Cellular and Molecular biology in oesophageal cancer
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INTRODUCTION

Histological differences

Oesophageal cancers comprises cancers of different histological types with diverse cellular and molecular bases [1-2]. The major histological types of oesophageal cancers are either squamous cell carcinoma or adenocarcinoma. It is important to note that there are histological variants of squamous cell carcinoma and adenocarcinoma like basaloid squamous cell carcinoma, spindle cell carcinoma, mucoepidermoid carcinoma and adenosquamous carcinoma [3-5]. In addition, neuroendocrine neoplasms such as small cell carcinoma of the oesophagus accounted for approximately 1% of primary oesophageal carcinoma [6]. All these carcinomas have distinct clinicopathological features. Limited studies revealed that the cellular and molecular biology of these uncommon types of oesophageal carcinomas are different from oesophageal squamous cell carcinoma or adenocarcinoma [3, 7-8].

In practice, the current understanding of cellular and molecular biology of oesophageal cancers focuses on oesophageal squamous cell carcinoma and oesophageal adenocarcinoma. The difference in prevalence of these two major histological types in different geographic regions is likely as a result of the complex interactions of genetic and environmental factors. In general, oesophageal squamous cell carcinoma predominates in areas with high incidence of oesophageal cancer whereas oesophageal adenocarcinoma is more common in areas with low incidence of oesophageal cancer. Also, the genetic mechanisms of oesophageal squamous cell carcinoma are complex as multiple genetic factors have been proposed for carcinogenesis of the squamous cell
carcinoma [2]. On the other hand, most of the oesophageal adenocarcinomas are known to be related to the genetic changes of the progression of lesions related to acid reflux. The histological progression from reflux oesophagitis to Barrett’ metaplasia to dysplasia to adenocarcinoma is well known.

**Applications of molecular and cellular biology**

Oesophageal cancer remains one of the leading causes of cancer death worldwide despite the improvements in surgical and adjuvant therapies. The only hope to further increase the quality of life of patients with oesophageal cancer is to understand and apply the knowledge of cellular and molecular biology in the clinical management of these cancers. Thus, the study of cellular and molecular biology in oesophageal cancer could potentially serve the following purposes: (1) to establish the presence or absence of an infectious co-factor; (2) to understand the genetic mechanisms; (3) to provide prognostic information; and (4) to predict response to medical therapies and new modalities of treatment. In performing researches, interpreting and applying the knowledge in this area, it is important to bear in mind the histological difference in oesophageal cancers. At current, most of the studies in this cancer are still on the understanding the genetic mechanisms rather than the applications of the knowledge.

**ESTABLISH INFECTIOUS CO-FACTOR**

**Human papilloma virus**

In oesophageal cancers, the main infectious co-factor under intensive studies was human papilloma virus (HPV). In squamous cell carcinomas from the upper aerodigestive tract, in particular in oral regions and oropharynx, identification of presence of human papilloma virus (HPV) in the carcinomas is of important values [9-11]. Patients with
cancers having HPV detected have better prognosis when compared to patients with cancers without HPV. The detection of HPV in squamous cell carcinomas from the oral and oropharynx also predicts better response to radiotherapy. Also, the detection of HPV in clinical setting is indirectly by the identification of increased expression of p16 protein by immunohistochemistry.

Oesophagus is distal to the oropharynx and histologically lined by stratified squamous epithelium as in the oral regions or oropharynx. Researches to investigate HPV in squamous cell carcinomas in oesophagus were noted for 30 years [12-13]. Thus, there is considerable data on the role of HPV infection in the development of oesophageal cancer. Majority of the studies were performed in oesophageal squamous cell carcinoma.

It is worth noting some data were obtained in oesophageal adenocarcinoma. Pooled-analysis from the literature revealed that HPV prevalence in oesophageal adenocarcinoma was 35.0% and HPV-16 prevalence was 11.4% [14]. Due to the limited number of studies on oesophageal adenocarcinoma, no detailed analysis of the impact was available. Nevertheless, it has postulated that progressive acid damage to the oesophagus increases the likelihood of mucosal breaks and allows the virus to enter the basal layer of the transformation zone. Recently, transcriptionally active HPV was noted be strongly associated with Barrett dysplasia and oesophageal adenocarcinoma, suggesting a potential role of HPV in oesophageal carcinogenesis.

In oesophageal squamous cell carcinoma, summarised HPV prevalence from both early and recent meta-analysis in oesophageal squamous cell carcinoma was 22% [14]. In general, HPV prevalence was found to be higher in the studies some countries from Asia and was much lower in studies reported in western countries such as in Europe and America [2]. Stratified analysis by localisation of cancer showed that oesophageal squamous cell carcinoma in was only slightly higher but not significantly higher than the
middle or lower portion of oesophagus [14-15].

With respect to HPV DNA detection in meta-analysis, the prevalence of oesophageal squamous cell carcinoma produced by type-specific primer PCR method (30.4%) was observed to be significantly higher than that by broad-spectrum primers (20.8%) [14]. P16 protein detection in by immunohistochemical method was not often studied. Nevertheless, the current data on using p16 detection in oesophageal squamous cell carcinoma did not reflect the HPV status in the cancer [16].

From pooled data, HPV-16 was the most frequently observed subtype with a summarised prevalence of 11.4% [2, 14]. The other six most frequent HPV individual types identified in oesophageal squamous cell carcinoma, in order of decreasing prevalence, were HPV-18 (2.9%), HPV-6 (2.1%), HPV-11 (2.0%), HPV-52 (1.1%), HPV-33 (0.8%) and HPV-31 (0.6%).

Overall, human papillomavirus infection was observed to be associated with an increased risk of oesophageal squamous cell carcinoma. However, the association was not as strong as oral/oropharyngeal squamous cell carcinoma or cervical squamous cell carcinoma. In addition, it is unlikely that HPV detection and/or p16 expression will predict radiotherapy response as it does for upper aerodigestive tract squamous cell carcinoma. At this stage, routine evaluation of HPV status and/or p16 is not required in management of the cancer.

**Epstein bar virus**

The association of Epstein bar virus (EBV) has also been studied in oesophageal cancer. The results are variable with prevalence detected ranging from 0% to 35% [17-18]. The differences are likely to be related to variety of racial, geographical and detection methods used. It is worth noting the lymphocytes in the cancer stroma can harbour EBV and
thus detection of virus in oesophageal cancer by PCR based methods may show false-positive results [18]. On the other hand, immunohistochemistry in situ hybridization may provide false negative result due to higher rate of RNA degradation. Most studies have shown that EBV-associated oesophageal cancer demonstrates similar morphologic findings to undifferentiated carcinoma of nasopharynx which is known to be associated with EBV. At the current status, more data is needed for the study of EBV in oesophageal cancer. It has no clinical application at the moment.

**Bacteria**

*Helicobacter pylori* is a Gram-negative spiral bacterium that is present in nearly half the world’s population. It is the major cause of peptic ulcer disease and a recognized cause of gastric carcinoma. Some strains of *Helicobacter pylori* may protect patients from gastroesophageal reflux disease and oesophageal adenocarcinoma [17]. Overall, there is no consensus on the role of *Helicobacter pylori* in oesophageal/cardia adenocarcinoma, with substantial differences between Asian and Western studies. Other than this, many bacteria have been identified in the oesophagus by metagenomics [17, 19]. Metagenomics is the study of microbiota in their natural habitat using next-generation sequencing through a PCR-based analysis of bacterial 16S rRNA genes. Two distinct clusters: a predominantly Gram-positive cluster (type I) and a predominately Gram-negative cluster (type II) were found. Type II cluster may stimulate expression of different proteins and genes leading to reflux and trigger the process of adenocarcinoma.

**UNDERSTANDING GENETIC MECHANISMS**

**Genetic profiles**

The genetic profiles of oesophageal cancers have been studied for decades. The
roles of oncogenes, tumour suppressor genes, metastatic genes, apoptosis genes, proliferation related factors, epigenetic factors as well as proteins related to metastases have been studied in both oesophageal squamous cell carcinoma and oesophageal adenocarcinoma [2, 20]. It is worth noting that adenocarcinoma from the oesophagogastric junction share similar histological features and profiles. However, some of the genetic changes may be related to gastric cardia. In general, for both histological subtypes, p53 mutation is an important genetic change [21-22].

Starting from around 2000, comparative genomic hybridization (CGH) and expression array were used to identify the different in genetic profiles of oesophageal cancer when compared to non-cancer oesophageal tissue [23-26]. Chromosomal regions with amplification may harbor oncogenes, and chromosomal regions with deletion may harbor tumour-suppressor genes. CGH can identify the whole profile of cytogenetic changes in an individual cancer. By this approach, novel genes were identified in both oesophageal squamous and oesophageal adenocarcinoma [27-31]. These provide more information of the carcinogenesis of oesophageal cancers as well as finding gene candidates as prognostic markers and molecular targets for therapy.

The introduction of next-generation sequencing has brought forth the ability to generate vast quantities of genetic data at a low cost [32]. These recent technologies allow researchers to sequence DNA and RNA much more quickly and economically than the previously used Sanger sequencing, and as such have revolutionised the study of genomics and molecular biology. The first commercial available next-generation sequencer was available in 2007. In addition, the International Cancer Genome Consortium (ICGC) has been organized to launch and coordinate a large number of research projects that have the common aim of elucidating comprehensively the genomic changes present in many cancers [33]. The preliminary meeting was held in 2007 and the consortium launched to
the public notice in 2010. The primary goals of the ICGC are to generate comprehensive
catalogues of genomic abnormalities (somatic mutations, abnormal expression of genes,
epigenetic modifications).

The oesophageal squamous cell carcinoma genome study is being taking care by
researchers in China whereas the oesophageal adenocarcinoma genome study is by
researchers in United Kingdom. It is likely the more comprehensive knowledge of the
genetic events in the oesophageal carcinoma will be available soon. The whole genome
sequencing data on oesophageal cancer started to appear in the literature in 2010 [34-37].
At current, a few studies were noted in literature on both oesophageal squamous cell
carcinomas and adenocarcinomas. Data were seen in the website of the ICGC. A large
volume of information is available for both histological subtypes of oesophageal cancer.
They provide huge resources for future research directions for better management of
patients with oesophageal carcinoma

In oesophageal squamous cell carcinoma, eight significantly mutated genes, of
which six are well known tumour-associated genes (TP53, RB1, CDKN2A, PIK3CA,
NOTCH1, NFE2L2), and two have not previously been described in oesophageal
squamous cell carcinomas (ADAM29 and FAM135B) [34]. Genomic analyses suggest that
oesophageal squamous cell carcinoma and head/neck squamous cell carcinoma share
some common pathogenic mechanisms.

In oesophageal adenocarcinoma, genes like p53 and SMAD4 were confirmed to be
implicated in stage-specific manner and confined to high grade dysplasia and
adenocarcinoma [35]. Many of the recurrently mutated genes in oesophageal
adenocarcinoma were also mutated in non-dysplastic Barrett epithelium. Thus, mutations
in oesophageal adenocarcinoma driver genes generally occur exceptionally early in
disease development with profound implications for diagnostic and therapeutic strategies.
Also, new significantly mutated genes include chromatin-modifying factors and candidate contributors *SPG20, TLR4, ELMO1 and DOCK2* were also identified.

**MicroRNAs (miRNAs)**

MicroRNAs (miRNAs) are a class of small, well-conserved, non-coding RNAs that regulate the translation of RNAs. Their biological and pathological roles and have been studies in numerous cancers [38-41]. Research works were also being done to see if the miRNA can be detected in serum of the patients with oesophageal cancer [42-43]. Studies showed that there are a number of candidate miRNAs that have so far been found to be up or down-regulated at various stages in the progression from Barrett oesophagus to oesophageal adenocarcinoma [44-47]. In oesophageal squamous cell carcinoma, many miRNAs were identified as diagnostic and prognostic markers [45-47]. It may be related to the fact that oesophageal squamous cell carcinoma has a more complex carcinogenesis than oesophageal adenocarcinoma. However, further work is required in order to be able to use miRNAs for clinical use.

**Cancer stem cells**

Cancer stem cells, as a subgroup of cancer cells, resemble critical properties of embryonic stem cells such as self-renewal and maintenance of stemness state. Only the cancer stem cells (CSCs) have tumour-initiating properties [48]. They play an important role in the resistance to adjuvant therapies for cancer. Also, epithelial-mesenchymal transition (EMT) may be involved in epithelial cell immortalization and enrichment of stemness. These immortal cells may regain their original properties via mesenchymal-epithelial transition (MET) and maintain epithelial stem cell properties [49]. In oesophageal squamous cell carcinoma, a few stem cell markers like CD44, ALDH1A1, SALL4 and SOX2 have been
identified [50]. The expressions of some of these markers were noted to be correlated to cancer staging. Cancer stem cells or tumour-initiating cells are present in oesophageal adenocarcinoma [51]. However, antibodies directed against novel surface antigens are needed to detect them.

Development of specific markers and signalling molecules to target the stem cells of oesophageal carcinomas and the validation of these stem cells might provide the basis for a revolutionary treatment approach for the elimination and/or differentiation of cancer stem cells in oesophageal cancer. Emerging therapeutic tools based on specific properties and function of CSCs may improve clinical output of the diseases. Therefore, innovative insight into CSC biology and CSC-targeted therapies will help to achieving effective management of oesophageal cancers.

**PROGNOSTIC INFORMATION**

**Prediction of progression**

Aneuploidy (detected by FISH/flow cytometry), promoter hypermethylation (methylation profiles such as p16, APC, etc) and cyclin A protein expression has been shown to be correlated to the progression from Barrett oesophagus to oesophageal adenocarcinoma [52]. Despite these results, there is generally lack of large prospective study to validate the use of these markers in clinical practice. The likely candidate of clinical application is p53 protein over-expression as determined by immunohistochemistry. Overexpression of p53 protein was noted to be correlated with the neoplastic progression to oesophageal adenocarcinoma. It could be a useful adjunct to determine the grade of dysplasia in Barrette oesophagus. Also the results have been validated in some studies and the procedure used is simple.

Many data exists for oesophageal adenocarcinoma and has found that expression or
identification of cellular and molecular markers could predict survival of the patients with oesophageal adenocarcinoma [53, 54]. Some of the more commonly described markers are epidermal growth factor receptors (1 and 2); Transforming growth factor (TGF α and β1); P53, Ki-67, cyclin dependent kinase inhibitor 1 (p21); B-cell lymphoma 2 (bcl-2); Cyclooxygenase-2 (COX-2); Nuclear factor-κB (NF-κB); vascular endothelial growth factor (VEGF); tissue inhibitor of metalloproteinase (TIMP) and microsatellite instability (MSI). At present, routine testing for all or any of these markers is not warranted as none of these markers have been validated adequately in prospective studies.

In oesophageal squamous cell carcinoma, many molecular and cellular markers have been proposed to be related to the prognosis of the patients with cancer [54-59]. Expressions of p21, p53, cyclin D1, Ki-67, E-cadherin and VEGF have been shown to provide some prognostic information. However, this method has not been widely used.

GUIDELINES FOR MEDICAL THERAPIES

Predict response to medical therapies

Pre-operative chemo-radiation is adopted as a standard way of treatment of oesophageal cancers. In patients who underwent neoadjuvant chemo-radiation therapy, histological regression of the primary cancer indicated by percentage of residual viable cells is an important prognostic factor in addition to nodal status and gender [60].

It is thus important to have means to predict the response for chemo-radiation. It has been reported that grade of oesophageal squamous cell carcinoma can predict the response to pre-operative chemotherapy [61]. Many molecular markers have been studied [62-65]. p53 protein is expected to be a representative biomarker. The cell cycle markers- CDC25B and 14-3-3sigma have potential as response biomarkers independent of the p53 status. The DNA repair markers, p53R2 or ERCC1, VEGF and hedgehog
signalling pathway factor Gli-1 also have potential as predictive biomarkers. However, proper validations are needed. In oesophageal adenocarcinoma, expression of EGFR, vascular endothelial growth factor, nuclear factor-xB and cDNA microarray are noted to acting as predictive factor for pre-operative chemo-radiation.

**Predictors for targeted therapy**

Targeted therapy is currently being investigated as an addition to these regimes and for the treatment of metastatic disease. The therapy usually involves targeting a specific gene mutation in the oesophageal cancer. Promising results were noted in melanoma, breast cancer and colorectal cancer [66-70]. Testing the cancer tissues for molecular markers is useful to predict the response of the patients to these targeted therapies.

Of the potential targets trialled to date in oesophageal cancer, epithelial growth factor receptor 2 - EGFR (HER1 and HER2) and vascular endothelial growth factor – VEGF surface receptor antagonists have shown the most promise [71-75]. Of these, Her 2 over expression has been most extensively studied. Trastuzumab-based therapy offered a significant survival advantage for patients with HER2 overexpressing locally advanced, recurrent or metastatic gastric and gastro-oesophageal junctional adenocarcinomas compared to conventional therapy alone. Currently the relevance in oesophageal adenocarcinomas and squamous carcinomas is unclear. It is likely that pathologists will be required to determine the HER 2 status in biopsy or resection material in gastroesophageal junction as well as in metastatic sites. HER 2 status could be assessed by immunohistochemistry and FISH testing. Other targeted therapies in oesophageal cancers are less well studied. For instance, over expression of EGFR -1 is found in 1/3 to 2/3 of oesophageal adenocarcinoma and squamous cell carcinoma. To date, clinical trials using the EGFR-1 antagonist, cetuximab, have revealed varied results. VEGF is over expressed in 30 – 60%
of oesophageal adenocarcinoma. VEGF antagonist bevacizumab has shown promising results when added to multimodality treatment. However, at this stage, there is not much data of the use of these therapies and predictive markers in oesophageal cancers.

**RESEARCH SOURCES OF MOLECULAR AND CELLULAR STUDIES IN OESOPHAGEAL CANCERS**

**Tissues studies**

Human cancer can be studied at tissue level when it is surgically removed from human body. These cancer tissues are without blood supply and degeneration will quickly occur. Cancer studies on these tissues can be performed in several ways. In clinical settings, the cancer tissues were fixed in formalin and embedded in paraffin. They were useful for different molecular studies. In fact, many of the research findings in oesophageal cancers were obtained by this method. This approach has the benefit of providing superior morphological features for studying the histological features as well as localization of biomarkers in the cellular level when compared with other methods (Figure 1).

In the recent years, tissue microarray (TMA) was increasing use in testing molecular markers in large number of samples by either immunohistochemistry or FISH (Figure 2). In the tissue microarray technique, a hollow needle is used to remove tissue cores as small as 0.6 mm in diameter from regions of interest in paraffin-embedded tissues. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern.

The drawback of working on paraffin-embedded tissues is formalin irreversibly cross-links proteins via the amino groups thus preserving the structural integrity of the cells so they can be stained with dyes used to analyse for abnormalities in the tissue that indicate cancer. The effect of these cross-linking fixatives on the nucleic acids and proteins will impair the molecular works. In recent decades, oesophageal cancer tissues were prospectively collected.
The cancer tissue snapped frozen the oesophageal cancer tissues in liquid nitrogen and stored the tissue in -80°C. The collection included a lot of clinical and scientific efforts and the tissues were superior in quality for molecular works. On the other hand, the morphological features are inferior to those obtained by paraffin embedded sections (Figure 3).

It is worth noting that research by these approaches could not provide functional dynamic studies of oesophageal cancers.

**Cancer cell lines**

For functional studies in oesophageal cancer, the works could be done on cancer cell lines derived from tissues obtained fresh after the operation. Cancer cell lines need proper medium to grow. Cancer cell lines often grow without attaching to a surface and they can proliferate to a very much higher density in a culture dish. The resulting transformed cancer cell lines, in reciprocal fashion, can often cause tumours if injected into a susceptible animal for making animal model. Cancer cell can be harvested from the animal and forming a more stable cell cancer cell line. In oesophageal cancers, some of the common used cell lines actually are secondary cell line. The cancer cell lines can allow functional studies to be done. They can be stored in liquid nitrogen for an indefinite period and retain their viability when thawed.

In oesophageal cancers, there are published cancer cell lines available for both adenocarcinoma and squamous carcinoma [76-79]. When compared to oesophageal squamous cell carcinoma, oesophageal adenocarcinoma is more uniform in characteristics as the risk factors and pathogenesis more established. Model research on oesophageal adenocarcinoma relies almost entirely on a relatively small set of established cancer cell lines. The high genomic similarities between the oesophageal cell lines and their original
cancers provide rational for their use. Nonetheless, cancer cell lines nearly always differ in important ways from the original cancer from which they were derived.

**Animal model**

Animal model is important to study the effects of cancer in vivo and also production of cancer cell lines. In more clinical relevant applications, animal model is a must for developing therapeutic strategies. Cancer development is a complex process with accumulation of genetic alterations and their downstream effects as well as interactions of the microenvironment in different tissues. The cancer microenvironment and its interactions with the cancer are important in determining growth dynamics of different cancers.

Injection of cancer or cancer cells in the subcutaneous tissue of the skin of immunodeficiency mice is a common practice to produce a cancer model in animal (Figure 4). In oesophageal cancers, this approach cannot recapitulate the microenvironment of the oesophagus as well as the response to the targeting carcinogens. The current approach is to make an orthotopic (occurring at normal place) model for both oesophageal squamous cell carcinoma and oesophageal adenocarcinoma [80-81]. The orthotopic model provides the most optimum environment for cancer growth and drug testing. In the anatomical setting of oesophageal cancer, the site is very difficult to approach surgically. Different approaches have been applied but many of these had some shortcomings. The establishment of these orthotopic models need to involve radiological guidance (magnetic resonance imaging and fluorescence imaging) so the cancer and the metastases can be visualized in real time [82]. In addition, pathological examination is important to clarity the histological typing, microscopic location and the microenvironment of the cancer in the animal [83].
Figures Legends

Figure 1. Histological features of oesophageal cancer prepared by formalin fixation and paraffin embedded revealing a well-differentiated squamous cell carcinoma with good histological details.

Figure 2. Tissue microarray of oesophageal squamous cell carcinoma showing multiple cores of cancer tissue in a block to allow testing of molecular markers simultaneously of many cases in one run of experiment.

Figure 3. Histological features of oesophageal cancer prepared by sectioning of frozen tissues showing inferior histological details when compared to Figure 1.

Figure 4. Tumour produced on an immunodeficiency mouse after injection of primary oesophageal squamous cell carcinoma in the subcutaneous
REFERENCES


