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ORIGINAL PAPER

Promoter methylation status of *MGMT*, *hMSH2*, and *hMLH1* and its relationship to corresponding protein expression and *TP53* mutations in human esophageal squamous cell carcinoma

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Abstract To determine the relevance of *O*-6-methylguanine-DNA methyltransferase (*MGMT*), human mutS homolog 2 (*hMSH2*), and human mutL homolog 1 (*hMLH1*) in *TP53* mutations in esophageal squamous cell carcinoma, we employed methylation-sensitive high-resolution melting technology and methylation-specific polymerase chain reaction (PCR) to analyze promoter hypermethylation of *MGMT*, *hMSH2*, and *hMLH1*, respectively, in 51 paired tumors and their adjacent normal tissues. The protein expression of the three proteins was also evaluated by Western blot analysis, and the PCR products of *TP53*, from exon 5 to exon 8, were directly sequenced to measure the mutation spectrum. Esophageal tumor tissues embraced statistically higher *MGMT* and *hMSH2* promoter methylation level than normal tissue. The promoter methylation status of MGMT and hMSH2 corresponds positively with the protein expression level of MGMT and hMSH2. However, such relevance was not found for hMLH1. Furthermore, *TP53* mutation status was well associated with *MGMT* and *hMSH2* promoter methylation status, indicating that silencing of the two genes could lead to *TP53* mutation in ESCC.

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Introduction

The incidence rate of esophageal squamous cell carcinoma (ESCC) is extremely high in certain parts of China, including the Huaian area, a region located north of the JiangSu province, where approximately 5,500 cases are diagnosed each year within a population of five million. Our previous epidemiological and ecological investigations in the Huaian area revealed that exposure to environmental carcinogens such as nitrosamines [22, 38], which commonly exist in the daily diet of the local residents, contribute much to the etiology of esophageal carcinogenesis. Nitrosamines induce alkylated DNA bases, including the highly pro-carcinogenic adduct *O*-6-methylguanine (O6MeG), which is most notorious because of its ability to mispair with thymine during replication, leading to G:C–A:T mutations [23]. These mutations lead to carcinogenesis if they occur in tumor-related genes [34] such as *TP53*. The repair of O6MeG is specifically executed by *O*-6-methylguanine-DNA methyltransferase (MGMT) through a so-called suicide process, in which the alkyl group from oxygen atoms within the DNA is transferred to a cysteine residue in the active site of the MGMT enzyme, thereby inactivating it and restoring the DNA to its original state. If not removed by MGMT, replication of DNA containing O6MeG gives rise to O6MeG/thymine mispair, which is recognized and excised by the DNA mismatch repair system (MMR) [4]. The mechanism described above shows that MGMT and MMR are extremely important in repairing O6MeG mutations, which has also been directly or indirectly demonstrated by the relationship between the aberrant functional expression of *MGMT*, *MMR,* and carcinogenesis found

in many studies over the past two decades [7, 20, 26, 27, 31]. In addition, the functional interaction between MGMT and MMR has also been extensively documented in transgenic animals and cell lines [12, 18]. Furthermore, our previous case–control study in the Huaian area indicated that low expression of *MGMT* and *hMSH2* (human mutS homolog 2) mRNA leads to an increased risk of local esophageal cancer occurrence (OR value was 6.65 and 1.30, respectively) [21], which indicates that these two DNA repair systems may play crucial roles in this disease.

To further investigate the roles that *MGMT* and *MMR* play in esophageal squamous cell carcinogenesis in the Huaian region, we selected two key genes of the *MMR* system, such as *hMSH2* and *hMLH1* (human mutL homolog 1), and compared the expression levels of MGMT, hMSH2, and hMLH1 (human mutL homolog 1) proteins in 51 paired ESCC and corresponding normal adjacent tissues. In addition, we measured the promoter methylation status of the three genes to determine whether it regulates the expression of functional proteins, as it is widely regarded that the loss of *MGMT* and *MMR* expression is rarely due to deletions, mutations, or rearrangements, but rather is due to the methylation of discrete regions of the CpG islands of the genes. Moreover, the correlation between the epigenetic change and expression levels of the three proteins was also examined. In this study, we selected the mutation spectrum of the tumor-related gene, *TP53*, as an end point to evaluate the collaborative effect of *MGMT* and *MMR* on mutations in 51 paired esophageal squamous cell cancer samples.

Materials and methods

Tumor samples and DNA extraction

Fifty-one pairs of ESCC and corresponding normal adjacent tissues adjacent to the tumors were obtained from surgically removed specimens of individual patients who underwent esophagectomies at The First People's Hospital of Huaian, China. None of these patients received antitumor treatment before the operation, and the diagnosis as ESCC was histologically confirmed. The paired normal tissues adjacent to the tumor were sampled at least 5 cm away from the center of the tumor. All samples were frozen in liquid nitrogen immediately after resection or biopsy and stored at −80 °C until processing. DNA was extracted using a standard method that was previously described [1].

Sodium bisulfite treatment

For subsequent PCR analysis, sample DNA was treated using the EpiTect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's instructions.

Methylation-sensitive high-resolution melting technology (MS-HRM)

Considering the significantly high OR value of *MGMT*, we measured the methylation level of *MGMT* promoter methylation status using methylation-sensitive high-resolution melting technology (MS-HRM) in the LightCycler 480 system (Roche, Germany). In brief, each sample was assessed by comparing the PCR product melting profiles between each sample and the standards with a known ratio of methylated and unmethylated templates, as previously described [36]. The melting profiles of samples were compared to the melting profiles of PCR products derived from the mixes of 100, 75, 50, 25, 5, and 0 % of fully methylated template in an unmethylated background and scored as being methylated at four levels: Grade I (0–25 %), Grade II (25–50 %), Grade III (50–75 %), and Grade IV (75–100 %). Published *MGMT* primer sequences were used [37].

Methylation-specific PCR (MSP)

DNA methylation patterns in the CpG islands of *hMSH2* and *hMLH1* were determined by MSP as described [14]. Primer sequences of *hMSH2* and *hMLH1* used for unmethylated and methylated reactions are given in Table 1. All samples were first amplified with flanking PCR primers that amplify bisulfite-modified DNA, but that would not preferentially amplify methylated or unmethylated DNA. The primers used were 5′-GAGTAGTTTTTTTTTAGGA GTGAAG-3′ (sense) and 5′-AAAAACTATAAAACCC TATACCTAATCTA-3′ (antisense). Human placental DNA treated in vitro with excessive SssI methyltransferase (New England Biolabs, England), generating DNA completely methylated at all CpG sites, served as the positive control [15]. Each PCR was loaded onto non-denaturing 6 % polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Western blot analysis

Tissue protein was extracted in lysis buffer (50 mM Tris–HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 % Triton X-100, and protease inhibitor cocktail). The protein concentration in the supernatants was evaluated using the BCA method. After addition of SDS-PAGE sample buffer and boiling, 20 μg of denatured proteins was separated on 12 % SDS-PAGE gels and then transferred to nitrocellulose membranes. After being incubated in blocking buffer, the membranes were incubated with the appropriate primary antibodies, which included monoclonal anti-MGMT (Santa Cruz Biotechnology, USA), monoclonal anti-β-actin

Table 1 Primer sequences for MSP

Gene	Sense	Antisense	Size (bp)
hMSH2			
U	5′-GGTTGTTGTGGTTGGATGTTGTTT-3′	5′-CAACTACAACATCTCCTTCAACTACACCA-3′	143
M	5′-TCGTGGTCGGACGTCGTTC-3′	5′-CAACGTCTCCTTCGACTACACCG-3′	134
hMLH1			
U	5′-TTTTGATGTAGATGTTTTATTAGGGTTGT-3′	5′-ACCACCTCATCATAACTACCCACA-3′	124
M	5′-ACGTAGACGTTTTATTAGGGTCGC-3′	5′-CCTCATCGTAACTACCCGCG-3′	115

M methylated, *U* unmethylated

Table 2 Primer sequences for *TP53* PCR products

Region of *TP53*	Sense	Antisense
Exon 5	5′-TGCCCTGACTTTCAACTCTG-3′	5′-GCTGCTCACCATCGCTATC-3′
Exon 6	5′-CTGATTCCTCACTGATTGCT-3′	5′-AGTTGCAAACCAGACCTC-3′
Exon 7	5′-CCTGTGTTATCTCCTAGGTTG-3′	5′-GCACAGCAGGCCAGGTGCA-3′
Exon 8	5′-GACCTGATTTCCTTACTGC-3′	5′-TCTCCTCCACCGCTTCTTGT-3′

(Santa Cruz), monoclonal anti-hMSH2 (Abcam, USA), and monoclonal anti-hMLH1 (Cell Signaling Technology, USA). Secondary antibodies (HRP conjugated) were purchased from Jackson Immuno Research Laboratories. Immunolabelling was visualized using the ECL procedure (Thermo, USA). Bands were quantified using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). Normalization was made against β-actin expression.

TP53 mutation analysis by direct sequencing

For *TP53* mutation analysis, target sequences were amplified by PCR using the given primer pairs shown in Table 2. PCR products of *TP53* from exon 5 to exon 8 were sequenced by an automated sequencing system (3,100 Avant Genetic Analyzer, Applied Biosystems, Hitachi, Japan). All *TP53* mutations were confirmed by direct sequencing of both strands.

Statistical analysis

The Wilcoxon rank sum test and Chi square analysis were used to compare the distribution of methylated *MGMT, hMSH2,* and *hMLH1* promoters among the tumor and normal tissues, respectively. Moreover, the Pearson χ^2 test was introduced to assay the correlation between *MGMT, hMSH2,* and *hMLH1* promoter status and their corresponding protein expression. All statistical methods were carried out using SPSS software, version 13.0 (SPSS Inc., Chicago, IL), and $P < 0.05$ was considered statistically significant.

Results

MGMT promoter methylation level was measured using MS-HRM technology, and the melting curves of standard methylated genomic DNA and representative samples are displayed in Figs. 1 and 2, respectively. The *MGMT* methylation level was divided into four grades: Grade I (0–25 %), Grade II (25–50 %), Grade III (50–75 %), and Grade IV (75–100 %). The methylation grade in normal tissue was generally statistically lower than that in tumor tissue (Table 3). Normal tissue had a lower grade of *MGMT* methylation than its tumor counterpart in 31 paired ESCC samples (60.78 %), while 14 pairs of ESCC samples (27.45 %) displayed the same grade in both tissues; however, the remaining 6 tumor tissues (11.76 %) showed a lower grade of *MGMT* methylation compared to normal tissue. The rate of *hMSH2* promoter hypermethylation in tumor tissue was 80.39 % (41/51) higher than that of the normal counterpart, which was 7.84 % (4/51). Considering individual samples, 38 paired samples (74.51 %) embraced a M/U methylation model that *hMSH2* hypermethylation was detected in tumor tissue but not in its paired normal counterpart. In addition, U/U (both unmethylated in tumor and normal tissue) and M/M (both methylated in tumor and normal tissue) models were found in nine and three paired samples, respectively (Fig. 3; Table 4). In terms of the *hMLH1* gene, the promoter methylation level was similar

in both tumor and normal tissue, as U/U and M/M models were discovered in 41 and four paired samples, respectively; four paired samples displayed the M/U model in which the *hMLH1* promoter was methylated in tumor tissue and unmethylated in it normal counterpart, while the remaining one paired sample had the opposite result, namely the U/M model, in which the promoter was methylated in normal tissue but was unmethylated in tumor tissue (Fig. 4; Table 4).

A positive association between *MGMT*, *hMSH2* promoter methylation status, and their corresponding protein expression was observed (Tables 5, 6). The levels of MGMT and hMSH2 protein in promoter hypermethylation samples were notably less than levels in less methylated or unmethylated promoter samples (Fig. 5), indicating that methylation status might be important in regulating

Table 3 *MGMT* methylation status in tumor and corresponding normal tissue of 51 paired samples

	MGMT methylation status				*Z* value	*P* value
	Grade I	Grade II	Grade III	Grade IV		
Tumor tissue	2	20	3	26	−5.468	<0.001
Normal tissue	17	28	1	4		

The distribution of 4 grades *MGMT* methylation status in tumor and normal tissue of 51 paired ESCC samples as shown above: the percentage of methylated *MGMT* of Grade I and II was significantly lower in tumor samples compared with normal tissue in the periphery of tumor samples. The methylation level in tumor tissue was significantly higher than normal control, with P value < 0.001 analyzed with Wilcoxon rank sum test

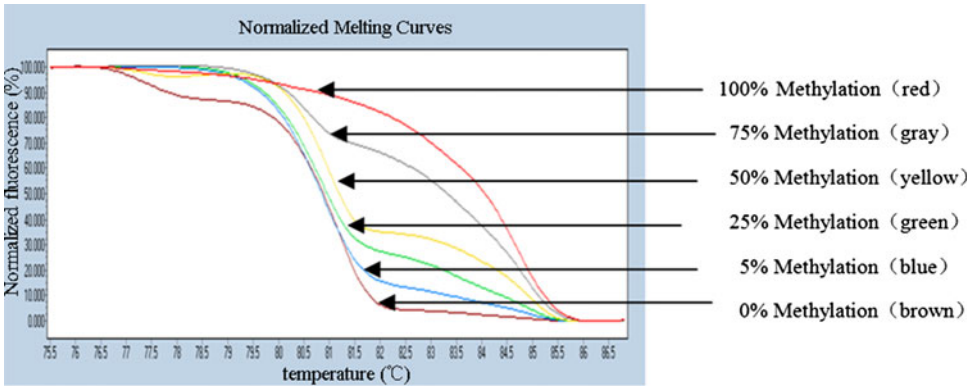

Fig. 1 Standard melting curve was achieved by the analysis of "fully" methylated genomic DNA and unmethylated genomic DNA

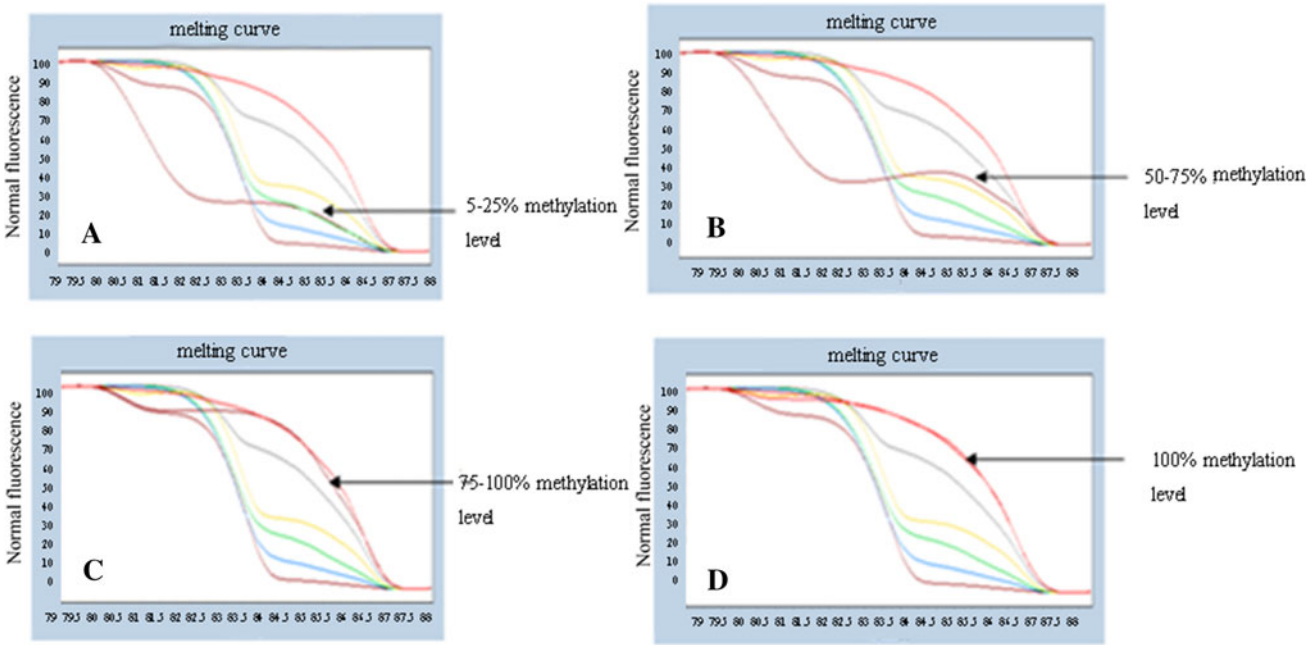

Fig. 2 Representative samples of different *MGMT* promoter methylation levels: **a** 5–25 % methylation level (No. 10.N), **b** 50–75 % methylation level (No. 26.T), **c** 75–100 % methylation level (No. 18.T), **d** 100 % methylation level (No. 43.T). *N* normal tissue adjacent to the tumor tissue, *T* ESCC tissue

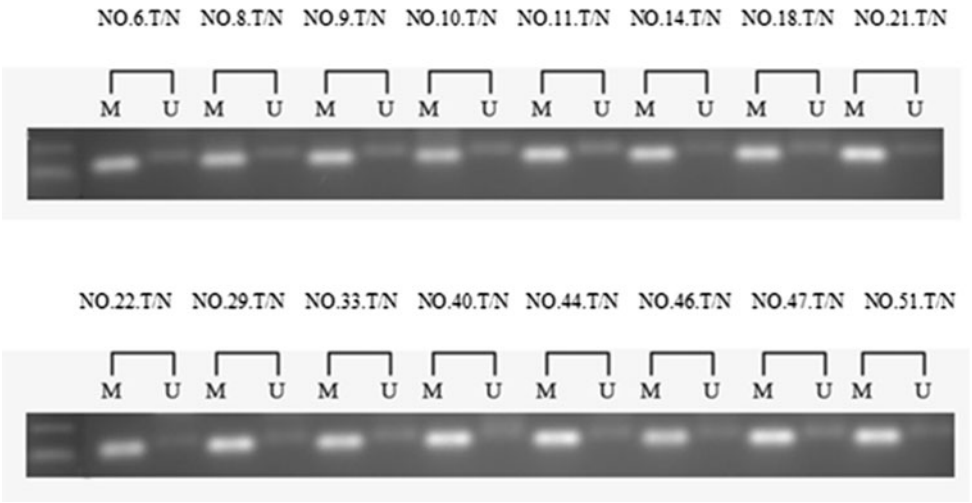

Fig. 3 We studied the MSP with both primers (*M* and *U*) for both normal and tumor tissue. Tumor tissue had methylated *hMSH2* while most normal tissue had an unmethylated promoter. In this figure, we selected representative samples to describe the phenomenon. In individual samples, the *left* electrophoresis line displayed tumor tissue amplified by primers and the *right* electrophoresis line under the same sample displayed the corresponding normal tissue amplified by the *U* primer. *N* normal tissue adjacent to the tumor tissue, *T* ESCC tissue, *M* methylated, *U* unmethylated, T/N: for every sample, T means tumor tissue in the *left line*/N means normal tissue in the *right line*

Table 4 Promoter methylation status of *hMSH2* and *hMLH1* in 51 paired esophageal samples

	hMSH2 hypermethylation			hMLH1 hypermethylation		
	M	*U*	*P* value	*M*	*U*	*P* value
Tumor tissue	80.39 % (41/51)	19.61 % (10/51)	<0.01	17.65 % (9/51)	82.35 % (42/51)	0.45
Normal tissue	7.84 % (4/51)	92.16 % (47/51)		11.76 % (6/51)	88.24 % (45/51)	

About 82.35 % of tumor tissue displayed methylated *hMSH2,* far higher than normal tissue did, which with the rate of 11.76 %, also $P < 0.05$; however, the distribution of *hMLH1* promoter status had no significance in both normal and tumor tissue

Statistical analysis: Wilcoxon signed rank test

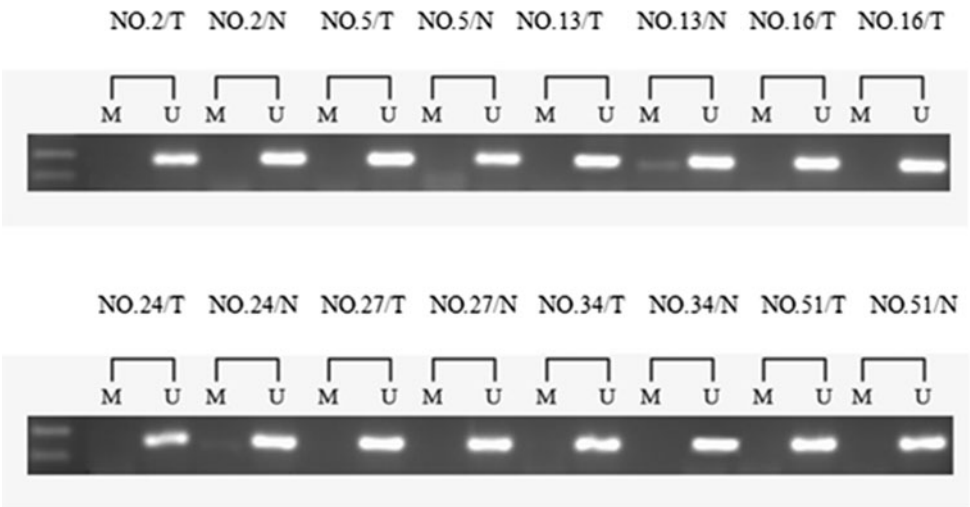

Fig. 4 Representative samples of different *hMLH1* promoter methylation status. No significance of the distribution of *hMLH1* methylation status was found between tumor and normal tissue. *N* normal tissue adjacent to the tumor tissue, *T* ESCC tissue, *M* methylated, *U* unmethylated

MGMT and hMSH2 protein expression. However, a statistically significant association was not found between *hMLH1* promoter status and protein expression (Table 7; Fig. 5).

The *TP53* mutation spectrum was analyzed through direct sequencing of exon 5 to exon 8. Different mutation models were detected in 33 paired samples (64.71 %), including 27 tumor tissues (52.94 %), one normal tissue

Table 5 Correlation between *MGMT* promoter methylation status and MGMT protein expression in 51 paired esophageal samples

MGMT promoter methylation level	MGMT protein expression			*R*	*P*
	+++	++	+		
Grade I	13	1	5	0.34	0.04
Grade II	14	17	19		
Grade III	1	1	3		
Grade IV	8	7	13		

The level of MGMT protein expression was divided into three degrees (+++, ++, +), and *MGMT* promoter methylation level was presented as four spans; obviously, the MGMT protein expression level is inversely proportional to *MGMT* promoter methylation status, and the correlation has statistical significance with $P < 0.05$

Table 6 Concordance analysis between *hMSH2* promoter methylation status and hMSH2 protein expression in 51 paired esophageal samples

Protein expression	Promoter methylation		*P* value	*R* value
	Methylated	Unmethylated		
Aberrant	40 (39.22 %)	5 (4.90 %)	0.00	0.59
Normal	8 (7.84 %)	49 (48.04 %)		
Concordant versus discordant	Concordant: 89 (87.25 %)[a]	Discordant: 13 (12.75 %)[+]		

The hMSH2 protein expression level is inversely proportional to *hMSH2* promoter methylation status, and actual numbers and percentages of cases in a total of 102 samples (including tumor and normal tissues) were presented

[a] Concordant: methylated/aberrant and unmethylated/normal. [+] Discordant: unmethylated/aberrant and methylated/normal. $P < 0.05$ for correlation between *hMSH2* promoter methylation and protein expression

(1.96 %), and five (9.80 %) of both tissues (Table 8). The mutation rate of tumor tissue was clearly much higher than that in corresponding normal tissue ($P = 0.00$). More interestingly, the number of mutation in all samples was well associated with the promoter status of *MGMT* and *hMSH2* genes ($P = 0.00$, OR = 3.33, 11.86, respectively), but there was no association with *hMLH1* promoter status ($P = 0.30$). Considering the special mutation model against MGMT and MMR system, we paid special attention to G:C–A:T transition, but this mutation model had no statistical correlation with the status of the three genes.

Discussion

For the past 30 years, a classical clonal genetic model has been prominently used to carcinogenesis, supported by the discovery of genetic alterations of dominantly acting oncogenes and recessively acting tumor-suppressor genes, such as *BCR and ABL* in CML [32] and *APC*, *KRAS, and TP53* in colorectal cancer [3, 8]. However, this model is limited to exploring the relationship between mutations and carcinoma because progression-related genetic changes are usually inconsistent. Moreover, age and environmental insults also make the time sequence complicated for identifying the precise time of tumor occurrence, especially those with dim latency, such as esophageal cancer. Studies have found that genetic mutations are not the only way cause of gene disruption in cancer. Pathological epigenetic non-sequence-based alterations are increasingly being regarded as alternatives to mutations and chromosomal alterations in disrupting gene function [5], and are composed of global DNA hypomethylation, hypermethylation, and hypomethylation of specific genes, chromatin alterations, and loss of imprinting. All of these can give rise to aberrant activation of growth-promoting genes and abnormal silencing of tumor-suppressor genes [9]. Consequently, the epigenetic progenitor model of cancer was established as a surrogate alteration for genetic changes that circumvent the limitations of genetic models [10]. In addition, the importance of epigenetic changes in carcinogenesis is supported by evidence found in various tumors [6, 13], especially hypermethylation of tumor-suppressor genes, such as the DNA repair system [25], among which hypermethylation of *MGMT* and *MMR* system has been widely discussed in variant cancers as the epigenetic change most widely regarded as the main cause of gene silencing [7, 20, 26, 27, 31]. In addition, the functional interaction between MGMT and DNA mismatch repair has been extensively demonstrated in transgenic animals and cell lines [12, 18], in addition to several solid tumors [11, 24]. However, there is less evidence available exploring the roles of promoter methylation status of the two DNA repair systems in esophageal carcinogenesis or their interactive functions in this disease.

Based on several years of investigation on esophageal cancer in the Huaian area, we garnered data suggesting that *MGMT* and *MMR* play crucial roles in the local high occurrence of this disease. First, the outcome of our previous case–control study revealed that low expression of *MGMT* and *hMSH2* mRNA leads to an increased risk of local esophageal cancer occurrence (OR value was 6.65 and 1.30, respectively). Secondly, epidemiological and ecological investigations demonstrated that exposure to environmental premutagens and precarcinogens, such as nitrosamines, significantly contribute to the etiology of the disease, since nitrosamines commonly exist in the daily diet of the local residents who prefer to eat pickles, and nitrosamines induce *O*-6-methylguanine, which is repaired by the *MGMT* and *MMR* system. Moreover, the influence of the two DNA repair systems on the local disease was also indirectly demonstrated in this study, as the protein

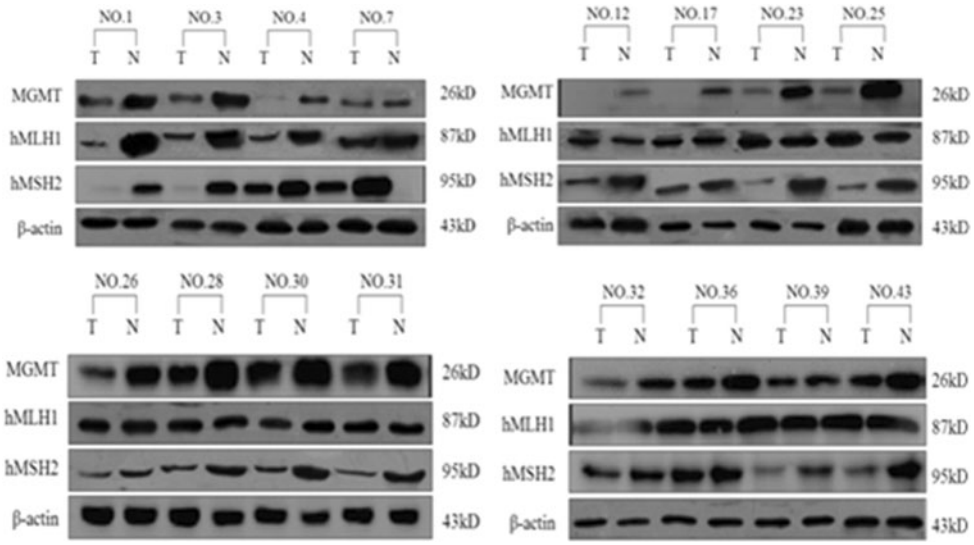

Fig. 5 Representative samples of MGMT, hMSH2, and hMLH1 protein expression. MGMT and hMSH2 were lower expressed in tumor tissue than in normal tissue, but no significance of hMLH1 protein was found in the two kinds of tissues. *N* normal tissue adjacent to the tumor tissue, *T* ESCC tissue

Table 7 Concordant analysis between *hMLH1* promoter methylation status and hMLH1 protein expression in 51 paired esophageal samples

Protein expression	Promoter methylation		*P* value	*R* value
	Methylated	Unmethylated		
Aberrant	6 (5.88 %)	41 (40.20 %)	0.24	0.17
Normal	9 (8.82 %)	46 (45.10 %)		
Concordant versus discordant	Concordant: 52 (50.98 %)[a]	Discordant: 50 (49.02 %)[+]		

hMLH1 protein expression level was not correlated with *hMLH1* promoter methylation status, with $P > 0.05$

[a] Concordant: methylated/aberrant and unmethylated/normal. [+] Discordant: unmethylated/aberrant and methylated/normal

expression of MGMT and hMSH2 was statistically more proficient in normal tissue than in its tumor counterpart, which was consistent with the previous mRNA data. Such common loss of expression of MGMT and hMSH2 proteins in tumor tissue seems to be regulated by the promoter methylation status of the two genes, as a remarkable correlation between *MGMT* and *hMSH2* promoter status, and their corresponding protein expression was confirmed, implying that silencing of the two genes was due to their high methylated promoter. Regarding the remarkable contribution loss of MGMT expression in the Huaian area, we used MS-HRM technology to detect MGMT promoter methylation status, which was more sensitive than MSP, and more importantly, could quantify methylation level. The results of the *MGMT* promoter study were in accordance with other groups [2, 19]; however, the outcome of *hMSH2* and *hMLH1* promoter status in this study had some conflict with other studies. For example, Tzao et al. [35] found that hMLH1 protein expression associated with promoter status and seemed to be a better prognostic predictor in esophageal squamous cell cancer, but hMSH2 protein expression did not correlate with its promoter methylation status. Nie et al. [29] and Vasavi et al. all detected high methylation of *hMLH1* in ESCC samples, but *hMSH2* was not analyzed in their study. The variation in these results may result from the span of culture and social economic levels of different people, or more importantly, from the special diet and genetic background in the Huaian area. As described above, loss of MGMT expression, which leads to mutation accumulation, increased ESCC occurrence in our study area. hMSH2 might be more likely to correlate with MGMT in regard to its ability to recognize G:C–A:T mismatches, but a larger sample size will be needed to verify this.

We selected the mutation spectrum of the tumor suppress gene, *TP53*, as an end point to evaluate the effect of MGMT, hMSH2, and hMLH1 on mutations (defending) in the 51 paired samples. Reasonably, the rate of *TP53* mutations in tumor tissue was 62.75 %, which was significantly higher than that in the normal counterpart (11.76 %), indicating that *TP53* mutations may be a remarkable end point generated in the carcinogenesis process. Moreover, *TP53* mutation levels associated with the status of *MGMT* and *hMSH2* promoter status, with OR values of 3.33 and 11.86, respectively, demonstrating the significant roles of the two genes in generating these mutations. Considering the mechanism of MGMT and MMR in creating *O*-6-methylguanine mutations, we also explored the effects on G:C–A:T mutations. *O*-6-methylguanine is the most critical lesion because of its tendency to mispair with thymine, preferentially resulting in G → A transition or G:C–A:T mutations. Moreover, the repair process of *O*-6-methylguanine carried out by MGMT protein has been exhaustively documented [16]. MGMT repairs *O*-6-methylguanine in a one-step reaction, whereby

Table 8 *TP53* mutation spectrum and *MGMT, hMSH2, hMLH1* promoter methylation status in 51 paired esophageal samples

Patient	Normal tissue					Tumor tissue				
	MGMT (Grade)	*hMSH2*	*hMLH1*	Number of mutation	Location of G:C–A:T	*MGMT (Grade)*	*hMSH2*	*hMLH1*	Number of mutation	Location of G:C–A:T
No. 1	I	U	U	–	–	IV	M	U	3	Exon 6
No. 4	II	U	U	–	–	III	M	U	3	–
No. 5	II	U	U	1	–	IV	M	U	4	Exon 6
No. 6	I	U	U	–	–	IV	M	U	2	Exon 6
No. 7	II	U	U	–	–	IV	U	U	2	–
No. 8	II	U	U	1	–	IV	M	U	1	Exon 6
No. 9	I	U	U	–	–	II	M	U	2	–
No. 10	III	U	M	–	–	II	M	U	1	–
No. 11	II	U	M	1	–	IV	M	U	2	Exon 5
No. 12	I	U	M	–	–	IV	M	M	1	Exon 6
No. 13	I	U	U	–	–	IV	M	U	3	Exon 8
No. 16	II	U	U	1	–	I	U	U	–	–
No. 17	II	U	U	–	–	II	M	U	1	–
No. 18	I	U	U	–	–	IV	M	U	1	Exon 5
No. 23	I	U	M	–	–	IV	M	M	2	Exon 7
No. 24	II	U	U	–	–	II	M	U	1	–
No. 25	II	U	U	–	–	II	M	U	1	–
No. 26	I	U	U	–	–	IV	M	U	3	Exon 7
No. 29	II	U	U	–	–	IV	M	U	1	Exon 8
No. 30	I	U	U	3	–	IV	M	U	1	Exon 6
No. 32	II	U	U	–	–	IV	M	U	3	Exon 6
No. 33	I	U	M	–	–	II	M	M	2	–
No. 36	I	U	U	–	–	IV	M	U	3	Exon 7
No. 39	II	U	U	–	–	IV	M	U	2	Exon 6
No. 40	I	U	U	–	–	IV	M	U	2	Exon 6
No. 41	IV	M	U	1	Exon 8	IV	M	U	2	Exon 6
No. 42	II	U	U	–	–	IV	U	M	1	Exon 6
No. 44	II	U	U	–	–	IV	M	M	1	Exon 6
No. 45	II	M	U	–	–	III	M	U	2	–
No. 46	II	U	U	–	–	IV	M	U	1	Exon 5
No. 48	II	U	U	–	–	II	M	U	1	–
No. 49	IV	U	U	–	–	III	M	U	1	Exon 5
No. 51	II	U	U	–	–	II	M	U	1	–

TP53 mutations were detected in 33 samples including 27 in only tumor tissues, one in only normal tissue, and five in both kinds of tissues. The mutation status was associated with the promoter status of *MGMT* and *hMSH2* promoter methylation. Interestingly, G:C–A:T mutation model had no correlation with the promoter methylation status of the three genes; however, all G:C–A:T existed in samples in which the *MGMT* promoter methylation was IV grade, and simultaneously, all had a methylated *hMSH2* promoter

the alkyl group from oxygen in the DNA is transferred to a cysteine residue in the catalytic pocket of the protein, thereby restoring DNA and inactivating itself. If not repaired by MGMT, replication of DNA containing *O*-6-methylguanine gives rise to O6MeG/thymine mispairs, which are recognized and excised by the DNA MMR. However, MMR is not able to process the lesion correctly, leading to a futile MMR cycle [17] with an unknown frequency. Such function of MMR could result in the generation of tertiary lesions, presumably gapped DNA, which gives rise to DNA double-strand breaks (DSBs) during replication, leading to cell death. This has been shown to occur in MGMT-deficient, but not in MGMT-proficient or tolerant cells following *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) treatment [30], indicating that the status of MGMT and MMR collaboratively determines the sensitivity of cells to alkylated agents and even their resulting fate [28, 33]. Since MGMT- and MMR-proficient

cells are much more likely to die, the MGMT- and MMR-deficient models are tolerant of the cytotoxic effects of alkylated agents, giving rise to mutations, especially in some tumor-related genes. Although no correlation between G:C and A:T transition and the promoter status of the three genes was found in this study, we found that all G:C–A:T mutations existed in samples with Grade IV *MGMT* methylation levels, and simultaneously, all samples with G:C–A:T mutations had a methylated *hMSH2* promoter.

In conclusion, the lower expression of MGMT and hMSH2 proteins is frequently associated with the promoter hypermethylation status of the two genes in Huaian ESCC, and *TP53* mutation status significantly correlates with *MGMT* and *hMSH2* promoter methylation level. The G:C–A:T transition might be a special mutation mode in samples with high methylated *MGMT* and *hMSH2* promoter. Our outcome implied that the methylation status of *MGMT* and *hMSH2* promoter may be attributed to the relative high occurrence of ESCC in the Huaian area.

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Conflict of interest None.

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