

galanin are considered a reflection of the extent of neurogenic inflammation associated with LDH, which takes place as a healing process following this internal traumatic event.

### ■ Key Points

- Plasma levels of CGRP, galanin, neuropeptide Y, and substance P were evaluated in 27 patients with lumbar disc herniation before and 3 weeks after lumbar discectomy.
- Preoperative plasma levels of CGRP were correlated significantly with the extent of sciatica as determined by VAS.
- Plasma levels of CGRP and galanin significantly decreased after lumbar discectomy in line with the disappearance of pain symptoms.
- These findings suggest the role of plasma CGRP and potentially galanin as a systemic neurochemical signature of pain states in patients with LDH.

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## Mapping of susceptibility and protective loci for acute GVHD in unrelated HLA-matched bone marrow transplantation donors and recipients using 155 microsatellite markers on chromosome 22

Tomoki Kikuchi · Taeko K. Naruse · Makoto Onizuka ·  
Suyun Li · Tetsuaki Kimura · Akira Oka ·  
Yasuo Morishima · Jerzy K. Kulski · Shingo Ichimiya ·  
Noriyuki Sato · Hidetoshi Inoko

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**Abstract** Despite matching donors and recipients for the human leukocyte antigens (HLAs) expressed by the major histocompatibility genomic region of the short arm of

chromosome 6, several recipients still develop acute graft-versus-host disease (aGVHD) after bone marrow transplantation (BMT). This is possibly due to non-HLA gene polymorphisms, such as minor histocompatibility antigens (mHAs) and genes coding for cytokines. However, a detailed genetic background for aGVHD has not yet been established. To find novel susceptibility and/or protective loci for aGVHD, a whole genome-wide association study of donors and recipients needs to be performed. As the first step to such a study, we retrospectively analyzed polymorphisms of 155 microsatellite markers spread across the long arm of chromosome 22 in 70 pairs of HLA-matched unrelated BMT donors and recipients. We performed individual typing and then compared the markers' allele frequencies (1) between all the aGVHD (grades III and IV GVHD) and GVHD-free (grade 0 GVHD) groups in donors and recipients and (2) between the aGVHD and aGVHD-free groups in donor/recipient pairs that were matched and mismatched for the microsatellite marker's allele. Screening of the microsatellite markers revealed five loci with a significant difference between the aGVHD and GVHD-free groups and revealed eight loci on chromosome 22, where the microsatellite allele mismatched markers were associated with aGVHD. This screening analysis suggests that several aGVHD-associated susceptible and protective loci exist on chromosome 22, which may encompass novel gene regions that need to be elucidated for their role in aGVHD.

T. Kikuchi · S. Ichimiya · N. Sato  
Department of Pathology,  
Sapporo Medical University School of Medicine,  
Hokkaido, Japan

T. K. Naruse  
Department of Molecular Pathogenesis,  
Division of Pathophysiology Medical Research Institute,  
Tokyo Medical and Dental University,  
Tokyo, Japan

T. Kikuchi · S. Li · T. Kimura · A. Oka · J. K. Kulski ·  
H. Inoko (✉)  
Department of Molecular Life Science,  
Course of Basic Medical Science and Molecular Medicine,  
Tokai University School of Medicine,  
Kanagawa, Japan  
e-mail: hinoko@is.icc.u-tokai.ac.jp

M. Onizuka  
Department of Hematology and Oncology,  
Tokai University School of Medicine,  
Kanagawa, Japan

Y. Morishima  
Department of Hematology and Cell Therapy,  
Aichi Cancer Center,  
Aichi, Japan

J. K. Kulski  
Centre for Comparative Genomics,  
School of Information Technology, Murdoch University,  
Murdoch, Western Australia, Australia

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## Introduction

The occurrence of acute graft-versus-host disease (aGVHD) is still a major cause of mortality in the bone marrow transplantation (BMT) recipients who are not related familiarly to donors. Despite successfully matching the human leukocyte antigen (HLA) alleles of donors and recipients for hematopoietic stem cell transplantation, a significant proportion of transplantation recipients develop aGVHD because of genetic differences attributed to minor histocompatibility antigens (mHa) (Chao 2004; Falkenburg et al. 2003), non-HLA genes coding for cytokines, and other molecules involved in the pathogenesis of aGVHD (Charron 2003; Kallianpur 2005; Dickinson and Charron 2005; Mullighan et al. 2004).

Genetic association studies of aGVHD can be performed at least in two ways: the candidate gene approach and genome-wide approach. The former approach is hypothesis-driven and dependent on the systematic knowledge of the aGVHD biological process. By using the candidate gene approach, single nucleotide polymorphisms (SNPs) were found within cytokine or cytokine receptor genes, which affect the aGVHD (Charron 2003; Kallianpur 2005; Dickinson and Charron 2005; Mullighan et al. 2004). However, aGVHD is a complex pathophysiological disease, and undoubtedly, a number of unknown genes contribute to or affect the GVHD mechanism. In this regard, the candidate gene approach would fail to find novel genes that are not already reported or thought to be immunoregulatory genes involved with aGVHD. In comparison, the genetic association studies using the genome-wide approach and genetic markers to test all possible variants systemically across the whole genome would be a more experimentally ideal approach to find novel genes involved with aGVHD. In addition, genomic matching by using SNP and/or microsatellite markers for finding compatibility of minor antigens in BMT may improve survival and other clinical outcomes.

Microsatellites and SNPs are two types of genetic markers that can be applied to genome-wide disease association studies, with each type of marker presenting certain advantages as well as inconveniences. Microsatellites are direct tandem-repeated sequences of DNA with a repeat size ranging from 2 to 6 bp. The number of repeats within a microsatellite sequence is usually less than 100. Because the microsatellite polymorphism is based on the differences in number of repeats, microsatellites are highly polymorphic with a high degree of heterozygosity. Polymorphic microsatellites are fewer in number than SNPs, but like SNPs, they are widely distributed across the human genome enabling efficient and accurate calculations of linkage disequilibrium (LD) between pairs of microsatellite loci separated by less than 100 kb of genomic sequence.

Indeed, we have already established and described a set of 27,039 microsatellite markers for the systematic analysis of the whole human genome and, together with SNP analysis, revealed at least seven potential susceptibility gene loci of rheumatoid arthritis (Tamiya et al. 2005). Therefore, the main advantage of using microsatellites as the primary or “first pass” genotyping method is that they allow for a genome association analysis to become an immediate and efficient reality.

To date, there are only a few association studies using microsatellite analysis to determine the potential clinical outcomes in hematopoietic stem cell transplantation, and these studies are limited mainly to the cytokine genes and the HLA region (Karabon et al. 2005; Li et al. 2004; Cullup et al. 2003; Nordlander et al. 2002; Witt et al. 1999). As a set of 27,039 microsatellite markers for the systematic analysis of the whole human genome has been established, we decided to use them in a genome-wide search of allele frequency differences to find and map novel susceptibility and/or protective loci for aGVHD. Although our ultimate goal is a complete genome-wide study, we have started our search for aGVHD susceptibility/protective loci within chromosome 22 (chr 22) for simplicity and economic convenience. A number of studies (Abecasis et al. 2001; Keicho et al. 2000; Oka et al. 1999; Ota et al. 1999; Li et al. 2004) suggest that association analysis using microsatellite markers as a first step of the genome-wide approach is a useful way to find candidate genes and specifically the mHa genes on chr 22 of BMT donors and recipients.

Human chr 22 is the second smallest of the autosomes comprising 1.6–1.8% of the genomic DNA (Dunham et al. 1999). There is no evidence to indicate the presence of any protein coding genes on the short arm of chr 22 (22p). In contrast, the long arm of the chr 22 (22q) is rich in genes compared with other chromosomes. In addition, alteration of gene dosage on the part of 22q is responsible for the etiology of 29 Mendelian disorders and a number of congenital abnormality disorders including cat eye syndrome and DiGeorge syndrome (McDermid and Morrow 2002). Linkage studies have shown an association of chr 22 loci to several disorders, such as schizophrenia, epilepsy, multiple sclerosis, and myopia (DeLisi et al. 2002; Berkovic et al. 2004; Liguori et al. 2004; Stambolian et al. 2004).

Interestingly, two recent reports have highlighted that there are many signal transducers and activators of transcription (STAT) and NF-kappaB-binding sites distributed across chr 22 (Martone et al. 2003; Hartman et al. 2005). STAT and NF-kappaB family members play an essential role in regulating the induction of genes involved in physiological processes, such as apoptosis, immunity, and inflammation, and they may also affect immunoregulatory genes relevant to the recognition and rejection of

foreign tissue. In addition, Gubarev et al. (1996) reported the localization of a gene encoding mHa to chr 22. On the basis of these reports and in an attempt to improve efficiency by screening chromosomal regions of high gene density, chr 22 is a very attractive target for genome-wide association research of GVHD and other immune-related diseases.

As the first step to our genome-wide study, we retrospectively genotyped 155 microsatellite markers on chr 22 in 70 HLA-matched unrelated BMT recipient and donor pairs and associated at least eight significant allele frequency differences with aGVHD. In accordance with our previous study using microsatellite markers to identify mHa (Li et al. 2004), we performed individual DNA typing to investigate the association between statistically significant donor/recipient microsatellite marker mismatches.

## Materials and methods

### Recipient and donor pairs

A total of 70 unrelated donor/recipient pairs after BMT who were treated through the Japan Marrow Donor Program and completely allele-matched for the HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 genes at the high resolution level were enrolled for this study after BMT (Sasazuki et al. 1998; Morishima et al. 2002). All 70 recipients underwent BMT from 1995 to 2000 for hematopoietic malignancy. None of the recipients received T-cell-depleted grafts. Patient, transplantation, and treatment information data are summarized in Table 1. All the donors and recipients provided informed consent for study, which was approved by the relevant institutional ethical committee.

### Diagnosis and evaluation of the acute GVHD study group

Acute GVHD was diagnosed clinically and classified into four groups according to standard criteria (grades 0, I, II, III, and IV; Glucksberg et al. 1974; Thomas et al. 1975). The 30 recipients who experienced grades III and IV were designated in this study as the aGVHD group. The 40 recipients who had not developed aGVHD (grade 0) were designated as the aGVHD-free group. The recipients with GVHD grades I or II were excluded from this analysis to differentiate more efficiently between the aGVHD and aGVHD-free groups.

### Microsatellite markers set

The association study was performed using 155 microsatellite markers spanning the long arm of chromosome 22.

**Table 1** Numbers and ratios for the major clinical characteristics

Clinical characteristics	Number or Ratio
Recipient's age (median year, range)	27.5, 1–50
Donor's age (median year, range)	33.9, 21–52
Recipient's sex (M/F)	38:32
Sex combination (recipient/donor)	M/M 29 M/F 19 F/F 13 F/M 9
Diagnosis	
Acute myeloid leukemia	28
Acute lymphoblastic leukemia	21
Chronic myeloid leukemia	21
Conditioning regimen	
CY+TBI	26
CY+CA+TBI	17
BU+CY+CA	2
BU+CY	7
CY+BU+TBI	3
CY+VP+TBI	4
BU+CY+TLI	1
LP+TBI	2
BU+VP+LP	1
CA+VP+TBI	1
CA+TBI	2
VP+TBI	1
BU+LP+TBI	1
CA+TBI	2
aGVHD frequency	
Grade 0	40
Grade III	20
Grade IV	10
GVHD prophylaxis	
CsA+MTX	64
FK+MTX	2
FK+PDR	1
CsA	1
CsA+MTX+PDR	1
CsA+MTX+FK	1

M Male, F female, CY cyclophosphamide, TBI total body irradiation, CA cytosine arabinoside, BU busulfan, VP etoposide, TLI total lymph node irradiation, LP melpharan, CsA cyclosporine A, MTX methotrexate, FK tacrolimus hydrate, PDR prednisolone

These markers were selected from Japan Biological Information Research Center (JBIRC) database (<http://jbirc.jbic.or.jp/gdbs/>). The markers covered the human genome from 15647099b (D22S0283i) to 49510061b (D22S0211i) on 22q with an average spacing of 200 kb.

### Microsatellite genotyping

Genomic DNA was isolated from the peripheral blood lymphocytes of patients and donors. The PCR procedure was performed in 10 µl reactions using fluorescent-dye conjugated PCR primers that were unilaterally labeled at



the 5'-end with the fluorescent reagent, 6-FAM (Applied Biosystems Japan, Tokyo, Japan). The PCR reaction mixture contained 10 ng of genomic DNA, 1  $\mu$ l of deoxyribonucleotide triphosphate (5 mM each), 1  $\mu$ l of 10 $\times$  buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>), and 20 pmol of forward and reverse primers as well as 0.5 unit of Ampli Taq Gold DNA polymerase (Applied Biosystems Japan). After initial denaturation for 5 min at 96°C, amplification was carried out in an automated thermal cycler (Applied Biosystems Japan Co.) for 40 cycles of 1 min at 96°C, 45 s at 57°C, and 45 s at 72°C with a final extension of 7 min at 72°C. Each PCR product was diluted 1:40 with water. The samples containing 1  $\mu$ l of the diluted PCR product, 10  $\mu$ l Hi-Di formamide (Applied Biosystems Japan Co.) and 0.1  $\mu$ l GeneScan-500LIZ size standard (Applied Biosystems Japan) were denatured for 3 min at 95°C, separated on capillary gels using an ABI PRISM 3730 automated sequencer, and the electrophoretic runs were analyzed with GeneMapper software (Applied Biosystems Japan).

#### Statistical analysis

Microsatellite allele frequency was calculated by direct counting. The strength of association was expressed by odds ratio (O.R), which was calculated from 2 $\times$ 2 contingency tables. Statistical significance was examined by the Fisher's double-sided exact test and the  $m\times n$  contingency table. Univariate analysis was performed to determine the association between microsatellite mismatch and aGVHD incidence. The *P* value, except when comparing a mismatch, was corrected by multiplying the number of microsatellite alleles (corrected *P<sub>c</sub>* value). The *P<sub>c</sub>* value less than 0.05 was accepted as statistically significant, and the *P<sub>c</sub>* value between 0.05 and 0.1 was indicative of a trend.

#### Definition of a microsatellite mismatch

Donor/recipient pairs were classified as matched or mismatched at each microsatellite marker locus. Pairs were defined as mismatched only when one or more recipient alleles are not shared by the corresponding donor (direction aGVHD).

## Results

The overall genotyping results for paired transplantation donors and recipients

A total of 155 microsatellite markers spanning the long arm of chromosome 22 were used to genotype 70 pairs of transplantation patients and donors. Of the 70 transplanta-

tion recipients, 30 (42.8%) developed aGVHD with GVHD grade III in 20 patients and GVHD grade IV in 10 patients. The genotyping results obtained for the 70 transplantation recipients and the 70 transplantation donors were then analyzed and compared between the aGVHD-free group (grade 0 aGVHD) and the severe aGVHD group (group III to IV aGVHD).

Of the 155 markers, there were three markers with significant allele frequency differences between all donors and all recipients (D22S0052i-385; D22S0099i-412; D22S0115i-225; data not shown). As these three markers have different allele distribution between all donors and all recipients, they were considered to be inappropriate markers for the comparison between the aGVHD-free and the aGVHD group and were therefore excluded from further analysis. The remaining 152 markers were retained for further analysis in this study because they showed no significantly different allele distribution between all donors and recipients (data not shown).

#### Recipient age and GVHD prophylaxis

The recipient age was not significantly higher in the patients with aGVHD than the aGVHD-free group (*P*=0.27 Student's *t* test). In regard to GVHD prophylaxis (Table 1), there was no significant association (*p*>0.07) of aGVHD factor risk between the patients in the total body irradiation (TBI) group and those in the non-TBI group.

#### Comparison of allele frequency differences between the aGVHD-free and the aGVHD group for the microsatellite polymorphisms in donors and recipients

The frequency differences for the microsatellite alleles between the aGVHD-free group and the aGVHD group were compared separately for the donors and recipients. The significant association (*P*<0.05) of markers with the occurrence of aGVHD was found for five markers (Table 2), with a significant difference (*P* and *P<sub>c</sub>*<0.05) for two donor markers (D22S283 and D22S0141i) and for three recipient markers (D22S0021i, D22S0199i, D22S0222i). The comparison of individual allele frequencies of the microsatellite markers in the grade 0 (aGVHD-free) and grade III+IV (aGVHD) groups revealed the presence of possible risk (R) alleles (O.R>1) and protective (P) alleles (O.R<1; Table 2). In the donors, the allele D22S0141i-431 was increased significantly (*P<sub>c</sub>*=0.049) and the allele D22S283-132 was decreased significantly (*P<sub>c</sub>*=0.008) in aGVHD when compared to the aGVHD-free group. Both of the marker loci were in position 22q12.3. In the recipients, the frequency of the allele D22S0021i-348 was significantly increased (*P<sub>c</sub>*=0.035) and three alleles (D22S0021i-357, D22S0199i-444,

**Table 2** Statistically significant alleles associated with aGVHD grade in patients and donors, respectively

Marker	Position	No. of alleles	Significant allele	aGVHD grade (N=40)	aGVHD grade III+IV (N=30)	Odds ratio (95% confidence interval)	Protective (P) or at risk (R)	P value	Pc
<b>Donor</b>									
D22S283	22q12.3	12	132	35 (87.5%)	15 (50.0%)	0.14 (0.04–46)	P	0.0007	0.008
D22S0141i	22q12.3	7	431	4 (10.0%)	14 (46.7%)	7.87 (2.24–27.7)	R	0.007	0.049
<b>Recipient</b>									
D22S0021i	22q13.2	5	348	21 (52.5%)	25 (83.2%)	4.52 (1.44–14.2)	R	0.007	0.035
			357	38 (90.0%)	18 (67.7%)	0.16 (0.06–0.48)	P	0.004	0.020
D22S0199i	22q13.2	4	444	22 (55.0%)	7 (23.3%)	0.25 (0.09–0.72)	P	0.007	0.028
D22S0222i	22q13.3	7	258	17 (42.5%)	4 (13.3%)	0.21 (0.06–0.71)	P	0.007	0.049

and D22S0222i-258) were significantly decreased in aGVHD when compared to the aGVHD-free group.

In regard to the D22S0021i locus, we found both a risk allele (D22S0021i-348) and a protective allele (D22S0021i-357). On the basis of a genotype analysis, there was a significant association ( $P=0.001$ ) between the D22S0021i genotype and aGVHD occurrence (Table 3).

Comparison of differences between the aGVHD-free and the aGVHD groups for microsatellite alleles that were matched or mismatched in donors and recipients

As a further comparison between the aGVHD-free and the aGVHD groups, we determined the significant differences between the number of alleles of the aGVHD-free and the aGVHD groups that were matched and mismatched for the donor and recipient pairs. We estimated that there were eight significant marker mismatches for an association with the occurrence of aGVHD (Table 4). Of these eight markers, three (D22S0267i, D22S0220i, and D22S683) were more often mismatched in the severe GVHD group ( $O.R>1$ ), and therefore, these markers appear to be protective against the occurrence of severe aGVHD. As D22S0220i and D22S683 are located in a relatively close position to each other on 22q12.3 where they are 780 kb apart, we selected an additional five markers between D22S0220i and D22S683. As a result, three markers (Z67524,  $P=0.09$ ,  $O.R=0.35$ ; D22S0132i,  $P=0.07$ ,  $O.R=2.54$ ; D22S0075i,  $P=0.07$ ,  $OR=0.03$  in order from the centromere to telomere) showed a tendency of association with aGVHD (Fig. 1).

**Table 3** Univariate analysis of D22S0021i genotype

Allele genotype	aGVHD grade 0 (N=40)	aGVHD grade III+IV (N=30)	P value
348/348	2	10	0.001
348/357	19	15	
357/357	17	3	

On the other hand, five markers (D22S0152i,  $P=0.0005$ ; D22S0145i,  $P=0.017$ ; Z66750,  $P=0.014$ ; D22S0085i,  $P=0.035$ ; D22S0197i,  $P=0.005$ ) were more often mismatched in the aGVHD-free group ( $O.R<1$ ), suggesting that they are significant susceptibility markers for aGVHD. Of these markers, D22S0152i and D22S0145i were located in a relatively close position to each other on 22q11.23 where they were 960 kb apart. We, therefore, genotyped an additional six markers (D22S0068i, D22S0186i, D22S0163i, D22S0169i, D22S0184i, and D22S1174) but found that none of them were significantly associated with aGVHD (data not shown).

Candidate genes within the aGVHD susceptibility regions

Table 5 lists the candidate susceptibility genes that are located within or near to the genomic susceptibility region which was identified by microsatellite genotyping. These genes are in the close vicinity of the significant microsatellite markers that were found within intron 3 of CACNG2, intron 3 of PEX26, intron 4 of KIAA0376, intron 7 of LARGE, and intron 8 of TOM1. Other genes, such as MYH9, EP300, TCF20, ARSA, FLJ31568, EMID1, APOL3, and FLJ44385, are located within 10 kb to 172 kb of the significantly associated microsatellite markers.

Genomic map of the association of microsatellite polymorphisms on 22q12.3 with the occurrence of aGVHD

The  $P$  values for comparing the matching of microsatellite marker alleles between those of the aGVHD-free group and the aGVHD group were determined and plotted as a  $P$  value plot against the physical location of the microsatellite markers and the known genes on 22q12.3. Figure 1 shows a  $P$  value plot and the gene map of one of the aGVHD susceptibility regions determined by the association analysis using the microsatellite markers from D22S0220i to D22S683 and beyond the border of 22q12.3 and 22q13.1. The figure shows that the genes TOM1, HMOX1, and

**Table 4** Correlation between matched mismatch donor–recipient pairs and aGVHD grade for each of the significant microsatellite markers on chromosome 22

Marker	Position	aGVHD grade 0		aGVHD grade III+IV		Odds ratio (95% CI)	Protective (P) or at risk (R)	P value
		Matched	Mismatched	Matched	Mismatched			
D22S0267i	22q11.21	38	2	23	7	5.78 (1.10–30.24)	P	0.028
D22S0152i	22q11.23	25	15	29	1	0.05 (0.01–0.41)	R	0.0005
D22S0145i	22q11.23	8	32	14	16	0.29 (0.10–0.82)	R	0.017
Z66750	22q12.1	28	12	28	2	0.17 (0.03–0.81)	R	0.014
D22S0085i	22q12.3	6	34	11	19	0.30 (0.10–0.96)	R	0.035
D22S0220i	22q12.3	17	23	4	26	4.80 (1.41–16.35)	P	0.008
D22S683	22q12.3	6	34	0	30	11.86 (0.64–219.35)	P	0.027
D22S0197i	22q13.33	16	24	22	8	0.24 (0.087–0.67)	R	0.005

MCM5 are in the region of the most significant *P* values and in close vicinity to the protective microsatellite marker D22S0220i.

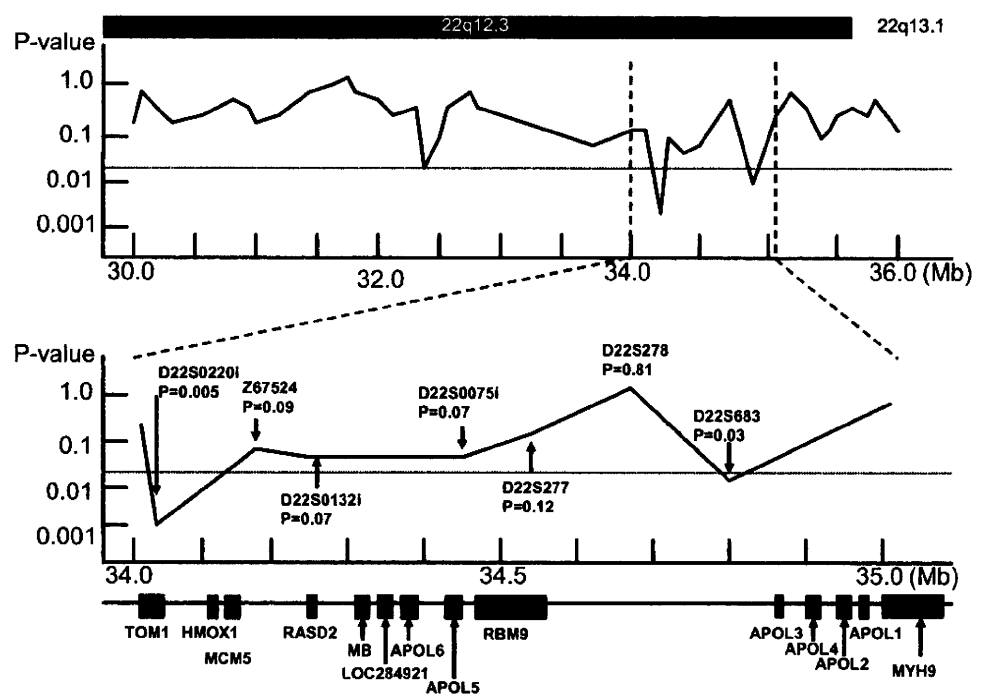
## Discussion

Of the 155 markers analyzed for differences between the aGVHD-free group and the aGVHD group and separately for the recipients and donors, only five markers on chr 22 (Table 2) were found to be significantly associated with aGVHD ( $P < 0.05$ ). Interestingly, of these five positive markers, the donor positive marker D22S283 was previously reported to be associated with schizophrenia (DeLisi et al. 2002), Sorsby's fundus dystrophy (Assink et al. 2000), and CDAGS (Mendoza-Londono et al. 2005).

Although the susceptibility genes on chr 22 for those diseases are still unknown, the positive microsatellite marker D22S283 is located within the SC2D4 schizophrenia susceptibility locus (NCBI GeneID 6379) and could be associated with neuropsychological impairment that may evolve with aGVHD (Sostak et al. 2003).

The other four positive markers, D22S0141i, D22S0021i, D22S0199i, and D22S0222i, which were associated with aGVHD (Table 2) had not been previously associated with any human disease. However, these markers are located in a region of human chr 22 that was previously associated with the presence of strong mucosal and T-cell immune response against HIV-1 (Kanari et al. 2005) and, therefore, that could also affect the aGVHD. The p300 gene, which is a transcriptional factor located 10 kb from D22S0021i, is believed to participate in the activities of hundreds of

**Fig. 1** aGVHD susceptibility gene mapping by association analysis using microsatellite markers on 22q12.3. *P* value (*y*-axis) was plotted against physical location of the microsatellite markers on 22q12.3 (*x*-axis), their distance (in Mb) in order from the centromere to the telomere. All markers were plotted according to their genetic map position taken from JBIRC database (<http://jbirc.jbic.or.jp/gdbs/>). The gene map at the bottom of the figure shows the representative genes that are indicated by black boxes on the locus near the two positive markers D22S0220i and D22S683 on 22q12.3. The dotted horizontal line shows the threshold for 5% significance





**Table 5** Candidate genes close to the positive microsatellite markers

Marker	Position	Distance	Neighboring gene	Description	Function
<b>Donor allele</b>					
D22S283	22q12.3	172 kb	MYH9	Myosin, heavy chain 9, nonmuscle	Cytoskelton
D22S0141i	22q12.3	Intron 3	CACNG2	Calcium channel, voltage dependent, gamma-2 subunit	Cell signaling
<b>Recipient allele</b>					
D22S0021i	22q13.2	10 kb	EP300	E1A binding protein, 300 KD	Transcription
D22S0199i	22q13.2	40 kb	TCF20	Transcription factor 20	Transcription
D22S0222i	22q13.33	45 kb	ARSA	Arylsulfatase A	Enzyme
<b>Mismatching allele</b>					
D22S0267i	22q11.21	Intron 3	PEX26	Peroxisome biogenesis factor 26	Protein degradation
D22S0152i	22q11.23	20 kb	FLJ31568	Hypothetical protein	Unknown
D22S0145i	22q11.23	Intron 4	KIAA0376	Hypothetical protein	Unknown
Z66750	22q12.1	10 kb	EMID1	BMI domain containing protein 1	Unknown
D22S0085i	22q12.3	Intron 7	LARGE	Acetyl glucosaminyltransferase-like protein	Unknown
D22S0220i	22q12.3	Intron 8	TOM1	Target of myb 1	Immuno regulation
D22S683	22q12.3	20 kb	APOL3	Apolipoprotein L-III	Lipid metabolism
D22S0197i	22q13.33	10 kb	FLJ44385	Hypothetical protein	Unknown

different genes (Vo and Goodman 2001). The p300 protein, together with the adenovirus serotype 5 E1A, has been reported to regulate the NKG2D ligand, NK cell lysis, and tumor rejection (Routes et al. 2005). In addition, p300-binding domains are known to interact with STAT1, 2, and 3, which play an important role in cytokine signal transduction (Pfitzner et al. 2004). Therefore, the p300 gene might be associated haplotypically with the D22S0021i marker, which has both a protective and risk allele for aGVHD (Table 3). These facts, together with our association results, strongly suggest that the loci at position 22q12–13 could affect the development of aGVHD. Whereas three of these positive markers are located 10 kb to 45 kb from any of the known genes, the positive microsatellite marker D22S0141i is located within intron 3 of the CACNG2 gene that encodes the calcium channel, voltage dependent, gamma-2 subunit (Table 5). This protein appears to interact with neural proteins (Black and Lennon 1999; Chen et al. 2000), and it might have a role in neurological complications arising from aGVHD (Sostak et al. 2003).

We found eight microsatellite markers that were significantly different between the aGVHD-free group and the aGVHD group when matched or mismatched between the recipient and donor groups. Three of the eight markers, D22S0267i, D22S0220i, and D22S683, are considered to be protective because they were more often mismatched in the severe aGVHD group ( $OR > 1$ ), suggesting the existence of one or more protective candidate genes in close vicinity. Two of these markers, D22S0220i and D22S683, were approximately 780 kb apart (D22S0085i and D22S0220i) with another three markers (Z67524, D22S0132i, and D22S0075i) located between them that showed a positive trend ( $P < 0.1$ ) of association (Fig. 1). Interestingly, Gubarev

et al. (1996) reported the localization of a gene encoding mHa on 22q12.3 in close vicinity to our significant markers by using T-cell clone and linkage-analysis. This report, which used different methods from our genome-wide approach, therefore strongly supports our results.

The highly significant protective microsatellite marker D22S0220i is located within intron 8 of the gene TOM1. The specific function of this gene has not yet been determined, but Tom1 may be a negative regulator of interleukin-1 and tumor necrosis factor-induced signaling pathways (Yamakami and Yokosawa 2004), and, therefore, affect aGVHD. D22S0220i is also located near to the HMOX1 gene (NCBI Gene ID 3162) that encodes the heme oxygenase (decycling) 1 protein. This association is biologically significant because HMOX1 (alias HO-1) is known to be a protective protein with anti-inflammatory and antiapoptotic properties (Willis et al. 1996; Brouard et al. 2002). Moreover, induction of HMOX1 in recipient mice of a BMT model resulted in a reduction in aGVHD and improved survival (Gerbitz et al. 2004). Therefore, HMOX1 is an excellent protective candidate gene for further aGVHD association studies specifically at the level of gene SNP analysis.

Another potential protective microsatellite marker D22S683 is located ~172.2 kb from the MYH9 gene (MIM 160775) and the Epstein syndrome locus (MIM 153650). The MYH9 mutations are known to result in the autosomal dominant giant-platelet disorders such as the May–Hegglin anomaly, the Fechtner syndrome, and the Sebastian syndrome (Seri et al. 2000). In addition, the MYH9 or the motor protein non-muscle heavy chain II A has been associated with the chemokine receptor CXCR4 in the T cell (Rey et al. 2002) and with the modulation of T cell motility (Jacobelli et al.



2004). Considering that one of the alleles of the microsatellite marker D22S283 is located within 172.2 kb of the MYH9 gene of the transplantation donors that were positively associated with aGVHD, then it can be envisaged that a neighboring SNP may affect the donor T cell behavior in a protective role against the occurrence and/or maintenance of aGVHD.

The five ‘disease-negative’ markers shown in Table 4 were associated with a risk of aGVHD because they were more often mismatched in aGVHD grade 0 group ( $OR < 1$ ) than the aGVHD group. This result seems to be paradoxical when considering the concept of a minor antigen mismatch, but it suggests that some gene products might need to be mismatched to prevent the development of disease. For example, it has been reported that the killer cell immunoglobulin-like receptor ligand (KIR-ligand), when mismatched between the donor and recipient, is associated with improved survival after stem cell transplantation for acute myeloid leukemia (Ruggeri et al. 2002). In this regard, the product of an unknown gene located near the ‘disease-negative’ microsatellite markers, when mismatched between donor and recipient, might help to prevent the development of aGVHD in a way that is analogous to the unique KIR-ligand mismatch involved with the NK-KIR biological system in response to transplantation (Malmberg et al. 2005).

To identify the candidate genes that are located within close vicinity to the significant microsatellite markers, we searched the human genome sequence deposited at NCBI for locations and annotations of genes in both directions of the microsatellite markers (Table 5). Interestingly, many of the genes that we identified near the associated markers, such as MYH9, CACNG2, EMID1, LARGE, and TCF20, have proximal STAT1- and STAT2-binding sites. Many DNA binding sites for STAT1 and STAT2 have been identified distributed across chr 22 in interferon-treated cells (Hartman et al. 2005). The STAT family proteins mediate transcriptional responses to many cytokines and are a useful system for studying inducible gene regulation. In addition, APOL3, EMID1, and LARGE exhibit IFN-sensitive expression changes. Considering the complex roles of cytokines, such as IFN, in the aGVHD occurring phase after BMT, the cytokine inducible candidate genes may play an important role in aGVHD.

The results of our study are largely dependant upon the hypothesis that microsatellite markers in LD will reveal an association between polymorphisms and the functional risk conferred by the variants or relevant genes so that certain marker alleles will be over represented in the aGVHD donors or patients compared with the GVHD-free donors or recipients (Ohashi and Tokunaga 2003; Zapata et al. 2001). In this study, we used 155 microsatellite markers whose spacing average was about 200 kb on the basis of the

knowledge accumulated from a large number of recent data that the average length of LD between disease susceptible SNPs and nearby microsatellite alleles is  $\geq 100$  kb (Abecasis et al. 2001; Keicho et al. 2000; Oka et al. 1999; Ota et al. 1999). Although the LD pattern is variable between different regions of human genome depending on several factors such as allele frequency, mutation and recombination, and ethnic population, the 200 kb interval between markers is likely to be of sufficient distance for LD coverage of chr 22 in this study.

The multiple testing issues and the restricted sample size of our study limit the statistical power to find conclusive evidence of association particularly in the case of susceptibility genes with minor effects. It is statistically possible that at a probability level of less than 0.05 that 1 in 20 of our markers will represent false positives. We have analyzed 155 different microsatellite markers for association with aGVHD, and therefore, we could expect about eight false positive markers distributed randomly across the 40 Mb of the long arm of chr 22. Of the 13 microsatellite markers that were significantly different between the GVHD-free group and the GVHD severe group, the location of three of the markers, D22S0220i, D22S683, and D22S283, were relatively close to each other, which increases the probability that they represent a true association. Moreover, this GVHD susceptibility locus, from D22S0220i and D22S283, spans approximately 1 MB of genomic sequence and contains at least 14 candidate genes, including TOM1 and HMOX1 and MYH9, near the APOL1 to APOL6 gene cluster (Fig. 1).

In conclusion, we used 155 microsatellite markers distributed across the long arm of chr 22 and the ‘genome-wide approach’ in this genetic association study of aGVHD to identify and map potential aGVHD susceptibility and resistant regions on the basis of a small number of significant markers. It now remains to use the ‘candidate gene approach’ and investigate the SNPs and haplotypes of the candidate genes, such as TOM1, HMOX, MCM5, and MYH9, which are located closely to the most significant microsatellite markers.

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# Identification of CLUAP1 as a human osteosarcoma tumor-associated antigen recognized by the humoral immune system

HISAMITSU ISHIKURA<sup>1,5</sup>, HIROAKI IKEDA<sup>1</sup>, HIROYUKI ABE<sup>1</sup>, TAKAYUKI OHKURI<sup>1</sup>,  
HIROAKI HIRAGA<sup>2</sup>, KAZUO ISU<sup>2</sup>, TOMOHIDE TSUKAHARA<sup>3,4</sup>, NORIYUKI SATO<sup>4</sup>,  
HIDEMITSU KITAMURA<sup>1</sup>, NORIMASA IWASAKI<sup>5</sup>, NAOKI TAKEDA<sup>6</sup>,  
AKIO MINAMI<sup>5</sup> and TAKASHI NISHIMURA<sup>1</sup>

<sup>1</sup>Division of Immunoregulation, Institute for Genetic Medicine, Hokkaido University; <sup>2</sup>Division of Orthopaedic Surgery, National Hospital Organization, Hokkaido Cancer Center; Departments of <sup>3</sup>Orthopaedic Surgery, <sup>4</sup>Pathology, Sapporo Medical University School of Medicine; <sup>5</sup>Department of Orthopaedic Surgery, Hokkaido University Graduate School of Medicine; <sup>6</sup>Hokkaido University College of Medical Technology, Sapporo, Japan

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**Abstract.** Since the prognosis of human osteosarcoma in advanced stage remains poor, the development of new and effective therapies including immunotherapy is required. To identify tumor-associated antigens of osteosarcoma applicable to the immunotherapy of this malignancy, we employed the serological analysis of recombinant cDNA expression library (SEREX) technique that defines tumor antigens recognized by the humoral immune system. Screening a cDNA library derived from an osteosarcoma cell line MG63 with sera from osteosarcoma patients identified 43 positive clones, representing 14 distinct antigens. Among them, CLUAP1 (clusterin-associated protein 1) was highly expressed in osteosarcoma tissue samples and cell lines. Overexpression of CLUAP1 was observed in other malignancies including ovarian, colon, and lung cancers. Our results suggest that CLUAP1 may be useful as a prognostic/diagnostic marker and/or for a target of immunotherapy of osteosarcoma.

## Introduction

Osteosarcoma is the most common primary bone tumor, typically occurring in children and young adults with frequency of about six children per million a year (1). Although the recent chemotherapy regimens and operating procedures have drastically improved the prognosis of the patients with non-metastatic osteosarcoma, the prognosis of the patients with recurrence or metastasis is still poor (2). The overall survival

with an aggressive chemotherapy regimen before and after surgery remains between 50 and 65% (3). Therefore, development of more effective and less toxic therapeutic approaches including immunotherapy is required.

While a number of tumor-associated antigens were identified for many types of solid tumors (4-6), the reports for the tumor antigens of human osteosarcoma are limited. Some of the tumor-associated antigens such as melanoma-associated antigen (MAGE) (7), squamous cell carcinoma antigen recognized by T cells (SART) 1 (8), SART 3 (9), or papillomavirus binding factor (PBF) (10) were reported to be expressed in osteosarcoma. Antigenic peptides derived from SART 3 or PBF were shown to be recognized by CD8<sup>+</sup> cytotoxic T lymphocytes (CTL) from patients with osteosarcoma in MHC class I-restricted manner (9,10).

Accumulating evidence from the studies on both human and animal models has indicated that CD4<sup>+</sup> T cells play an important role in anti-tumor immune responses (6,11,12). The information of tumor-associated antigens of osteosarcoma recognized by CD4<sup>+</sup> T cells, however, has not been reported. The serological analysis of recombinant cDNA expression library (SEREX) has identified a broad range of tumor-derived proteins capable of eliciting humoral immune response in tumor patients (5,13,14). Since SEREX antigens were identified by the high-titer immunoglobulin (Ig) G responses that rely on cognate T cell help in patients *in vivo*, the use of SEREX provides a direct route to the analysis of the CD4<sup>+</sup> T cell repertoire against tumor antigens (15). On the other hand, novel as well as previously defined tumor antigens have been identified using the SEREX method, including MAGE-1 and tyrosinase, both originally identified by expression cloning of epitopes recognized by CTL (13,16,17). Thus, SEREX is considered to be able to define immunogenic tumor antigens that elicit cellular as well as humoral immunity.

In this study, we aimed to identify tumor-associated antigens of osteosarcomas by the use of SEREX analysis. We examined the expression of one defined antigen, CLUAP1, in osteosarcoma and other malignancies to evaluate the potential of CLUAP1 as a marker of the tumor-specific immune

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*Correspondence to:* Dr Takashi Nishimura, Division of Immunoregulation, Institute for Genetic Medicine, Hokkaido University, N-15, W-7, Kita-Ku, Sapporo 060-0815, Japan  
E-mail: tak24@igm.hokudai.ac.jp

*Key words:* tumor-associated antigen, SEREX, osteosarcoma



response and/or a target for the immunotherapy of cancer patients.

## Materials and methods

**Cell lines, sera, and tissues.** Osteosarcoma cell lines (MG63, Saos2, HuO3N1, HuO9N2, OS2000, HOS) were kindly provided from Cell Resource Center for Biomedical Research, Tohoku University and Department of Pathology, Sapporo Medical University School of Medicine. They were cultured in RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. Sera were obtained from 11 osteosarcoma patients attending National Sapporo Hospital. In addition, sera of 10 healthy individuals were obtained from volunteers at Division of Immunoregulation, Institute for Genetic Medicine, Hokkaido University. Tumor tissues were obtained from patients who underwent surgery at Hokkaido University Hospital. All samples were collected from patients or healthy volunteers after obtaining informed consents concerning the use of material for scientific research and appropriate ethical approval for the projects.

**cDNA library construction.** Total RNA was isolated from  $1 \times 10^7$  MG63 cells with TRIzol reagent (Invitrogen, Rockville, MD, USA). mRNA was isolated by mRNA purification kit (Amersham Biosciences, Buckinghamshire, UK) following the manufacturer's instruction. Double-strand cDNA was synthesized from 5 µg of poly (A) RNA using SMART cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA). Then ligated into ZAP Express λ phage vector (Stratagene, La Jolla, CA, USA). The titer of the constructed cDNA library was  $1 \times 10^6$  pfu/ml.

**Serological screening of expression library with patient sera.** A total of  $1 \times 10^6$  recombinants were screened by each serum. The constructed library was transfected into XL1 Blue in agar plates, and cultured at 37°C for 5 h. The nitrocellulose filters, impregnated with isopropyl-β-D-thiogalactopyranoside (IPTG), were laid on top of the developing plaque on the agar surface and were incubated at 37°C for 15 h. The filters were peeled off after incubation and rinsed in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T). Subsequently, they were agitated in TBS-T and 5% non-fat dried milk (blocking buffer) for 1 h. The filters were screened by 2 patient sera, which were pre-treated with *Escherichia coli* (*E. coli*) for absorption of anti-*E. coli* natural antibodies. Each serum was diluted by TBS at 100-fold dilution. After rinsing 3 times in TBS-T, 2000-fold diluted horseradish alkaline phosphatase-conjugated goat anti-human Ig G (Jackson Immunoresearch, West Grove, PA, USA) was added to the filter. Antibodies binding to proteins on the filter surface were detected by nitro blue tetrazolium chloride/5-bromo-4chloro-3-indolylphosphate color development.

**Cloning and sequencing.** The plaque corresponding to the positive spot in the previous immunoscreening was cultured and stocked in SM buffer. Positive clones were converted into pTriplEx phagemid vector from the λTriplEx vector using

the *in vivo* excising protocol supplied by Clontech Laboratories. Cloned DNAs were sequenced with vector specific primers, the ABI PRISM Dye terminator sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA), and an ABI automated DNA sequencer (Applied Biosystems).

**RNA extraction and RT-PCR.** RNA was isolated from frozen tumor tissues or from cultured tumor cell lines using ISOGEN (Nippon Gene Ltd., Tokyo, Japan) according to manufacturer's instruction. Total RNA (5 µg) was primed with an oligo (dT) 18 oligonucleotide and reverse-transcribed with Superscript II (Invitrogen) according to the manufacturer's instructions. Obtained cDNA was tested for integrity by amplification of β-actin and transcripts in a 30-cycle PCR reaction as described elsewhere (18).

Gene specific primers were designed to amplify fragments of 500-600 and synthesized commercially (Hokkaido System Science, Sapporo, Japan) as followed: HU-OS-12, forward primer 5'-GGCAGATTAACCCCTCAGACTT-3', reverse primer 5'-GTATTGTGAACATGCAGACGAAA-3'; HU-OS-1, forward primer 5'-TCCAGTGAATTTGAAGTTAGGAGAC-3', reverse primer 5'-CATCAAATATTCCCTTTCTTCCTT-3'; HU-OS-5, forward primer 5'-CTAATGCTA ACTGGAAAGCACTAA-3', reverse primer 5'-CCTCCC CATAACTCTCTCATATTT-3'; HU-OS-8, forward primer 5'-AATCATCCAGCAGGAGCAAG-3', reverse primer 5'-AGTTAGAAGCTGGGCAGCAA-3'; HU-OS-9, forward primer 5'-TGTGGGATAAAATCCATTTAGAAAA-3', reverse primer 5'-TATATACCCAGTCAGTTGTCTGCAA-3'; β-actin, forward primer 5'-TTAAGGAGAAGCTGTGCTACGTC-3', reverse primer 5'-ATCTTGTTTCTGCGCAAGTTAG-3'.

**Quantitative RT-PCR analysis.** The relative expression of CLUAP1 mRNA was measured by quantitative PCR using mRNA extracted from tumor tissues and cell lines. Total RNA (5 µg) was reverse transcribed using an oligo (dT) 18 oligonucleotide and reverse-transcribed with Superscript II (Invitrogen). Tissue cDNA panels (Clontech Laboratories) was used as normal tissue-derived cDNA. β-actin was used to normalize the target gene expression. Quantitative RT-PCR was performed using the PRISM 7000 (Applied Biosystems) with QuantiTect Probe PCR master mix (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification conditions were: 95°C (10 min), 45 cycles of 95°C (15 sec) and 60°C (1 min). The primer and probe sequences for detecting CLUAP1 were as followed: Forward primer: 5'-CCAAGCCACAGACAGCCAT-3'; Reverse primer: 5'-TCTCCACCTTGCATCGTGC-3'; Probe: 5'-CAAGGAA GACCTGGCAAACGCAAACGCATTGT-3'. Taqman gene expression assay (Applied Biosystems) was used as the primers and the probe to evaluate β-actin expression. Expression of CLUAP1 was normalized by β-actin value.

## Results

**Serological identification by SEREX and sequence analysis of osteosarcoma-associated antigens.** Recombinant clones ( $1.0 \times 10^6$ ) of cDNA library derived from one of the well-characterized osteosarcoma cell lines, MG63 (19), were

Table I. SEREX analysis by sera of patients with osteosarcoma.

Patient	Age	Sex	Clinical stage	Prognosis	Number of serum positive clones	Number of different antigens
A	14	M	IV A	DOD	25	8
B	12	F	II B	NED	19	7

M, male; F, female; DOD, died of disease; NED, no evidence of disease. Clinical stage was defined by Enneking stage.

Table II. Antigens obtained by SEREX analysis of osteosarcoma-derived cDNA library and patient sera.

Antigen	Frequency of isolated clones	Identity	UniGene No.	Proposed function
HU-OS-1	18/43	Sorting nexin 7 (SNX7)	Hs.197015	Intracellular trafficking
HU-OS-2	1/43	Nucleolar protein 8 (NOL8)	Hs.442199	Regulation of gene expression, possible involvement in tumorigenesis
HU-OS-3	1/43	Microtubule-associated protein 1B (MAP1B)	Hs.584777	Development and/or repairing of neurons
HU-OS-4	2/43	Eukaryotic initiation factor 2B, subunit 5 $\epsilon$ , 82 kDa (EIF2B5)	Hs.283551	Exchange of eukaryotic initiation factor 2-bound GDP for GTP
HU-OS-5	1/43	Tetratricopeptide repeat 5 (TTC5)	Hs.102480	Mediating protein-protein interaction
HU-OS-6	2/43	Nexilin (F actin binding protein) (NEXN)	Hs.632387	Mediating cell motility
HU-OS-7	1/43	Angiogenic factor with G patch and FHA domains 1 (AGGF1)	Hs.213393	Promotion of angiogenesis
HU-OS-8	1/43	Oral-facial-digital syndrome 1 (OFD1)	Hs.6483	Differentiation of metanephric precursor cells
HU-OS-9	1/43	PC4 and SFRS1 interacting protein 1 (PSIP1)	Hs.493516	Transcriptional coactivator involved in neuroepithelial stem cell differentiation and neurogenesis
HU-OS-10	7/43	Brix domain containing 2 (BXDC2)	Hs.38114	Mediating biogenesis of the 60S ribosomal subunit
HU-OS-11	1/43	$\alpha$ thalassemia/mental retardation syndrome X-linked (ATRX)	Hs.533526	Transcriptional regulation and modification of gene expression by affecting chromatin remodeling
HU-OS-12	6/43	Clusterin associated protein 1 (CLUAP1)	Hs.155995	Mediating cell proliferation and apoptosis, possible involvement in tumorigenesis
HU-OS-13	1/43	Heat shock 70-kDa protein 4 (HSPA4)	Hs.90093	Inhibition of protein aggregation, possible involvement in tumorigenesis
HU-OS-14	1/43	Huntingtin interacting protein 1 (HIP1)	Hs.329266	Regulation of cell filament networks, possible involvement in tumorigenesis

screened by 2 individual sera from patients suffering from osteosarcoma to identify tumor-associated antigens. Table I shows the number of immunoreactive clones obtained from the screening with each serum. In total, 43 positive cDNA clones representing 14 different antigens, designated HU-OS-1 through HU-OS-14, were identified. Table II summarizes the characteristics of the genes encoding the antigens identified

based on the information derived from the NCBI BLAST, UniGene database. Only HU-OS-1 was recognized by two sera preparation. The 14 genes are all reported with known function as shown in Table II.

*Expression of mRNA encoding SEREX-defined osteosarcoma antigens in normal tissues.* We examined the mRNA expression

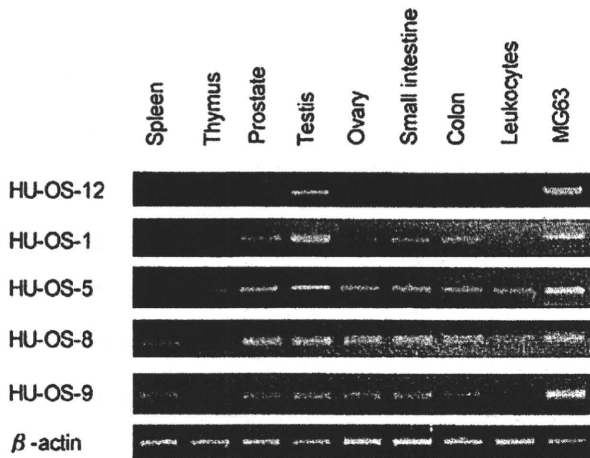


Figure 1. mRNA expression of identified antigens in normal tissues. The expression of HU-OS-12 (CLUAP1) and other representative antigens (HU-OS-1, HU-OS-5, HU-OS-8, and HU-OS-9) in 8 adult normal tissues and MG63 cells were analyzed by conventional reverse transcription-PCR.  $\beta$ -actin housekeeping gene was used as an internal control.

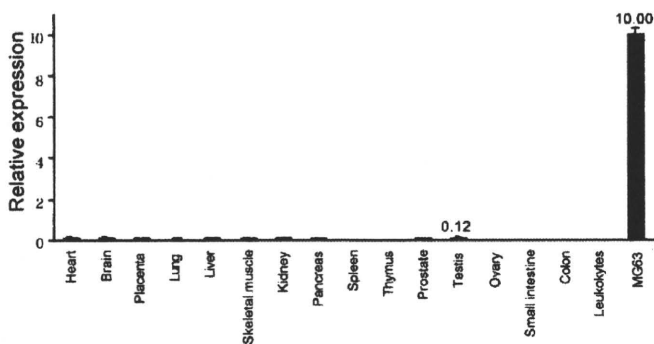


Figure 2. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in normal tissues. The expression of CLUAP1 in 16 adult normal tissues and MG63 cells were analyzed by quantitative RT-PCR. Expression of CLUAP1 was normalized by  $\beta$ -actin value. The results are presented as mean  $\pm$  SD of 3 measurements.

patterns of each antigen identified in normal tissues using a panel of 8 adult normal tissues (spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes) and MG63 as positive control by conventional semi-quantitative RT-PCR. As shown in Fig. 1, only HU-OS-12 that was encoded by the gene of *CLUAP1* showed faint expression in normal tissues with the exception of testis. All of other antigens were expressed in multiple organs (Fig. 1 and data not shown). This result led us to focus on CLUAP1 for further analysis. Sequence analysis revealed that isolated clones encoding *CLUAP1* did not have any mutations (data not shown).

**mRNA expression of CLUAP1 in normal tissues and osteosarcomas.** By the use of quantitative RT-PCR, we examined the expression of CLUAP1 within adult normal tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes), osteosarcoma cell lines, and osteosarcoma tissues. Among the normal tissues tested, testis appeared to

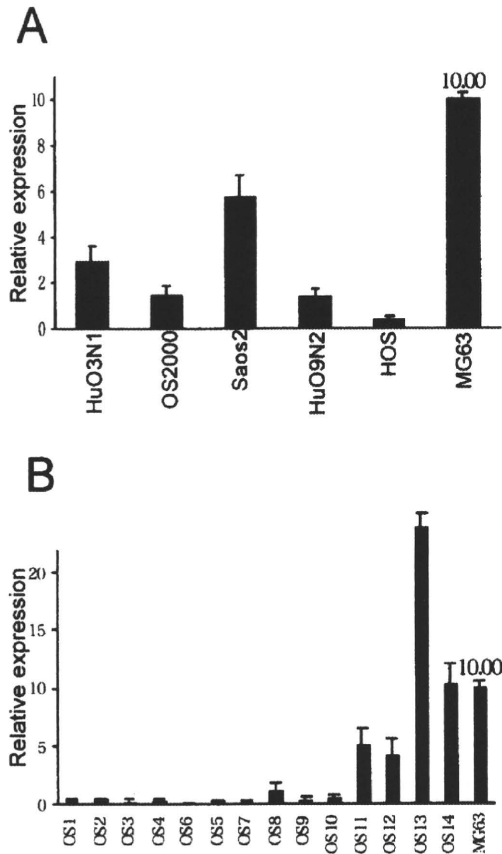


Figure 3. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in osteosarcoma cell lines and tissues. (A) Relative expressions of CLUAP1 in 6 osteosarcoma cell lines were 2.98 (HuO3N1), 1.42 (OS2000), 5.69 (Saos2), 1.39 (HuO9N2), 0.35 (HOS), and 10.00 (MG63). (B) Relative expressions of CLUAP1 in 14 osteosarcoma tissues were measured by quantitative RT-PCR. OS1 (relative expression; 0.32), OS2 (relative expression; 0.33), OS8 (relative expression; 1.18), OS9 (relative expression; 0.30), OS10 (relative expression; 0.49), OS11 (relative expression; 5.01), OS12 (relative expression; 4.08), OS13 (relative expression; 23.84), and OS14 (relative expression; 10.26) showed high expression of CLUAP1. Expression of CLUAP1 was normalized by  $\beta$ -actin value. The results are presented as mean  $\pm$  SD of 3 measurements.

express CLUAP1 most abundantly. However, the osteosarcoma cell line MG63 that was used for the cDNA library construction expressed CLUAP1 80-2000 times more compared to the normal tissues as shown in Fig. 2. We examined CLUAP1 expression in 6 osteosarcoma cell lines including MG63. While the relative expression of CLUAP1 varied among these 6 cell lines, all of the 6 cell lines expressed CLUAP1 >2-fold of the expression of the testis that appeared to express CLUAP1 most abundantly among the normal tissues tested (Fig. 3A). In 14 osteosarcoma tissues (OS1 through OS14), 9 cases (64%) expressed CLUAP1 >2-fold of the testis expression (Fig. 3B).

**mRNA expression of CLUAP1 in tumors other than osteosarcoma.** We next extended the analysis of mRNA expression of CLUAP1 to tumors other than osteosarcoma. Frozen section samples obtained from surgical resection of ovarian cancer, colon cancer, non-small cell lung cancer (NSCLC), esophageal cancer, and bile duct cancer were used for the quantitative RT-PCR analyses of CLUAP1 expression. None

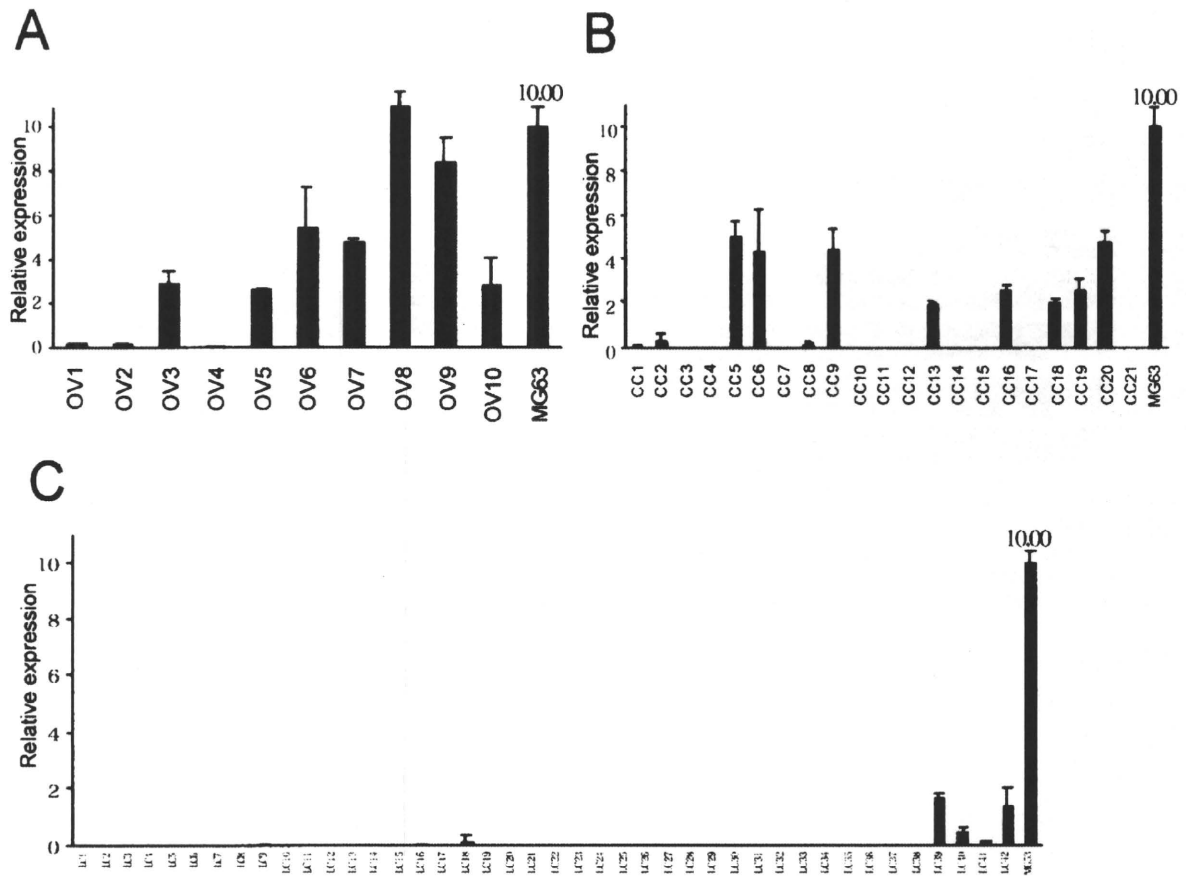


Figure 4. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in ovarian, colon, and lung cancers. (A) Relative expressions of CLUAP1 in 10 ovarian cancer tissues with MG63 as a positive control were measured by quantitative RT-PCR. OV3 (relative expression; 2.88), OV5 (relative expression; 2.53), OV6 (relative expression; 5.50), OV7 (relative expression; 4.78), OV8 (relative expression; 10.98), OV9 (relative expression; 8.34), and OV10 (relative expression; 2.77) showed high expression of CLUAP1. (B) Relative expressions of CLUAP1 in 21 colon cancer tissues with MG63 as a positive control were measured by quantitative RT-PCR. CC2 (relative expression; 0.28), CC5 (relative expression; 4.99), CC6 (relative expression; 4.32), CC9 (relative expression; 4.45), CC13 (relative expression; 1.87), CC16 (relative expression; 2.49), CC18 (relative expression; 1.97), CC19 (relative expression; 2.49), and CC20 (relative expression; 4.72) showed high expression of CLUAP1. (C) Relative expressions of CLUAP1 in 42 NSCLC tissues with MG63 as a positive control were measured by quantitative RT-PCR. LC39 (relative expression; 1.67), LC40 (relative expression; 0.50), and LC42 (relative expression; 1.39) showed high expression of CLUAP1. Expression of CLUAP1 was normalized by  $\beta$ -actin value. The results are presented as mean  $\pm$  SD of 3 measurements.

of the 15 cases of esophageal cancer or the 16 cases of bile duct cancer tested showed high expression of CLUAP1 (data not shown). In contrast, 7 out of 10 cases (70%) of ovarian cancer (OV1 through OV10), 9 out of 21 cases (43%) of colon cancer (CC1 through CC21), and 3 out of 42 cases (7.1%) of NSCLC (LC1 through LC42) expressed CLUAP1 >2-fold of the testis expression, as shown in Fig. 4.

## Discussion

A number of tumor-associated antigens of human malignancies have been identified (4-6), and many of these antigens have been currently applied to the clinical vaccine trials with successful induction of immune response or tumor regression (20). However, limited antigens of osteosarcoma have been identified to date. One clear reason for this delay is the technical difficulty to establish the pairs of osteosarcoma cell lines and their autologous T cell lines, associated with the relatively poor adaptability of osteosarcoma to *in vitro* culture (21,22). Another reason is the absence of appropriate candidate genes for a reverse immunotherapy such as a tumor-specific

altered gene (23,24). Among the limited reports of tumor-associated antigens of osteosarcoma, peptides derived from squamous cell carcinoma antigen recognized by T cells (SART) 3 and papillomavirus binding factor (PBF) were shown to be recognized by CTL derived from patients with osteosarcoma in MHC class I-restricted manner (9,10). Nabeta *et al* reported that self HLA-Cw\*0102 molecule and smooth muscle myosin light chain (SMML) was identified by SEREX analysis of an osteosarcoma cell line (22), although tumor-specific expression of these genes are unlikely or not established. Melanoma-associated antigen (*MAGE*) (7), *SART 1* (8), *surviving* (25,26) and *SSX* genes (27) were shown to be expressed in osteosarcoma, suggesting that these antigens may be potential targets of tumor immunotherapy of this malignancy.

Despite the increasing evidence supporting the importance of CD4<sup>+</sup> T cells in anti-tumor immune responses (6,11,12), tumor-associated antigens recognized by CD4<sup>+</sup> T cells in patients with osteosarcoma is unknown. Since CLUAP1 is identified by SEREX technique that screens Ig G response of patient sera to the clones from cDNA library, CD4<sup>+</sup> T cells



were likely to respond to CLUAPI *in vivo*. The frequency and the degree of the immune response to CLUAPI in osteosarcoma patients are unclear at present. In our preliminary screening of patient sera with plaque hybridization method, 1 out of 11 sera from osteosarcoma patients but none of the serum from 10 healthy volunteers reacted to CLUAPI. However, precise evaluation should be undertaken after establishing materials necessary for the ELISA assay of antibody response against CLUAPI in patients and healthy individuals.

CLUAPI, assigned to chromosomal band 16p13, was identified as a gene frequently transactivated in colon cancer by analyzing the expression profiles of colorectal cancers using a genome-wide cDNA microarray containing 23040 genes (28). Clusterin was identified as CLUAPI-interacting protein by the yeast two-hybrid system. Chen *et al* revealed that Clusterin was also upregulated in murine intestinal neoplasias and human colorectal tumors (29). Suppression of CLUAPI by short interfering RNAs (siRNAs) resulted in growth retardation in the transfected tumor cells. Moreover, expression of CLUAPI was induced in S phase of cell-cycle progression, suggesting that its elevated expression was relevant to cellular proliferation (28).

In this study, we found CLUAPI expression most abundantly in testis among the normal tissues tested. This result was consistent with the report from Takahashi *et al.* However, we found that other tissues such as brain or heart also express CLUAPI to some extent while they did not detect CLUAPI expression in heart or brain by Northern blot analysis (28). The reason for this discrepancy is unclear, although the difference in the methodology used in expression analyses might be responsible. Expression of CLUAPI in restricted normal tissues suggests that immunological therapy targeting CLUAPI should be evaluated with the effect in normal tissues. This may be assessed by the use of animal model since CLUAPI has its homologues in many animals including mouse with 89% homology in protein level (28). It is notable that at least one of the currently utilized targets of antibody therapy with successful effect, HER2/neu, is widely expressed in normal tissues but overexpressed in malignancies (30).

We found that many of the osteosarcoma cell lines and tissues express much higher CLUAPI mRNA compared to testis that expressed CLUAPI most abundantly among the normal tissues. This result strongly suggests that CLUAPI expression is frequently upregulated in osteosarcoma. In addition to the overexpression in colon cancer that is consistent with previous report (28), we found that CLUAPI was overexpressed in ovarian cancer and lung cancer. Our result may be related to the previous finding that Clusterin was overexpressed in human ovarian cancer (31), and may suggest that interaction of CLUAPI and Clusterin plays an important role in carcinogenesis.

Among the antigens identified in this study other than CLUAPI, nucleolar protein 8 (NOL8), heat shock 70-kDa protein 4 (HSPA4) (heat shock protein 70 family) and huntingtin interacting protein 1 (HIP1) were previously reported as tumor-associated proteins. Jinawath *et al* identified NOL8 that overexpressed in diffuse type stomach cancer by microarray (32). They indicated that suppression of NOL8

expression induced apoptosis by siRNAs specific to NOL8. Heat shock protein (HSP) 70 is a potent anti-apoptotic HSP, and its overexpression allows cells to survive in the variable conditions (33,34). There are several reports on HSP70 expression in malignant tumors, such as breast cancer (35), lung cancer (36), oral squamous cell carcinoma (37), prostate cancer and carcinoma of uterine cervix (33,34). The majority of the published results demonstrated that HSP70 overexpression correlated with poor prognosis and resistance to therapy (35,37-39). HIP1 was reported to be expressed in myelomonocytic leukemia, prostate cancer, and colon cancer (40,41). The regulation of HIP1 expression and the mechanism by which it is increased in tumors are unclear at present.

In conclusion, we searched for tumor-associated antigens of osteosarcoma using SEREX methodology, and identified at least one potential target, CLUAPI, that may be applicable to monitoring of immunological response to this tumor *in vivo* and/or immunotherapy of this malignancy. Since it is possible that CLUAPI provides antigens for CD4<sup>+</sup> T cell recognition, combination with other antigens for CD8<sup>+</sup> T cells may be effective in immunotherapy. High expression of CLUAPI was found not only in osteosarcomas but also in ovarian, colon, and lung cancers. These results suggest that CLUAPI may play an important role in carcinogenesis of multiple types of tumors and may be useful as a tumor-associated antigen in multiple malignancies.

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# Clonal T-Cell Response Against Autologous Pleomorphic Malignant Fibrous Histiocytoma Antigen Presented by Retrieved HLA-A\*0206

Shigeharu Kimura,<sup>1</sup> Yuriko Kozakai,<sup>1</sup> Satoshi Kawaguchi,<sup>1</sup> Tomohide Tsukahara,<sup>1</sup> Kazunori Ida,<sup>1</sup> Masaki Murase,<sup>1</sup> Tadaki Matsumura,<sup>1</sup> Mitunori Kaya,<sup>1</sup> Toshihiko Torigoe,<sup>2</sup> Takuro Wada,<sup>1</sup> Noriyuki Sato,<sup>2</sup> Toshihiko Yamashita<sup>1</sup>

<sup>1</sup>Department of Orthopaedic Surgery, Sapporo Medical University School of Medicine, Sapporo, 060-8543, Japan

<sup>2</sup>Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, 060-8556, Japan

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**ABSTRACT:** Towards the goal of identifying tumor-rejection antigens on eradicated tumors in bone and soft tissue sarcomas, we evaluated the immune response against antigens presented by lost HLA class I molecules. Tumor specimens and peripheral blood samples were obtained from a 70-year-old woman with pleomorphic malignant fibrous histiocytoma. Over 1-year culture, a tumor cell line (MFH2004) was established. A B-cell line infected with Epstein–Barr virus (B2004-EBV) was developed from the blood samples. HLA genotypes of B2004-EBV cells were A\*0206/2402, B\*4006/4601, and C\*0102/0801, whereas MFH2004 cells were defective for A\*0206, B\*4006, and C\*0102. Loss of HLA-A2 expression was also proved immunohistochemically in the primary tumor tissues. Lost HLA-A2 in MFH2004 cells was retrieved by transfection of HLA-A\*0206 cDNA to develop MFH2004-A2. Attempts to induce CTLs by mixed culture with autologous T cells and MFH2004 cells resulted in failure. In contrast, those with MFH2004-A2 induced CTL clones CTL2004-c6 and CTL2004-c17. These CTL clones specifically killed MFH2004-A2 but not MFH2004 or B2004-EBV in an HLA-A2-restricted manner. These findings suggest that CTL2004-c6 and CTL2004-c17 recognize autologous tumor-rejection antigens presented by HLA-A\*0206, which may have been expressed by tumor cells that had been eradicated by the host's immunosurveillance system. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 26:271–278, 2008

**Keywords:** human leukocyte antigen (HLA) class I; malignant fibrous histiocytoma; tumor-associated antigen; immunotherapy; cytotoxic T lymphocyte

## INTRODUCTION

After a nearly 30-year interval from the initial immunotherapeutic trials for patients with osteosarcoma,<sup>1,2</sup> clinical trails of immunotherapy for bone and soft tissue sarcomas restarted recently with advanced cellular and molecular technologies.<sup>3–7</sup> Despite the use of antigen-specific peptides<sup>4,5,7</sup> or antigen-stimulated dendritic cells<sup>3</sup> as vaccines, only a few tumors have shown responses.<sup>3,4</sup> Such dismal outcomes have also been seen in vaccination trials for other types of solid tumors,<sup>8–10</sup> together suggesting the importance in identifying further efficacious therapeutic antigens.

A dilemma in current antigen identification approaches is that most antigens have been defined from established tumors. These tumors have

developed as a result of escape from immune surveillance of the host. Thus, antigens expressed by these tumor cells do not represent immunodominant antigens. In contrast, the tumor cells that had already been eradicated by the host immune surveillance theoretically had expressed tumor-rejection antigens. Identification of such antigens on the eradicated tumor cells may provide a clue towards efficacious immunotherapy. In this regard, a trace of eradicated tumor cells can be found in the established tumors as a loss of antigen-presenting human leukocyte antigen (HLA) class I molecules.<sup>11,12</sup> According to the concept of immunologic sculpting,<sup>13</sup> the tumor cells expressing normal HLA class I have been eradicated by the host T cells during development. Therefore, lost HLA class I molecules in the established tumor may have presented tumor-rejection antigens.<sup>14</sup> While the concept of immunologic sculpting has been well proved in animal models,<sup>13</sup> there is little evidence with clinical samples.<sup>15</sup>

Towards the goal of identifying antigens on eradicated tumors in bone and soft tissue sarcomas,

Correspondence to: Satoshi Kawaguchi (Telephone: 81-11-611-2111, ext. 3333; Fax: 81-11-641-6026; E-mail: kawaguch@sapmed.ac.jp)

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the primal question is whether such immunologic sculpting exists in patients with these tumors. To answer this question, we developed a cell line from pleomorphic malignant fibrous histiocytoma (MFH), which showed haplotype loss of HLA class I alleles. We hypothesize that the haplotype loss may have resulted from eradication of the tumor cells that express the HLA class I allele presenting the tumor-rejection antigen. This hypothesis can be proved by demonstration of (i) failure of killing activity of autologous T cells against the tumor cells with haplotype loss and (ii) successful induction of cytotoxic T lymphocyte (CTL) clones that react with the tumor cells where the lost HLA class I allele putatively presenting the tumor-rejection antigen is retrieved.

## MATERIALS AND METHODS

This study was approved under institutional guidelines for the use of human subjects in research. The patient gave informed consent for the use of tissue specimens and blood samples in our research.

### Patient

The patient was a 70-year-old woman suffering a tumor in the left elbow. Magnetic resonance imaging (MRI) revealed a multinodular mass with isointensity in T1 weighted images and mixed intensity in T2 weighted images. Metastatic lesions were not detected by lung and abdominal computed tomography (CT) scans or gallium scintigraphy. Biopsy specimens of the tumor showed a mixture of spindle and histiocytelike cells, with atypia and mitoses of nuclei (Fig. 1). Bundles of spindle cells were arranged in a storiform pattern. Immunohistochemistry revealed that the tumor cells were diffusely positive for vimentin and CD68 and focally positive for smooth muscle actin, whereas desmin, cytokeratin, S-100, HHF35, caldesmon, EMA, and CD34 were all negative. Upon these findings, the tumor was diagnosed as pleomorphic MFH. The tumor was excised along with brachioradialis and extensor carpi radialis muscles, and a part of the lateral epicondyle of the left humerus on January 17, 2000, to achieve a wide surgical margin. The skin defect was covered by a mesh skin graft. Postoperatively, neither local recurrence or metastasis have been observed for 7 years.

### Establishment of Cell Lines

Fresh specimens taken at surgery from the tumor were minced into small pieces and separately cultured with Iscove's modified Dulbecco Eagle's medium (IMDM; GIBCO BRL, Grand Island, NY), containing 10% fetal calf serum, in a 5% CO<sub>2</sub> incubator. Over 1 year of continuous passages, a cell line was established and designated MFH2004. Epstein-Barr virus-transformed

B cells were also established from a blood sample of the same patient and designated B2004-EBV according to a method described previously.<sup>16,17</sup>

### Xenotransplantation Model

MFH2004 cells ( $2 \times 10^7$ ) were injected subcutaneously into the backs of three 6-week-old NOD-SCID mice (NOD.CB17.prdkc<sup>scid</sup>/J, The Jackson Laboratory, Bar Harbor, ME). On day 30, the tumor was resected and fixed in formalin. Paraffin-embedded sections were made and stained with hematoxylin and eosin (HE).

### Immunostaining

For immunostaining, anti-vimentin (Nichirei Corp., Tokyo, Japan), anti-CD68 (Dako, Glostrup, Denmark), anti-EMA (Nichirei Corp.), anti-S-100 (Dako), anti-CD34 antibody (Nichirei Corp.), anti-desmin (Dako), anti-cytokeratin (BioGenex, San Ramon, CA), anti-HHF35 (BioGenex), anti-caldesmon (BioGenex), anti-pan HLA class I (EMR8-5<sup>12</sup>), and anti-HLA-A2 antibodies 0397HA and 0791HA (One Lambda, Canoga Park, CA) were used.

Deparaffinized formalin-fixed paraffin-embedded sections of the original tumor and xenotransplanted tumor tissues were boiled by microwave for antigen retrieval. The sections were blocked and stained by the standard ABC method, as previously described.<sup>12</sup> For staining of HLA-A2, both 0397HA and 0791HA were used in combination as the primary antibodies according to the method described by Zhu and colleagues.<sup>18</sup> Hematoxylin was used for counterstaining.

### Analysis of HLA Genotype

HLA genotypes of MFH2004 and the blood sample were tested by Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan).

### Cloning of HLA-\*A0206 cDNA and Transfection

Total RNA was extracted from B2004-EBV cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA). PCR was performed with KOD plus DNA polymerase (Toyobo, Tokyo, Japan), using forward primer 5'-GGGCGATATCGGACTCAGAATCTCCCCA-GACGCCGAG-3' and reverse primer 5'-ATGCGGCCGC-TCACACTTTACAAGCTGTGA-3'. The forward primer contained the EcoRV site (underlined) and the reverse primer contained the NotI site (underlined) for digestion. The mixture was denatured at 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, 53°C for 30 s, and 68°C for 2 min. The PCR products were ligated into EcoRV and NotI sites of the pIRESpuo expression vector (BD Biosciences Clontech, Palo Alto, CA). Cloned cDNAs were sequenced with an ABI DNA sequencer (PerkinElmer Japan, Yokohama, Japan) and proved to be identical to the sequence of HLA-\*A0206 cDNA (gene bank number M24042).