possible explanation is the difference in the antibodies used. Berghuis et al. [8] have used two mAbs, HC10, and HCA2. HC10 was shown to react with HLA-B and C allele proteins, but barely with HLA-A [25]. In contrast, HCA2 preferentially reacts with HLA-A allele proteins except for HLA-A24 [26]. EMR8-5mAb used in the present study reacts all alleles of HLA-A, B, and C, including HLA-A24 [12,25]. Because HLA-A24 is shared by nearly half of Japanese and 17% of Caucasians [27], one analysis including HLA-A24 and the other analysis excluding HLA-A24 could show different results. In addition, differences in distribution of HLA types and fusion gene subtypes between the study groups could also affect the prognostic impact of HLA class I expression. In the present study, well-known prognostic factors of ESFTs such as tumor size, stage, and surgical margin have also shown significant prognostic impact, supporting the validity of our patient population.

Down-regulation of HLA class I molecule in tumor cells serves as a major mechanism of escaping from both natural immune surveillance and T cell-based immunotherapy [25,28]. Nevertheless none of the immunotherapy trials including those in patients with ESFT [29,30] have yet examined the status of HLA class I in the corresponding tumors. In the study by Mackall et al. [29] with translocation breakpoint peptide vaccines in 20 patients with ESFT, immune responses to the peptide vaccines were noted in 39% of the participants. The efficacy of those therapeutic vaccines can be further improved by restoration of HLA class I expression on tumor cells. In this regard, Berghuis et al.[8] reported that stimulations with interferon gamma have increased HLA class I expression in all six Ewing's sarcoma cell lines examined. Restoration of HLA class I expression can also be achieved with histone deacetylation inhibitors such as tricostatin-A and valporoic acid, provided that loss of HLA class I is due to reduced DNA methylation [25].

In conclusion, we revealed for the first time that the status of HLA class I expression affects the survival of patients with ESFT. This emphasizes the importance of HLA class I molecules in natural and therapeutic immune surveillance in patients with ESFT.

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Autologous CTL response against cancer stem-like cells/cancer-initiating cells of bone malignant fibrous histiocytoma

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Malignant fibrous histiocytoma (MFH) of the bone is an aggressive tumor with high rates of local recurrence and metastasis. The development of novel therapeutic approaches is critical to improve the prognosis of patients with MFH. We reported previously that the side population (SP) cells of the MFH2003 bone MFH cell line have the characteristics of cancer stem-like cells (CSC)/cancer-initiating cells. In the present study, to establish immunotherapy targeting CSC, we analyzed cell surface immune molecules on SP cells of the MHF2003 cell line, as well as autologous CTL responses against these SP cells in the tumor microenvironment and peripheral circulating lymphocytes, using autologous tumor-infiltrating lymphocytes and autologous CTL clones derived from peripheral blood, respectively. We found that the SP cells expressed human leukocyte antigen (HLA) Class I molecules on the cell surface. The autologous tumor-infiltrating lymphocyte line TIL2003 recognized both the SP and main population cells of the MFH2003 cell line. Next, we induced the CTL clone Tc4C-6 by mixed lymphocyte tumor cell culture using autologous peripheral blood mononuclear cells and freshly isolated SP cells, followed by a limiting dilution procedure. The Tc4C-6 clone showed specific cytotoxicity against the SP cells. Moreover, the cytotoxicity against SP cells was blocked by the anti-HLA Class I antibody W6/32. In conclusion, the findings of the present study support the idea that CSC of bone MFH are recognized by autologous CTL in the tumor microenvironment and peripheral circulating lymphocytes. Thus, CTL-based immunotherapy could target CSC of bone sarcoma to help prevent tumor recurrence. (*Cancer Sci* 2011; 102: 1443–1447)

Malignant fibrous histiocytoma (MFH) of the bone is a rare primary neoplasm, accounting for <5% of primary bone malignancies.[1,2] Histologically, MFH of the bone is composed of fibroblasts and pleomorphic cells with a prominent storiform pattern. It is an aggressive tumor, with high rates of local recurrence and metastasis and a poor prognosis; the 5-year survival has been reported to be <60%.[3,4] Therefore, the development of novel therapeutic approaches is critical to improve the outcomes of patients with MFH.

It was thought that all neoplastic cells within a tumor were capable of tumorigenic growth. However, recent studies have demonstrated that malignant tumors can be generated by a distinct subpopulation of tumor cells, the so-called cancer stem cells (CSC)/cancer-initiating cells (CIC), which have self-renewal ability, differentiation potential, and tumorigenic capacity.[5,6] Thus, CSC could be a therapeutic target for the complete eradication of tumor cells. However, CSC have been reported to be resistant to standard therapeutic modalities, including radiation and drugs.[7,8]

Recently, many clinical trials of CTL-based immunotherapy using peptide vaccination have demonstrated the potency of this new therapeutic modality for various cancers that are resistant to standard chemotherapy.[9] However, it remains unknown whether CTL-based immunotherapy can kill CSC. Previously, we demonstrated that the side population (SP) cells from the bone MFH cell line MFH2003 have CSC characteristics.[10] The SP cells of the MFH2003 cell line exhibited cancer-initiating activity, with *in vitro* sphere formation and *in vivo* tumorigenesis in NOD/SCID mice. In the present study, to characterize the immunogenicity of CSC, we analyzed autologous CTL responses against SP cells of the MFH2003 cell line in the tumor microenvironment, as well as in peripheral circulating blood, using autologous tumor-infiltrating lymphocytes and a CTL clone.

Materials and Methods

The present study was approved under the institutional guidelines for the use of human subjects in research. The patients and their families, as well as healthy donors, provided informed consent for the use of blood samples and tissue specimens in our research.

Cell lines and culture. The cell lines used in the present study were a bone human MFH cell line (MFH2003), an erythroleukemia cell line (K562), and Epstein-Barr virus-transformed B cell lines (LG2-EBV, B2003-EBV). The OS2000, KIKU, MFH2003, and B2003-EBV cell lines were established in our laboratory.[11] The K562 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). ThILG2-EBV cell line was donated by Dr. PG Coulie (Christian de Duve Institute of Cellular Pathology, University of Louvain, Brussels, Belgium). The MFH2003 cells were cultured in Iscove's modified Dulbecco's Eagle's medium (IMDM; Gibco BRL, Grand Island, NY, USA) containing 10% FBS. The LG-2-EBV, B2003-EBV, and K562 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS. All other cell lines were maintained in DMEM (Sigma-Aldrich) containing 10% FBS in a 5% CO_2 incubator at 37°C.

Purification of side population cells. The CSC of the MFH2003 cell line were purified by side population analysis, as described previously.[10] Briefly, cell suspensions were labeled with Hoechst 33342 dye (Cambrex Bio Science, Walkersville, MD, USA) at a final concentration of 5.0 μg/mL in the presence or absence of verapamil (75 μM; Sigma-Aldrich) as an inhibitor of the ATP-binding cassette (ABC) transporter. Cells were incubated at 37°C for 90 min with continuous shaking. At the end of the incubation period, cells were washed with ice-cold PBS with 5% FBS, centrifuged at 440*g* for 5 min at 4°C, and resuspended in ice-cold PBS containing 5% FBS. Propidium iodide (final concentration 1 μg/mL; Life Technologies, Carlsbad, CA, USA) was used to gate viable cells. Flow cytometry and cell sorting were performed using a FACSAria II cell sorter (BD

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Biosciences, Bedford, MA, USA). The Hoechst 33342 dye was excited at 357 nm and its fluorescence was analyzed using dual wavelengths (blue, 402–446 nm; red, 650–670 nm).

When the proportion of SP cells was low (≤5%), the SP cells were sorted and subjected to *in vitro* culture in 10 mL IMDM containing 10% FBS for enrichment. Then, after at least 14 days culture, SP analysis and cell sorting were performed again.

Analysis of expression of cell surface molecules. Expression of cell surface molecules was assayed as described previously[11] using an anti-human leukocyte antigen (HLA)-A24 mAb (C7709A2.6), anti-HLA-B&C mAb (B1.23.2), anti-HLA Class I mAb (W6/32), anti-HLA-Class II mAb (L243), and an anti-CD80 mAb (Hybridoma cells for C7709A2.6 were donated by Dr. PG Coulie [Christian de Duve Institute of Cellular Pathology, University of Louvain, Brussels, Belgium] and those for B1.23.2 and L243 were purchased from the American Type Culture Collection [Manassas, VA, USA]. An anti-CD80 mAb was purchased from Immunotech [Marseille, France].). SP, and main population (MP) cells of the MFH2003 cell line, as well as LG2-EBV, B2003-EBV, and K562 cells, were incubated with appropriate mAb for 40 min on ice. Then, the cells were incubated with FITC-labeled secondary antibodies and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

ELISA. Target cells ($1–2 \times 10^4$) were plated in flat-bottomed 96-microwell plates (Corning, Corning, NY, USA) in DMEM containing 10% FBS. Then, TIL2003 cells (5×10^4) in AIM-V medium were added. After 24 h incubation at 37°C, the amount of granulocyte–macrophage colony stimulating factor (GM-CSF) in the supernatant (100 μL) was measured using an ELISA Development kit (TechneCorp, Minneapolis, MN, USA) according to the manufacturer's instructions. All experiments were performed in duplicate.

Establishment of autologous CTL clones against SP cells of the MFH2003 cell line. Autologous CTL clones against SP cells of the MFH2003 cell line were established as described previously. Briefly, peripheral blood mononuclear cells (PBMC) were obtained from an MFH2003 donor patient. The $CD8^+$ T cells were collected from PBMC using magnetic anti-CD8 beads (Miltenyi Biotec, Gladbach, Germany). A total of 5×10^5 irradiated (100 Gy) SP cells of the MFH2003 cell line and 5×10^6 $CD8^-$ T cells were distributed into five wells of a 24-well flat-bottomed culture plate containing 2 mL/well AIM-V and cultured at 37°C. The following day, 20 U/mL recombinant human interleukin-2 (rhIL-2; a kind gift from Takeda Chemical Industries, Osaka, Japan) and 10% AB human serum (HS) were added. The stimulation of T cells was repeated at intervals of 7–10 days using SP cells. After the fourth stimulation, the CTL were plated from all five culture wells at various dilutions in round-bottomed 96-microwell plates (Corning) in AIM-V supplemented with rhIL-2 (200 U/mL) and phytohemagglutinin (PHA; 5 μg/mL; Wako Chemicals, Osaka, Japan). Irradiated LG-2 EBV cells (1×10^4 cells/well) and allogeneic PBMC (1×10^5 cells/well) were added as feeder cells. Cells were incubated at 37°C. After 42 days, three resultant CTL clones were used in the cytotoxicity assays. One CTL clone showing specific cytotoxicity against SP cells of the MFH2003 cell line was selected and designated Tc4C-6. The cytotoxicity assay was performed as described below. Cell surface phenotypes of Tc4C-6 were assayed using an FITC-conjugated anti-CD3 antibody (BD Biosciences), phycoerythrin (PE)-conjugated anti-CD4 antibody (BD Biosciences), PE-Cy5-conjugated anti-CD8 antibody (eBioscience, San Diego, CA, USA), FITC-conjugated anti-CD45RA (BD Biosciences) antibody, and PE-conjugated anti-CCR7 antibody (BD Biosciences). The Tc4C-6 clone and healthy donor PBMC were incubated with these antibodies for 30 min on ice in the dark. After washing with PBS, cells were fixed with 1% paraformaldehyde in PBS and analyzed by flow cytometry.

Cytotoxicity assay. The specific cytotoxicity of CTL clones was measured using the non-radioactive aCella-TOX assay (Cell Technology, Mountain View, CA, USA) according to the manufacturer's instructions. Target cells were plated in triplicate (5000 cells/well) in round-bottomed 96-well microwells in IMDM containing 50 U/well rhIL-2. Effector cells were added at various effector:target (E/T) ratios, as indicated. Spontaneous effector and target cell death was achieved by including control wells of effector and target cells at numbers corresponding to those of their various E/T ratios. To determine maximum release, calculated as total glyceralehyde-3-phosphate dehydrogenase (G3PDH) released, 10 μL lysis reagent (0.5% Nonidet P-40 per 100 μL sample) was added to the target cell positive control 10 min after the end of the assay incubation. After 12 h incubation at 37°C, the culture supernatant from each well was transferred into a corresponding well containing Enzyme Assay Reagent reacting against G3PDH on a white OptiPlate-96 (PerkinElmer, Waltham, MA, USA) and detection reagent was added to each well. The luminescence of each well was analyzed immediately using an ARVO MX/Light 1420 Multilabel Luminescence Counter (PerkinElmer). All experiments were performed in triplicate. Cytotoxicity (%) was calculated as [(experimental G3PDH release − spontaneous G3PDH release from effector cells − spontaneous G3PDH release from target cells)/(maximum G3PDH release from target cells − spontaneous G3PDH release from target cells) × 100].

In blocking experiments, the target cells was incubated with an anti-HLA Class I mAb (W6/32) or anti-HLA Class II mAb (L243) for 30 min at 37°C before the cytotoxicity assay was performed.

Results

Enrichment of SP cells in the MFH2003 cell line. To isolate SP cells as CSC/CIC, SP analysis and cell sorting were performed 95 times. As shown in Figure 1, in independent experiments the proportion of SP cells in the MFH2003 cell line ranged from 0.3% to 7.2% (mean 4.0 ± 1.7%). Figure 1(a) shows typical results from two independent SP analysis experiments. The number of SP cells isolated from bulk MFH2003 cells ranged from 0.1 to 6.7×10^5 (mean $2.4 \pm 1.4 \times 10^5$). Because more than 5×10^5 SP cells were required for each experiment in the present study, the variance in the proportion of SP cells and the low number isolated sometimes made it difficult to complete the experiments. To overcome these problems, we enriched SP cells using *in vitro* SP cell culture. After 7–10 days, the proportion of SP cells increased to between 9.4% and 36.2% (mean 18.6 ± 7.4%). In addition, the resultant number of sorted SP cells increased more than sixfold, ranging from 3.3 to 38.2×10^5 (mean $15.3 \pm 7.7 \times 10^5$; Fig. 1a,b). This improvement in the isolation efficiency of SP cells was useful for further experiments.

Expression profiles of immune molecules on SP and MP cells of the MFH2003 cell line. First, we analyzed the immune molecules on SP and MP cells of the MFH2003 cell line (Fig. 2). The SP cells expressed HLA Class I, HLA-A24, B, and C molecules on their cell surface. The expression of these molecules was greater than that for MP cells. Although MHC Class I and CD80, which provide costimulatory signals necessary for T cell activation and survival, were not expressed on SP cells, the higher expression of MHC Class I on SP cells suggests that CSC can be recognized by the host cellular immunity.

Autologous TIL2003 recognized both SP and MP cells of MFH2003. Next, to evaluate whether CTL can recognize SP cells in the tumor microenvironment, we assessed the response of the autologous tumor-infiltrating lymphocyte line TIL2003 against SP and MP cells. The TIL2003 cell line is a CTL line we established previously from the metastatic lymph nodes of

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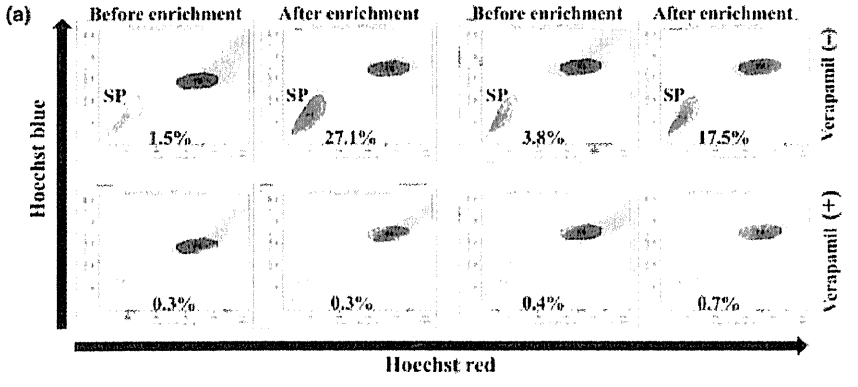

(b)

	No. experiments	% SP cells	Input : bulk ($\times 10^7$ cells)	Output : sorted SP ($\times 10^5$ cells)
Before enrichment	72	4.0 ± 1.7 (0.3–7.2)	1.7 ± 0.8 (0.6–5.7)	2.4 ± 1.4 (0.1–6.7)
After enrichment	23	18.6 ± 7.4 (9.4–36.2)	2.2 ± 1.0 (0.7–4.0)	15.3 ± 7.7 (3.3–38.2)

Fig. 1. Enrichment of the side population (SP) cells of the MFH2003 cell line. (a) The SP cells before and after enrichment, in the presence or absence of verapamil, in two independent experiments. The SP cells are encircled by black lines. The proportion of SP cells among total living cells is indicated in each case. (b) Summary of the enrichment of SP cells giving the mean proportion (%) of SP cells in the MFH2003 cell line, the mean number of bulk MFH2003 cells stained with Hoechst 33342 dye, and the mean number of sorted SP cells. Data show the mean ± SD with the range given in parentheses.

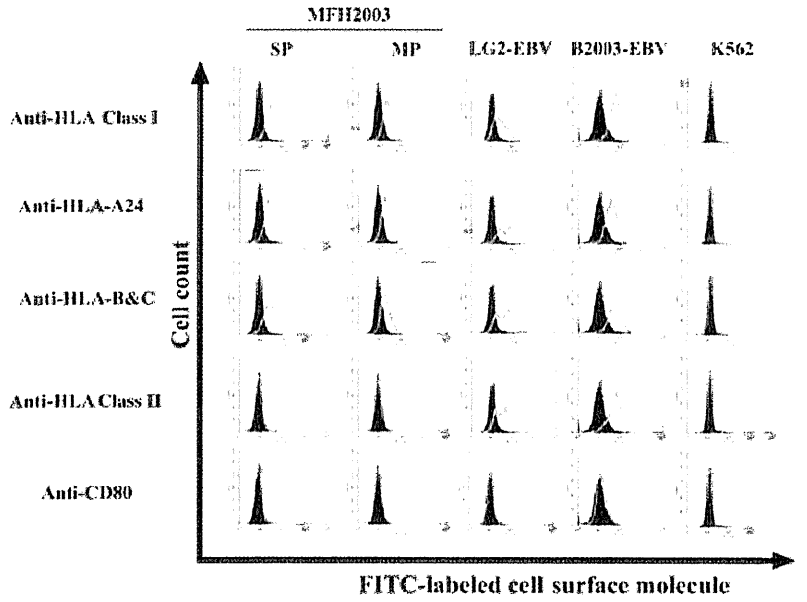

Fig. 2. Expression profile of immune molecules on side population (SP) and main population (MP) cells showing the cell surface expression of human leukocyte antigen (HLA) Class I (HLA-A24, B&C), HLA Class II, and CD80 molecules on MFH2003 (bulk, SP, and MP cells), LG-2, EB-B (B2003-EBV) and K562 cells.

the MFH2003 patient.[11] As shown in Figure 3, TIL2003 cells recognized both SP and MP cells. Although we could not completely rule out the possibility that MP cells triggered the immune response against both SP and MP cells in the context of some antigens expressed in both SP and MP cells, the results do suggest that the CTL response against SP cells was triggered by SP cells in the tumor microenvironment.

Specific response of the CTL clone derived from peripheral blood against SP cells. To detect the peripheral specific CTL response against SP cells of the MFH2003 cell line, we attempted to induce an autologous CTL clone that recognized the SP cells using SP cells as the stimulatory antigen. Cells were stimulated four times by mixed lymphocyte–tumor cell culture using purified SP cells of the MFH2003 cell line and autologous PBMC. Subsequently, conventional limiting dilution was performed. As a result, we obtained one CTL clone, namely Tc4C-6, which showed specific cytotoxicity against SP cells of the MFH2003 line. The Tc4C-6 clone expressed a single Vβ-chain

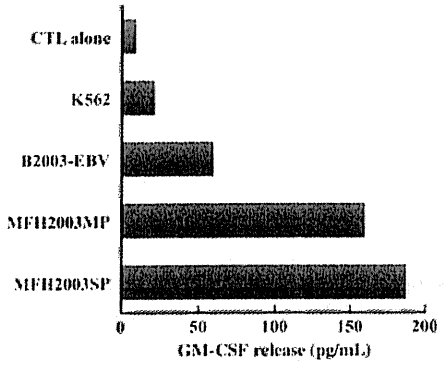

Fig. 3. Autologous tumor-infiltrating lymphocyte TIL2003 cells recognized both side population (SP) and main population (MP) cells. Freshly isolated SP and MP cells from the MFH2003 cell line, autologous EB-B cells (B2003-EBV), and negative control K562 cells were cocultured with TIL2003. After 24 h, the culture supernatant was harvested and the granulocyte–macrophage colony stimulating factor (GM-CSF) released from the TIL2003 was determined using ELISA.

(Vb5.2–3) mRNA, which was also expressed by TIL2003 cells (data not shown). The phenotype of the Tc4C-6 clone was $CD3^+CD4^-CD8^+CCR7^-CD45RA^+$, a typical effector phenotype (Fig. 4a). Moreover, the Tc4C-6 clone exhibited higher cytotoxicity against SP cells than MP cells of the MFH2003 cell line, as purified by cell sorting (Fig. 4b,c). In addition, the anti-HLA Class I W6/32 antibody apparently blocked the cytotoxicity of the Tc4C-6 clone against MFH2003SP cells (Fig. 4d). These results suggest that SP cells can be killed by autologous CTL in an HLA Class I-restriction manner.

Discussion

In the present study, we showed that: (i) SP cells, as CSC of the MFH200 cell line, expressed more HLA Class I on their cell surface than did MP cells (non-CSC); (ii) SP cells could be recognized by autologous tumor-infiltrating lymphocytes; and (iii) an autologous CTL clone could be induced by mixed lymphocyte–tumor cell culture using SP cells as antigens and that this induced clone killed SP cells rather than MP cells. These results indicate that CTL-recognizing CSC certainly exist in the tumor microenvironment and circulating peripheral blood and that SP cells can be killed by CTL. Thus, CTL-based immunotherapy against the CSC of bone sarcoma is a promising option.

Previous reports have suggested that CSC may be a candidate target for immunotherapy. For example, Pellegatta *et al.*[12] reported that dendritic cell based vaccine therapy resulted in an efficient anti-tumor immune response against glioma stem cells; Todaro *et al.* showed that γδ T cells killed human colon CSC; Pietra *et al.* demonstrated that natural killer (NK) cells killed human melanoma CSC;[13,14] and Weng *et al.*[15] induced CTL against ovarian CSC from HLA-$A2^+$ healthy donors using CSC-DC fusion cells and demonstrated that the CTL killed ovarian CSC. However, until now, the autologous CTL response against CSC had not been investigated.

It is well documented that tumors can escape T cell-mediated elimination by downregulating molecules essential for immune recognition.[16] The downregulation of HLA Class I molecules

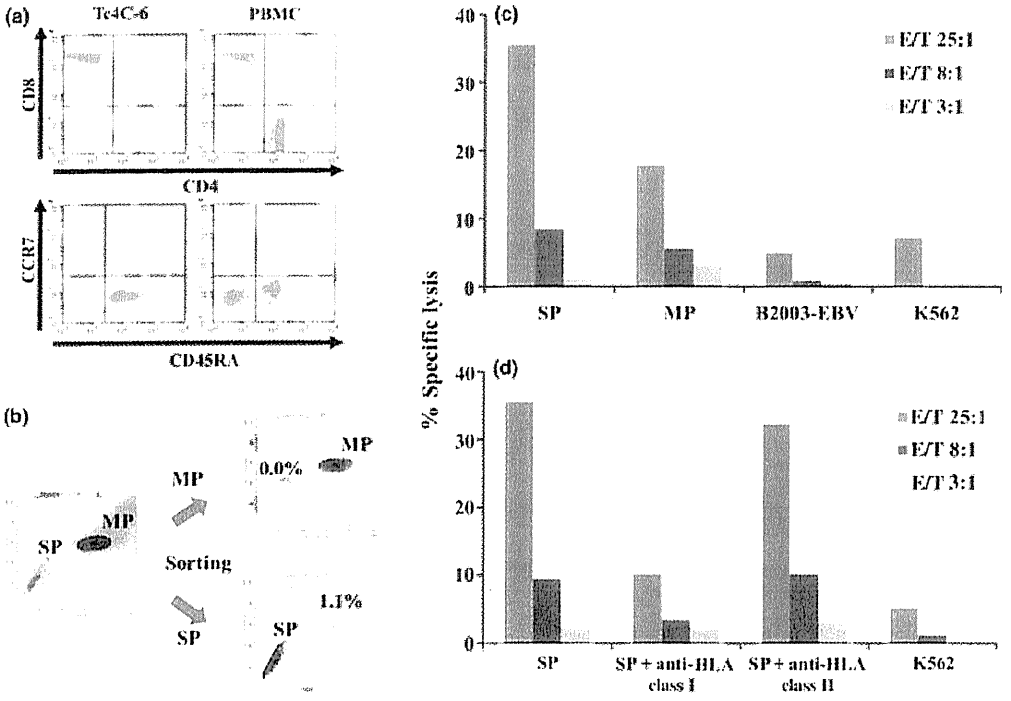

Fig. 4. The autologous CTL clone Tc4C-6 recognized side population (SP) cells. (a) Cell surface expression of CD4, CD8, CCR7, and CD45RA on Tc4C-6 and allogeneic peripheral blood mononuclear cells (PBMC) from a healthy donor. (b) Reanalysis of sorted SP and main population (MP) cells. (c) Cytotoxicity of the CTL clone Tc4C-6. Freshly isolated SP and MP cells, autologous EB-B cells (B2003-EBV), and negative control K562 cells were used as target cells and cocultured with Tc4C-6 at the specified effector:target (E/T) ratios. After 12 h, CTL-mediated cytotoxicity was measured using the aCella-TOX assay (Cell Technology), as described in the Materials and Methods. (d) Blocking assay of Tc4C6-mediated recognition of SP cells from the MFH2003 cell line using anti-human leukocyte antigen (HLA) Class I (W6/32 and anti-HLA Class II (L243) mAbs. Cytotoxicity was also measured with the aCella-TOX assay.

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in tumor tissues is a major prognostic factor and has an important role in tumor immune escape.[17] We have reported previously on the relationship between downregulation of HLA Class I and the poor prognoses of patients with osteosarcoma and Ewing's sarcoma.[18,19] However, as shown in the present study, the expression profile of immune molecules, including HLA Class I molecules on CSC, is preserved. Therefore, CSC may not escape from cellular immune surveillance activated by CTL-based immunotherapy.

The identification of CSC-associated antigens recognized by autologous CTL is very important, especially for the establishment of CTL-based immunotherapy in the adjuvant setting for the prevention of recurrence and metastasis. To this end, establishment of anti-CSC-specific CTL lines is a prerequisite. Although Weng *et al.*[15] assessed the CTL response against allogeneic ovarian CSC, there are no reports regarding CTL lines induced by autologous CSC. Therefore, the CTL clone Tc4C-6 is the first CTL clone against CSC induced by autologous CSC and could serve as a good probe against autologous CTL clone-defined CSC-associated antigen. We are currently trying to isolate the cDNA of the T-cell receptor (TCR) α- and β-chains to develop a permanent probe for cDNA library expression cloning.

In the present study, we evaluated SP cells of the MFH2003 cell line in 72 independent experiments. The proportion of SP cells in the MFH2003 cell line varied among experiments and this was often the main obstacle to completing the experiments using SP cells; thus, we needed to enrich the SP cells. The isolation of SP cells requires high-level technical skills and intensive, hard laboratory work. Although we do not know why the proportion of SP cells in the MFH2003 cell line is so variable, differentiation of SP cells into MP cells in cell culture *in vitro* may contribute to the variance in the proportion of SP cells. Recently, the dynamic regulation theory of cancer stem cells was proposed.[20] A subpopulation of SP cells that were jumonji AT-rich interactive domain 1B (JARID1B) positive was shown to have high proliferative ability. However, not only could individual JARID1B-positive cells become JARID1B negative, but individual JARID1B-negative cells could become JARID1B positive and acquire tumorigenicity. Such a dynamic change in the characteristics of SP and MP cells is another possible reason for the variability of the proportion of SP cells among independent experiments.

In conclusion, we have demonstrated the immunogenicity of CSC of bone MFH using autologous tumor-infiltrating lymphocytes and a peripheral CTL clone. On the basis of our results, we propose that CTL-based immunotherapy could target the CSC of bone sarcoma to prevent tumor recurrence.

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Disclosure Statement

All authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Bone and/or joint attachment is a risk factor for local recurrence of myxofibrosarcoma

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Abstract
Background Myxofibrosarcoma is characterized by a high local recurrence rate despite optimal surgical treatment. The definition of prognostic factors for recurrence offers high-risk patients a closer follow-up and a multidisciplinary therapeutic approach.
Methods A cohort of 23 patients treated for primary myxofibrosarcoma was retrospectively analyzed. The patients (sex and age), tumors (size, stage, tumor location, bone and/or joint attachment), radiological findings (abnormal signal extension in MRI), histological findings (FNCLCC grade and microscopic extension along the muscle fascia), and treatment (surgical margin) characteristics were included in univariate prognostic factor analysis.
Results After a median follow-up of 63.3 months (range 15–191), the overall recurrence rate was 34.7%. Median time between initial surgery and recurrence was 24.8 months (range 8–52). Inadequate surgical margins ($p = 0.026$) and bone and/or joint attachment ($p = 0.001$) were associated with an increased recurrence rate.
Conclusion For the further improvement of local recurrence-free survival of patients with myxofibrosarcoma, accurate diagnosis of the tumor extension and adequate planning for the surgical margin should be focused on in cases with bone and/or joint attachment.

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Introduction

Myxofibrosarcoma is one of the most common sarcomas in elderly patients that arise in the limbs, including limb girdles. About two-third of cases develop in dermal/subcutaneous tissue, with the remainder occurring in the underlying fascia and skeletal muscles [1]. Complete surgical resection is the cornerstone of the treatment, but negative margins are difficult to obtain because myxofibrosarcoma has an unusual infiltrative growth pattern along fascial planes [2, 3]. This infiltrative growth pattern of myxofibrosarcoma can result in anatomically deceptive boundaries at surgery because of microscopic extension into the dermis and skeletal muscle. Therefore, the outcomes are characterized by a high local recurrence rate leading to poor overall survival [4, 5]. The aim of this study was to investigate the influence of clinical, histological, and surgical variables on recurrence in a cohort of 23 patients with myxofibrosarcoma. The final objective was to identify patients with a high recurrence risk to offer appropriate follow-up and a multidisciplinary therapeutic approach.

Patients and methods

A total of 23 patients with myxofibrosarcoma treated between 1992 and 2007 were included in this retrospective study. This study did not receive institutional review board approval because our institution does not require such approval for retrospective studies. All patients had planned wide local excision with an adequate (>50 mm) tumor-free margin. If the tumor showed evidence of cortical erosion or signal change within the bone marrow cavity, the bone segment involved by the tumor was resected. If the MRI showed that the lesion was adjacent to bone, careful physical

Table 1 Clinical informations of 23 patients with myxofibrosarcoma

Case	Age	Sex	Tumor locations		FNCLCC grade	AJCC stage	Margin	Histological infiltration	MRI extension	Size (cm)	Bone/joint attachment	Recurrence	Metastasis	Follow-up periods (months)	Outcome
			Parts	Depths											
1	75	M	Inguinal	Intramuscular	2	3	Adequate	+	+	7	–	–	–	84	CDF
2	51	M	Thigh	Intermuscular	2	3	Inadequate	+	+	7	+ (Hip)	2[a] (15)[b]	–	90	NED
3	74	M	Thigh	Subcutaneous	2	1	Inadequate	+	+	4	–	–	–	71	CDF
4	64	M	Upper arm	Intermuscular	2	3	Adequate	+	+	3	+ (Humerus)	1 (8)	–	83	NED
5	53	M	Forearm	Intermuscular	1	1	Adequate	+	+	6	–	–	–	63	CDF
6	77	M	Back	Intermuscular	1	1	Adequate	+	+	5	+ (Scapula)	2 (32)	–	61	CDF
7	48	F	Thigh	Intermuscular	1	1	Adequate	+	–	3	–	–	–	53	CDF
8	46	M	Upper arm	Intramuscular	1	1	Adequate	+	+	6	–	–	Lung	48	NED
9	75	F	Buttock	Intramuscular	2	1	Adequate	+	–	6	+ (Hip)	–	–	41	CDF
10	81	M	Thoracic	Intramuscular	2	1	Inadequate	+	+	5	+ (Rib)	1 (31)	Lung	39	NED
11	71	F	Thigh	Intermuscular	2	1	Adequate	+	–	3	–	–	–	36	CDF
12	78	F	Thigh	Intermuscular	2	1	Inadequate	+	+	5	–	–	–	35	CDF
13	78	F	Thoracic	Intermuscular	2	1	Inadequate	+	+	3	+ (Sternum)	3 (52)	–	191	NED
14	68	M	Thigh	Intermuscular	2	3	Adequate	+	+	10	+ (Femur)	–	–	170	CDF
15	48	F	Forearm	Subcutaneous	2	2	Inadequate	+	+	4.5	+ (Ulna)	2 (29)	–	130	NED
16	75	F	Thigh	Subcutaneous	2	3	Inadequate	+	+	4.5	–	–	–	84	CDF
17	79	M	Lower leg	Subcutaneous	2	1	Adequate	+	+	5	–	–	Liver	27	NED
18	67	F	Forearm	Subcutaneous	1	1	Adequate	+	+	5	+ (Ulna)	2 (12)	–	72	NED
19	87	F	Forearm	Intermuscular	2	3	Inadequate	+	+	5	+ (Ulna)	1 (10)	Lung	20	DOD
20	82	M	Forearm	Intermuscular	2	2	Adequate	+	+	5	–	–	–	15	CDF
21	69	M	Lower leg	Subcutaneous	3	2	Adequate	+	+	5	–	–	–	21	CDF
22	74	M	Neck	Subcutaneous	2	2	Adequate	+	+	3	–	–	–	29	CDF
23	83	F	Axillar	Intermuscular	3	2	Adequate	+	+	7	–	–	–	15	CDF

[a] Number of local recurrences

[b] Times of the first local recurrence from the initial surgery

examination was used to determine whether the tumor moved freely over the periosteal surface of the bone or whether the tumor appeared fixed to the bone. This evaluation was repeated in the operating room after the fascia was opened circumferentially around the bone deep to the tumor. If the sarcoma was fixed to the bone even after the fascia had been opened, the bone segment was resected. Resection was considered complete if microscopic margins were negative. The clinical information was obtained from medical records, radiographic imaging, and pathologic reports. The patients' information, including age, gender, and anatomical location of the tumor, is presented in Table 1. The stage of the primary tumor was determined according to the staging system of the American Joint Committee on Cancer, 6th edition [6]. The specimens were assigned to the French Federation of Cancer Center Sarcoma Group (FNCLCC) classification. This classification is based on the mitotic index, necrosis extension, and histologic differentiation of the tumor [7]. The recurrence rate after surgery was used as an end point and calculated from the date of the first treatment. In the absence of any events, the date of last follow-up was considered as end point. The patients (sex and age), tumors (size, stage, tumor location, bone and/or joint attachment), radiological findings (abnormal signal extension in MRI), histological findings (FNCLCC grade and microscopic extension along the muscle fascia), and treatment (surgical margin) characteristics were included in univariate prognostic factor analysis. Tumor location was classified as subcutaneous or deep. If the MRI showed that the lesion was attached to the bone and/or joint capsule, the lesion was judged as the tumor having bone and/or joint attachment. The curve for overall survival was drawn according to the Kaplan-Meier method, and differences were analyzed by applying the log-rank test. Quantitative variables were compared using the log-rank test. All statistical analysis was performed using SPSS® 10-0 for Windows (SPSS, Chicago, IL). Statistical significance was defined as $p < 0.05$.

Results

A total of 23 patients (13 men and 10 women) with a median age of 68.3 years (range 51–87) were included in the study. The median follow-up period was 63.3 months

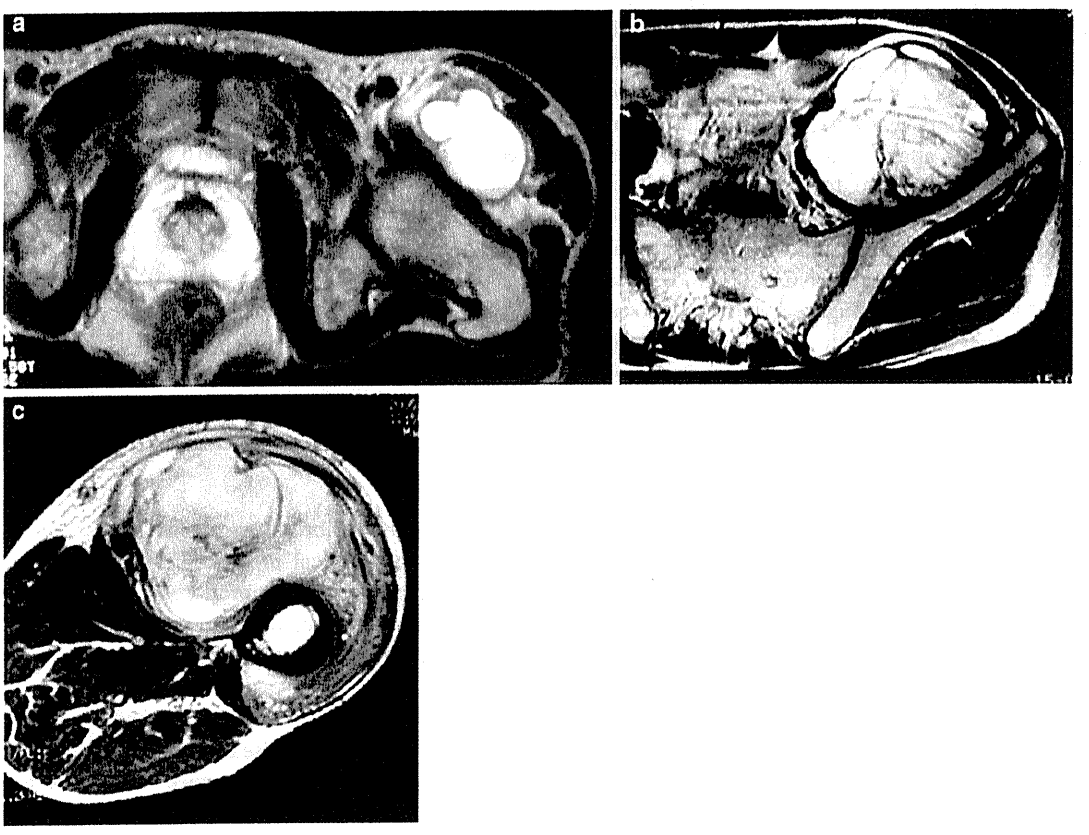

Fig. 1 A 51-year-old male with myxofibrosarcoma of the left thigh. The tumor was attached to the joint capsule of the hip joint at the initial presentation (**a**). The first local recurrence was 15 months after the initial operation. The tumor relapsed at the proximal part of the initial tumor (**b**). The second local recurrence was 51 months after the initial operation. The tumor relapsed at the distal part of the initial tumor (**c**)

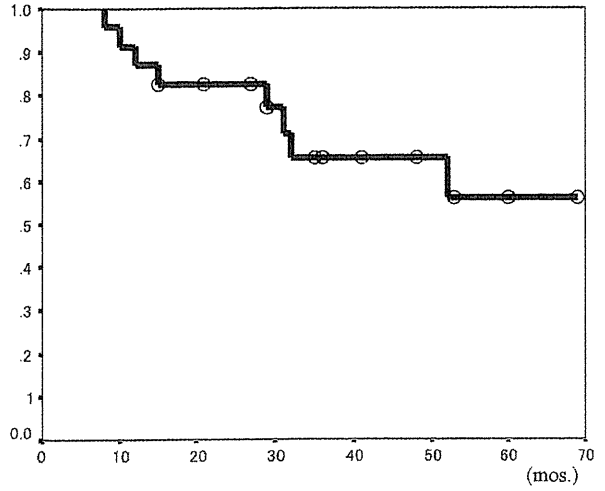

Fig. 2 Kaplan-Meier local recurrence-free survival curves of all patients with myxofibrosarcoma

(range 15–191). The mean radiological size of the tumors was 5.2 cm (range 3–10). There were 7 patients who presented with subcutaneous tumors and 16 patients with deep-seated tumors. Ten tumors had bone and/or joint attachment (Fig. 1). In eight out of these ten patients with the tumor attached to the bone/joint, the tumor was resected by stripping the periosteum of the bone deep to the tumor. Partial scapulectomy was performed on the patient (case 6) with the tumor attached to the scapula. In the patient with a tumor attached to the hip joint (case 2), partial capsulectomy was performed. Despite extensive surgery, an adequate tumor-free margin was only obtained in 16 (69.5%) patients. A high FNCLCC grade was observed in 18 (78.2%) patients. Figure 2 shows the local recurrence-free survival curves of all 23 patients. Eight of the 23 patients developed local recurrence at a mean of 24.8 months (range 8–52) after surgical resection. The 5-year local recurrence-free survival was 55.9%.

In univariate analysis, inadequate surgical margins ($p = 0.026$) and bone and/or joint attachment ($p = 0.001$) were associated with an increased recurrence rate (Table 2). When stratified into those with inadequate and adequate surgical margins, 5-year relapse-free survival rates were 77.8% for those with adequate surgical margins and 21.4% for patients with inadequate surgical margins (Fig. 3a). There was also a significant difference in the local recurrence-free survival between the patients with tumors with and without bone and/or joint attachment. Of the ten patients with a tumor with bone and/or joint attachment, eight developed local recurrence. The local recurrence-free survival rate for the patients with a tumor with bone and/or joint attachment was 15.0%. In comparison, none of the 13 patients with a tumor without bone and/or joint attachment developed local recurrence (Fig. 3b).

Table 2 Univariate analysis of risk factor for local recurrence

Clinicopathological feature		5-year local recurrence-free survival (%)	*p* value
Age			
<70 years	9	54.3	
>70 years	14	53.3	0.41
Gender			
Female	10	45.7	
Male	13	60.4	0.76
Location			
Trunk	7	30.0	
Extremity	16	60.6	0.82
Grade			
Stage 1	5	60.0	
Stage 2 and 3	18	53.8	0.95
Surgical margin			
Inadequate	7	77.8	
Adequate	16	21.4	0.026
Stage			
Stage 1 and 2	17	57.0	
Stage 3	6	40.0	0.16
MRI single extension			
+	20	48.8	
–	3	100	0.19
Size			
>5 cm	15	60.0	
<5 cm	8	56.2	0.89
Depth			
Subcutaneous	7	53.1	
Deep	16	64.2	0.91
Bone joint involvement			
+	10	15.0	
–	13	100	0.001

Discussion

In the case of myxofibrosarcomas, wide excision is the usual goal of the surgical procedure. Whenever possible, negative surgical margins are preferred and are associated with lower rates of tumor recurrence [5]. In addition, we have demonstrated that bone and/or joint attachment was also associated with an increased rate of local recurrence in the present study. These results clearly indicated that the local aggressiveness of the disease led to a high frequency of local recurrence. Large clearance clearly is a gold standard procedure in surgical oncology. However, surgical rules are often difficult to respect for myxofibrosarcoma because of its unusual characteristic of multidirectional spreading along the fascial planes. Indeed, negative margins are not easily obtained despite preoperative planning

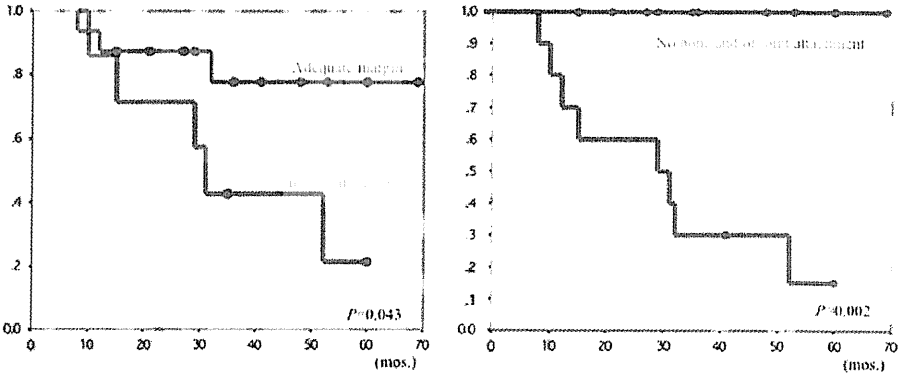

Fig. 3 Kaplan-Meier local recurrence-free survival curves of patients with myxofibrosarcoma by **a** surgical margin, **b** bone and/or joint attachment

of wide local excision with an adequate tumor-free margin. In particular, as observed in our previous study, the full extent of the tumor may be difficult to determine even with MRI [8]. This often makes surgical planning difficult.

A negative margin was not obtained in half of the patients with bone and/or joint attachment. This could be the reason for the high incidence of local recurrence of myxofibrosarcoma with bone and/or joint attachment. Because of the anatomical characteristics, accurate evaluation of the extension of such a tumor is difficult even with MRI. In addition, the spreading of the tumor with bone and/or joint attachment may be wider than believed, including the possibility of skip metastases. Furthermore, wide local excision of a tumor that involves the bone and/or joint is technically difficult, which increases the risk of tumor dissemination.

Based on these results, the optimal treatment of patients with myxofibrosarcoma should be based on radical surgery to minimize the risk of a positive margin. In high-risk patients (surgical positive margin, bone and/or joint involvement), close follow-up is mandatory to offer subsequent optimal surgical procedures. In addition, a multidisciplinary therapeutic approach is necessary. However, the impact of radiotherapy or chemotherapy on relapse-free and overall survival remains to be proved.

We are aware that our findings require more detailed evaluation with a larger sample size before their ultimate significance can be determined. The small sample size in this study limits out ability to conduct multivariate analysis. Nevertheless, we hope that our study will prompt investigators to design new studies to better understand the risk factors for local recurrence of myxofibrosarcoma. Although our study may be limited by the small sample size, the results reported here should provide useful prognostic information for the management of myxofibrosarcoma. New larger, prospective studies specially designed to address this issue are warranted.

In conclusion, myxofibrosarcoma with no bone and/or joint attachment can be safely managed by wide local excision. For the further improvement of local recurrence-free survival of patients with myxofibrosarcoma, accurate diagnosis of the tumor extension and adequate planning for the surgical margin should be focused on in patients with bone and/or joint attachment.

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VI. がんワクチン療法の進歩

骨・軟部肉腫に対するペプチドワクチン療法

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Peptide vaccination therapy for bone and soft tissue sarcoma

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Abstract

Bone and soft tissue sarcomas are relatively difficult to treat or cure. Despite a multidisciplinary approach involving surgery, chemotherapy, and radiotherapy, metastatic or relapsed diseases are difficult to cure. Therefore, novel therapeutic options need to be explored. We investigated the use of peptide vaccination therapy for synovial sarcoma and osteosarcoma. The present trial for synovial sarcoma showed the safety, immunogenic properties, and partial clinical efficacy of peptides. However, the power of the vaccine, at least in the present trial, is not strong enough to suppress tumor growth in the presence of gross residual tumors. Therefore, the use of the vaccination therapy as an adjuvant to chemotherapy for the treatment of refractory tumors may be a promising tool.

Key words: bone and soft tissue sarcoma, peptide vaccination, osteosarcoma, synovial sarcoma

はじめに

骨・軟部肉腫とは間葉系組織に由来する悪性腫瘍である．その発生頻度は癌腫に比較して極めて低く，悪性骨腫瘍，悪性軟部腫瘍の国内における2000年度の発生頻度はそれぞれ人口10万人対0.8，2.0人である[1]．治療法の基本は外科的切除であり，化学療法や放射線治療を合わせた集学的治療により，ここ20年で生存率は向上している．しかし組織型が多岐に及び，また低悪性から高悪性のものまで多彩で診断が困難である．また化学療法や放射線療法に対する感受性が異なり治療に難渋する．これまで以上に治療成績を向上させるためには新規治療の開発が必要である．

著者らの施設では滑膜肉腫，骨肉腫に対する新規治療としてペプチド免疫療法を開発した．滑膜肉腫に対しては2002年より，骨肉腫に対しては2008年より第1相臨床試験を開始している．

本稿ではこれらの肉腫に対する標準治療を概説し，ペプチドワクチン療法のこれまでの結果と今後の展望に関して述べる．

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表1 滑膜肉腫，骨肉腫に対する第1相臨床試験概要

対象がん	滑膜肉腫	骨肉腫
年齢(歳)	10-85	10-80
対象病期	根治手術不能	根治手術不能
HLA型	A2402	A0201あるいはA2402
使用ペプチド	Bペプチド(GYDQIMPKK), K9I(GYDQIMPKI)	PBF A2.24(AYRPVSRNI), PBF A2.2(ALPSFQIPV)
投与間隔	1回/2週×6回	1回/2週×6回
ペプチド投与量	1mg, 10mg	1mg, 10mg
併用薬	IFNα	なし

表2 滑膜肉腫，骨肉腫に対する第1相臨床試験結果

対象	ペプチド	プロトコール	症例数	有害事象(症例数)	抗腫瘍効果(症例数)	進行状況
滑膜肉腫	B	ペプチド単独	6	発熱(1)	PD(5), SD(1)	終了
	K9I	ペプチド単独	3	発熱(1)	PD(3)	終了
	B	ペプチド+アジュバント+IFNα	6	発熱(6)	PD(3), SD(3)	終了
	K9I	ペプチド+アジュバント+IFNα	4	発熱(4), 脳出血(1)	PD(2), SD(2)	進行中
骨肉腫	A2.2	ペプチド+アジュバント	1	なし	PD(1)	進行中
	A24.2	ペプチド+アジュバント	1	なし	PD(1)	進行中

PD: progressive disease. SD: stable disease.

1. 滑膜肉腫，骨肉腫に対するペプチドワクチン療法の第1相臨床試験

本試験の概要と結果を表1，2に示す．

a. 滑膜肉腫

滑膜肉腫は若年成人の四肢関節近傍に好発し，軟部肉腫の約10％を占める頻度の高い軟部肉腫である[2]．治療は外科的切除に化学療法，放射線療法を組み合わせた集学的治療が行われる．他の軟部肉腫と比べて，アドリアマイシン(ADM)，イフォマイド(IFO)に高い感受性を有する[3]．しかし5年生存率は36-76％とされ，手術や化学療法が無効な進行期例に対する有効な治療法はないのが現状である[2]．

滑膜肉腫には染色体転座によって18番染色体とX染色体転座による融合遺伝子SYT-SSXがほぼ全例に存在する[4]．この特異的な融合遺伝子が滑膜肉腫の発生にかかわることが示されている[5]．融合遺伝子は正常組織には存在しないため，融合部分をまたぐペプチドは腫瘍に特異的に発現する．著者らはSYT-SSXのアミノ酸配列からHLA-A24と結合するBペプチド(GYDQIMPKK)と，Bペプチドの第9番アミノ酸をチロシンからイソロイシンに改変してHLA-A24との結合能を高めたK9I(GYDQIMPKI)をペプチドワクチンとして選択し[6,7]，2002年より第1相臨床試験を開始している[8]．

対象は根治手術が不可能(遠隔転移例，再発例，体幹発生例)で，腫瘍がSYT-SSX転座遺伝子を発現しており，HLA-A2402陽性の患者である．HLA-A24拘束性Bペプチドあるいは K9Iと不完全フロイントアジュバント(IFA)およびサイトカインを2週間に1回，計6回投与する．これまで計19人に投与した．

安全性に関しては投与局所の発赤，硬結を全例に認めた．Grade 2以下の発熱を12例，脳出血を1例に認めた．脳出血症例は鼠径部発生の滑膜肉腫であり，腫瘍切除の際に人工血管置換術を施行され抗凝固療法を行っていた．ペプチド療法との因果関係は不明であった．Bペプチド単独を投与した群では6例中3例で，末梢血中にBペプチド特異的CTLの頻度の増加が認

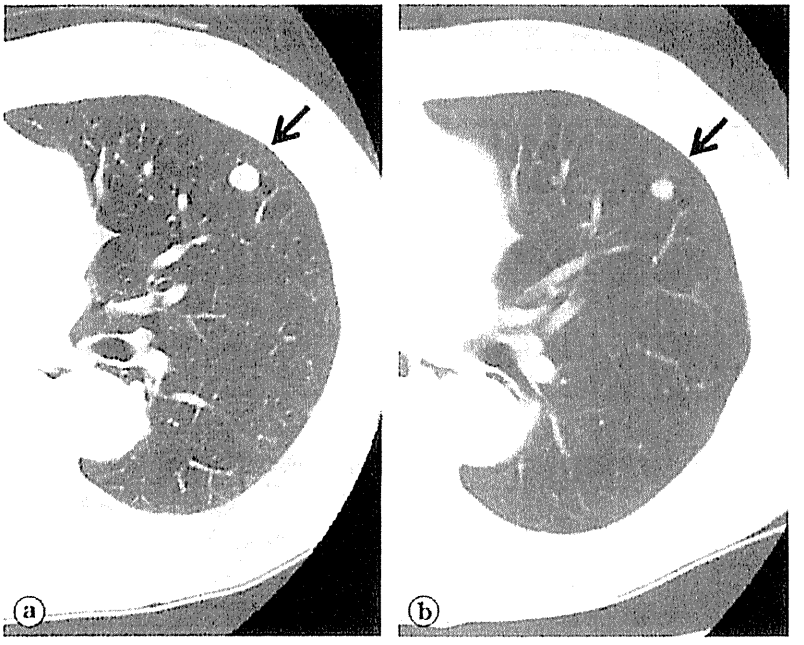

図1　66歳 男性，滑膜肉腫肺転移症例
a. ワクチン接種前.
b. ワクチン4回接種後，肺転移巣の縮小を認めた(矢印)．縮小率は48％であった.

められた．Response Evaluation Criteria in Solid Tumors(RECIST)基準による抗腫瘍効果判定では，progressive disease(PD)13例，stable disease(SD)6例であった．K9IとIFNαのプロトコールで腫瘍の縮小が認められた(図1)．

b. 骨肉腫

骨肉腫は骨に発生する肉腫の中で最も頻度が高く，腫瘍細胞が直接骨を産生することが特徴である．我が国における年間登録数は約200例である[9]．骨肉腫は10歳代の小児の膝・肩周囲に好発する．治療は術前化学療法，手術，術後化学療法による集学的治療が基本である．化学療法のkey drugはメトトレキサート大量療法(HD-MTX)，シスプラチン(CDDP)，ADM，IFOの4剤であり，これら4剤を中心とした多施設共同プロトコールNECO-95Jが骨肉腫の標準的プロトコールとして位置づけられ，5年累積生存率は78％である[9]．しかし初診時遠隔転移症例や再発症例に対しては，可能なかぎり外科的切除による治療と，前述した4剤以外のsecond line chemotherapyが行われるが，2年生存率は約20％程度と極めて予後不良である[10]．したがって新規治療の開発が急務であり，著者らは骨肉腫に対してもペプチドワクチン療法を開発した．

2000年に16歳の大腿骨骨肉腫患者から骨肉腫細胞株OS2000を樹立した．また同時に本患者の末梢血からTリンパ球を培養して，OS2000を特異的に傷害するクローンTcOScl303を樹立した．これらを用いてcDNA発現クローニングを行い，骨肉腫抗原ペプチドpapillomavirus binding factor(PBF)を同定した[11]．続いてPBFの全アミノ酸配列からHLA-A24/A2に強力に結合できるペプチドを作製し，免疫原性が高かったPBF A2.24(AYRPVSRNI)，PBF A2.2(ALPSFQIPV)をペプチドワクチンとして選択し，2008年より第1相臨床試験を開始している．これまで2人に投与した．安全に関しては投与局所の発赤，硬結を認めたのみで，そのほかの毒性は観察されなかった．RECIST基準による抗腫瘍効果判定ではいずれもPDであった．今後更なる症例の蓄積が望まれる．

2. 今後の課題

本試験によりペプチドワクチンの安全性と免疫学的有効性ならびに臨床効果が示された．本試験は第1相臨床試験であるため対象症例が進行期で，大きな腫瘍塊を有し全身状態が不良であることが多い．ワクチン療法は本来，臨床的に腫瘍塊を認めない状態あるいは腫瘍量が少ない状態で最も効果を発揮できると考えられる．著者らのこれまでの第1相臨床試験の結果からも，ペプチドワクチン療法が効果的であった症例は腫瘍径が1 cm以下と小さいものであった．したがって化学療法との併用による補助療法として用いることでより大きな効果につながる可能性がある．化学療法と併用したペプチドワクチンの補助療法としての利用法が，がん免疫療法の新たなブレイクスルーとなることを期待している．

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Involvement of Cancer Biomarker C7orf24 in the Growth of Human Osteosarcoma

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Abstract. *Background: Up-regulation of the expression of the gene C7orf24, encoding γ-glutamyl cyclotransferase, is a common event in cancers derived from various tissues, but its involvement in osteosarcomas (OS) has not yet been demonstrated. Materials and Methods: The expression of C7orf24 was analyzed in human OS cell lines and primary tumor samples. The biological effects of C7orf24 on growth, motility, and invasion in the OS cell lines were investigated using siRNA for C7orf24. Genes related to the function of C7orf24 were sought by genome-wide gene expression profiling. Results: The level of C7orf24 expression was much higher in the OS cell lines and OS primary tumors than in normal osteoblasts. Down-regulation of C7orf24 expression inhibited the growth of the cell lines in association with enhancement of cell-clustering. Treatment with C7orf24-siRNA inhibited cell motility and invasion. Gene ontology suggested the function of C7orf24 to be related to cell adhesion and protein transport. Conclusion: C7orf24 is also involved in the growth of OS, and is a potential biomarker for this type of tumor.*

Osteosarcoma (OS) is a primary bone malignancy generally affecting the young, with 60% of cases occurring before the age of 25 years and peak incidence at 15 years (1). Current standard treatment for OS involves neoadjuvant (preoperative) chemotherapy, definitive surgery on the primary tumor, and adjuvant (postoperative) chemotherapy, and the survival rate has improved significantly and reached more than 70% at 5 years (2-5). Further improvement, however, may be difficult without developing novel approaches such as molecular target therapy. A number of studies have been performed to identify molecules involved in the malignant phenotype of OS cells (6). The expression level of Ezrin, an adaptor protein linked to the cell membrane, correlated with the metastatic activity of OS (7), which led to clinical trails of the mammalian target of rapamycin (mTOR) inhibitor for OS patients (6). Inhibition of growth factors such as insulin-like growth factor (IGF), either by an antibody or by inhibitor for IGF receptors, prevented the growth of OS (8, 9). Although the functional involvement is not known, the expression level of the chemokine (C-X-C motif) receptor, CXCR4, correlated with the incidence of metastasis (10). Recent genome-wide gene expression analyses identified the receptor tyrosine kinase-like orphan receptor 2 (*ROR2*) gene as being up-regulated in OS tumors, and that the signal through a putative ligand, WNT5B, to ROR2 was involved in the growth of OS (11). Array-based analyses identified some miRNAs related to the resistance of chemotherapy such as miR-140 (12), and miR-92a, miR-99b, miR-132, miR-193a-5p and miR-422a (13). It remains to be resolved how these multiple factors affect the overall phenotype of OS, and whether any other molecules are also involved.

We have identified chromosome 7 open reading frame 24 (C7orf24) as an up-regulated protein of unknown function in bladder cancer (14), which was independently identified as

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Key Words: C7orf24, γ-glutamyl cyclotransferase, osteosarcoma.

a 21-kDa cytochrome *c*-releasing factor in the cytosolic fraction of human leukemia U937 cells after treatment with geranylgeraniol (15). Geranylgeraniol has potent apoptosis-inducing activity in various tumor cell lines, implicating the C7orf24 protein in apoptotic pathways of cancer cells (15). *In silico* analyses utilizing a panel of gene expression profiles of cancer cells also identified *C7orf24* as a gene up-regulated in many types of cancer (16, 17). Here we investigated *C7orf24* expression in human OS, and its association with cell motility and invasion.

Materials and Methods

Tissue specimens and cell lines. Tumor tissues were obtained at either biopsy or resection surgery and kept at -80°C. Informed consent was obtained from each patient, and tumor samples were approved for analysis by the Ethics Committee of the Faculty of Medicine, Kyoto University. The human OS cell lines Saos2, HuO, HOS, MG63, U2OS, and G292 were obtained from the ATCC (Rockville, MD, USA) or the Japanese Cancer Research Resources Bank (Tokyo, Japan). OS690 cells were established in our laboratory from a tumor of a 10-year-old girl with an osteoblastic OS in the femur. Normal human osteoblasts (NHOst) were obtained from TaKaRa (TaKaRa Bio, Shiga, Japan). These cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 100 units/ml of penicillin and 100 g/ml of streptomycin.

Reverse transcription (RT-PCR). All RT reactions were performed using 1 μg of total RNA with the Superscript III first-strand system (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR was performed for the *C7orf24* and β-actin (*ACTB*) genes using standard procedures. PCR products were loaded on 1% agarose gel and visualized by ethidium bromide staining. A pair of intron-spanning primers specific for human C7orf24 cDNA (GenBank accession number NM_024051; sense primer, 5'-ACAAGTCAAACTTGGCATGGAG-3'; antisense primer, 5'-TCTTGATACTCC AGCGGCAAAC-3') was used to amplify the 296 bp product.

RT-quantitative PCR (RT-qPCR). The relative amount of *C7orf24* mRNA was assessed by TaqMan real-time PCR with the ABI PRISM 7700 sequence detection system (Life Technologies). A 75-bp fragment from +293 (exon 2) to +408 (exon 3) of the *C7orf24* cDNA was amplified using specific primers (sense, 5'-TCCCAAGGCAAAACAAGTCAA-3'; antisense, 5'-TTAACCC CTTCTTGCTC ATCCA-3') and labeled with a TaqMan probe (5'-FAM-CACCATTTTTCAGAG TCCTG-GCGATGA-3'-TAMRA). NHOsts were used as the internal control, and all of the reactions were run in duplicate. The ratio of *C7orf24* of OS cell lines and sample/NHOst in each sample was calculated, and the expression level of *C7orf24* was demonstrated as a relative value using the *C7orf24/18S* ratio as a standard.

Western blotting. Whole-cell lysate in SDS sample buffer was prepared from each cell line. Aliquots of the extracts were electrophoresed in 15% polyacrylamide gels. Subsequently, proteins were transferred onto Immobilon-P Transfer Membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk, membranes were probed with an anti-human C7orf24 monoclonal antibody, 6.1E (14), at a 1:40,000 dilution overnight. After 1 h of incubation at room temperature with secondary antibody (horseradish peroxidase-conjugated rabbit IgG against mouse Ig; Dako, Kyoto, Japan) at 1:20,000, immunoreactive bands were detected with ECL Western Blotting Buffer Detection Reagents (GE Healthcare, Biosciences, Piscataway, NJ, USA). The densitometric analysis was conducted using ImageJ (http://rsb.info.nih.gov/ij/) and values were normalized with those of NHOst.

siRNA synthesis and transfection. The following target sequences were used to generate siRNA (Qiagen, Chatworth, CA, USA): sequence no. 1 (057), 5'-CUUUGCCUACGGCAGCAAC-3' (nucleotides 184-202); sequence no. 2 (498), 5'-UGACUAUACAGGAAAGGUC- GA-3' (nucleotides 625-643); sequence no. 3 (570), 5'-CAUAACA GAAUAUAU- CUAA-3' (nucleotides 697-715); GL3 (firefly luciferase), 5'-CUUACGCUGAGU-ACUUCUUCGA-3'. The synthetic siRNA duplexes were transfected to cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. For validation of the knockdown effect, 5×10^5 cells in a 60 mm dish were transfected with siRNAs. The medium was changed 24 h after the transfection, and the cells were harvested at 48 h.

Water-soluble tetrazolium salts (WST)-1 assay. The antiproliferative activities of siRNAs were measured by WST-1 assay (Dojindo, Kumamoto, Japan). Appropriate numbers of cells were seeded into 96-well plates 24 h after the transfection of siRNAs. The WST-1 assay was carried out 48 h after seeding, and repeated every 24 h until the 144 h mark. Colorimetric measurements at 450 nm were made in a microplate reader (Thermo Labsystems, Waltham, MA, USA).

Cytochemistry. Cells were seeded in 8-well chamber slides 24 h after the transfection of siRNAs (10 nM). After a 48-h culture, slides were washed with phosphate-buffered saline (PBS) and fixed with 4% para-formaldehyde in PBS. They were then incubated with rhodamine-phalloidin conjugate (10 U/ml) (Life Techonologies) at room temperature for 30 min. After washing with PBS, slides were counterstained with 4,6-diamidino-2-phenylindole, and viewed under fluorescence microscopy.

Matrigel invasion assay. Cell motility and invasion were assayed using BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA). Twenty-four hours after the transfection of *C7orf24*- or *GL3*-siRNA, cells (2.5×10^4) suspended in serum-free DMEM were placed in the upper chamber of 8 μm Control Cell Culture Inserts (BD Biosciences), and DMEM plus 5% FBS was placed in the lower chamber as a source of chemoattractant. Cells were allowed to migrate through a porous, uncoated membrane for 24 h at 37°C. The cells remaining in the upper chamber were then removed with a cotton-tip applicator. The cells on the lower surface were fixed with methanol and stained with 1% toluidine blue. The number of migrating cells was determined by counting in five randomly chosen fields under a magnification of ×100. For invasion assays, cells (2.5×104/well) in serum-free DMEM were seeded in the upper chamber coated with Matrigel. DMEM plus 5% FBS was placed in the lower chamber. Incubation was carried out for 24 h at 37°C. The membrane was processed as described for the motility assay. Cell invasiveness was calculated by dividing the number of cells invading through the Matrigel membrane by the number invading the control insert. Experiments were performed three times in triplicate.

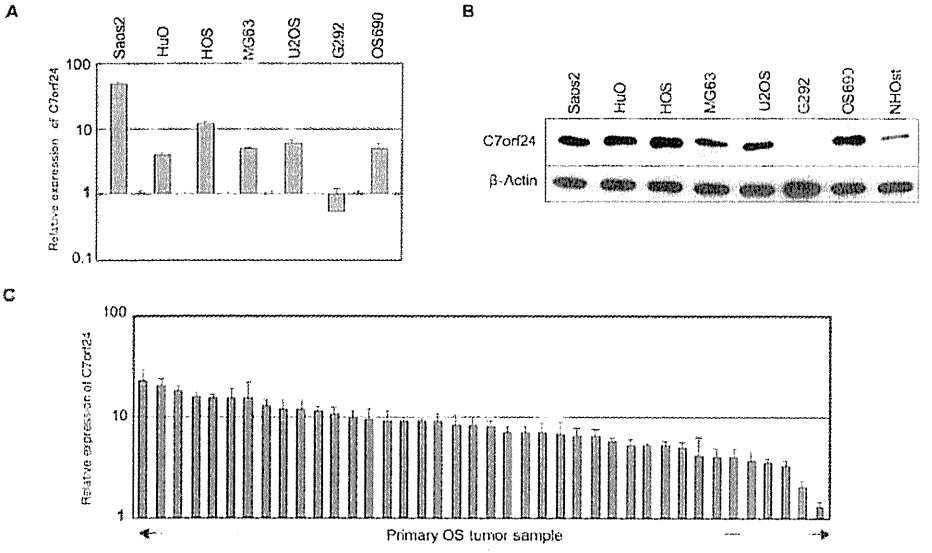

Figure 1. *Expression of C7orf24 in OS cell lines and primary tumors. mRNA expression of the C7orf24 gene in OS cell lines (A) and primary tumors (C). Expression levels of the C7orf24 gene in OS cell lines are indicated relative to that in NHOst. B: Expression of C7orf24 protein in OS cell lines and NHOst.*

Gene expression on transfection with siRNA. RNA was extracted from HOS cells 36 h or 72 h after the transfection of either *C7orf24*- or *GL3*-siRNA (10 nM), and processed for the microarray analyses using a GeneChip Human Genome U133 Plus 2.0 Array containing 54,675 probes (Affymetrix, Santa Clara, CA, USA).

Statistical analysis. Statistical analyses were performed using StatView software (Abacus Concepts Inc., Piscataway, NJ, USA). For comparisons of two individual data points, a two-sided Student's *t*-test was applied to assess statistical significance. An ANOVA with post hoc testing was used for comparisons of more than three data points.

Results

Expression of C7orf24 mRNA in human OS cell lines and primary tumors. Expression of the *C7orf24* mRNA was investigated by RT-qPCR in seven OS cell lines (Saos2, HuO, HOS, MG63, U2OS, G292, and OS690), as well as in normal human osteoblasts (NHOst). Relative to the value in NHOst, the level of *C7orf24* gene expression in the OS cell lines, except for G292 was increased by 3- to 55-fold (Figure 1A). The up-regulation of *C7orf24* expression was further confirmed at the protein level. The amount of C7orf24 protein was higher in OS cell lines, except G292, than in NHOst (Figure 1B), which corresponded to the results of the mRNA analyses (Figure 1A). The expression of *C7orf24* mRNA in tumor tissues was also analyzed by RT-qPCR using 40 primary OS samples. Relative to the expression in NHOst, the level of *C7orf24* was 2- to 24-fold higher in OS tumors (Figure 1C).

Knockdown of the expression of C7orf24 by siRNA. To knockdown the expression of *C7orf24*, three siRNA targeting *C7orf24* were designed and transfected into HOS by lipofection. An siRNA targeting the firefly luciferase gene was used as a control (*GL3*-siRNA). The efficacy of transfection was more than 70% based on the number of positive cells transfected with the fluorescence-labeled gene (data not shown). The expression of *C7orf24* was relatively unchanged in cells transfected with *GL3*-siRNA, but significantly down-regulated in cells transfected with 498- and 570-siRNA at 48 h, and the siRNA remained effective until 96 h after transfection (Figure 2A). Transfection of 498-siRNA also reduced the expression of the *C7orf24* gene in MG63, Saos2, and OS690 (Figure 2B) and also in U2OS, HuO, and G292 (data not shown). Based on these results, 498-siRNA was used in subsequent experiments as *C7orf24*-siRNA.

Down-regulation of C7orf24 expression inhibited the growth of OS cell lines. Either *C7orf24*-siRNA or *GL3*-siRNA was transfected into seven OS cell lines, as well as NHOst, and growth profiles were examined by WST-1 assay. No significant change in growth was observed in any cell line transfected with *GL3*-siRNA (data not shown). The growth profile of NHOst transfected with *C7orf24*-siRNA showed no change even at the highest concentration. G292 cells, which expressed *C7orf24* at the lowest level among the OS cell lines, showed no significant change either. In MG63 cells, the growth-

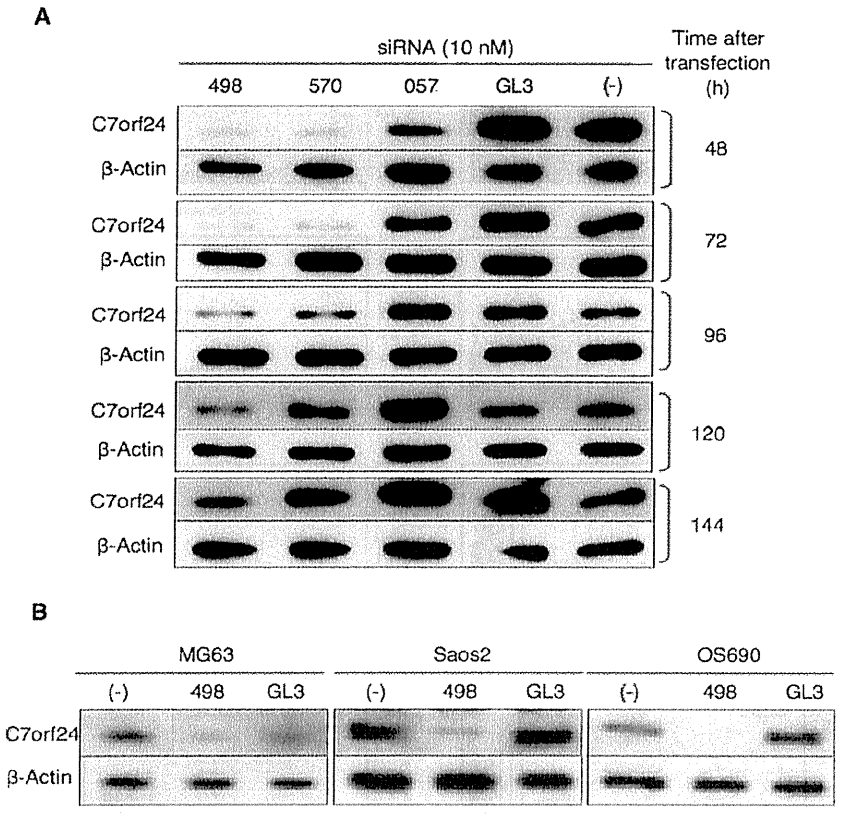

Figure 2. *Knockdown of the expression of C7orf24 by siRNA. A: mRNA expression of the C7orf24 gene in HOS cells after the transfection of siRNAs. RNAs were extracted at the indicated time points after transfection of siRNAs targeting C7orf24 (498, 570 or 057) or GL3. B: mRNA expression of the C7orf24 gene in OS cell lines after the transfection of siRNAs. RNAs were extracted from MG63, Saos2, and OS690 cells 48 h after the transfection of siRNAs targeting C7orf24 (498) or GL3.*

inhibitory effect of *C7orf24*-siRNA was observed only after 144 h at the highest concentration. In contrast, a time- and dose-dependent reduction in growth was observed in the other five OS cell lines (Figure 3).

Down-regulation of C7orf24 expression induced clustering in OS cell lines. Parental HOS cells retained their original spindle shape and proliferated with less cell-to-cell contact until confluent (Figure 4A, left), which was also observed in HOS cells transfected with *GL3*-siRNA (Figure 4A, middle). In contrast, HOS cells transfected with *C7orf24*-siRNA were polygonal to cuboidal in shape and tended to cluster via cell-to-cell attachments (Figure 4A, right). This change was more clearly observed when actin fibers were stained (Figure 4B). The morphological change gradually reversed with time (Figure 4C), which seemed to correspond to the loss of inhibitory effect of siRNA (Figure 1A). Similar but less significant changes were observed in U2OS, SaOS2 and OS690 cells, the growth of which was inhibited even at lower concentrations of *C7orf24*-siRNA (Figure 3). In contrast, no obvious morphological changes were observed in MG63 and G292 cells, in which the growth inhibitory effect of C7orf24 was not remarkable (Figure 3).

Down-regulation of C7orf24 expression reduced the motility and invasiveness of OS cell lines. The morphological changes induced by *C7orf24*-siRNA suggested that *C7orf24* down-regulation affects cell motility. The migration and invasion by HOS cells transfected with either *C7orf24*- or *GL3*-siRNA were evaluated using the matrigel invasion chambers. The number of cells passing through the control membrane was counted as an index of cell motility. The introduction of *C7orf24*-siRNA significantly reduced the number of cells passing through the control membrane (Figure 5A). Cell invasion was also assayed using a matrigel-coated membrane. As well as cell motility, cell invasion was reduced by the introduction of *C7orf24*-siRNA (Figure 5B).

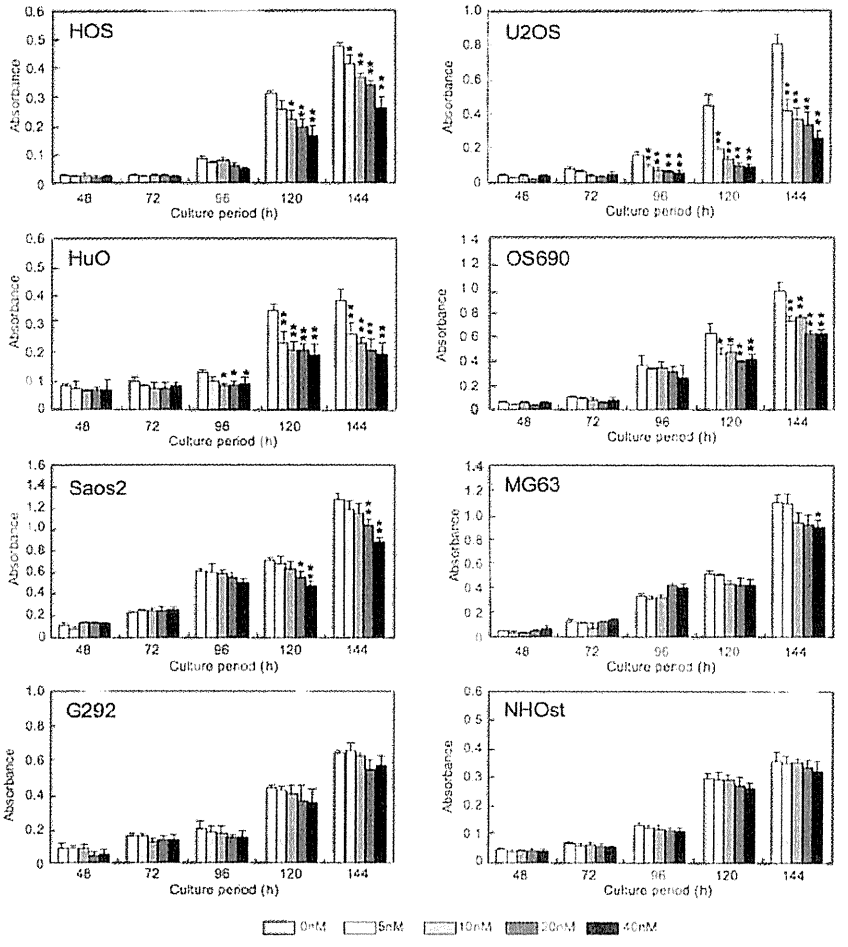

Figure 3. *Growth profiles of OS cell lines treated with C7orf24-siRNA. C7orf24-siRNA in the indicated concentration was transfected into OS, as well as NHOst, cell lines. Cell numbers were evaluated by the WST1 assay 48 h after transfection and every 24 h thereafter. *p<0.05; **p<0.01.*

Genes up- or down-regulated by the knockdown of C7orf24. To elucidate the functional relevance of *C7orf24*, the gene expression profiles of HOS cells transfected with *C7orf24*-siRNA and *GL3*-siRNA were compared, and genes up- or down-regulated by the knockdown of *C7orf24* were identified. RNAs were isolated at two time points (36 and 72 h after transfection of each siRNA), and used for the Affymetrix gene chip. The criteria for up- and down-regulated genes were an expression level higher by more than two-fold and lower by less than half in *C7orf24*-siRNA-treated cells than in *GL3*-siRNA-treated cells at both time points, respectively. One hundred and ninety-seven genes were identified as being up-regulated, and the ontological analyses revealed that the biological function of the genes with the highest *p*-value was cell adhesion followed by system development (Table I). Two hundred and seventy-seven genes were identified as being down-regulated, and the biological function of the genes with the highest *p*-value was intracellular protein transport followed by protein localization (Table I).

Discussion

We performed a proteomic analysis of bladder cancer using narrow range pH two-dimensional gel electrophoresis (2DE) to find new proteins that can be used for cancer diagnosis or treatment (14). Fifteen spots were identified as proteins up-regulated in cancerous tissue, including C7orf24. Functional analyses using expression vectors and siRNA revealed that C7orf24 was involved in the growth of cancer cells (14). Xu *et al.* identified *C7orf24* as 1 out of 46 genes that form a common cancer signature in a study that directly merged