

## ORIGINAL ARTICLE

# Loss of heterozygosity at the human leukocyte antigen locus in thymic epithelial tumors

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## Keywords

Human leukocyte antigen (HLA); loss of heterozygosity (LOH); microsatellite; thymic epithelial tumor (TET).

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Received: 8 November 2014;

Accepted: 5 January 2015.

doi: 10.1111/1759-7714.12252

Thoracic Cancer 6 (2015) 749–753

## Abstract

**Background:** To study the relationship between loss of heterozygosity (LOH) at the human leukocyte antigen (HLA) locus and the pathogenicity and clinicopathological features of thymic epithelial tumors (TET).

**Methods:** Tumor and adjacent normal tissues were isolated from 36 TET patients. Five microsatellite loci (D6S1666, D6S265, D6S273, DS6276, and D6S291) within the HLA locus were amplified by polymerase chain reaction. DNA sequencing was used to measure the frequency of microsatellite LOH.

**Results:** LOH was identified in at least one locus in 83.6% of TET patients. LOH frequency at D6S1666, D6S265, D6S273, D6S276, and D6S291 was 44.4%, 16.7%, 30.5%, 38.9%, and 36.1% respectively. There was no significant association between LOH frequency in TET with tumor severity, or in the presence or absence of myasthenia gravis.

**Conclusions:** D6S1666, D6S265, D6S273, DS6S276, and D6S29 are sensitive loci for studying microsatellite LOH in TET. LOH within the HLA complex is implicated in the occurrence and development of TET, with the HLA-DQA1 gene likely involved. However, an understanding of the relationship between LOH and the clinicopathological features of TET requires a larger sample size than that of the present study.

## Introduction

Thymic epithelial tumors (TET) are the most common neoplasm of the anterosuperior mediastinum, accounting for approximately 20% of all mediastinal tumors.<sup>1</sup> However, the pathogenesis of TET is unclear. The human leukocyte antigen (HLA) complex is located at 6p21.3, and downregulation or loss of HLA class I expression on tumor cells is a known mechanism of cancer immune evasion.<sup>2</sup> Loss of heterozygosity (LOH) is the most common mechanism of HLA haplotype absence in a malignant tumor, and the frequency of LOH-6p21 has been reported in many cancer types.<sup>3</sup> Furthermore, LOH has been implicated in carcinogenesis and its presence is a useful prognostic marker in many malignant tumors. Multiple instances of the tumor suppressor gene (TSG) LOH in cancer have been described, and inactivation of TSGs may contribute to pathogenesis in TETs.<sup>4</sup> However, there are few reports concerning microsatellite LOH in TET at present. Therefore, we investigated the frequency of microsatellite LOH at the HLA locus using samples from 36

patients with TET. We examined the relationship between LOH, tumorigenesis, and clinicopathological features of TET.

## Methods

### Clinical data

Tumor tissue samples were obtained from 36 TET patients with a definite pathological diagnosis who had not received preoperative chemoradiotherapy but underwent surgery at the Department of Cardiovascular and Thoracic Surgery at Tianjin Medical University General Hospital from 2008–2012. The 16 male and 20 female patients had an average age of  $53 \pm 10.7$  years, and 23 of these patients had concurrent myasthenia gravis (MG). TET samples were classified according to the World Health Organization (WHO) histological classification for TET (2004), with four cases of type A, six of type AB, four of type B1, seven of type B2, 10 of type B3, and five cases of thymic carcinoma. Of these, types A, AB, B1, and

**Table 1** Primer sequences and their amplicon size for five microsatellite loci

Microsatellite	Primer sequences	Amplicon (bp)
D6S1666	Forward: 5'-CTGAGTTGGGCAGCATTG-3' Reverse: 5'-ACCCAGCATTTGGAGTTG-3'	113–151
D6S291	Forward: 5'-CTCAGAGGATGCCATGTCTAAAATA-3' Reverse: 5'-GGGGATGACGAATTACTACTAACT-3'	198–210
D6S273	Forward: 5'-GCAACTTTTCTGTCAATCCA-3' Reverse: 5'-ACCAAACCTCAAATTTTCGG-3'	120–140
D6S265	Forward: 5'-ACGTTCTGACCCATTAACCT-3' Reverse: 5'-ATCGAGGTAACAGCAGAAA-3'	122–144
D6S276	Forward: 5'-TCAATCAAATCATCCCCAGAAG-3' Reverse: 5'-GGGTGCAACTTGTCCTCTCT-3'	197–230

B2 are considered benign tumors, whereas types B3 and thymic carcinoma are considered malignant tumors.

### Selection of microsatellite loci

Based upon previous studies of microsatellite LOH in TET and other tumors, we selected five highly polymorphic microsatellite loci internal to or either side of the HLA genes: D6S1666, D6S291, D6S273, D6S265, and D6S276.<sup>5–9</sup> Primer sequences and expected amplicon length were based upon sequence data deposited in the National Center for Biotechnology Information database (Table 1). The positions of these microsatellite loci are listed in Table 2 and depicted in Figure 1.

### DNA extraction

Frozen tissue samples were resuspended in 500  $\mu$ L TE buffer, followed by the addition of 50  $\mu$ L 10% sodium dodecyl

sulfate and 2.5  $\mu$ L 20 mg/mL proteinase K. DNA extraction was performed using a standard phenol-chloroform method.

### Polymerase chain reaction (PCR) amplification

Polymerase chain reaction (PCR) primers (listed in Table 1) were synthesized by the Beijing Microread Gene Technology Co., Ltd (Beijing, China) and one oligonucleotide of each primer pair was labeled with fluorescent phosphoramidites. Multiplex PCR reactions were performed using AmpliTaq Gold (Microread Gene Technology Co., Ltd. Beijing, China) and 1.0  $\mu$ g of genomic DNA. Amplification was performed using a GeneAmp 9600 PCR instrument (Applied Biosystems, Carlsbad, CA, USA) in a total volume of 15  $\mu$ L, with an initial denaturation of 10 minutes at 95°C, and 40 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 58°C, and 30 seconds extension at 72°C. Products were separated on 1.2% agarose gels stained with ethidium bromide.

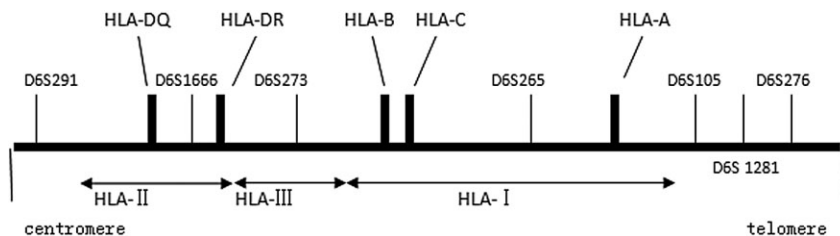
**Table 2** Specific location of five microsatellite markers on chromosome 6

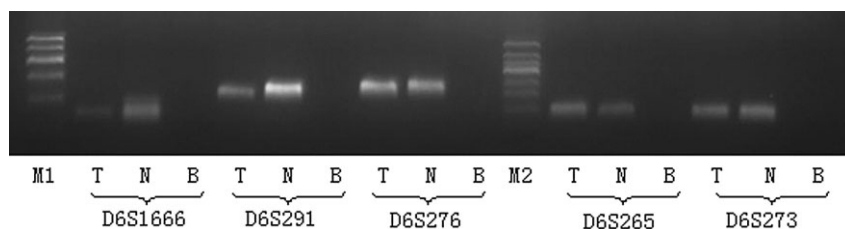
STR	Location on chromosome 6	
	Start	End
D6S1666	32542253	32542453
D6S265	30127492	30127613
D6S291	36265516	36265715
D6S276	24185863	24186082
D6S273	31791664	31791799

STR, short tandem repeats.

### DNA sequencing and data analysis

Sequencing reactions containing 1.0  $\mu$ L PCR product, 0.5  $\mu$ L, ROX-500 internal standard, and 8.5  $\mu$ L formamide were assembled and processed on a 3730XL DNA sequencer (Applied Biosystems). A single peak at a microsatellite locus was regarded as homozygous, while two peaks were regarded as heterozygous. LOH was defined as a decreased ratio of

**Figure 1** Graphic representation of human leukocyte antigen (HLA) microsatellite loci on the short arm of chromosome 6.



**Figure 2** Agarose gel electrophoresis of polymerase chain reaction products following amplification of five microsatellite regions within the human leukocyte antigen gene. M: marker, T: tumor tissue, N: normal tissue, B: blank. M<sub>1</sub> from top to bottom: 600, 500, 400, 300, 200, and 100 bp; M<sub>2</sub> from top to bottom: 500, 400, 350, 300, 250, 200, 150, 100, and 50 bp.

allele peak height that exceeded 40% in the tumor tissue.<sup>6</sup> All aberrations were confirmed twice. The missing rate was calculated by  $1 - \text{Ht ratio}$  (Ht ratio = [peak height of normal allele 2/peak height of normal allele 1]/[peak height of tumor allele 2/peak height of tumor allele 1]).

### Statistical analysis

Statistical analysis was conducted using the chi-squared test, with a  $P$  value  $< 0.05$  considered statistically significant.

## Results

### Microsatellite PCR amplification electrophoresis results

Five polymorphic microsatellite regions within the HLA locus were amplified effectively. PCR products from amplification reactions were of the expected size (see Table 1) and could be clearly visualized following electrophoresis (Fig 2).

### Loss of heterozygosity (LOH) at human leukocyte antigen microsatellite loci in thymic epithelial tumors (TET)

Loss of heterozygosity within the HLA locus in the sample group was identified at 33.3% (60/180) of microsatellite loci. In the 129-information idiomotype, the LOH frequency was 46.5% (60/129). LOH was detected in at least one locus in 83.6% (30/36) of patients. At specific loci, LOH was: 44.4%

(16/36) at D6S1666, 16.7% (6/36) at D6S265, 30.5% (11/36) at D6S273, 38.9% (14/36) at D6S276, and 36.1% (13/36) at D6S291.

### TET LOH is not correlated with the presence of myasthenia gravis

Loss of heterozygosity at the HLA locus in TET patients with MG was 82.6% (19/23), while 76.9% (10/13) TET patients without MG exhibited LOH. Chi-squared analysis revealed no relationship between LOH at the loci tested and the presence of MG ( $\chi^2[1, N = 36] = 0.17, P = 0.68$ ).

### LOH is not associated with World Health Organization histological classification of TET

Thymic epithelial tumor tissues were classified according to WHO histological classification, which defines types A, AB, B1, and B2 as benign and types B3 and C as malignant.<sup>10</sup> LOH in benign and malignant TET were 85.7% (18/21) and 73.3% (11/15), respectively (Table 3). There was no statistically significant difference ( $\chi^2[1, N = 36] = 0.86, P = 0.35$ ).

## Discussion

We selected five highly polymorphic microsatellite loci within the HLA locus that were reported to have a high incidence of LOH in tumors. LOH at D6S1666 has been previously studied in the context of TET, while LOH at the remaining four

**Table 3** Relationship of microsatellite LOH with MG and thymus tumor properties

	N	n	Ratio	D6S1666	D6S265	D6S276	D6S273	D6S291	$P$ value
Total LOH frequency	36	29	83.6%	16	6	11	14	13	
MG (+)	23	19	82.6%	13	5	11	6	7	0.68
MG (-)	13	10	79.6%	3	1	3	5	5	
Benign	21	18	85.7%	9	4	9	5	6	0.35
Malignant	15	11	73.3%	7	2	5	6	6	

N, total cases; n, loss of heterozygosity (LOH) in at least one microsatellite locus. MG, myasthenia gravis.

microsatellite loci has been reported in other tumor types, but not in TET.<sup>11,12</sup> LOH was detected at all five selected microsatellite loci. Moreover, LOH in at least one locus was identified in 83.6% (30/36) of patients with TET. For the 129 individual information genotype, LOH was 46.5%. This is slightly higher than the frequency of LOH within the third highest risk area for TET development (which contains the HLA gene complex), reported by Inoue *et al.* to be 30%.<sup>7</sup> This increased frequency of LOH may be related to the simultaneous analysis of five regions with high LOH frequency. This high level of LOH suggests that HLA gene mutation and TET pathogenesis are closely related. As the five microsatellite loci examined lie either inside or adjacent to the HLA class I, class II, or class III genes, LOH at these sites often causes inactivation of these TSGs. TET pathogenesis is complex and its development likely involves more than one mechanism; however, TSG inactivation is the most common mechanism implicated in cancer development. Therefore, identification of TSGs that have been inactivated in thymic tumors requires that we establish their location in the genome first. Our findings suggest that the HLA locus contains important TSGs, and that LOH at this region is associated with TET development. Furthermore, multiple TSGs may exist within the HLA locus.

The highest frequency of LOH was identified at the D6S1666 locus. Inoue *et al.* have previously studied LOH at this locus in TET.<sup>7</sup> D6S1666 is located within the HLA class II gene, adjacent to the HLA-DQA1 gene, and LOH at this location frequently leads to deletion of HLA-DQA1.<sup>13</sup> Microsatellites D6S265, D6S273, D6S276, and D6S291 also had a high frequency of LOH, and several TSGs (e.g. p21) implicated in cancer are located nearby.<sup>14</sup> Therefore, our study provides a basis for further screening to identify TSGs associated with the pathogenesis of TET.

We frequently identified microsatellite LOH within or adjacent to HLA class I, class II, or class III genes. Widespread LOH at these sites could affect gene structure and eventually lead to the downregulation or complete lack of expression of HLA molecules, enabling tumor cells to escape immune surveillance. Deletion of HLA genes may, thus, facilitate the clonal expansion of tumor cells. This selective pressure could explain why LOH within the HLA locus was found at such a high frequency in our TET study sample. The outcome of this gene deletion could be the abnormal selection of T cells within the thymus. We speculate that the loss of HLA molecules from the TET cell surface facilitates development of autoaggressive T cells and subsequent autoimmune disease.

HLA absence or downregulation has been identified in a wide variety of human tumors, with LOH being the main mechanism responsible for this loss.<sup>15</sup> Immune dysfunction and TET result from abnormalities within chromosome 6.<sup>16</sup> However, we did not identify any relationship between microsatellite LOH at HLA loci in TET and the presence of

MG. This lack of association could be attributed to the small sample size of this study, and further work is required to verify this. We also found no correlation between HLA microsatellite LOH and TET classification, and two reasons may account for this observation. First, the ratio of thymic epithelial cells and lymphocytes in different classes of TET was different, and micro cutting cannot completely rule out contamination by lymphocytes. Therefore, different types of TET could affect the results. Second, normal control tissues were taken from sites adjacent to tumor tissues; therefore, normal tissues from different classifications of TET may have different molecular features, which would interfere with LOH analysis.

## Conclusion

D6S1666, D6S265, D6S273, D6S276, and D6S291 are sensitive loci for studying microsatellite LOH in TET. LOH within the HLA complex is implicated in the occurrence and development of TET, with the HLA-DQA1 gene likely involved. However, an understanding of the relationship between LOH and the clinicopathological features of TET requires a larger sample size. Our study provides a basis for further screening to identify TSGs associated with the pathogenesis of TET.

## Acknowledgments

The authors would like to thank Dr Guo Yuhong of the Tianjin Medical University Cancer Institute and Hospital for contributions to research directions in this manuscript.

## Disclosure

No authors report any conflict of interest.

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