

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 1×10^7 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 1×10^6 pfu/ml.

Serological screening of expression library with patient sera. A total of 1×10^6 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-4-chloro-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'-GGCAGATTAAACCCCTCAGACTT-3', reverse primer 5'-GTATTGTGAACATGCAGACGAAA-3'; HU-OS-1, forward primer 5'-TCCAGTGAATTTGAAGTTAGGAGAC-3', reverse primer 5'-CATCAAAATATTCCTTTCTTCCTT-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'; β-actin, forward primer 5'-TTAAGGAGAAGCTGTGCTACGTC-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. β-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 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 (1.0×10^6) 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 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

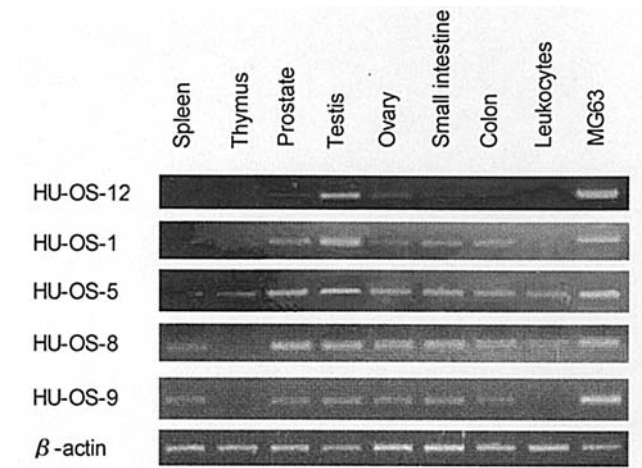


Figure 1. mRNA expression of identified antigens in normal tissues. The expression of HU-OS-12 (CLUAP1) and other representative antigens (HU-OS-1, HU-OS-5, HU-OS-8, and HU-OS-9) in 8 adult normal tissues and MG63 cells were analyzed by conventional reverse transcription-PCR. β-actin housekeeping gene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

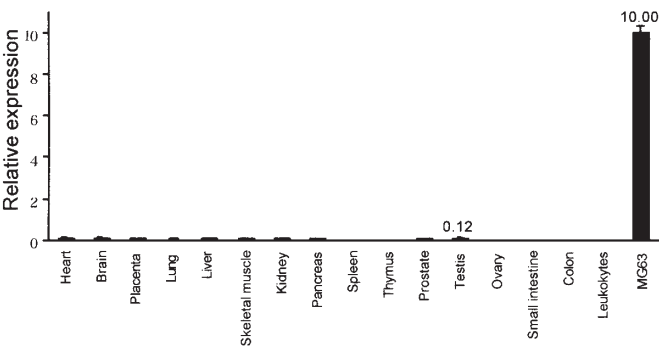


Figure 2. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in normal tissues. The expression of CLUAP1 in 16 adult normal tissues and MG63 cells were analyzed by quantitative RT-PCR. Expression of CLUAP1 was normalized by β-actin value. The results are presented as mean ± SD of 3 measurements.

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 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

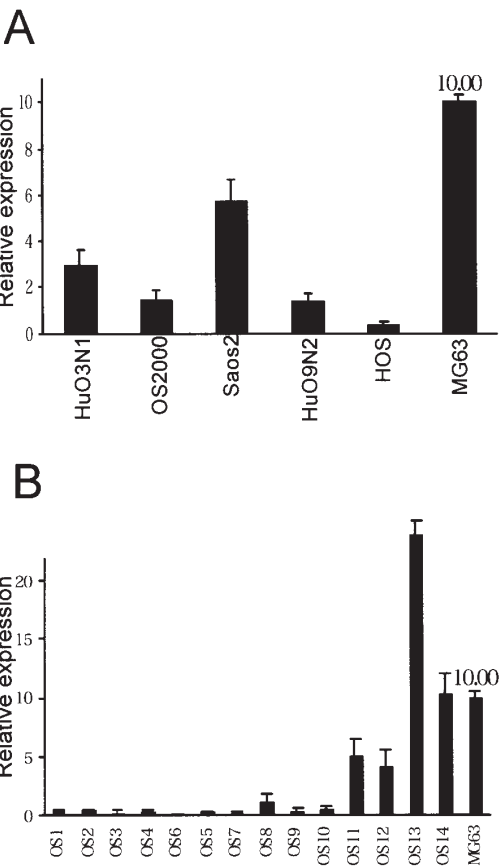


Figure 3. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in osteosarcoma cell lines and tissues. (A) Relative expressions of CLUAP1 in 6 osteosarcoma cell lines were 2.98 (HuO3N1), 1.42 (OS2000), 5.69 (Saos2), 1.39 (HuO9N2), 0.35 (HOS), and 10.00 (MG63). (B) Relative expressions of CLUAP1 in 14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

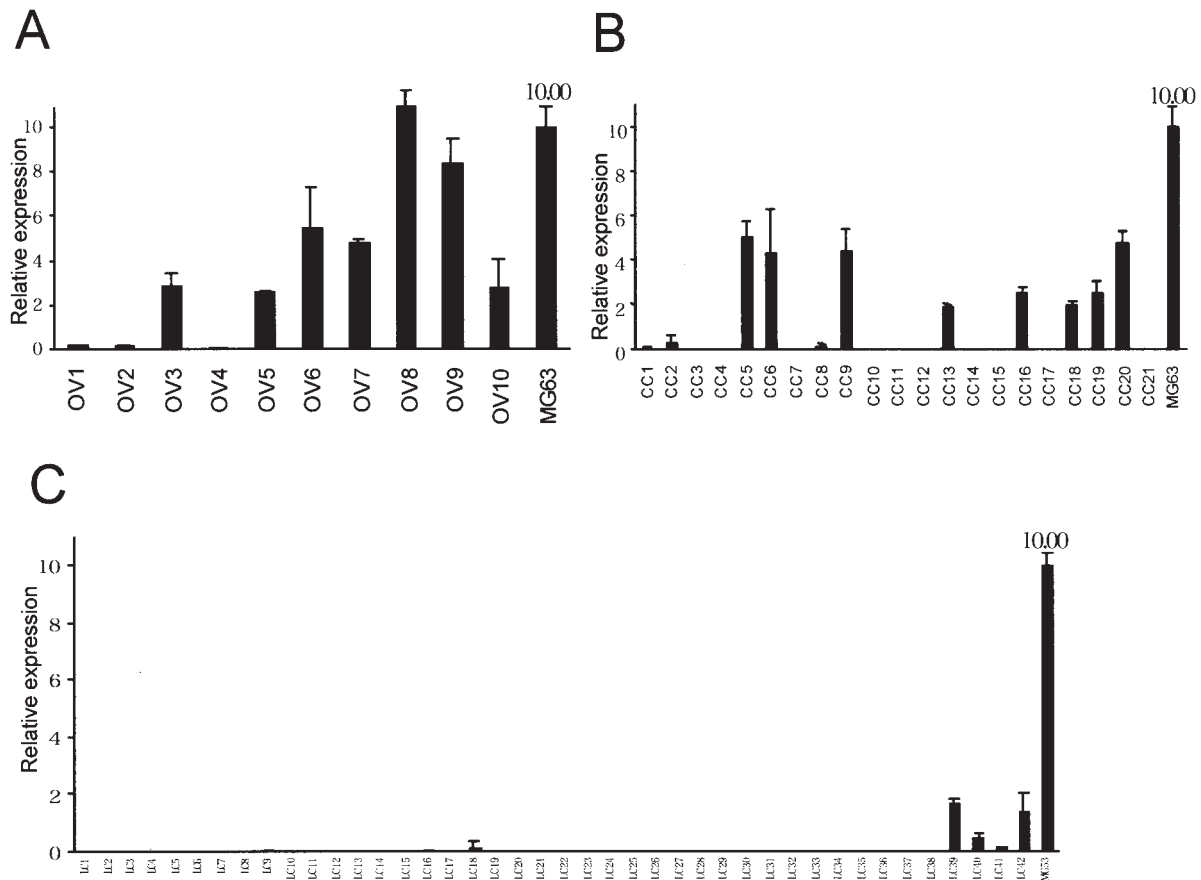


Figure 4. Quantitative RT-PCR analysis of mRNA encoding CLUAP1 in ovarian, colon, and lung cancers. (A) Relative expressions of CLUAP1 in 10 ovarian cancer tissues with MG63 as a positive control were measured by quantitative RT-PCR. OV3 (relative expression; 2.88), OV5 (relative expression; 2.53), OV6 (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 β -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⁺ 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.

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