Review

Aberrant DNA Methylation in Esophageal Squamous Cell Carcinoma and its Clinical Implications in Systemic Chemotherapy

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Abstract

Esophageal cancer is a significant global health concern, with esophageal squamous cell carcinoma being the predominant subtype in high-incidence regions like China. Despite advances in multidisciplinary treatments, the prognosis for ESCC remains poor, with systemic chemotherapy facing the challenge of drug resistance. Epigenetic alterations, particularly DNA methylation, play a crucial role in ESCC carcinogenesis and therapeutic response. Aberrant DNA methylations, including global hypomethylation and promoter-specific hyper-methylation, disrupt critical pathways such as cell cycle regulation, apoptosis, and DNA repair, contributing to chemoresistance. Several studies have identified methylation markers that predict treatment response, particularly for chemotherapy, targeted therapy and immunotherapy, such as *p16* and *GPX3* for cisplatin, *MTHFR* for 5-FU, *CHFR* for paclitaxel. DNA methyltransferase inhibitors and other epigenetic therapies are being explored to reverse these methylation changes and enhance therapeutic efficacy. However, the clinical utility of these markers remains limited due to the lack of large-scale validation and concerns over off-target effects. This review aims to summarize all aberrant methylation alterations in ESCC and the clinical implications of aberrantly methylated candidate genes identified in ESCC systemic chemotherapy, with the goal of further understanding the underlying molecular mechanisms, refining methylation-targeting therapies, and integrating them with conventional treatments to improve patient outcomes.

Keywords: esophageal squamous cell carcinoma, DNA methylation, systemic chemotherapy, therapeutic response, epigenetic therapy

Introduction

Esophageal cancer ranks as the 11th most common newly diagnosed cancer and is the 7th leading cause of cancer mortality worldwide, with approximately 511,000 new cases and 445,000 deaths reported in 2022 according to the latest global cancer statistics [1]. Esophageal cancer (EC) is histologically classified into esophageal squamous cell carcinoma (ESCC), esophageal adenocarcinoma (EAC) and other subtypes [2]. ESCC and EAC have significant differences in many features such as geographic distribution, etiologies, pathogenesis, physiological and molecular mechanisms [3-6]. Cigarette smoking, alcoholic abuse and inappropriate dietary pattern (e.g. nutritional deficiencies, nitrosamines, betel quid chewing, pickled vegetables and hot food/beverages) are the major risk factors for ESCC [5, 7]. Meanwhile EAC accounts for nearly two-thirds of esophageal cancer cases in high-income countries with excess body weight, gastroesophageal reflux disease, and Barrett's esophagus as the major risk factors [7].

Esophageal squamous cell carcinoma is the predominant subtype in China, accounting for approximately 90 percent of esophageal cancer and ranking the 7th in incidence and 5th in mortality [8]. Multidisciplinary treatments including surgery, radiotherapy, chemotherapy, molecular-targeting therapy and immunotherapy have improved overall survival of esophageal cancer patients, nonetheless the prognosis is still poor with overall 5-year survival ranging from 15% to 25% [9, 10].

Systemic chemotherapy which mainly consists of cytotoxic agents, molecular-targeted agents and immune checkpoint inhibitors play an essential role in the treatment of ESCC patients. Cytotoxic agents, such as cisplatin (DDP) and 5-fluorouracil (5-FU), have been used as the standard treatment for decades [11]. However, their limited efficacy due to resistance and inevitable adverse effects significantly impact the long-term survival of ESCC patients. In terms of immunotherapy, the Food and Drug Administration (FDA) has approved the PD-1 inhibitor pembrolizumab for second-line treatment of PD-L1 positive, advanced or metastatic ESCC [12, 13]. Based on phase 2 and phase 3 clinical trials, nivolumab as another PD-1 inhibitor was found to outperform conventional chemotherapy irrespective of PD-L1 status [14, 15]. Multiple molecular-targeted agents against EGFR, HER-2, VEGFR, c-Met and other oncogenes were also investigated in ESCC treatment. As vital candidates, anti-EGFR inhibitors such as cetuximab and nimotuzumab have some promising results in clinical trials [16, 17]. However, most molecular-targeted agents haven't demonstrated clinical utility in randomized phase 3 trials of ESCC so far.

Negative results from multiple systemic chemotherapy clinical trials suggest the importance of identifying predictive biomarkers of responses to specific agents. Nevertheless, definitive predictive biomarkers to determine right patient candidates remain unclear for conventional chemotherapy, targeted therapy and immunotherapy in patients with ESCC. Recently intriguing findings have emerged that both genetic and epigenetic alterations equally contribute to ESCC with collusion between them, which provides novel theories and understandings about therapeutic effect evaluation, potential therapeutic targets and prognostic prediction in ESCC patients [18].

DNA methylation, along with other epigenetic alterations such as histone modification, chromatin remodeling and the expression of microRNAs, plays an important role in transcriptional regulation, thus interfering driver genes expression [19, 20]. The best-understood epigenetic mechanism is DNA methylation, which mainly involves global hypomethylation and specific gene promoter hypermethylation especially of tumor suppressor genes (TSGs). Sufficient evidence has suggested that TSGs transcriptional silence by promoter hypermethylation and increased chromosome instability by global hypomethylation were critical steps in ESCC carcinogenesis [21]. Besides, epigenetic alterations of upstream transcription factors have been proved to cause tumor suppressor network silence as well. Silenced genes with aberrant methylation occurred in almost every significant molecular pathway associated with systemic chemotherapy, such as cell cycle regulation, cell apoptotic, DNA damage repair, cell adhesion and immune response regulation pathways [22].

DNA methylation has a crucial role in esophageal carcinogenesis, which was indicated in previous researches. More specifically, aberrant methylated genes have been detected in fields of early screening, diagnosis, prognosis and chemo-sensitivity prediction. Promising predictive markers have great potentiality to improve limited therapeutic effect and poor prognosis in ESCC. In this review, we aim to summarize the molecular mechanisms of aberrant methylated candidate genes in ESCC systemic chemotherapy and their clinical applications on biomarkers of chemotherapy response and therapeutic potentials.

Aberrantly methylated genes in ESCC

Numerous DNA methylation profiling researches have revealed that, similar to other cancers, ESCC genome exhibits widespread regions of hypomethylation and focal regions of hypermethylation, when compared with adjacent normal tissues or noncancer controls [23, 24]. Although the general patterns of hyper- and hypomethylation are similar, the distribution of specific methylation sites differs from that observed in other related cancers (e.g. head and neck squamous cell carcinoma). Large-scale genomic and epigenomic data further confirm that ESCC and EAC exhibit nearly mutually exclusive sets of driver genes and significantly distinct epigenetic alterations. Hence, a focused summary of aberrant methylation alterations in ESCC is both crucial and indispensable.

As illustrated in **Figure 1**, these alterations in the epigenetic landscape, characterized by global genomic DNA hypomethylation and specific genes promoter hypermethylation, both contribute to the pathogenesis and development of ESCC via distinct mechanisms [25]. Besides those, the occasional hypomethylation of specific genes has also seen some research progress, which will be detailed in the following sections.

Genome-wide Hypomethylation
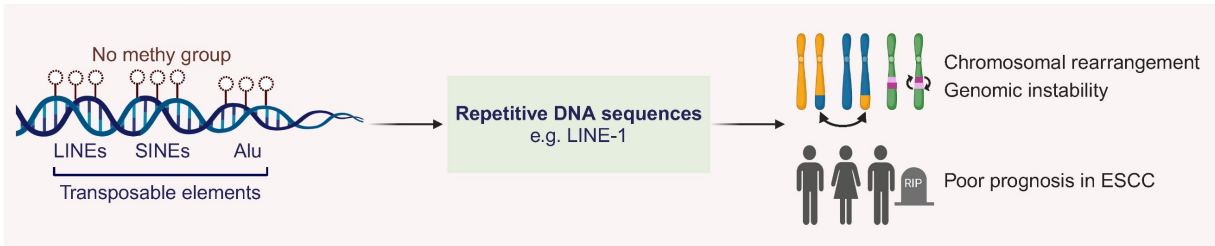
Promoter-specific Hypermethylation
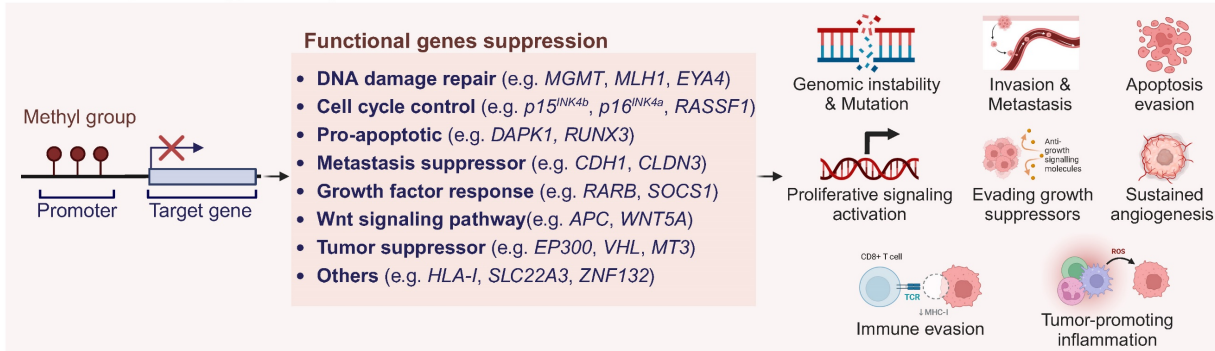
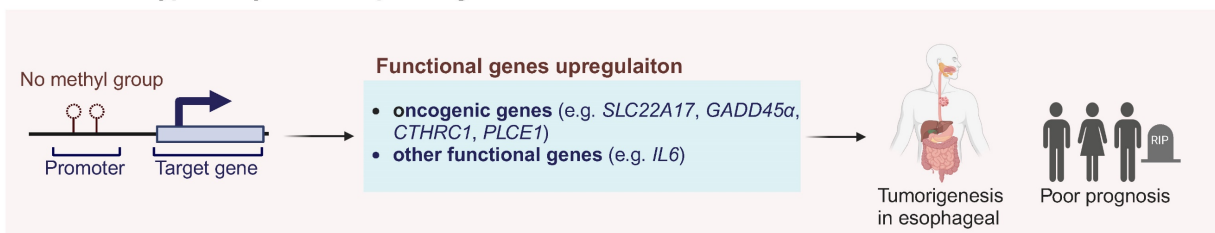**Occasional hypomethylation of specific genes**

Figure 1. Aberrant DNA methylation patterns in ESCC. Promoter hypermethylation silencing tumor suppressor genes and hypomethylation of repetitive DNA sequences lead to genomic instability and tumorigenesis. Occasional hypomethylation of specific genes were also reported to contribute to ESCC development. Abbreviations: LINEs, long interspersed nuclear elements; SINEs, short interspersed nuclear elements; LINE-1, long interspersed nuclear element-1; ESCC, esophageal squamous cell carcinoma; TCR, T-cell receptor; MHC-I, major histocompatibility complex class I; ROS, reactive oxygen species. Created in BioRender. Li, YK. (2025) https://BioRender.com/z74h803 (accessed on 25 January 2025).

Global genomic DNA hypomethylation

Feinberg et.al [26] reported global genomic DNA hypomethylation in tumor tissues as early as 1983. Gaudet et.al [27] managed to induce tumorigenesis in mice by genomic hypomethylation, which validated the correlation between genomic hypomethylation and tumorigenesis. However, the underlying mechanism of global genomic DNA hypomethylation in ESCC is still equivocal. Current studies showed that DNA hypomethylations, especially those located in long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and other highly repetitive DNA sequences like Alu elements, could destabilize chromosome and cause rearrangements [28, 29]. LINE-1 elements constitute nearly 17% of human genome as important parts, so methylation of LINE-1 indicates the global DNA methylation level [21]. Variable studies showed that LINE-1 methylation in ESCC ranged from 25% to 92%, and low methylation level of LINE-1 was indicated to

correlated significantly with poor prognosis [30, 31]. Global genomic DNA hypomethylation could still influence tumorigenesis through some other mechanisms that require further investigations [25].

Promoter-specific hypermethylation in ESCC

DNA methylation has become the best-studied epigenetic field due to the stable characteristic and credible detection techniques. Previously published studies have shown that as much as hundreds of genes have been found to be silenced by promoter hypermethylation. Numbers of evidences have suggested that the promoter hypermethylation of tumor suppressor genes is the critical step in carcinogenesis of ESCC [32]. In the Table 1, we summarized all reported TSGs in ESCC which play significant roles in signal pathways including cell-cycle regulation, apoptosis control, cell adhesion, metastasis, DNA damage repair, growth factor response, etc. The other functional genes which are silenced by promoter hypermethylation, accompanied

with detailed gene information and corresponding methylation frequency in tumor tissues, were also listed as below.

Rare hypomethylation of specific genes in ESCC

According to the comprehensive analysis of the DNA methylation profile in ESCC, only 12.4% out of 26081 differentially methylated regions exhibited hypomethylation while 87.6% showed hypermethylation [106]. In ESCC carcinogenesis, the phenomenon of occasional hypomethylation of specific genes has been a subject of intense research [38, 106]. Cui *et al.* found that the hypomethylation of *NGALR* (*SLC22A17*) gene contributes to its overexpression in ESCC, implicating its role in tumorigenesis [107]. *GADD45a*, involved in DNA repair, is also overexpressed due to promoter hypomethylation in ESCC [108]. The DNA polymerase iota (*Polι*) gene, overexpressed in ESCC, is linked to hypomethylation, potentially driving genomic instability [109]. The *IGF2* gene is influenced by hypomethylation at its DMR0, correlating with poor prognosis [110]. Similarly, the *OCT1* and *CTHRC1* genes are upregulated in ESCC due to hypomethylation, activating MAPK/MEK/ERK pathway and facilitating tumor progression [111, 112]. The hypomethylation-associated upregulation of *PLCE1* has been linked to tumorigenesis, tumor angiogenesis and poor prognosis in ESCC [113]. The *IGFBP7* gene shows lower promoter methylation, with its unmethylated state correlating with increased globulin levels and reflux in ESCC patients [114]. Notably, the gene *CDKN1C*, a putative tumor suppressor, exhibits diminished expression associated with the loss of methylation at the differentially methylated region (DMR)-LIT1, rather than its own promoter CpG methylation [115]. Lastly, the interleukin 6 (*IL6*) gene, which is known to activate the JAK/STAT3 pathway, is implicated in ESCC and influenced by hypomethylation [45]. These findings underscore the complex interplay between hypomethylation and the dysregulation of key genes in ESCC pathogenesis.

Clinical implications of aberrantly methylated genes in systemic chemotherapy

DNA methylation and therapeutic response prediction in ESCC

As mentioned in the above review, DNA methylation has been implicated in silencing apoptotic genes, disrupting DNA repair mechanisms, dysregulating cell cycle and even enhancing drug efflux, all of which contribute to chemotherapy resistance. Notably, these specific genes epigenetically modified by DNA hypermethylation regulate cancer response to systemic chemotherapy, making them potential predictive markers for therapy response, as shown in **Figure 2**.

In 2006, Hamilton *et al.* firstly identified a combined 9-gene panel (*p16, REPRIMO, p57, p73, RUNX-3, CHFR, MGMT, TIMP-3,* and *HPP1*) to predict adjuvant chemotherapy (DDP and 5-FU) responses based on methylation levels from 12 ESCC patients and 23 EAC patients [116]. Among these genes, *REPRIMO* and *p16*, key regulators of the cell cycle, exhibited significantly higher methylation rates in esophageal cancer patients non-responsive to chemoradiation, and promoter methylation of both genes impairs the ability to arrest at the G2/M checkpoint, where cisplatin exerts their effects, leading to chemoresistance [116, 117]. The study conducted by Chang *et al.* in 2017 stands out for establishing a six-CpG panel, including *IFNGR2, KCNK4, NOTCH4, NPY, PAX6,* and *SOX17*, which predicts treatment response to chemoradiation (DDP, 5-FU and concurrent radiotherapy). This panel's risk score equation, derived from the methylation status of selected genes in 91 ESCC patients, demonstrated a remarkable power in discriminating poor responders from good responders, with an AUC of 0.930, underscoring its potential in personalized medicine [118]. Iwabu *et al.* identified *FGF5* as an oncogenic growth factor and its methylation as a predictive marker for definitive chemoradiotherapy (dCRT) sensitivity from 117 ESCC patients, with low methylation linked to poor response (sensitivity 45%, specificity 90%), highlighting its potential clinical utility. FGF5 could be induced by dCRT in ESCC when it is unmethylated, supporting cell survival and leading to resistance [119]. In a comprehensive study by Salta *et al.* to identify DNA methylation-based biomarkers for early detection and prediction of response to therapy in ESCC patients, the methylation of *GPX3* has been identified as a potential marker for predicting responses to therapy. *GPX3* encodes glutathione peroxidase, which mitigates oxidative damage. Methylation-induced inactivation, along with compensatory overexpression of other antioxidants and increased genomic instability, further enhances cisplatin resistance in cancer cells [120]. Based on molecular profiles from 36 ESCC patients in 2021, de Klerk *et al.* revealed that *TP63* amplification and *TFPI2* gene promoter methylation are associated with unfavorable responses to neoadjuvant chemoradiotherapy which consists of carboplatin, paclitaxel and concurrent radiotherapy [121]. Huang *et al.* demonstrated that *PON3*

hypermethylation downregulates its expression, contributing to multidrug resistance in ESCC, although the underlying mechanisms remain to be further elucidated [122]. The study by Min *et al.* sheds light on the epigenetic evolution of acquired resistance to combination therapy (cisplatin and verapamil) in ESCC. *SLC8A3* was identified as a potential multidrug resistance gene from 6 ESCC patients' WGBS results, whose promoter descending methylation dynamics were associated with resistance to chemotherapy [123].

Table 1. Hypermethylated functional genes in esophageal squamous cell carcinoma

Classification	Year	Gene Symbol	Description	Major functions	Location	Methylation rate in tumor (%)	References
DNA damage repair genes	1998	FHIT	Fragile Histidine Triad Diadenosine Triphosphatase	DNA damage response, cell-cycle regulation	3p14.2	14-33, 50	[33, 34]
	2003	MGMT	O-6-Methylguanine-DNA Methyltransferase	DNA damage repair	10q26	27-72	[35, 36]
	2005	MLH1	MutL Homolog 1	DNA mismatch repair, cell-cycle regulation	3p22.3	3-62	[37-39]
	2005	MSH2	MutS Homolog 2	DNA mismatch repair, cell-cycle regulation	2p21	29-32	[40]
	2006	BRCA1	BRCA1 DNA Repair Associated	DNA damage repair, cell-cycle regulation, transcriptional activator	17q21.31	26-28	[38]
	2015	HIC1	HIC ZBTB Transcriptional Repressor 1	DNA damage response, apoptosis, Wnt signaling pathway, tumor suppressor	17p13.3	84.2	[41]
	2018	EYA4	EYA Transcriptional Coactivator and Phosphatase 4	DNA damage repair, migration and invasion, epithelial-mesenchymal transition, AKT signaling pathway, tumor suppressor	6q23.2	78-85.7	[42]
Cell cycle control genes	1999	CDKN2A (p16^{INK4a})	Cyclin Dependent Kinase Inhibitor 2A	Cell-cycle regulation, tumor suppressor	9p21	12-88	[35, 37, 43-45]
	1999	CDKN2B (p15^{INK4b})	Cyclin Dependent Kinase Inhibitor 2B	Cell-cycle regulation	9p21	13-18	[43]
	2003	RASSF1	Ras Association Domain Family Member 1	Cell-cycle regulation, apoptosis	3p21.3	14-53	[34]
	2015	CHFR	Checkpoint With Forkhead and Ring Finger Domains	Cell cycle and apoptosis, tumor suppressor	12q24.33	45	[46]
Pro-apoptotic genes	2005	UCHL1 (PGP9.5)	Ubiquitin C-Terminal Hydrolase L1	Ubiquitin regulation, cell growth inhibition, apoptosis, tumor suppressor	4p13	42	[47]
	2006	DAPK1	Death Associated Protein Kinase 1	Cell survival, apoptosis, and autophagy	9q21.33	26-38	[38, 44]
	2010	ZNF382	Zinc Finger Protein 382	Pro-apoptotic transcription factor, tumor suppressor	19q13.12	89	[48]
	2011	RUNX3	RUNX Family Transcription Factor 3	Cellular growth and differentiation, Notch and TGF-β signaling pathway, tumor suppressor	1p36.11	35.8-51.4	[49, 50]
Metastasis suppressor genes	2003	CADM1 (TSLC1)	Cell Adhesion Molecule 1	Migration and invasion, cell proliferation, tumor suppressor	11q23.3	50	[51]
	2004	CDH1 (E-cadherin)	Cadherin 1	Cell adhesion, proliferation, metastasis, epithelial-mesenchymal transition	16q22.1	14-61	[38, 44, 52]
	2004	CDH13	Cadherin 13	Cell adhesion and recognition, tumor growth, cell cycle, tumor suppressor	16q23.3	14-39.4	[53]
	2006	PCDH10	Protocadherin 10	Cell-cell interaction, cell migration, tumor suppressor	4q28.3	51-81	[54]
	2006	CLDN3	Claudin 3	Cell-cell adhesion, cell tight junctions	7q11.23	69	[35]
	2008	DCC	DCC Netrin 1 Receptor	Cell adhesion, cell proliferation and differentiation, apoptosis, tumor suppressor	18q21.2	74	[55]
	2010	UPK1A	Uroplakin 1A	Cell motility, metastasis, tetraspanin superfamily, tumor suppressor	19q13.12	62	[56]
	2011	CLDN4	Claudin 4	Cell-cell adhesion, cell tight junctions	7q11.23	41.7	[57]
	2012	CDH11	Cadherin 11	Cell adhesion, migration and invasion, Wnt/β-catenin signaling pathway	16q21	93	[58]
Growth factor response-related genes	2003	RARB	Retinoic Acid Receptor Beta	Growth factor response, cellular growth and differentiation	3p24	25, 39, 67-70	[34, 35, 44]
	2005	RBP1	Retinol Binding Protein 1	Retinoid signaling	3q23	17.9-31	[35, 59]
	2005	RARRES1 (TIG1)	Retinoic Acid Receptor Responder 1	Retinoid signaling	3q25.32	17.9	[59]
	2007	CRABP1	Cellular Retinoic Acid Binding Protein 1	Cell cycle, proliferation, tumor suppressor	15q25.1	52.8	[60]
	2011	SOCS1	Suppressor Of Cytokine Signaling 1	Negative regulator of JAK/STAT pathway and interferon signaling, tumor suppressor	16p13.13	45.3	[61]

Classification	Year	Gene Symbol	Description	Major functions	Location	Methylation rate in tumor (%)	References
Wnt signaling related genes	2007	*APC*	APC Regulator of WNT Signaling Pathway	Wnt signaling pathway, cell polarity and chromosome segregation	5q21-q22	27-46	[37]
	2007	*SFRP1*	Secreted Frizzled Related Protein 1	Wnt signaling modulator	8p11.21	29.6-64.3	[37, 44, 50]
	2007	*SFRP2*	Secreted Frizzled Related Protein 2	Wnt signaling modulator	4q31.3	19.6	[37]
	2007	*WIF1*	WNT Inhibitory Factor 1	Wnt signaling pathway inhibitor	12q14.3	27-35	[44, 62]
	2011	*DKK3*	Dickkopf WNT Signaling Pathway Inhibitor 3	Wnt signaling inhibitor, tumor suppressor	11p15.3	37.4	[50]
	2012	*SOX17*	SRY-Box Transcription Factor 17	Cell proliferation, Wnt signaling pathway, transcription regulator	8q11.23	65	[63]
Genes with tumor suppressive functions	2003	*ECRG4*	ECRG4 Augurin Precursor	Cell proliferation, cell cycle, senescence, tumor suppressor	2q12.2	80	[64]
	2003	*PRSS3 (Trypsinogen 4)*	Serine Protease 3	Proteolytic activity, tumor suppressor	9p13.3	50	[65]
	2003	*VHL*	Von Hippel-Lindau Tumor Suppressor	Ubiquitin ligase component, tumor suppressor	3p25.3	36	[34]
	2005	*MT3*	Metallothionein 3	Cellular growth inhibition, intracellular metal homeostasis	16q13	52	[66]
	2006	*GRIN2B (NMDAR2B)*	Glutamate Ionotropic Receptor NMDA Type Subunit 2B	Apoptosis, signal transduction, tumor suppressor	12p13.1	95	[67]
	2007	*DLC1*	DLC1 Rho GTPase Activating Protein	Cytoskeleton organization, Ras-mediated signaling pathways, cell adhesion, cell cycle, tumor suppressor	8p22	51	[68]
	2007	*CDX2*	Caudal Type Homeobox 2	Cellular growth and differentiation, transcription factor	13q12.2	49	[69]
	2007	*PLCD1*	Phospholipase C Delta 1	Phospholipase, cell proliferation, cell cycle, migration and invasion, tumor suppressor	3p22.2	38	[70]
	2007	*ADAMTS18*	ADAM Metallopeptidase with Thrombospondin Type 1 Motif 18	Tumor microenvironment modulator, cell proliferation, tumor suppressor	16q23	52-88	[71]
	2007	*EP300*	E1A Binding Protein P300	Transcription regulator activity, tumor invasion and metastasis	22q13.2	42	[72]
	2008	*GNG7*	G Protein Subunit Gamma 7	Signal transduction, tumor suppressor	19p13.3	33.3	[73]
	2008	*SST*	Somatostatin	Somatostatin hormone, tumor suppressor	3q27.3	53.8	[74]
	2008	*HOPX*	HOP Homeobox	Tumor suppressor, serum response factor-related pathway	4q12	50	[75]
	2008	*ITGA4*	Integrin Subunit Alpha 4	Cell attachment to ECM, signal transduction, tumor suppressor	2q31.3	21	[44]
	2008	*IRF8*	Interferon Regulatory Factor 8	Transcriptional factor, IFN-γ signaling pathway, immune response, cellular growth and differentiation, tumor suppressor	16q24.1	58	[76]
	2008	*ENG*	Endoglin	TGF-β signaling pathway, angiogenesis, tumor suppressor	9q34.11	46.2	[77]
	2009	*KAT2B (PCAF)*	Lysine Acetyltransferase 2B	Transcription regulator activity, histone acetylation, cell cycle, tumor suppressor	3p24.3	70	[78]
	2010	*NEFH*	Neurofilament Heavy Chain	Mitochondrial function, glycolysis, Akt/β-catenin pathway, tumor suppressor	22q12.2	65	[79]
	2011	*HSPB2*	Heat Shock Protein Family B (Small) Member 2	Stress response and resistance, heat shock protein activity, tumor suppressor	11q23.1	95.7	[80]
	2012	*PTK6*	Protein Tyrosine Kinase 6	Cell proliferation, cellular differentiation, apoptosis, migration and invasion, tumor suppressor	20q13.33	40	[81]
	2012	*TFPI2*	Tissue Factor Pathway Inhibitor 2	Proteolytic activity, ECM degradation, tumor suppressor	7q21.3	67	[82]
	2012	*RAB25*	RAB25, Member RAS Oncogene Family	Cell survival, migration, invasion and angiogenesis, MAPK/ERK signaling pathway, tumor suppressor	1q22	75-100	[83]
	2013	*DIRAS1*	DIRAS Family GTPase 1	Apoptosis, cell motility, ERK and p38 MAPK signaling pathways, tumor suppressor	19p13.3	40	[84]
	2014	*DACH1*	Dachshund Family Transcription Factor 1	TGF-β signaling, transcription factor, tumor suppressor	13q21.33	61.5	[85]
	2014	*RASSF10*	Ras Association Domain Family Member 10	Cell proliferation, cell cycle regulation, microtubule stability, tumor suppressor	11p15.3	44.3	[86]
	2014	*ADAMTS8*	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 8	Cell proliferation, apoptosis, EGFR-MEK-ERK signaling pathway, tumor suppressor	11q24.3	22	[87]
	2014	*SPINT2*	Serine Peptidase Inhibitor, Kunitz Type 2	Cell proliferation, apoptosis, tumor suppressor	19q13.2	52.08	[88]

| Classification	Year	Gene Symbol	Description	Major functions	Location	Methylation rate in tumor (%)	References
	2015	*RASSF5A*	Ras Association Domain Family Member 5A	Apoptosis, tumor suppressor	1q32.1	47.1	[89]
	2017	*BIN1*	Bridging Integrator 1	Cell proliferation, tumor suppressor	2q14.3	62	[90]
	2017	*PAX1*	Paired Box 1	Transcription factor, tumor suppressor	20p11.22	96-100	[91, 92]
	2018	*RHCG*	Rh Family C Glycoprotein	Ammonium transporter, invasion, metastasis, NF-κB signaling pathway, tumor suppressor	15q26.1	86.4	[93]
	2019	*CHL1*	Cell Adhesion Molecule L1 Like	Cell proliferation, cell cycle, metastasis, AKT signaling pathway, tumor suppressor	3p26.3	59.0	[94]
	2019	*SEMA3B*	Semaphorin 3B	Cell proliferation, invasion, tumor suppressor	3p21.31	54.3	[95]
	2024	*PREX2*	Phosphatidylinositol-3,4,5-Trisphosphate Dependent Rac Exchange Factor 2	Migration, invasion, tumor suppressor	8q13.2	14.6	[96]
Other functional genes	2006	*GATA4*	GATA Binding Protein 4	Zinc-finger transcription factor	8p23.1	61	[97]
	2006	*GATA5*	GATA Binding Protein 5	Zinc-finger transcription factor	20q13.33	32	[97]
	2006	*MT1G*	Metallothionein 1G	Cellular stress response, metal metabolism, detoxification	16q13	62	[35]
	2007	*TAC1*	Tachykinin Precursor 1	Neurotransmitter	7q21.3	50	[98]
	2007	*CACNA1G*	Calcium Voltage-Gated Channel Subunit Alpha1 G	Calcium-dependent process, hormone or neurotransmitter release, cell proliferation, cell motility and cell death	17q21.33	23.2	[37]
	2008	*SCGB3A1 (HIN-1)*	Secretoglobin Family 3A Member 1	Signal transduction, cytokine activity, cellular growth	5q35.3	50-72	[99]
	2011	*HLA-I*	Major Histocompatibility Complex, Class I	Immune response, immune evasion, metastasis	6p21-22	70.1	[100]
	2011	*PTX3*	Pentraxin 3	Innate immune defense, inflammatory reactions, angiogenesis	3q25.32	85	[101]
	2011	*GPX3*	Glutathione Peroxidase 3	Antioxidative defense	5q33.1	54.8-71.4	[102]
	2017	*PAX5*	Paired Box 5	Cell proliferation, cell cycle, chemosensitivity, transcription factor	9p13.2	85.9	[103]
	2017	*ZNF582*	Zinc Finger Protein 582	Transcriptional regulator	19q13.43	85.7-93.2	[91, 92]
	2018	*ZNF132*	Zinc Finger Protein 132	Cell proliferation, apoptosis, migration, transcription factor	19q13.43	70.8	[104]
	2018	*SLC22A3*	Solute Carrier Family 22 Member 3	Antioxidative defense, DNA damage repair	6q25.3	NA	[105]
	2019	*SOX1*	SRY-Box Transcription Factor 1	Transcription factor	13q34	89.2	[92]
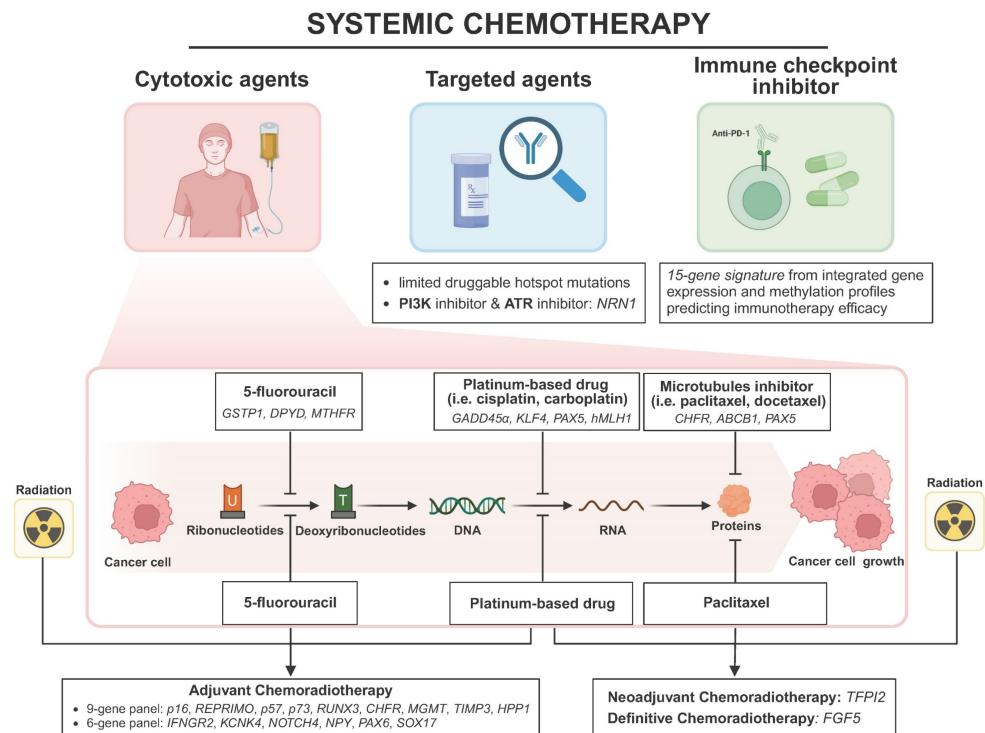
Figure 2. Overview of DNA methylation markers in ESCC systemic chemotherapy, including cytotoxic agents, targeted agents and immune checkpoint inhibitors. Abbreviations: PI3K, phospho-inositol-3 kinase; ATR, ataxia telangiectasia and Rad3-related protein kinase. Created in BioRender. Li, YK. (2025) https://BioRender.com/d45q992 (accessed on 25 January 2025).

Other findings about methylation markers in predicting platinum-based drug efficacy are as follows. Wang *et al.* linked *GADD45a* overexpression to promoter hypomethylation, affecting cisplatin sensitivity through the disruption of apoptotic pathways [108]. Lin *et al.* demonstrated that long-term cisplatin exposure induces *OCT1* methylation, causing cisplatin resistance [124]. Both *KLF4* and *PAX5* gene methylation were discovered to predict cisplatin sensitivity and clinical outcomes. KLF enhances cisplatin sensitivity in ESCC cells through apoptosis induction and cell cycle arrest, whereas PAX5 contributes to cisplatin resistance by regulating GLUT1, a known chemoresistance factor in cancer cells [103, 125]. Cao *et al.* confirmed the role of *hMLH1* promoter methylation in cisplatin resistance through mismatch repair deficiency, suggesting demethylation treatment may enhance conventional chemotherapy efficacy [126].

About the response to 5-fluorouracil, *GSTP1* encoding a detoxifying enzyme was found to be methylated by DNMTs which were recruited by the long noncoding RNAs LINC01270 and LINC01419, thereby reducing the chemosensitivity of ESCC to 5-FU [127, 128]. Meanwhile, dihydropyrimidine dehydrogenase (DPYD) as a 5-FU degrading enzyme, was repressed by LINC00261 overexpression via the methylation-dependent manner, ultimately enhancing the 5-FU response in ESCC [129]. Moreover, *MTHFR* methylation mediated by lncRNA HOTAIR inhibits 5-FU sensitivity by regulating the systemic exposure of ESCC cells to 5-FU, suggesting that targeting HOTAIR or *MTHFR* could potentially overcome chemoresistance [130].

As for the efficacy of microtubule inhibitors, Yun *et al.* reported that *CHFR* methylation sensitizes ESCC cells to docetaxel and paclitaxel. This occurs because the CHFR protein's role at the prophase checkpoint, where it delays mitotic entry in response to microtubule poisons, thereby attenuating the cytotoxic efficacy of microtubule inhibitors [46]. Sumarpo *et al.* found that *ABCB1* gene amplification and promoter demethylation contribute to the acquisition of taxane resistance in ESCC cell lines [131]. Additionally, Zhang *et al.* revealed *PAX5* as a tumor suppressor gene regulated by promoter methylation, sensitizing ESCC cell lines to docetaxel and 5-FU by promoting p53 signaling activity [132].

With regards to molecular-targeted therapies, few studies on ESCC exist, most of which target EGFR and limited druggable hotspot mutations. Du *et al.* found that NRN1 methylation is a sensitive marker for PI3K-Akt-mTOR and ATR inhibitors, implying that demethylation of NRN1 could enhance the efficacy of these inhibitors in ESCC [133].

Recent multiple randomized studies have shown that immune checkpoint inhibitors (ICIs), especially PD-1/PD-L1 inhibitors, exhibit antitumor activity in advanced ESCC [134, 135]. Nonetheless, the predictive value of existing maker for immunotherapy like PD-L1 status in ESCC is unsatisfactory [136]. Identifying sensitive and solid predictive markers is essential to improve response and prognosis. Zheng *et al.* integrated gene expression and DNA methylation profiles to characterize two molecular subtypes of ESCC associated with distinct immune-related pathways and clinical outcomes. The 15-gene expression signature could predict the response rate to immunotherapy [137].

Researches on methylation biomarkers for predicting therapeutic response in ESCC are still ongoing. The studies mentioned above contribute to stratifying ESCC patients for personalized therapy and provide new strategies for overcoming chemotherapeutic drug resistance.

DNA methylation as a therapeutic target in ESCC

DNA methylations differ from genetic alterations by being reversible, making them promising targets for therapeutic intervention [138]. DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B, catalyze DNA methylation process. Nucleoside analogs such as 5-azacytidine and its derivative 5-aza-2'-deoxycytidine (decitabine) inhibit DNMT activity and are currently approved for treating myelodysplastic syndrome (MDS), significantly improving patient survival. Theoretically, DNMT inhibitors can reverse the hypermethylation-mediated silencing of tumor suppressor or other functional genes. Consequently, multiple preclinical and clinical trials are carried out to assess the efficacy of these agents in solid tumors, including ESCC [139, 140]. To be noted, in a phase Ib/II study by Chen et al. low-dose decitabine-primed chemoimmunotherapy re-sensitized resistant cancer cells and showing promising safety and efficacy in ESCC patients, suggesting DNMT inhibitor as a potential therapeutic agent in relapsed/refractory ESCC patients [140]. Two independent researches both highlighted that low-dose decitabine epigenetically upregulates MAGE-A antigens, enhancing T cell-mediated tumor recognition and antigen-specific responses. These findings suggest that combining decitabine with immunotherapy may be a promising strategy for treating advanced ESCC [141, 142].

Recently Chang et al. demonstrated that Nutlin-3, a murine double min 2 (MDM2) small molecule inhibitor, could function as a novel DNMT

inhibitor via enhancing p53 and RB expression, making it a promising DNMT inhibitor for sensitizing chemoradiation-resistant ESCC to therapy [143]. Besides that, natural compounds have demonstrated potential as DNMT inhibitors. Fang et al. reported that (-)-epigallocatechin-3-gallate (EGCG), a major green tea extract, inhibits DNMT1 activity, reversing methylation and restoring the expression of tumor suppressor genes such as *CDKN2A*, *RARB*, *MGMT*, and *MLH1* in ESCC cell lines [144]. Similarly, genistein, a soy-derived isoflavone, was found to reverse the hypermethylation of TSGs via a direct inhibition of DNA methyltransferase, suggesting these natural extracts could contribute to the chemoprevention of ESCC [145]. Huang *et al.* indicated that black raspberries (BRBs) could reduce DNMT1 and DNMT3b mRNA levels, thereby demethylating Sfrp4, inhibiting WNT signaling and suppressing esophageal tumorigenesis in rats, a model akin to human ESCC. This suggests that BRBs may prevent the aberrant DNA methylation in ESCC development, offering a natural approach to target epigenetic alterations in cancer prevention [146].

Nevertheless, currently available DNMT inhibitors are not sufficiently selective, leading to off-target adverse effects including global DNA hypomethylation, oncogene activation and increased genomic instability. Therefore, further researches are needed to develop targeted therapies that specifically reverse the hypermethylation of tumor suppressor genes, thereby improving therapeutic efficacy.

In addition to DNMTs, TET1 as a CpG demethylase was reported to have the catalytic domain to inhibit the CpG methylation of TSG promoters and reactive their expression [147]. Interestingly, *TET1* underwent promoter CpG methylation-mediated silencing in human cancers including ESCC, leading to elevated methylation levels in tumor cells via a DNA methylation feedback loop, which suggests targeting *TET1* as a therapeutic target is worth further research. UHRF1, a multidomain nuclear protein that recruits DNMT1 to maintain DNA methylation has also been identified as a potential therapeutic target in ESCC patients, particularly those with elevated *UHRF1* expression [148].

Regarding the repurposing of non-cancer drugs, there have been some interesting findings. In a pre-clinical trial, Liu et al. showed that the nonsteroidal anti-inflammatory drug celecoxib demethylated TSGs and boosted apoptosis, highlighting its potential as an epigenetic therapy [149]. The study by Wang *et al.* revealed that metformin, a first-line drug for diabetes, could counteract nicotine-upregulated CHRNA7 expression in ESCC by enhancing promoter hypermethylation, thereby inhibiting JAK2/STAT3/SOX2 signaling pathway and tumor progression. The finding underscores the therapeutic potential of metformin in modulating epigenetic changes and offers a novel strategy for ESCC treatment, especially in smoking patients [150].

Discussion and Perspectives

Despite continuous advancements and improvements in multidisciplinary treatments, esophageal squamous cell carcinoma remains one of the most lethal malignancies in China and worldwide. DNA methylation, a major component of epigenetic modifications, plays a crucial role in the initiation and progression of ESCC. Compared to message RNA and protein, DNA is more stable biochemically, and tumor-derived DNA can be readily obtained from patient specimens including plasma, stool, sputum and urine after being released into the circulation. Moreover, techniques to detect DNA methylation like pyrosequencing, methylated DNA immunoprecipitation sequencing (MeDIP-Seq) and methylation-specific PCR (MSP), provide significant technical advantages. Taken together, the detection of aberrantly methylated genes can be exploited as potent predictive methods for clinical translation.

The first part of this review provides a comprehensive summary of all aberrantly methylated genes identified in ESCC to date and their associated characteristics. Previous studies and reviews have repeatedly highlighted the clinical applications of DNA methylation biomarkers in ESCC, particularly for early screening, early diagnosis and prognostic prediction. However, given the limited efficacy of systemic chemotherapy in ESCC and lack of reliable predictive biomarkers and novel therapeutic targets, there is a pressing need for further exploration. From the perspectives of significance and necessity, the latter part of this review meticulously discusses the predictive value of aberrant DNA methylation for therapeutic efficacy in systemic treatments and its potential as a promising therapeutic target.

Through extensive review of the literature, we have found that the major obstacle preventing DNA methylation markers from becoming truly useful biomarkers in ESCC is the lack of suitable large-scale, prospective clinical trial validation. In the future, we will focus on more large-sample prospective cohort studies and even initiate large-scale investigator-driven clinical trials based on our promising preliminary findings to identify and verify DNA methylation markers that can genuinely improve treatment outcomes and prognosis in clinical settings. Besides that, we have observed that

epigenetic patterns like DNA methylation, exhibit considerable plasticity and undergo dynamic changes during tumor progression or under therapeutic selection pressure. Therefore, it is essential to adopt a more dynamic perspective to evaluate DNA methylation markers, paying close attention to their temporal changes. By flexibly interpreting thresholds and incorporating these dynamic processes, we can achieve more precise treatment and timely therapeutic adjustments.

ESCC exhibits a relatively high proportion of methylation abnormalities, and epigenetic therapies targeting these aberrations hold substantial potential, with multiple clinical studies currently underway. It is important to note, however, that epigenetic therapies generally lack specificity, leading to considerable off-target effects and toxicity. As a result, most methylation-targeting drugs are currently administrated at low doses and are used in combination with conventional chemotherapy agents. Future research needs to develop more specific methylation-targeting drugs to enhance both efficacy and safety, as well as exploring the synergistic effects of these drugs with other conventional therapies, with the aim of significantly improving treatment outcomes and reversing drug resistance. It is anticipated that large-scale clinical trials in ESCC along with further exploration of the regulatory mechanisms of DNA methylation will discover more effective predictors of therapeutic response and identify novel therapeutic targets for eventual clinical application.

Conclusion

This review underscores the pivotal role of DNA methylation in ESCC, particularly its involvement in systemic chemotherapy. The key findings highlight the identification of numerous aberrantly methylated genes that disrupt critical pathways in ESCC. Clinically, DNA methylation markers hold promise for predicting therapeutic responses, though their utility is hindered by insufficient validation and off-target effects. Future research should prioritize large-scale validation of methylation markers through prospective trials and the development of more targeted epigenetic therapies. Integrating dynamic changes in methylation during treatment could enhance precision medicine. Further exploration of methylation mechanisms may reveal new therapeutic targets and improve clinical outcomes in ESCC.

Abbreviations

ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma; DDP: Cisplatin; 5-FU: 5-fluorouracil; FDA: Food and drug administration; TSG: Tumor suppressor gene; LINEs: Long interspersed nuclear elements; SINEs: Short interspersed nuclear elements; IL6: Interleukin 6; dCRT: Definitive chemoradiotherapy; WGBS: Whole genome bisulfite sequencing; DNMTs: DNA methyltransferases; DPYD: dihydropyrimidine dehydrogenase; ATR: Ataxia Telangiectasia and Rad3-related protein kinase; ICI: Immune checkpoint inhibitor; MeDIP-Seq: Methylated DNA immunoprecipitation sequencing; MSP: Methylation-specific polymerase chain reaction.

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Author contributions

Z.L. and Y.Z. had the idea for the review. Z.L. and X.C. performed the literature search, data analysis and table preparation. YK.L. and Y.Z. designed and created illustrations. Z.L. and Y.Z. drafted the original manuscript. Y.Z. and Y.X. critically reviewed and revised the work.

Competing Interests

The authors have declared that no competing interest exists.

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