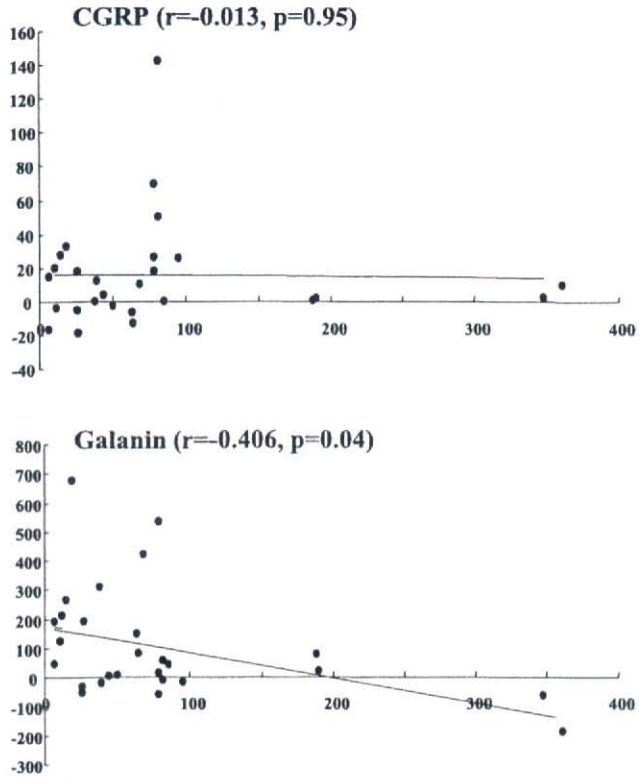

Figure 4. Correlation between duration of symptoms and postoperative changes of plasma CGRP and galanin. x-axis indicates the duration of symptoms from onset to lumbar discectomy (days). y-axis indicates the extent of postoperative changes of neuropeptides (pg/mL) calculated as preoperative concentration of the peptide − postoperative concentration. Negative values indicate that the level of plasma neuropeptide increased after surgery. The correlation between the duration of symptoms and postoperative changes of plasma neuropeptide levels was analyzed by the Pearson correlation method. Values in parentheses in each neuropeptide indicate a correlation coefficient (r) and a probability (*P*). There was a significant negative correlation between the extent of postoperative changes of plasma galanin levels and the duration of symptoms.

samples taken at an interval of 3 weeks from conservatively treated patients could provide insights into the magnitude of the changes in plasma neuropeptides.

Presently, little is known of the origin of neuropeptides in the peripheral blood. However, it is well established that CGRP and galanin are synthesized by DRG neurons, transported centrally and peripherally, and released at both terminals.[6,22,23] Centrally transported peptides may in part be released into the CSF. In this regard, Lindh *et al*[17] reported lower CSF levels of CGRP in patients with LDH than those in healthy individuals. The authors of the current study also found no significant correlation between plasma levels and CSF levels of CGRP, galanin, and NPY in 12 patients with LDH (Takeuchi *et al*, unpublished observation). These findings indicate that the plasma levels of neuropeptides in patients with LDH do not reflect centrally released neuropeptides.

Since a herniated disc sensitizes both nerve endings and nerve roots, peripherally transported neuropeptides are most likely released from both the nerve endings surrounding the herniated disc and the sensory nerve endings of the affected roots in the lower extremities. Indeed, CGRP and SP were detected immunohistochemically in the anulus fibrosus of rat intervertebral disc specimens[20] and human herniated lumbar disc specimens.[24] Notably, in the study of 12 herniated lumbar disc specimens by Ashton *et al*,[24] CGRP immunoreactivity was found not only in fine nerve fibers but also in the vicinity of blood vessels and nonperivascular locations. In contrast, SP immunoreactivity was faintly detected and confined to a few nerve fibers. These findings are consistent with the higher plasma levels of CGRP than SP in the current study.

It is generally known that neuropeptides released from the peripheral terminals exert their biochemical effects over various types of cells in its microenvironment.[6] Such biochemical events are termed as neurogenic inflammation. In LDH, they can take place at peripheral nerve terminals in the anulus fibrosus and the posterior longitudinal ligament injured by a herniated disc. While CGRP serves as a potent vasodilator and modulator of immune system activity,[6,25] galanin has trophic effects on sensory neurons.[26] It is likely that galanin released around the herniated nucleus pulposus may activate the regeneration of injured peripheral nerves in the anulus fibrosus and ligament tissues. Also, the CGRP stimulation of chemotaxis of immune cells leads to an infiltration of these cells into the herniated disc. As our group[27,28] and others[29,30] have demonstrated, such inflammatory infiltration plays a pivotal role in spontaneous regression of the herniated disc. Collectively, neurogenic inflammation associated with LDH occurs primarily to provide optimal microenvironments for healing and regeneration. However, this inflammatory response simultaneously facilitates sensitization of the afferent neurons in the affected nerve root.

There are several limitations to the present study. First, the present study includes a small number of patients. Therefore, the low participation of candidates with large changes of plasma neuropeptides after lumbar discectomy can determine the overall trend of our findings. The results might have been different if 2 patients who continued with pain after surgery were included. Second, only 4 representative neuropeptides were examined. Third, only one single time point was chosen to evaluate postoperative neuropeptide levels. And finally, it would also have been more practical to comparatively analyze plasma levels of neuropeptides and their local expressions in the herniated disc materials. Nevertheless, difficulties in the evaluation of pain states in animal models for LDH and the presence of species-dependent variations of neuropeptide expressions in injured nerves[23] highlight the importance in analysis of clinical materials in pain research.

■ Conclusion

This is the first study that shows the role of plasma CGRP and galanin as the biochemical signature of pain states in patients with LDH. Plasma levels of CGRP and

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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ORIGINAL PAPER

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

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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.

Keywords Microsatellite · Bone marrow transplantation · Acute GVHD · Chromosome 22 · Non-HLA

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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 familially 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* methotorexate, *FK* tacrolimus hydrate, *PDR* predonisolone

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 μl of deoxyribonucleotide triphosphate (5 mM each), 1 μl of 10× buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 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 ul of the diluted PCR product, 10 μl Hi–Di formamide (Applied Biosystems Japan Co.) and 0.1 μl 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 2×2 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 Pc value). The Pc value less than 0.05 was accepted as statistically significant, and the Pc 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 transplantation 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 Pc<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 (OR<1; Table 2). In the donors, the allele D22S0141i-431 was increased significantly (Pc=0.049) and the allele D22S283-132 was decreased significantly (Pc=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 (Pc= 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
Donor									
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
Recipient									
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	
348/357	19	15	
357/357	17	3	
			0.001

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 (Pc<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

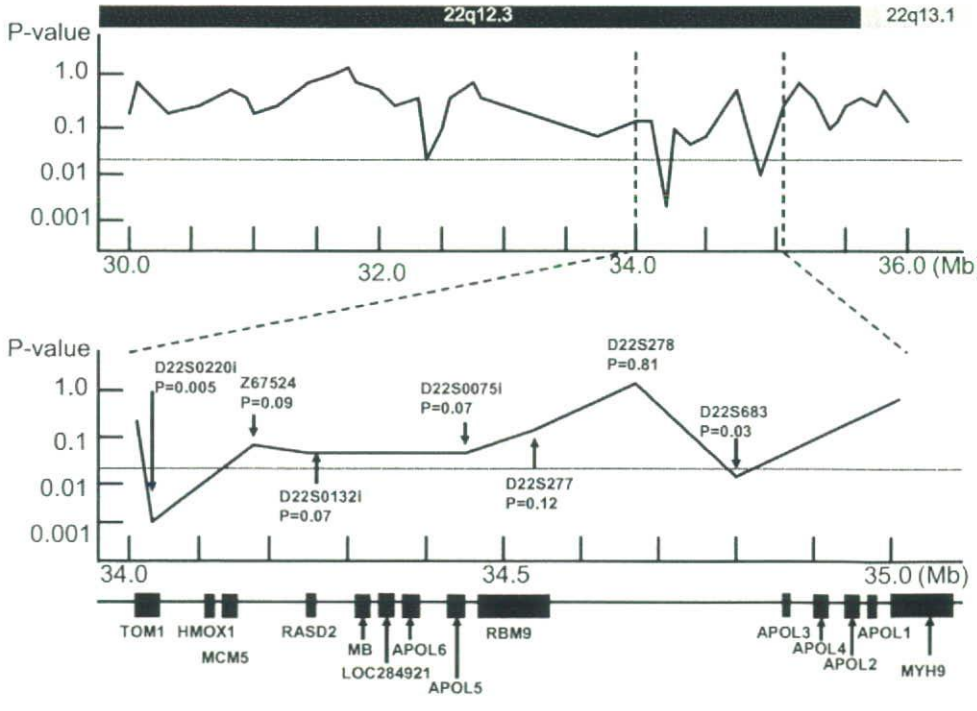

Fig. 1 aGVHD susceptibility gene mapping by association analysis using microsatellite markers on 22q12.3. *P* value (*y-axis*) was plotted against physical location of the microsatellite markers on 22q12.3 (*x-axis*), their distance (in Mb) in order from the centromere to the telomere. All markers were 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
Donor allele					
D22S283	22q12.3	172 kb	MYH9	Myosin, heavy chain 9, nonmuscle	Cytoskelton
D22S0141i	22q12.3	Intron 3	CACNG2	Calcium cannel, voltage dependent, gamma-2 subunit	Cell signaling
Recipient allele					
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
Mismatching allele					
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	Apolipoprotien 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 D2250141i 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 ≥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

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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 $CD8^+$ 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 $CD4^+$ T cells play an important role in anti-tumor immune responses (6,11,12). The information of tumor-associated antigens of osteosarcoma recognized by $CD4^+$ 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 $CD4^+$ 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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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 $1x10^7$ 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 $1x10^6$ pfu/ml.

Serological screening of expression library with patient sera. A total of $1x10^6$ 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-ß-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 λTripleEx 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 ß-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'-GGCAGATTAAAACCCTCAGACTT-3', reverse primer 5'-GTATTGTGAACATGCAGACGAAA-3'; HU-OS-1, forward primer 5'-TCCAGTGAATTTGAAGTTAG GAGAC-3', reverse primer 5'-CATCAAAATATTCCCTTT CTTCCTT-3'; HU-OS-5, forward primer 5'-CTAATGCTA ACTGGGAAAGCACTAA-3', reverse primer 5'-CCTCCC CATAACTCTCTTCATATTT-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'; ß-actin, forward primer 5'-TTAAGGAGAAGCTG TGCTACGTC-3', reverse primer 5'-ATCTTGTTTTCT GCGCAAGTTAG-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. ß-actin was used to normalize the target gene expression. Quantitative RT-PCR was performed using the PRISM 7000 (Applied Biosystems) with QuantiTec 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 ß-actin expression. Expression of CLUAP1 was normalized by ß-actin value.

Results

Serological identification by SEREX and sequence analysis of osteosarcoma-associated antigens. Recombinant clones ($1.0x10^6$) 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 definded 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	Microtuble-associated protein 1B (MAP1B)	Hs.584777	Development and/or repairing of neurons
HU-OS-4	2/43	Eukaryotic initiation factor 2B, subunit 5 ε, 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	α 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

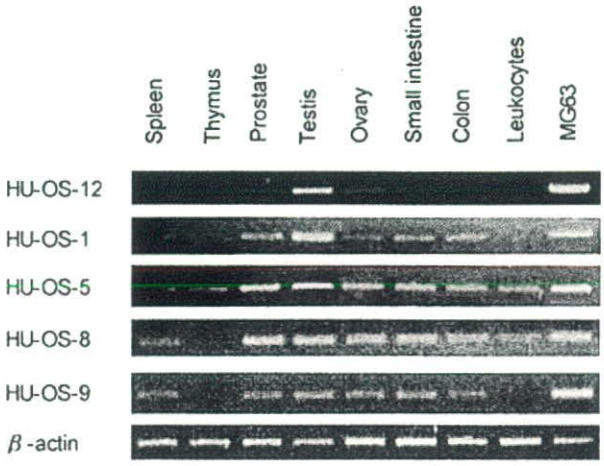

Figure 1. mRNA expression of identified antigens in normal tissues. The expression of HU-OS-12 (CLUAP1) and other representative antigens (HU-OS-1, HU-OS-5, HU-OS-8, and HU-OS-9) in 8 adult normal tissues and MG63 cells were analyzed by conventional reverse transcription-PCR. ß-actin housekeeping gene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

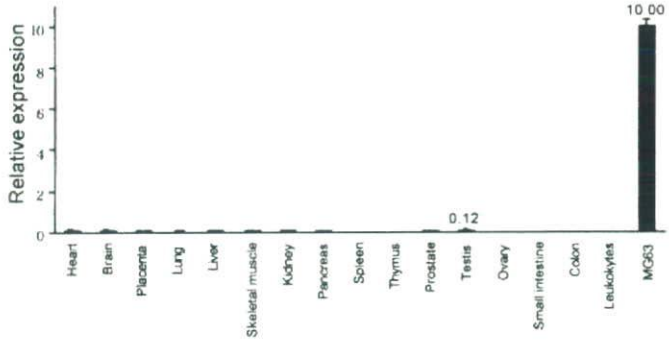

Figure 2. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in normal tissues. The expression of CLUAP1 in 16 adult normal tissues and MG63 cells were analyzed by quantitative RT-PCR. Expression of CLUAP1 was normalized by ß-actin value. The results are presented as mean ± SD of 3 measurements.

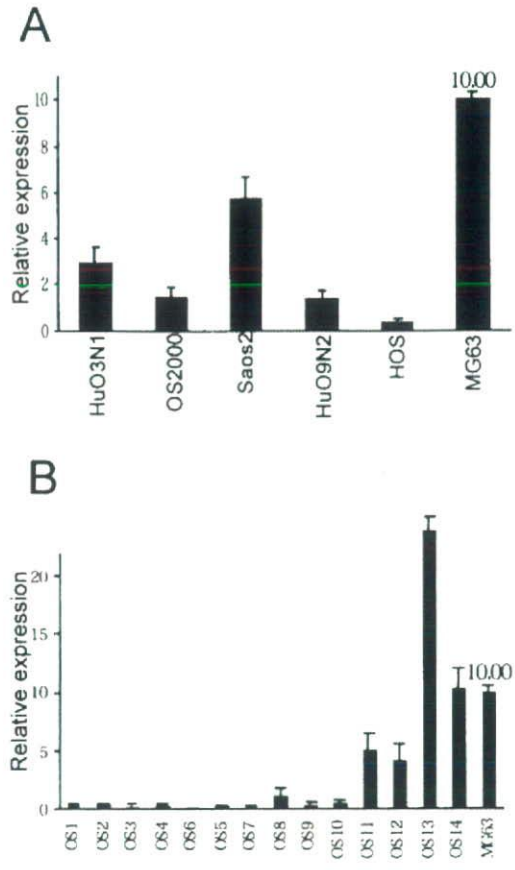

Figure 3. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in osteosarcoma cell lines and tissues. (A) Relative expressions of CLUAP1 in 6 osteosarcoma cell lines were 2.98 (HuO3N1), 1.42 (OS2000), 5.69 (Saos2), 1.39 (HuO9N2), 0.35 (HOS), and 10.00 (MG63). (B) Relative expressions of CLUAP1 in 14 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 ß-actin value. The results are presented as mean ± 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 encording *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 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

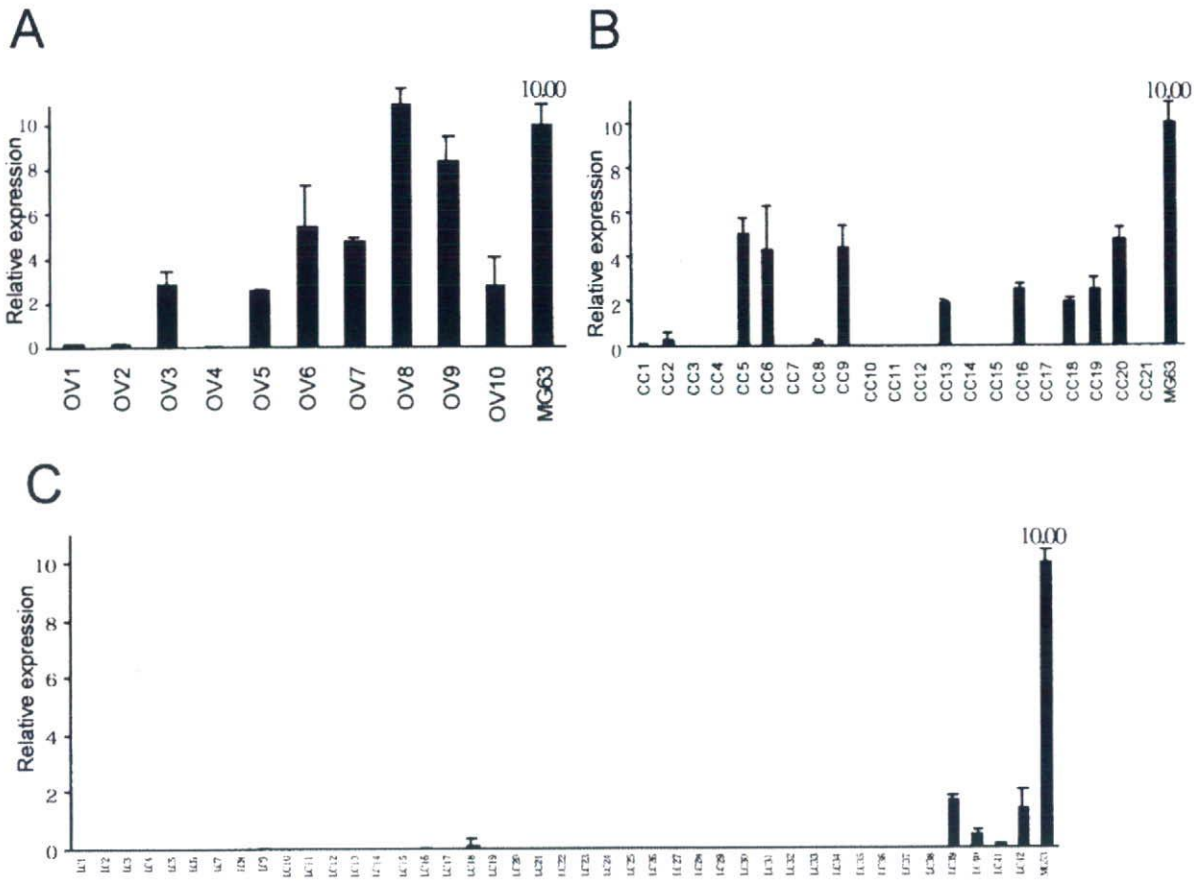

Figure 4. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in ovarian, colon, and lung cancers. (A) Relative expressions of CLUAP1 in 10 ovarian cancer tissues with MG63 as a positive control were measured by quantitative RT-PCR. OV3 (relative expression; 2.88), OV5 (relative expression; 2.53), OV6 (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 ß-actin value. The results are presented as mean ± 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 $CD4^+$ T cells in anti-tumor immune responses (6,11,12), tumor-associated antigens recognized by $CD4^+$ 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, $CD4^+$ T cells

were likely to respond to CLUAP1 *in vivo*. The frequency and the degree of the immune response to CLUAP1 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 CLUAP1. However, precise evaluation should be undertaken after establishing materials necessary for the ELISA assay of antibody response against CLUAP1 in patients and healthy individuals.

CLUAP1, 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 CLUAP1-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 CLUAP1 by short interfering RNAs (siRNAs) resulted in growth retardation in the transfected tumor cells. Moreover, expression of CLUAP1 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 CLUAP1 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 CLUAP1 to some extent while they did not detect CLUAP1 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 CLUAP1 in restricted normal tissues suggests that immunological therapy targeting CLUAP1 should be evaluated with the effect in normal tissues. This may be assessed by the use of animal model since CLUAP1 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 CLUAP1 mRNA compared to testis that expressed CLUAP1 most abundantly among the normal tissues. This result strongly suggests that CLUAP1 expression is frequently upregulated in osteosarcoma. In addition to the overexpression in colon cancer that is consistent with previous report (28), we found that CLUAP1 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 CLUAP1 and Clusterin plays an important role in carcinogenesis.

Among the antigens identified in this study other than CLUAP1, 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, CLUAP1, 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 CLUAP1 provides antigens for $CD4^+$ T cell recognition, combination with other antigens for $CD8^+$ T cells may be effective in immunotherapy. High expression of CLUAP1 was found not only in osteosarcomas but also in ovarian, colon, and lung cancers. These results suggest that CLUAP1 may play an important role in carcinogenesis of multiple types of tumors and may be useful as a tumor-associated antigen in multiple malignancies.

Acknowledgements

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p63 Induces CD4$^+$ T-Cell Chemoattractant TARC/CCL17 in Human Epithelial Cells

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To preserve immunosurveillance, epithelial cells support T-cell trafficking toward inflammatory foci. However, how epithelial cells are enrolled in recruiting T cells has not been fully elucidated. In this study we investigated the function of p63, a p53 family member, in the regulation of the expression of various types of chemokine ligands by focusing on the property of p63 as an epitheliotropic transcription factor. As assessed by experiments using three different human epithelial cell lines with small-interfering RNAs or plasmids of p63, certain CC chemokine ligands were found to be under the control of p63. In these CC chemokine ligands, p63 had the common capacity to upregulate TARC/CCL17 in the different cell lines, whose receptor CCR4 was preferentially presented on CD4$^+$ T cells such as memory, regulatory, IL-17-producing and type II helper T cells. More interestingly, when cells were stimulated with transforming growth factor-β (TGF-β) or epidermal growth factor (EGF) as observed during tissue repair process, the expression of p63 and TARC/CCL17 was concomitantly suppressed. This implies that, in local inflammatory regions with general epithelial tissue remodeling, the p63-TARC/CCL17 axis may participate in the engagement of efficient immune reactions by specified T-cell subsets.

Introduction

It is well recognized that epithelial cells play an important role in the immune system as the front line of defense against external pathogens (Hayday and others 2001). Anatomically, epithelial cells of tissues such as lung, skin, and intestines tightly connect to each other to form a sheet structure with various junctional molecules (Tsukita and Furuse 2000). These epithelial networks eventually help defend against pathogens and also evoke cellular and molecular interactions of immune responses by recruiting a multitude of inflammatory cells like lymphocytes and dendritic cells (von Andrian and Mackay 2000). In addition to the mechanisms of innate immunity, acquired or adaptive immunity is essential for specific responses to various immunologic insults around epithelial tissues. The mechanism of homing of inflammatory cells to epithelial cells has been extensively investigated and soluble factors, including chemokine ligands, are known to have a pivotal function, but the secretion mechanism by which epithelial cells foster regional lymphocytes is still unclear.

Chemokine ligands are secreted polypeptides that trigger an elaborate process whereby inflammatory cells presenting chemokine receptors of seven-transmembrane G-protein-coupled receptors can promptly channel them to the foci requiring immune and inflammatory reactions (Kunkel and Butcher 2002; Milligan and Smith 2007). Based on their structural characteristics with regard to the positions of a cysteine residue, chemokine ligands are classified into two major subfamilies: CC and CXC chemokine ligands. To date many different CC and CXC chemokine ligands have been discovered and some of them derived from epithelial tissues are known to play cardinal roles in the emergence of allergic or atopic inflammatory disorders and cancer development (Juremalm and others 2005; Ruffini and others 2007).

Accumulating evidence has indicated that p63, a p53-like molecule, is a usual epithelial constituent and determines properties of epithelial stem cells (Truong and others 2006). Indeed, loss of p63 leads to severe epithelial tissue hypoplasia due to the inhibition of both stratification and differentiation of keratinocytes (Koster and others 2007). Various genes, encoding JAG1, PERP, p21, or 14–3-3σ, are transactivated by p63, but it is not well investigated whether p63 is involved in the expression of chemokine ligands in epithelial cells (Sasaki and others 2002; Westfall and others 2003; Ihri and others 2005).

In this study we assessed the role of p63 in the regulation of the transcription of a series of chemokine ligands on three different types of human epithelial cells with

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