**Molecular pathways in the development and treatment of oesophageal cancer.**

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**Abstract**

The molecular pathways involved in the development and treatment of oesophageal cancer are complex. Recent large-scale genome sequencing studies have delivered novel insights into aetiology and possible targeted treatments. Oesophageal squamous cell carcinoma (OSCC) and adenocarcinoma (OAC) are distinct entities. At the molecular level OSCC is more similar to squamous cell cancers in other organs than OAC. Whilst considerable heterogeneity exists in both tumour types new data suggests that driver gene events and mutational signatures may be able to categorise tumours into potentially actionable subtypes. Taken together these findings not only suggest new avenues for treatments in a cancer type with appalling outcomes, but also a new era of molecular rather than purely anatomical classification and staging of oesophageal cancer.

Cancer of the oesophagus is the seventh most common cancer worldwide and the sixth most common cause of cancer death accounting for 572,034 new diagnoses and 508,585 deaths in 2018(1). Curable disease is found in less than 40% of patients(2) and the best 5 year survival rates approach but do not exceed 50%(3–5). Current gold standard treatment consists of perioperative chemotherapy with or without chemoradiotherapy(3,6,7) but neoadjuvant therapy is ineffective for the majority(8,9) and highly morbid. Mortality from neoadjuvant treatment for locally advanced OAC approaches 2%(10,11). Curative surgery continues to improve in safety but also carries a mortality of 2%(2) and a morbidity of 59%(12). There has been only one successful trial of precision therapy in oesophageal cancer wherein the researchers reported a modest 2.7 month increase in median overall survival(13). A systematic review in 2015 focussing on adenocarcinoma alone found no clinically useful molecular biomarkers for guiding treatment and prognosis(14).

Several studies have attempted to understand the molecular pathways corrupted in the development of oesophageal cancer in an attempt to identify commonly mutated driver genes. A driver gene mutation is a mutation that confers a selectional or evolutionary advantage to a cell through either a loss of function (e.g. a missense single nucleotide variation(SNV)) or an increase in function (e.g. a copy number amplification). These driver mutations impact on the clinical course of the disease by influencing tumour aggressiveness, response to treatment and resistance and are therefore targets for therapy. They represent a small number of the genetic events in cancer and therefore with current technology many thousands of tumours need to be sequenced in order to identify all of the potential driver genes in any given cancer.

This analysis has revealed two very different diseases squamous cell carcinoma (OSCC) and adenocarcinoma (OAC), which were hitherto largely treated as one entity determined by their anatomic location(1,15). While they may share mutations in similar pathways the affected genes and the predominant mutational signatures are distinct. For example, CDKN2A **(**cyclin-dependent kinase Inhibitor 2A) is frequently mutated in both OSCC and OAC and in the precursor lesions of squamous dysplasia/intraepithelial neoplasia and Barrett’s Oesophagus respectively. However, in OAC the CDKN2A aberration is more commonly a silencing mutation and in OSCC more commonly a deletion event. Taking an integrated “omic” approach to cancer, OSCC shares striking similarities to head and neck squamous cell cancer and is distinct from OAC and gastric adenocarcinoma(16).These findings support the assertion that OAC and OSCC are distinct diseases with different aetiologies, which may inform treatment strategies, and in this chapter, they shall be described separately.

**Oesophageal Squamous Cell Carcinoma**

**Progression from squamous epithelium to invasive carcinoma**

Oesophageal squamous cell carcinoma arises from the normal stratified squamous epithelium of the oesophagus. The only pre-cursor lesion is basal cell hyperplasia. In response to chronic inflammatory insult the normal cells acquire somatic mutations, copy number variations(CNVs) and chromosomal aberrations such as aneuploidy and arm level aberrations, progressing in a stepwise fashion from phenotypically normal epithelium to simple basal cell hyperplasia, through low-grade and high grade intra-epithelial neoplasia (dysplasia) and finally invasive carcinoma(17,18)(Figure 1).

The normal oesophageal mucosa is already composed of mutated clones of phenotypically normal squamous cells(19). Due to Darwinian evolution mutations which confer a survival advantage are positively selected and these by definition include cancer driver genes. In fact the normal oesophageal mucosa contains more mutations in cancer genes than UV damaged skin cells despite the fact that those skin cells have a higher overall mutational burden (19). These very recent findings ask profound questions regarding our understanding of the drivers and requirements for cancer initiation and progression and might suggest that the fate of mutated cells is determined, at least in part, by the microenvironment.

It is clear that the majority of mutations occur early in the disease at the progression from normal oesophageal mucosa to intra-epithelial neoplasia. The tumour suppressor gene TP53 remains the most frequently mutated (70% of dysplastic lesions and 80% of malignant lesions) gene. KMT2D(15.2%), NOTCH1(13.2%), NFE2L2(9.3%), ZNF750(9%), FAT1(8.8%) and PIK3CA(8.2%) are also commonly mutated in OSCC(16,20–26).

Frequently mutated genes are however shared at the dysplastic stage and in invasive cancer(27). Large scale chromosomal deletions and amplifications in regions containing cancer genes such as CDKN2A, ASCL3, FEV, CCND1, NFE2L2 and SOX2 are common to both dysplastic and invasive lesions. While copy number variations are not significantly increased between dysplastic and malignant cells, it has been suggested that several known cancer genes (ATR, MECOM, PIK3CA, BCL6, MYC and CCND2) are more frequently affected by CNVs in malignant than dysplastic cells. NOTCH signalling, ERBB2-PI3K signalling and DNA damage repair subnetworks are frequently dysfunctional across invasive and pre-invasive stages of disease. Many of these commonly mutated genes are shared with other squamous cell carcinomas of the head and neck and of the lung(28,29). It is therefore probable that cancers that arise from similar lineages are more molecularly alike than cells that arise from different lineages within the same organ. This may in turn mean that biomarkers and therapeutic targets for cancer will be shared between tumour types based on cell of origin rather than anatomic location (26).

Risk-stratifying patients during progression from normal mucosa to invasive OSCC is made difficult by the lack of clear genomic aberrations that mark the transition between stages. This results in differing therapeutic recommendations(30) with over and under-treatment in some patients. Two potential protein biomarkers have been identified whose expression increases during the transition from normal tissue to invasive cancer via dysplasia (TNFAIP3 and CHN1) (31) but their use has not been validated in a prospective study.

In the absence of clearly defined targetable pathways common to many patients, alternative strategies have been used in an attempt to stratify patients (and tumours) to aid clinical decision making.

**Mutational Signatures**

Tumours exhibit characteristic mutational signatures, combinations of mutation types arising from a specific mutagenic process that may inform aetiology and give insight into therapeutic potential(32). In OSCC, signatures 1, 2, 4, 5 and 13 are the most prevalent(16). Signature 1, characterised by C>T mutations is the most common in OSCC and correlates strongly with aging. These C>T mutations result from spontaneous deamination of 5-methylcytosine accumulated over a lifetime(32). Signature 5 is characterised by T>C mutations and is also thought to be an aging signature. Signatures 2 and 13 are characterised by C>T and C>G mutations. This pattern is associated with the activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide like (APOBEC). APOBEC converts cytidine to uracil and activates the base excision repair pathway and its activity is high in OSCC(22,33). Finally signature 4, characterised by C>A substitutions is associated with tobacco exposure. Tobacco smoke contains over 60 carcinogens and signatures 1 and 2 were strongly correlated with tobacco exposure in the original Alexandrov paper(32), in addition signature 5 was found to be associated with lung adenocarcinoma and therefore may be associated with the activity of tobacco smoke carcinogens.

**Molecular Subtypes**

The Cancer Genome Atlas Research network used a bioinformatic technique (iCluster) to group oesophageal squamous cell carcinoma into 3 molecular subtypes. The first, ESCC1, was defined by alterations in the NRF2 pathway which regulates adaptation to oxidative stress. This group also contained a higher frequency of SOX2 and TP63 amplifications. Geographically ESCC1 included 66% of the Asian population studied.

ESCC2 contained many of the eastern European and south American patients. Their tumours were characterised by higher rates of ZNF750 and NOTCH1 mutation, inactivation of the chromatin modulators KDM6A and KDM2D, CDK6 amplification and inactivation of the PIK3CA suppressors PIK3R1 and PTEN.

There were only four ESCC3 cases in the TCGA dataset and all four were from North America. Mutations in the cell cycle pathway were conspicuously absent in this subtype and only 1 patient had a TP53 mutation. They all contained mutations predicted to activate the RTK/RAS/PI3K pathway and 3 out of 4 contained somatic mutations in chromatin remodelling.

**Targetable Pathways**

A better understanding of the driver gene events, mutational signatures and molecular subtypes of OSCC has led to the recognition of cellular pathways suitable for targeted treatments. The major candidate pathways are discussed below.

**Cell Cycle**

Over 90% of OSCC samples in the cancer genome atlas (TCGA) contain alterations in the Cell Cycle pathway but across all studies this may be as high as 98%. Cell cycle suppressor genes such as CDKN2A and RB1 (which codes for Retinoblastoma protein) are commonly deleted. The cyclins CCNE1, CCND1, CCND2 and CDK4 and 6 are commonly amplified. This suggests a potential role for cell cycle kinase inhibitors in OSCC(34,35). Palbociclib is approved by the FDA for treatment of HER2(ERBB2) positive breast cancer. It is a specific inhibitor of CDK4 and CDK6 although it may have other anti-tumour effects(36). It is now being trialled in squamous cell carcinoma of the lung and positive results from these trials could lead to trials in oesophageal cancer.

**RTK/RAS/PI(3)K**

The receptor tyrosine kinase-ras-phosphatidylinositol-3-kinase pathway is an important intracellular signalling pathway that regulates cell proliferation. OSCC contains a high number of alterations in the PI3K pathway affecting 59% of samples in TCGA. It is activated by EGFR, FGFR1 and PIK3CA, and suppressed by PTEN and PIK3R1. EGFR is amplified in 19% of squamous cell cancers. A number of RTK/RAS/PI(3)K targeting drugs exist.

**Hedgehog Signalling**

Protein patched homolog 1 (PTCH1) in its active form inhibits the activity of smoothened (SMO). In normal tissue the activity of PTCH1 is switched off by sonic hedgehog (SHH). Smoothened promotes the downstream signalling of the hedgehog pathway which is involved in many of the hallmarks of cancer(37) including increased cell cycling, angiogenesis, inhibiting apoptotic signals and promoting stem cell self-renewal. Snail proteins activated by SMO have been shown to promote epithelial to mesenchymal transformation(EMT)(38). In 6% of OSCCs PTCH1 contains an inactivating mutation which may lead to increased SMO downstream signalling. Several components of the Hedgehog pathway (SHH, SMO, and GLI1/2) are viable therapeutic targets for anti-cancer therapies(39).

**Squamous Cell Maturation**

Amplifications of the 3q chromosome which contain TP63 and SOX2 were found in 43% of OSCC tumours in TCGA, in up to 68% in other studies and in 70% of dysplastic squamous epithelia(27). SOX2 is a transcription factor that plays a significant role in pluripotent stem cells and promotes the development and maintenance of squamous epithelia. SOX2 dependent pathways may be a targetable vulnerability in SCC. SOX2 preferentially binds with p63 in SCC cells as opposed to OCT4 which is its preferred binding partner in embryonic stem cells. ﻿SOX2 and p63 jointly regulate gene expression, including the oncogene ETV4(40).

ZNF750 is a transcriptional regulator of squamous cell differentiation and a tumour suppressor(22,41,42). Its mutation and therefore under-expression in TCGA and other datasets is associated with poorer prognosis and correlates with metastasis. Restoration of wildtype ZNF750 protein supresses the malignant phenotype of SCC cells and appears to inhibit cell migration.

**Chromatin Modelling**

KDM6A, KMT2D(MLL2) and KMT2C(MLL3) are histone modifying enzymes that are frequently inactivated via deep deletions or truncating mutations in OSCC. Their function appears to be demethylation of the histones H3 and H4. Methylated H3 (H3K27me3) has been shown to be prognostic in OSCC and is associated with over-expression of enhancer of zeste homology 2 (EZH2)(43). An effective means of targeting chromatin remodelling has not been successfully trialled. Although EZH2 inhibitors have been developed the contradictory oncogenic and tumour suppressive roles of EZH2 have not been clarified(44).

**Summary of OSCC**

OSCC is a relatively radio-sensitive tumour type that at the molecular level has more in common with squamous cell cancers in other organs that other tumour types in the oesophagus. Recent comprehensive genomic and molecular characterisation of OSCC has identified new opportunities for treatment advances.

**Oesophageal Adenocarcinoma**

**Barrett’s Oesophagus**

The precursor lesion to adenocarcinoma of the oesophagus is Barrett’s oesophagus, a metaplastic transition from squamous to columnar epithelium in the distal oesophagus associated with bile and gastric acid reflux(45,46). Like many other epithelial tumours OAC progresses from non-dysplastic Barrett’s oesophagus through low- and high-grade dysplasia to invasive adenocarcinoma. Barrett’s oesophagus cells carry a high mutational burden of 5.5-7 SNV’s per megabase of DNA, which is lower than OAC(10 SNVs/MB) but higher than many other cancers(47,48). The mutational burden in coding regions of the genome (exons) correlated with phenotypic dysplasia in one study(48) but this finding was not confirmed in larger studies of the whole cancer genome(47). Two models have thus far been described to explain the progression (Figure 2).

In the first, somatic mutations resulting in loss of function in tumour suppressor genes such as TP53, CDKN2A and SMAD4 accumulate in stepwise fashion and confer an evolutionary advantage on a clone of Barrett’s cells which then acquire further SNVs leading to an accumulation of cancer gene mutations and invasive carcinoma(49). The second proposes that large-scale chromosomal instability follows the loss of TP53 and the progression to OAC is marked by copy number variations and genome doubling(48). Multiple clones are formed and are subjected to selective and evolutionary pressures resulting in a far more heterogenous clonality. Since this model was proposed further studies have demonstrated the clonality of Barrett’s oesophagus and demonstrated significant heterogeneity between clones and even between Barrett’s cells and adjacent OAC(47).

The most significant molecular change from Barret’s oesophagus to OAC seems to be in copy number alterations with large amplifications of regions containing known driver genes in OAC such as ﻿GATA4, KLF5, CCND1, and VEGFA which may be targetable.

**Oesophageal adenocarcinoma**

Oesophageal adenocarcinoma can be classified as a C-type cancer(50) because of its dramatic chromosomal instability, copy number changes and structural variations(16,51–53). Several frequently mutated genes have been identified including TP53, CDKN2A, SMAD4, ARID1A, ERBB2, KRAS, PIK3CA and CTNNB1.

**Overlap with Gastric Cancer**

The distinction between oesophageal and gastric cancer is undetermined and controversial. This is especially so at the oesophago-gastric junction (OGJ) where purely anatomical definitions, such as the Siewert classification of OGJ tumours (54)have been used for many years. However, the latest AJCC/UICC staging manual (8th Edition) has changed the definition of OGJ tumours based on their anatomic epicentre(55). Fortunately, recent large-scale molecular investigation of oesophageal and gastric cancer is beginning to shed light on the biological sub-types of cancer within the upper gastrointestinal tract. The Cancer Genome Atlas study of gastric cancer identified 4 subtypes; Genomically Stable (GS, a group consisting mostly of diffuse histological pattern tumours), Chromosomally Instable (CIN), Epstein-Barr virus positive (EBV) and a group characterised by micro-satellite instability (MSI)(56). When TCGA authors compared these subtypes with OAC they found OAC and CIN gastric cancers clustered together in a group distinct from their other three subgroups (Figure 3). The CIN gastric cancers increased in frequency as tumour location moved more proximally towards the oesophago-gastric junction. Pure oesophageal tumours, distinct from tumours of the oesophago-gastric junction, contained no tumours from MSI or EBV subgroups. At the OGJ, however some EBV and MSI tumours were identified. Given the different molecular features and therefore targetable vulnerabilities of OGJ tumours this suggests that staging based on anatomical location alone is probably insufficient. Despite some differences in frequently mutated genes, there are no molecular features that dichotomise gastric CIN from oesophageal CIN.

**Mutational Signatures**

The hallmark Alexandrov(32) signature of Oesophageal Adenocarcinoma is signature 17 ﻿dominated by T>G substitutions in CTT codons and probably associated with gastric acid reflux(51,57,58). Analysis from the international cancer genome consortium has identified a total of six prominent signatures in OAC(51). The authors subdivided signature 17 into the classic 17A signature and a second 17B which was characterised by T>C mutations rather than T>G. The other signatures included signature 1, the predominant signature found in OSCC; ﻿signature 2, with C>T mutations in a TCA/TCT context, an APOBEC driven pathway also seen in OSCC. Signature 3 is a complex mutational signature seen as a result of defects in the BRCA 1/2 DNA damage response pathway. A ﻿signature 18 – like pattern was identified, C>A/T dominant in a GCA/TCT context.

When they analysed the dominant mutation signatures in each patient they were able to assign patients to one of three groups (Figure 4). The S1, S2 and S18 signatures coalesced into a C>A/T dominant group for which conventional chemotherapy may be the only current option, with targeted ERBB2/MET inhibition in selected cases.

A DNA damage repair(DDR) deficient group was identified. The mutational signature of this group was similar to BRCA1/2 mutations described previously in other cancers, but contained very few mutations in BRCA genes. Instead a 4.3 fold increase in mutations in the homologous recombination (HR) pathway was observed, suggesting that this mutational signature is due to mutations in the HR pathway as a whole rather than in the specific BRCA genes. The DDR deficiency in these tumours could be exploited by using DNA damaging agents such as PARP inhibitors which create double strand breaks which cannot be repaired if the HR pathway is dysfunctional leading to death of cancer cells (synthetic lethality)(59).

Finally, the predominant group with signature 17 type mutations carried a significantly higher mutational burden than the others. This was matched by a 1.5 fold higher neoantigen burden. Both mutational burden and neoantigen burden have been shown to predict an improved response to immune checkpoint inhibitors in other highly mutated cancers such as melanoma(60–62). These findings open the door to a new era of immunotherapy combination treatments for oesophageal and OGJ tumours. The cell cycle checkpoint regulators WEE1 and CHK1/2 have been targeted in recent studies with inhibitors resulting in an antitumorigenic effect in other highly mutated cancers(63,64). In cell lines characterised as mutagenic (MFD-1)(65) the authors were able to demonstrate increased sensitivity to these drugs.

The most comprehensive analysis oesophageal carcinoma to date includes 551 tumours investigated with whole genome sequencing(66). For the first time a catalogue of driver gene events has been made possible. A total of 77 driver genes and 21 non-coding driver elements have now been identified. These events cause aberrations in a restricted number of major regulatory pathways, giving cause for hope of better targeted treatments. A summary of the potentially actionable events is described below.

**Targetable Pathways**

**Cell Cycle**

67% of 551 OAC samples contained mutations in the cell cycle pathway. CDKN2A was frequently deleted or otherwise mutated in 29% of cases. Using the Cancer Genome Interpreter(67) the authors were able to hypothesise response to each drug class in the database. CDK4/6 inhibitors were predicted to be the most significantly effective. Sensitising events in receptor tyrosine kinase and cell cycle pathways were identified. *In vitro* experiments have shown that cell lines and 3D organoid models(68) predicted to respond due to sensitising mutational events did respond and those with resistance mutations (CCNE1 enables bypassing of CDK4/6) demonstrated resistance. These predictions need to be tested in clinical trials.

**Receptor Tyrosine Kinase**

The RTK pathway is dysregulated in 60-75% of cases. ERBB2 (18%-30%), VEGF(<28%), KRAS(14-19%) and EGFR(10-15%) amplifications and gains are common. ERBB2, EGFR and KRAS amplifying mutations tend to be mutually exclusive suggesting that these genes have a common downstream tumour promoting effect perhaps through the activity of CCND1 and CDK4 and 6 which promote cell cycling. This finding suggests that CDK4/6 inhibitors can be used to target amplifications and gains in RTK pathways by obviating their downstream effects.

**Prognostic Markers**

SMAD4 is often affected by inactivating mutations in OAC. Mutations in this gene have been shown to be exclusive to OAC in the progression from Barrett’s Oesophagus. Its role is to activate the tumour suppressor CDKN2A and it has been shown to be prognostic in OAC. GATA4, GATA6 and MUC6 are associated with GI cell differentiation. GATA4 and GATA6 tend to be mutually exclusive mutations suggesting a common pathway but their precise role is not clear. Accumulations of activating mutations seem to be negative prognostic markers in OAC and other cancers.

**Barriers to Precision Therapy in OAC**

The rational design of precisions therapies is complicated by non-linear associations between gene copy number and protein expression. This means that sequencing data alone may not be sufficient to guide treatment(66). The best way to identify targetable pathways in a clinically meaningful timeframe has yet to be established and in highly heterogeneous cancers such as oesophageal a large gene panel with immunohistochemical validation may be required for clinical use. In addition, precision targets are not consistent between clones in OAC(69) (Figure 5). Recently analysis of metastases from multiple sites over time and including warm autopsy has shown that metastases occur in multiple sites involving multiple clones and subclones(70). If clones do not contain the same driver mutations as their parent tumours then our sampling techniques may need to be adapted in order to improve precision therapeutics in OAC.

**Summary of OAC**

OAC is highly heterogeneous and difficult to treat with conventional methods. The traditional anatomical classification of tumours will rapidly be replaced with molecular staging to inform the rational application of novel treatments. Until very recently we were unable to categorise tumours based on driver events, but emerging data suggests a limited number of pathway aberrations that may be suitable for therapy. Immunotherapy combinations are likely to represent the next generation of treatments for widespread use, possibly targeted to the mutagenic subtype.

Practice Points

* Oesophageal cancers are heterogeneous with complex molecular features
* Genomic lesions are commonly found in precursor lesions suggesting that the determinants of progression may exist in the microenvironment
* Oesophageal squamous cell carcinoma and adenocarcinoma are distinct biological entities
* Large-scale genomic sequencing and molecular phenotype studies have enabled the recategorization of oesophageal cancers by molecular sub-type rather than anatomy

Research Agenda

* The rational design of precisions therapies is complicated by non-linear associations between gene copy number and protein expression
* The best way to identify targetable pathways in a clinically meaningful timeframe has yet to be established
* Actionable events may differ between the primary tumour and metastasis meaning that multiple therapies may be required in the same patient
* Current trials are focused on understanding the optimum place for immunotherapy
* Future collaborative efforts will require real-time sampling and genomic analysis coupled with rapid interpretation of actionable targets to direct patients to appropriate adaptive clinical trials

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**Figure legends**

Figure 1: Model for the stepwise progression from normal squamous epithelium to invasive squamous carcinoma.

Figure 2: Model of the genetic events leading to oesophageal adenocarcinoma. Multiple clones accumulate mutations in tumour supressive driver genes over time. Reproduced with permission from Gregson et al(71)

Figure 3: Subtypes of oesophagogastric malignancies shift in proportion associated with distal progress in the upper gastrointestinal tract. Reproduced wiht permission from Kim et al.(16)

Figure 4. Sub-classification of OAC based on mutational signatures and potential therapies. Adapted from Secrier et.al.(52)

Figure 5. Comparison of shared mutations and amplifications between primary and metastatic OAC (70).