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Etiology, cancer stem cells and potential diagnostic biomarkers for esophageal cancer

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ABSTRACT

Esophageal cancer (EC) has been a leading cause of cancer death worldwide in part due to late detection and lack of precision treatment. EC includes two major malignancies, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). Recent studies reveal that ESCC and EAC have distinct cell of origin and contain cancer stem cells (also known as tumor initiating cells) expressing different cell surface markers. These biomarkers have potentially important values for both early detection and finding effective therapy. In this review we summarize the updated findings for cell of origin and provide an overview of cancer cell biomarkers that have been tested for ESCC and EAC. In addition, we also discuss recent progress in the study of molecular mechanisms leading to these malignancies.

1. Background

Esophageal cancer (EC) is a leading cause of cancer death worldwide, and esophageal squamous cell carcinoma (ESCC) is the predominant histological sub-type of EC in the developing countries. For example, ESCC accounts for 90% of cancer-related deaths in China. By contrast, esophageal adenocarcinoma (EAC) has become a rapidly increasing malignancy in developed countries, and the US has seen a more than 6-fold increase during the recent three decades [1,2].

ESCC has been closely associated with life and environmental factors such as drinking and smoking that likely alter the genomic landscape genetically and epigenetically. The progression of ESCC mainly involves five stages, including simple epithelial hyperplasia, dysplasia, pre-invasive carcinoma, invasive carcinoma, and metastatic carcinoma. Dysplasia is a critical step during ESCC development. Histological analysis revealed that the significant feature of dysplasia is basal cell hyperplasia with irregular epithelial stratification and increased normal and abnormal mitosis. Dysplasia is also featured with enlarged nucleoli, individual cell keratinization, loss or reduction of cell cohesion and loss of cell polarity prior to malignant transformation. In clinics ESCC can be divided into five stages based on the malignant status, namely, stage 0, stage I, stage II, stage III, and Stage IV. Stage 0 tumors are intramucosal tumors that do not invade the lamina propria; stage I tumors invade the lamina propria without lymph-node or distant involvement; stage II tumors extend to the muscle layer either without (IIA) or with (IIB) lymph-node involvement; stage III tumors invade through the muscular layer and involve lymph nodes or other adjacent structures; and stage IV tumors spread to distant organs or lymph nodes [2].

By contrast, EAC is closely associated with gastroesophageal reflux (GERD) which promotes the development of Barrett's esophagus, an only known risk factor for EAC [3]. EAC also progresses through dysplasia, pre-invasive carcinoma, invasive carcinoma, and metastatic carcinoma. Recent studies with mouse models and human biopsies have shown that ESCC and EAC have distinct cells of origin [4–6] (Fig. 1). Significantly, some of these models implicate malignant transformation of stem/progenitor cells, leading to sequential progression towards cancer [4,6].

Cancer stem cells (CSCs, also known as tumor initiating cells) are associated with treatment resistance and cancer relapse in many tissues [7]. These CSCs can be detected in the tissue where tumor arises and in the circulation system [8]. Importantly, the circulating CSCs offer a window for non-invasive diagnosis. Multiple CSC populations have

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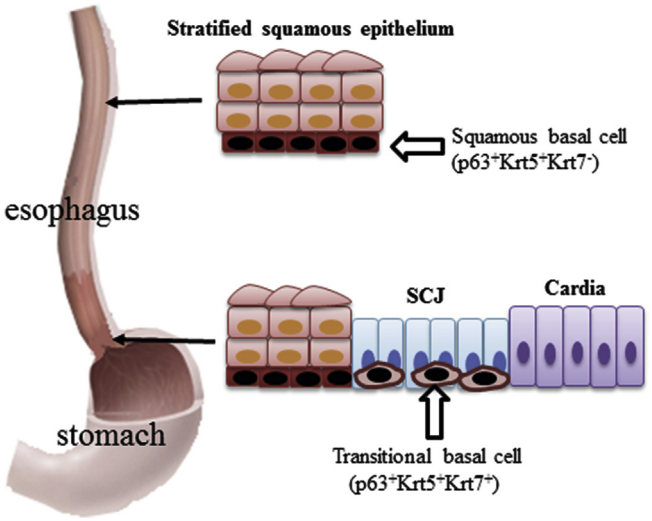

Fig. 1. Distinct cell of origin for ESCC and EAC.
Squamous basal cells are considered as the cell of origin for ESCC [4]. By contrast, EAC and its precursor Barrett's esophagus (BE) are associated with the transitional basal cells [5] and gastric cardia [6].

been identified with cell surface markers including proteins, lncRNA, and miRNA in both ESCC and EAC. It is of great importance to achieve consensus on the characteristic of CSCs and their biomarkers. In this review we focus on the etiology of ESCC and EAC, especially the recent progress achieved using clinical samples and animal models. We also highlight the advances in the identification of diagnostic biomarkers which would be helpful for the early detection and treatment of EC.

2. Recent progress in the etiology of EC

2.1. Genetic etiology of ESCC

Previous studies have demonstrated that ESCCs are associated with multiple environmental and genetic factors, including tobacco smoking, alcohol consumption [9–11], drinking tea at high temperature [12], diet with low intake of fresh fruits and vegetables, or with hot foods, or with pickled vegetables, or with red and processed meat, or with vitamin and mineral deficiencies [9,11,13–17], exposure to chemical factors such as polycyclic aromatic hydrocarbons, N-nitroso compounds, acetaldehyde and fumonisins [9,18,19], infection with human papillomavirus [9,19–21], family history [19,22], and genetic changes [19,23] (Fig. 2).

In vitro modelling with cell lines revealed some of these environmental factors alter cellular activities, e.g. cell proliferation [24]. Carcinogen and genetic mouse models further support that basal cells respond to the challenges and behave abnormally. Basal cells are the progenitor cells of the squamous epithelium [4,5,25–27]. We used lineage tracing to show that these basal cells give rise to the differentiating suprabasal cells [4]. Upon inflammatory challenges through interleukin-1β (IL-1β)/interleukin-6 (IL-6), these basal cells can be transformed, resulting in ESCC. Our further analysis demonstrated that the inflammatory mediator Stat3 cooperates with SOX2 during the transformation. Conversely, downregulation of SOX2 or Stat3 reduces the growth of mouse and human ESCC. Significantly, we found that increased levels of phosphorylated STAT3 and SOX2 protein are closely associated with a poor prognosis in ESCC patients [4].

Recent genomic studies (e.g. exome sequencing) further suggested that the mutational profile of ESCC is rather diverse, involving genes regulating cell cycle, apoptosis, and histone modification. Some of the mutations lead to dysregulation of the HIPPO and NOTCH signalling pathways [28]. In another study, 8 significantly mutated genes were identified in Chinese patients with ESCC through a combined whole-genome and exome sequencing. Among these 8 genes, 6 genes are well-known tumor-associated genes, including *TP53, RB1, CDKN2A, PIK3CA, NOTCH1* and *NFE2L2*, and the other two, *ADAM29* and *FAM135B* are novel genes. Additionally, several important histone regulator genes, such as *MLL2, ASH1L, MLL3 (KMT2C), SETD1B, CREBBP*, and *EP300* are frequently altered in ESCC, suggesting that gene mutation and amplification are pivotal for ESCC development, and they are also closely associated with the poor prognosis [29]. It is noteworthy that the squamous epithelium contains relatively common mutations in some genes including *NOTCH1* and *TP53* in the normal esophagus. Mutations of these genes likely alter the clonal expansion of basal progenitor cells [30]. However, whether and how clonal selection leads to the initiation of malignancy remains to be explored.

Single nucleotide polymorphism was also found to be involved in the initiation of ESCC. New evidence showed that two SNPs, including rs7447927 and rs1642764, which are located in *TMEM173* and *ATP1B2* genes, respectively, achieved genome-wide significance after joint analysis of three genome-wide association studies of ESCC; additionally, a locus in the HLA class II region at 6p21.32 (rs35597309) achieved a genome-wide significance in the two populations associating with the highest risk for ESSC [31]. In addition, the rs1154402C > G in intron-1 of the *ADH5* gene substantially reduces the expression levels of *ADH1A*. The suppressive effects caused by rs1154402 in *ADH5* and another SNP (rs11066015 in ALDH2) can substantially increase the risk of ESCC [32]. Moreover, a previous study revealed 6 new susceptibility loci in *CCHCR1, TCN2, TNXB, LTA, CYP26B1* and *FASN*. The study also showed that low levels of all-trans-retinoic acid in the serum of individuals with the rs138478634-GA genotype as compared to the rs138478634-GG genotype in the *CYP26B1* gene. These alternations may have some influence in the progression of ESCC [33].

2.2. Current understanding of the cell of origin for BE and EAC

Barrett's esophagus (BE) is the only known risk factor of EAC, and BE develops following chronic and erosive reflux [34,35]. EAC pathogenesis has also been associated with decreased estrogen exposure [36], single-nucleotide polymorphisms in cancer-related genes [37] and obesity [38]. In addition, EAC progression can be further influenced by helicobacter pylori infection [39], gene methylation [40,41], and ectopic expression of some genes, such as *GATA6* and *CDK6* [42,43].

BE pathogenesis is closely associated with inflammation resulting in metaplastic changes in the epithelium lining the squamous columnar junction (SCJ). Thus far several models have been established to study the cell of origin and disease mechanism of BE, which was summarized in our previous studies [5]. One of these models suggests that mutation in the *Trp63* gene is associated with intestine-like metaplasia of squamous epithelium. The group further showed that a residual embryonic epithelium ($Krt7^+$ $Trp63^-$) located at the SCJ can serve as the cell source for BE [44]. However, our recent study demonstrated that a transitional epithelium with distinct basal progenitor cells ($p63^+$ $Krt5^+$ $Krt7^+$) at the SCJ serves as progenitor cells for BE following inflammatory challenges or genetic manipulations. We used multiple mouse models including mice undergoing anastomosis surgery combined with lineage tracing to show that the transitional epithelium generates Barrett's epithelium [5].

3. Recent advances in the diagnosis and biomarker discovery of EC

Early diagnosis is essential for effective therapeutic management of EC. During malignant transformation, the levels of several cellular components may change, in which some of them are available for tumor detection, and even for monitoring malignant status and prognosis. Here, we focus on tissue biomarkers for cancer stem cells during EC development and these biomarkers can potentially facilitate non-

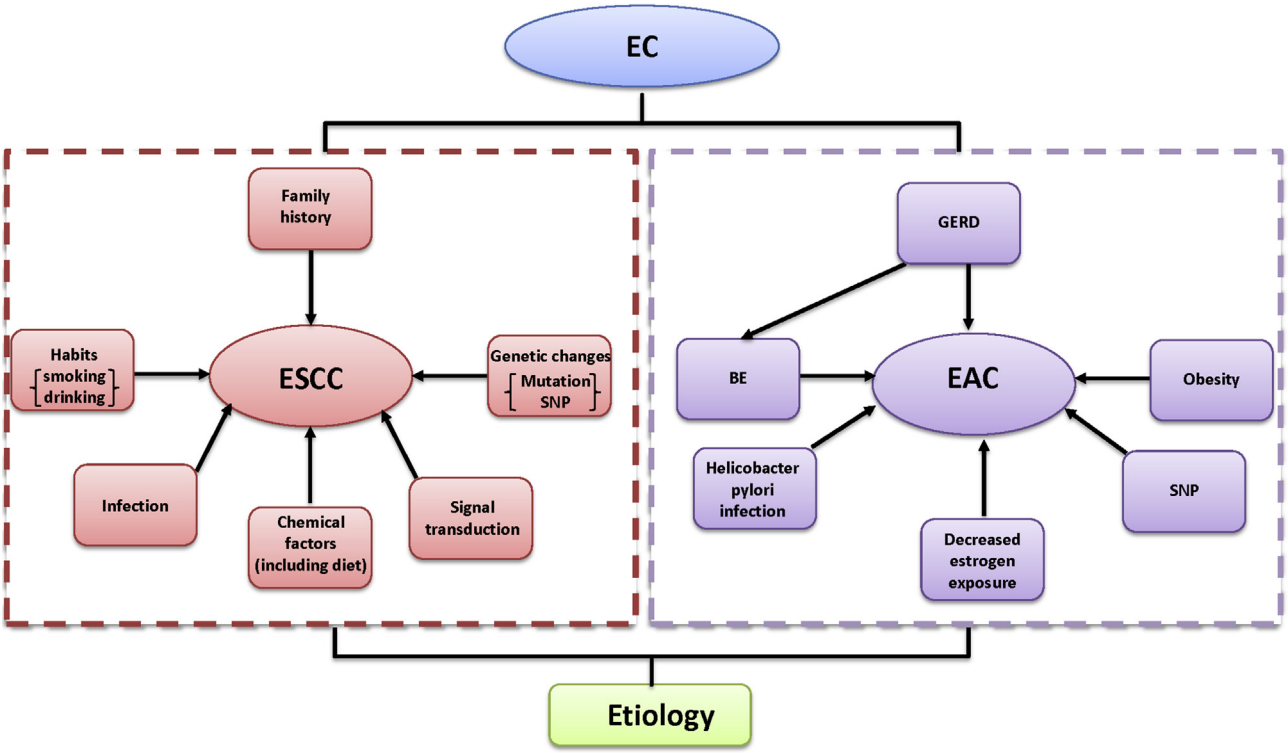

Fig. 2. Factors associated with the incidence of ESCC and EAC.
Distinct environmental factors and genetic mutations are associated with the development of ESCC and EAC.

invasive diagnosis of ESCC and EAC.

3.1. Diagnostic biomarkers on the surface of esophageal CSCs

CSCs can take different forms at different stages of tumor development. They can be mitotically quiescent and can also possess a more aggressive phenotype with active self-renewal and differentiation associated with a greater therapeutic resistance, and metastasis. Therefore targeting CSCs could provide an effective treatment for EC [45,46].

A series of studies have revealed the existence of CSCs in ESCC [45,47–49]. However, thus far consensus on the common biomarkers for esophageal CSCs has yet to be achieved. At the very beginning Hoechest 33342 dye was used as marker for isolating CSCs from EC9706 and EC109 cell lines [50]. These esophageal CSCs show strong dye efflux activity and high clone formation efficiency. However, p75NTR positive cells in ESCC cell lines also exhibit a number of characteristics, such as self-renewal and effective response to chemotherapy [51]. For the past two decades CSCs in ESCC have been shown to express multiple cell surface markers including p75NTR (CD271) [51–53], CD44 [54], aldehyde dehydrogenase (ALDH) [48], aldehyde dehydrogenase 1 (ALDH1) [55], aldehyde dehydrogenase 1 family member A1 (ALDH1A1) [45,56], CD90 [57], intercellular adhesion molecule 1 (ICAM1) [58], Cripto-1 [47], SCAR Homolog (WASH) [59], CD133 and CXCR4 [60], and adenosine triphosphate-binding cassette superfamily G member 2 (ABCG2) [49,61] (Table 1). Significantly, expression of some biomarkers is correlated with poor prognosis for EC [55,62], and these biomarkers can be used for predicting therapeutic responses [63]. In addition, high levels of the surface biomarkers are closely associated with different stages of cancer development, differentiation degree, invasion depth and lymph node metastasis [55,56,59]. They can also serve as important parameters for prognosis prediction [56,59]. A comprehensive understanding of these biomarkers and their associated cellular functions is expected to provide insights into the development of useful therapies. Use of cell lines has been instrumental in this direction [64,65].

Compared to ESCC, our understanding of CSCs in EAC remains limited, and we just began to know the existence of CSCs in EAC [66,67]. Interestingly, studies have shown that CSCs in EAC do not express the common biomarkers for CSCs [68], which is consistent with the different cells of origin for EAC and ESCC. Recently, Lgr5 and musashi-1 were proposed as the potential biomarkers for CSCs in EAC [69–71]. In a separate study, CSCs in EAC have been shown to express high levels of the transcription factor SOX9 accompanied by increased YAP signalling activities [72]. This study further showed that YAP-driven SOX9 expression is important for the acquisition of CSC properties in EAC, thereby providing a potential means of blocking EAC through the inhibition of the YAP/SOX9 axis.

3.2. Blood diagnostic biomarkers for EC

Circulation blood has become more and more useful for cancer diagnosis. Previously, pathological examination of tissue markers in biopsy was a principal method for tumor detection. Protein biomarkers such as carcinoembryonic antigen (CEA), CA199 and squamous cell carcinoma antigen (SCCA) were commonly used for diagnosis of ESCC in clinics. In the past ten years or so protocols have been optimized to successfully isolate circulating cell-free DNA, exosomes or circulating tumor cells that are originated from the malignancies [73–75]. In addition, cancer cells secrete several other components including miRNAs, lncRNA, and circRNA which can also be explored for diagnostic purpose in a non-invasive manner. A variety of proteins and RNAs have been assessed as potential diagnostic biomarkers in EC patients [76–79]. These proteins and RNAs are readily detected in the blood (Table 2). It is noteworthy that several lncRNAs (e.g. POU3F3) were proposed to serve as potential diagnostic markers for ESCC [80]. Additionally, plasma miR-25 and miR-18a have been shown to be helpful in the detection and monitoring tumor progression in ESCC patients [81,82].

We just began to identify biomarkers for EAC detection (Table 3). Interestingly, while NY-ESO-1 autoantibody and miR-375 were both

Table 1
Biomarkers for esophageal CSCs.

Biomarkers for CSCs	Methods	References
p75NTR(CD271)	Immunohistochemistry, fluorescence activated cell sorting, quantitative real time RT-PCR, sphere formation assay, DDP sensitivity assay, 64copper accumulation assay, tumorigenicity assay/immunohistochemistry, flow cytometric analysis, fluorescence activated cell sorting, cell cycle analysis, colony formation, chemoresistance assay, tumorigenicity assay/Immunohistochemistry, RT-PCR, Cell fractionation, immunocytochemistry, flow cytometry, colony formation	[51–53]
CD44	Tumorigenicity assay, flow cytometry, immunohistochemistry, fluorescence activated cell sorting, differentiation induction	[54]
ALDH	Fluorescence activated cell sorting, esospheres formation, tumorigenicity assay	[48]
ALDH1	Immunohistochemistry	[55]
CD90	Integrative RNA-Seq analysis, quantitative real time RT-PCR, flow cytometry and cell sorting, sphere formation, differentiation assay, chemoresistance assay, tumorigenicity and metastasis assay	[57]
ICAM1	Comparative membrane proteomic approach, quantitative PCR, western blot, sphere formation and drug resistance assay, tumorigenicity assay, metastasis assay	[58]
Cripto-1	Quantitative real time RT-PCR, western blot, fluorescence activated cell sorting, colony and limiting dilution sphere formation, xenograft, knockdown, metastasis assay	[47]
WASH	Sphere formation assay, quantitative real time RT-PCR, Immunohistochemistry, knockdown, overexpression, tumorigenicity assay	[59]
ALDH1A1	ALDH1A1 activity assay, fluorescence activated cell sorting, sphere formation assay, microarray analysis/immunohistochemistry, ALDEFLUOR assay and flow cytometry, limiting dilution assay, colony formation, xenograft, metastasis assay	[45,56]
CD133 and CXCR4	Immunohistochemical staining, flow cytometry, fluorescence activated cell sorting, colony-forming and transwell assay	[60]
ABCG2	Knockdown, overexpression, proliferation and migration assay	[49,61]

found in patients with ESCC and EAC, biomarkers for ESCC and EAC seem vastly different. For example, judged from Tables 2 and 3, autoantibodies against Trp53, Peroxiredoxin VI, and lncRNA POU3F3 were found in patients with ESCC, but not EAC. This may again indicate different cell of origin for ESCC and EAC.

4. Conclusion and remarks

ESCC and EAC are the two major subtypes of EC that seriously impact public health. Recent studies using animal models, cell lines and human biopsies began to shed insights into the cell of origin and potential diagnostic markers for both ESCC and EAC. ESCC and EAC have different cell of origin. Consequently, drastic differences have been identified in terms of gene expression and molecular profiling. ESCC and EAC are also associated with distinct risk factors which likely impact stem/progenitor cells, resulting in tumor initiation. In addition, although CSCs have been identified for both malignancies, it seems that they do not share many common cell surface markers which may correlate with their different cell origins.

Alterations in the levels of several molecules that are exclusively

Table 2
Potential diagnostic biomarkers for ESCC.

Types	Biomarkers	Methods	Tested Sample	References
Protein	L1-cell adhesion molecule	ELISA assay	blood	[83]
	Autoantibody against p53, NY-ESO-1, MMP-7, Hsp70, Prx VI, Bmi-1	ELISA assay	blood	[76]
	Stathmin-1	Competitive AlphaLISA and western blot	blood	[84]
	NY-ESO-1 autoantibody	ELISA assay	blood	[85]
	Angiopoietin-Like Protein 2	Quantitative real time RT-PCR, immunohistochemistry and ELISA assay	blood	[86]
	YKL-40, SCCA	ELISA assay, western blot, quantitative real time RT-PCR, immunohistochemistry and electrochemiluminescence	blood	[87]
	Macrophage inhibitory factor 1	ELISA assay, western blot, immunohistochemical staining and quantitative real time RT-PCR	blood	[88]
	Ghrelin	Radioimmunoassay	blood	[77]
	HSP70, HMGB1	Two-dimensional PAGE, MALDI-TOF MS, ELISA assay immunohistochemistry,	blood	[89]
	Hsp70 autoantibody	Two-dimensional PAGE and western blot, MALDI-TOF/TOF-MS, immunohistochemistry, ELISA assay	blood	[90]
	DKK1	Immunohistochemical staining and ELISA assay	blood	[91]
	Peroxiredoxin VI autoantibody	Two-dimensional PAGE and western blot, MALDI-TOF/TOFMS, immunohistochemistry, quantitative real time RT-PCR	blood	[92]
	Thomson-Friedenreich antigen	Peanut agglutinin -ELLA and Immunohistochemical staining	blood	[93]
	C-reactive protein	Immunohistochemical staining and latex photometric immunoassay	blood	[94]
	p53 antibody	ELISA assay and immunohistochemical staining	blood	[95]
LncRNA	HOTAIR	Quantitative real time RT-PCR	blood	[96]
	Linc00152, CFLAR-AS1, POU3F3	LncRNA microarray and quantitative real time RT-PCR	blood	[97]
	POU3F3, SCCA	Quantitative real time RT-PCR	blood	[80]
miRNA	MicroRNA-146a	Quantitative real time RT-PCR	blood	[78]
	MiR-25	Integration of two miRNA array-based approaches and quantitative real time RT-PCR	blood	[81]
	MiR-25, miR-100, miR-193-3p, miR-194, miR-223, miR-337-5p, miR-483-5p	TaqMan Low Density Array and quantitative real time RT-PCR	blood	[98]
	MiR-18a	Quantitative real time RT-PCR	blood	[82]
	MiR-1246	miRNA microarray and quantitative real time RT-PCR	blood	[99]
	MicroRNA-1322	Real time RT-PCR	blood	[100]
	MiR-21, miR-375	Quantitative real time RT-PCR	blood	[79]
	MiR-31	Real time RT-PCR	blood	[101]
	MiR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, miR-127-3p	Solexa sequencing	blood	[102]

Table 3
Potential diagnostic biomarkers for EAC.

Types	Biomarkers	Methods	Tested Sample	References
Protein	Complement C9	Lectin magnetic bead array (LeMBA), multiple reaction monitoring (MRM)-mass spectrometry	blood	[103]
	Amino acid L-proline, ketone body 3-hydroxybutyrate, carbohydrate D-mannose	Gas- and liquid- chromatography in combination with mass spectrometry, LC-MS/MS	blood	[104]
	Doublecortin-like kinase 1	Immunohistochemistry, western blot and ELISA assay	blood	[105]
	Biglycan, annexin-A6, myeloperoxidase, and protein S100-A9	Mass spectrometry-based proteomics discovery study	blood	[106]
	Mesothelin	Immunohistochemistry and double determinant (sandwich) immunoassay	blood	[107]
	FasL, NY-ESO-1 autoantibody	Luminex LabMAP protein array and CellCorrect microarray	blood	[108]
miRNA	miR-25-3p, miR-151a-3p, miR-100-5p, miR-375	Solexa deep sequencing	blood	[109]
	RNU6-1/miR-16-5p, miR-25-3p/miR-320a, let-7e-5p/miR-15b-5p, miR-30a-5p/miR-324-5p, miR-17-5p/miR-194-5p	TaqMan OpenArray miRNA RT-PCR	blood	[110]

observed in patients with EC can also be used for the development of diagnostic biomarkers. Previous studies have revealed that methylation and hypermethylation show different patterns in BE, ESCC, and EAC [111,112]. For instance, the *APC* gene becomes abnormally methylated and is detected in the plasma DNA in patients with EAC, suggesting that the hypermethylation status of *APC* is an indicator of aggressive disease in patients with EC [113]. With the advances in the development of high throughput technology in omics, such as genomics, proteomics, transcriptomics, RNomics, and immunomics, one would expect new biomarkers with high specificity and sensitivity will be identified [114]. Future studies are also needed to develop biomarkers for monitoring each stage of EC and response to chemotherapy [115].

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Abbreviations

BE	Barrett's esophagus
CEA	Carcino-embryonic antigen
CSCs	Cancer stem cells
EAC	Esophageal adenocarcinoma
EC	Esophageal cancer
ESCC	Esophageal squamous cell carcinoma
ICAM1	Intercellular adhesion molecule 1
SCJ	squamous columnar junction
TICs	Tumor-initiating cells

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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