# DNA Methylation and Esophageal Squamous Cell Carcinoma: Special Reference to Research in China

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**Abstract**: Genetic abnormalities of proto-oncogenes and tumor suppressor genes have been demonstrated to be involved frequently in esophageal carcinogenesis, especially the hypermethylation of CpG islands. Accumulated evidences indicate that hypermethylation of CpG islands in the promoter regions are one of the important mechanisms to silence the expression of many important genes and may play an important role in esophageal carcinogenesis. In this review, evidences for gene hypermethylation in human esophageal precancerous and cancerous lesions with special reference to research in China and their correlations with other populations in Asia were summarized to provide molecular clues for identifying the biomarkers for high-risk subject screening and early diagnosis. [Life Science Journal. 2006;3(2):1-11] (ISSN: 1097-8135).

**Keywords**: methylation; esophageal squamous cell carcinoma; carcinogenesis; CpG islands

**Abbreviations**: APC: adenomatous polyposis coli; BCH: basal cell hyperplasia; CIS: carcinoma in site; CRBP1: cellular retinol-binding protein 1; DNMT: DNA methyltransferase; DYS: dysplasia; EAC: esophageal adenocarcinoma; LOH: loss of heterozygosity; MGMT: $O^6$-methylguanine-DNA methyltransferase; NSCLC: non-small cell lung cancer; RARβ2: retinoic acid receptor-beta 2; RASSF1: Ras-association domain family 1; SCC: squamous cell carcinoma; TIG1: tazarotene-induced gene 1; TSG: tumor suppressor genes; TSLC: tumor suppressor in lung cancer

## 1 Introduction

Esophageal cancer is one of the most common malignant diseases, with a remarkable geographical distribution and poor prognosis[1]. The five-year survive rate is only 10%. However, the five-year survive rate for the patients with the early esophageal cancer is more than 90%. But in clinical, more than 85% of the esophageal cancer patients are diagnosed at the late stage. Lack of early specific symptoms and diagnosis biomarker remains the leading cause of late diagnosis for esophageal cancer. Therefore, the current challenges in esophageal cancer research are to obtain a better understanding of the underlying molecular alterations to establish the strategies for high-risk subject screening and early diagnosis. Cancer of the esophagus exists in two main forms with different etiological and pathological characteristics, i. e. esophageal squamous cell carcinoma and esophageal adenocarcinoma[2]. It has been well recognized that esophageal carcinogenesis is a multistage and progressive process with a sequence of from basal cell hyperplasia to dysplasia to carcinoma in site and esophageal carcinoma. A variety of genetic lesions has been demonstrated to be involved in esophageal carcinogenesis, including p53-Rb pathway with gene amplifications, loss of heterozygosity or homozygous deletions, point mutations, and chromosomal rearrangements[2-4]. Besides, the accumulated evidences in the epigenetic inactivation of genes have shown their importance in esophageal carcinogenesis, as important a driving force as the inactivation of genes by mutation[5].

"Epigenetic" events, i.e. heritable changes in gene function which can not be explained by changes in DNA sequence, are composed of histone acetylation, the chromatin structure and DNA methylation[5]. DNA methylation seems to be the most important mechanism for "epigenetic change" at present[5,6]. Through a process of covalent modification catalyzed by DNA methyltransferase, the DNA of mammalian cells contains a "fifth base", namely 5-methylcytosine. The most frequent target for this modification is cytosine in the context of the dinucleotide CpG[5]. Throughout the genome CpG dinucleotides are found at one-fifth of their

predicted frequency[7]. In marked contrast to the genome wide under representation of CpGs, there are regions of the genome termed CpG islands which have maintained their expected frequency of this dinucleotide. And the CpG islands are often found within the promoter of the genes[7,8]. There is an inverse relationship between the density of promoter methylation and the transcriptional activity of a gene[9,10]. The mechanism of gene silencing by promoter hypermethylation has recently been shown to be related with the recruitment of repressor protein complex, resulting in deacetylation of the chromatin and histone, thus barring access of the active transcription complex. However, the actual mechanisms by which DNA methylation modulates gene expression are largely unknown[11,12]. The assays for detection of cytosine methylation could be divided into two groups: restriction enzyme-based and bisulfite treatment-based[13,14]. The former employs the inhibition of certain restriction enzymes by methylation of their recognition sites as an indicator for the presence of methylation. The latter translates the epigenetic information of cytosine methylation in primary sequence differences by converting unmethylated cytosine to uracil whereas methylated cytosine remains unaltered. The bisulfite-converted genomic DNA can be analyzed by a wide variety of PCR-based methods of which direct sequencing of the PCR products or sequencing of individual PCR product clones gives the most detailed information about the methylation pattern in the CpG islands under study[14,15].

Methylation is needed for the normal development of cells. Genome stability and normal gene expression are largely maintained by a fixed and predetermined pattern of DNA methylation[15]. Aberrant methylation confers a selective growth advantage that results in cancerous growth[15]. From various lines of evidence, it is known that the methylation pattern of the cancerous cell is associated with a broad genomic hypomethylated state that is often accompanied by a more regional and locus-specific hypermethylated pattern[7]. The presence of alterations in the profile of DNA methylation in cancer was initially thought to be exclusively a global hypomethylation of the genome that would possibly lead to massive overexpression of oncogenes whose CpG islands were normally hypermethylated[14]. Nowadays, however, this is considered to be an unlikely or, at best, incomplete scenario[14]. The popularity of the concept of demethylation of oncogenes leading to their activation is in clear decadency[14,15]. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is now firmly established as an important mechanism for gene inactivation[16-18].

The particular genes that are hypermethylated in tumor cells are strongly specific to the tissue of origin of the tumor. A profile of CpG island hypermethylation exists according to the tumor type[15]. The mechanism responsible for this type of pattern remains unclear. Moreover, accumulating evidence indicates that CpG island hypermethylation is an early event in cancer development and, in some cases, may precede the neoplastic process[19]. Therefore, such profiles would provide invaluable insight into mechanisms underlying the evolution of each tumor type and will provide new molecular markers. This review will focus on the current understanding of DNA methylation abnormalities in esophageal cancer and provide molecular clues for identifying the biomarkers for high-risk subject screening and early diagnosis.

## 2 Studies of Genes Promoter Hypermethylation in SCC

Table 1 complied genes[20-55] that have been extensively studied in the past with a special reference to Chinese population, especially for those from Linxian, the highest incidence area for esophageal cancer in Henan, northern China. Putative TSG, involving apoptosis, cell adherence, DNA repair, and the cell cycle, have been investigated for hypermethylation by various techniques in esophageal cancer.

### 2.1 $p14^{ARF}$, p15 and p16

The 9p21 chromosomal band is one of the most frequently altered genomic regions in human cancers[56]. Within a short distance of 50 kb, a gene cluster consisting of three genes, $p14^{ARF}$, p15 and p16, are harbored. All of which have putative tumor suppressor roles[56-58]. Inactivation of $p14^{ARF}$, p15 and p16 genes has been observed in many types of human cancers including SCC[20,22,26,57,58]. For example, our results from immunohistochemical analysis indicated that p16 expression was present in only 3 out of 22 SCC cases[59]. Some studies showed that germline mutations in p16 gene might be related to familial melanoma[60], but our study found the mutation of p16 gene in esophageal cancer was rare[20]. Hemizygous and homozygous deletion at 9p21 are widely considered to be one of the primary mechanisms of p16/p15 inactivation[20]. Recently, however, aberrant methylation of the CpG island at the pro-

moter regions of p16 and p15 genes was reported in SCC and was associated with loss of transcription[20-31]. By analyzing the $p14^{ARF}$, p15 and p16 genes individually in 40 SCC, we detected aberrant promoter methylation of the p16 gene in 16 (40%), of $p14^{ARF}$ in 6 (15%), and of p15 in 5 (12.5%) tumor samples. We further detected homozygous deletion of p16 in 7 (17.5%), of $p14^{ARF}$ in 13 (33%), and of p15 in 16 (40%) tumor samples, and detected no mutation in the $p14^{ARF}$ and p16 genes[20, 26]. The above results suggest that $p14^{ARF}$, together with p15, is a primary target of homozygous deletion, whereas p16 is the hypermethylation hotspot in human esophageal cancer. Johanna *et al* analyzed methylation of CDKN2A ($p16^{INK4\alpha}$ and $p14^{ARF}$) individually in 40 SCC from Linxian, and detected aberrant promoter methylation of the $p16^{INK4\alpha}$ gene in 4 (19%), of $p14^{ARF}$ in 11 (52%), in 21 tumor samples[22]. Although samples in two studies above collected were all from the same area, the results were different markedly. The difference probably results from: 1) Regions for the patients studied are not completely the same. In Johanna's study, part of the SCC patients was from Linzhou; in the other two studies, all the SCC patients were from Linzhou. 2) Different methylation primes and the different sites to be detected. The 5′ position of the sense unmethylated and methylated primers of $p14^{ARF}$ gene corresponds to bp 227 and 225 of GenBank sequence number L41934 in our study, which respectively amplify a 165-bp and 160-bp product[22,26]. However, the 5′ position of the sense unmethylated and methylated primers of $p14^{ARF}$ gene corresponds to bp 195 and 201 of GenBank sequence number L41934 in the latter, which respectively amplify a 132-bp and 122-bp product[22,26]. 3) Difference in histopathological types of collected samples. In our study, pathology grades of the samples enrolled were unknown. But in the latter, the majority tumors analyzed were of Ⅰ-Ⅱ grade (15/21), and 4 cases belonged to grade Ⅲ, for two cases grades were not listed[20]. 4) Different criteria in the process of enrolling samples. In Yan's study, since DYS and CIS are pathologically similar, CIS were combined into group DYS[26]. Guo *et al* analyzed methylation of p16 gene in 37 SCC from Hebei, and detected aberrant promoter methylation of the p16 gene in 21 (64.9%)[29]. The similar results were observed for Japanese people[23-25,28]. The p16 methylation in SCC was higher in Japanese people than in Chinese people, suggesting the possibility of different carcinogenic factors and mechanisms involved in different populations.

### 2.2 The FHIT gene

The FHIT gene is located at chromosome 3p14.2 and encodes a polypeptide of 147 amino acids[61]. FHIT allelic deletions and reduced or absent FHIT protein expression have been observed in a variety of tumors suggesting a putative tumor suppressor function[61, 62]. In SCC, the hypermethylation of CpG island in the FHIT promoter region was significantly correlated with the deletion of FHIT protein expression[26,29,42,47,52]. Methylated SCC cell lines exhibit re-expression of the FHIT gene and demethylation in the CpG island after treatment with demethylating agent 5-aza-2′-deoxycytidine[63]. These findings suggest that methylation of the 5′ CpG island of the FHIT gene is closely associated with transcriptional inactivation and might be involved in tumor development of the esophagus.

### 2.3 The RARβ2 gene

The retinoic acid receptor-beta 2 gene located at 3p24 has been intensively studied in many cancers and found to have defective function, thus making it a candidate TSG[64]. We found that $RAR\beta_2$ was detected in 36% (18/50) of normal esophageal tissues, and that 14 of 20 (70%) SCC samples had hypermethylation of the $RAR\beta_2$ promoter[40]. Another group of study reported that 27 of 47 (55%) primary resected SCC samples showed $RAR\beta_2$ methylation[42]. Liu *et al* analyzed the methylation status of $RAR\beta_2$ promoter region and its expression in 51 SCC tissue samples with their adjacent normal epithelia and two normal esophageal epithelia, and found that there was a statistically significant correlation between methylation status of $RAR\beta_2$ and tumor grade[33]. Moreover, a relationship between methylation status and decreased $RAR\beta_2$ expression was found only in G (2) stage tumors. Methylation of $RAR\beta_2$ promoter regions was detected in 26/51 (51%) of the primary tumors; moreover in seven tissue samples with their adjacent normal epithelia, methylation of this locus was found in both the adjacent normal epithelia and matched tumor tissues; and in other 19 tissue samples methylation of this locus only existed in the primary esophageal tumors; and in two cases, hypermethylation was observed in the adjacent normal epithelia, but not in the corresponding SCC samples[33]. Thus, they considered that $RAR\beta_2$ methylation is a common neoplastic feature of SCC. These results identified methylation as the underlying mechanism for this frequent loss of $RAR\beta_2$ in esophageal cancer[33,34,39,40,42].

**Table 1.** Compilation of genes hypermethylated in SCC by regions and methods

| Genes | Regions for the patients studied | Incidence of Methylation | | Methods for methylation | References |
|---|---|---|---|---|---|
| | | Cancer tissue n/N (%) | ANT# n/N (%) | | |
| p14ARF | Linxian (T#) | 6/40(15%) | ND# | MSP, Sequencing | 20 |
| | Linxian (T) | 11/21(52%) | ND | MSP, HPLC | 22 |
| | Linxian (T) | 6/21(28.6%) | 10/40(25%) | MSP | 26 |
| p15 | Linxian (T) | 5/40(12.5%) | ND | MSP, Sequencing | 20 |
| | Linxian (T) | 2/25(8%) | 3/75(4%) | MSP | 26 |
| p16 | Linxian (T) | 16/40(40%) | ND | MSP, Sequencing | 20 |
| | Cell lines | 4/30(13%) | ND | MSP | 21 |
| | Linxian (T) | 4/21(19%) | ND | MSP, HPLC | 22 |
| | Japan (T) | 6/31(19%) | ND | Sequencing | 23 |
| | Japan (T) | 30/42(71.4%) | 24/30(80%) | MSP | 24 |
| | Japan (T) | 31/38(82%) | ND | MSP | 25 |
| | Japan (S#) | 7/31(23%) | ND | MSP | 25 |
| | Linxian (T) | 7/21(33.3%) | 12/40(30%) | MSP | 26 |
| | Sporadic (T) | 5/34(15%) | ND | MSP in situ | 27 |
| | Iran (T) | 22/30(73.3%) | ND | MSP | 28 |
| | Iran (B#) | 13/30(43.3%) | ND | MSP | 28 |
| | Iran (S) | 8/30(26.6%) | ND | MSP | 28 |
| | Iran(family)(B) | 18/28(64.3%) | ND | MSP | 28 |
| | Hebei (T) | 21/37(64.9%) | 5/79(6.3%) | MSP | 29 |
| | Hubei (S) | 34/56(61%) | ND | nMSP | 30 |
| | | 15/56(27%) | | MSP | |
| NMDAR2B | USA(T) and cell lines | 95% | ND | MSP | 31 |
| RASSF1A | Hong Kong (T) | 22/64(34%) | 3/64(5%) | MSP | 32 |
| | Cell lines | 3/7(43%) | | Sequencing | |
| | Japan (T) | 24/47(51%) | 2/47(4%) | MSP | 32 |
| | Cell lines | 16/22(73%) | | | |
| | Japan (T) | 25/48(52%) | ND | MSP | 36 |
| | Cell lines | 17/23(74%) | | | |
| | Japan (T) | 13/55(24%) | ND | MSP | 73 |
| RARβ2 | Linxian and Anhui (T) | 26/51(51%) | 9/51(18%) | MSP | 33 |
| | Cell lines | 4/6(67%) | | MSP | 39 |
| | Linxian (T) | 14/20(70%) | 18/50(36%) | MSP | 40 |
| | Japan (T) | 7/28(25%) | 1/10(10%) | MSP | 34 |
| | Japan (T) | 27/47(55%) | 18/47(38%) | MSP | 42 |
| CRBP1 | Japan (T) | 5/28(17.9%) | 0/28(0) | MSP | 34 |
| TIG1 | Japan (T) | 5/28(17.9%) | 0/28(0) | MSP | 34 |
| MGMT | Linxian (T) | 13/18(72%) | 23/49(47%) | MSP | 35 |
| | Sporadic (T) | 46/119(38.7%) | 5/22(22.7%) | MSP | 43 |
| hMLH1 | Japan (T) | 6/9(66.7%) | ND | MSP | 36 |
| | Japan (T) | 0/30(0) | ND | MSP | 45 |
| | Linxian (T) | 5/25(20%) | 7/75(9%) | MSP | 26 |
| | Sporadic (T) | 30/92(32.6%) | ND | MSP | 53 |
| E-cadherin | Japan (T) | 25/41(61%) | ND | MSP | 37 |

| | | | | | |
|---|---|---|---|---|---|
| | Linxian (T) | 4/25(16%) | 0/75(0) | MSP | 26 |
| | Hong Kong (T) | 16/20(80%) | ND | MSP | 49 |
| LRP1B | Japan (T) | 5/34(14.7) | ND | Bisulfite-PCR Sequencing | 38 |
| Trypsin | USA(T) | 5/10(50%) | ND | MSP | 41 |
| TSLC1 | Japan (T) | 28/56(50%) | ND | MSP | 68 |
| VHL | Japan (T) | 6/47(13%) | 0/47(0) | MSP | 42 |
| | Linxian (T) | 2/25(8%) | 0/75(0) | MSP | 26 |
| FHIT | Japan (T) | 21/47(45%) | 14/47(30%) | MSP | 42 |
| | Japan (T) | 25/36(69.4%) | ND | MSP | 47 |
| | Linxian (T) | 5/25(20%) | 3/75(4%) | MSP | 26 |
| | Japan (T) | 5/35(14%) | 0/35(0) | MSP | 52 |
| | Hebei (T) | 25/37(67.6%) | 3/79(3.8%) | MSP | 29 |
| ECRG4 | Linxian (T) | 12/20(60%) | 3/20(15%) | MSP, DHPLC | 44 |
| HLA class I | Linxian (T) | 19/29(66%) | 0/29(0) | MSP | 50 |
| HLA-A | Linxian (T) | 2/25(4%) | 0/75(0) | MSP | 26 |
| | Linxian (T) | 7/29(24%) | 0/29(0) | MSP | 50 |
| HLA-B | Linxian (T) | 5/25(20%) | 5/75(7%) | MSP | 26 |
| | Linxian (T) | 11/29(38%) | 0/29(0) | MSP | 50 |
| HLA-C | Linxian (T) | 5/25(20%) | 2/75(3%) | MSP | 26 |
| | Linxian (T) | 9/29(31%) | 0/29(0) | MSP | 50 |
| Chfr | Japan (T) | 7/43(16.3%) | ND | MSP | 48 |
| | Cell lines | 4/15(26.7%) | | | |
| APC | USA(T) | 16/32(50%) | ND | MSP | 51 |
| | USA(P#) | 2/32(6.3%) | | | |
| MT3 | Hebei (T) | 10/47(21.3%) | ND | COBRA | 54 |
| GATA-4 | Henan (T) | 27/44(61%) | 0/44(0) | MSP | 55 |

#ANT, adjacent nonmalignant tissue; ND, not done; T, tumor tissue; S, serum sample; B, blood sample; P, plasma sample

Retinoic acid receptor-beta, cellular retinol-binding protein 1, and tazarotene-induced gene 1 have been linked to retinoic acid signaling. Little is known about the involvement of these three genes in SCC. Mizuiri *et al* found that DNA hypermethylation of $RAR\beta_2$ existed in seven (25.0%) of the 28 SCC, of CRBP1 in five (17.9%), and of TIG1 in five (17.9%). DNA methylation of $RAR\beta_2$ was identified in one of 10 samples of corresponding non-neoplastic mucosa (10.0%), whereas no DNA methylation of CRBP1 or TIG1 was detected. No correlation was found between the DNA methylation status of RARβ and clinicopathological factors such as depth of invasion, lymph node metastasis, or tumor stage. In contrast, DNA methylation of both CRBP1 and TIG1 was observed only in stage Ⅲ SCC[34]. These results showed that inactivation of the retinoic acid signaling-associated genes $RAR\beta_2$, CRBP1, and TIG1 by DNA methylation occurred frequently in SCC.

## 2.4 The APC gene

The adenomatous polyposis coli gene, located on chromosome 5q21, is a TSG in the WNT signaling pathway[65]. We found that APC shows frequent LOH in esophageal carcinomas, and the prevalence of mutations in the APC gene in esophageal carcinomas is low. Hypermethylation of the promoter region of the APC gene occurred in abnormal esophageal tissue in 16 (50%) of 32 patients with SCC, but not in matching normal esophageal tissues[51]. So methylation of the promoter region of this gene constitutes an alternative mechanism of gene inactivation in esophageal carcinoma.

## 2.5 The MGMT gene

The human enzyme $O^6$-methylguanine-DNA methyltransferase protects the cell from guanine methylation by irreversibly transferring the alkyl group of the $O^6$-methylguanine to a specific cysteine residue within the molecule[66]. Approximately 20% of tumor cell lines lack MGMT activity and

are highly sensitive to alkylating agents[66,67]. In established cancer cell lines, MGMT expression appears to be correlated with methylation in the promoter of the gene[35,66]. The development of SCC has been linked to exposure to carcinogens such as nitrosamines that cause various alkyl DNA damages and MGMT is a primary defense against alkylation-induced mutagenesis and carcinogenesis. We found that 18 (72%) SCC samples had DNA hypermethylation in the MGMT promoter region, and that the frequency of the loss of MGMT mRNA and protein expression was highly correlated with MGMT promoter hypermethylation according to Fisher's exact tests[35]. The gene has been shown to be methylated in 46/119 SCC (38.7%), but all 21 normal esophageal tissues had unmethylated MGMT[43].

**2.6 The E-cadherin gene**

E-cadherin is a *Mr* 120,000 transmembrane glycoprotein expressed on the surface of epithelial cells. In epithelial tissues, E-cadherin mediates homophilic, $Ca^{2+}$-dependent intercellular adhesion that is essential for the maintenance of normal tissue architecture[49]. Loss of E-cadherin expression occurs in a variety of human tumors and is correlated with invasion and metastasis, and activation of E-cadherin results in the growth inhibition of tumor cell lines[37]. E-cadherin can be targeted by both genetic and epigenetic means. Moreover, the hypermethylation of E-cadherin was seen frequently in most tumor types, but mutations only frequent in a small number of specific subtypes[37]. E-cadherin, a cell adhesion molecule, is regarded as an invasion-suppressor molecule and a prognostic marker in many types of human cancers. In esophageal carcinoma, downregulation of E-cadherin is common and is associated with an increase in invasive and metastatic potential, but mutations of the gene are rare[49]. E-cadherin was methylated in 16 of 20 (80%) SCC samples in Hong Kong people and 4 of 6 SCC cell lines[49]. And treatment of E-cadherin negative carcinoma cells with the demethylating agent, 5-aza-2′-deoxycytidine, induced re-expression of the gene[49]. In Japan, E-cadherin was methylated in 25 of 41 (61%) SCC samples[37]. However, we found that E-cadherin was methylated in 4 of 25 (16%) SCC clinical samples in Linxian, Northern China[26]. These data suggest that epigenetic silencing via aberrant methylation of the E-cadherin promoter is the critical mechanism for inactivation of this gene in esophageal cancer, and that the frequency of the hypermethylation of E-cadherin varied with the patients from different regions.

**2.7 The TSLC1 gene**

Tumor suppressor in lung cancer was first characterized as a TSG in human non-small cell lung cancer and termed TSLC1[68]. The tumor suppressor role of this gene has been demonstrated in the cell lines of NSCLC, hepatocellular carcinoma, pancreatic cancer and SCC[68,69]. Loss of TSLC1 expression was observed in 75% of the SCC cell lines and 50% of the primary tumors from SCC patients[69]. Kaganoi *et al*[68] examined the methylation status of six cytosine residues of CpG sites in a putative promoter sequence upstream from the translation initiation site by bisulfite sequencing in four cell lines, including KYSE270, which expressed TSLC1, and KYSE410, KYSE520, and KYSE960, which did not express it, and found that all of the cytosine residues in KYSE270 DNA were unmethylated, whereas all of the six cytosine residues in KYSE520 DNA and five residues in KYSE410 and KYSE960 DNA were methylated. Especially, the cytosine residues in KYSE520 DNA were all hypermethylated.

**2.8 The RASSF1A gene**

Many known Ras effectors are oncoproteins on their own. Less is known about Ras effectors possessing tumor suppressor properties[42]. Recently, a new family of genes encoding a putative Ras effector, the Ras-association domain family 1 gene, has been identified within the critical lung and breast cancer deletion region at 3p21.3. The RASSF1 locus encodes several major transcripts by alternative promoter selection and alternative mRNA splicing: RASSF1A, RASSF1B and RASSF1C. Many studies have suggested that RASSF1A was a new putative TSG[32,42,46]. RASSF1A acts as a negative effector of Ras in a pro-apoptotic signaling pathway. Interestingly, mutational inactivation of this gene is very rare ($<$ 2%), and the main mechanism of its inactivation is through promoter methylation and LOH[46]. The RASSF1A isoform is highly epigenetically inactivated in lung, breast, ovarian, kidney, prostate, thyroid, esophagus and several other carcinomas[32]. Hypermethylation of RASSF1A was detected in 73% of SCC cell lines and 51% of primary SCC from Japan, whereas only 4% of RASSF1A hypermethylation were detected in corresponding noncancerous tissues[42]. There was a statistically significant correlation between the presence of hypermethylation and tumor stages[27]. Hypermethylation of RASSF1A was found in 3/7 (43%) of SCC cell lines and 22/64 (34%) of primary SCC from Hong Kong peoples[32]. These findings sug-

gest that epigenetic silencing of RASSF1A gene expression by promoter hypermethylation could play an important role in SCC carcinogenesis.

Besides the above mentioned genes, there are hypermethylation of some other genes involved in esophageal cancer, including hMLH1 (0 – 66.7%)[26,36,45,53], VHL (8 – 13%)[26,42], MT3 (21.3%)[54], NMDAR2B (95%, USA)[31], LRP1B (14.7%, Japan)[38], Trypsin (50%, USA)[41], ECRG4 (60%)[44], GATA-4 (61%)[55], Chfr (16.3%, Japan)[48] and HLA class I[26,50] including HLA-A (4 – 24%), HLA-B (20 – 38%) and HLA-C (20 – 31%). Because of the limited case number and low frequency of methylation or the populations outside Asia, the discussion was not expanded on these studies.

## 3 Methylation in Serum from the Esophageal Cancer Patients

DNA methylation of the promoter region of certain cancer-associated genes is one potential early detection biomarker[25,30,51]. Genetic analysis has shown that cell-free circulating DNA in plasma or serum of cancer patients share similar genetic alterations to those described in the Table 1[25,28,30,51]. Numerous studies have demonstrated the presence of promoter hypermethylation of tumor suppressor genes in the serum DNA of patients with various cancers[30]. Hypermethylated APC DNA was observed in the plasma of two of 32 (6.3%) SCC[51]. Hibi *et al*[25] found that aberrant promoter methylation of the p16 gene was detected in 31 of 38 (82%) SCC, and 7 of these 31 (23%) patients with a p16 alteration in the primary tumor had the same methylation changes in the corresponding serum DNA. This study yielded a promising result: a tumor associated DNA alteration could be detected in the serum of 18% of SCC patients (7 of 38 patients) using p16 methylation as a target. Moreover, the clinical sensitivity of this assay can be potentially improved by incorporating other possibly methylated target genes, which has been estimated in other tumor types. For example, Fukami *et al*[69] analyzed primary NSCLC and serum from 22 patients for the methylation pattern of four TSG (DAPK, GSTP1, p16 and MGMT). Methylation of at least one of these genes was detected in 68% of NSCLC. Comparing primary tumors with methylation and matched serum samples, 73% of the matched serum samples were found to be methylation. In addition, none of the sera from the patients with tumors not demonstrating methylation was positive[69]. Yao *et al* detected 61% p16 methylation in 56 serum samples of SCC by nMSP. In contrast, 27% p16 methylation in 56 serum samples of SCC was detected by MSP[30]. Therefore, combined detection of aberrant promoter hypermethylation of cancer-related genes in serum may be useful for esophageal cancer diagnosis or the detection of recurrence. Improved method to detect methylation would increase sensitivity.

### 3.1 Early aberrant DNA methylation in esophageal carcinogenesis

In many tumors, it has been proved that aberrant DNA methylation frequently occurs in precancerous tissue as well as cancer tissue, and both factors, genetic and epigenetic, lie at the origin of carcinogenesis[59]. The relative contribution of each varies significantly in different human tumors[59]. In our previous report, we compared hypermethylation of p16, p15, p14, HLA-A, -B, -C, hMLH1, E-cadherin, FHIT and VHL genes in SCC tumor, neighboring normal and precancerous tissues. We found that in 48 biopsy samples with BCH or DYS, the most frequent hypermethylated genes were p16 (18.8%) and $p14^{ARF}$ (14.6%), and seventeen out of these 48 samples (35.4%) contained hypermethylation of at least one gene[26]. In the resected tissue samples, 52% of the BCH and 81% of the tumors showed hypermethylation of at least one gene. Moreover, genes hypermethylated in earlier stage lesions were always found hypermethylated at the later stage lesions in the same patient[26]. In another study, we reported that two of 17 (12%) normal, 9 of 21 (43%) BCH, 7 of 12 (58%) DYS, and 14 of 20 (70%) SCC samples had hypermethylation of the $RAR\beta_2$ promoter region[40]. As to progression of EAC, it has been reported that, methylation of the p16 promoter was detected in 18 of 22 (82%) EAC and 10 of 33 (30%) premalignant lesions, whereas no methylation of the p16 promoter was found in normal esophageal epithelia[59]. These data suggest that aberrant DNA methylation participates early in the development of esophageal cancer. Recent studies showed that epigallocatechin-3-gallate (EGCG), the major polyphenol from green tea, inhibited DNMT activity and reactivate several methylation-silenced genes, including p16, $RAR\beta_2$, MGMT and hMLH1, in human esophageal cancer KYSE 510 cells, accompanied by the expression of mRNA of these genes[70]. The result suggests that methylation might be a new target of chemopreventive activity. In the last two decades, it has been proved that many drugs, such as tamoxifen, aspirin, COX-2 inhibitors, possess positive chemopreventive

activity against esophageal cancer[4]. However, the related mechanisms have not been elucidated so far. Therefore, it will be very attractive to examine the effect of these drugs on promoter methylation status of key genes in esophageal precancerous lesions.

### 3.2 Hypermethylation as a target of therapeutic intervention

It has been reported that demethylating agents 5-azacytidine and 5-aza-2′-deoxycytidine can reactivate the demethylated state of several TSG and increase their expression in various cancers, including esophageal cancer, *in vitro* and *in vivo*[21,22,33,35,71,72]. Since methylation and transcriptional status are inversely correlated, the use of demethylating agents appears to be a promising option for the treatment of tumors. Methylation of genes in tumor cells could provide a tumor specific target for new therapies[33,35,71]. In fact, these demethylating agents have exhibited significant activity in the treatment of patients with myelodysplastic syndrome, chronic myeloid leukaemia and acute myeloid leukaemia[24,71]. However, preliminary experience with these agents in solid tumors has been relatively poor[20,33]. Esophageal tumor shows a high prevalence of TSG hypermethylation, and the above studies demonstrated that gene expression could be restored after treatment of esophageal tumor cells with demethylating agents *in vitro*. However, up to date the clinical trial about demethylating agents in esophageal cancer is unavailable. Although it is too early to make any expectation about the effect of these drugs on esophageal cancer, this is a very promising concept and needs to be tested in further studies[72].

### 3.3 Significance of methylation in clinical application

Emerging evidence suggests a possible prognostic value of gene promoter hypermethylation. Kawakami *et al*[51] reported that high plasma levels of methylated APC DNA were statistically significantly associated with reduced patient survival. Schulmann *et al*[74] analyzed the methylation status of ten genes (HPP1, RUNX3, RIZ1, CRBP1, 3-OST-2, APC, TIMP3, p16, MGMT, p14) of 77 EAC samples and found that DNA methylation of some genes individually showed only trends toward diminished survival, whereas patients whose tumors had $>50\%$ of their gene profile methylated had both significantly poorer survival and earlier tumor recurrence than those without positive methylation. The data suggest that combined detection of methylation status for multiple genes is a effective strategy to predict esophageal tumor behavior, to help staging esophageal cancer, to detect recurrent disease, and to monitor disease progression or treatment response. Although some genes that are frequently inactivated by methylation and are of prognostic impact for esophageal cancer patients have already been found, additional genes need to be identified. Thus, patients with a worse prognosis could be selected. These patients might benefit from a more aggressive treatment strategy.

Unlike genetic changes, epigenetic changes could potentially be reversed using DNA methyltransferase inhibitors such as 5-aza-dc in cancer. Clinically, 5-aza-dc and its analogs have been used to treat leukemia and lung cancer[75, 76]. The clinical benefit observed has been associated with the restoration of previously silenced genes. Additionally, one potential advantage of using gene methylation as a biomarker is the fact that its presence or absence is easily established by use of MSP. Hypermethylation biomarkers, in combination with other molecular markers, such as p53 mutation, for predicting early outset of SCC would be valuable for a follow-up study in high-risk area for esophageal cancer.

## 4 Conclusion and Perspectives

Esophageal carcinogenesis is a multistep process of accumulation of genetic and epigenetic abnormalities. It has become clear that promoter hypermethylation of TSG are important for this progressive process. The steadily growing list of genes inactivated by promoter hypermethylation in esophageal carcinoma provides not only new insights into the molecular basis of the diseases but also a long list of interesting candidate genes for the development of molecular biomarkers for high-risk subject screening and early diagnosis. In addition, the fact that methylation could be reversed *in vitro* raise a new treatment strategy for esophageal cancer treatment and prevention. It is much desirable to develop methylation biomarker from cell free circulating blood samples which is of apparent significantly in large-scale mass survey for high-risk subject screening in the high-risk areas for esophageal cancer. Furthermore, It is crucial to establish standard method in methylation detection to make a fundamental conclusion.

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## References

1. Wang LD, Hong JY, Qiu SL, et al. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. Cancer Res 1993; 53: 1783－7.
2. Enzinger PC, Mayer RJ. Esophageal cancer. N Eng J Med 2003; 349: 2241－52.
3. Chen X, Yang CS. Esophageal adenocarcinoma: a review and perspectives on the mechanism of carcinogenesis and chemoprevention. Carcinogenesis 2001; 22: 1119－29.
4. Wang LD, Zhou Q, Feng CW, et al. Intervention and follow-up on human esophageal precancerous lesions in Henan, northern China, a high-incidence area for esophageal cancer. Gan to Kagaku Ryoho 2002; 29:159－72.
5. Momparler RL. Cancer epigenetics. Oncogene 2003; 22: 6479－83.
6. Farrell WE, Clayton RN. Epigenetic change in pituitary tumorigenesis. Endocr Relat Cancer 2003;10: 323－30.
7. Chim CS, Liang R, Kwong YL. Hypermethylation of gene promoters in hematological neoplasia. Hematol Oncol 2002; 20: 167－76.
8. Garinis GA, Patrinos GP, Spanakis NE, et al. DNA hypermethylation: when tumor suppressor genes go silent. Hum Genet 2002; 111: 115－27.
9. Jain PK. Epigenetics: the role of methylation in the mechanism of action of tumor suppressor genes. Ann N Y Acad Sci 2003; 983: 71－83.
10. Lehmann U, Brakensiek K, Kreipe H. Role of epigenetic changes in hemato-logical malignancies. Ann Hematol 2004; 83: 137－52.
11. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Eng J Med 2003; 349: 2042－54.
12. Fraga MF, Esteller M. DNA methylation: a profile of methods and applications. Biotechniques 2002; 33: 632－49.
13. Esteller M. Relevance of DNA methylation in the management of cancer. Lancet Oncol 2003; 4: 351－8.
14. Esteller M. DNA methylation in cancer: too much, but also too little. Oncogene 2002; 2: 5400－13.
15. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002; 21: 5427－40.
16. Jones PA, Laird PW. Cancer epigenetics comes of age. Nat Genet 1999; 21: 163－7.
17. Momparler RL, Bovenzi V. DNA methylation and cancer. J Cell Physiol 2000; 183: 145－54.
18. Esteller M. Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv Exp Med Biol 2003; 532: 39－49.
19. Nephew KP, Huang TH. Epigenetic gene silencing in cancer initiation and progression. Cancer Lett 2003; 190: 125－33.
20. Xing EP, Nie Y, Song Y, et al. Mechanisms of inactivation of p14ARF, p15INK4b, and p16INK4a genes in human esophageal squamous cell carcinoma. Clin Cancer Res 1999; 5: 2704－13.
21. Tanaka H, Shimada Y, Imamura M, et al. Multiple types of aberrations in the p16 (INK4a) and the p15 (INK4b) genes in 30 esophageal squamous-cell-carcinoma cell lines. Int J Cancer 1997; 70: 437－42.
22. Smeds J, Berggren P, Ma X, et al. Genetic status of cell cycle regulators in squamous cell carcinoma of the oesophagus: the CDKN2A (p16 (INK4a) and p14 (ARF)) and p53 genes are major targets for inactivation. Carcinogenesis 2002; 23: 645－55.
23. Maesawa C, Tamura G, Nishizuka S, et al. Inactivation of the CDKN2 gene by homozygous deletion and de novo methylation is associated with advanced stage esophageal squamous cell carcinoma. Cancer Res 1996; 56: 3875－8.
24. Tokugawa T, Sugihara H, Hattori T. Modes of silencing of p16 in development of esophageal squamous cell carcinoma. Cancer Res 2002; 62: 4938－44.
25. Hibi K, Taguchi M, Nakase T, et al. Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res 2001; 7: 3135－38.
26. Nie Y, Liao J, Zhao X, et al. Detection of multiple gene hypermethylation in the development of esophageal squamous cell carcinoma. Carcinogenesis 2002; 23: 1713－20.
27. Zhang F, Wang L, Wu PP, et al. In situ analysis of p16/INK4 promoter hypermethylation in esophageal carcinoma and gastric carcinoma. Chin J Dig Dis 2004; 5: 149－55.
28. Abbaszadegan MR, Raziee HR, Ghafarzadegan K, et al. Aberrant p16 methylation, a possible epigenetic risk factor in familial esophageal squamous cell carcinoma. Int J Gastrointest Cancer 2005; 36: 47－54.
29. Guo XQ, Wang SJ, Zhang JH, et al. CpG island methylation of p16 and FHIT gene in tissues of the esophageal precancerous lesions. Chin Clin Cancer 2005; 32: 554－7.
30. Yao QF, Kang XJ, Hao QL, et al. Detection of promoter hypermethylation in the serum of esophageal squamous cell carcinoma patients by nested methylation-specific-polymerase chain reaction. Cancer Res on Prev and Treat 2005; 32: 463－6.
31. Kim MS, Yamashita K, Baek JH. N-methyl-D-aspartate receptor type 2B is epigenetically inactivated and exhibits tumor-suppressive activity in human esophageal cancer. Cancer Res 2006; 66: 3409－18.
32. Wong ML, Tao Q, Fu L, et al. Aberrant promoter hypermethylation and silencing of the critical 3p21 tumor suppressor gene, RASSF1A, in Chinese esophageal squamous cell carcinoma. Int J Oncol 2006; 28: 767－73.
33. Liu Z, Zhang L, Ding F, et al. 5-Aza-2′-deoxycytidine induces retinoic acid receptor-beta (2) demethylation and growth inhibition in esophageal squamous carcinoma

cells. Cancer Lett 2005; 230: 271–83.
34. Mizuiri H, Yoshida K, Toge T, et al. DNA methylation of genes linked to retinoid signaling in squamous cell carcinoma of the esophagus: DNA methylation of CRBP1 and TIG1 is associated with tumor stage. Cancer Sci 2005; 96: 571–7.
35. Fang MZ, Jin Z, Wang Y, et al. Promoter hypermethylation and inactivation of $O^6$-methylguanine-DNA methyltransferase in esophageal squamous cell carcinomas and its reactivation in cell lines. Int J Oncol 2005; 26: 615–22.
36. Kubo N, Yashiro M, Ohira M, et al. Frequent microsatellite instability in primary esophageal carcinoma associated with extra-esophageal primary carcinoma. Int J Cancer 2005; 114: 166–73.
37. Takeno S, Noguchi T, Fumoto S, et al. E-cadherin expression in patients with esophageal squamous cell carcinoma: promoter hypermethylation, snail over-expression, and clinicopathologic implications. Am J Clin Pathol 2004; 122: 78–84.
38. Itaru S, Issei I, Jun I, et al. Frequent silencing of low density lipoprotein receptor-related protein 1B (LRP1B) expression by genetic and epigenetic mechanisms in esophageal squamous cell carcinoma. Cancer Res 2004; 64: 3741–7.
39. Liu ZM, Ding F, Guo MZ, et al. Down regulation of retinoic acid receptor-beta (2) expression is linked to aberrant methylation in esophageal squamous cell carcinoma cell lines. World J Gastroenterol 2004; 10: 771–5.
40. Wang Y, Fang MZ, Liao J, et al. Hypermethylation-associated inactivation of retinoic acid receptor beta in human esophageal squamous cell carcinoma. Clin Cancer Res 2003; 9: 5257–63.
41. Yamashita K, Mimori K, Inoue H, et al. A tumor-suppressive role for trypsin in human cancer progression. Cancer Res 2003; 63: 6575–8.
42. Kuroki T, Trapasso F, Yendamuri S, et al. Allele loss and promoter hypermethylation of VHL, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. Cancer Res 2003; 63: 3724–8.
43. Zhang L, Lu W, Miao X, et al. Inactivation of DNA repair gene $O^6$-methylguanine-DNA methyltransferase by promoter hypermethylation and its relation to p53 mutations in esophageal squamous cell carcinoma. Carcinogenesis 2003; 24: 1039–44.
44. Yue CM, Deng DJ, Bi MX, et al. Expression of ECRG4, a novel esophageal cancer-related gene, downregulated by CpG island hypermethylation in human esophageal squamous cell carcinoma. World J Gastroenterol 2003; 9: 1174–8.
45. Hayashi M, Tamura G, Jin Z, et al. Micro-satellite instability in esophageal squamous cell carcinoma is not associated with hMLH1 promoter hypermethylation. Pathol Int 2003; 53: 270–6.
46. Kuroki T, Trapasso F, Yendamuri S, et al. Promoter hypermethylation of RASSF1A in esophageal squamous cell carcinoma. Clin Cancer Res 2003; 9: 1441–5.
47. Noguchi T, Takeno S, Kimura Y, et al. FHIT expression and hypermethylation in esophageal squamous cell carcinoma. Int J Mol Med 2003; 11: 441–7.
48. Shibata Y, Haruki N, Kuwabara Y, et al. Chfr expression is down-regulated by CpG island hypermethylation in esophageal cancer. Carcinogenesis 2002; 23: 1695–9.
49. Si HX, Tsao SW, Lam KY, et al. E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. Cancer Lett 2001; 173: 71–8.
50. Nie Y, Yang G, Song Y, et al. DNA hypermethylation is a mechanism for loss of expression of the HLA class I genes in human esophageal squamous cell carcinomas. Carcinogenesis 2001; 22: 1615–23.
51. Kawakami K, Brabender J, Lord RV, et al. Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. J Natl Cancer Inst 2000; 92: 1805–11.
52. Tanaka H, Shimada Y, Harada H, et al. Methylation of the 5′ CpG islands of the FHIT gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. Cancer Res 1998; 58: 3429–34.
53. Zhang JH, Liu FR, Ma L, et a1. Effects of hMLH1 promoter methylation OR esophageal carcinoma. Chin J Public Health 2005; 21: 7780–1.
54. Tian ZQ, Liu JF, Zhang YF, et al. Clinical significance of CpG island methylation of MT3 gene in squamous cell carcinoma of esophagus. Journal of Practical Oncology 2004; 19: 386–9.
55. Guo MZ, Michael GH, Yoshimitsu A, et al. Hypermethylation of the GATA gene family in esophageal cancer. Chin J Gastro Hepa 2003; 12: 130–7.
56. Carnero A, Hannon GJ. The INK4 family of CDK inhibitors. Curr Top Microbiol Immunol 1998; 227: 43–55.
57. Kamb A. Cyclin-dependent kinase inhibitors and human cancer. Curr Top Microbiol Immunol 1998; 227: 139–48.
58. Ortega S, Malumbres M, Barbacid M. CyclinD-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta 2002; 1602: 73–87.
59. Bian YS, Osterheld MC, Fontolliet C, et al. p16 inactivation by methylation of the CDKN2A promoter occurs early during neoplastic progression in Barrett's esophagus. Gastroenterology 2002; 122: 1113–21.
60. Platz A, Hansson,J, Mansson-Brahme E, et al. Screening of germline mutations in the CDKN2A and CDKN2B genes in Swedish families with hereditary cutaneous melanoma. J Natl Cancer Inst 1997; 89: 697–702.
61. Pekarsky Y, Zanesi N, Palamarchuk A, et al. FHIT: from gene discovery to cancer treatment and prevention. Lancet Oncol 2002; 3: 748–54.
62. Sozzi G, Huebner K, Croce CM. FHIT in human cancer. Adv Cancer Res 1998; 74: 141–66.
63. Tanaka H, Shimada Y, Harada H, et al. Methylation of the 5′ CpG islands of the FHIT gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. Cancer Res 1998; 58: 3429–34.
64. Qiu H, Zhang W, El-Naggar AK, et al. Loss of retinoic acid receptor-beta expression is an early event during esophageal carcinogenesis. Am J Pathol 1999; 155: 1519–23.
65. Fearnhead NS, Britton MP, Bodmer WF. The ABC of APC. Hum Mol Genet 2001; 10: 721–33.
66. Esteller M, Hamilton SR, Burger PC, et al. Inactivation of the DNA repair gene $O^6$-methylguanine-DNA methyl-

transferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 1999; 59: 793-7.

67. Nakamura M, Watanabe T, Yonekawa Y, et al. Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C→A:T mutations of the TP53 tumor suppressor gene. Carcinogenesis 2001; 22:1715-9.
68. Kaganoi J, Kan T, Watanabe G, et al. Involvement of TSLC1 in progression of esophageal squamous cell carcinoma. Cancer Res 2003; 63: 6320-6.
69. Fukami T, Fukuhara H, Kuramochi M, et al. Promoter methylation of the TSLC1 gene in advanced lung tumors and various cancer cell lines. Int J Cancer 2003; 107: 53-9.
70. Fang MZ, Wang Y, Ai N, et al. Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 2003; 63:7563-70.
71. Fang MZ, Chen D, Sun Y, et al. Reversal of hypermethylation and reactivation of p16INK4a, RAR beta, and MGMT genes by genistein and other isoflavones from soy. Clin Cancer Res 2005; 11: 7033-41.
72. Yamashita K, Upadhyay S, Osada M, et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell 2002; 2: 485-95.
73. Yamaguchi S, Kato H, Miyazaki T, et al. RASSF1A gene promoter methylation in esophageal cancer specimens. Dis Esophagus 2005; 18: 253-6.
74. Schulmann K, Sterian A, Berki A, et al. Inactivation of p16, RUNX3, and HPP1 occurs early in Barrett's-associated neoplastic progression and predicts progression risk. Oncogene 2005; 24: 4138-48.
75. Momparler RL, Bouffard DY, Momparler LF, et al. Pilot: phase I-II study on 5-aza-20-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 1997; 8: 358-61.
76. Rivard GE, Momparler RL, Demers J, et al. Phase I study on 5-aza-20-deoxycytidine in children with acute leukemia. Leuk Res 1981; 5: 453-8.

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