Molecular landscape of esophageal cancer: implications for early detection and personalized therapy

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Esophageal cancer (EC) is one of the most lethal cancers and a public health concern worldwide, owing to late diagnosis and lack of efficient treatment. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are main histopathological subtypes of EC that show striking differences in geographical distribution, possibly due to differences in exposure to risk factors and lifestyles. ESCC and EAC are distinct diseases in terms of cell of origin, epidemiology, and molecular architecture of tumor cells. Past efforts aimed at translating potential molecular candidates into clinical practice proved to be challenging, underscoring the need for identifying novel candidates for early diagnosis and therapy of EC. Several major international efforts have brought about important advances in identifying molecular landscapes of ESCC and EAC toward understanding molecular mechanisms and critical molecular events driving the progression and pathological features of the disease. In our review, we summarize recent advances in the areas of genomics and epigenomics of ESCC and EAC, their mutational signatures and immunotherapy. We also discuss implications of recent advances in characterizing the genome and epigenome of EC for the discovery of diagnostic/prognostic biomarkers and development of new targets for personalized treatment and prevention.

Keywords: esophageal cancer; genomics; epigenomics; early detection; personalized therapy

Introduction

Esophageal cancer (EC) is a highly aggressive, lethal malignancy with over 400,000 deaths annually, representing a major public health concern worldwide. EC is classified into two main histopathological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). 1,2 Although both subtypes share poor outcomes with a 5-year overall survival rate of approximately 15%, 3 they are distinct diseases in terms of the cell of origin, incidence, epidemiology, and molecular signatures. ESCC is the predominant subtype that usually arises from squamous epithelial cells of the esophagus, whereas EAC originates from glandular cells present near the stomach and is believed to be largely related to acid exposure of the lower esophagus. 2 The incidence rates and patterns vary greatly among the two subtypes, with ESCC being more prevalent in the developing countries of Southeastern and Central Asia, Southeastern Africa, and South America, of which the Asian countries contribute to nearly 79% of the global ESCC cases. 4 EAC is a major subtype in Northern and Western Europe, Northern America, and Oceania, constituting around 46% of global
adenocarcinoma (AC) cases. A high male to female ratio is a characteristic of EC and although it is more pronounced in EAC than ESCC (4.4 in EAC versus 2.7 in ESCC), the sex ratios differ significantly by geographical regions in both the subtypes. The geographical variations in incidence rates, pattern and sex ratios are often attributed to differences in environmental and dietary factors, but the exact cause remains poorly understood.

EAC is associated with obesity, gastroesophageal reflux disease (GERD), and often arises from a premalignant condition of metaplastic epithelial cells in the lower esophagus termed Barrett's esophagus (BE). BE is a metaplastic change of the lining of the esophagus, where the normal squamous epithelium is replaced by specialized columnar epithelium. The condition is usually a complication of chronic GERD and it is one of the common risk factors of EAC. In contrast, ESCC is thought to have a more exposure-driven causality, such as tobacco consumption, alcohol intake, hot beverage drinking, and poor nutrition. Direct and prolonged exposure of the squamous epithelium of the upper digestive tract to various carcinogenic compounds is likely to modulate genetic and epigenetic makeup of exposed cells, thereby facilitating neoplastic transformation. Therefore, identification of molecular aberrations has been a major focus of modern technologies capable of genome-wide analysis with the final goal of having a deeper understanding of disease subtypes, risk factors, prognosis, and identifying molecular targets for the development of biomarkers for early diagnosis and therapy. Large-scale comprehensive molecular characterization has complemented many of these goals in several cancer types. One of several examples includes the comprehensive molecular study of 164 ECs in the Cancer Genome Atlas Research Network (TCGARN), where ESCC emerged as a disease closer to other squamous cell carcinomas (SCCs) than EAC, which better resembled the chromosomal instability subtype (CIN) gastric cancer subtypes. This questioned the premise of envisioning esophageal carcinoma as a single entity and combining EAC and ESCC for clinical trials of various therapeutic regimens.

Therefore, in the era of emerging dynamics of technological advancements and their application to cancer care, this review focuses on the genomic and epigenomic landscape of EC that provides insights into the mechanism of carcinogenesis and the translational discoveries targeting such molecular characteristics.

Genomics, mutations, and deregulated pathways

Genomics of EC

The last 7 years have seen the emergence of several studies reporting on the genomics of EC based on approaches such as whole genome sequencing (WGS), whole exome sequencing (WES), chromosomal analysis, RNA copy analysis, and methylation status. These studies provide a better understanding of both EAC and ESCC, with potential application for future therapeutic opportunities.

Separating EAC and ESCC. Compelling evidence based on recent genomic analysis shows that EAC and ESCC are different cancer entities. Although TP53 is the most commonly altered gene in both EAC and ESCC (with alterations observed in > 80% of all samples analyzed), there is consensus that the profile of genomic alterations in EAC and ESCC vary considerably. In EAC, the genes that are altered more frequently than in ESCC include ERBB2, KRAS, EGFR, SMAD4, FGFR3/4/19, VEGFA, CCNE1, and GATA4/6, whereas PIK3CA, CCND1, PTEN, NFE2L2, NOTCH1, MLL2, SOX2, FGFR1, and RB1 are altered more frequently in ESCC.

TCGARN recently published an elegant study that used genomic analysis to characterize tumors derived from various locations in the esophagus and stomach, largely to better separate EAC and gastric ACs. Their study shows that EAC is more closely aligned with gastric cancers—more specifically, gastric cancer with chromosomal instability (GC-CIS), the largest subtype of gastric cancer. Based on an analysis of 90 ESCC, the authors categorized ESCC into three molecular subtypes—ESCC1 (50/90), ESCC2 (36/90), and ESCC3 (4/90), where ESCC1 most closely resembled the molecular profile of the classical subtypes of squamous carcinomas of the lung and head and neck cancers and not EAC.

Studies with a genome-wide approach also supported the TCGARN findings with a larger sample size showing contrasting differences between ESCC and EAC both at genomic and epigenomic levels, and revealed novel molecular features for further delineating these cancers. They also reported two significantly
mutated genes \textit{CUL3} and \textit{ZFP36L2}, which were functionally validated as important tumor suppressors specific to the ESCC subtype.\cite{24} In a separate study, WES of ESCC and EAC exhibited substantial disparity in the spectrum of mutations, with more indels and NOTCH1 loss-of-function mutations specific to ESCCs. They also identified the overlapping mutations between EACs and matched BE.\cite{25} Another study on WES of ESCC re-established the fact that ESCC closely resembled those of SCCs of other tissues but differed from that of EAC. This study found EP300 mutations were associated with poor survival and Hippo and Notch pathways, and epigenetic modulators were frequently deregulated by mutations that could have prognostic and potential therapeutic implications.\cite{26}

EAC. Genomic analysis performed on EAC samples provide interesting clues about the tumorigenic processes in EAC and future developments in this field. In a study that analyzed 149 tumor samples, Dulak et al.\cite{27} generated a list of 26 significantly mutated genes that include \textit{TP53}, \textit{CDKN2A}, \textit{SMAD4}, and \textit{PIK3CA}, \textit{ELMO1}, and \textit{DOCK2} (mediators of \textit{RAC1}) were also significantly altered in EAC (17\% samples tested), implicating \textit{RAC1} mediated motility pathways in EAC tumorigenesis. A later report suggested that many of the putative driver mutations such as \textit{SMAD2}, \textit{TLI}, \textit{TLR4}, and \textit{DOCK2} probably appear later in the evolution of the tumor.\cite{28} Their evidence suggests a considerable level of intratumor heterogeneity in EAC, with chromosomal instability and associated genome doubling constituting a defining characteristic of EAC. Genome instability is proposed to occur as an early event in EAC tumorigenesis.\cite{19, 28} Except for \textit{TP53}, very few genes are altered by point mutations in multiple EAC tumor samples analyzed—most gene alterations occurred as a result of chromosomal instability resulting in gene loss or duplication events.\cite{29} There is an intriguing suggestion that it is the heterogeneity in EAC, manifested as the amplification of multiple receptor tyrosine kinases (RTKs) expressing genes and genes involved in downstream mitogenic pathways that may be responsible for the poor response of EACs to drugs targeting isolated RTKs and mitogenic pathways. As a potential solution to this problem, it is proposed that the dominant mutational profile of EAC patients should be determined, and that the patients then be stratified into one of three groups for targeting with specific therapeutic interventions.\cite{29} Challenges associated with targeted therapies of EAC identified extensive differences in genomic alterations, including discrepancies in potentially clinically relevant alterations by multiregion sequencing.\cite{30} Moreover, profiling paired primary and metastatic tumors, and cell-free circulating DNA (cfDNA) from patients, suggested the potential for cfDNA to enhance the selection of therapy.\cite{30} A study on EAC with multiple WES before and after platinum-containing neoadjuvant chemotherapy (NAC) revealed the presence of a platinum signature with enrichment of C>A mutations within a CpC context following NAC. The study also suggests early chromosomal instability leading to amplifications of oncogenes that persist through chemotherapy and could be potentially significant targets for future therapeutic approaches.\cite{28}

ESCC. ESCC occurs with a very high frequency in several specific geographical regions, with Asian countries contributing nearly 79\% of global ESCC cases, so it is not surprising that many of the genomic studies on ESCC have been conducted in China. A recent review article highlighted WES of ESCCs that revealed many crucial driver mutations and also mentioned WES for elucidating inter- and intratumor heterogeneity. This article also discusses the epigenetic alterations in ESCC emphasizing a major challenge of understanding epigenetic mechanisms contributing to carcinogenesis.\cite{31} A couple of studies summarized and re-analyzed the genomic data from several recent genomic studies of large cohorts of ESCC patients.\cite{32, 33} The re-analysis by Du et al. identified recurrent mutations in approximately 18 genes, 15 of which had been reported previously (\textit{TP53}, \textit{AJUBA}, \textit{CDKN2A}, \textit{KMT2D} (\textit{MLL2}), \textit{ZNF750}, \textit{FAT1}, \textit{NOTCH1}, \textit{NOTCH3}, \textit{PIK3CA}, \textit{NFE2L2}, \textit{RB1}, \textit{KDM6A}, \textit{FBXW7}, \textit{CREBBP}, and \textit{TGFB2}), with three significantly mutated novel genes (\textit{CUL3}, \textit{PTEN}, and \textit{DCDC1}).\cite{33} Furthermore, a recent study reported 26 significantly mutated genes, including eight novel (\textit{NAV3}, \textit{TENM3}, \textit{PTCH1}, \textit{TGFB2}, \textit{RIPK4}, \textit{PBRM1}, \textit{USP8}, and \textit{BAP1}) and 18 that have been previously reported. They also identified \textit{TENM3} mutations and the \textit{TP53} hotspot mutation p.R213* are independent prognosticators for poor survival in ESCC.\cite{34} Pathway analysis indicated that these alterations affected cell cycle and apoptosis, PI3Kinase signaling, histone
modification, the p53 signaling pathway, the NOTCH and WNT pathways, and Hedgehog signaling, thus, identifying potential therapeutic targets for ESCC. Another study also identified high activity of hedgehog signaling and the PI3K pathway in approximately 60% of 104 ESCC tumors suggesting therapies targeting these pathways might be particularly promising strategies for ESCC.\textsuperscript{35} Also, WES and targeted deep sequencing of more than 300 ESCC cases identified previously uncharacterized mutated genes such as FAT1, FAT2, ZNF750, and KMT2D in addition to those already known such as TP53, PIK3CA, and NOTCH1. This study also identified drug target candidates such as XPO1 that was further explored as a therapeutic target because it exhibited both gene mutation and protein overexpression.\textsuperscript{36}

The genomic analysis also identified copy number alterations in many of the cohorts, and Du et al. show that this approach can be used to stratify ESCC into three subtypes, with subtype 3 having the highest copy number alterations and the poorest prognosis.\textsuperscript{33} They also observed significant differences in the expression profile of specific genes between subtypes; for example, a high frequency of PIK3CA amplification in subtype 3 (72%) compared to subtype 2 (16%), suggesting the targeted application of therapeutic interventions for specific subtypes (in this case a PI3K inhibitor for subtype 3 ESCC). Additional studies with WGS and WES of ESCC identified multiple cancer-related genes with frequent mutations. Among these mutations in VANG1\textit{L1} was found to be associated with accelerated growth and miR-4707-5p and MYBL2 were associated with somatic copy-number alterations (SCNAS), cell proliferation, and metastasis.\textsuperscript{37} In a separate study, WGS of ESCC and gastric cancer revealed mutations in certain common cancer-related genes such as TP53, JAK3, BRCA2, FGF2, FBXW7, MSH3, PITCH, NFI, ERBB2, and CHEK2, in addition to potentially novel cancer-associated genes KISS1R, AMH, MNX1, WNK2, and PRKIR.\textsuperscript{36,38}

Different geographic populations may display different genomic alterations, with mutations detected in AJUBA, ZNF750, FAT1, and FBXW7 in a cohort of ESCC patients drawn from a high-risk region in northern China that were not detected in ESCC patients drawn from a high-risk region in southern China.\textsuperscript{35,39} Another WES study also highlighted the population-specific variation in genomic alterations with key genetic differences between the American and Chinese ESCCs.\textsuperscript{25} Zhang et al. link this to the exposure to different risk factors in southern China where epidemiological studies independently associate ESCC with chewing fermented areca nut, whereas in northern China ESCC was linked to consumption of hot food and N-nitroso compounds, in addition to the other common risk factors.\textsuperscript{35} Evidence produced by TCGAR supports the contention that different populations may display slightly different mutation profiles.\textsuperscript{19} Furthermore, in the only large-scale genomic analysis performed on ESCC subjects from sub-Saharan Africa (59 ESCC cases from Malawi), the authors were unable to show the typical mutational signature associated with tobacco smoking, but identified an unusual mutational signature previously observed in a small number of oropharyngeal squamous carcinoma cases.\textsuperscript{40} These observations underscore the need to perform a more detailed genomic analysis of ESCC cases located in those regions that have been poorly sampled.

A recent study showed that ESCC displays the highest level of intratumor heterogeneity (ca. 90%), compared to other cancers, including EAC (56%), high-grade serous ovarian cancers (52%), and clear cell renal carcinoma (~35%).\textsuperscript{41} A couple of other studies analyzed intratumor heterogeneity with multiregion WES of ESCC tissues identified more than one-third of heterogeneous somatic mutations and clonal driver mutations in tumor-suppressor genes such as TP53, KMT2D, and ZNF750.\textsuperscript{42,43} Considering that a higher intratumor heterogeneity in EAC has been associated with poor responses to NAC, the high intratumor heterogeneity in ESCC, may in part, explain the poor overall 5-year survival rates and adopting multiple targeted therapies in a combinatorial approach may be more effective.\textsuperscript{28,41}

Mutational signature and response to treatment in EAC

EAC is a solid malignancy with the fastest rise in the incidence rate in the last four decades.\textsuperscript{44} Unfortunately, recent refinements of oncological protocols and surgical management have failed to improve patient outcomes. Similarly to other types of cancers, EAC is molecularly heterogeneous; however, unlike breast, pancreatic, and colon cancers, where specific subtypes with therapeutic implications have
has been characterized, we have a poor understanding of the clinical significance of EAC heterogeneity. Hence, the key clinical questions are: (1) How can we classify EAC in a clinically useful way? (2) How can we improve response to conventional oncological therapies? (3) How can we learn from the genomic diversity of EAC to tailor targeted therapy?

EAC is one of the cancers with the highest mutation frequency, second only to melanoma and lung cancer, which are linked to exposures to known mutagens.27 This suggests that the noxious effect of acid reflux has a role in the acquisition of a high mutational burden. On the other hand, sequencing of multiple regions from the same tumor demonstrated that EAC is characterized by high level of spatial and temporal heterogeneity, with up to 47% of putative driver mutations occurring in phylogenetic branches.28 This constitutes a potential problem when trying to target individual mutations for patient-tailored therapies. Currently, a standard treatment for EAC is NAC (±radiotherapy) followed by surgery, with or without adjuvant chemotherapy. The rate of complete pathological response to neoadjuvant treatment ranges from 0% to 23% depending on the regimen adopted.45–47

So far, among the few dozens of targeted agents trialled in the EAC, only two agents have been approved for treatment in the metastatic setting, the anti-HER2 monoclonal antibody trastuzumab and ramucirumab, a recombinant antibody that binds vascular endothelial growth factor receptor 2 (VEGFR-2).48 Despite positive oncological trials, an average survival advantage from the addition of these agents to standard palliative chemotherapy is 2 months, with substantially increased toxicity.49,50 This calls for a novel approach to usefully classify EAC in order to inform therapeutic management.

As cancer develops through DNA damage from exogenous (e.g., smoking, acid reflux) and endogenous processes (e.g., DNA repair defects), retracing its etiology can unveil distinct mechanisms of tumor formation and potentially clinically relevant features. Evidence for such carcinogenic processes acting throughout the lifetime of the cancer can be reconstructed from whole-genome/exome sequencing data by linking context-based nucleotide substitution patterns (termed mutational signatures) with previously reported risk factors. Dulak et al.27 were the first to describe a pattern of frequent A>C transversions at AA dinucleotides (S17 signature) in EAC. Recently, WGS on 129 EAC cases uncovered three subtypes with distinct etiology based on the prevalence of individual mutational signatures (Fig. 1). These subgroups are (1) mutagenic, characterized by a S17-dominant signature, high mutational and neoantigen burden; (2) DNA damage repair (DDR)-impaired, with frequent defects in the homologous recombination machinery; and (3) a subgroup with a C>A/T dominant base substitution landscape linked primarily to aging.29

Limited experimental validation to date suggests that this genomic classification may inform treatment options in addition to the standard of care (Fig. 1). The DDR-impaired subgroup may be amenable to PARP inhibitors and other drugs targeting this pathway, while the mutagenic patients are possible candidates for immunotherapy or Wee1/Chk1 inhibitors. RTKs were found frequently coamplified in EAC (potentially explaining the low success rate of RTK monotherapies) and these events had a trend of higher prevalence in the C>A/T dominant subgroup.29 Combinations of RTK inhibitors could therefore be an option for these patients, who are also more likely to show better responses with the current standard of care.

While these are likely to be the dominant mutational exposure groups in EAC, a larger scale analysis will be better powered to uncover additional subtypes, and may change the relative prevalence of current signatures. As suggested by other studies,51,52 additional signatures related to smoking or to the activity of the cytosine deaminase apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) may prove central to the etiology of EAC (Fig. 1).

Undoubtedly, more research is needed before transitioning a mutational signature-based classification into a routine clinical practice. However, with the upcoming developments in shallow genome sequencing and with its advantage of being unbiased by tumor heterogeneity, this technique could prove valuable in the longer term and shows promise for a more accurate, holistic classification of EAC for therapeutic action.

Deregulated pathways and therapeutic significance in EC

Although ECs have been grouped together based on their anatomic location, it is now clear that
ESCC and EAC are distinct pathologies. This is a consequence not only of their cell of origin and associated risk factors, but also of the molecular alterations each histological subtype harbors, which has a direct impact on treatment response and eligibility. While altered pathways bring ESCC together with other squamous carcinomas, EAC closely resembles gastric ACs. Therefore, these marked differences should be taken into account when treating EC patients.

Cell cycle regulation is probably the most important disrupted pathway that brings ESCC and EAC together, even though some players and/or molecular alterations differ. As mentioned earlier, TP53 is the most frequently mutated gene in both cases, reaching a frequency of 91% in ESCC and 71% in EAC. The loss-of-function of the kinase inhibitor CDKN2A occurs at similar rates (approximately 80%), although through different mechanisms, with deletions being more common in ESCC and epigenetic silencing affecting EAC cases. Gene amplifications are also associated with the disruption of the cell cycle in these tumors. CCND1 is more commonly affected in ESCC than in EAC (57% and 15%, respectively), while CCNE1 gain-of-function occurs more often in EAC (14% versus 4% in ESCC). Put together, these molecular alterations result in the disruption of the pathway in about 90% of EC cases—numbers that could even be underestimated since other regulatory mechanisms have not been considered in this analysis. It has been shown, for example, that the downregulation of miR-503 and miR-200b and the consequent upregulation of their targets, CCND1 and CDK2, respectively, take place in ESCC. Therefore, these alterations point to a potential benefit of inhibitors of cell cycle kinases, especially cyclin-dependent kinases, in the treatment of EC patients. Despite such promise, only two clinical trials, phase 1 and 2 studies have been carried out with Flavopiridol, the first CDK inhibitor in

Figure 1. Mutational signatures in EAC with potential therapeutic implications. Well-studied and emerging mutational signatures associated with various risk factors in EAC are highlighted, along with potential treatment options, which could be tailored to the biology of the respective signature. Only the “stem” of the signature is depicted, indicating the main nucleotides and context that are mutated in the respective signature. The likely associated risk factors for each signature are indicated with an asterisk in gray (with a question mark for the cases where the association has not been clearly proven but is likely). Left panel: The most prevalent mutational signatures identified in EAC patients to date, and the corresponding subtype previously categorized in the literature. Right panel: Mutational signatures previously reported in EAC for which the prevalence and relevance to therapy need to be confirmed in larger studies.
human clinical trials to treat esophageal neoplasms (ClinicalTrials.gov accessed in February 2018).

On the other hand, a number of clinical trials involving targeted therapy against RTKs have been carried out in EC. Although alterations of these pathway activators are not as common as cell cycle disruption, drugs targeting EGFR, HER2, and VEGFR have been approved or currently under trials for EAC treatment.\(^{55}\) This is a good example of how differences between ESCC and EAC should be taken into account when choosing the appropriate treatment. It has recently been shown that HER2 and VEGFR amplification or mutations may affect up to 32% and 28% of EAC cases, respectively, while for ESCC these numbers are below 5%.\(^{18}\) The same study showed a similar frequency of EGFR alterations for both tumors (15–20%), but it had been previously shown that a high EGFR protein expression affects only 4% of ESCC cases.\(^{56}\) Therefore, although EAC patients may benefit from targeted therapy against these receptors, ESCC patients do not seem to be eligible for such therapies. An alternative would be targeting other RTKs, such as c-MET. It has been shown that c-MET protein overexpression affects 43–69% of ESCC patients and is usually associated with a poor prognosis.\(^{57–60}\) Although no clinical trials have been carried out with c-MET inhibitors in ESCC, in vitro results have shown that they are capable of inhibiting the invasive capacity and radiosensitizing ESCC cell lines by prompting apoptosis and G2/M arrest.\(^{57,61}\)

Going downstream on the pathways activated by RTKs, KRAS, and BRAF alterations do not seem to be common in ESCC, while mutations or amplification in KRAS are observed in 14% of EAC.\(^{19,56}\) Besides, PIK3CA activating mutations are observed in 13% of ESCC and PTEN inactivation affects 9% of the cases.\(^{19}\) Although these numbers may seem low, the pathway could be activated by other mechanisms such as gene overexpression. Upregulation of PIK3R3, a regulatory subunit that forms heterodimers with PIK3CA, leads to the activation of the PI3K/AKT pathway in ESCC and may be an independent prognostic biomarker (unpublished results). Currently, several drugs inhibiting this pathway are in development and several clinical trials are ongoing for different types of solid tumors.\(^{62}\) Although the first results using these drugs as monotherapy were disappointing, the combination with drugs targeting other oncogenic pathways to counteract feedback mechanisms seems to be promising. For ESCC, resistance to PI3Ka inhibition is suggested to be mediated by AXL upregulation. When overexpressed, AXL interacts with EGFR leading to a PI3K-independent mTOR activation, which is mediated by PKC. As a consequence, inhibition of any of these players, AXL, EGFR, or mTOR, was capable of reversing resistance to PI3Ka inhibition.\(^{63}\)

Another pathway often shown to be activated in ESCC is the Wnt signaling. While in colorectal tumors this pathway is most commonly deregulated by genetic alterations, with APC mutations being detected in 70% of all cases, in ESCC the upregulation of Wnt activators and/or downregulation of inhibitors of the pathway seem to be the mechanisms of activation.\(^{19,39,64,65}\) This pathway is of special interest not only because of the high frequency of alterations found in ESCC (up to 86% of the cases\(^{39}\)), but also because of its intimate association with cell stemness. It has been shown that the stimulation of the pathway using soluble factors in Oct4/Sox2/Klf4-transfected cells is capable of reprogramming somatic cells to pluripotency.\(^{56}\) In ESCC, WNT10A overexpression was shown to promote migration, invasion and proliferation of transformed esophageal cells, and to induce a greater population of putative cancer stem cells.\(^{67}\) Therefore, targeting this pathway for therapy may result in the reduction of both bulky tumor cells and cancer stem cells population, a major cause of tumor recurrence, progression and resistance to therapy. Currently, two phase 1 clinical trials in EC involving Wnt modulators are recruiting participants (ClinicalTrials.gov accessed in February 2018), although much remains to be learned.

Oxidative stress and mutational signatures induced by APOBEC activity represent other pathways more commonly affected in ESCC than in EAC. In fact, alterations of these pathways distinguish molecular ESCC subtypes according to one of the most comprehensive genomic and epigenomic studies carried out so far for this tumor type.\(^{19}\) The subtype ESCC1 has been characterized by alterations of oxidative stress machinery in up to 54% of the cases, with NFE2L2 being affected by amplification or nonsense mutations in 30% of the tumors. NFE2L2 encodes a transcription factor that regulates the expression of many detoxifying and antioxidant genes and its gain of
function has been associated with resistance to stressors, including chemoradiotherapy.\textsuperscript{68–70} The presence of \textit{NFE2L2} mutation was associated with tumor recurrence and poor prognosis in ESCC and the mutant \textit{NFE2L2} confers resistance to 5-fluorouracil (5-FU) and \textgamma-irradiation in \textit{in vitro} models.\textsuperscript{69} Based on these findings, mutations of this stress sensor could be a useful tool in predicting response to therapy of ESCC patients and targeting the activated protein could represent a new treatment approach for this neoplasm.

Finally, the molecular subtype ESCC2 has been characterized, among other alterations, by a high APOBEC mutational signature fraction.\textsuperscript{19} This signature is characterized by substitutions of cytosine by thymine or guanine in \textit{TpC} context and has been shown to be highly prevalent in ESCC by different authors.\textsuperscript{70,71} The activation of AID/APOBEC enzymes and their mutagenic potential are also related to intratumoral heterogeneity.\textsuperscript{72} This is of special clinical interest because it has already been shown that the degree of intratumoral heterogeneity may affect patient’s prognosis and response to treatment. In early stages of nonsmall-cell lung carcinoma, a greater number of subclones were observed in patients that showed relapse in comparison with those free of disease.\textsuperscript{73} Therefore, different authors have proposed the inhibition of APOBECs as a new therapeutic approach. Another possibility is the use of the already available inhibitors of DNA repair, such as PARP inhibitors, with the intent of inducing a level of hypermutability that would not be compatible with cell viability.\textsuperscript{74}

\textbf{Epigenomics of EC}

During tumor development, cells undergo several genetic and epigenetic alterations that mutually contribute to tumorigenesis. These molecular alterations such as large-scale DNA alterations, mutations, methylation, and RNA or protein expressions might complement histological analysis to improve accuracy and can evolve as more useful biomarkers. In addition to the large number of DNA sequence changes found in EC, epigenetic changes play prominent roles in EC pathogenesis. One of the common molecular mechanisms that mediate epigenetic phenomena is DNA methylation, where methyl groups are added to the DNA sequences. This methylation primarily occurs on cytosine bases in tracts of cytosine–guanine (CPG) dinucleotides, where hypermethylation of these CpG islands on the promoter regions of genes results in transcriptional silencing, while hypomethylation results in increased transcription. The identification of specific DNA methylation sites could not only provide significant biological insights into the development and progression of cancer but also discover novel biomarkers for early detection, prognosis and novel therapeutic targets of cancer.

\textbf{Exposure-specific DNA methylation change in EC}

The fact that alterations in DNA methylation affect gene expression and can be influenced by environmental factors makes it the marker of choice to study the causal associations of such factors with disease risk. There is an overall paucity of environment epigenetic interaction studies in ECs; the few studies conducted either had modest numbers of samples or only included a few genes.\textsuperscript{75} Alcohol consumption is a major risk factor of ESCC.\textsuperscript{76} One of the mechanisms of alcohol-induced carcinogenesis may be the inhibition of DNA methylation.\textsuperscript{77} Hypo- and hypermethylation of genes are among the most important mechanisms of transcription regulation.\textsuperscript{78} Alcohol inhibits S-adenosyl-methionine (SAM) synthesis, a universal methyl group donor and enzyme activator in methyl transfer reactions.\textsuperscript{79} In hepatic cells, alcohol-mediated inhibition of SAM synthesis was found to cause global DNA hypomethylation, resulting in the upregulation of oncogenes and downregulation of tumor-suppressor genes.\textsuperscript{80} Although such alcohol-induced global hypomethylation was not elucidated in esophageal tumors, LINE-1 hypomethylation, a surrogate marker of global hypomethylation, was suggested to be an important event during ESCC carcinogenesis.\textsuperscript{81–83} ESCC has also been associated with exposure to nitrosamines,\textsuperscript{84} which leads to alkyl-related DNA damage that is normally repaired by enzymes such as O(6)-methylguanine DNA methyltransferase (MGMT).\textsuperscript{85} Therefore, inactivation of MGMT by aberrant DNA methylation might favor the progression of esophageal squamous epithelium to ESCC. In fact, aberrantly methylated MGMT has been shown in 33–39\% of ESCC cases, and can be associated with a reduction in MGMT protein levels.\textsuperscript{86,87} Certain tobacco-derived carcinogens, such as nicotine-derived nitrosamine ketone (NNK) and benzo[a]pyrene, were found to be
capable of modulating DNA methylation in cultures, animal models, as well as some tobacco-related cancers.\textsuperscript{88–90} NNK could induce RARB promoter hypermethylation through upregulation of DNMT1 in esophageal squamous epithelial cells, resulting in enhancement of cell proliferation and inhibition of apoptosis.\textsuperscript{90} Nicotine induced the methylation of FHIT and attenuated FHIT protein in association with increased expression of DNMT3a in human esophageal squamous epithelial cells, a process important in early carcinogenesis.\textsuperscript{91} Alterations in DNA methylation is a frequent event in the formation of BE, and its progression to EAC.\textsuperscript{92–94} An epigenome-wide study identified several differentially methylated functional genes mapping to meaningful pathways associated with obesity and tobacco smoking that influence the risk of developing BE/EAC.\textsuperscript{95} Immortalized esophageal epithelia induced with cigarette smoke were found to contribute to the pathogenesis of EAC by epigenetic repression of miR-217 via upregulation of KLK7.\textsuperscript{96} Therefore, environmental exposures might affect the development of BE and EAC through influencing the epigenetic status of specific loci that have a biologically plausible role in neoplastic transformation.

**Tumor-specific DNA methylation**

**Tumor-specific DNA methylation in EAC.** Although global methylation studies are sparse in EAC, earliest genome-wide studies aimed at understanding the role of aberrant methylation in malignant transformation of BE to EAC found considerable differences between the DNA methylation patterns in normal esophageal tissues, BE and EAC, with BE being more representative of the DNA methylation patterns found in tumors.\textsuperscript{97,98} The study not only confirmed several of the previously reported hypermethylated genes but also identified a large number of novel hypermethylated genes in BE and EAC tissues, particularly genes encoding disintegrin and metalloproteinase (ADAM) peptidase proteins, cadherins and proteocadherins, and potassium voltage-gated channels. Moreover, close clustering of BE and EAC tissues suggested key methylation events to occur early during the progression of EAC.\textsuperscript{97} Another study determining the methylation landscape of EAC and its impact on gene expression identified distinct methylation patterns pertaining to subtypes of EAC, one similar to the CpG island methylator phenotype that was potentially associated with a worse clinical outcome.\textsuperscript{99} Apart from widespread hypermethylation of genes, global hypomethylation was found to be an early event in EAC development, even observed within the first visible metaplastic lesions of the squamous esophagus.\textsuperscript{100,101} Hypomethylation has been hypothesized to lead to carcinogenesis by encouraging genomic instability, aberrant activation of oncogenes, or transcriptional upregulation during multistep progression to high-grade dysplasia and cancer.\textsuperscript{100,102,103} These studies provided new insights into the contribution of epigenetics to EAC carcinogenesis and clinical outcome.

**Tumor-specific DNA methylation in ESCC.** DNA methylation occurs in several key components of cancer-related signaling pathways. It affects the genes involved in cell cycle, DDR, Wnt, TGF-β, and NF-κB signaling pathways, including PI6, MGMT, SFRP2, DACH1, and ZNF382.\textsuperscript{104} One of the preliminary high-throughput DNA methylation profiling arrays was Illumina GoldenGate\textsuperscript{™} Assay for Methylation consisting of more than 1500 CpG sites spanning 800 genes. The first study to address methylation changes in ESCC in a large set of genes conducted using the technique on around 10 ESCC tumor versus adjacent normal tissues identified 37 differentially methylated CpG sites, including genes involved in IL-10 anti-inflammatory signaling and cell communication. Moreover, methylation of TFF1 was identified as a potential early marker for ESCC in this study.\textsuperscript{105} A subsequent study interrogating approximately 450,000 CpG sites on a small set of samples comparing ESCC tissues paired normal surrounding tissues and normal mucosa from healthy individuals; it identified 168 genes with differentially methylated promoter CpG and a gene expression pattern inverse to the direction of change in DNA methylation involved in several cancer-related pathways.\textsuperscript{106} High-throughput sequencing techniques such as methylated DNA immunoprecipitation sequencing (MeDIP-Seq) and RNA-Seq were also used to investigate whole-genome DNA methylation patterns and the genome expression profiles in ESCC samples. The study mapped differentially methylated regions to cell cycle, adhesion, proliferation, and apoptosis pathways. Expression levels of several genes like MLH1, TWIST1, and CDX1 were consistent with their DNA
methylolation profiles. The identification of whole-genome DNA methylation patterns in ESCC provides new insight into the carcinogenesis of ESCC and might prove a promising avenue to investigate novel biomarkers, prognostic and therapeutic targets.

**Epigenetic biomarkers in EC**

In recent years, many studies have identified cancer-specific epigenetic alterations for exploring them as cancer biomarkers for diagnosis and/or prognosis. This has a great significance because of its potential clinical implication and improving overall patient outcome. DNA methylation signatures can also be used to determine positive and negative prognoses and provide the possibility to identify relatively indolent or aggressive tumors. This may help in decision making regarding the selection of more aggressive or less aggressive treatment and monitoring of the case.

Targeted promotor methylation detection revealed a set of DNA repair genes hMLH1, hMSH2, and MGMT that are frequently methylated in ESCC holding promise as a potential predictive factor in primary cases. Another recent study using Illumina Infinium HumanMethylation450 BeadChip array suggested that HOXB2 and SEPT9 may be useful epigenetic biomarkers for the prediction of the presence of lymph node metastasis in ESCC. Cell-free DNA (cfDNA) released from dying cells is emerging as a diagnostic tool for investigating cancer-associated dynamics, providing it as a minimally invasive technique for diagnosis and monitoring of patients. Investigation of genome-wide cfDNA methylation profiles was found to be highly consistent with DNA methylation profiles detected in corresponding tumor tissues. This supports the utility of differential cfDNA methylation profiling as a useful approach for the noninvasive screening of EAC.

**Future perspectives**

**Immuno-oncology in ECs: new promising strategies and therapeutic options**

Cancer immunotherapy is a major scientific and medical breakthrough, currently driven by the clinical development of monoclonal antibodies that release cellular brakes on T cells, like the inhibitors of the cytotoxic T lymphocyte–associated antigen (CTLA-4) or the programmed cell death protein 1 (PD-1) and its ligand (PD-L1). The use of these immune checkpoint inhibitors (ICIs) institutes a new therapy for gastrointestinal (GI) malignancies after the recent FDA approvals of PD-1 inhibitors for microsatellite instable (MSI) tumors. Since the first ICIs were approved, clinical research also evaluates other targets within the “cancer immunity cycle” and investigating ICIs in combination with numerous other established or novel drugs. The first clinical evidence of phase II trials and phase I expansion cohorts at the end of 2017 is available for atezolizumab, avelumab, durvalumab, nivolumab, and pembrolizumab.

Results from two single-arm trials with 64 patients with nivolumab and 23 patients with pembrolizumab are reported. Doi et al. included only PD-L1 positive patients (PD-L1 cutoff value ≥1%), 74% of them with SCC; patients with AC histology from the distal esophagus and patients with gastroesophageal junction (GEJ) tumors were enrolled as well (Fig. 2).

The reported objective response rate (ORR) of 30% (28% for SCC, 40% for AC) in this patient cohort (87% had received ≥2 prior therapies) was in the range of the 17% reported by Kudo et al. in a PD-L1 all-comer SCC cohort of Japanese patients who received nivolumab after a median of three prior therapies. Similarly, median duration of response was 11.2 and 9.3 months in the nivolumab and pembrolizumab trials, respectively, in which only one half of patients originated from Asia. Treatment-related adverse events of grade ≥3 were detected only in 17% of patients with no treatment-related deaths.

Several ICI phase III in EC are ongoing with the first results expected in 2018. For unresectable advanced or recurrent EC in patients that failed standard chemotherapy (CTx), confirmative trials testing the use of nivolumab or pembrolizumab as monotherapy in second-line patients are directly competing. The progression-free survival and overall survival (OS) are also used as co-primary end points in the phase-III trials of pembrolizumab in the second-line (KeyNote-181) and in the first-line KeyNote-590 (NCT03189719) studies.

Nivolumab is investigated in two large phase II trials in the first-line as well as in the adjuvant settings. The first-line trial CheckMate-648 (NCT03143153)
adds nivolumab to cisplatin plus fluoropyrimidine standard regimen and compares—the like the respective pembrolizumab KeyNote-590 trial—the experimental regimen against the standard regimen, but investigates in a second experimental arm the activity of combined immune checkpoint blockade with nivolumab plus CTLA-4 antagonist ipilimumab. The CheckMate-577 (NCT02743494) phase III trial is testing the adjuvant use of nivolumab in patients with resected esophageal or GEJ cancer is already recruiting patients, the first results for the primary outcomes measure are expected after September 2020. The first neoadjuvant trials of ICI are ongoing, too: an investigator-sponsored U.S. trial (IST) (NCT02998268) compares the concomitant versus sequential use of pembrolizumab as part of an induction chemoradiation regimen prior to surgery in patients with the locally advanced EAC, followed by the adjuvant use of this ICI monotherapy after surgery. The addition of an ICI to chemoradiation prior to surgery will be tested in two phase-I trials of anti-PD-L1 durvalumab as well (NCT02962063; NCT02735239).

So far, the impact of biomarker to predict response to ICI in the esophageal population is still difficult to assess.\textsuperscript{120} No comparative biomarker analyses are available from different company studies (Attraction-01 and KeyNote-028). The interpretation of present clinical data remains difficult: For heavily pretreated patients with SCC histology that received nivolumab, a promising median OS of 10.8 months is reported in the all-comer population (PD-L1 $\geq$).\textsuperscript{119} The old benchmark to interpret these ICI efficacy data are the median OS of 7.6 months for previously CTx-untreated SCC patients receiving cisplatin + 5-FU.\textsuperscript{121} For patients progressing after primary CTx, a standard given the median OS of $\sim$4 months in a phase III trial comparing gefitinib after CTx with best supportive care, with mixed AC/SCC histology population for both treatment arms.\textsuperscript{122}

In 2017, the important approvals of ICI for use in GI cancers were granted namely for: (1) PD-1 inhibitors nivolumab (in the United States for metastatic mismatch repair deficient (dMMR) or microsatellite-instable-high (MSI-H) colorectal carcinoma, and in Japan for gastric cancer), and (2) pembrolizumab (in the United States for use in gastric, and for dMMR/MSI-H tumors including colorectal cancer). The complex interactions between
cancer and the immune system at the individual level demand additional new therapeutic strategies.\textsuperscript{123}

Hence, biomarker development and refinement, also in relation to the role of high tumor mutational burden as a potential predictive and prognostic marker, constitutes another research priority\textsuperscript{124} to improve the outcome of PD-1 inhibition. The use of ICI in tumors with higher mutational load was already associated with improved OS,\textsuperscript{125} providing the rationale for their use in upper GI cancers. Comprehensive phase III programs have been initiated in esophageal and gastric cancer.

**Implications for improving treatment and prognosis**

In this review, we have primarily focused on the molecular landscape of EC and recent advances in the field of genomics and epigenomics that have provided valuable insights into the genes and pathways deregulated by genetic and epigenetic changes and suggested potential mechanisms of the development and progression of EC. These current progresses hold great potential for molecular subtyping of the cancer, identification, and development of biomarker panels for early detection, screening, risk stratification, cancer prevention, and treatment of both ESCC and EAC. While recent progress suggested a high potential of comprehensive multiomics data for molecular subtyping of ESCC, their limitation lay in the fact that the cases originate from the regions with moderate or lower ESCC incidence. Therefore, these findings are important to be replicated, including in high incidence populations of Asia (China, Bangladesh, India, Iran), Southeastern Africa, and South America (especially Brazil).\textsuperscript{4}

Identification of certain molecular alterations in esophageal tumors could provide targets for existing targeted therapies. For instance, one-third of the EACs with alterations in the gene \textit{ERBB2} (also called \textit{HER2}) might be good candidates for the drug trastuzumab (Herceptin\textsuperscript{TM}), which blocks the extracellular part of the transmembrane receptor protein product of this gene.\textsuperscript{126,127} However, several studies also recommend that ESCC and EAC should be separated in the clinical setting so that each can be targeted according to its specific genomic features.\textsuperscript{2,128}

Molecular characterization of premalignant esophageal lesions and healthy normal cells from esophageal lining could help to determine early molecular deregulation driving cancer development and progression. The Cytosponge\textsuperscript{TM} device-based esophageal sample collection is less invasive, which provides an opportunity to collect esophageal cells from patients with premalignant lesions and normal cells of esophageal lining of healthy individuals without the need to

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*Figure 3. Role of molecular characterization in addressing major challenges of esophageal cancer.*
Molecular landscape of esophageal cancer

Table 1. Advances in noninvasive esophageal cell sampling devices for detection of esophageal abnormalities using molecular markers

| Techniques                 | Utility                                      | Molecular markers                                      | Additional markers                                      | References |
|----------------------------|----------------------------------------------|--------------------------------------------------------|---------------------------------------------------------|------------|
| Nonendoscopic balloon sampling | Detection of Barrett’s esophagus             | Two DNA methylation markers: within CCNA1 and VIM locus |                                                         | 121        |
| Cytosponge                 | Risk stratification and surveillance of patients with Barrett’s esophagus | Three protein markers: P53, c-Myc, and Aurora kinase A. Two DNA methylation markers: within MYOD1 and RUNX3 locus Mutation status: TP53 | Cytology: glandular atypia                                | 122        |

undergo endoscopy. Molecular characterization of premalignant esophageal lesions and healthy normal cells from esophageal lining could help to determine early molecular deregulation driving cancer development and progression. BE is one of the precursors for EAC and has been extensively studied to identify early events of carcinogenesis and differences between the progressing and nonprogressing types of BEs. Some of these studies identified different mutation, DNA methylation, and SCNA patterns among the progressing and nonprogressing types of BE suggesting a window of opportunity for early detection with multimomics approach. A couple of recent reports using molecular biomarker-based nonendoscopic method (Table 1) enables an efficient, well-tolerated, sensitive, and specific method of screening at-risk populations for BE. Moreover, exposure-associated genetic and epigenetic changes may prove instrumental in evaluating the cancer risk in heavily exposed individuals. A large-scale study using combinations of biomarker on Cytosponge TM collected cancerous, dysplastic, and normal samples could validate the use of this technique in early detection and risk stratification of EC.

Among the molecular markers, DNA methylation biomarkers have been extensively studied in the recent years due to their potential utility to develop exposure-specific biomarkers. The fact that DNA methylation changes occur at a higher rate than mutations makes them suitable for both mechanism-based biomarkers and early cancer detection and screening markers. However, the validation of methylation-based biomarkers in larger cohorts of EC is still lacking. Although many previous studies for discovery of diagnostic or prognostic markers included both primary cancer tissues and surrogate tissues, they tend to be limited by their small sample sizes. In addition, there is lack of studies with significant sample size investigating tumor specific methylation events in tumor and adjacent normal tissues for both EAC and ESCC. In addition, regardless of many candidate epigenetic biomarkers, only a few of these markers have been adequately validated for routine clinical practice. Possible reasons for the current limitations in epigenetic biomarker development are application of different assays for methylation profiling, few comparative data, and a frequent lack of concordance among studies.

Major challenges associated with ECs are its aggressive progression and late diagnosis leading to poor prognosis and high mortality. As described in Figure 3, a combination of the omics approaches for identifying biomarkers for early diagnosis and prognosis of the disease will also aid in personalized risk-stratification profile for each patient, enabling an early intervention and the possibility of improved prevention strategy. Moreover, understanding the cancer status at a molecular level with different omics approaches will help to untangle the mechanism of carcinogenesis and develop personalized therapy and prevention based on the molecular feature of individual cancer cases. To achieve this, the major task is the establishment of funds at major medical centers, where genome-assisted medicine is likely to be practiced for proper understanding of the cancer status and then design better treatment selection for precision therapy. However, implementing these programs in the low- and medium-income regions of the world, where this cancer is most prevalent, remains a major challenge.
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Competing interests
The authors declare no competing interests.

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