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Review

Promoter methylation of tumor suppressor genes in esophageal squamous cell carcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) is a prevalent and fatal cancer in China and other Asian countries. Epigenetic silencing of key tumor suppressor genes (TSGs) is critical to ESCC initiation and progression. Recently, many novel TSGs silenced by promoter methylation have been identified in ESCC, and these genes further serve as potential tumor markers for high-risk group stratification, early detection, and prognosis prediction. This review summarizes recent discoveries on aberrant promoter methylation of TSGs in ESCC, providing better understanding of the role of disrupted epigenetic regulation in tumorigenesis and insight into diagnostic and prognostic biomarkers for this malignancy.

Key words Tumor suppressor gene, CpG island, promoter methylation, esophageal squamous cell carcinoma, tumor marker

Esophageal cancer is the sixth most common cancer worldwide but has a unique geographic and ethnic distribution[1], with a higher incidence in Asia than in the West. In some endemic districts in northern and central China, its incidence exceeds 100 cases per 100 000 people per year, comprising 78% of annual new cases and 76% of annual deaths of total carcinoma cases[2]. Esophageal cancer has two main types with different etiologic and pathologic characteristics: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma[3]. Notably, ESCC is the predominant type and accounts for approximately 90% of esophageal cancer cases worldwide[4]. Although the overall effectiveness of surgical and medical treatments for ESCC has improved in recent years, its prognosis still remains poor, with a 5-year survival rate of less than 10% for the patients[5]. Thus, elucidating the molecular mechanisms of ESCC pathogenesis will help to identify specific tumor markers for early detection, risk assessment, and therapeutic targeting.

Both genetic and epigenetic alterations contribute to the initiation and progression of ESCC. Genetic abnormalities involved in ESCC tumorigenesis include chromosomal loss and gain, loss of heterozygosity (LOH), and gene amplification and mutation[6]. Recently, epigenetic disruptions, including promoter CpG island methylation of tumor suppressor genes (TSGs) and microRNA methylation[7,8], have been recognized as key events in ESCC development. Here, we provide an overview of aberrant promoter methylation of critical TSGs in ESCC and the potential of these alterations as both tumor markers and therapeutic targets for ESCC.

TSGs Silenced by Promoter Methylation in ESCC

We briefly summarized the epigenetically silenced TSGs in ESCC according to their biological functions, such as apoptosis, cell cycle control, cell adhesion, and DNA repair (Table 1). Major functional groups are briefly reviewed below.

Cell cycle control genes

p16^{INK4a} and *p14*^{ARF}, transcripts of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus on chromosome 9p21, are two well-studied TSGs that are

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Table 1. Summary of tumor suppressor genes (TSGs) silenced by promoter methylation in esophageal squamous cell carcinoma (ESCC)

Classification	Gene name	Full name	Location	Major functions	Reference(s)
Cell cycle control genes	*CHFR*	Checkpoint with forkhead and ring finger domains	12q24.33	Cell cycle control	[16]
	$p14^{ARF}$/CDKN2A	Cyclin-dependent kinase inhibitor 2A	9p21	Stabilizing p53, cell cycle control	[11,19]
	$p15^{INK4b}$/CDKN2B	Cyclin-dependent kinase inhibitor 2B	9p21	Cell cycle control	[11]
	$p16^{INK4a}$/CDKN2A	Cyclin-dependent kinase inhibitor 2A	9p21	Cell cycle control	[11,19]
	RASSF1A	RAS association domain family 1A	3p21.3	Cell cycle control, apoptosis	[14]
Pro-apoptotic genes	*DAPK*	Death-associated protein kinase	9q34.1	Apoptosis	[19,67]
	RUNX3	Runt-related transcription factor 3	1p36	Transcription factor	[14,21,22]
	UCHL1	Ubiquitin carboxyl-terminal hydrolase L1	4p14	Cell growth inhibition, apoptosis	[23,24]
	ZNF382	Zinc finger protein 382	19q13.12	Pro-apoptotic transcription factor	[77]
Metastasis-antagonizing genes	*CDH1*	Cadherin 1, E-cadherin	16q22.1	Cell adhesion, proliferation, metastasis	[27–29]
	CDH11	Cadherin 11, OB-cadherin	16q21	Cell adhesion, proliferation, metastasis	[30]
	CDH13	Cadherin 13, H-cadherin	16q23.3	Cell adhesion, proliferation, metastasis	[31]
	CLDN3	Claudin 3	7q11.23	Cell-cell adhesion	[32]
	CLDN4	Claudin 4	7q11.23	Adhesion melocule	[33]
	DCC	Deleted in colorectal carcinoma	18q21.3	Cell adhesion, differentiation, apoptosis	[34]
	LRP1B	Low density lipoprotein receptor-related protein 1B	2q21.2	Migration	[35]
	PCDH10	Protocadherin 10	4q28.3	Cell-cell connection	[36]
	PCDH17	Protocadherin 17	13q21.1	Cell-cell connection	[37]
	TSLC1	Tumor suppressor in lung cancer 1	11q23.2	Cell adhesion	[38]
	UPK1A	Uroplakin-1A	19q13.13	Tetraspanin cell surface receptor	[72]
DNA repair genes	*FHIT*	Fragile histidine triad	3p14.2	Cell cycle control, DNA-damage response	[53–55]
	MGMT	O6-methylguanine-DNA methyltransferase	10q26	DNA repair	[41–44]
	MLH1	Human mutL homolog 1	3p21.3	DNA repair, cell cycle control	[47–49]
	MSH2	Human mutS homolog 2	2p21	DNA mismatch repair, cell cycle control	[50]
Growth factor response-related genes	*CRBP1*	Retinol-binding protein 1, cellular	3q23	Retinol transport	[78]
	CRABP1	Cellular retinoic acid-binding protein 1	15q24	Differentiation and proliferation	[79]
	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	5p13	Growth factor response, blocks Ras activity	[80]
	RARB	Retinoic acid receptor, beta	3p24	Cell growth and differentiation	[3,58–61]
	RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	3q25.32	Retinoid signaling	[81]
	SOCS1	Suppressor of cytokine signaling 1	16p13.13	Negative regulator of JAK/STAT pathway	[78]
WNT signaling-related genes	*APC*	Adenomatous polyposis coli	5q21–q22	Cell polarity and chromosome segregation	[69]
	SFRP1	Secreted frizzled-related protein 1	8p11.21	Antagonist of WNT protein receptors	[19,82]
	SFRP2	Secreted frizzled-related protein 2	4q31.3	Antagonist of WNT protein receptors	[19]
	SOX17	SRY box 17	8q11.23	WNT antagonist	[83]
	WIF1	Wnt inhibitory factor 1	12q14.3	WNT-signaling pathway inhibitor	[84]
	WNT5A	Wingless-type MMTV integration site family, member 5A	3p21–p14	WNT-signaling pathway inhibitor	[85]
Other genes with tumor suppressive functions	*ADAMTS9*	ADAM metallopeptidase with thrombospondin type 1 motif, 9	3p14.1	Metallopeptidase activity	[86]
	ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	16q23	Metallopeptidase activity	[87]
	BLU/ZMYND10	Zinc finger, MYND-type containing 10	3p21.3	Stress-response, transcription factor	[88]
	CACNA1G	Calcium channel, voltage-dependent, T type, alpha 1G subunit	17q22	Cell proliferation and cell death	[19]
	CDX2	Caudal type homeobox 2	13q12.3	Transcription factor activity	[89]

(*To be continued*)

Table 1. Summary of tumor suppressor genes (TSGs) silenced by promoter methylation in esophageal squamous cell carcinoma (ESCC) (*continued*)

Classification	Gene name	Full name	Location	Major functions	Reference(s)
Other genes with tumor suppressive functions	*CMTM3*	CKLF-like MARVEL transmembrane domain containing 3	16q21	Chemokine activity	[90]
	CMTM5	CKLF-like MARVEL transmembrane domain containing 5	14q11.2	Chemokine activity	[91]
	DLC1	Deleted in liver cancer 1	8p22	Cytoskeleton organization, signal transduction, cell adhesion	[92]
	DLEC1	Deleted in lung and esophageal cancer 1	3p22–p21.3	Signal transduction	[93]
	ECRG4	Esophageal cancer-related gene 4 protein	2q12.2	Unknown	[94]
	EDNRB	Endothelin receptor type B	13q22	G-protein-coupled receptor activity	[95]
	EMP3	Epithelial membrane protein 3	19q13.3	Unknown	[96]
	ENG	Endoglin	9q33–q34.1	Signal transduction	[97]
	GATA4	GATA-binding protein 4	8p23.1–p22	Zinc-finger transcription factor	[98]
	GATA5	GATA-binding protein 5	20q13.33	Zinc-finger transcription factor	[98]
	GPX3	Glutathione peroxidase 3	5q23	Catalyzes the reduction of hydrogen peroxide	[99]
	GSTP1	Glutathione S-transferase pi 1	11q13	Glutathione transferase activity	[100]
	HIN1/SCGB3A1	Secretoglobin, family 3A, member 1	5q35–qter	Signal transduction	[101]
	HLA–I	HLA class I	6p21.3	Immune response	[102]
	HLTF	Helicase-like transcription factor	3q25.1–q26.1	Helicase and ATPase activities	[103]
	HOPX	HOP homeobox	4q12	Regulation of gene expression	[104]
	HSPB2	Heat shock 27kDa protein 2	11q22–q23	Heat shock protein activity	[105]
	ITGA4	Integrin, alpha 4	2q31.3	Cell communication, signal transduction	[29]
	IRF8	Interferon regulatory factor 8	16q24.1	Transcription factor activity	[106]
	MT1G	Metallothionein 1G	16q13	Cellular stress response	[32]
	MT3	Metallothionein 3	16q13	Growth inhibition	[107]
	NMDAR2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	12p12	Signal transduction	[70]
	NEFH	Neurofilament, heavy polypeptide	22q12.2	Cell growth and/or maintenance	[108]
	NELL1	NEL-like 1	11p15.1	Cell growth regulation and differentiation	[109]
	p300/EP300	E1A-binding protein p300	22q13.2	Transcription regulator activity	[110]
	PCAF/KAT2B	K(lysine) acetyltransferase 2B	3p24	Transcription regulator activity	[111]
	PLCD1	Phospholipase C, delta 1	3p22–p21.3	Phospholipase activity	[112]
	SST	Somatostatin	3q28	Somatostatin hormone	[113]
	TAC1	Tachykinin, precursor 1	7q21–q22	Tachykinin peptide hormone	[65]
	THSD1	Thrombospondin, type I, domain containing 1	13q14.3	Unknown	[71,114]
	TIMP3	TIMP metallopeptidase inhibitor 3	22q12.3	Metalloproteinase inhibitor	[71]
	TPEF/TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2	2q32.3	Transmembrane protein	[115]
	Trypsinogen 4	Trypsinogen 4	9p11.2	Proteolytic activity	[116]
	VHL	von Hippel-Lindau tumor suppressor	3p25	Ubiquitin ligase component	[117]

ADAM, disintegrin and metalloprotease domain; CKLF, chemokine-like factor; HLA, human leukocyte antigen; HOP, homeodomain-only protein; MYND, myeloid, Nervy, and DEAF-1; NEL, neural epidermal growth factor-like; SRY, sex-determining region Y; TIMP, tissue inhibitor of metalloproteinase 1.

inactivated by genetic or epigenetic alterations in multiple malignancies [9,10]. In ESCC, *p16*^{INK4a} was methylated in 40% –61% of primary tumors and was less frequently inactivated due to homozygous deletion or mutation [11,12], whereas *p14*^{ARF} was methylated at a low frequency (13% –15%) and was mainly inactivated due to homozygous deletion [11]. These results suggest that promoter methylation is the predominant mechanism for *p16*^{INK4a}

inactivation but not *p14*^{ARF} during ESCC pathogenesis [11].

As a gatekeeper for G_1/S cell cycle progression, the RAS association domain family 1A (*RASSF1A*) gene is epigenetically inactivated in a broad spectrum of tumors[13]. In ESCC, *RASSF1A* was methylated in 51% of primary tumors, but rarely in matched non-cancerous tissues [14]. In addition, *RASSF1A* methylation was correlated with the clinical stage of ESCC[14]. Remarkably, the frequency of *RASSF1A* methylation in Chinese ESCC patients was relatively lower than that in Japanese ESCC patients[15], indicating that a possibly different mechanism is involved in *RASSF1A* methylation among these populations. Other cell cycle control genes silenced by promoter methylation have also been reported in ESCC, such as *p15*^{INK4b} and checkpoint with forkhead and ring finger domains (*CHFR*)[11,16] (Table 1).

Pro-apoptotic genes

Death-associated protein kinase (*DAPK*), a gene that encodes a pro-apoptotic serine/threonine kinase, participates in various apoptotic pathways in response to tumor necrosis factor-α (TNF-α), Fas ligand, ceramide, tumor growth factor-β (TGF-β), arsenic trioxide, and detachment from the extracellular matrix [17,18]. Promoter methylation of *DAPK* was frequently detected in intraepithelial lesions and primary ESCC[19], but rarely in normal and non-neoplastic epithelia, suggesting a role of methylation-mediated *DAPK* silencing in ESCC progression.

The runt-related transcription factor 3 (*RUNX3*) gene encodes RUNX3, a pro-apoptotic factor in the TGF-β signaling pathway that is commonly silenced in a variety of human tumors [20]. In ESCC, *RUNX3* silencing by promoter methylation [21] induced tumor progression and worsened patient prognosis[22]. As different frequencies of *RUNX3* methylation were reported in ESCC, the precise CpG region at which the *RUNX3* promoter is methylated for silencing needs to be further confirmed.

In addition, other novel methylated pro-apoptotic genes have been identified in ESCC. For instance, ubiquitin carboxyl-terminal hydrolase L1 (*UCHL1*), located on chromosome 4p14, can induce apoptosis through the intrinsic, caspase-dependent pathway [23]. Studies showed that *UCHL1* was methylated in 40% of primary ESCCs but not in the paired adjacent non-tumor tissues[23]. Furthermore, *UCHL1* methylation was correlated with regional lymph node metastasis [24]. These findings indicate that *UCHL1* may serve as an independent prognostic factor for ESCC patient survival.

Metastasis-antagonizing genes

Cadherin 1 (*CDH1*), which encodes a transmembrane glycoprotein, is a classic TSG at 16q22.1 and acts as a key cell-cell adhesion molecule to maintain normal tissue architecture and inhibit tumor initiation[25]. The inactivation of *CDH1* occurs at different stages of tumorigenesis, even at an early stage[26]. *CDH1* silencing with promoter methylation was detected in 41%–80% of primary ESCCs, which is related with poor survival of patients with stage I and stage II ESCC[27-29]. Similarly, other genes related to cell adhesion silenced by promoter methylation, such as cadherin 11 (*CDH11*)[30], cadherin 13 (*CDH13*)[31], claudin 3 (*CLDN3*)[32], claudin 4 (*CLDN4*)[33], deleted in colorectal carcinoma (*DCC*) [34], low density lipoprotein receptor-related protein 1B (*LRP1B*) [35], protocadherin 10 (*PCDH10*)[36], protocadherin 17 (*PCDH17*)[37], and tumor suppressor in lung cancer 1 (*TSLC1*)[38], have already been determined to be involved in tumor invasion and metastasis of ESCC (Table 1).

DNA repair genes

The product of the O-6-methylguanine-DNA methyl-transferase (*MGMT*) gene mediates a unique DNA repair pathway by removing methyl/alkyl groups from O-6-alkyl-guanine (G) and thus protects cells from mutagenic and cytotoxic effects of alkylating agents [39]. *MGMT* was reported to be epigenetically silenced in about 30% of human cancers due to promoter methylation[40]. In ESCC, *MGMT* methylation was increased along with tumor progression[41]. Notably, *MGMT* methylation was associated with *TP53* mutations [42] or the C677T polymorphism of 5,10-methylenetetrahydrofolate (*MTHFR*) in ESCC patients[43,44], suggesting a synergistic effect of both epi-genetic and genetic mechanisms in ESCC pathogenesis.

Mismatch repair gene mutL homolog 1 (*MLH1*) was reported to be inactivated by genetic or epigenetic alterations in multiple human cancers [45,46]. Promoter methylation of *MLH1*, which reduced its protein expression level, was detected in 62% of ESCCs [47]. Interestingly, epigenetically silenced *MLH1* was always associated with microsatellite instability in ESCC [48,49], indicating that *MLH1* plays a critical role in ESCC progression. *MSH2*, another important DNA mismatch repair gene, was also silenced by promoter methylation in 32% of ESCCs but none of the matched normal tissues[50].

The fragile histidine triad (*FHIT*) gene, located at 3p14.2 [51], plays an essential role in chromosomal abnormality and DNA damage [52]. *FHIT* was methylated in 69% of ESCCs but not in the matched normal tissues, and this methylation was responsible for decreased FHIT protein level [53]. Loss of FHIT expression was usually observed at initial stages of ESCC [54] and thus might serve as an independent prognostic marker and as a marker for early detection of ESCC[55]. In addition, aberrant methylation of *FHIT* can also be induced by nicotine [56], indicating its role in smoking-related ESCC tumorigenesis.

Growth factor response-related genes

Retinoids play an important role in growth arrest and apoptosis via binding to specific nuclear retinoid receptors, such as retinoic acid receptor β (RARβ)[57]. Loss of expression of *RARB*, the gene encoding RARβ, was observed in 54% of ESCCs and 57% of dysplastic lesions [58], with no LOH detected[59]. Frequent promoter methylation of *RARB* was detected in primary ESCC tumors (70%), dysplastic lesions (58%), and basal cell hyperplasia (43%) but rarely in normal tissues, and methylation was related with ESCC grade[60]. Moreover, *RARB* expression could be reactivated by pharmacologic demethylation treatment [61]. These data suggest that *RARB* silencing by promoter methylation is an early event in ESCC development.

Promoter Methylation of TSGs as Tumor Markers for ESCC

Detecting promoter methylation of TSGs has advantages compared to protein or RNA analysis. First, DNA can be released outside of the tumor mass and is more stable than RNA or protein, which makes DNA-based markers easier to obtain from distinct types of biological fluid (such as sputum, pancreatic juice, and urine), blood and tissues (including 10% formaldehyde-fixed samples)[62]. Second, PCR-based analyses of DNA methylation have relatively high sensitivity. For example, methylation-specific PCR is able to detect a single methylated allele among 1000 unmethylated alleles, even in the presence of an abundance of normal DNA[63]. Third, because DNA used for methylation analysis is chemically stabilized, sample handling requirements are not rigid[64]. Thus, DNA methylation assays can be exploited as potent noninvasive diagnostic methods for clinical applications.

Given the high mortality, early detection or diagnosis is essential for successful treatment of ESCC. Promoter methylation of multiple TSGs, including $p16^{INK4a}$, $p14^{ARF}$, *FHIT*, *RARB*, *MGMT*, and tachykinin1 (*TAC1*), was detected in precancerous basal cell hyperplasia or dysplastic lesions, indicating their early diagnostic values in ESCC[19,41,61,65]. Furthermore, a panel of four methylated genes, aryl-hydrocarbon receptor repressor (*AHRR*), $p16^{INK4a}$, metallothionein 1G (*MT1G*), and *CLDN3*, was used to successfully screen esophageal balloon cytology samples with much better specificity and sensitivity compared with single-gene methylation[66]. Another panel of methylated genes, *RARB*, *DAPK*, *CDH1*, $p16^{INK4a}$, and *RASSF1A*, had a diagnostic sensitivity of 82.2% and a specificity of 100% for ESCC in detecting serum DNA of ESCC patients[67]. These findings suggest that a cluster of methylated TSGs is more efficient for early detection of ESCC than single-gene methylation.

Since TNM staging has a limited capacity in assessing tumor prognosis, many studies have been performed to establish a reliable technique with which to predict prognosis in human cancers. Recently, the feasibility of TSG methylation as a predictor of clinical outcome after radical surgery has been studied in ESCC. For example, promoter methylation of *CDH1*[29], *FHIT* [55], and integrin alpha 4 (*ITGA4*)[29] can be used to stratify patients with stage I and II ESCC. Promoter methylation of *CDH1*[68] and *ITGA4*[29] have been linked to tumor recurrence, and methylation of other genes including adenomatous poly-posis coli (*APC*)[69], N-methyl D-aspartate 2B (*NMDAR2B*)[70], tachykinin 1 (*TAC1*)[65], TIMP metallopeptidase inhibitor 3 (*TIMP3*)[71], *UCHL1*[24], and uroplakin 1A (*UPK1A*) [72] have been linked to shorter survival.

Translational Applications of DNA Demethylation in ESCC Treatment

Epigenetic reagents intended to reactivate epigenetically silenced TSGs or tumor antigens are being tested for their anticancer effects. Nucleoside analogues 5-azacytidine (azacytidine) or 5-aza-2'-deoxycytidine (decitabine) can effectively reverse silencing of multiple TSGs by blocking the activity of DNA methyltransferase (DNMT) in tumor cells, thereby exhibiting significant tumor suppressive activity[73]. These drugs have been approved by the US Food and Drug Administration (FDA) for treating myelodysplastic syndrome, a pre-leukemia disease. Recently, several novel DNMT inhibitors have also been reported for future clinical use, such as 5-fluoro-2'-deoxycytidine (Zebularine), epigallocatechin-3-gallate (EGCG), and RG108[64]. However, due to lack of specificity for target genes, more studies of demethylation therapy are currently being performed to prove the efficacy of this approach on solid tumors [74]. Although clinical trials using demethylation reagents have not been reported in ESCC yet, combining DNA demethylation agents with traditional chemotherapy drugs should be a promising prospect for ESCC treatment in future.

Conclusions

ESCC pathogenesis is a multistep process controlled by both genetic and epigenetic mechanisms. Silencing TSGs by promoter methylation plays essential roles in ESCC initiation and development. Numerous methylated genes have been identified in ESCC in recent years and thus provide new insights into the molecular mechanism

of ESCC pathogenesis and expand the knowledge of tumor markers for clinical application. However, some issues remain to be solved in the future. For example, few methylated genes have been identified in ESCC by a single group, with the methylation frequency of some TSGs varying widely in different labs, probably due to different patient cohorts or detection methods[75]. With the use of genome-wide epigenomic approaches[76], the more reliable identification of methylated genes or gene panels might improve the early detection and prognosis of ESCC in future.

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