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

Provisional list and orders of the authors:

Talukdar, F. R.[1], di Pietro, M.[2] , Secrier, M.[3] , Moehler, M.[4] , Goepfert, K.[4] , Lima, S. S.[5], Pinto, L. F.[5], Hendricks, D.[6] , Parker, M. I.[6] and Herceg, Z.[1]

Affiliations:
[1]Section of Mechanisms of Carcinogenesis, International Agency for Research on Cancer (WHO), Lyon, France.
[2]MRC Cancer Unit, University of Cambridge, Cambridge, UK.
[3]Department of Genetics, Evolution and Environment, University College London, London, UK.
[4]First Department of Internal Medicine, Johannes Gutenberg-University of Mainz, Mainz, Germany.
[5]Molecular Carcinogenesis Program, National Cancer Institute, Rio de Janeiro, Brazil.
[6]Division of Medical Biochemistry & Structural Biology, University of Cape Town, Cape Town, South Africa

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Abstract

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 a 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 tumour 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, which help understanding molecular mechanism and critical molecular events driving the progression and pathological features of the disease. Here 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.

Introduction *(ZdenkoHerceg and Fazlur Rahman Talukdar)*

Esophageal cancer (EC) is a highly aggressive, lethal malignancy with over 400,000 deaths annually which represent a 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 five-year overall survival rates of approximately 15% [3], they are distinct diseases in terms of cell or 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 varies greatly among the two subtypes, with ESCC being more prevalent in the developing countries of South-Eastern and Central Asia, South-Eastern Africa and South America, of which the Asian countries contribute to nearly 79% of the global ESCC cases [4]. EAC is the major subtype in Northern and Western Europe, Northern America and Oceania, constituting around 46% of global adenocarcinoma cases [5]. A high male to female ratio is characteristics of EC and although it is more pronounced in EAC than ESCC (4.4 in EAC vs 2.7 in ESCC) the sex ratios differ significantly by geographical regions in both the subtypes[4]. The geographical variations in incidence rates, pattern and sex ratios are often attributed to differences in environmental and dietary factors [6-9], 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)[9, 10]. In contrast, ESCC is thought to have a more exposure driven causality, such as tobacco consumption, alcohol intake, hot beverage drinking, and poor nutrition [11-13]. 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[14]. 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 develoment of biomarkers for early diagnosis and therapy. Large-scale comprehensive molecular

characterization has complemented many of these goals in several cancer types[15-17]. One of the 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 than EAC, which better resembled the 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 regimes[18]. 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.

1. Genomics, mutations and deregulated pathways

1. a) Genomics of esophageal cancer *(Iqbal Parker and Denver Hendricks)*

The last seven years have seen the emergence of several studies reporting on the genomics of esophageal cancer 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 show that EAC and ESCC are different cancer entities [18, 19]. Although *TP53* is the most commonly altered gene in both EAC and ESCC (with alterations observed in >80% of all samples analysed), there is consensus that the profile of genomic alterations in EAC and ESCC vary considerably[20]. In EAC, the genes that are altered more frequently than in ESCC include, *ERBB2, KRAS, EGFR, SMAD4*, *FGF3/4/19, VEGFA*, *CCNE1* and *GATA4/6*, whereas PIK3CA, *CCND1, PTEN, NFE2L2*, *NOTCH1, MLL2, SOX2*, *FGFR1* and *RB1* are altered more frequently in ESCC[18].

TCGARN recently published an elegant study which used genomic analysis to characterise tumours derived from various locations in the esophagus and stomach, largely to better separate EAC and gastric adenocarcinomas[21, 22]. Their study shows that EAC is more closely aligned with gastric cancers – more specifically, gastric cancer with chromosomal instability (GC-CIS), the largest sub-type of gastric cancer identified. Based on an analysis of 90 ESCC, the authors categorised ESCC into 3 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 cancer and not EAC[15, 18].

EAC

Genomic analysis performed on EAC samples provide interesting clues about the tumorigenic processes in EAC and future developments in this field. Dulak and colleagues[23] (in a study that analysed 149 tumour samples) generated a list of 26 significantly mutated genes that include *TP53, CDKN2A, SMAD4* and *PIK3CA*. *ELMO1* and *DOCK2* (mediators of RAC1) were also significantly altered in EAC (17% samples tested), implicating *RAC1* mediated motility pathways in EAC tumorigenesis. A later report suggested that many of the putative driver mutations such as *SMAD2, TLL1, TLR4* and *DOCK2* probably appear later in the evolution of the tumour[24]. Their evidence suggests a considerable level of intra-tumour 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[18, 24]. Except for TP53, very few genes are altered by point mutations in multiple EAC tumour samples analysed – most gene alterations occurred as a result of chromosomal instability resulting in gene loss or duplication events [25]. There is an intriguing suggestion that it is the heterogeneity in EAC, manifested as the amplification of multiple RTK expressing genes and genes involved in downstream mitogenic pathways that may be responsible for the poor response of EACs to drugs targeting isolated RTK's 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 [25].

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.

Two recent articles summarised and re-analysed the genomic data from several recent genomic studies of large cohorts of ESCC patients[26, 27]. The re-analysis by Du et al identified recurrent mutations in approximately 18 genes, 15 of which had been reported previously (*TP53, AJUBA, CDKN2A, KMT2D (MLL2), ZNF750, FAT1, NOTCH1, NOTCH3, PIK3CA, NFE2L2, RB1, KDM6a, FBXW7, CREBBP* and *TGFBR2*), with three novel significantly mutated genes (*CUL3, PTEN* and *DCDC1*)[27]. Further, a recent study reported 26 significantly mutated genes, including eight novel (*NAV3, TENM3, PTCH1, TGFBR2, RIPK4, PBRM1, USP8* and *BAP1*) and 18 that have been previously reported. They also identified TENM3 mutations and the TP53 hotspot mutation p.R213*

are independent prognosticators for poor survival in ESCC[28]. Pathway analysis indicated that these alterations affected cell cycle and apoptosis, PI3Kinase signalling, histone modification, the p53 signalling pathway, the NOTCH pathway, WNT pathway and Hedgehog signalling; 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[29].

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 3 subtypes, with subtype 3 having the highest copy number alterations and the poorest prognosis[27]. 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).

Different geographic populations may display different genomic alterations with mutations having being 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[29, 30]. Zhang et al links 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[29]. Evidence produced by TCGARN supports the contention that different populations may display slightly different mutation profiles[18]. Furthermore, in the only large scale genomic analysis performed on ESCC subjects from sub-Saharan Africa (59 ESCC samples from patients in Malawi), the authors were unable to show the typical mutational signature associated with tobacco smoking, but did identify an unusual mutational signature previously observed in a small number of oropharyngeal squamous carcinoma cases [31]. These observations underscore the need to perform 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 intra-tumor heterogeneity (ca 90%) compared to other cancers, including EAC (56%), high grade serous ovarian cancers (52% and clear cell renal carcinoma (ca 35%) [32]. Considering that higher intra-tumor heterogeneity in EAC has been associated with poor responses to neoadjuvant chemotherapy, the high intra-tumor 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[24, 32].

1. b) Mutational signature and response to treatment in esophageal adenocarcinoma

(Massimiliano Di Pietro and Maria Secrier)

Esophageal adenocarcinoma (EAC) is the solid malignancy with the fastest rise in the incidence rate in the last four decades[33]. 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, pancreas and colon cancers, where specific subtypes with therapeutic implications have been characterized, we have poor understating about the clinical significance of EAC heterogeneity. Hence, the key clinical questions are: *i.* how can we classify EAC in a clinically useful way? *ii.* How can we improve response to conventional oncological therapies? *iii.* 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 [23]. 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 same tumor has 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[24]. This constitutes a potential problem when trying to target individual mutations for patient-tailored therapies. Currently, standard treatment for EAC is neo-adjuvant chemotherapy (+/- radiotherapy) followed by surgery, with or without adjuvant chemotherapy. The rate of complete pathological response to neo-adjuvant treatment ranges from 0 to 23% depending on the regimen adopted [34-36]. 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 rumucirumab, a recombinant antibody that binds VEGFR-2[37]. Despite positive oncological trials, the average survival advantage from the addition of these agents to standard palliative chemotherapy is 2 months, with substantially increased toxicity [38, 39]. 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 [23]were the first to describe a pattern of frequent A>C transversions at AA dinucleotides (S17 signature)in EAC, likely associated with gastro-esophageal acid reflux. Recently, whole-genome sequencing on 129 EAC cases has uncovered three subtypes with distinct etiology based on the prevalence of individual mutational signatures (figure). These subgroup are (1) *mutagenic*, characterized by S17-dominant signature, high mutational and neoantigen burden; (2) *DNA damage repair (DDR) impaired*, with frequent defects in the homologous recombination machinery; (3) a subgroup with a *C>A/T dominant* base substitution landscape linked primarily to ageing[25].

Limited experimental validation to date suggests that this genomic classification may inform treatment options in addition to the standard of care (figure). 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. Receptor tyrosine kinases (RTKs) were found frequently co-amplifiedin 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[25]. 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[40, 41], additional signatures related to smoking or to the activity of the cytosine deaminase APOBECs may prove central to the etiology of EAC (figure1). Undoubtedly, more research is needed before transitioning a mutational signature-based classification into routine clinical practice. However, with upcoming developments in the area of 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.

1. c) Deregulated pathways and therapeutic significance in EC (*Luis Felipe Ribeiro Pinto and Sheila Soares Coelho Lima***)**

Although ECs have been grouped together due to their anatomic location, it is now clear that esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (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 adenocarcinomas[18]. 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 together ESCC and EAC, even though some players and/or molecular alterations differ. *TP53* is the most frequently mutated gene in both cases, reaching a frequency of 91% in ESCC and 71% in EAC [18]. 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 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 esophageal cancer 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[42, 43]. 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, a phase 1 and a phase 2 study, have been carried out with Flavopiridol, the first CDK inhibitor in human clinical trials, in esophageal neoplasms (ClinicalTrials.gov accessed on 02/2018).

On the other hand, a number of clinical trials involving targeted therapy against tyrosine kinase receptors have been carried out in esophageal cancer. Although alterations of these pathway activators are not as common as cell cycle disruption, drugs targeting EGFR, HER2 and VEGFR have been approved for EAC treatment[44]. This is a good example of how differences between ESCC and EAC should be taken into account when choosing the appropriate treatment. It has been recently shown that *HER2* and *VEGFR* amplification or mutations may affect up to 32% and 28% of EAC cases, respectively, while for ESCC these numbers do not reach 5%[18]. The same study showed a similar frequency of *EGFR* alterations for both tumors (15-20%), but it has been previously shown that a high EGFR protein expression affects only 4% of ESCC cases[45]. Therefore, although EAC patients may benefit from targeted therapy against these receptors, ESCC patients do not seem to be entitled for such therapies. An alternative would be targeting other tyrosine kinase receptors, 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[46-49]. Although no clinical trials have been carried out with c-MET inhibitors in ESCC, *in vitro* results have shown they are capable of inhibiting the invasive capacity and radiosensitizing ESCC cell lines by prompting apoptosis and G2/M arrest[46, 50].

Going downstream on the pathways activated by tyrosine kinase receptors, *KRAS* and *BRAF* alterations do not seem to be common in ESCC, while mutations or amplification in *KRAS* are observed in 14% of EAC[18, 45]. Besides, *PIK3CA* activating mutations are observed in 13% of ESCC and *PTEN* inactivation affects 9% of the cases [18]. Although these numbers may seem low, the pathway could be activated by other mechanisms such as gene overexpression. Our group has recently shown that the upregulation of *PIK3R3*, a regulatory subunit that forms heterodimers with PIK3CA, leads to the activation of the PI3K/AKT pathway in ESCC and is an independent prognostic biomarker (under submission). Currently, a number of drugs inhibiting this pathway are in development and several clinical trials are ongoing for different types of solid tumors[51]. 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 has been 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[52].

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[18, 30, 53, 54]. This pathway is of special interest not only because of the high frequency of alterations found in ESCC (up to 86% of the cases[30]), 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-infected cells is capable of reprogramming somatic cells to pluripotency[55]. In ESCC, *WNT10a* overexpression was shown to promote migration, invasion and proliferation of transformed esophageal cells, as well as to induce a greater population of putative cancer stem cells[56]. 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 on 02/2018), although much remains to be learnt.

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[18]. The subtype ESCC1 has been characterized by alterations of oxidative stress machinery in up to 54% of the cases, with *NFE2L2* gene being affected by amplification or missense 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[57-59]. The presence of *NFE2L2* mutation was associated with tumor recurrence and poor prognosis in ESCC and the mutant *NFE2L2* confers resistance to 5-fluorouracil and γ-irradiation in *in vitro* models[58]. 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 neoplasia.

Finally, the molecular subtype ESCC2 has been characterized, among other alterations, by a high APOBEC mutational signature fraction[18]. This signature is characterized by substitutions of cytosine by thymine or guanine in TpC context and has been shown to be highly prevalent in ESCC by different authors[59, 60].The activation of AID/APOBEC enzymes and their mutagenic potential are also related to intratumor heterogeneity[61]. This is of special clinical interest because it has already been shown that the degree of intratumor heterogeneity may affect patient's prognosis and response to treatment. In early stages of non-small-cell lung carcinoma, a greater number of subclones was observed in patients that showed relapse in comparison with those free of disease[62]. The presence of subclonal driver mutations was associated with a shorter time to retreatment or death in chronic lymphocytic leukemia[63]. 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[63].

Recently, a great advance has been made in identifying important molecular differences between ESCC and EAC that may help not only the comprehension of the signaling pathways responsible for the development of each tumor type, but also the identification of new molecular targets. Besides, genome-wide studies have started to suggest the occurrence of distinct molecular ESCC subtypes, which may lead to more specific treatments and consequent better response to therapy. Despite such advances, most clinical trials still group together the two EC histological subtypes, what may

confound results. Furthermore, surrogate markers of eligibility and response to therapy still need to be characterized.

2. Epigenomics of EC (*Zdenko Herceg and Fazlur Rahman Talukdar*)

2. a) Exposure specific DNA methylation change in EC

The fact that alterations in DNA methylation affects 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 were either with modest numbers of samples or including only a few genes[64]. Alcohol consumption is a major risk factor of ESCC[65]. One of the mechanisms of alcohol induced carcinogenesis may be inhibition of DNA methylation[66]. Hypo and hypermethylation of genes are among the most important mechanisms of transcription regulation [67]. Alcohol inhibits S-Adenosyl-methionine (SAM) synthesis, a universal methyl group donor and enzyme activator in methyl transfer reactions[68]. 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[69]. Although such alcohol induced global hypomethylation was not elucidated in esophageal tumours, LINE-1 hypomethylation, a surrogate marker of global hypomethylation, was suggested to be an important event during ESCC carcinogenesis[70-72]. ESCC has also been associated with exposure to nitrosamines [73], which leads to alkyl-related DNA damage that is normally repaired by enzymes such as O(6)-methylguanine DNA methyltransferase (*MGMT*)[74]. Therefore, inactivation of *MGMT* by aberrant DNA methylation might favour 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 [75, 76]. Certain tobacco derived carcinogens such as NNK (Nicotine-derived nitrosamine ketone), BAP (Benzo-a-Pyrene) were found to be capable of modulating DNA methylation in cultures, animal models as well as some tobacco-related cancers[77-79]. NNK could induce *RARbeta* promoter hypermethylation through upregulation of *DNMT1* in esophageal squamous epithelial cells, resulting in enhancement of cell proliferation and inhibition of apoptosis [79]. Nicotine induced the methylation of *FHIT* gene and attenuated Fhit protein in association with increased expression of DNMT3a in human esophageal squamous epithelial cells, a process important in early carcinigenesis[80]. Alterations in DNA methylation is a frequently event in the formation of BE and its progression to EAC [81-83]. An epigenome-wide study identified several differentially methylated functional genes mapping to meaningful pathways associated with obesity and tobacco smoking which influences the risk of developing BE/EAC [84]. Immortalized esophageal epithelia (IEE)

induced with cigarette smoke was found to contribute to the pathogenesis of EAC by epigenetic repression of miR-217 via upregulation of *KLK7*[85]. 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.

2. b) Tumor specific DNA methylation marks in EC

During tumor development cells undergo several genetic and epigenetic alterations which mutually contribute to tumorigenesis. These molecular alterations such as large-scale DNA alterations, mutations, methylations and RNA or protein expressions might complement histological analysis to improved 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 (Fig. 1). One epigenetic mechanism 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 as well as novel therapeutic targets of cancer.

DNA methylation in EAC: Although global methylation studies are sparse in EAC, earliest genome-wide studies aimed at understanding the role of aberrant methylations in malignant transformation of BE to EAC found considerable differences between the DNA methylation patterns in non-malignant esophageal tissues, BE and EAC, with BE being more representative of the DNA methylation patterns found in tumours[86, 87]. 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 ADAM (A Disintegrin and Metalloproteinase) peptidase proteins, cadherins and protocadherins, 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[86]. Another study determining the methylation landscape of EAC and its impact on gene expression identifieddistinct methylation patterns pertaining to subtypes of EAC, one similar to the CpG Island Methylator Phenotype (CIMP) which was potentially associated with worse clinical outcome[88].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[89, 90]. 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[89, 91, 92]. These studies provided new insights into the contribution of epigenetics to EAC carcinogenesis and clinical outcome.

DNA methylation in ESCC: DNA methylation occurs in several key components of cancer-related signaling pathways. It affects the genes involved in cell cycle, DNA damage repair, Wnt, TGF-beta, and NF-kappa B signaling pathways, including *P16*, *MGMT*, *SFRP2*, *DACH1*, and *ZNF382*[93]. One of the preliminary high-throughput DNA methylation profiling arrays was Illumina Golden-Gate array consisting of more than 1500CpG 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 signalling and cell communication. Moreover, methylation of TFF1 was identified as a potential early marker for ESCC in this study[94]. 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 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 [95]. High throughput sequencing techniques like 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 identified differentially methylated regions (DMRs) mapping to cell cycle, adhesion, proliferation and apoptosis pathways. Expression levels of several genes like *MLH1*, *TWIST1* and *CDX1* were consistent with their DNA methylation profiles[96]. The identification of whole-genome DNA methylation patterns in ESCC provide new insight into the carcinogenesis of ESCC and might prove a promising avenue to investigate novel biomarkers, prognostic and therapeutic targets.

2. c) 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[97]. DNA methylation based 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 [98].

Targeted promotor methylation detection revealed a set of DNA repair genes *hMLH1*, *hMSH2*, and *MGMT* which are frequently methylated in ESCC holding promise as a potential predictive factor in primary cases[99]. 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[100]. Cell-free circulating 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[101]. Investigation of genome-wide cfDNA methylation profiles was found to be highly consistent with DNA methylation profiles detected in corresponding tumor tissues. This support the utility of differential cfDNA methylation profiling as a useful approach for the non-invasive screening of EAC[102].

The utility of innovative, less invasive and cost-effective technique for esophageal sample collection can largely improve early detection and risk stratification. This is evident from the findings of a recent study where a combination of biomarkers (P53 abnormality, glandular atypia, and AurKA staining) was used with clinical variables (age, length of Barrett's oesophagus, and obesity). Based on the biomarkers, patients were stratified to determine with high confidence a group with Barrett's esophagus at low risk of progression for whom endoscopy could be avoided [103].

3. Future perspectives

3. a) Immuno-oncology in oesophageal cancers: new promising strategies and therapeutic options *(Markus Moehler)*

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 [104], like inhibitors of the cytotoxic T-lymphocyte-associated antigen (CTLA-4) or the programmed cell death protein 1 (PD-1) and its ligand (PD-L1)[105, 106]. The use of these immune checkpoint inhibitors (ICI) institutes a new therapy of gastrointestinal (GI) malignancies after the recent FDA approvals of PD-1 inhibitors for microsatellite instable (MSI) tumors. Since the first ICI were approved, clinical research also evaluates other targets within the 'cancer immunity cycle' and investigating ICI in combination with numerous other established or novel drugs [105, 107, 108]. 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[109]and 23 patients with pembrolizumab[110] are reported. Doi *et al* included only PD-L1 positive patients (PD-L1 cut-off value ≥1%), 74% of them with squamous cellcarcinoma (SCC); patients with adenocarcinoma (AC) histology from the distal esophagus and patients with gastro-esophageal junction (GEJ) tumors were enrolled as well (Figure 2) are reported. Doi et al included only PD-L1 positive patients (PD-L1 cut-off value ≥1%), 74% of them with squamous cell carcinoma (SCC); patients with adenocarcinoma (AC) histology from the distal esophagus and patients with gastro-esophageal junction (GEJ) tumors were enrolled as well (Figure 2).

The reported ORR of 30% (28% for SCC, 40% for AC) in this patient cohort (87% had received ≥2 prior therapies) was in the range than 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 3 prior therapies. Similarly, median duration of response was 11.2 months and 9.3 months in the pembrolizumab trial respectively, in which only one half of patients was originating from Asia. Treatment-related adverse events (TRAE) of grade ≥ 3 were detected only in 17 % of patients with no treatment-related deaths reported.

Several ICI phase III in esophageal cancer are ongoing with first results expected for year 2018. For unresectable advanced or recurrent esophageal cancer in patients that failed standard chemotherapy (CTx), confirmative trials testing the use of nivolumab or pembrolizumab mono in second-line patients are directly competing. CheckMate-473 (NCT02569242) and KeyNote-181 (NCT02564263) are globally recruiting in biomarker-unselected all-comer populations: taxanes constitute the predominant competitors for both trials. For pembrolizumab, a supportive phase II trial in 100 patients (KeyNote-180; NCT02559687) with at least two prior therapies will generate up-front efficacy data that might allow gaining accelerated approval in the United States. The PFS and OS are also used as co-primary endpoints in the phase-III trials of pembrolizumab in second-line (KeyNote-181) as well as in first-line KeyNote-590 (NCT03189719).

Nivolumab is investigated in two large phase II trials in first-line as well as in the adjuvant setting. The first-line trial CheckMate-648 (NCT03143153) adds nivolumab to cisplatin plus fluoropyrimidine standard regimen and compares – 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 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, first results for the primary outcomes measure are expected after September 2020. First neo-adjuvant

trials of ICI are ongoing too: an investigator-sponsored US trial (IST) (NCT02998268) compares the concomitant versus sequential use of pembrolizumab as part of an induction chemoradiation regimen prior to surgery in patients with locally advanced esophageal adenocarcinoma, followed by adjuvant use of this ICI mono 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 [110]. No comparative biomarker analyses are available from different company studies (Attraction-01, KeyNote-028). The interpretation of present clinical data remains difficult: For heavily pre-treated patients with SCC histology that received nivolumab, a promising median OS of 10.8 months is reported in the all-comer population (PD-L1±)[109]. 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 [111]. For patients progressing after primary CTx, a standard given the median OS of ~ 4 months in a phase III trial comparing gefitinib after CTx with best supportive care (BSC), with mixed AC/SCC histology population for both treatment arms[112].

In 2017, the important approvals of ICI for use in GI-tract cancers were granted: namely for PD-1 inhibitor nivolumab (in the US for metastatic mismatch repair deficient (dMMR) or microsatellite-instable-high (MSI-H) colorectal carcinoma (CRC) and in Japan for gastric cancer), and for the PD-1 inhibitor pembrolizumab: in the US for use in gastric as well as 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[113].

Hence, biomarker development and refinement, also in relation to the role of high tumor mutational burden (TMB) as a potential predictive and prognostic marker, constitutes another research priority [114] to improve the outcome of PD-1 inhibition. The use of ICI in tumors with higher mutational load was already associated with improved OS [115], providing the rationale for their use in upper GI-tract cancers. Comprehensive phase III programs have been initiated in esophageal and gastric cancer.

3. b) Implications for improving treatment and prognosis *(ZdenkoHerceg and Fazlur Rahman Talukdar)*

In this review, we have primarily focused on the molecular landscape of EC and recent advances in the field of genomics and epigenomics which has 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 multi-omics data for molecular subtyping of ESCC, their limitation lay in the fact that the cases originates from the regions with moderate or lower ESCC incidence. Therefore, these findings are important to be replicated including high incident populations of Asia (China, Bangladesh, India, Iran etc.), South-Eastern Africa and South America (especially Brazil) [4].

Identification of certain molecular alteration in esophageal tumors could provide targets for existing targeted therapies. For instance, one-third of the esophageal adenocarcinomas with alterations in the gene *ERBB2* (also called *HER2*) might be good candidates for the drug trastuzumab (Herceptin®), which blocks the extracellular part of the trans-membrane receptor protein product of this gene[116, 117]. 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[2, 118].

The Cytosponge 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 undergo endoscopy[119,120]. 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. 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 collected cancerous, dysplastic and normal samples could validate the use of this technique in early detection and risk stratification of EC[103, 121].

Among the molecular markers, DNA methylation-based 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 occurs at a higher rate than mutations makes them suitable for both mechanism-based biomarkers as well as early cancer detection and screening markers. However, validation of methylation-based biomarkers in larger cohorts of EC is still lacking. Although many previous studies for discovery of diagnostic or prognostic markers have been 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 frequent lack of concordance among studies [122].

Major challenges associated with ECs are its aggressive progression, late diagnosis leading to poor prognosis and high mortality. As described in Figure 3, a combination of omics approaches for identifying the biomarkers for early diagnosis and prognosis of the disease will also aid in personalized risk-stratification profile for each patient, enabling time for early intervention and the possibility of improved prevention strategy. Moreover, understanding the cancer status at 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 centres, 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[123]. However, implementing these programmes in low and medium resource regions of the world, where this cancer is most prevalent, remains a major challenge.

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Figure legends

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 grey (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 needs to be confirmed in larger studies.

Figure 2: Clinical trials with PD-1/PD-L1 inhibitors in esophageal cancer

Figure 3: Role of molecular characterization in addressing major challenges of esophageal cancer

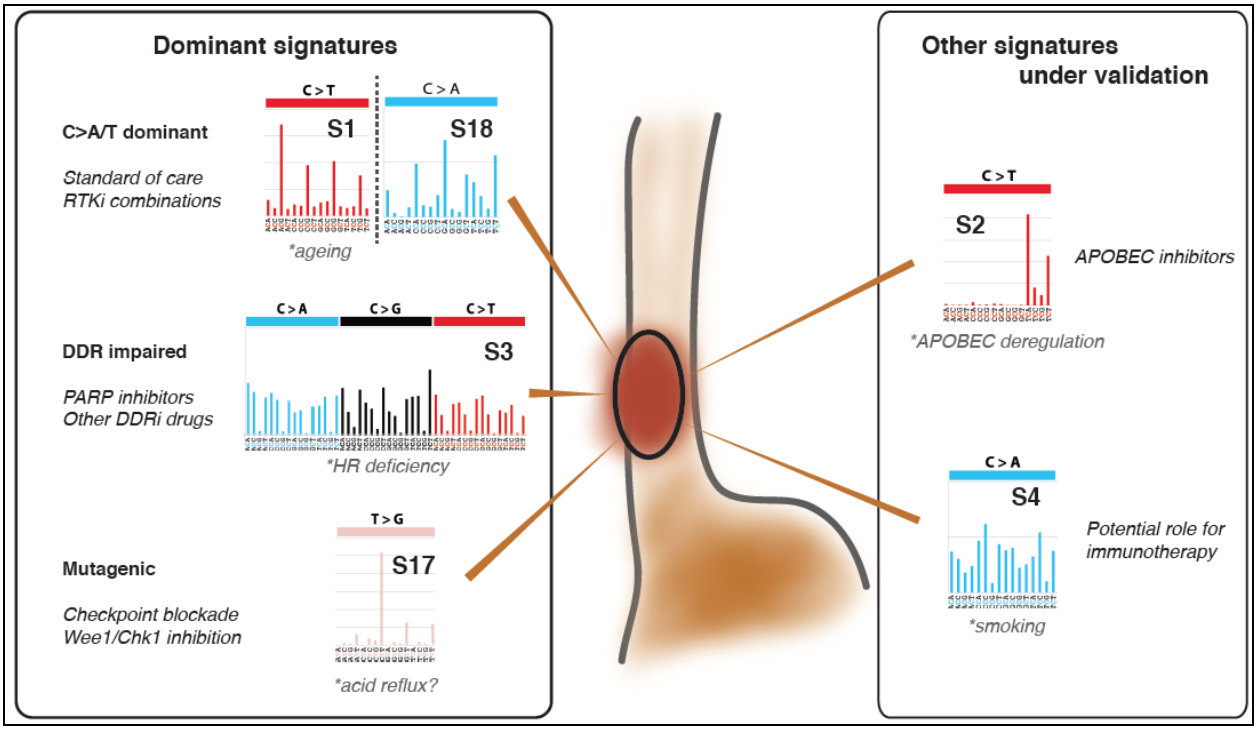
Dominant signatures
C>A/T dominant
Standard of care
RTKi combinations
C>T
S1
C>A
S18
*ageing
DDR impaired
PARP inhibitors
Other DDRi drugs
C>A
C>G
C>T
S3
*HR deficiency
Mutagenic
Checkpoint blockade
Wee1/Chk1 inhibition
T>G
S17
*acid reflux?
Other signatures
under validation
C>T
S2
APOBEC inhibitors
*APOBEC deregulation
C>A
S4
Potential role for
immunotherapy
*smoking

Figure 1

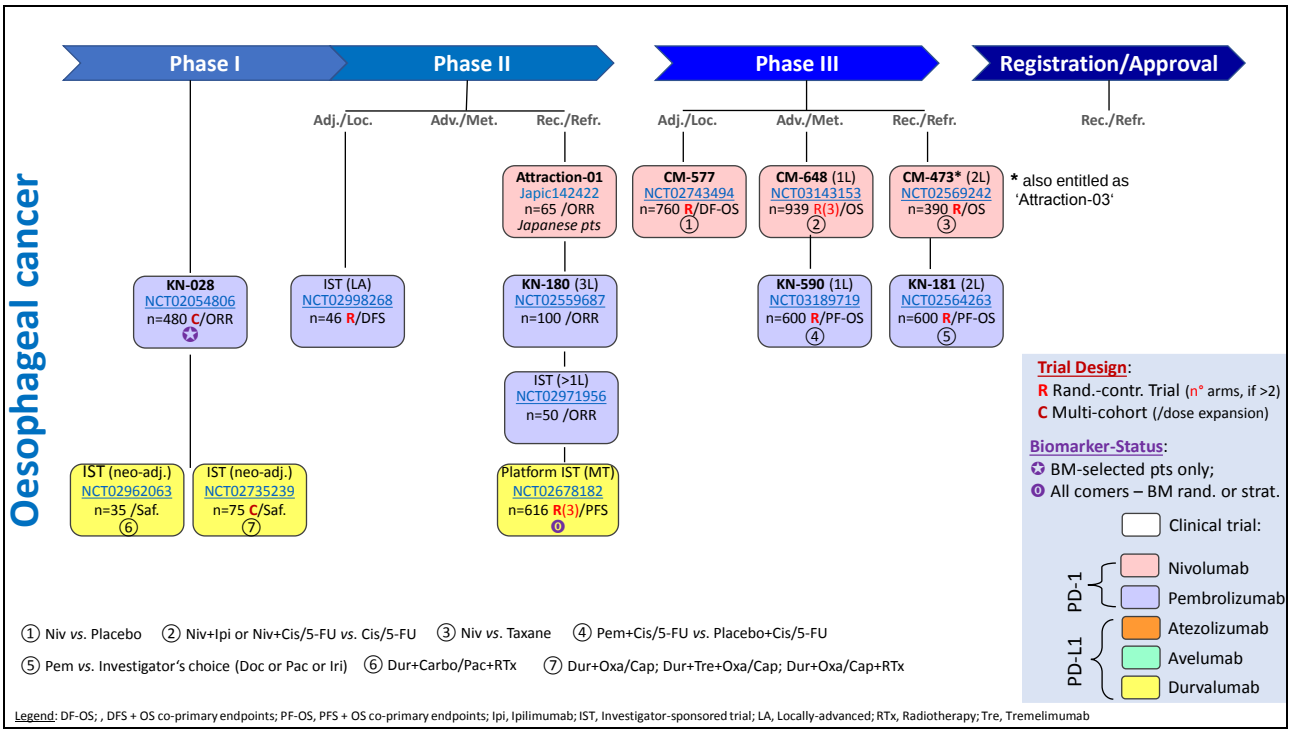
Phase I
Phase II
Phase III
Registration/Approval
Adj./Loc.
Adv./Met.
Rec./Refr.
Adj./Loc.
Adv./Met.
Rec./Refr.
Rec./Refr.
Oesophageal cancer
KN-028
NCT02054806
n=480 C/ORR
IST (LA)
NCT02998268
n=46 R/DFS
Attraction-01
Japic142422
n=65 /ORR
Japanese pts
KN-180 (3L)
NCT02559687
n=100 /ORR
IST (>1L)
NCT02971956
n=50 /ORR
Platform IST (MT)
NCT02678182
n=616 R(3)/PFS
CM-577
NCT02743494
n=760 R/DF-OS
①
CM-648 (1L)
NCT03143153
n=939 R(3)/OS
②
KN-590 (1L)
NCT03189719
n=600 R/PF-OS
④
CM-473* (2L)
NCT02569242
n=390 R/OS
③
KN-181 (2L)
NCT02564263
n=600 R/PF-OS
⑤
* also entitled as 'Attraction-03'
IST (neo-adj.)
NCT02962063
n=35 /Saf.
⑥
IST (neo-adj.)
NCT02735239
n=75 C/Saf.
⑦
Trial Design:
R Rand.-contr. Trial (n° arms, if >2)
C Multi-cohort (/dose expansion)
Biomarker-Status:
BM-selected pts only;
All comers – BM rand. or strat.
Clinical trial:
PD-1
Nivolumab
Pembrolizumab
PD-L1
Atezolizumab
Avelumab
Durvalumab
① Niv vs. Placebo
② Niv+Ipi or Niv+Cis/5-FU vs. Cis/5-FU
③ Niv vs. Taxane
④ Pem+Cis/5-FU vs. Placebo+Cis/5-FU
⑤ Pem vs. Investigator's choice (Doc or Pac or Iri)
⑥ Dur+Carbo/Pac+RTx
⑦ Dur+Oxa/Cap; Dur+Tre+Oxa/Cap; Dur+Oxa/Cap+RTx
Legend: DF-OS; , DFS + OS co-primary endpoints; PF-OS, PFS + OS co-primary endpoints; Ipi, Ipilimumab; IST, Investigator-sponsored trial; LA, Locally-advanced; RTx, Radiotherapy; Tre, Tremelimumab

Figure 2

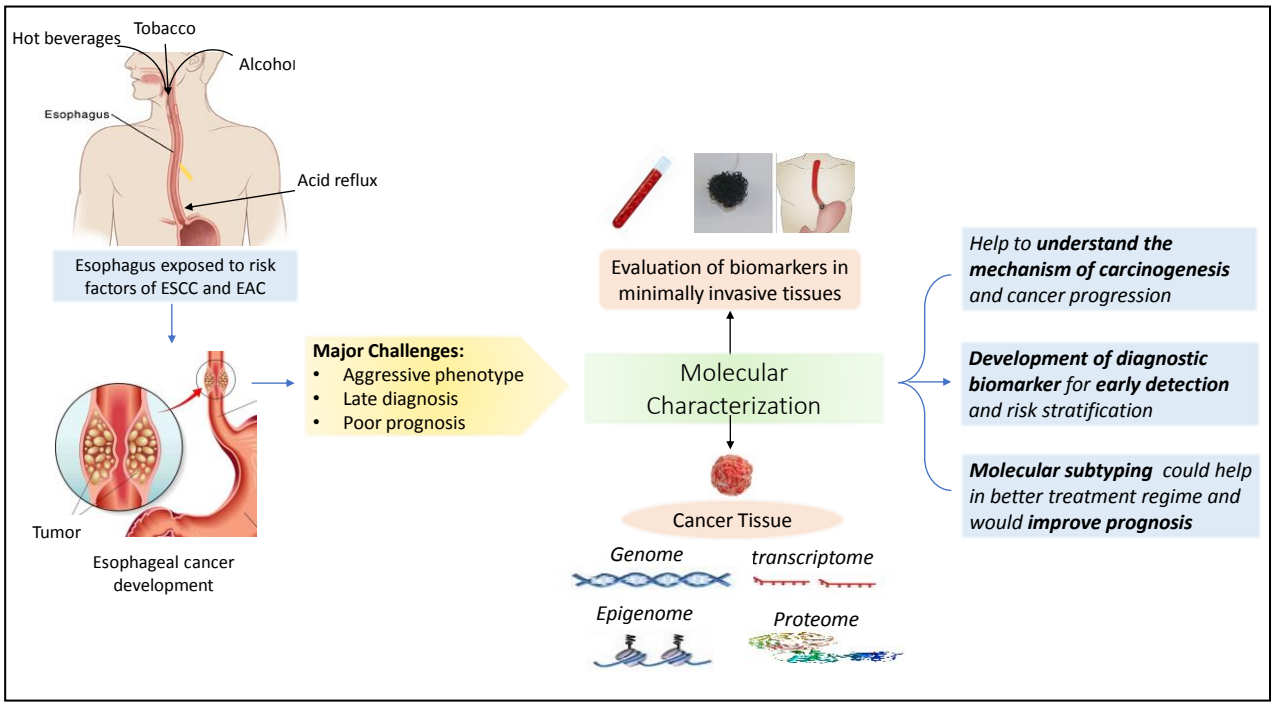
Hot beverages
Tobacco
Alcohol
Esophagus
Acid reflux
Esophagus exposed to risk factors of ESCC and EAC
Tumor
Esophageal cancer development
Major Challenges:
• Aggressive phenotype
• Late diagnosis
• Poor prognosis
Evaluation of biomarkers in minimally invasive tissues
Molecular Characterization
Cancer Tissue
Genome
transcriptome
Epigenome
Proteome
Help to understand the mechanism of carcinogenesis and cancer progression
Development of diagnostic biomarker for early detection and risk stratification
Molecular subtyping could help in better treatment regime and would improve prognosis

Figure 3