitant increase in cleaved PARP in all HPV cell lines tested by 48 hours drug treatment. This was not detected in the non-HPV C33A line (Fig. 5B and Supplementary Fig. S11). The increased PARP cleavage was associated with an increase in the cleavage and activation of caspase 3. The levels of the antiapoptotic Mcl-1, Bcl-2, and Bcl-XL proteins varied between each cell line. Alisertib treatment had little effect on the levels of Bcl-2 and Bcl-XL, but there was up to 50% reduction in the level of Mcl-1 in three of the HPV-transformed lines. Alisertib treatment had little effect on either the relatively insensitive SiHa or the non-HPV C33A line. Tumor-suppressor p53 was only readily detectable by immunoblotting in the non-HPV cell lines and was not restored in the HPV-transformed lines by Alisertib treatment. The expression of p53-regulated proapoptotic proteins PUMA, NOXA, and BAD was not affected by Alisertib treatment. An increase in the level of Mcl-1-selective BIM in the majority of cell lines treated was observed (Fig. 5B and C). The consistently reduced level of Mcl-1 in the Alisertib treated HPV-transformed cell lines together with the increased level of BIM and the resistance to Alisertib of Hela cells overexpressing Mcl-1 indicates that the reduced Mcl-1 and increased BIM levels are the mechanism by which apoptosis is induced by Alisertib treatment.

**Aurora A inhibition targets host interaction with HPV E7**

To determine whether Alisertib sensitivity was a direct consequence of expression of HPV oncoproteins, C33A non-HPV cervical cancer and SCC25 non-HPV SCC cell lines were transfected with HPV16 E7 oncogene, then assessed for their sensitivity to Alisertib. Expression of E7 in SCC25 cells (SCC25 HPV16-E7)
Figure 4.
Asenirib treatment of HPV-transformed cancer cells promotes apoptosis and delays HPV-transformed cancer cell mitosis. A, HPV-transformed cancer cells were treated with 5 μM Asenirib and sampled at intervals over 72 hours and analyzed by flow cytometry for DNA content. The data are the mean and SD of three independent experiments. The data for only Caski and C33A are shown. See Supplementary Figs. S1A and S1B for other cell lines. ME180 and C33A cell line data are shown in Supplementary Fig. S4A. B, quantitation of the time in mitosis for each cell line with and without 5 μM Asenirib. Treatment determined from the time-lapse experiments. The data are the mean and 95% confidence interval for >100 cells in each case.

resulted in >39-fold reduction of the IC50 value (from 1.5 to 0.5 μM) whereas E7 expression in C33A cells (C33A-HIV1.6-E7) resulted in approximately 50% IC50 reduction (Fig. 6A and Supplementary Fig. S1D). Indicating that E7 expression induces the observed sensitivity. Our previous experiments hint that the level HPV E7 expression appeared to be correlated with sensitivity to Asenirib as lowest E7 expressing line, SiHa (28) was the least sensitive (Fig. 2). To assess whether the level of HPV E7 expression influences Asenirib sensitivity, SCC25 lines were transduced with lentivirus HPV18 E7 as an IRES GFP labeled expression construct. The resultant population was FACS sorted into low and high GFP expression as a direct marker of HPV E7 expression (Supplementary Fig. S1C; ref. 14). These populations were treated with 5 μM Asenirib, and the delay in mitosis and changes in apoptotic proteins assessed as before. We observed a dose-dependent increase in mitotic delay between the low and high-expressing cells upon Asenirib treatment (Fig. 6B), which was highly significant (P < 0.005) in the E7 high-expressing population. Increased BIM levels were observable in all Asenirib-treated population, but a reduced Mcl-1 level (to 50% control) was only observed in E7 high-expressing population at 3 days.

Discussion

Here, we have used siRNA kinome screening to identify Aurora A as a molecular target for killing HPV-driven cervical cancer cells. Previously, siRNA-based kinome screens from the Harlow and
Aurora A Inhibitor Targets Cervical Cancer

Munger laboratories have explored the role of E7 synthetic lethality, but only 130 kinases were screened and these did not include any of our seven top hits (27).

The Aurora A Inhibitor, Alisertib, has been used in over 35 clinical trials; however, it has so far only elicited modest responses in a range of tumor settings (20, 28, 29). Targeting Aurora A using Alisertib demonstrated selectivity for HPV-transformed cancer cells both in vitro and in vivo. In vitro, the major difference observed was that the HPV cell lines failed cytokinesis and died whereas non-HPV cervical cancer lines failed cytokinesis but remained viable. In xenograft experiments, we observed that non-HPV cervical cancer was somewhat sensitive to Alisertib treatment, showing delayed tumor growth and eventual recovery, a typical response reported for Alisertib in other cancer models (30, 31). By contrast, no tumor was detectable in either HeLa or CaSkI cells, even 50 days after treatment. Although Alisertib had a more modest effect in mouse Ki67-grafted model, the lack of effect on the adjacent wild-type graft demonstrates the same selectivity observed in the cancer models. The increased apoptosis and most cell infiltrate point to Alisertib treatment having an HPV-E7–directed effect even in this premalignant setting. Together, these data provide strong evidence that Aurora A selectively targets HPV E7–expressing cells in vitro and in vivo.

This observation is supported by the mechanistic studies, which show Aurora A depletion/inhibition selectively kills HPV-transformed cervical cancer cells, and this is dependent on HPV E7 expression. The expression HPV E7 in HPV-negative C33A and SCC25 sensitized them to Alisertib and the level of E7 correlated with sensitivity. HPV E7 expression has been reported to increase Aurora A levels (32), though we found little difference in the level of Aurora A in HPV and non-HPV cancer cell lines. HPV E7 can upregulate the expression of the p33-regulated p73 activator phosphatase enzyme activity, which can transactivate the expression of purified proteins PUMA and NOSA. Aurora A has been reported to phosphorylate p73, an HPV E7–regulated gene (33), and inhibit its transcriptional activity, thus inhibiting the expression of p73-regulated PUMA and NOSA-promoting apoptosis (25, 34). However, we found no evidence of this mechanism in Alisertib-dependent death in the HPV-transformed cancer cell lines.

A consistent difference between the Alisertib-treated HPV-transformed and non-HPV cell lines is the time to mitosis, with the HPV-transformed lines all delaying up to five minutes longer in mitosis as non-HPV cells. These data show that a majority of cells required to transit through one or two mitoses before undergoing apoptosis suggested that the extended mitotic delay was a major contributor to the selective killing observed. A number of components of the apoptotic pathway have been reported to be regulated by mitosis. Mcl-1 is destabilized by CDK1–Cyclin B (35, 36), and Bim, an Mcl-1 selective Bcl2–only protein (37), is phosphorylated by Aurora A, which promotes its degradation in mitosis. Our data indicate that the apoptosis promoted by Alisertib treatment in the HPV-transformed cells is dependent on Mcl-1. The mechanism by which Aurora A regulates Mcl-1 stability is linked to the increased delay in mitosis found with Aurora A inhibition in the HPV-transformed cells. The longer delay in mitosis increases the destruction of Mcl-1. In addition to this reduction in antipapoptotic signal, inhibition of Aurora A directly stabilizes Bim, increasing proapoptotic signalling. Others have shown that Bim, depletion, reduced the sensitivity of HeLa cells to Aurora A Inhibitor (38), supporting the role for BIM in this
apoptotic signalling. The combined effect of reduced Mcl-1 and maintenance of Bim levels, which bound by Mcl-1, results in elevated Bim not bound in an antiapoptotic Mcl-1 complex. The degree of Mcl-1 destruction during the mitotic delay may be the factor determining whether one or two mitoses are required for triggering apoptosis as observed in the time lapse experiments. CDK1 - Cyclin B phosphorylation of Bcl-2 and Bcl-XL has also been reported to inhibit their antiapoptotic activity (39), and the combined effect of reduction of antiapoptotic Bcl-2, Bcl-XL, and Mcl-1, with maintenance of Bim levels promoting an overall proapoptotic signal.

It may be that the level of E7 expression directly influences CDC20 expression and thereby Mcl-1 stability in mitosis. The level of HPV E7 expression contributes to length of the mitotic delay in A2780 tumor cells. Low level E7 expression had little effect on time of E7 transduced SCC25 cells in mitosis with A2780 treatment, but high level expression in these cells delayed significantly the mitotic delay. Only the high-level HPV E7-expressing SCC25 cells showed significant reduction in Mcl-1 levels, consistent with this proposition. The mechanism underlying the effect of Aurora A inhibition in the E7 expressing cells is at present unknown. In summary, we have identified inhibition of Aurora A as a synthetic lethal target in the presence of the HPV E7 oncogene and demonstrated that inhibiting Aurora A with the small-molecule inhibitor Aurora selectively and effectively targeting HPV E7 - expressing cells in vivo. The data presented here strongly suggest that targeting Aurora A with drugs such as Alisertib may be an effective therapy for recurrent cervical cancer, and may be useful in treating other HPV transformed cancer types.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
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References

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APPENDIX – 3
Dear Emeritus Professor Johnson

Your application for ethical review for the project, "The role of Human papillomaviruses in the aetiopathogenesis of head and neck cancer in South Asia, and approaches to treatment" (GU Ref No: DOH/13/14/HREC) has been received by the Office for Research. We will notify you of the outcome of your application as soon as possible. Please note that you cannot commence this research until you are issued with authorisation to do so.

If you have any further questions about this matter please do not hesitate to contact us.

Regards

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Ethical Clearance Certificate

The Ethical Committee of Dhaka Medical College Approved the Following Research Protocol in time.

Title of the Research Work: “The role of Human papillomaviruses in the aetiopathogenesis of head and neck cancer in South Asia, and approaches to treatment”.

Principal Investigator: Emeritus Professor Newell W. Johnson Honorary Professor of Dental Research Menzies Health Institute Queensland Griffith University, Queensland, Australia.

Supervisor: Self

Place of Study: Department of Pathology Dhaka Medical College, Dhaka


Prof. (Dr) K.M. Shahidul Islam Head of the Department of Microbiology & Chairman, Ethical Review Committee Dhaka Medical College, Dhaka.