RESEARCH COMMUNICATION

Loss of Heterozygosity at 6p21 and HLA Class I Expression in Esophageal Squamous Cell Carcinomas in China

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Abstract

Background and objective: The loss or downregulation of human leukocyte antigen (HLA-I) has been proposed to contribute to immune evasion by cancer cells. Since the human leukocyte antigen (HLA-I) complex is located at 6p21.3, loss of heterozygosity of this region may alter HLA class I tumor phenotypes. The aim of this study was to analysis loss of heterozygosity (LOH) of chromosome 6p in ESCC samples and correlate this with HLA class I expression. Materials and methods: A total of 87 formalin-fixed, paraffin-embeded and frozen-fresh of ECSS lesions were collected. HLA-Iand antigen-processing machinery component expression was investigated by immunohistochemistry with anti-HLA classI monoclonal antibody and a panel of 49 ESCCs with downregulated HLA class I expression wereselected for LOH studies using 3 microsatellite markers located at 6p21.3 (D6S105,D6S265,D6S273). Results: HLA-Iantigen,TAP1 and LMP were lost or down-regulated in 57.5, 29.8 and 47.0% of the ESCC lesions, respectively. In 23/49(46.9%) of the ESCCs, allelic loss for at least one locus at 6p21.3 was found. Conclusions: Our data show that downregulation of HLA class I expression is correlated with loss of heterozygosity regions at 6p21.3 in ESCC.

Keywords: Esophageal squamous cell carcinoma - HLA class I - immune surveillance - loss of heterozygosity

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Introduction

Esophageal squamous cell carcinoma (ESCC) is a very aggressive disease with a poor prognosis. Despite aggressive treatment modalities such as surgical tumor resection with extensive lymphadenectomy and chemo-radiation therapy, only 20–36% of them survive at 5 years (Goldminc et al., 1993). In recent year immunotherapy such as the utilization of anti-tumor T cells or antibodies induced by cancer vaccination has become an attractive option for the revival of the role of immune surveillance in the control of tumor growth (Dunn et al., 2002). It is well known that HLA class I molecules are critical for the presentation of antigen peptides, including those derived from tumor cells, to cytotoxic T lymphocytes and the downregulation of HLA class I on the tumor allows it to evade cytotoxic T lymphocytes-mediated anti-tumor immunity, leading to variant cancer cells that arise from the parent tumor during tumor progression at both primary and metastatic sites (Mizuki et al., 2007). Loss of HLA class I antigen expression can occur at the genetic, transcriptional and post-transcriptional levels. Multiple molecular mechanisms are responsible for these altered HLA class I tumor phenotypes. Since the human leukocyte antigen (HLA) complex is located at 6p21.3, so loss of heterozygosity of this region may lead to lost or downregulation of HLA class I . The variable percentages of LOH at chromosome 6p in tumor derived from different tissues have reported: 50% in cervical carcinoma cell lines (Koopman et al., 1998), 49% for head and neck carcinomas (Feenstra et al., 1999), 13.8% in colorectal carcinomas,17.6% in larynx tumors, and 15.3% in melanomas (Tamiya et al., 1998). The aim of this study was to analyse loss of heterozygosity (LOH) of chromosome 6p in paired PBL and esophageal sqmamous cell carcinoma samples and correlate this with HLA class I expression. Furthermore, we evaluated the correlation between LOH of HLA class I and the clinicopathological status or the prognosis in patients with ESCC.

Materials and Methods

ESCC specimen preparation and DNA extraction

Eighty-seven primary ESCC specimens containing neighboring epithelial tissue were collected at the Second Hospital of Shandong University in Jinan (Table 1). The samples were frozen in liquid nitrogen within 1 h after surgical resection and were stored in liquid nitrogen, on dry ice of at –80°C until use. For each specimen, two pieces of tissue, one from the tumor mass, were dissected

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and embedded with tissue freeze medium (ODC). Ten micrometer thick serial sections were cryosected and used for DNA extraction. The presence of tumor or normal tissue in the samples was confirmed histopathologically on slides stained with hematoxtlin and eosin. Cancerous lesions were microdissected using a laser capture microdissection system for the genetic assay About 500 cells were dissected for each lesion. DNA was extracted with a Qiagen Tissue Kit following the manufacturers procedure. The DNA was stored in aliquots at –20°C until use. None of the patients received radiotherapy, chemotherapy, or other medical interventions before the study. This study was approved by the Ethical Committee of the Shandong University, and written informed consent was obtained from all individuals.

Immunohistochemical staining

Immunohistochemical staining was performed by using the PV6000 IHC Kit. Briefly, sections were cut at 5 mm from the TMA blocks. After being dewaxed in xylene and rehydrated in alcohol and distilled water, antigen was retrieved by microwave oven heating (10 min) at middle power in 0.01 mol/l sodium citrate buffer (pH 6.0). The sections were then incubated with 3% hydrogen peroxide in absolute methanol at room temperature for 10 min, to block endogenous peroxidase activity. After three rinses (each for 5 min) in phosphate-buffered saline (PBS), the sections were incubated sequentially with mouse anti-human HLA class I antibody (1:100; Proteintech Group), a rabbit anti-human TAP1 antibody (1:100, Proteintech Group), a mouse anti-human LMP2 antibody (1:100; Proteintech Group) diluted in PBS overnight at 48°C, with 150 ml polymerized HRP-anti mouse/rabbit IgG for 30 min at room temperature. The reaction products were visualized with diaminobenzidine (DAB Peking Zhongshan), and slides were counterstained with hematoxylin, dehydrated, and evaluated under light microscope. Negative controls were done by omitting primary antibodies. For the evaluation of HLA class I expression, two independent observers (YCH and PCL) assessed HLA class I positivity, without previous knowledge of clinicopathological data.

A consensus number was reached for each tumor sample between the two investigators. Normal lymphocytes and vessel endothelia were used in each specimen as internal controls. Tumor specimens were scored as positive, heterogeneous, or negative when the percentage of stained tumor cells in an entire lesion was >75, 25–75 and <25%, respectively, according to the criteria established by the HLA and cancer component of the 12th International Histocompatibility Workshop (Garrido et al., 1997).

Polymerase chain reaction(PCR)

For the amplification of microsatellite CA dinucleotide markers on chromosome 6p (D6S105,D6S265,D6S273). Details of the selected primers are given in Table 2. PCR was performed in 50 ul reactions containing 1xPCR buffer, 100ng DNA, 10 pmol of each primer combination, 1 mm Dntp's, 0.3ul Amplitaq DNA polymerase (5U/ul) and 2.5-4mm MgCl2 . Amplification was performed in 30 Cycles in a PE 480 thermal cycler as follows: 94°C(30s), 55°-64°,72°C (45s). $MgCl_2$ concentration and annealing temperature was optimized for each primer set 2. Amplification was preceded by a denaturating step (94°C, 4min)and completed with an extension step (72°C,5min). Loss of heterozygosity was assigned when more than 25% of signal reduction of one allele was shown in the tumour sample compared to the control PBL sample.

Statistical analysis

Statistical analysis was performed by using SPSS 12.0. Chi-squared test was used to compare the expression of HLA class I, LMP2, TAP1 of HLA-I and the frequencies of genetic alteration of ESCC between primary tumors and lymph node metastases of ESCC and to assess theirs correlations between HLA class I antigen expression and LOH of ESCC and clinicopathological characteristics. A p value of less than 0.05 was considered of statistical significance, and all statistical tests were two-side tests.

Table 1. Characteristics of Patients with ESCC

Characteristics	Category	n (%)
Gender	Male	61 (70.1%)
	Female	26 (29.9%)
Age (years)	≥60	54 (62.1%)
	<60	33 (37.9%)
Tumor differentiation	Well	32 (36.8%)
	Moderate	19 (21.8%)
	Poor	36 (41.4%)
Depth of invasion	T1–T2	34 (39.1%)
	T3–T4	53 (60.9%)
Tumor size (cm)	≥5	39 (44.8%)
	<5	48 (55.2%)
Lymph node status	Negative	52 (59.8%)
	Positive	35 (40.2%)
Stage	Low (IandII)	33 (37.9%)
	High (IIIandIV)	54 (62.1%)

Results

Expression of HLA class I and APM components in esophageal lesions

All the antibodies were first tested on normal esophageal tissues. As shown in Figure 1, esophageal surface epithelium and stromal cells were homogeneously stained. Anti-HLA class I showed strong staining of cell membranes, whereas anti-TAP1, anti-LMP2, primarily stained the intracellular regions. Immunohistochemical staining of 87 primary ESCC lesions (figure 1) was done with

Table 2. Characteristic of the Microsatellites

STR	Sequence CA-primer	Sequence GT-primer	Range(bp)	Location	Alleles
D6S273	5'GCAACTTTTCTGTCAATCCA3'	5'ACCAAACTTCAAATTTTCGG3'	120-140	6p21.3	8
D6S265	5'ACGTTCGTACCCATTAACCT3'	5'ATCGAGGTAAACAGCAGAAA3'	118-140	6p21.3	12
D6S105	5'GCCCTATAAAATCCTAATAAC3'	5'GAAGGAGAATTGTAATTCCG3'	116-138	6p21.3	12

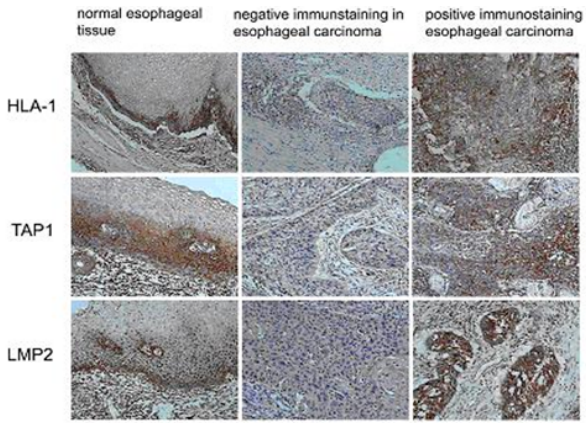

Figure 1. Representative Staining Patterns of Primary ESCC Lesions with HLA Class I, TAP1, LMP2 with Antigen Processing-1-specifci Monoclona Antibody. Intense immunostaining for the HLA-1 , TAP1 and LMP2 were observed in most normal esophageal and partly esophageal carcinoma epithelia.Negative immunostaining for the HLA-1 , TAP1 and LMP2 were observed in most esophageal carcinoma.

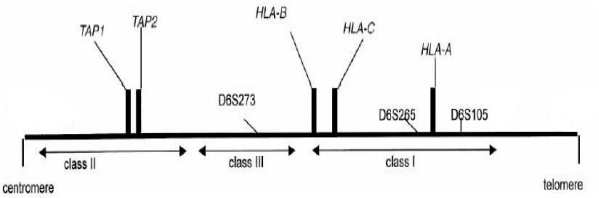

Figure 2. Schematic Presentation of the Location of the STRs on Chromosome 6p and their Relative Position to the HLA-A,-B and –C and TAP1 and TAP2 Genes.

the aforementioned antibodies. Staining of lymphocytes was used as a reference of the immunohistochemical staining in each tissue section. Tumor lesions were found to have a highly variable expression profile ranging from a total loss, heterogeneous but decreased expression, to normal expression. As shown in Table 2 and Figure 1, among the three proteins examined, HLA class I showed the greatest reduction in expression, negative in 34.5% and downregulated in 23.0% of the lesions. TAP1 showed the smallest reduction, negative in 37.9% and downregulated in 20.7% of the lesions. The expression levels of LMP2 was intermediate when compared to other proteins. LMP2 was negative in 24.1% and downregulated in 29.9% of the lesions (Table 3). Thus, HLA class I antigen and APM components were all downregulated in ESCCs.

Association of HLA presentation with APM components

LMP2 participates in the proteosomal degradation of antigens. TAP1 is responsible for the transport of the antigen-derived peptide to the lumen of the endoplasmic reticulum, where they are loaded on the newly synthesized b2m-associated HLA class I heavy chains, and transported via Golgi to the cell surface (Flutter and Gao, 2004). Therefore, it is conceivable that abnormal expression of any of the APM components may lead to defects in the expression of HLA class I peptide complexes. To determine whether the expression of APM components affect the presentation of HLA class I molecules, we carried out a correlation analysis. As shown in Table 4,

Table 3. Expression of HLA-1 and APM Components in ESCC Cells (Positive)

IHC parameter	Tumor samples (n=87)
HLA-1	37 (42.5%)
TAP1	36 (41.4%)
LMP2	40 (45.9%)

Table 4. The Expression Correlation of HLA Class I with APM Component ($p < 0.05$)

HLA-1	TAP1		LMP2	
	Positive	Negative	Positive	Negative
Positive	29	8	23	14
Negative	19	27	17	33

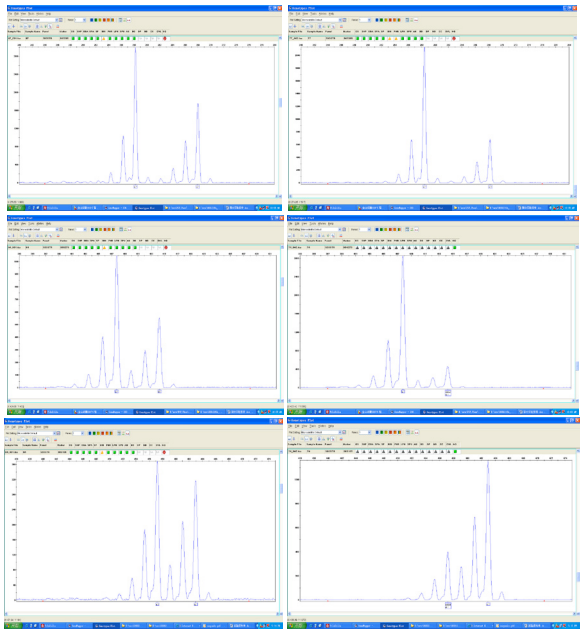

Figure 3. Allelic Loss at D6S105, D6S265, D6S273 in ESCC. Loss of heterozygosity was assigned when more than 25% of signal reduction of one allele was shown in the tumor compared to the normal sample.

the levels of all the examined APM components were correlated to those of HLA class I molecules, suggesting that the reduced expression of HLA class I in tumor cells is partially attributed to the absence or reduction of the components involved in the steps of antigen processing and transport. In fact, a correlation between HLA class I expression and the levels of various APM components was previously observed in laryngeal squamous cell carcinomas and ovarian cancers (Ogino et al., 2006). These results suggest that pathways leading to the loss or reduction of HLA class I expression in cancer cells are probably shared among the different types of cancer.

Association of HLA class I and APM components with clinicopathological characteristics

The association between the expression of HLA class I and APM components and clinicopathological characteristics was investigated in ESCC lesions. As can be seen in Table 2, the expression levels of HLA class I molecules are significantly associated with tumor grade, the depth of tumor invasion, the status of lymph nodes, and the clinical stage, but are independent of age and tumor size. TAP1 expression is significantly associated

Table 5. Correlation of HLA Class I, TAP, LMP with Clinicopathological Characteristics in ESCC

Characteristic		HLA class I				TAP1				LMP2			
		+	±	—	P	+	±	—	P	+	±	—	P
Gender	Male	26	14	21	0.908	23	12	26	0.725	31	16	14	0.203
	Female	11	6	9		13	6	7		9	10	7	
Age(years)	≥60	24	10	20	<0.001	21	10	23	0.424	29	16	9	0.174
	<60	13	10	10		15	8	10		11	10	12	
Tumor	Well	13	10	9	<0.050	15	8	9		13	11	8	<0.050
differentiation	Moderate	9	4	6		7	2	10	0.306	8	6	5	
	Poor	15	6	15		14	8	14		19	9	8	
Depth of	T1-T2	20	9	5	0.047	16	6	12	0.818	15	11	8	0.206
invasion	T3-T4	17	11	25		20	12	21		25	15	13	
Tumor size	≥5cm	18	6	15	0.284	12	5	20	0.096	18	9	12	0.196
	<5cm	19	14	15		24	13	13		22	17	9	
Lymph node	Positive	15	6	14	0.002	11	8	16	0.188	17	10	8	0.013
status	Negative	22	14	16		25	10	17		23	16	13	
Stage	I-II	17	10	6	0.029	18	7	8	0.282	17	9	8	0.034
	III-IV	20	10	24		18	11	25		23	17	13	

+, positive; ±, downregulation; -, negative; ++*, HLA-A, -B, -C or two of them; +*, HLA-A or -B or -C

Table 6. Correlation of HLA Class I Expression and Chromosomal Deletions at 6p

H6p Markers HLA class I / 6p	D6S273	D6S265	D6S105
HLA-/ no LOH	21	22	20
HLA-/ LOH	12	13	12
HLA*/ no LOH	12	11	11
HLA*/ LOH	0	0	1
P=value	<0.05	<0.05	<0.05

Down-regulation of HLA class I was correlated with LOH at 6p; The numbers of tumor with downregulated HLA class I expression and (HLA-/ LOH)at 6p are correlated with the numbers of tumor with downregulated HLA class I expression and no LOH(HLA-/ no LOH),normal HLA class I expression and no LOH and normal HLA class I expression and LOH. -= downregulated expression; *normal expression

with grade, stage, and lymph node status of tumor. LMP2 was significantly associated with tumor stage and lymph node status of tumor. Interestingly, the expression level of LMP2 tended to be lower in patients aged 60 years or older. All the proteins examined, were associated with tumor stage and lymph node status of tumor. Tumor stage and lymph node status of tumor were consistently associated with HLA class I molecules, and the APM components (Table 5).

LOH in 6p21 region of ESCC

We examined paired PBL and tumor DNA from 49 ESCC with downregulated HLA class I expression, for microsatellite alterations at 6p.LOH at 6p was studied using 3 STR markers covering the 6p arm including the HLA complex at 6p21.3. Figure 2 showed the location of the 3 STRs at 6p.Twenty-three tumors (23/49)showed allelic loss at 6p for at least one marker, of which showed allelic loss specifically at 6p 21.3. A number of tumours containing LOH at 6p showed complex LOH patterns, in the tumours allelic loss non-continuously distributed along the 6p arm. Figure 3 shows an example of an electropherogram of paired PBL an tumour DNA from for marker D6S265 . the tumor sample shows signal reduction of one allele compared to the PBL sample. For example , HN21 showed allelic retention at markers D6S273 and D6S105 ,while allelic losses were found on informative loci flanking these markers at both sides. The STR marker D6S105 located at the 6p telomere showed the highest frequency of loss(42%). A high frequency of allelic loss was also found at D6S265(49%),which located centromeric to the TAP genes. The lowest frequency of allelic loss was found at the HLA class I located D6S273 marker(21%).

Discussion

The HLA class I antigens, HLA-A, HLA-B and HLA-C, which form the class I major histocompatibility complex in humans, take part in the recognition of virally infected, grafted or transformed cells by cytotoxic T cells (Mizuki et al., 2007). The HLA genes are located on chromosome 6p21 and are expressed in most somatic tissues. Selective loss of expression of these loci has frequently been observed in human tumors and this event is thought to help tumor cells escape immune surveillance by T cells and natural killer cells (Ogino et al., 2006; Rui-Cabello e tal., 2006). The downregulation of HLA class I has been observed in kidney, prostate, stomach, colon and germ cell testicular cancer and has been associated with tumor invasiveness and aggressiveness (Ogino et al., 2006). In our study, we found that HLA class I was downregulated or even lost in 78.2% of primary ESCC lesions, and was downregulated in 87% of metastatic lymph nodes.This frequency in primary ESCC lesions is comparable to those reported in previous studies (Nie et al, 2001). Those studies and our results clearly showed that there is a marked reduction in the expression of HLA class I molecules in ESCC lesions. And in our studies, it showed that the reduction of HLA class I presentation was significantly associated with the metastasis, tumor grade and poor survival of the ESCC. This is also comparable to those reported in previous studies (Hosch et al., 1997).

Loss of HLA class I antigen expression can occur at the genetic, transcriptional and post-transcriptional levels. Genetic alterations can result in inactivation of proto-oncogenes or functional loss of tumour suppressor

genes (Dutrillaux, 1995). In addition,genetic alterations can affect expression of genes involved in immune responses, providing tumour cells with a mechanism to become invasive and metastatic by escaping rejection by the immune system. Loss of a 6p haplotype reduces the cell's heterozygous advantage to present a wide variety of tumour antigens, in the context of HLA, for recognition by cytotoxic T cells (Boon and van der Bruggen, 1994). Loss at 6p was previously shown in follicular centre cell lymphoma, ovarian cancer, pancreas cancers and melanoma (Randerson et al., 1996; Real et al., 1998). In this study, 49 ESCC with downregulated HLA class I expression were investigated for LOH at 6p. Paired normal and tumour DNA was studiedusing 3 STR markers covering the 6p arm from centromere to telomere ,including the HLA complex at 6p21.3. Our data demonstrate that LOH for 6p21.3 is a frequent event occurring in 23/49 (46.9%) of the tumours with downregulated HLA class I expression. Downregulation of HLA class I expression and loss at 6p21.3,considering all loci used in this study together, is associated ,albeit considered quite significant (Table 6 $p<0.05$) . When analysed for individual loci, downregulation or loss of HLA class I expression is associated with loss at locus D6S105,D6S265, and D6S273. Correlation was considered significant for those locus too ($p<0.05$).

In summary, 49 fresh samples of ESCC were examined with IHC and PCR–LOH analysis. Downregulation and/or loss of expression of HLA class I molecules were found, and correlations between abnormal HLA class I expression or HLA gene alterations and clinical data such as patients' age, tumor's location, and stage were confirmed. The much higher frequencies of detection of abnormal HLA class I expression and HLA gene alterations reported may attribute to the larger samples and more informative STR markers used in this study. 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 clinical trials.

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