RESEARCH ARTICLE

Epigenetic Changes within the Promoter Regions of Antigen Processing Machinery Family Genes in Kazakh Primary Esophageal Squamous Cell Carcinoma

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Abstract

The esophageal squamous cell carcinoma (ESCC) is thought to develop through a multi-stage process. Epigenetic gene silencing constitutes an alternative or complementary mechanism to mutational events in tumorigenesis. Posttranscriptional regulation of human leukocyte antigen class I (HLA-I) and antigen processing machinery (APM) proteins expression may be associated with novel epigenetic modifications in cancer development. In the present study, we determined the expression levels of HLA-I antigen and APM components by immunohistochemistry. Then by a bisulfite-sequencing PCR (BSP) approach, we identified target CpG islands methylated at the gene promoter region of APM family genes in a ESCC cell line (ECa109), and further quantitative analysis of CpG site specific methylation of these genes in cases of Kazakh primary ESCCs with corresponding non-cancerous esophageal tissues using the Sequenom MassARRAY platform. Here we showed that the development of ESCCs was accompanied by partial or total loss of protein expression of HLA-B, TAP2, LMP7, tapasin and ERp57. The results demonstrated that although no statistical significance was found of global target CpG fragment methylation level sof HLA-B, TAP2, tapasin and ERp57 genes between ESCC and corresponding non-cancerous esophageal tissues, there was significant differences in the methylation level of several single sites between the two groups. Of thesse only the global methylation level of LMP7 gene target fragments was statistically higher (0.0517±0.0357) in Kazakh esophageal cancer than in neighboring normal tissues (0.0380±0.0214, $p<0.05$). Our results suggest that multiple CpG sites, but not methylation of every site leads to down regulation or deletion of gene expression. Only some of them result in genetic transcription, and silencing of HLA-B, ERp57, and LMP7 expression through hypermethylation of the promoters or other mechanisms may contribute to mechanisms of tumor escape from immune surveillance in Kazakh esophageal carcinogenesis.

Keywords: Esophageal squamous cell carcinoma - HLA-I - APM - DNA methylation - mass ARRAY

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Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies (Sarah et al., 2008). Xinjiang, in Western China, is one of the highest prevalence of ESCC in the world, and the prevalence and mortality of ESCC in Kazak ethnic group is very high and above the average level in China (Zhang, 1988). Despite improvements in multimodality treatment such as surgical tumor resection with extensive lymphadenectomy and chemo-radiation therapy that have been made over the past decade, the overall survival rate for patients with ESCC has not changed significantly (Kleinberg et al., 2007). The high mortality rate of ESCC occurs primarily due to the majority of patients being diagnosed at an advanced stage of the disease when the 5-year survival rate not exceeding 10% (Zheng et al., 2010; Jemal et al., 2010). Tumor development is a multifactorial process, which including the activation of oncogenes, inactivation of tumor-suppressor genes as well as including immune evasion of cancer cells. The formation and survival of a tumor cell is a sign of successful immune escape and a failure in host immune surveillance and elimination. For the antitumor T cell responses, the interaction of the T cell receptors with their specific human leukocyte antigen class I (HLA-I) complex is required. While, the expression of multiple components of the antigen-processing machinery (APM) is a prerequisite for constitutive HLA class I surface expression and necessary for recognition of non-self antigens by CD8+ cytotoxic T lymphocytes (Jensen, 2007; Raghavan et al., 2008). Therefore, the HLA class I antigen-processing machinery plays a crucial role in mediating immune responses by the assembling, loading, and presenting endogenous antigenic peptide as well as regulating cellular and humoral immunity [Liu et al., 2009; Garrido et al., 2010; Barbara, 2012]. However, Tumor cells

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can be able to escape specific immune responses mediated by down regulation of the surface expression of HLA-Imolecules and it is frequently found in many different types of human tumors (Helen et al., 2010; Garrido et al., 2010; Ayshamgul et al., 2011).

Recently, the molecular mechanisms underlying altered HLA-I surface expression have been attributed to impaired expression of the transporter associated with antigen processing (TAP) 1 and TAP2, the low molecular weight proteins (LMP)-2, LMP7, and LMP10, the β2-microglobulin (β2-m), and the HLA-I heavy chain (Cabrera et al., 2003; Chang et al., 2006). Our previous protein expression studies show that loss expression of HLA-I was associated with down-regulation of APM components and which contribute significantly to risk for ESCC in Kazakh populations (Ayshamgul et al., 2011). The molecular mechanisms underlying the relatively low level of HLA-I and APM components in cancer cells are largely unexplained. Epigenetic events play an important role in gene transcription and protein expression and also contribute to tumor progression. DNA methylation, especially 5_-CpG methylation, is an important mechanism in silencing the expression of genes (Sugimura et al., 2000). Epigenetic, transcriptional, and/or posttranscriptional control of several APM components has been described in malignant cells (Seliger, 2008). In addition, methylation was found with a low frequency for LMP7 and TAP1 in tumors and was associated with a decreased HLA-I surface expression (Campoli et al., 2008; Sers et al., 2009; Ye et al., 2009). Down-regulation of HLA-I expression caused by hypermethylation of HLA-B genes has been documented in esophageal squamous cell carcinoma (Nie et al., 2001; Yan et al., 2002).

In the present study, we hypothesized that the absence of HLA class I and low expression of APM components in esophageal cancer cells was regulated through epigenetic mechanisms in Kazakh ESCC patients. To address this problem, we focused on down-regulated or loss protein expressed 5 candidate genes belonging to the APM family and to evaluate the aberrant methylation of CpG islands at promoter regions. To attain this goal, Human esophageal cancer cell line Eca109 were firstly used to screen specific methylated CpG fragments, cloning into vector and sequencing of corresponding genes to identify CpG sites related to gene promoter methylation and then quantitative detection of gene methylation in clinical esophageal tissue by mass spectrometry (Sequenom MassARRAY) approach, to find out the molecular mechanism of the relationship between esophageal carcinogenesis and to understand the esophageal cancer pathogenesis related to the function of antigen presentation.

Materials and Methods

Clinical characteristics and tissue samples

Primary tumor samples and corresponding non-cancerous esophageal mucosa obtained during surgery from 30 Kazak ESCC patients undergoing tumor resection at the Department of Thoracic Surgery of the First Affiliated Hospital in Xinjiang Medical University (Xinjiang, China) were frozen immediately in liquid nitrogen and stored at -80°C until required, and their corresponding formalin-fixed, paraffin-embedded (FFPE) tissues samples had been obtained at the First Affiliated Hospital in Xinjiang Medical University between March 2010 and June 2011.Relevant clinical data were available for all patients. Of the 30 ESCC patients (none of whom had received pre-operative radiotherapy or chemotherapy). The mean age of the patients was 53.5 years; the youngest patient was 42 years old, and the oldest patient was 73 years old at the time of surgery. Each specimen was histologically examined, and the tumor was graded by at least two experienced pathologists. The main characteristics of ESCC patients, including tumor grade, stage, and lymph node status of the tumor, were categorized according to the TNM (American Joint Committee on Cancer, 4th edition) as follows: (1) 10 cases as clinical stage I; (2) 12 cases as stage II; and (3) 8 cases as stage III. Among the 30 tumors were 12 well-differentiated Tumors, 7 moderately differentiated tumors, and 11 poorly differentiated tumors. Eighteen patients had lymph node metastases. All patients provided informed written consent, and the study was approved by the Ethical Committee of the Medical University of Xinjiang.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed with formalin-fixed paraffin embedded tissue sections. Briefly, 3-μm-thick sections were cut from the paraffin-embedded tissue blocks. After being dewaxed in xylene and rehydrated in alcohol and distilled water, antigen was retrieved by heating in the microwave oven for 15 min at 95°C in EDTA buffer ((pH 8.0; Zhong Shan Goldenbridge Biotechnology Co. Ltd., China). After cooling and rinsing in distilled water, endogenous peroxidase activity was blocked by incubating sections for 15 min in 3% H2O2, followed by rinsing in 0.01 M PBS (pH 7.4) for 10 min. Samples were preincubated with a protein blocking solution for 10 min and the sections were incubated at 4°C overnight in a humid chamber with the indicated antibodies (anti-HLA-B antibody, 1:300; TAP2 antibody 1:300, Santa Cruz Biotechnology, respectively), LMP7 and Tapasin antibody (1:200; Abcam, Cambridge, MA, USA,), ERp57 antibody (1:300, Santa Cruz Biotechnology). Slides were washed three times in PBS and then incubated with a biotinylated secondary antibody (Zhong Shan Goldenbridge Biotechnology Co. Ltd., China) for 15 min at room temperature. The reaction products were visualized with diaminobenzidine (DAB Kit; Zhongshan Goldenbridge Biotechnology). PBS was used in place of the primary antibody as a negative control and slides were counterstained with hematoxylin, dehydrated, and evaluated under light microscope.

The percentage and intensity of positively stained tumor cells in each lesion was investigated by two pathologists who had no knowledge of the patients' characteristics. A consensus number was reached for each tumor sample between the two investigators. Results were scored on a scale from 0 to 3 by the percentage and intensity of positive cells among tumor cells. The percentage of positive cells was scored as 0 for ≤10%, 1 for 11-25%, 2 for 26-50%, 3 for 51-75%, 4 for ≥76%. The

intensity of staining is as follows: 0 indicates absence of staining, 1 indicates weak staining, 2 indicate moderate staining, and 3 indicate intense staining. The sum of both scores was used to identify three categories of expression: normal expression (total score 7–8), partial loss of expression (3–6), and total loss of expression (0–2). IHC staining demonstrated strong expression of HLA-Iin stromal tissue and tumor-infiltrating inflammatory cells, thereby providing an internal positive control, as suggested by a previous study (Akash et al., 2008).

Cell lines

The human esophageal carcinoma cell line (ECa109) was obtained from the Wuhan University (WuHan China). Cells were grown in RPMI 1640 media (Invitrogen) supplemented with 5% fetal bovine serum and penicillin/streptomycin in a 5% CO_2 humidified incubator at 37°C. Before transfection, Eca109 cells were seeded in 6-well plates, the cells transfection were performed when the cells grew up at 60% confluence.

DNA extraction, bisulfite treatment, and bisulfite-sequencing PCR (BSP)

DNA was extracted from (ECa109) cells using a DNA extraction kit (QIAGEN, Valencia, CA, USA), and genomic DNA (500 ng) was bisulfite-modified using the EZ Methylation Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. CpG island fragment specific primers were designed by scanning gene promoter regions using specialized Methyl Primer Express software (ABI company) based on genetic information obtained from the Genbank database. Bisulfite treated DNA from esophageal cancer cell line was PCR amplified. Complete bisulfite modification was confirmed by sequence analysis. BSP amplifications were performed in 50-μl reaction mixtures containing 2 μl bisulfite-modified genomic DNA, 2 μl dNTPs, 1.2 μl primers, 2 μl MgCl2, 20 nM ammonium sulfate, . 75 nM Tris-HCl (pH 8.3), and 3 U of Taq DNA polymerase. The touch-down PCR scheme was applied to amplify with the following cycling conditions: 95°C denaturation for 15 min, 95°C for 20 sec, annealing temperatures ranging from 62°C to 56°C for 1 min, extension at 72°C for 1 min for 45 cycles, and final incubation at 72°C for 7 min. Annealing temperatures were as follows: HLA-B: 60°C, TAP1: 62°C, TAP2: 56°C, ERP57: 56°C, LMP2: 60°C, LMP7: 58°C, tapasin: 58°C and ERAP1: 62°C. PCR was followed by cloning into vectors and sequencing to identify CpG sites related to gene promoter methylation.

Quantitative DNA methylation analysis

For quantitative detection of methylated DNA, we used MassARRAY (Sequenom, San Diego, CA, USA) to analyze the ESCC tissue DNA for CpG content. Target gene specific primer pairs were used to compare methylation levels of target fragments and CpG sites among different samples according to the manufacturer's instructions and as described previously. The primers used were designed according to Sequenom Standard EpiPanel (Sequenom, November 2007 version and Supplemental Table 1). PCR amplification was performed with the following parameters: the PCR mixture contained 10 ng bisulfite-treated DNA, 200 mM dNTPs, 0.2 U of Hot Start Taq DNA polymerase (QIAGEN), and 0.2 mM forward and reverse primers in a total volume of 5μl . The cycles included a hot start at 94°C for 15 min, followed by denaturation at 94°C for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min (45 cycles), with a final incubation at 72°C for 3 min. Unincorporated dNTPs were dephosphorylated by adding 2 ml of premix including 0.3 U shrimp alkaline phosphate (SAP; Sequenom). The reaction mixture was incubated at 37°C for 40 min and SAP was then heat inactivated for 5 min at 85°C. After SAP treatment, 2 ml of the PCR product was used as a template for *in vitro* transcription and RNase a cleavage was used for the reverse reaction, per the manufacturer's instructions (Sequenom). The samples were conditioned and spotted on a 384-pad Spectro-CHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Irvine, CA, USA), followed by spectral acquisition on a MassARRAY analyzer compact MALDI-TOF mass spectrometer (Sequenom). The methylation analyses were carried out using the EpiTYPER application (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites.

Statistical analyses

All statistical analyses were performed with the SPSS Version 17 software package. All *P* values were two-sided and the significance level was $P<0.05$. Mann-Whitney test were used to test continuous variables for differences in immunohistochemical staining scores between tumor and normal tissues for the HLA-I and APM components. Quantitative DNA methylation data derived from MassARRAY were treated as continuous variables and missing measurements were imputed into multivariable regression analyses using samples with replacement for the nonmissing values (single imputations).

Results

HLA-I and APM components proteins expression in Kazak esophageal lesions

Immunohistochemical staining of 30 primary tumor

Table 1. Expression of HLA-I and APM Components in Kazak Esophageal Lesions

	Normal Adjacent Tissue (n=30)			ESCC (n=30)			*p*
	Narmal Expression	Partial Loss	Total Loss	Narmal Expression	Partial Loss	Total Loss	
HLA-B	29 (96.7)	1 (3.3)	0 (0)	6 (20.0)	6 (20.0)	18 (60.0)	0.000
TAP2	17 (56.7)	8 (26.7)	5 (16.7)	4 (13.3)	12 (40.0)	14 (46.7)	0.001
LMP7	15 (50.0)	9 (30.0)	6 (20.0)	4 (13.3)	8 (26.7)	18 (60.0)	0.002
ER_p57	24 (80.0)	5 (16.7)	1 (3.3)	14 (46.7)	6 (20.0)	10 (33.3)	0.006
Tapasin	26 (86.7)	3 (10.0)	1 (3.3)	10 (33.3)	8 (26.7)	12 (40.0)	0.000

tissue and normal esophageal tissue were done with anti HLA-B, TAP2, LMP7, ERp57 and Tapasin proteins (Table 1). Representative staining patterns for HLA-B are shown in Figure 1. Of the 30 cases, 6 (20%) samples show normal expressed, 6 (20%) partial expression and 18 (60%) total loss of HLA-B.HLA-B also demonstrated strong positive expression in stoma cells and tumor-infiltrating inflammatory cells.APM component-specific antibodies showed variable expression of TAP2, LMP7, ERP57 and Tapasin ranging from a total loss to partial loss to normal expression (Figure 1). The loss expression of TAP2, LMP7, ERp57 and Tapasin in ESCC were 14 (46.7), 18 (60%), 10 (33.3%), and 12 (40%), respectively. whereas, 4 (13.3%), 4 (13.3%), 14 (46.7%), and 10 (33.3%) positive expression respectively. The expression of HLA-I antigen and APM components were frequently down-regulated in ESCCs.

Methylation of the APM family gene CpG island in ESCC cell line

We used bisulfite-sequencing PCR (BSP) to map the methylation status of CpG sites in APM family gene promoters in ESCC cell lines. CpG islands are small regions of DNA ranging from 0.5 to 3-5 kb in length where the GC content is more than 60% and the CpG : CpG ratio is more than 0.6, using these criteria, we have conducted a search for well preserved CpG islands in genes that have been reported to be downregulation in Kazak esophageal cancers. All eight APM component genes showed exceptionally high CpG:CpG ratio, as well as a GC content more than 60% (Figure 2). First, we designed eight BSP primer pairs to amplify the gene promoter region sequences spanning 3 kb around the transcription start site. The promoters studied included HLA-B, TAP1, TAP2, LMP2, LMP7, ERAP1, tapasin, and ERp57. These candidate genes are defectively expressed in ESCC. Therefore, we design eight primer pares of BSP to amplify the gene promoter region sequences, respectively. The Esophageal cancer cell line was used to amplify each candidate gene. The products of BSP were cloned into vectors and sequenced. We identified various extents of CpG site methylation at promoter regions of HLA-B, TAP2, LMP7, tapasin, and ERp57. All of the CpG sites of methylated candidate genes are shown in Table 2. The underlined sequence indicates location of the primers; the italicized sequence indicates CpG sites, with bold sequence indicating methylated CpG sites.

Methylation of the APM family gene promoter region in primary Kazak ESCC

To determine the extent to which aberrant methylation of HLA-B, TAP2, LMP7, ERp57 and Tapasin might be also involved in silencing of the gene's expression in primary ESCCs, we quantitative analysed the methylation status of above 5 genes in 30 frozen sections of Kazak primary ESCCs with corresponding non-cancerous esophageal tissues by Sequenom MassARRAY platform. A mass spectrometry approach, is based on the detection of methylation state of a single CpG site at a target fragment (CpG island) and generates a data representing the ratio or frequency of the methylation events on a CpG site in all samples DNA. The methylation levels of every CpG site within HLA-B, TAP2, LMP7, ERp57 and Tapasin were evaluated. As shown in Table 3, methylation levels of five genes varied at the different CpG sites. The single CpG site methylation of HLA-B has shown that the methylation level between two groups CpG sites (CpG_1.2, CpG_12, and CpG_13) from CpG_3, CpG_4, CpG_5.6, and CpG_7.8.9, CpG_10.11, and CpG_12 significant

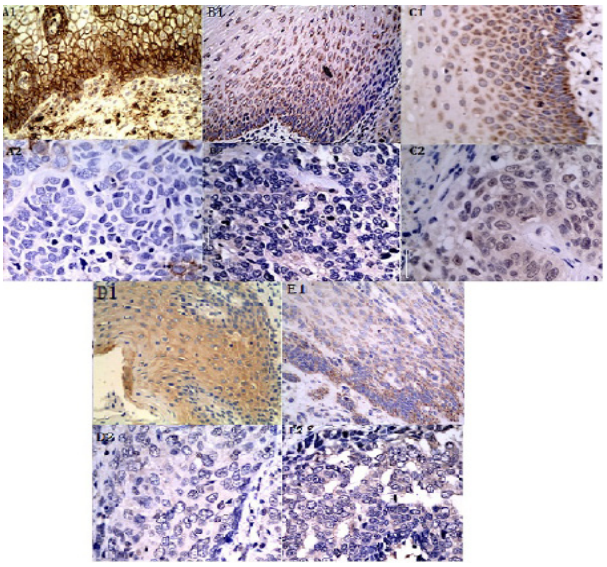

Figure 1. IHC Staining of Esophageal Lesions with HLA-B, TAP2, LMP7, ERp57and Tapasin-specific mAbs. Panels A1–E1 depict normal adjacent tissues with normal protein expression. Panels A2-E2 shows ESCC with weak or total loss of protein expression (original magnification, ×400)

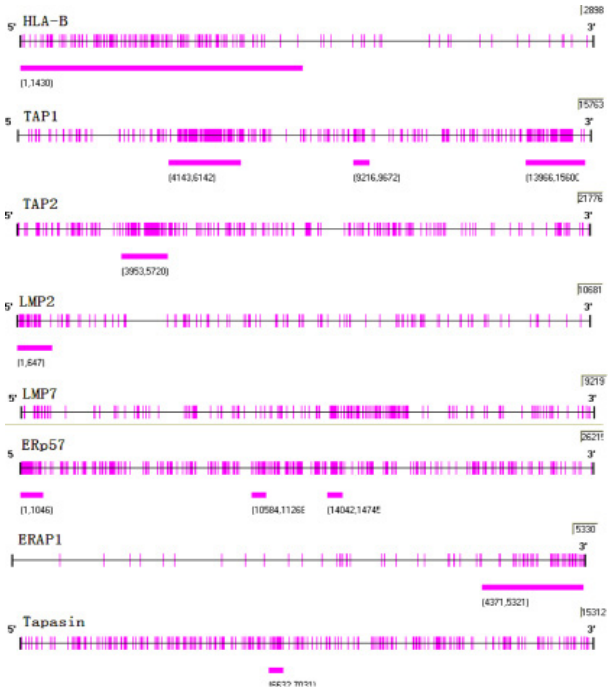

Figure 2. Schematic Map of the CpG-rich Region Around Exon 1 of HLA-B, TAP1, TAP2, LMP2, LMP7, ERAP1, ERp57and Tapasin Genes. Each vertical indicate an individual CpG site. Solid line positions of CpG Island and the first solid line are the positions of BSP primers. All the BSP primers are designed to cover the transcriptional start site should be close to transcriptional start site

Table 2. The CpG Methylation Sites in Target Gene Promoter Fragments

Target gene	Target gene promoter fragments	Total CpG sites	Methylated CpG sites
HLA-B	GAGGTAGGGAGTTTAGTTTAGGGTGGGGACGCGTCACGAGTATCCTGG AGAAGGACCCGACACAAGTTGGGAGAAGAAGTGAAACTCAGGGGAGTG GGAATCCCCAACGCTGCGCCTCCCCATTGCAGACGCGGCCCTCGGAGC TGAGACCCTGAGAGCCCCGTCCGGGACCTGGGACTTCGTCCTGATCCC CTTCTCCTACACCAAGCCTCTTTGTCACACTGTCTGCCTGAGTCCTGG ACAAGGATCTGTCTGTGGAAACCAGGGAGAGACCCCCAGGCTGCGCCC GCCCCTTCCCCTTCACTTCTCGTCCTGGAATCCCTGTCCCTGTGGGT ATTGGATATTTAGAGAAGTTA	14	11
TAP2	AAGGTTTTGGGTTAGGAAGGATATTTAAAAATTTAATTATTGTTTTA AATAGTGTTAAAATAGAATAAGAATTAAAGTTTAGTACGGGGTATTT TTTTTATAGGTTGAAGGTGCGTTTAATATAATTTGGAGTATAGATTT AGAGGTATTTGAATACGCGTTAGTTTAAGGTGTTTCGGTTGAGAAGG ACGGATGAAGATGAACGTTTAGGGTTTATTAAATTTAAAGTTTGTACG TGAAAATTTTTTTTGGTTTGGTG	8	8
LMP7	TGTGATGGTTTTGGTTTAGGTATTAATTGTTTTTTTTTCGGAAAAGGT AGGGGGATGTGGAAAAGAGTTTTGTTTTTTTTTTTTCGATTTGTGGTT TTCGTTTTTATTTTTTTTTTCGAGAGCGGATAGATTTTTGGGTGTTGGG CGGTTATGGCGTTATTAGATGTATGCGGAGTTTTTCGAGGGTAGCGGT CGGAATCGGTTTTTTCGGTTGCGGGAAGCGGGCGTCGTTCGGATTTAG GATATTATAGTTTTTTTATGCGATTTTTAGAGTTCGTTTTATTTCGGG GAATGTAGGTCGGGGTAGTAGGGAAGTTTTTAGGGATGTAGGGAG	22	2
Tapasin	GAAAAGTAAGGTTAGGTGTGGTGGTTTACGTTTGTAATTTTAGAATTT TGGGATATCGAGGTCGGTGGATTATTTGAGGTTAGGAGTTTGAGATTA GTTTGGTTAATACGGTGAAATTTCGTTTTTATTAATATTAAAATTAG TCGGGTATGGTGGCGGGTGTTTGTAATTTTAGTTACGAGGGAGGTTGA GGTAGGAGAATCGTTTGAATTCGGAAGGTAGAGGTTGCGGTGAGTCG AGATTATATTATGGTATTTTAGTTTGGGTAATAAG	12	5
ERp57	AAGATTTAGGGTTTTTTGAAATAAAAGGGGTTAAGAGTGGTAAAGAT ATTGAGTAGTCGATTAAGTGGTTAGGTATTTTTATTTTGTGACGGTA TTATTAATTAAAAGTTTATTAAAAAAAAAAAAAAAAAAAAAAAGT TCGGGCGTGGTGGTTTACGTTTGTAATTTTAGTATTTTGGAATGTCG AGGCGAGCGGATTACGAGGTTAGGAGATAGAGATTATTTTGGTTAAT ACGGTGAAATTCGGTTTTTATTAAAAATATAAAAAATTAGTCGGGCG TGGTGGCGGGCGTTTGTAGGTTTAGTTATTCGGGAAGTTGAGGTAGGA GAATGGCGTGAATTCGGGAGGTAGAGTTTGTAGTGA	18	18

Table 4. Correlation between Target Gene Methylation and Corresponding Protein Expression in ESCC

protein expression	Methylation levels (x±s)				
	HLA-B	TAP2	LMP7	ERp57	Tapasin
Positive	0.0195 ±0.0110	0.8329±0.04697	0.0421±0.0213	0.6914±0.11206	0.9011±0.05697
heterogeneous	0.0295 ±0.0198	0.8529±0.04259	0.0494±0.0393	0.7114±0.10106	0.8957±0.08036
negative	0.0695 ±0.0213	0.9001±0.09697	0.0568±0.0358	0.8038±0.19720	0.9129±0.04697
P	0.034	0.059	0.041	0.048	0.651

differences between ESCC and normal esophageal mucous epithelia. We used MassARRAY analysis to examine the methylation status of 11 CpG sites in the promoter of the TAP2 gene. Although no statistical significance were found of whole target CpG fragment methylation level of TAP2 between two groups, there was significant differences in the methylation level of CpG_7 sites between ESCC and corresponding non-cancerous esophageal tissues. LMP7 gene promoter contains 22 CpG sites and the methylation level between CpG_5, CpG_9, CpG_20, CpG_21, and CpG_22 were significant differences between two groups. ERp57 gene promoter contains 9 CpG sites, of which CpG_1 was significant differences between ESCC and corresponding non-cancerous esophageal tissues.Tapasin gene promoter contains 12 CpG sites, of which CpG_1 and CpG_6 were significant differences between ESCC and corresponding non-cancerous esophageal tissues (shown in Table 3). We also analyzed the global methylation level of target fragments of HLA-B, TAP2, LMP7, ERp57 and Tapasin genes. From them only the LMP7 gene target fragments global methylation level was statistically higher (0.0517±0.0357) in Kazak esophageal cancer than in neighboring normal tissues (0.0380±0.0214, $P<0.05$).

Association between DNA methylation and the APM family gene protein expression

Further analyze the correlation between protein expressions with DNA methylation in ESCC tissue; the results demonstrated an inverse correlation of altered CpG island methylation of HLA-B, ERp57, and LMP7 with changes in protein expression of corresponding genes. However, the TAP2 and Tapasin protein expression in tumor was not associated with methylation levels (in Table 4).

Table 3. Quantitative Analysis of HLA-B, TAP1, LMP7, ERp57and Tapasin Genes Single CpG Site Methylation by Sequenom MassARRAY.

CpG site	Methylation levels (x±s)		t	*P*
	Tumor tissues	normal adjacent tissues		
HLA-B_CpG_1.2*	0.0704±0.02808	0.0370±0.01772	4.984	0
HLA-B_CpG_3	NA	NA		
HLA-B_CpG_4	NA	NA		
HLA-B_CpG_5.6	0.0113±0.01254	0.0077±0.01105	1.266	0.219
HLA-B_CpG_7.8.9	0.0404±0.02989	0.0390±0.03981	0.257	0.8
HLA-B_CpG_10.11	0.0113±0.01254	0.0077±0.01105	1.266	0.219
HLA-B_CpG_12*	0.0265±0.02288	0.0139±0.01033	2.522	0.019
HLA-B_CpG_13*	0.0865±0.05630	0.0604±0.02531	2.127	0.045
HLA-B_CpG_14	0.0083±0.01054	0.0067±0.00905	0.132	0.896
TAP2_CpG_1	0.8529±0.04697	0.8457±0.08036	0.258	0.8
TAP2_CpG_2	0.9129±0.04697	0.8957±0.08036	0.358	0.712
TAP2_CpG_3.4	0.7358±0.12038	0.7500±0.09809	0.456	0.652
TAP2_CpG_5	0.9129±0.04697	0.8957±0.08036	0.358	0.712
TAP2_CpG_6	NA	NA		
TAP2_CpG_7*	0.8458±0.01981	0.8079±0.06312	2.554	0.02
TAP2_CpG_8	0.6216±0.19581	0.5642±0.10875	1.088	0.291
TAP2_CpG_9.10.11	1	1	0	1
LMP7_CpG_1	0.0295±0.01802	0.0276±0.01814	0.384	0.765
LMP7_CpG_2	0.0532±0.07235	0.0532±0.05313	-0.066	0.948
LMP7_CpG_3	0.0718±0.20801	0.0305±0.01812	0.95	0.353
LMP7_CpG_4	0.0557±0.05306	0.0433±0.06591	0.677	0.506
LMP7_CpG_5*	0.0736±0.01620	0.0323±0.02224	7.124	0
LMP7_CpG_6	0.0524±0.08608	0.0167±0.3979	1.713	0.102
LMP7_CpG_7	0.0695±0.01322	0.0681±0.01662	0.289	0.775
LMP7_CpG_8	0.0055±0.01335	0.0023±0.00861	-0.892	0.382
LMP7_CpG_9*	0.0918±0.07601	0.0577±0.0411	4.564	0.033
LMP7_CpG_10.11	0.0268±0.01985	0.0241±0.0151	0.597	0.557
LMP7_CpG_12.13.14	0.0273±0.02381	0.0159±0.00982	1.421	0.176
LMP7_CpG_15.16.17.18	0.1473±0.02963	0.1377±0.03624	0.993	0.332
LMP7_CpG_19	0.0368±0.02982	0.0368±0.0163	0	1
LMP7_CpG_20*	0.0300±0.00143	0.0014±0.0021	2.414	0.026
LMP7_CpG_21*	0.0587±0.0123	0.0174±0.0162	3.565	0.0123
LMP7_CpG_22*	0.0391±0.0075	0.0223±0.0308	2.478	0.022
ERp57_CpG_1*	0.7314±0.10106	0.5995±0.15377	2.805	0.011
ERp57_CpG_2	0.7024±0.10246	0.6795±0.15067	0.805	0.411
ERp57_CpG_3.4	0.6914±0.11206	0.6095±0.14377	0.915	0.311
ERp57_CpG_5	0.7238±0.16720	0.6729±0.19782	0.983	0.337
ERp57_CpG_6	0.7514±0.1012	0.6995±0.15377	1.105	0.211
ERp57_CpG_7	0.7938±0.17720	0.7729±0.19782	0.283	0.787
ERp57_CpG_8	0.8138±0.19720	0.7701±0.18756	0.383	0.713
ERp57_CpG_9	1	1	0	1
Tapasin_CpG_1*	0.9250±0.08697	0.8920±0.08336	2.123	0.016
Tapasin_CpG_2	NA	NA		
Tapasin_CpG_3	0.9040±0.07501	0.9050±0.08036	0.458	0.965
Tapasin_CpG_4	0.9130±0.07697	0.9150±0.08036	0.488	0.642
Tapasin_CpG_5	0.9129±0.04697	0.9057±0.08036	0.158	0.836
Tapasin_CpG_6*	0.9490±0.10469	0.8810±0.08036	2.666	0.026
Tapasin_CpG_7	0.9129±0.04697	0.8957±0.08036	0.358	0.712
Tapasin_CpG_8	NA	NA		
Tapasin_CpG_9	0.9490±0.10469	0.9157±0.08036	0.458	0.635
Tapasin_CpG_10	0.9129±0.04697	0.9130±0.07697	0.158	0.912
Tapasin_CpG_11	0.8957±0.08036	0.8152±0.08112	0.312	0.803
Tapasin_CpG_12	0.9040±0.07501	0.8810±0.08036	0.558	0.512

NA, the corresponding normal DNA is not available; **P* values are from t test and were statistically significant at <0.05 (two sided)

Discussion

The purpose of the present study was to elucidate whether epigenetic modifications influence HLA class I antigen and APM components expression in Kazak esophageal cancer cells. To this aim, we used bisulfite-sequencing PCR (BSP) to map the methylation status of CpG sites in APM family gene promoters in ESCC cell lines and determine the sequence of candidate gene methylation then quantitative verify the methylation levels within tissue specimens from patients with ESCC who had not received clinical treatment.

Considering ESCC specimens as solid tumor tissues, the molecular analyses may be affected by tissue heterogeneity due to the presence of necrotic areas and non-tumor cells, such as tumor-infiltrating leukocytes, endothelial cells and fibroblasts (Ingrid et al., 2011). Furthermore, heterogeneity of surgical tumor specimens can influence the sequencing. Thus, to overcome the problem of tissue heterogeneity, it is necessary to have a viable and more homogeneous cell material retaining the phenotypic and genomic profile of original tissue. Therefore, the cytological composition should be as homogeneous as possible to help identify the aberrant methylation target CpG fragments at promoter regions. To attain this goal, we first used bisulfite-sequencing PCR (BSP) to map the methylation status of CpG sites in APM family gene promoters in ESCC cell lines and determine the target sequences of candidate gene methylation, and then quantitatively verified the methylation levels in tissue specimens from patients with ESCC who had not received clinical treatment.

In present study, we used MassARRAY analysis to examine the methylation status of every single CpG site in the promoter of the HLA-B, TAP2, LMP7, ERp57, and tapasin gene. The result demonstrated that various extents of CpG site methylation at promoter regions of above described genes in ESCC. Although no statistical significance were found of whole target CpG fragment methylation level of HLA-B, TAP2, ERp57, and tapasin between Kazak esophageal cancer and corresponding non-cancerous esophageal tissues, hypermethylation occur on certain CpG islands and sites. The results indicate that there are multiple CpG sites, and not methylating of every site leads to down regulation of gene expression or deletion of expression. Only some of them produce genetic transcription (D'Alessio et al., 2006). The sites which were methylated are the key CpG sites. Therefore, the overall methylating level can not represent the clinical value. In fact, only the accurate information of CpG sits methylating level represent the clinical application value. In the current study, we also found that an inverse correlation of altered CpG island methylation of HLA-B, ERp57, and LMP7 with changes in protein expression of corresponding genes. Our results suggest that the methylation status of the HLA-B, ERp57, and LMP7 promoter region may be important for the control of HLA-B, ERp57, and LMP7 expression in Kazak esophageal cancer. HLA-B has been reported to be methylated in ESCC (Nie et al., 2001), in their study, DNA hypermethylation occurred in ESCC but not methylated in earlier stages and demethylation and re-expression of HLA-B gene was obtained by treatment with 5-aza-2-deoxycytidine in one of the esophageal cells, suggesting that DNA methylation was responsible for mRNA inactivation in ESCC cell line (Nie et al., 2001; D'Alessio et al., 2006). Differences in methylation within the 5' regulatory region of HLA-G were detected between normal ovarian surface epithelial cells and ovarian cancers that was loss expression in ovarian cancers (Griffioen et al., 1999). But they found that no correlation exists between methylation and HLA-G gene expression between ovarian tumor samples and OSE, suggests that changes in methylation may be necessary but not sufficient for HLA-G expression in ovarian cancer. It is consisted with our results that TAP2 and Tapasin promoter methylation were without correlation with protein expression. It is should be mind that the speculated significance of methylation changes in gene regulatory regions is the hypomethylation or hypermethylation access which provides for the binding of proteins or protein complexes that regulate gene expression. Therefore, although methylation changes in gene regulatory regions may be necessary for subsequent changes in levels of expression, these changes in methylation alone may not be sufficient to effect expression changes (Laura et al., 2008). The fact that the significant differences in single CpG site methylation we observed between esophageal cancer and normal samples do not correlate with differences in TAP2 and Tapasin protein expression may serve to underlie this distinction. Multiple mechanisms may be involved in the inactivation of APM components, including genomic alteration, transcriptional regulation and protein transportation. Our results may consistent with a theory whereby changes in methylation in esophageal cancer cells potentiate them for TAP2 and Tapasin transcriptional activation by regulatory proteins induced by micro-environmental or mutational changes in specific tumor cell. In previous studies, gene promoter hypermethylation was associated with downregulation of LMP7 protein expression in esophageal squamous cell carcinoma, colon cancer, renal cancer, and melanoma. These effects were reversible upon treatment with methyltransferase inhibitors, providing evidence that APM gene expression is regulated by methylation (Tomasi et al., 2006). These was also re-expression of LMP2/LMP7 genes altered by epigenetic modifications can be achieved by inhibitors of the DNA methyltransferases, such as 5-aza-2_-deoxycytidine (DAC) or 5-aza-cytidine (5-aza-C) in a murine tumour cell line expressing human E6 and E7 human papilloma virus 16 (HPV16) oncogenes and deficient in HLA class I expression, as a result of impaired antigen-presenting machinery (Manning et al., 2008).

In our results, although global methylation level of LMP7 gene promoter significantly higher in ESCC than corresponding non-cancerous esophageal tissues, hypermethylation occur only on certain CpG islands and sites. This indicates that the regulation of expression by DNA methylation is not CpG islands-specific, but varies in individual CpG site. This was also reported that Methylation of CpG near the transcriptional start site was inversely correlated with MUC1 gene expression (Yamada et al., 2008). Similarly, MUC4 expression was also regulated by methylation of CpG sites near the transcriptional start site (N Yamada et al., 2009).

In summary, our results shown that HLA-B and APM component gene's certain CpG sites methylation were associated with loss protein expression, which result in escape from immunesurveillance. However, since the regulation of immune response is a complicated process involved in numerous genes, it would be expected that individual genes might have only limited effect to disease susceptibility. Therefore, further studies with larger numbers of patients are warranted to confirm our findings.

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