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Cellular and Molecular Biology of Esophageal Cancer

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ABSTRACT

The study of cellular and molecular biology in esophageal carcinomas could serve the following purposes: (1) to establish the presence or absence of an infectious co-factor such as human papilloma virus; (2) to understand the genetic mechanisms of disease such as genetic mutations, changes in microRNAs changes as well as the roles of cancer stem cells; (3) to provide prognostic information; and (4) to predict response to medical therapies and new modalities of treatment. In recent years, major genomic information obtained from the whole genomic sequencing of prospectively collected frozen samples of esophageal carcinomas open the field for in-depth understanding of the complex molecular pathway of the cancer. The Anti-Her 2 therapy was approved internationally as targeted therapy for esophageal adenocarcinoma at the gastroesophageal junction. Assessment of Her 2 expression is the most important molecular test to be performed properly in clinical settings. The study of cellular and molecular biology in esophageal carcinomas depends on the properly collected formalin fixed as well as snap frozen tissues and blood from the patients. Tissue microarray and whole-slide scanning technologies allow tissue research in esophageal carcinomas to be progressed in a more efficient way. Cancer cell lines and animal models could use to study the functional aspects of the various cellular and molecular changes in esophageal carcinomas.

Keywords: esophageal; squamous cell carcinoma; adenocarcinoma; HPV; gene; prognosis; TMA; pathology; cell lines; animal model; molecular biology

INTRODUCTION

Histological differences

Esophageal cancers comprise cancers of different histological types of diverse cellular and molecular bases [1-2]. The two major histological types of esophageal cancers are squamous cell carcinoma and adenocarcinoma. It is important to note that there are histological variants of squamous cell carcinoma and adenocarcinoma such as basaloid squamous cell carcinoma, spindle cell carcinoma, mucoepidermoid carcinoma and adenosquamous carcinoma [3-6]. In addition, neuroendocrine neoplasms such as small cell carcinoma of the esophagus account for approximately 1% of primary esophageal carcinoma [7]. All these carcinomas have distinct clinicopathological features. Limited studies have revealed that the cellular and molecular biology of these uncommon types of esophageal carcinomas are different from those of esophageal squamous cell carcinoma or adenocarcinoma [4, 8-9].

The current understanding of the cellular and molecular biology of esophageal cancers focuses on esophageal squamous cell carcinoma and esophageal adenocarcinoma. The difference in prevalence of these two major histological types in different geographic regions is likely due to the complex interactions of genetic and environmental factors. In general, esophageal squamous cell carcinoma predominates in areas with high incidence of esophageal cancer whereas esophageal adenocarcinoma is more common in areas with low incidence of esophageal cancer. In addition, the genetic mechanisms of esophageal squamous cell carcinoma are complex with multiple genetic factors proposed [2]. On the other hand, most esophageal adenocarcinomas show genetic changes of the progression of lesions related to acid reflux. The histological progression from reflux esophagitis to Barrett's metaplasia to dysplasia to adenocarcinoma is well known.

Applications of molecular and cellular biology

Esophageal cancer is one of the leading causes of cancer death worldwide despite recent improvements in surgical and adjuvant therapies. The only hope to further increase the quality of life of patients with esophageal 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 esophageal cancer could serve the following purposes: (1) to establish the presence or absence of an infectious co-factor; (2) to understand the genetic mechanisms of disease; (3) to provide prognostic information; and (4) to predict response to medical therapies and new modalities of treatment. In performing research, interpreting and applying knowledge in this area, it is important to bear in mind the histological difference in esophageal cancers. Currently, many studies on this cancer focused on the understanding genetic mechanisms of the cancer but there are current applications of this knowledge.

ESTABLISHMENT OF AN INFECTIOUS CO-FACTOR

In esophageal adenocarcinoma, gastroesophageal reflex and the resulting Barrett's esophagus (intestinal metaplasia) is the most important risk factor for esophageal adenocarcinoma [1]. Obesity, tobacco use, drugs and dietary factors also play roles as risk factors. For esophageal squamous cell carcinoma, smoking, alcohol consumption and dietary factors are risk factors [10]. Besides these, the role of infection in the development of esophageal cancer has long been suspected and in particular the role of human papilloma virus.

Human papilloma virus

In esophageal cancers, the main infectious co-factor under intensive study is human papilloma virus (HPV). HPV is a non-enveloped double-stranded DNA virus that could infect the basal cells of the skin or mucosa. Sexual contact is the exclusive mode to acquire the disease. However, the majority of patients with HPV infections are asymptomatic. After the infection, approximately 10% of patients may have persistent infection, which may lead to cancer [11]. In squamous cell carcinomas from the upper aerodigestive tract, in particular in oropharynx, identification of the presence of HPV in the carcinomas is of important value [12]. In these areas, patients with HPV-positive cancers have better prognosis when compared to patients with HPV-negative cancers. The detection of HPV in oropharyngeal squamous cell carcinomas also predicts better response to radiotherapy. The detection of HPV in clinical settings is indirectly by the identification of expression of p16 protein by immunohistochemistry [13].

The esophagus is distal to the oropharynx and histologically lined by stratified squamous epithelium as in the oropharynx. Studies to investigate HPV in esophageal squamous cell carcinomas have been underway for 30 years [14-15]. Thus, there is considerable data on the role of HPV infection in the development of esophageal cancer. The majority of studies were in esophageal squamous cell carcinoma.

Pooled analysis of five studies (in the years 2006 to 2013) from the literature revealed that HPV prevalence in esophageal adenocarcinoma was 35.0% (range, 1% to 90%) and HPV-16 prevalence was 11.4% [16]. Due to the limited number of studies on esophageal adenocarcinoma, no detailed analysis of the impact was available. Nevertheless, the hypothesis is that progressive acid damage to the esophagus 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's dysplasia and esophageal adenocarcinoma, suggesting a potential role of HPV in esophageal carcinogenesis. The involvement of HPV is with wide-type p53 and aberrations of the retinoblastoma protein pathway [17]. On the other hand, Antonsoon and colleagues in 2016 showed no evidence of HPV DNA in a large cohort (n= 233) of histologically confirmed archived esophageal adenocarcinomas [18]. Thus, HPV alone is unlikely to cause esophageal adenocarcinoma.

In esophageal squamous cell carcinoma, summarized HPV prevalence from both early and recent meta-analysis was 22% [16]. In general, HPV prevalence was higher in studies from some Asian countries and was much lower in studies reported in Western countries such as in Europe and America [2]. Stratified analysis by localization of cancer showed that esophageal squamous cell carcinoma was only slightly higher in cervical portion but not significantly higher than the middle or lower portion of the esophagus [18].

With respect to HPV DNA detection in meta-analysis, the prevalence of esophageal squamous cell carcinoma produced by type-specific primer PCR method (30.4%) was significantly higher than that by broad-spectrum primers (20.8%) [16]. There are limited studies on the p16 protein detection by immunohistochemical method in studying the HPV infection in esophageal carcinoma. Nevertheless, the current data using p16 detection in esophageal squamous cell carcinoma did not reflect the HPV status in the cancer [20]. Detection of HPV DNA is the preferred means of study HPV in esophageal carcinoma.

Human papillomaviruses are a group of more than 100 subtypes of viruses [11]. Slightly more than 30 are oncogenic in human beings. They are high-risk types and low-risk types for cancers [21]. From pooled data, HPV-16 was the most frequently observed subtype with a summarized prevalence of 11.4% [2, 16]. The other six most frequent HPV individual types identified in esophageal 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%). Apart from HPV-6 (low-risk type), all the detected types belong to high-risk carcinogenic HPV types. HPV16 could induce cancer stem-like cells phenotypes in esophageal squamous cell carcinoma through the activation of p13K/AKT signaling pathway [22].

Overall, HPV infection was associated with an increased risk of esophageal squamous cell carcinoma. However, the association was not as strong as that for oropharyngeal squamous cell carcinoma or cervical squamous cell carcinoma. The impacts on survival of patients with esophageal squamous cell carcinoma have not been clearly determined. Patients with HPV-positive esophageal squamous cell carcinoma had better respond to chemo radiation [23, 24]. Wang and colleagues also reported better 3-year survival in patients with HPV-positive cancers [24]. On the other hand, de Costa and colleagues showed no predictive values of HPV, p16 and p53 status on the cancer survival of patients with esophageal squamous cell carcinoma in a recent multivariate analysis [25]. At this stage, routine evaluation of HPV or p16 status is not required in the management of esophageal cancer.

The importance of studying the pathogenesis of HPV in cancers also stems from the availability of effective vaccines against HPV in the market. Prophylactic HPV vaccine was now in second generation [26]. The vaccine was useful to prevent premalignant genital and anal lesions arising from infection with HPV when given to young females. Australia is the first country to offer complimentary HPV vaccines to boys and girls. The clinical impacts of the vaccination program are already visible in the population. Although there is no data from clinical trials for the efficacy of the vaccines for HPV-related cancers outside the genital tract, it is likely that the universal vaccination could affect the prevalence of the HPV-related esophageal cancers in the future.

Epstein-Barr virus

The detection rates of Epstein-Barr virus (EBV) in esophageal cancer are variable with a detected prevalence ranging from 0% to 35% [27-29]. The differences are likely resulting from differences in racial, geographical and detection methods used. It is worth noting that lymphocytes in the cancer stroma can harbor EBV and thus detection of virus in esophageal cancer by PCR-based methods may show false-positive results [28]. On the other hand, *in situ* hybridization may provide false negative results due to higher rate of RNA degradation. Most studies have shown that EBV-associated esophageal cancer demonstrates similar morphologic findings to undifferentiated carcinoma of the nasopharynx, which is associated with EBV. At the current time, the identification of EBV in esophageal carcinoma has no clinical application.

Bacteria

Helicobacter pylori, previously known as Campylobacter pylori, is a Gram-negative microaerophilic spiral bacterium, which 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 esophageal adenocarcinoma [27, 29]. The effect may be the bacterium decreases the acid production through the production of cytokines [29]. It is worth noting that the worldwide decreased prevalence of *Helicobacter pylori* because of use of antibiotics parallel with the increased prevalence of esophageal adenocarcinoma [29]. Overall, there is no consensus on the role of *Helicobacter pylori* in esophageal adenocarcinoma, with substantial differences between Asian and Western studies. Metagenomics identified many other types of bacteria in the esophagus [27, 30]. 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) noted. The 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

Esophageal carcinomas are biologically aggressive cancers and thus the genetic profiles of esophageal cancers are complex. Oncogenes, tumor suppressor genes, metastatic genes, apoptosis genes, proliferation related factors, epigenetic factors as well as proteins related to metastases have roles in the pathogenesis of both esophageal squamous cell carcinoma and esophageal adenocarcinoma [2, 31]. In the recent years, in esophageal squamous cell carcinoma, many components of the *P13/AKT* (*phosphatidylinositol 3-kinase/protein kinase B)* pathway appeared to be important in the pathogenesis of the cancer. The expressions of different markers such as E-cadherin, N-cadherin, p120, DNAJB6 (DnaJ homolog subfamily B member 6), phosphorylated AKT play roles in progression of the cancer as well as predicting the prognosis of the patients with the cancer [32-34]. Oncogenic proteins such as receptors for VEGF (vascular endothelial growth factor) and CAPN10 (calpain 10) which is *regulated by GAEC1 (gene amplified in esophageal cancer 1)* are related to the clinical progression of esophageal squamous cell carcinoma [35, 36]. In addition, epigenetic changes such as promotor methylation of NID2 (nidogen-2, produce a key component of the basement membrane) could suppress the EGFR (epidermal growth factor receptor) /AKT metastasis related pathway and control cancer metastases [37]. In general, for both esophageal squamous and adenocarcinoma, p53 mutation is an important genetic change [38-39].

DNA copy number alterations and methylation analysis could detect many of the

genetic and epigenetic changes in esophageal carcinomas [40-43]. Starting from around 2000, comparative genomic hybridization (CGH) and expression array could identify the differences in genetic profiles between esophageal cancer and non-cancerous esophageal tissue [44-47]. Chromosomal regions with amplification may harbor oncogenes, and chromosomal regions with deletion may harbor tumor-suppressor genes. Comparative genomic hybridization could identify the whole profile of cytogenetic changes in an individual cancer. By this approach, researchers identified many new cancer-related genes in both esophageal squamous cell carcinoma and esophageal adenocarcinomas [48-54]. These provide more information regarding the carcinogenesis of esophageal cancers as well as defining gene candidates as prognostic markers and molecular targets for therapy.

Traditional way of detecting genetic mutation in by sequencing the gene is by Sanger sequencing [55]. The introduction of next-generation sequencing in research and clinical practice led has to sequencing many new genes and generate vast quantities of genetic data at a low cost [56, 57]. These recent technologies allow researchers to sequence DNA much more quickly and economically than the previously used Sanger sequencing, and as such have revolutionized the study of genomics and molecular biology. The first commercially available next-generation sequencer was available in 2007. Many new versions of sequencer were then available with the ability to detect many genes at one run of experiment in equipment of small size (Figure 1). Using the new robust sequencing platforms, whole exome sequencing and whole genome sequencing of patients in esophageal carcinoma are possible. In the literature, reports of whole exome sequencing noted mainly in esophageal squamous cell carcinoma and occasionally in esophageal adenocarcinoma [58-67]. There are many novel mutations and genetic pathways detected which could help us to understand the pathogenesis of this group of cancers with complex

genetic alternations (Table 1).

The International Cancer Genome Consortium (ICGC) coordinates a large number of research projects that have the common aim of comprehensively elucidating the genomic changes present in many cancers [68]. The preliminary meeting was in 2007 and the consortium launched to 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). In esophageal cancer, the genomic study in esophageal squamous cell carcinoma was by researchers in China whereas in esophageal adenocarcinoma was by researchers in the United Kingdom.

The whole genome sequencing data on esophageal cancer started to appear in the literature in 2013 [69-83]. A large volume of information is available for the two major histological subtypes of esophageal cancer, which provides substantial resources for future research directions for the better management of patients with esophageal carcinoma (Table 1). The information included: (1) many novel driver genes' mutations first reported; (2) the relevant frequencies of the key mutations in esophageal carcinomas recognized; (3) the predominant mutation pathways in esophageal cancers identified; (4) the mutational signatures related to risk factors recognized; (5) the progression of the cancer as well as change related to adjuvant chemotherapy were noted. It is worth noting that as predicted from the biological aggressiveness of the esophageal cancer, the genomic changes obtained are very complex. It still takes times for research works of the functional aspects of these genomic changes in order to apply the knowledge for clinical managements of patients with this group of cancer.

MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a class of small, well-conserved, non-coding RNAs that regulate the translation of RNAs. Many carcinomas showed that miRNA have important biological and pathological functions [84-96]. miRNAs affect variety of biological process in the body as well as acting as oncogenes, tumour suppressor genes or regulation of cancer stem cells. Due to their small size, there are established means of detection methods (traditional and new) of them in serum, cell lines as well as human tissues in esophageal carcinoma [97-98]

In esophageal adenocarcinomas, altered expressions of different sets of miRNAs are present in the development of adenocarcinoma from Barrett's esophagus. In different studies, miRNAs such as miRNA-192, miRNA-196 and miRNA-21 were frequently up-regulated whereas miRNA-203, miRNA-205 and miR-let-7 were commonly down-regulated during the development of Barrett's esophagus to esophageal adenocarcinoma [99]. In addition, changes in the expression of miRNAs are associated with the predication of metastasis, prognosis and response to chemo-radiation in the patients with esophageal adenocarcinoma. Similarly, in esophageal squamous cell carcinoma, many miRNAs are involved in the pathogenesis of the cancer. miRNAs have oncogenic or suppressor roles as well as potential roles as diagnostic and prognostic markers in the cancer. Many more miRNAs were identified in esophageal squamous cell carcinoma as the carcinoma has a more complex carcinogenesis than esophageal adenocarcinoma [100, 101,102].

Experimental studies in manipulating the miRNAs in cancer cell lines could provide hints for therapeutics for the cancer. However, further works such as how to deliver the miRNA in the cancer tissue are required in order to be able to apply miRNAs for clinical use.

Cancer stem cells

Cancer stem cells (CSCs) are subgroup of cancer cells, with properties resembling the critical properties of embryonic stem cells such as self-renewal and maintenance of stemness [103-106]. Only cancer stem cells have tumor-initiating properties. They are cancer cells that are responsible for initiation, progression, metastases, and recurrence in cancer. They play an important role in the resistance to adjuvant therapies for cancer. The other important contributing factor for cancer stem cells in cancer management is their function in causing treatment resistance and recurrence in cancer via their activation of different signaling pathways such as Notch, Wnt/β-catenin, TGF-β, Hedgehog, PI3K/AKT/mTOR and JAK/STAT pathways [105,106]. In addition, 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 [107].

Identification of cancer stems is important in cancer. Identification of these cells is challenging. The identification most often is by detecting the expression of their antigens in a group of stem cells [108]. There are many surface markers, which can detect the cancer stem cells by directly targeting their specific antigens present in cells. In addition, multiple analytical methods and techniques including functional assays, cell sorting, filtration approaches, and xenotransplantation methods could identify cancer stem cells.

In esophageal squamous cell carcinoma, there are several markers such as CD44, ALDH, Pygo2, MAML1, Twist1, Musashi1, Side population (SP), CD271 and CD90 that could identify the cancer stem cells in individual cancer masses. In addition, stem cell markers like ALDH1, HIWI, OCT3/4, ABCG2, SOX2, SALL4, BMI-1, NANOG, CD133 and podoplanin are associated with patient's prognosis, pathological stages, cancer recurrence and therapy resistance [109]. In esophageal adenocarcinoma, cancer stem cells are

responsible for intrinsic and acquired chemotherapy resistance, which is associated with epithelial mesenchymal transition regulation [110]. Like in esophageal squamous cell carcinoma, different methods including functional assays, cell sorting using various intracellular & cell surface markers and xenotransplantation techniques could identify and separate out the cancer stem cells. None of these methods solely can guarantee complete isolation of cancer stem cell population. Thus, a combination of methods may use to detect and isolate the cancer stem cells.

The development of specific markers and signaling molecules to target the stem cells of esophageal 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 esophageal cancer. Emerging therapeutic tools based on specific properties and function of cancer stem cells may improve clinical output of the diseases. Therefore, innovative insight into biology of cancer stem cells and targeted therapies to cancer stem cells will help to achieve effective management of esophageal cancers.

PROGNOSTIC INFORMATION

Predication of progression

Aneuploidy (detected by FISH/flow cytometry), promoter hypermethylation and cyclin A protein expression have been shown to be correlated with the progression from Barrett's esophagus to esophageal adenocarcinoma [111,112]. Despite these results, there is generally lack of large prospective studies to validate the use of these markers in clinical practice. The likely candidate for clinical application is p53 protein over-expression as determined by immunohistochemistry. Overexpression of p53 protein correlated with neoplastic progression to esophageal adenocarcinoma. It could be a useful adjunct to determine the grade of dysplasia in Barrett's esophagus. In addition, the

results validated in some studies and the procedure used is simple.

Expression or identification of cellular and molecular markers could predict the survival of patients with esophageal adenocarcinoma [113,114]. 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, there is no routine testing for these markers, as researchers have not validated these makers adequately in prospective studies.

In esophageal squamous cell carcinoma, many molecular and cellular markers related to the prognosis of the patients with cancer. Expressions of p21, p53, cyclin D1, Ki-67, E-cadherin provide some prognostic information [33, 34, 36,115,116]. However, this approach has not been widely used.

GUIDELINES FOR MEDICAL THERAPIES

Predict response to medical therapies

Pre-operative chemo-radiation is a standard treatment for esophageal cancers. In patients who undergo 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 [117].

It is thus important to have a means to predict the response to chemo-radiation. The grade of esophageal squamous cell carcinoma could potentially predict the response to pre-operative chemotherapy [118]. Many molecular makers have been studied [119-122]. 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

353 status. The DNA repair markers, p53R2 or ERCC1, vascular endothelial growth factor
354 (VEGF) and hedgehog signaling pathway factor Gli-1 also have potential as predictive
355 biomarkers. However, researchers need further studies to validate the findings. In
356 esophageal adenocarcinoma, expression of EGFR, VEGF, nuclear factor-κB and cDNA
357 microarray could act as predictive factors for pre-operative chemo-radiation.
358 It is important to aware of the histological changes after pre-operative chemo-
359 radiation [123]. In the current AJCC (American Joint Committee on Cancer) staging of
360 esophageal carcinoma, patients having pre-operative chemo-radiation had different
361 guidelines for pathological staging from those patients without the therapy [124].
362
363 **Predictors for targeted therapy**
364 Targeted therapy involves targeting a specific gene mutation in the cancer. In
365 clinical settings, oncologists use targeted therapies to treat melanoma, breast cancer and
366 colorectal cancer with promising results [125-129]. Testing the cancer tissues for
367 molecular markers is useful to predict the response of the patients to these targeted
368 therapies.
369 Of the potential targets trailed to date in esophageal cancer, EGFR (Her 1 and Her 2)
370 and VEGF surface receptor antagonists have shown the most promise results [130-134]. For
371 instance, over expression of EGFR-1 present in 1/3 to 2/3 of esophageal adenocarcinoma
372 and squamous cell carcinoma. Her 2 (also known as c-erbb2, CD340 and Neu) staining have
373 been demonstrated in esophageal squamous cell carcinoma [135]
374 The most important advance in the molecular biology and oncology in esophageal
375 adenocarcinoma at the gastroesophageal junction is the approval of anti-Her 2 therapy in the
376 treatment of the cancer [136]. On October 20, 2010, the Food and Drug Administration
377 (FDA) in USA granted approval for the use of a drug that target the Her 2 protein, known as

trastuzumab (Herceptin). The drug in combination with other chemotherapy is validated for the treatment of patients with Her 2 overexpressing metastatic esophageal adenocarcinoma at gastroesophageal junction who have not received prior treatment for metastatic disease. The approval based on the findings in many clinical trials that trastuzumab-based therapy offered a significant survival advantage for patients with Her 2 overexpressing locally advanced, recurrent or metastatic gastric and gastro-esophageal junctional adenocarcinomas when compared to conventional therapy alone. The approval of the use of the trastuzumab was followed by authorities in other countries, e.g. TGA (Therapeutic Goods Administration) in Australia.

Pathologists are required to determine the Her 2 status in biopsy or resection material in gastroesophageal junction as well as in metastatic sites. Immunohistochemistry and ISH (in situ hybridization) testing could assess expression of Her 2. Precise testing of the status of Her 2 in esophageal adenocarcinoma at the gastroesophageal junction is important, as Her 2 testing is the only biomarker established for patients with advanced the cancer. Pathologist should ensure that biopsies or resection specimens used for testing are properly fixed and pathological assessed [137]. In many clinical laboratories, the protocol adopted is a combination of testing of Her 2 by immunohistochemistry and ISH. Her-2 staining is membranous in cancer. They are scored as “negative, 1+, 2+ and 3+” depending on standard criteria. In many centers, for cases that are negative or “1+” by immunohistochemistry, the patients are not for anti-Her 2 therapy. In addition, for cases those are strongly positive (3+, as defined by strong and complete membranous reactivity), the patients could opt for anti-Her 2 therapy. The esophageal adenocarcinomas at the gastroesophageal junction that are equivocal (2+) in staining will be tested by ISH to make a decision for the targeted therapy.

RESEARCH SOURCES OF MOLECULAR AND CELLULAR STUDIES IN ESOPHAGEAL CANCERS

Tissues studies

Human cancer can be studied at the tissue level when it is surgically removed from the 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, cancer tissues are fixed in formalin and embedded in paraffin. Thin sections were cut from the paraffin-embedded tissues, stained by hematoxylin and eosin, and examined by pathologists under light microscope. They could be useful for different molecular studies. In fact, many of the research findings in esophageal cancers were from studies performed on paraffin-embedded tissues. This approach has the benefit of providing superior morphological features for studying histological features as well as localization of biomarkers at the cellular level when compared with other methods (Figure 2). It is worth noting that histological assessment is important before starting any further molecular research. It is important to confirm the presence of cancer and proportion of cancer cells on histological examination of the tissue. Proper dissection and histological examination of cancer tissue could provide variable information such as histological type, grading and pathological staging which are important parameters to determine the behavior of the cancer as well as the treatment options for the esophageal carcinoma [123,124,138].

In recent years, the use of tissue microarray (TMA) has increased for testing molecular markers in large numbers of samples by either immunohistochemistry or ISH (Figure 3). The testing of multiple samples in a block will allow fast screening of large number of patients as well as reducing the costs of reagents in the research on cancer. The use of tissue in the form of TMA saves using a large amount of invaluable patients' tissue for research tests, which may be needed for clinical uses. In the TMA technique, a hollow

needle is used to remove tissue cores as small as 0.6 mm in diameter from regions of interest in each paraffin block of cancer of interest. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced array pattern [139]. The core(s) of tissues in the recipient block are from different patients. There are some drawbacks as cancer is heterogeneous and small samples from a cancer may not have the information that could get in the whole section of the cancer. In addition, preparation and work-up on the TMA blocks require more technical expertise and time when compared to conventional tissue blocks.

The drawback of working on paraffin-embedded tissues is that 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 analyze for abnormalities in the tissue that indicate cancer. The effect of these cross-linking fixatives on the nucleic acids and proteins may impair the molecular interactions. To overcome this drawback, snap freezing in liquid nitrogen and storage at -80°C is used to collect esophageal cancer tissues for use in research. The collection included substantial clinical and scientific effort and provided tissues that are superior in quality for molecular studies. For instances, frozen samples are needed for whole genome or whole exome study in esophageal carcinomas. On the other hand, the morphological features are inferior to those obtained using paraffin embedded sections (Figure 4).

The staining of histological sections will fade in time. In addition, storage of large amount of histological sections are difficult. Whole-slide imaging allows scanning and storage of the histological slides in digital files [140]. This also allows long-term storage of the research data as well as computerized analysis of the histological parameters (Figure 5). The researchers could share information more easily with the digitalized slides.

The other human cancer sample used in patients with esophageal carcinomas is blood. It could be used to analysis the circulating DNA, mi-RNA or circulating tumour cells in esophageal carcinoma [141,142].

Cancer cell lines

It is worth noting that research with removed cancer tissue cannot provide functional dynamic studies of esophageal cancers. For functional studies in esophageal cancer, studies often performed in cancer cell lines derived from tissues obtained freshly from surgery. Different molecular approaches could block the genetic changes in the cancer [143]. For instances, RNA interference (RNAi) is a normal physiological mechanism in which a short effector antisense RNA molecule regulates target gene expression. It could silence a particular gene of interest in a sequence-specific manner and used to target against various molecular pathways in esophageal carcinoma by designing RNAi targeting key pathogenic genes. RNAi-based therapeutics against esophageal carcinoma could involve different strategies including inhibition of overexpressed oncogenes, blocking cell division by interfering cyclins and related genes or enhancing apoptosis by suppressing anti-apoptotic genes.

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 tumors if injected into a susceptible animal to generate an animal model. Cancer cells can be harvested from the animal and form a more stable cell cancer cell line. In esophageal cancers, some of the commonly used cell lines are actually secondary cell lines. Cancer cell lines can allow functional studies to be performed. They can be stored in liquid nitrogen for an indefinite period and retain their viability when thawed.

In esophageal cancers, there are published cancer cell lines available for both adenocarcinoma and squamous carcinoma [144-147]. When compared to esophageal squamous cell carcinoma, esophageal adenocarcinoma is relatively uniform in characteristics as the risk factors and pathogenesis are more established. Model research on esophageal adenocarcinoma relies almost entirely on a relatively small set of established cancer cell lines. The high genomic similarities between the esophageal cell lines and their original cancers provide rationale for their use. Nonetheless, cancer cell lines nearly always differ in important ways from the original cancer from which they derived from.

Animal models

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

Injection of cancer or cancerous cells in the subcutaneous tissue of the skin of immunodeficient mice is a common practice to produce a cancer model in animals (Figure 6A). In many instances, researchers use cancer cell line as it is easy to growth. However, if we would like to adopt a personalized approach for testing the cancer from a particular group of patient, we must try injection of cancer tissue. It is worth noting not it needs careful planning, highly experience personnel as well as having a high failure rate for the cancer to grow in the animal (when compared to using the commercial obtained cancer lines)

In esophageal cancers, this approach cannot recapitulate the microenvironment of the esophagus or the response to the targeting carcinogens. An approach is to make an orthotopic

501 (occurring at normal place) model for both esophageal squamous cell carcinoma and
502 esophageal adenocarcinoma [148-149] (Figure 6B). The orthotopic model provides the
503 optimum environment for cancer growth and drug testing. In the anatomical setting of
504 esophageal cancer, the site is very difficult to approach surgically. Different approaches were
505 use but many of these have some shortcomings. The establishment of these orthotopic
506 models needs to involve radiological guidance (magnetic resonance imaging and fluorescence
507 imaging) so the cancer and the metastases can be visualized in real time [150]. In addition,
508 pathological examination is important to clarify the histological typing, microscopic location
509 and the microenvironment of the cancer in the animal.
510
511

Figures Legends

Figure 1. Use of next generation sequencer in studying esophageal carcinoma
A chip (arrow) in which DNA to be sequenced is loaded. On the right side, the chip (arrow) is in the grounding plate on the benchtop sequencer.

Figure 2. Histological features of carcinomas from formalin fixation and paraffin embedded samples.
2A: well differentiated squamous cell carcinoma. 2B: well differentiated adenocarcinoma. 2C: lymph node with metastatic esophageal adenocarcinoma

Figure 3. Tissue microarray (TMA) of esophageal carcinoma
3A: Making of tissue microarray block by manual technique. 3B: A tissue microarray block with multiple tissue cores in the paraffin. 3C: Section stained by hematoxylin & eosin taken from the tissue microarray block of esophageal squamous cell carcinoma. 3D: Higher magnification of two of the cores of 3C. 3E: The TMA section used to test a biological maker.

Figure 4. Histological features of esophageal carcinoma prepared by sectioning of frozen tissues
The quality of the morphological features is inferior to those in Figures 2 or 3.
4A: Squamous cell carcinoma of esophagus. 4B: Non-neoplastic esophageal epithelium (control in research). 4C: Para-esophageal lymph node infiltrated by squamous cell carcinoma.

Figure 5. Whole-slide imaging of esophageal carcinoma
5A: the captured of the histological section of an esophageal squamous cell carcinoma frozen section by scanner.
5B: the image obtained from scanner of an esophageal adenocarcinoma. Arrow and scale added on the picture. Zooming of the image possible as noted on the right hand corner.

Figure 6. Animal models of esophageal carcinoma
6A. Tumor produced in an immunodeficient mouse after subcutaneous injection of primary esophageal squamous cell carcinoma from a patient (courtesy of A/Professor Johnny Tang from the Hong Kong Polytechnic University).
6B. An orthotopic nude model of esophageal squamous cell carcinoma (courtesy of Professor Maria Lung from University of Hong Kong). Histological section of the mouse's esophagus showing the squamous cell carcinoma (grew from cancer cell line surgically implanted in the wall of the esophagus) was successfully growth in the esophagus of the mouse. A carcinoma nodule is present in the lymphatic in the wall of the esophagus (arrow). L: lumen in the esophagus; E: esophageal epithelium. T: carcinoma.

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