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Aberrant DNA Methylation in Esophageal Squamous Cell Carcinoma: Biological and Clinical Implications

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Almost all cancer cells possess multiple epigenetic abnormalities, which cooperate with genetic alterations to enable the acquisition of cancer hallmarks during tumorigenesis. As the most frequently found epigenetic change in human cancers, aberrant DNA methylation manifests at two major forms: global genomic DNA hypomethylation and locus-specific promoter region hypermethylation. It has been recognized as a critical contributor to esophageal squamous cell carcinoma (ESCC) malignant transformation. In ESCC, DNA methylation alterations affect genes involved in cell cycle regulation, DNA damage repair, and cancer-related signaling pathways. Aberrant DNA methylation patterns occur not only in ESCC tumors but also in precursor lesions. It adds another layer of complexity to the ESCC heterogeneity and may serve as early diagnostic, prognostic, and chemo-sensitive markers. Characterization of the DNA methylome in ESCC could help better understand its pathogenesis and develop improved therapies. We herein summarize the current research and knowledge about DNA methylation in ESCC and its clinical significance in diagnosis, prognosis, and treatment.

Keywords: esophageal squamous cell carcinoma, aberrant DNA methylation, global DNA hypomethylation, promoter hypermethylation, heterogeneity, clinical significance

INTRODUCTION

As the ninth most common cancer in the world, esophageal carcinoma is also the sixth leading cause of cancer-related deaths (1). Esophageal squamous cell carcinoma (ESCC) is the predominant subtype of esophageal carcinoma and accounts for ∼90% of the cases (2). Although enormous progress has been made in early diagnosis and multimodal therapies, the prognosis of ESCC patients remains dismal, with the overall 5-year survival rate below 30% (2, 3). To achieve better clinical outcomes, a lot more work needs to be done to understand the pathogenesis of this disease thoroughly.

Intensive molecular biological studies have revealed that epigenetic dysregulation is a fundamental characteristic of nearly all human cancers (4). The most widely studied epigenetic modification is DNA methylation, which meanwhile is the most frequently found abnormal epigenetic change in human cancers. In mammals, DNA methylation occurs predominantly at the 5′ position of cytosine forming cytosine guanine dinucleotides. This modification is catalyzed

and maintained by enzymes known as DNA methyltransferases (DNMTs) (5). The two major DNA methylation changes that occur in human cancers, including ESCC, are global DNA hypomethylation and site-specific CpG island promoter hypermethylation (6, 7). Experimental studies indicate that DNA hypomethylation of repetitive sequences (i.e., long interspersed nucleotide elements, *LINEs*) may predispose cells to chromosomal defects and rearrangements that result in genetic instability. Thus, global DNA hypomethylation increases chromosomal instability, leading to cancer development (8, 9). On the other hand, hypermethylation of the CpG islands located in gene promoter regions may involve in carcinogenesis as a result of three possible mechanisms: (1) cytosine methylation facilitates gene mutation, as 5-methylcytosine is deaminated to thymine; (2) aberrant DNA methylation may be associated with allelic loss; (3) tumor suppressor genes (TSGs) may be inactivated by promoter hypermethylation (7, 10–12) (**Figure 1**).

In the context of tumor epigenetic heterogeneity, DNA methylation has also been the focus of intense research due to the quantitative nature of DNA methylation assays and the relative ease of obtaining sufficient stable genomic DNA (13, 14). Mechanistically, changes in DNA methylation contribute to the cancer development process through regulation of the spatial chromatin organization and alteration of the transcriptome at the right timing and environment (15). To date, DNA methylation heterogeneity has been quantified and been linked to clinical variables in various cancer types, including prostate cancer (16), chronic lymphocytic leukemia (17), glioblastoma (18), Ewing sarcoma (19), as well as ESCC (20). These findings demonstrate the power of DNA methylation sequencing in analyzing intratumor heterogeneity. At the same time, they suggest the extensive involvement of DNA methylation in tumor development.

In this brief review, we summarize recent advances regarding DNA methylation changes in the initiation and progression of ESCC. We also refer to their possible applications in the clinical management of ESCC patients.

GLOBAL DNA HYPOMETHYLATION AND PROMOTER CPG ISLAND HYPERMETHYLATION IN ESCC

Global DNA hypomethylation in cancer tissues was first observed more than 3 decades ago and has been recognized as a key mechanism involved in carcinogenesis (21, 22). Nevertheless, its presence and importance remain less well-understood in ESCC. To evaluate the global DNA methylation status in ESCC, several studies have measured the methylation level of the *LINE-1*. *LINE-1* constitutes a substantial portion (∼17%) of the human genome and has therefore attracted attention as a useful surrogate marker for global DNA methylation (23–27). Importantly, genome-wide hypomethylation was consistently observed in ESCCs, and *LINE-1* hypomethylation was strongly correlated with ESCC progression, leading to a poor prognosis among ESCC patients (23–26). However, this association still awaits further confirmation in larger cohorts, and potential mechanisms by which global DNA hypomethylation affects ESCC behavior have yet to be clearly revealed.

Compared with global genomic DNA hypomethylation, locus-specific DNA hypermethylation, which mostly occurs at promoter CpG islands, has been extensively studied in ESCC by candidate gene approaches. In ESCC, as well as other malignancies, aberrant hypermethylation in promoter CpG islands is involved in the major components of cell cycle regulation, DNA damage repair, and cancer-related signaling pathways. For example, cell cycle-related genes *CDKN2A*, *RASSF1A*, and *RASSF10* are frequently hypermethylated and transcriptionally silenced in ESCC (28–32). Inactivation of DNA repair genes *MGMT*, *MLH1*, and *MSH2* in ESCC mainly attributes to the methylation change in their promoter regions (33–35). Promoter hypermethylation in the genes of *APC*, *RUNX3*, and *ZNF382* respectively affects the Wnt/β-catenin, TGF-β, and NF-κB pathways in ESCC (36–40). Additionally, epigenetic studies on ESCC have discovered many known and putative TSGs that are frequently inactivated by promoter hypermethylation (**Table 1**). Although growing evidence has

FIGURE 1 | Schematic diagram of major DNA methylation changes in human cancer. In cancer cells, global hypomethylation at repetitive sequences might cause genetic instability, whereas site-specific CpG island promoter hypermethylation could lead to silencing of tumor suppressor genes (TSGs).
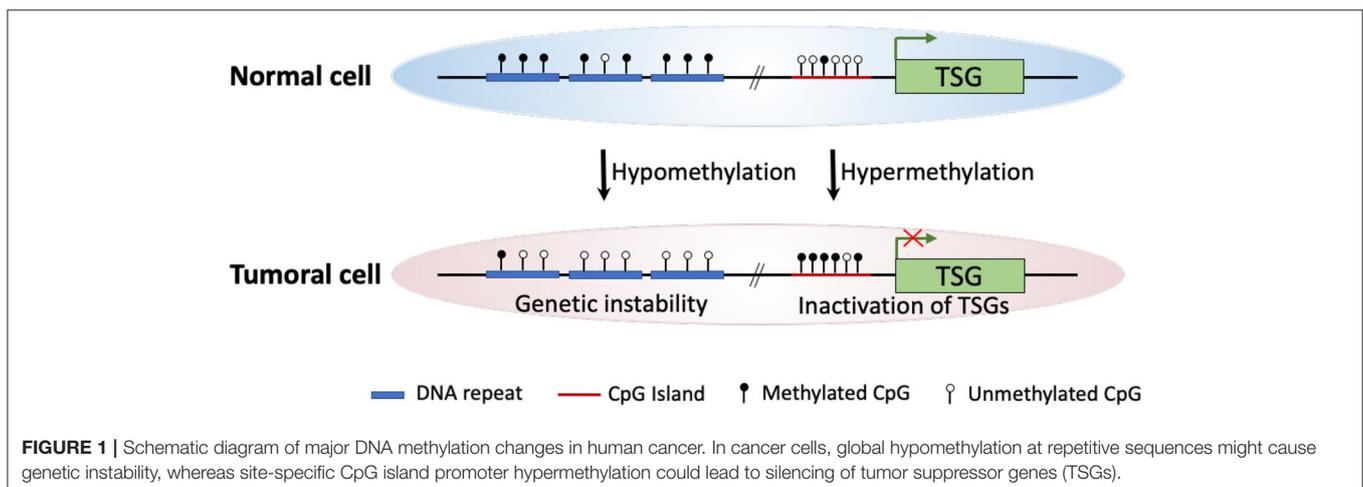
TABLE 1 | Aberrantly hypermethylated genes in ESCC.

Gene	Official full name	Function	References
RASSF1	Ras association domain family member 1	Cell cycle regulation	(24)
CDKN2A	Cyclin dependent kinase inhibitor 2A	Apoptosis Modulation and Signaling	(24)
BRCA1	BRCA1 DNA Repair Associated	DNA repair	(28)
CDH1	Cadherin 1	Cell adhesion	(28)
RAR-β	Retinoic acid receptor-beta	Nuclear receptor	(28)
DAPK	Death associated protein kinase	Programmed cell death	(28)
EPHA7	EPH receptor A7	Brain development	(28, 41)
MGMT	O-6-methylguanine-DNA methyltransferase	DNA damage reversal	(33–35)
MLH1	MutL homolog 1	DNA mismatch repair	(33, 35)
MSH2	MutS homolog 2	DNA mismatch repair	(33, 35)
APC	Adenomatous polyposis coli	Wnt signaling pathway	(36)
RUNX3	RUNX family transcription factor 3	Notch/TGF-beta receptor signaling pathway	(39)
ZNF382	Zinc finger protein 382	Transcription factor	(40)
FHIT	Fragile histidine triad diadenosine triphosphatase	Purine metabolism	(42)
TFF1	Trefoil factor 1	Secretory protein in gastrointestinal mucosa	(43)
HIN-1	Secretoglobin family 3A member (High in normal-1)	Secreted cytokine-like protein	(44)
ABCB4	ATP binding cassette subfamily B member 4	ATP-binding cassette transporter	(41)
PCDH10	Protocadherin 10	Cell adhesion	(41)
DOK1	Docking protein 1	Signal transduction	(41)
TAC1	Tachykinin precursor 1	Neurotransmitter	(45)
DKK3	Dickkopf WNT signaling pathway inhibitor 3	Wnt signaling modulator	(46)
SFRP1	Secreted frizzled related protein 1	Wnt signaling modulator	(47)
CHL1	Cell adhesion molecule L1 like	Signal transduction	(48)
RHCG	Rh family C glycoprotein	Ammonium transporter	(49)
PAX5	Paired box 5	Neural development; spermatogenesis	(50, 51)
CHFR	Checkpoint with forkhead and ring finger domains	Cell cycle progression	(52)
TFPI2	Tissue factor pathway inhibitor 2	Cell adhesion; plasmin signaling	(53)
BIN1	Bridging integrator 1	Neuroscience	(54)
ZNF132	Zinc finger protein 132	Transcription factor	(55)
PTX3	Pentraxin 3	Innate immune system; inflammatory reactions	(56)
RAB25	RAB25, member RAS oncogene family	Membrane trafficking	(57)
ECRG4	ECRG4 augurin precursor	Senescence	(58)
SPINT2	Serine peptidase inhibitor, Kunitz type 2	Transmembrane protein	(59)
EYA4	EYA transcriptional coactivator and phosphatase 4	DNA repair	(60)
SEMA3B	Semaphorin 3B	Neuronal development	(61)

demonstrated that tobacco smoking and alcohol consumption are the two major risk factors for ESCC, and correlations between gene promoter methylation and smoking exposure have also been observed in ESCC, the exact triggers and mechanisms responsible for aberrant ESCC DNA methylation changes remain elusive (62–65).

DNA METHYLATION CHANGE IS A CRITICAL EVENT IN THE PATHOGENESIS OF ESCC

The histologic progression of ESCC is a multi-step process that begins from normal squamous epithelium to low-grade dysplasia, high-grade dysplasia, carcinoma *in situ*, and invasive carcinoma (66, 67). In addition to genetic alterations, epigenetic abnormalities, in particular DNA methylation changes, have become widely accepted as common molecular features during ESCC development (68–70).

There is growing recognition that aberrant DNA methylation patterns occur in the earliest stage of ESCC and may parallel the histologic changes observed during ESCC carcinogenesis. For example, promoter methylation of TSGs, including *p16*, *p14*, *FHIT*, *MGMT*, and *TFF1* was detected in precancerous dysplastic lesions, indicating their early development in ESCC (42, 43). High in normal-1 (*HIN-1*) is a tumor suppressor gene that is highly expressed in many epithelial tissues. Methylation of this gene promoter occurs in the early stages of ESCC and accumulates with esophageal progression tendency during carcinogenesis, resulting in *HIN-1* silencing

in carcinomas-*in-situ* (44). By analyzing normal esophageal mucosa, esophageal dysplasia, and ESCC, Guo et al. also found clear evidence of accumulated methylation events during ESCC progression, which included accumulation of promoter region methylation of genes *MGMT*, *p16*, *BRCA1*, *MLH1*, *CDH1*, *RARβ2*, *DAPK*, and *APC* (28, 71). These findings are reminiscent of the idea that hypermethylation abnormalities may represent a "field cancerization" or "field defect" of preneoplastic changes, which occur early in carcinogenesis and predispose cells to subsequent malignant transformation (72–74).

From the standpoint of epigenetic heterogeneity, DNA methylation change is also involved in the evolutionary trajectory of ESCC. In 1976, Peter C. Nowell firstly proposed the Darwinian-like clonal evolution of tumor cells (75). Since then, models of tumor evolution derived from intratumor heterogeneity have greatly improved our understanding of tumorigenesis (76, 77). Investigations of intratumor heterogeneity at the DNA methylation level have provided unique opportunities in identifying epigenetic alterations associated with tumor evolution.

In ESCC, DNA methylation status within promoters of transcription factors SIM2 and SIX1 is strongly correlated with their heterogeneous expression pattern, which is further associated with ESCC differentiation and progression (78–80). Using the Illumina Human Methylation 450K (HM450) Bead array, Hao et al. performed multi-region global methylation profiling on 3 ESCC patients and found numbers of TSGs, including *EPHA7*, *ABCB4*, *PCDH10*, *DOK1*, etc., were heterogeneously hypermethylated at their promoters within tumors from the same individual (41). This observation suggests that intratumor heterogeneous DNA methylation may play a role in the subclonal diversification of ESCC tumors. Moreover, the construction of phylogenies independently from DNA methylation (phyloepigenetic tree) and somatic mutations (phylogenetic tree) yielded highly concordant as well as complementary evolutionary histories (41). This finding supports the concept of co-dependency of aberrant DNA methylation and genetic alterations and again demonstrates the involvement of DNA methylation heterogeneity in the ESCC evolutionary process. In an attempt to gain deeper insights into the DNA methylation heterogeneity of ESCC, most recently, Teng et al. generated single-base resolution whole-genome bisulfite sequencing data on 84 ESCCs and paired paraneoplastic tissues (20). Their analysis also identified numerous DNA methylation alterations associated with ESCC carcinogenesis and lymph node metastasis. In general, they found ESCC manifested substantial inter- and intratumor DNA methylation heterogeneity. Strikingly, the degree of intertumor DNA methylation heterogeneity of ESCC was even higher than that of prostate cancer or chronic lymphocytic leukemia, two well-recognized heterogeneous cancer types (16, 17). Furthermore, ESCC patients with a great extent of DNA methylation heterogeneity tended to experience more aggressive disease and worse overall survival (20).

Altogether, these data suggest the biological significance of DNA methylation change in ESCC. However, additional research is required to determine the extent to which intratumor

heterogeneity of DNA methylation reflects differences in regional or clone-specific driver or passenger events of ESCC, and to what extent the alterations to DNA methylation and genetics may be functionally related. It is also not yet known whether and how the pattern of DNA methylation change will evolve during ESCC progression.

CLINICAL IMPLICATIONS OF DNA METHYLATION CHANGE IN ESCC

In several cancer types, the methods of detecting aberrant DNA methylation changes have been applied to clinical fields related to early diagnosis, prognostic prediction, and personalized therapy.

In ESCC, as with other cancers, early detection is a prerequisite for treatment success and survival improvement. In fact, aberrantly methylated genes have been detected in the plasma or serum from ESCC patients, implying that they may serve as non-invasive diagnostic biomarkers. Using methylation-specific polymerase chain reaction, Das et al. detected the methylation status of *MGMT* from 100 newly diagnosed ESCC patients and reported that promoter hypermethylation of *MGMT* was present in 70% of serum samples (81). Zheng et al. evaluated the diagnostic role of *RUNX3* methylation in serum DNA of ESCCs and found that *RUNX3* hypermethylation was detectable in 51.4% (36/70) of the cases, which was significantly higher than that of the healthy donors (39). Moreover, Li et al. showed that the serum DNA detection of a panel of methylated genes, including *RAR-β*, *DAPK*, *CDH1*, *p16*, and *RASSF1A*, had a high diagnostic sensitivity of 82% and specificity of 100% for ESCC patients. They thereby suggested that it might be more efficient to early diagnose ESCC through integrative analysis of the methylation status of multiple TSGs (33). Of note, the early diagnostic value of aberrant methylation changes in serum DNA has been widely investigated by many other studies in ESCC. Such methylation events include hypermethylation of genes *SFRP1*, *CASZ1*, *CDH13*, *ING2*, *DKK-3*, *TAC1*, etc. (45–47, 82).

The aberrant methylation pattern has also been identified as a prognostic indicator for ESCC patients. Recently, promoter hypermethylation of *CHL1* and *RHCG*, two novel tumor suppressor candidates, has been reported to be associated with poor differentiation and increased invasion of ESCC, as well as advanced tumor stage and decreased overall survival (48, 49). Using DNA from the plasma of ESCC patients, Liu et al. analyzed the methylation status of Wnt antagonists *SFRP1*, *DKK3*, and *RUNX3* and showed that patients carrying two or three of these hypermethylated genes had a significantly elevated risk of cancer recurrence, compared with those without methylated genes (46). In an attempt to identify DNA methylation markers in predicting lymph node metastasis, the genome-wide methylation of 86 ESCC patients has been recently assessed, and a 10-probe lymph node metastasis-associated methylation signature has been established through stringent bioinformatics analyses (83). All in all, finding genes of prognostic impact aberrantly methylated allows one to customize therapy for ESCC patients: patients with a worse prognosis might benefit from a more aggressive

treatment strategy, while patients with low risk can forego radical surgery.

Methylation might result in the deactivation of genes that are responsive or unresponsive to chemotherapy and radiotherapy. Therefore, the methylation status of certain genes can be used to predict a patient's treatment response in advance (84). The best known example is *MGMT* promoter methylation and the resultant response to DNA alkylating agents in gliomas (85). However, ESCC studies in this regard are very limited. *PAX5* gene methylation was identified as an excellent marker for squamous cell carcinoma detection (50). According to Kurimoto et al.'s study, hypermethylation of *PAX5* was significantly correlated with increased ESCC cell proliferation and cisplatin resistance, leading to poor recurrence-free survival and overall survival (51). *CHFR* is an early mitotic checkpoint gene that controls cell cycle progression at the G2/M checkpoint and maintains chromosomal integrity (86). Promoter region methylation of *CHFR* was found frequently in ESCC and shown to sensitize ESCC to taxane treatment (52).

DISCUSSION

Epigenetic alterations have been recognized as key contributors to cancer initiation and progression. Amongst these, DNA methylation is one of the most extensively studied epigenetic modifications that occurs in the earliest stage of cancers (87). The aim of this review is to discuss the aberrant DNA methylation changes affecting ESCC carcinogenesis. However, many investigations into such epigenomic features of ESCC are still in their infancy.

Depending on the genomic location, DNA methylation may have different biological functions. Methylation in promoter CpG islands is typically associated with gene repression, while methylation in the gene body is usually linked to active gene expression. Accumulating evidence has shown that intergenic regions contain many regulatory elements, such as enhancers, silencers, and non-coding RNAs, and their functions may also be affected by DNA methylation (84). However, there is a current lack of knowledge with regards to these types of changes during ESCC development. Thus, the ESCC methylome landscape awaits further characterization through sequence-based approaches such as whole-genome bisulfite sequencing at single-base levels of resolution.

DNA methylation changes have emerged as clinically-relevant disease markers due to their chemical stability and measurable feasibility (88). As mentioned previously, there are numerous candidate methylation events that may serve as diagnostic, prognostic, and chemo-sensitive markers in

ESCC. However, the cancer type-specificity of many of these methylation changes remains to be determined in larger ESCC cohorts. Their predictive and prognostic value is also challenged by the property of tumor heterogeneity, especially when phenotypically diversified cancerous cells are unequally distributed and asynchronously evolving over space and time. This entails personalized and longitudinal studies assessing methylation changes in the course of ESCC development. It is also worth investigating whether the extent of DNA methylation heterogeneity by itself could be used as a clinically useful biomarker in ESCC.

Unlike genetic alterations that are essentially fixed, epigenetic changes, including DNA hypermethylation, are intriguingly dynamic and theoretically reversible. This makes them attractive targets for cancer therapy or chemoprevention (89). Indeed, several studies have demonstrated that TSG expression could be restored after treatment of cells with demethylating agents, and DNMT inhibitors, such as azacytidine and decitabine, are currently under preclinical and clinical investigations in various cancer types (e.g., ClinicalTrials.gov Identifier: NCT01193517, NCT03666559, and NCT04187703) (90–93). Nonetheless, up until now, very limited data have been shown concerning the effectiveness of DNMT inhibitors in ESCC. Future research on this field are required to gain a more in-depth insight into ESCC development and open a new area for ESCC treatment. Ideally, we will finally reach our goal of "epigenetic precision medicine" in ESCC.

AUTHOR CONTRIBUTIONS

LL conceived and wrote the review. XC and DY helped the revision. All authors contributed to the article and approved the submitted version.

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