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expression of Tyr527-phosphorylated SRC was detectable. Expression levels for CSK were strong in 30%, moderate in 17%, and weak in 50% of the samples, no CSK expression was found in one synovial sarcoma. Expression levels for PTP1B were strong in 13%, moderate in 37%, and weak in 40% of the samples, no expression of PTP1B was found in 10% of the samples. No significant correlation between SRC phosphorylation status and CSK or PTP1B protein expression levels was detected. Tyr416-phosphorylated SRC expression in the tumor samples did not correlate with the patients' age, sex, tumor location, and tumor size or tumor grade. As in the immunohistochemical analysis, no correlation between SRC activity and PTP1B or CSK levels was observed in the cell lines analyzed in Western blots (Fig. 1 and data not shown).

SRC activation in synovial sarcoma is induced by SS18/SSX translocation

To functionally understand the mechanism of SRC activation in synovial sarcoma, T-REx-293 cells were stably transfected with vectors containing *SS18/SSX1*, *SS18/SSX2*, *SSX1*, *SSX2*, or *SS18* cDNA to obtain an inducible cell culture model of the synovial sarcoma–specific chimeric translocation proteins. Western blot analysis showed elevated levels of activated p-(Tyr416)-SRC in T-REx-293 cells transfected with *SS18/SSX1* and *SS18/SSX2* (Fig. 2A); expression levels of PTP1B and CSK were not affected (data not shown). As it has been shown previously that receptor tyrosine kinase pathways including IGF-IR signaling are of particular importance in synovial sarcomas, we analyzed promoter-specific expression levels of *IGF2* showing upregulation of promoter P2- and P4-dependent *IGF2* transcripts in *SS18/SSX1* and *SS18/SSX2*-expressing T-REx-293 cells (Fig. 2B). Stimulation of 1273/99, FUJI and CME-1 synovial sarcoma cells with recombinant human IGF-II protein was associated with an increase of phosphorylation of IGF-IR at Tyr1131, AKT at Ser473, and SRC at Tyr416, which revealed IGF-IR signaling as a functionally relevant mechanism leading to SRC activation (Fig. 2C). A minor induction of phosphorylation of SRC at Tyr416 was observed upon SS18 overexpression alone as well; however, this activation was not associated with *IGF2* transcriptional induction. Inversely, siRNA knockdown of the IGF-IR in CME-1 cells was associated with a significant decrease of p-(Tyr416)-SRC levels (Supplementary Fig. S2).

SRC inhibition by dasatinib or RNA interference impairs growth of synovial sarcoma cells

siRNA-mediated knockdown of SRC resulted in a significant decrease of growth of CME-1 and 1273/99 cells in MTT assays (*t* test: $P < 0.001$; Fig. 3A and data not shown). All analyzed synovial sarcoma cell lines displayed dose-dependent growth inhibition upon treatment with the SRC inhibitor dasatinib (Fig. 3B). This effect was particularly distinct in nanomolar concentrations of the inhibitor. Among the 4 synovial sarcoma cell lines investigated CME-1 ($GI_{50} = 0.008$ μmol/L), FUJI ($GI_{50} = 0.01$ μmol/L), and SYO-1 ($GI_{50} = 0.013$ μmol/L) were found to be slightly more sensitive to dasatinib than 1273/99 cells ($GI_{50} = 0.077$ μmol/L).

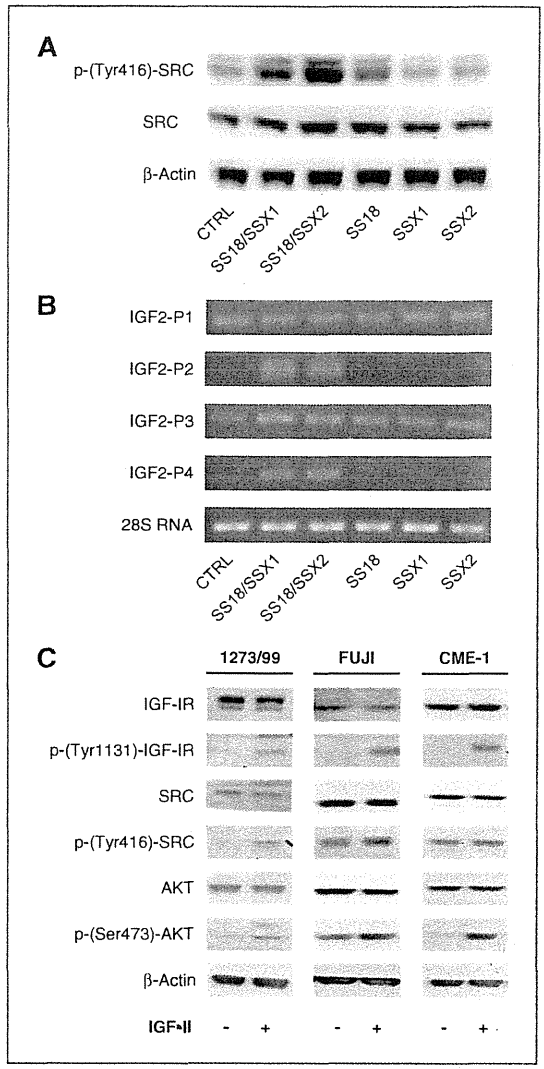

Figure 2. A, elevated levels of activated p-(Tyr416)-SRC in T-REx-293 cells expressing *SS18/SSX1* and *SS18/SSX2*. B, induction of promoter P2- and P4-dependent *IGF2* transcripts in *SS18/SSX1*- and *SS18/SSX2*-expressing T-REx-293 cells. C, induction of phosphorylation of IGF-IR (Tyr1131), SRC (Tyr416), and AKT (Ser473) upon stimulation of 1273/99, FUJI, and CME-1 cells with IGF-II.

Inhibition of SRC affects phosphorylation of its interaction partners

To assess the effect of SRC inhibition on its interaction partners in synovial sarcomas, cells were treated with increasing concentrations of dasatinib (0.01–3 μmol/L) for 60 minutes (Fig. 3C). Dose-dependent dephosphorylation of p-(Tyr416)-SRC, p-(Ser473)-AKT, p-(Tyr576/577)-FAK, p-(Tyr705)-STAT3, and p-(Tyr1131)-IGF-IR was observed in all synovial sarcoma cell lines with nanomolar concentrations of dasatinib. Similarly, siRNA knockdown of SRC led to the dephosphorylation of

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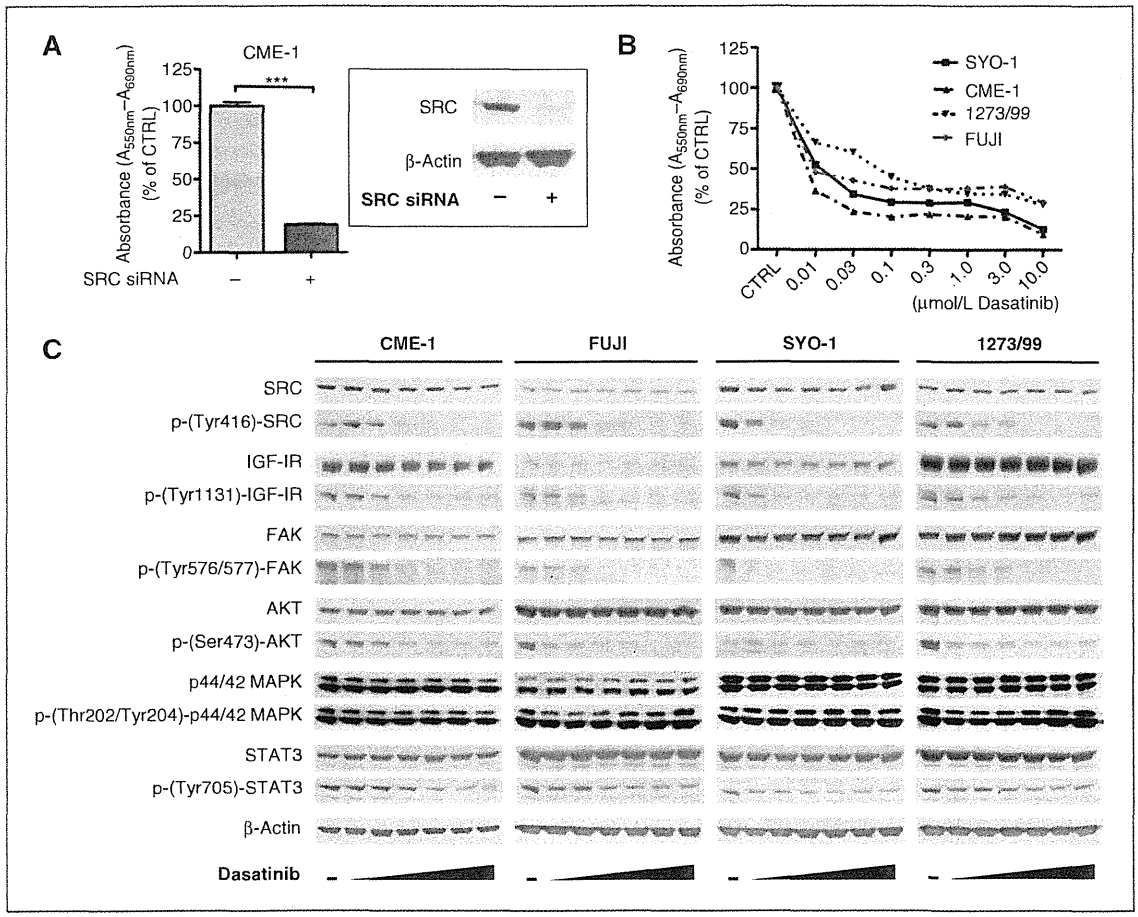

Figure 3. A, significant growth inhibition (MTT proliferation assay) upon siRNA-mediated knockdown of SRC (inset) in CME-1 synovial sarcoma cells. ***, $P < 0.001$, Student t test. B, dose-dependent growth inhibition (MTT proliferation assay) in 4 synovial sarcoma cell lines treated with the SRC inhibitor dasatinib. C, dose-dependent dephosphorylation of p-(Tyr416)-SRC, p-(Tyr1131)-IGF-IR, p-(Tyr576/577)-FAK, p-(Ser473)-AKT, and p-(Tyr705)-STAT3 in synovial sarcoma cells upon treatment with increasing concentrations of dasatinib (0.01–3 μmol/L).

FAK (data not shown). Interestingly, p-(Thr202/Tyr204)-p44/42 MAPK levels increased in 1273/99 and FUJI after treatment with higher doses of dasatinib.

Dasatinib treatment increases apoptosis and decreases mitotic rate in synovial sarcoma cells

To determine the effect of dasatinib on the apoptotic and mitotic rate of synovial sarcoma cells, flow cytometric analyses were conducted. Cleaved PARP (Asp214) was used as a marker of apoptosis, and phospho-(Ser10)-histone H3 was used as a marker of mitosis. CME-1, 1273/99, and SYO-1 cell lines showed significantly increased rates of apoptosis and decreased mitotic fractions after treatment with dasatinib in concentrations of 0.1 and 0.6 μmol/L (Fig. 4A, Supplementary Table S2). These results were confirmed by microscopic analyses of DAPI-stained synovial sarcoma cells treated with dasatinib, showing increasing rates of chromatin condensation and fragmentation in a dose-dependent manner in CME-1 and SYO-1 cells (Fig. 4B). As an indicator of SRC specificity of the effects observed, dasatinib treatment of CME-1 cells after SRC knockdown did not show significant effects in terms of proliferation and apoptosis in flow cytometry (Supplementary Fig. S1).

Combination of SRC inhibition and conventional chemotherapy results in additive effects on cell growth

To determine the effects of combinations of conventional chemotherapy (vincristine, doxorubicin, actinomycin D) and dasatinib on the growth of synovial sarcoma cells, we investigated 1273/99 cells, which were the least responsive to monotreatments with dasatinib. Cells were exposed to increasing concentrations of conventional cytotoxic drugs and to a concentration of dasatinib that led to 20% to 30% growth inhibition after 72 hours. The combinations did not fulfill the

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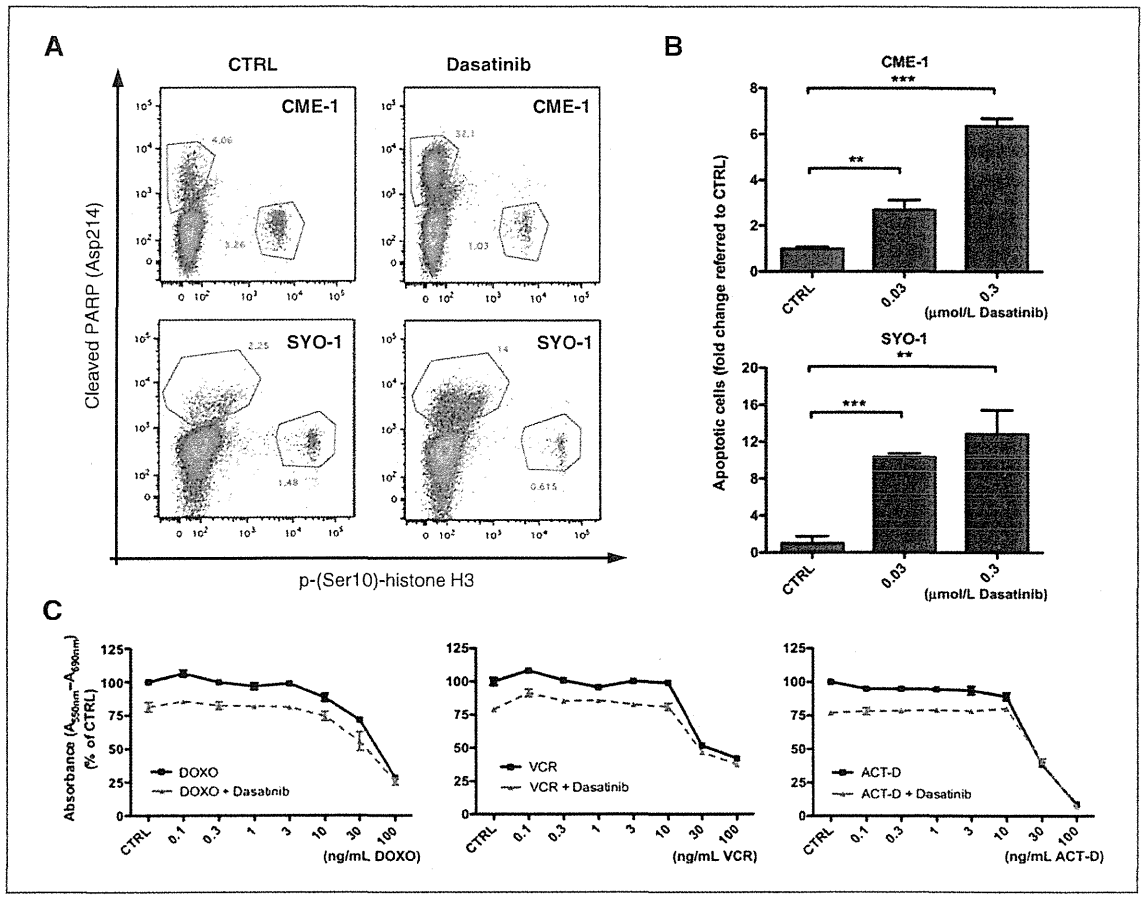

Figure 4. A, significantly increased rate of apoptosis [cleaved PARP (Asp214)] and decreased mitotic fraction [phospho-(Ser10)-histone H3] in CME-1 and SYO-1 synovial sarcoma cells upon treatment with 0.6 µmol/L dasatinib as determined by flow cytometry. B, microscopic analyses of DAPI-stained cells concerning chromatin condensation and fragmentation. **, $P < 0.01$; ***, $P < 0.001$, Student t test. C, coincubation of 1273/99 cells with conventional cytotoxic drugs and 0.0075 µmol/L dasatinib resulted in additive effects.

criteria of synergy as defined above; the effects observed resulted from an additive, obviously independent action of the SRC inhibitor and conventional chemotherapeutic agents (Fig. 4C).

Dasatinib inhibits motility and invasive potential of synovial sarcoma cells associated with an increased activity of RhoA and diminished Rac activity

As SRC has been shown to modulate motility and invasiveness of tumor cells, we investigated the effect of SRC inhibition by dasatinib on cell migration and invasion. CME-1, SYO-1, and 1273/99 synovial sarcoma cells (treated with dasatinib in doses not affecting cell viability) showed a dose-dependent decrease of migratory and invasive potential in Boyden chamber and invasion chamber assays. Accordingly, wound healing was impaired in scratch assays in CME-1 and SYO-1 treated with dasatinib. In ELISA-based RhoA and Rac activation assays, treatment with dasatinib resulted in significantly increased levels of activated RhoA and decreased levels of activated Rac in SYO-1 and CME-1 cells (Fig. 5A–D, data not shown). As an indicator of SRC specificity of the effects observed, dasatinib treatment of CME-1 cells after SRC knockdown did not show significant effects in terms of migration and invasion in Boyden chamber and invasion chamber assays (Supplementary Fig. S1).

Dasatinib displays antitumor activity in synovial sarcoma xenografts *in vivo*

The antitumor activity of dasatinib was tested *in vivo* in a xenograft model of SYO-1 synovial sarcoma cells. The inhibitor significantly reduced tumor growth rate (Fig. 6A). No significant changes in the weight of the tumor-bearing mice were observed (data not shown). Consistent with the *in vitro* results, treatment was associated with diminished levels of Tyr416-phosphorylated SRC, a significant reduction of the mitotic fraction (t test: $P < 0.001$) and a significant increase of the

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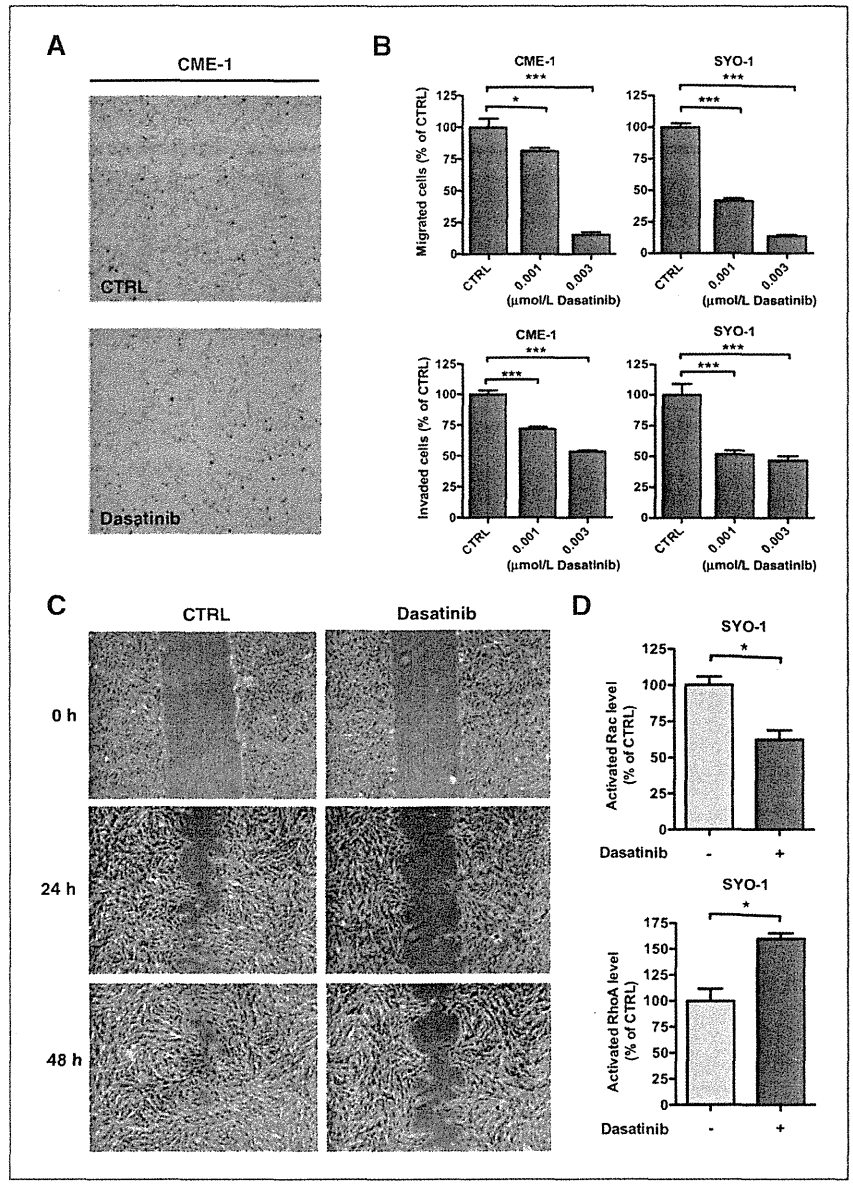

Figure 5. Inhibition of cellular motility and invasiveness of synovial sarcoma cells treated with dasatinib. A, Boyden chamber membranes of CME-1 cells treated with 0.003 μmol/L dasatinib for 24 hours. A and B, comparable reduction of motility and invasiveness in invasion chamber assays. C, representative wound scratches in SYO-1 synovial sarcoma cells treated with 0.003 μmol/L dasatinib (original magnification, ×20). D, significantly increased levels of activated RhoA and decreased levels of activated Rac in SYO-1 cells treated with dasatinib. *, $P < 0.05$; ***, $P < 0.001$, Student t test.

apoptotic fraction (t test: $P < 0.001$) compared with control tumors (Fig. 6B and C).

Discussion

Considerable progress has been made in the understanding of soft-tissue tumors in the recent years. However, apart from few examples such as c-KIT or platelet-derived growth factor (PDGF) receptor inhibition in GIST and dermatofibrosarcoma protuberans (5, 6), the translation of molecular results into clinical care in terms of molecularly based therapies is still rare in this group of neoplasias. Despite elaborate treatment protocols involving radical surgery and standardized chemo- and radiotherapy, prognosis is poor in advanced cases of synovial sarcoma. Therefore, the identification of molecular targets, which are at the same time biologically essential and accessible to specific therapeutic drugs, represents an important issue for the development of innovative therapeutic approaches.

On the basis of a phosphokinase screen, we identified the SRC tyrosine kinase as one of the most strongly phosphorylated kinases in synovial sarcoma cells. Its particular relevance was confirmed immunohistochemically in biopsies of 30 synovial sarcomas, in which Tyr416-phosphorylated, that is activated,

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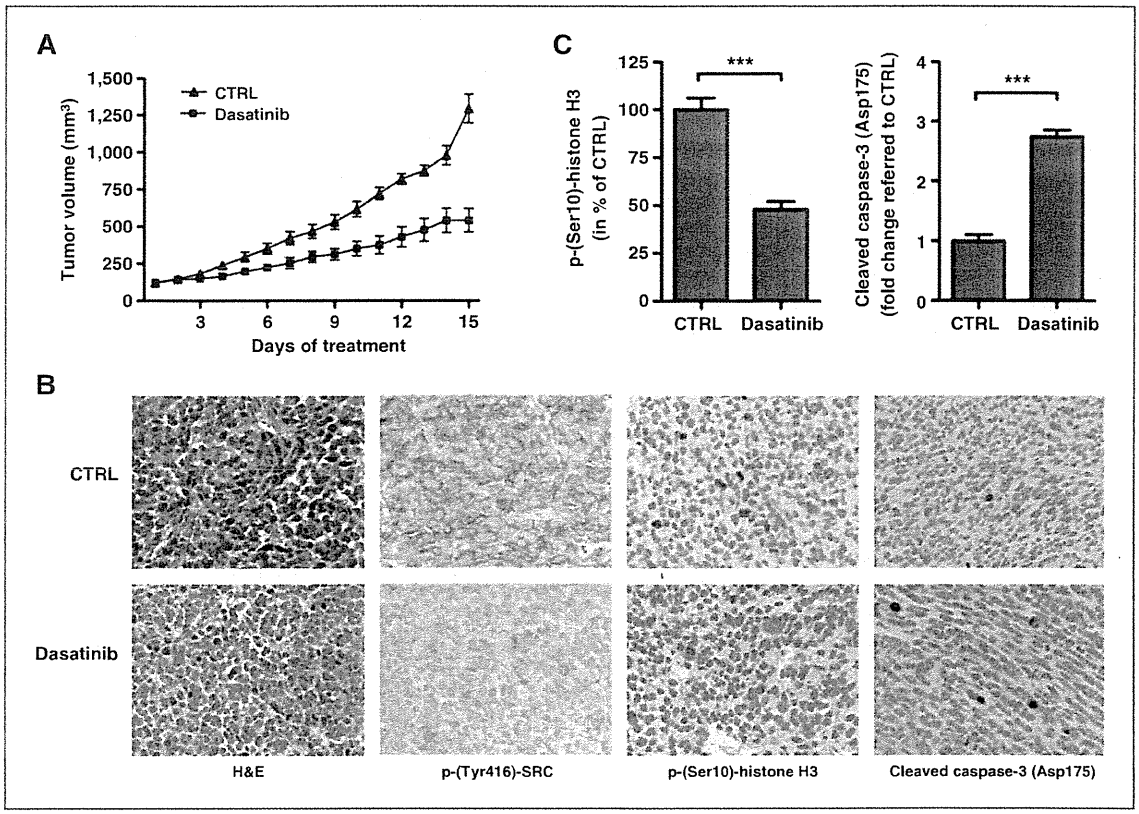

Figure 6. A, significantly reduced tumor growth *in vivo* in dasatinib-treated SYO-1 xenografts associated with diminished levels (B, C) of Tyr416-phosphorylated SRC, a reduction of the p-(Ser10)-histone H3–positive mitotic cell fraction, and an induction of the cleaved caspase-3 (Asp175)–positive apoptotic cell fraction. ***, $P < 0.001$, Student *t* test.

SRC was found to be expressed in the majority of the cases. A consistent pattern of dysregulation of the SRC-regulating proteins CSK and PTP1B, analogous to what has been shown in some epithelial tumors could be excluded in synovial sarcomas (15–17, 30). Interestingly, SRC was found to be activated through the SS18/SSX translocation proteins. This activation was associated with an IGF-IR–dependent mechanism based on transcriptional induction of *IGF2*, which links SRC activation to the characteristic molecular aberration of synovial sarcomas. As shown, expression of the transcriptional cofactor SS18 alone is capable to (indirectly) induce SRC phosphorylation at lower levels as well; however, this appears to be independent from *IGF2* induction. This finding underlines the oncogenic character of the SS18/SSX fusion proteins and distinguishes components of the IGF/SRC context from other therapeutic targets as molecularly based and tumor-specific. However, the finding of consistent expression of further growth factor receptors, such as PDGFR and EGFR, in synovial sarcomas makes it probable that other than IGF-IR–dependent pathways may mediate SRC activation in synovial sarcomas as well (31). Considering the IGF-IR and the SRC kinases as potential therapeutic targets, its central position within different oncogenic signaling pathways makes SRC an attractive candidate for specifically directed approaches. As shown here, synovial sarcomas display a fundamental dependence on SRC signals with regard to cellular proliferation and survival. This was observed *in vitro* in siRNA-mediated approaches and after pharmacologic intervention with the SRC inhibitor dasatinib as well as *in vivo* in murine synovial sarcoma xenografts. A role for dasatinib is clinically well-established in the treatment of chronic myelogenous leukemia and Philadelphia chromosome–positive acute lymphoblastic leukemia (ALL), in which the substance inhibits Abl kinases (32, 33). Effectivity of dasatinib has previously been shown for cells derived from solid tumors as well, including mesenchymal neoplasias, such as GIST and chondrosarcoma (34, 35). In chondrosarcoma, growth effects observed upon treatment with dasatinib were not consistently associated with diminished p-(Tyr416)-SRC levels, which makes SRC-independent modes of action probable (35). In contrast, in synovial sarcomas, dephosphorylation of SRC and its targets was a consistent feature detectable upon treatment with dasatinib. The low drug dosages resulting in dephosphorylation of the SRC targets argue in favor of SRC-dependent effects and against effects exerted through direct interaction of dasatinib with IGF-IR, FAK, and AKT (36). As an indirect proof of specific SRC-related

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action of dasatinib in synovial sarcoma cells, CME-1 did not display any significant effects upon dasatinib treatment after siRNA-mediated SRC knockdown. Interestingly, SRC inhibition was associated with a loss of phosphorylation of the IGF-IR at Tyr1131, which indicates activation of IGF-IR tyrosine kinase activity usually detectable upon ligand binding. This finding is particularly relevant for the option of IGF-IR directed therapeutic approaches, which have been proposed for synovial sarcomas recently (20, 37). Beyond that, because of the central position of SRC within intracellular signaling networks and its obvious capacity of cross-activating pathways as documented here, it is conceivable that targeting SRC as a central component integrating different signaling activities might be advantageous compared with individual receptor-directed approaches. As shown here, combined treatment of synovial sarcoma cells with chemotherapeutic drugs and dasatinib results in additive but not in synergistic effects. Therefore, SRC inhibitors might be useful in innovative therapeutic approaches, in which targeting of an activated pathway with specific inhibitory substances allows the reduction of the individual compounds' dosages, thereby minimizing toxicity. In our *in vivo* experiments, dasatinib was found to be highly effective with regard to tumor growth and was well tolerated by the animals.

As known for a variety of epithelial tumors (36, 38), on the basis of our data, the SRC signaling network appears to be of crucial relevance for cellular migration and invasion in synovial sarcomas. In all assays applied here, doses of dasatinib, which did not affect cellular proliferation, resulted in significantly impaired migratory and invasive capacities. These effects were associated with an SRC-dependent shift in activation levels of Rac and RhoA, small GTPases essentially involved in the regulation of cell mobility processes. This finding provides a functional background of the effects observed here and substantiates specificity, as increased levels of activated RhoA are associated with stress fiber formation, whereas diminished levels of activated Rac go along with the impairment of a "motile" phenotype (18). This finding is of particular importance with regard to therapeutic concepts, as prognostically unfavorable cases of synovial sarcomas frequently develop metastases. Using a dual-inhibition approach of SRC and Aurora kinases by SU6656, Arai and colleagues recently observed high antitumor effectiveness in synovial sarcoma xenografts involving antiangiogenic mechanisms. This finding provides further evidence of the crucial role of SRC with regard to complex aspects of tumor biology and underlines its role in a oncogenic signaling network (39).

In summary, our data in detail substantiate previous findings on the relevance of SRC in synovial sarcomas (40). For the first time, it is systematically shown that the SRC signaling network is commonly activated in synovial sarcomas and that targeting SRC results in substantial effects on tumor cell growth and motility. These findings argue in favor of SRC as a potential therapeutic target in synovial sarcomas.

Disclosure of Potential Conflicts of Interest

E. Wardelmann has honoraria from speakers' bureau from Novartis Oncology, MSD, and Eisai and is a consultant/advisory board member for Novartis Oncology and MSD. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Michels, E. Sievers, M. Renner, N. Friedrichs, P. Wurst, R. Penzel, O. Larsson, S. Tanaka, G. Mechtersheimer, E. Wardelmann, R. Büttner, W. Hartmann
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Michels, M. Trautmann, D. Kindler, S. Huss, P. Wurst, L. Heukamp, R. Penzel, R. Büttner, W. Hartmann
Writing, review, and/or revision of the manuscript: S. Michels, M. Trautmann, E. Sievers, D. Kindler, M. Renner, N. Friedrichs, O. Larsson, P. Schirmacher, E. Wardelmann, R. Büttner, W. Hartmann
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Trautmann, J. Kirfel, S. Steiner, A. Kawai, H. Sonobe, P. Schirmacher
Study supervision: W. Hartmann

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ORIGINAL ARTICLE – BONE AND SOFT TISSUE SARCOMAS

Prognostic Factors in Elderly Osteosarcoma Patients: A Multi-institutional Retrospective Study of 86 Cases

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ABSTRACT

Background. The occurrence of osteosarcoma in elderly patients has recently been increasing, and the outcome is poor. This multi-institutional retrospective study was conducted to investigate clinical features and prognostic factors in patients older than 40 years with osteosarcoma.

Methods. Patients with conventional high-grade osteosarcoma older than 40 years were recruited to this study. Secondary osteosarcoma arising from Paget's disease or irradiated bones was excluded. Information on tumor- and treatment-related factors was collected and statistically analyzed. The median follow-up was 57 months (range 8–244 months) for all surviving patients.

Results. A total of 86 patients were enrolled in this study. The median age at diagnosis was 61 years. Surgery and chemotherapy were conducted in 73 and 63 % of all patients, respectively. The 5-year overall and event-free survival rates were 38.8 and 34.0 %, respectively. Tumor site (extremity 57.9 %; axial 19.0 %; $p < 0.0001$), metastasis at diagnosis (yes 12.2 %; no 48.3 %; $p < 0.0091$), and definitive surgery (yes 56.2 %; no 10.6 %; $p < 0.0001$) were associated with overall survival. Although patients without metastasis who received definitive surgery were regarded as good candidates for chemotherapy, the addition of chemotherapy did not have any impact on the outcome (yes 63.4 %; no 65.2 %; $p = 0.511$).

Conclusions. The present study revealed the distinct clinical features, such as the high incidence of truncal tumors or metastasis at diagnosis, in patients older than 40 years with osteosarcoma. Additionally, prognostic factor analyses revealed that tumor site, metastasis at diagnosis, definitive surgery, and surgical margins were significant prognostic factors, whereas chemotherapy did not influence survival.

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Osteosarcoma, the most commonly diagnosed primary malignant bone tumor, has two peaks of incidence in early adolescence and the elderly. As a result of the rapid aging of the population and falling birthrates, osteosarcoma has been increasing in patients older than 40 years in Japan over the last 30 years, and ~30 % of all osteosarcoma patients were older than 40 years in 2007.[1] Most developed countries share these problems, and the need for a study on this specific patient group has been increasing.

Several reports have described the clinicopathological features, outcomes, and prognostic factors of osteosarcoma in the elderly.[2–10] Patients older than 40 years with osteosarcoma exhibit different clinical features than those of adolescents. However, regardless of multidisciplinary treatment including surgery and chemotherapy, the outcomes of elderly osteosarcoma patients are still poor, and no standard treatment strategy has been established. The efficacy of chemotherapy in this group of patients in particular is still controversial.[2–4,6,7,9]

The aim of this study was to investigate the clinical features and prognostic factors of patients older than 40 years with osteosarcoma.

PATIENTS AND METHODS

This study was designed as a multi-institutional retrospective study and carried out by the Higashi-nihon Orthopaedic and Pediatric Sarcoma group (HOPES). Three

tertiary musculoskeletal oncology hospitals, Chiba Cancer Center, National Cancer Center Hospital, and Kanagawa Cancer Center, participated in this study.

We retrospectively reviewed the records of each institute between November 1990 and January 2010. Patient eligibility criteria were: (1) diagnosis of conventional high-grade osteosarcoma with pathological confirmation by a musculoskeletal tumor pathologist at each institute; secondary osteosarcoma arising from Paget's disease or irradiated bones was excluded from this study; and (2) age at diagnosis older than 40 years. The rationale for the cutoff of older than 40 in this study was that most previous clinical trials for osteosarcoma set the inclusion criteria up to 40 years. We excluded patients without sufficient information. This study was approved by the Institutional Review Board at Chiba Cancer Center, and a waiver of informed consent was provided.

Information on tumor-related factors (age, sex, site of primary lesions, American Joint Committee on Cancer (AJCC) staging, metastasis at diagnosis) and treatment-related factors (type of local therapy, chemotherapy status), local and distant relapse, follow-up period, and outcome were collected anonymously.[11] Additional information, such as the surgical margin (Enneking criteria) in those who had surgery, the setting and course number of chemotherapies, radiological evaluation (RECIST1.1), and histological evaluation (Huvos criteria) in those who had chemotherapy, were also obtained.[12–14] The median follow-up period was 57 months (range 8–244 months) for all surviving patients. The 52 patients who died had a median follow-up of 18 months (range 1–203 months).

Overall survival (OAS) was defined as the time period from the date of diagnosis to that of death or the last follow-up. Event-free survival (EFS) was defined as the time period from the date of diagnosis to that of disease progression, recurrence, death from any cause, or the last follow-up for patients without events. OAS and EFS were calculated using the Kaplan–Meier product limit method. Differences in survival were assessed by the log-rank test and Cox proportional hazard regression method. Differences were considered significant when p values were <0.05. Statistical analysis was performed using JMP software (SAS Institute, Cary, NC, USA).

RESULTS

Patient Characteristics and Treatment

A total of 86 patients were enrolled in this study. The 39 men and 47 women had a median age of 61 years (range 41–87 years) at presentation. The sites of primary lesions were the extremities in 44 patients (51 %) and axial sites in 42 (49 %) including the pelvis in 27 (31 %) and trunk in 15 (17 %). According to the AJCC staging system, 15 patients were classified as IIA, 45 were IIB, 2 were III, 19 were IVA, and 5 were IVB, which indicated that metastasis at diagnosis was observed in 28 % of all patients.

Surgical treatment was performed in a total of 63 patients (73 %): 55 patients had surgery, and 8 received a combination of surgery and radiotherapy. Conventional radiotherapy and carbon-ion radiotherapy (conducted at the National Institute of Radiological Sciences, Chiba, Japan) were performed as a local treatment in 9 patients each.[15,16] Five patients did not receive any local treatment. Information of surgical margin analysis was available in 59 of the 63 patients who underwent surgery. An adequate margin, including a radical and wide margin, was achieved in 45 patients (71 %), and an inadequate margin, including a marginal and intralesional margin, was noted in 14 (22 %). From the viewpoint of surgical planning, we defined definitive surgery as surgery that was intended to achieve an adequate margin. Therefore, 52 patients (60 %) underwent definitive surgery.

Chemotherapy was given to a total of 54 patients (63 %): neoadjuvant chemotherapy, adjuvant chemotherapy, and chemotherapy after relapse were conducted in 20, 25, and 9 patients, respectively. The main reason for not giving chemotherapy was patient refusal. Another reason was serious comorbidity such as a renal failure or cardiac dysfunction. Various chemotherapy regimens including high-dose methotrexate, cisplatinum-doxorubicin, ifosfamide, and ifosfamide-doxorubicin were administered, and the median course number of chemotherapies was 6 (range 1–18). The details of chemotherapy received are shown in Table 1. Information on radiological evaluations after neoadjuvant chemotherapy or chemotherapy after relapse was available in 17 patients, and PR, SD, and PD were observed in 6, 6, and 5 patients, respectively. Histological evaluations were performed in 14 patients, who consisted of 4 good responders including grade 3 or 4, and 10 poor responders including grade 1 or 2.

Outcome

In total, 34 of 86 patients (40 %) were still alive at the time of analysis; 44 patients died of disease and 4 died of other causes. We could not follow up the outcomes of 4 patients at the time of analysis.

Local failure was observed in 12 patients (14 %). Of these, local treatment consisted of surgery in 8 patients, a combination of surgery and radiotherapy in 2, conventional radiotherapy in 1, and carbon-ion radiotherapy in 1. Distant failure was observed in 41 patients (48 %), in which the first site of metastasis was the lung in 33 patients, bone in 6, and both in 2. Thus, the 5-year OAS and EFS rates were

38.8 % (95 % CI 28.3–50.4) and 34.0 % (95 % CI 24.3–45.3), respectively (Fig. 1).

Prognostic Factor Analyses

Univariate analysis for potential prognostic factors for the 5-year OAS was performed in all 86 patients (Table 2). The sites of primary lesions, presence or absence of metastasis at diagnosis, and with or without definitive surgery were significantly correlated with OAS and EFS. Among the patients who underwent surgery, the surgical margin was a significant prognostic factor for OAS and EFS ($p = 0.0137$ and 0.026, respectively). Interestingly, subclass analysis with patients who had no metastasis at diagnosis and received definitive surgery and, thus, were regarded as good candidates for chemotherapy, demonstrated that the addition of adjuvant/neoadjuvant chemotherapy had no impact on the outcome (OAS $p = 0.511$, EFS $p = 0.923$) (Table 3).

Multivariate analysis revealed that metastasis at diagnosis and no definitive surgery was significantly correlated with poor OAS (hazard ratio [HR] = 2.29, $p = 0.0272$; HR = 2.24, $p = 0.0176$, respectively). However, chemotherapy did not significantly affect OAS or EFS ($p = 0.168$ and 0.227, respectively) (Table 4).

TABLE 1 Details of the various chemotherapy regimens used

Regimens	Patient numbers	Age (mean)
High-dose methotrexate	18	41–70 (51)
Cisplatinum–doxorubicin	25	41–72 (54)
Ifosfamide	31	41–73 (56)
Doxorubicin	7	45–68 (65)
Ifosfamide–doxorubicin	11	41–60 (53)
Doxorubicin–cyclophosphamide	10	47–77 (67)

The clinical characteristics between patients who did or did not receive adjuvant/neoadjuvant chemotherapy were compared among patients who underwent definitive surgery without metastasis at diagnosis (Table 5). The no chemotherapy group showed a relatively higher age, female dominance, or smaller tumor size. Relapse occurred equally, and outcomes were also similar in spite of the lower percentage of treatment after relapse in the no chemotherapy group.

DISCUSSION

In the present study, we reviewed the records of 86 osteosarcoma patients older than 40 years treated at 3 tertiary musculoskeletal oncology hospitals to demonstrate clinical features and prognostic factors. Our patient cohort showed disease characteristics distinct from common osteosarcomas arising in younger patients. In addition, our results of prognostic factor analyses indicated that the site of primary lesions, metastasis at diagnosis, definitive surgery, and surgical margins were significant prognostic factors, whereas chemotherapy did not influence survival.

Several studies have shown that patients with osteosarcoma older than 40 years exhibit various clinical features compared with adolescents.[2–10,17] Consistent with previous reports, tumors in axial sites were more frequent compared with younger patients.[3,4,6,9,10] The incidence of axial osteosarcoma in elderly patients has been reported as 19–38.2 %. Special features of osteosarcoma in elderly patients arising in association with Paget's disease or those developing after irradiation contribute to these findings to some extent.[18,19] Metastasis at diagnosis has also been frequently observed in elderly patients, with an incidence between 5 and 33 %.[2–4,6,7,9] In the present study, almost half of the patients had a tumor at an axial site, despite the exclusion of disease related to Paget's disease or irradiated

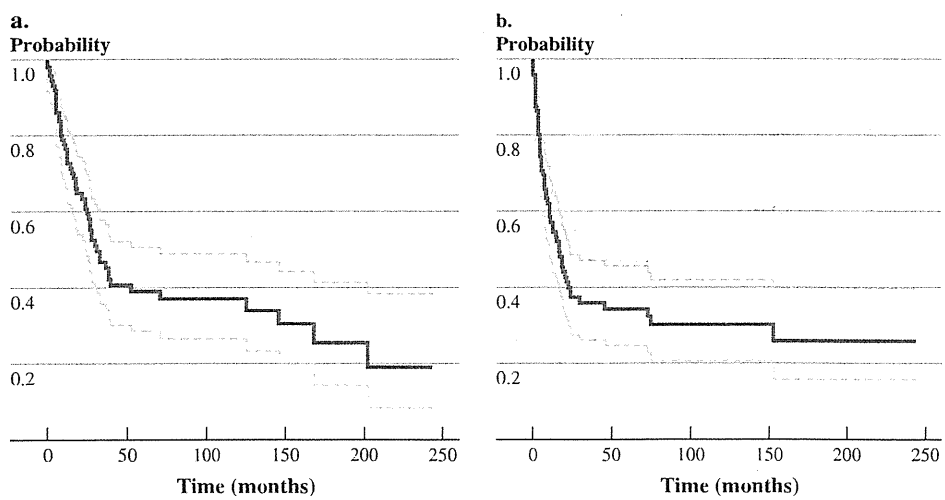

FIG. 1 Kaplan–Meier survival curve for (**a**) overall and (**b**) event-free survival. *Solid line* is median survival; *dotted lines* represent upper and lower 95 % CI

TABLE 2 Univariate analysis investigating prognostic factors with all patients

Factors	*N* (%)	OAS		EFS	
		5-year	*p* value	5-year	*p* value
Age					
40–55 years	30 (35)	46.7	0.180	36.4	0.123
56–70 years	39 (45)	41.8		40.0	
71 years and over	17 (20)	9.7		15.5	
Sex					
Male	39 (45)	43.6	0.691	35.7	0.845
Female	47 (55)	34.3		33.0	
Site of primary lesions					
Extremity	44 (51)	57.9	<0.0001*	46.0	0.0023*
Axial	42 (49)	19.0		21.5	
Metastasis at diagnosis					
No	62 (72)	48.3	0.0091*	41.1	0.0206*
Yes	24 (28)		12.2	15.5	
Definitive surgery					
Yes	52 (60)	56.2	<0.0001*	46.1	0.0006*
No	34 (40)	10.6		16.1	

OAS overall survival, *EFS* event-free survival

* Statistically significant

TABLE 3 Subclass analysis with patients who underwent surgery and definitive surgery without metastasis

Factors	*N* (%)	OAS		EFS	
		5-year	*p* value	5-year	*p* value
Patients who underwent surgery ($n = 63$)					
Surgical margins					
Adequate	45 (71)	57.0	0.0137*	44.7	0.0262*
Inadequate	14 (22)	0		0	
Not assessed	4 (7)				
Patients who underwent definitive surgery without metastasis ($n = 40$)					
Adjuvant/neoadjuvant chemotherapy					
Yes	21 (53)	63.4	0.511	54.4	0.923
No	19 (47)	65.2		49.4	

OAS overall survival, *EFS* event-free survival

* Statistically significant

bones. Additionally, a quarter of the patients had metastasis at diagnosis, which was relatively high. Bielack et al.[20] reported in the results of a COSS study that patients with osteosarcoma of the axial skeleton were older and more likely to show metastases at diagnosis than those with extremity osteosarcoma. Many studies have demonstrated that elderly patients with osteosarcoma show a poor prognosis, which may be explained by those distinct features of osteosarcoma in the elderly.[3–7,9,10]

TABLE 4 Summary of multivariate analysis investigating prognostic factors

Factors	Risk ratio	95 % CI	*p* value
OAS			
Site, axial	1.80	0.96–3.26	0.0646
Metastasis at diagnosis, yes	2.29	1.10–4.87	0.0272*
Definitive surgery, no	2.24	1.15–4.50	0.0176*
Chemotherapy, no	1.53	0.83–2.80	0.168
EFS			
Site, axial	1.64	0.82–3.31	0.160
Metastasis at diagnosis, yes	1.69	0.92–3.03	0.0913
Definitive surgery, no	1.83	0.96–3.53	0.0672
Chemotherapy, no	1.43	0.80–2.56	0.227

OAS overall survival, *EFS* event-free survival

* Statistically significant

Although patients without metastasis who have undergone definitive surgery are regarded as good candidates for chemotherapy, the addition of chemotherapy did not have any impact on their outcomes. The role of chemotherapy in elderly patients with osteosarcoma has remained controversial. Some reports stated that chemotherapy for osteosarcoma in the elderly is beneficial, while others did not.[2,3,7,9] Antman et al.[21] reported that a relatively lower effectiveness of chemotherapy was observed in adults with advanced disease. This may have arisen from two reasons: one is the biological feature of osteosarcomas in the elderly who have a lower sensitivity to standard chemotherapy agents than that of younger patients. The other reason is that the treatment was occasionally not used for some patients because of their refusal of treatment, health conditions that could not tolerate intensive chemotherapy, or a clinician's decision. In the present study, the 3 participating hospitals had similar therapeutic strategies including a chemotherapy protocol and surgical methods, and, thus, they showed a relatively higher rate of surgery and chemotherapy implementation. However, our statistical analysis revealed that chemotherapy did not affect the outcome. It should be noted that the high 5-year OAS rate was achieved by definitive surgery in patients without metastasis at diagnosis, regardless of the chemotherapy administration status (with chemotherapy, 63.4 %; without chemotherapy, 65.2 %). Additionally, we could not find any significant difference in the tumor site, stage, or surgical margins between patient groups with or without chemotherapy (Table 5). According to the results of the present study and previous reports concerning osteosarcoma in the elderly, metastasis at diagnosis and definitive surgery are critical factors for survival.[3,6,7,9,10] Nevertheless, no report has investigated the outcome of patients without metastasis at diagnosis and who underwent

TABLE 5 Clinical characteristics of patients who underwent definitive surgery without metastasis at diagnosis

Factors	Chemotherapy	No chemotherapy
Patient numbers	21	19
Follow-up period, months (mean)	8–244 (40)	1–194 (44)
Sex (male:female)	13:8	4:15
Age, years (median)	42–68 (53)	45–80 (61)
Site (extremity:axis)	15:6	16:3
Stage (IIA:IIB:III)	3:17:1	7:12:0
Margin (wide:marginal:NA)	19:0:2	16:2:1
Relapse	11 (52 %)	10 (53 %)
Treatment at relapse	7 (64 %)	2 (20 %)
Status at the last follow-up		
Alive without disease	8	9
Alive with disease	0	2
Dead	12	6
Lost to follow-up	1	2
5-year OAS	63.4	65.2
5-year EFS	54.4	49.4

OAS overall survival, *EFS* event-free survival, *NA* not assessed

definitive surgery, and the 5-year OAS of patients achieving an adequate margin reportedly ranged from 45 to 70 %, regardless of the presence or absence of metastasis at diagnosis.[3,9,10] These results have led to the hypothesis that some elderly patients with osteosarcoma without metastasis at diagnosis and who underwent definitive surgery do not necessarily require chemotherapy. To confirm this, a large multicentric prospective study of elderly patients with osteosarcoma is awaited.

One of the main limitations of the present study is its retrospective nature. Precise analyses concerning the chemotherapy type, dose, and intensity were lacking in this study, which are critically important as they affect the sensitivity of patients to chemotherapy. However, this study presents integrated data from multiple tertiary musculoskeletal oncology hospitals sharing common treatment strategies, and the relatively large patient number supports the results described previously.

In summary, the present study revealed the distinct clinical features, such as the high incidence of truncal tumors or metastasis at diagnosis, of patients older than 40 years with osteosarcoma. Additionally, prognostic factor analyses showed that the tumor site, metastasis at diagnosis, definitive surgery, and surgical margins were significant prognostic factors, whereas chemotherapy did not influence survival.

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DISCLOSURE The authors declare that they have no conflict of interest.

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Analysis of MicroRNAs Expressions in Chondrosarcoma

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ABSTRACT: MicroRNAs (miRNAs) are small non-coding RNAs capable of inhibiting gene expression post-transcriptionally and expression profiling can provide therapeutic targets and tools for cancer diagnosis. Chondrosarcoma is a mesenchymal tumor with unknown cause and differentiation status. Here, we profiled miRNA expression of chondrosarcoma, namely clinical samples from human conventional chondrosarcoma tissue, established chondrosarcoma cell lines, and primary non-tumorous adult articular chondrocytes, by miRNA array and quantitative real-time PCR. A wide variety of miRNAs were differently downregulated in chondrosarcoma compared to non-tumorous articular chondrocytes; 27 miRNAs: miR-10b, 23b, 24-1*, 27b, 100, 134, 136, 136*, 138, 181d, 186, 193b, 221*, 222, 335, 337-5p, 376a, 376a*, 376b, 376c, 377, 454, 495, 497, 505, 574-3p, and 660, were significantly downregulated in chondrosarcoma and only 2: miR-96 and 183, were upregulated. We further validated the expression levels of miRNAs by quantitative real-time PCR for miR-181a, let-7a, 100, 222, 136, 376a, and 335 in extended number of chondrosarcoma clinical samples. Among them, all except miR-181a were found to be significantly downregulated in chondrosarcoma derived samples. The findings provide potential diagnostic value and new molecular understanding of chondrosarcoma. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:1992–1998, 2013

Keywords: microRNA; chondrosarcoma; cartilage; sarcoma; malignancy

MicroRNAs (miRNAs) are a class of short non-coding RNA with length of 20–24 nucleotides. They function by binding to multiple target mRNAs, preferentially to their 3′ UTR with partial complementarity, thereby inhibiting the expression by destabilizing the mRNAs and/or disturbing translation. The expression of miRNAs is regulated tightly in a cell type, differentiation and activation-dependent manner. Growing lines of evidence indicate miRNAs play important roles both in physiological development and pathogenesis.[1]

Chondrosarcoma is the second most common primary malignant tumor of bone.[2] Chondrosarcomas are histologically subtyped into conventional (80–90%), dedifferentiated (10%), mesenchymal (3–10%), and clear cell chondrosarcomas (approximately 2% of all chondrosarcomas),[2] however, molecular pathophysiology of the disease has not been fully understood.

In the current study, we focused on the expression profile of miRNAs in conventional chondrosarcoma, and identified differently expressed miRNAs compared to other types of cells including primary non-tumorous human articular chondrocytes by miRNA array and quantitative real-time PCR.

METHODS

Patients and Tissue Samples

A total of 20 tissue samples from conventional chondrosarcoma patients along with two chondrosarcoma cell lines, namely SW1353 and OUMS-27 cell lines, and three primary non-tumorous chondrocyte preparations (PNC) derived from normal human articular cartilage were analyzed as cartilaginous samples. Normal skeletal muscle (NSM) derived RNA sample, NRS-1 rhabdomyosarcoma cells, and HEK293T cells were used as non-cartilaginous samples. NSM derived RNA and NRS-1 cell line were used as muscle related samples as chondrocyte free negative controls. HEK293T cells, deriving from human embryonic kidney and being one of the most intensively characterized cell lines, were also used as a negative control. All 20 chondrosarcoma samples were obtained at the time of biopsy or surgery with informed consent from patients who were treated at National Cancer Center Hospital (Tokyo, Japan). The ethical review board approved the project. All cases were reviewed and histopathologically diagnosed by a certified pathologist. Clinical staging was determined based on the criteria according to the Musculoskeletal Tumor Society Surgical Staging System.[3] The tumor tissues were snap-frozen immediately after excision and stored at −80°C until total RNA extraction.

Cell Culture and Other RNA Sources

SW1353 grade II conventional chondrosarcoma cells (ATCC no. HTB-94), OUMS-27 grade III conventional chondrosarcoma cells (JCRB no. IFO50488) NRS-1 human rhabdomyosarcoma cells (Riken BioResource Center, permission from Dr. Motoyama, no. RCB1188), and HEK293T cells were cultured according to providers' instruction. PNC were obtained from normal articular cartilages of the knee at autopsy from three donors: 45-, 50-, and 60-year-old males, and prepared according to the method described previously.[4] In brief, excised cartilages were finely chopped into fine pieces, followed by enzymatic digestion of associated extracellular matrix component, and then, separated chondrocytes were washed several times in Ham's-F12, counted the cell number, and checked for viability using trypan blue staining. The isolated chondrocytes were cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin solution (Life technologies, Grand Island, NY). These prima-

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ry chondrocytes within four passages were cultured until 60–80% confluent, and subjected to total RNA extraction. The expression levels of chondrocytic gene markers were assessed by RT-PCR. The primer sets used were as follows: COL1A1 5′-AGGGTCACCGTGGCTTCT-3′, CAGGAGCACCAGCAGAGC; COL2A1 5′-GGCAATAGCAGGTTCACGTACA-3′, 5′-CGATAACAGTCTTGCCCCACTT-3′; COL10A1 5′-GGCAGAGGAAGCTTCAGAAA-3′, 5′-AAGGGTATTTGTGGCAGCATA-3′; GAPDH 5′-CCTGGTCACCAGGGCTGC-3′, 5′-CGCTCCTGGAAGATGGTGATG-3′. Total RNA extracted from NSM was purchased from Clontech (Mountain View, CA).

miRNA Microarray Assay

The assays were performed with Agilent's human miRNA array system (Agilent Technologies, Santa Clara, CA), including probe sets for 723 miRNAs. A 100 ng total RNA sample was used according to the manufacturer's instruction. Following digitization of the primary scanned images, the data were further analyzed using NIA array analysis software.[5] The criteria for extracting differently expressed miRNAs were set to a condition: fold change was >10 in the digitized fluorescent intensity, and false discovery rate was <0.3. Heat map analysis and hierarchical clustering were carried out by TIGR-MEV ver. 4.4.[6]

Quantitative Real-Time PCR (qPCR)

TaqMan miRNA assay kits were purchased from Applied Biosystems (Carlsbad, CA): hsa-miR-181a (4373117), hsa-miR-let-7a (catalog no. 4373169), hsa-miR-100 (4373160), hsa-miR-222 (4395387), hsa-miR-136 (4373173), hsa-miR-376a (4373026), hsa-miR-335 (4373035), and RNU6B (4373381). A total of 0.6 ng of complementary DNA templates per sample were used for the reaction. Each sample was assayed in duplicate, and the data with more than 1.0 of difference in Ct values were excluded from further analysis. Fold change expression of each miRNA was calculated by ΔΔ-CT method compared to the mean expression level of PNC. RNU6B was used as an internal control.

Statistical Analysis

The data from miRNA microarray were analyzed by ANOVA on NIA array analysis. The statistical significance was calculated by the false discovery rate method. The actual criteria for extracting particular miRNAs have been described above. Statistically significant differences in qPCR were assessed by ANOVA followed by pairwise *t*-test with Bonferroni's correction. p-values < 0.05 were considered as significant. Statistical calculations in qPCR analysis were performed using StatView ver. 5.0 (SAS Institute, Cary, NC).

RESULTS

The samples for miRNA microarray assay included: two conventional chondrosarcomas (case no. CS01 and CS03, clinicopathological features including other chondrosarcoma patients in Table 1), SW1353 cells, and OUMS-27 cells, three PNC samples, NSM, NRS-1 cells, and HEK293T cells. All PNCs expressed articular chondrocyte marker COL2A1 mRNA and the semi quantitative ratios of COL2A1/COL1A1 were as follows: NC01, 1.47; NC08-41, 0.80; and NC08-42, 0.81, respectively (S-Fig. 1). The Hypertrophic chondrocyte marker COL10A1 was not detected in all primary articular chondrocytes. These data suggest that all PNCs kept hyaluronic cartilage status. Gene expression profiles of the above samples compared to PNC01 are shown as a scatter plots in S-Figure 2. Overall miRNA expression profiles of primary chondrocytes and chondrogenic tumor samples were significantly different (S-Fig. 2). The correlation matrix (Fig. 1a) shows significant correlations within each sample group; that is, PNC group for PNC01, PNC08-41, and PNC08-42; chondrosarcoma clinical samples (CS_clin) for CS01 and CS03; and chondrosarcoma cell lines (CS_cell) for SW1353, and OUMS-27. Hierarchical clustering of these samples using dendrogram (Fig. 1b) revealed that chondrosarcoma samples have a similar expression profile compared to other type of samples. To characterize differently expressed miRNAs between chondrosarcoma samples and PNC more accurately, we carried out supervised hierarchical clustering analysis using 117 probe sets, which were chosen with satisfactory criteria by ANOVA (Fig. 2). We further subdivided these 117 probe sets into six groups according to the expression profile focused on chondrosarcoma related samples compared with PNC (Fig. 3a,b and S-Table 1a,b). CS_clin showed 28 differently upregulated and 67 downregulated miRNAs versus PNC. CS_cell had 4 upregulated and 56 downregulated miRNAs. Among them, 2 and 27 miRNAs were differently upregulated and downregulated, respectively in both CS_clin and CS_cell. Commonly up-/down-

Table 1. Clinicopathological Features of Conventional Chondrosarcoma Patients

Case No.	Age Years	Sex	Primary Site	Grade	Follow-up Status
CS01#	33	M	Pelvis	1	NED
CS02	29	M	Scapula	1	NED
CS03#	58	M	Pelvis	1	NED
CS04	29	F	Femur	1	NED
CS05	47	M	Scapula	1	NED
CS06	44	M	Pelvis	1	NED
CS07	57	M	Vertebra	1	NED
CS08	60	M	Hand	1	NED
CS09	67	M	Rib	1	Meta.(+)
CS10	61	M	Femur	2	AWD
CS11	86	M	Knee	2	NED
CS12	51	F	Rib	2	NED
CS13	55	M	Tibia	2	NED
CS14	65	M	Humerus	2	AWD
CS15	59	F	Pelvis	2	NED
CS16	66	M	Hand	2	AWD
CS17	86	F	Knee	2	NED
CS18	75	F	Femur	2	NED
CS19	63	F	Humerus	3	NED
CS20	59	M	Pelvis	3	DOD

NED, no evidence of disease; Meta.(+), with metastasis at the final assessment; AWD, alive with disease; DOD, dead of disease. # indicates the cases whose sample was applied to the miRNA array screening.

a

		1	2	3	4	5	6	7	8	9	10
		PNC			CS_clin		CS_cell				
1. NC01	PNC	1.00	0.94	0.93	0.80	0.83	0.87	0.83	0.43	0.70	0.51
2. NC08-41	PNC	0.94	1.00	0.97	0.82	0.84	0.83	0.82	0.44	0.65	0.44
3. NC08-42	PNC	0.93	0.97	1.00	0.85	0.84	0.82	0.80	0.42	0.64	0.47
4. CS01	CS_clin	0.80	0.82	0.85	1.00	0.97	0.77	0.74	0.53	0.64	0.51
5. CS03	CS_clin	0.83	0.84	0.84	0.97	1.00	0.80	0.76	0.56	0.69	0.54
6. SW1353	CS_cell	0.87	0.83	0.82	0.77	0.80	1.00	0.81	0.45	0.64	0.62
7. OUMS-27	CS_cell	0.83	0.82	0.80	0.74	0.76	0.81	1.00	0.55	0.76	0.47
8. NSM		0.43	0.44	0.42	0.53	0.56	0.45	0.55	1.00	0.47	0.35
9. NRS-1		0.70	0.65	0.64	0.64	0.69	0.64	0.76	0.47	1.00	0.49
10. HEK293T		0.51	0.44	0.47	0.51	0.54	0.62	0.47	0.35	0.49	1.00

b

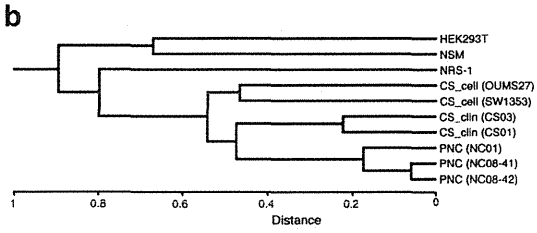

Figure 1. Correlations between samples on the miRNA arrays. (a) Correlation matrix for log-intensity among samples. (b) Hierarchical clustering of the arrays. The dendrogram shows the degree of similarity of miRNA expression pattern among samples. The chondrogenic samples, including both chondrosarcoma clinical samples and chondrosarcoma derived cell lines, showed a distinct expression pattern in miRNA from primary non-tumorous chondrocyte derived samples. CS_clin, chondrosarcoma clinical sample; CS_cell, chondrosarcoma derived cell line; PNC, primary non-tumorous articular chondrocytes.

regulated miRNAs in chondrosarcoma samples are: miR-96 and 183 as upregulated; miR-10b, 23b, 24-1*, 27b, 100, 134, 136, 136*, 138, 181d, 186, 193b, 221*, 222, 335, 337-5p, 376a, 376a*, 376b, 376c, 377, 454, 495, 497, 505, 574-3p, and 660 as downregulated.

Next, we validated the miRNA array data with some miRNAs differently expressed in CS_clin and/or CS_cell compared to PNC in a larger group of patients with chondrosarcoma by qPCR. Due to the limitation of total RNA quantity, we further examined the following miRNAs expression level by qPCR; miR-181a, let-7a, 100, 222, 136, 376a, and 335. MiR-181a was chosen because it is of interest as a rare upregulated miRNA and a potential regulator of, silent information regulator 1 (SIRT1). MiR-100, 222, 136, 376a, and 335 were included because they showed commonly decreased expressions in CS_clin and CS_cell. We also examined the expression of miR-let-7a, which was downregulated in chondrosarcoma samples, as a representative of let-7 group. In our miRNA array study, most of let-7 group miRNAs including let-7a, b, c, and i were downregulated in CS_cell. In addition, the downregulation of let-7 group miRNAs has been reported in various other types of tumors.

The results from qPCR experiments are shown in Figure 4 (detailed qPCR data in S-Table 2). Overall results from qPCR had the same tendency as miRNA array data. The expression of miR-let-7a, 100, 222, 136, 376a and 335 were significantly decreased in both CS_cell and CS_clin samples versus PNC. Neither CS_cell nor CS_clin samples showed significant differences compared to PNC concerning the expression of miR-181a, though CS_clin samples had an increased expression pattern version PNC.

DISCUSSION

Using the PNC as controls, the miRNA profiling analysis of CS samples has been performed and we found that some specific miRNAs expressions are different between CS and PMC (Fig. 1 and S-Fig. 2).

A total of 106 miRNAs were downregulated in either CS_clin or CS_cell compared to PNC, and 27 miRNAs were commonly downregulated in the array study. Further validation by qPCR analysis with expanded sample size proved the significant downregulation of miR-let-7a, 100, 222, 136, 376a, and 335 in CS_clin or CS_cell (Table 1).

MiR-96 and -183 were upregulated in chondrosarcoma samples (Fig. 3, Table 2). Downregulation of these miRNA expression has also been observed in other solid tumors.[7]

MiR-34 and miR-181a members have been reported to target SIRT1 and control cell survival under various stress condition.[8,9] In our array study, the expression of miR-34b, 34c-5p, and 181a were differently upregulated in CS_clin compared to PNC. These miRNAs may contribute to protecting host cells from tumor progression.

MiR-let-7 group (let-7s) is one of the most intensively studied miRNAs in carcinogenesis, generally functioning as tumor suppressors, and downregulated in various tumors. Let-7s directly target oncogenic genes Ras and HMGA2, and their loss of expression can lead to oncogenic transformation.[10,11] In the current study, chondrosarcoma derived samples (CS_clin and CS_cell) also showed downregulation of let-7s compared to PNC. In addition, miR-100, let-7a-2, and miR-125b-1 form one gene cluster, and this cluster has been reported to exert antagonistic effects on cell proliferation and carcinogenesis by regulating Myc activity.[12] In both miRNA array and qPCR experiments, miR-100 expression was shown to be downregulated in chondrosarcoma samples (CS_clin and CS_cell). Downregulation of let-7s and miR-100 would play a distinct role in tumor progression in chondrosarcoma.

MiR-221/222 has been reported to target tumor suppressor gene PTEN and enhance carcinogenesis by activating the AKT/PKB signaling pathway in lung and liver cancers.[13] Liu et al.[14] found miR-136, 376a, and 31 were overexpressed in murine and human lung cancers. Lee et al.[15] have shown that miR-136, 199a-3p, and 144 function as regulators of Rb1 and the versican-PTEN pathway. Versican is a relatively minor extracellular matrix protein in mature cartilage, but is highly expressed in chondrocyte lineage progenitor cells,[16,17] suggesting a role maintaining the immature status of chondrocyte lineage cells. Decreased

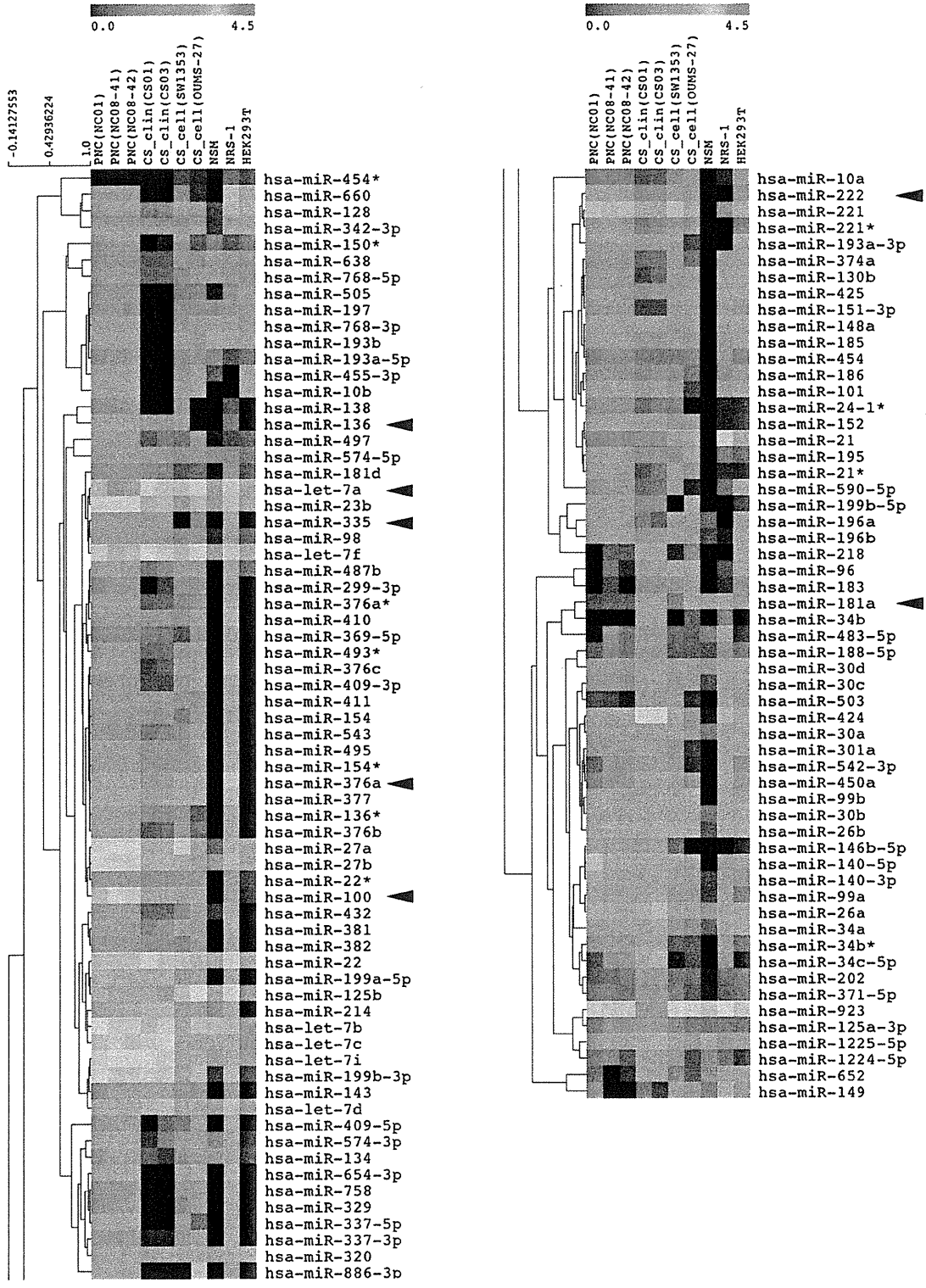

Figure 2. Expression pattern of the selected 117 miRNAs. The closed triangles show the miRNAs that were subjected to the further verification by quantitative real-time PCR.

expression of miRNA-376a has been reported to induce cell proliferation in hepatocellular carcinoma cell lines by targeting PKI3R1.[18] It has been reported that decreased expression of miR-335 is functionally associated with poor distal metastasis-free survival in breast cancer, and miR-335 inhibits metastasis in breast cancer by targeting SOX4 and TNC.[19] The decreased expressions of miR-222, 136, 376a, and 335 would also

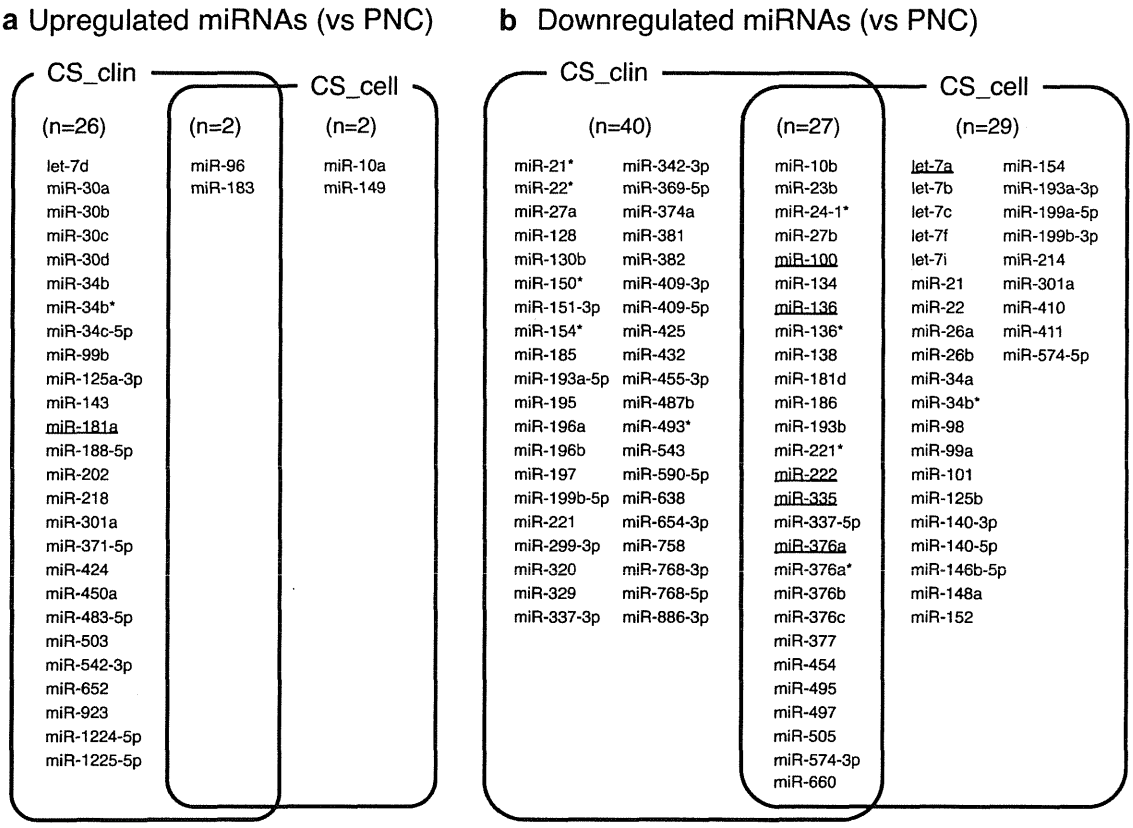

Figure 3. Differentially expressed miRNAs in chondrogenic samples. The expression levels of miRNAs in clinical chondrosarcoma samples and chondrosarcoma cell line samples were compared to primary non-tumorous articular chondrocytes. (a) The list of upregulated miRNAs in chondrosarcomas vs PNC (b) The list of downregulated miRNAs in chondrosarcomas vs PNC. The underlined miRNAs represent those used for verification by quantitative real-time PCR. CS_clin, chondrosarcoma clinical sample; CS_cell, chondrosarcoma derived cell line; PNC, primary non-tumorous articular chondrocytes.

play a specific role in malignant chondrogenic carcinogenesis.

It has been demonstrated that chromosomal amplification of 8q24,[20] 12p11, 12p13, and 12q13 loci[21] and a loss of 8q24,[22] and 11p11 locus[22] are associated with chondrosarcoma. Some miRNAs are located in these genomic regions, namely has-miR-5692a-2, 3926-1, and 3926-2 in 8q24; miR-613, 614, 141, 1244-3, 3649 and 200c in 12p13; miR-4698, 4494, 1291, 4701, 1293, 196a-2, 615, 3198-2, 148b, 1228, and 616 in 12q13,

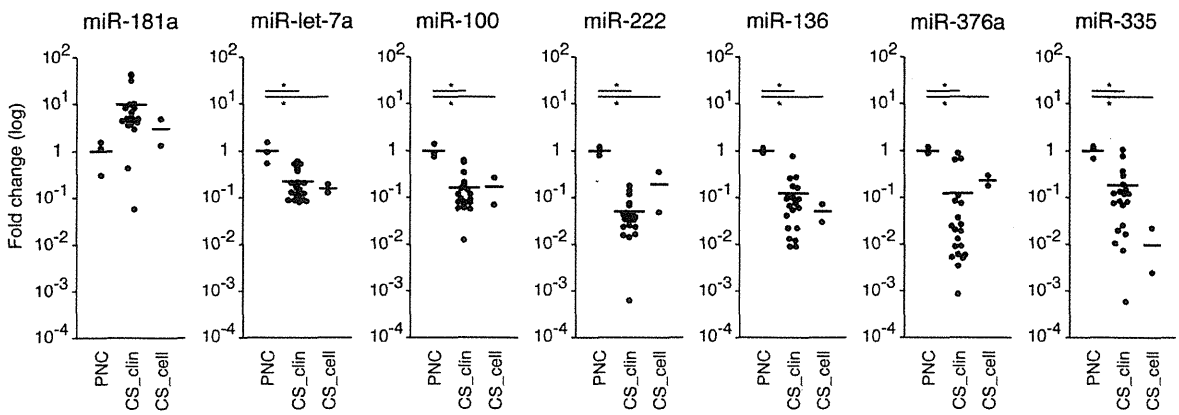

Figure 4. Expression of miRNAs in chondrosarcoma clinical samples in quantitative real-time PCR assay. All data are given as fold change expression levels compared to PNC. The expression level of each miRNA was normalized to RNU6B as an internal control. CS_clin, chondrosarcoma clinical sample; CS_cell, chondrosarcoma derived cell line; PNC, primary non-tumorous articular chondrocytes. The bar represents the mean. *$p < .05$ by ANOVA and pairwise t-test with Bonferroni's correction.

Table 2. Differentially Expressed miRNAs and Characteristics

miRNAs	Chromosome Locations	Expression (CS vs PNC)		Target Genes (Reported)	Functions	Refs.
		Array	qPCR			
miR-181a	1q32.1, 9q33.3	Up	Not significant	SIRT1	Cell survival, defense against oxidative stress	8,9
miR-let7a	9q22.3, 11q24.1, 22q13.31	Down	Down	Ras, HMGA2	Carcinogenesis	10,11
miR-100	11q24.1	Down	Down	Myc	Carcinogenesis	12
miR-222	Xp11.3	Down	Down	PTEN	Carcinogenesis	13
miR-136	14q32.2	Down	Down	Rb1, Versican	Carcinogenesis	14,15
miR-376a	14q32.31	Down	Down	P1K3R1	Cell proliferation	14,18
miR-335	7q32.2	Down	Down	SOX4, TNC	Tumor metastasis	19

respectively. Dysregulation of miRNAs' expressions, whichever they are up- or downregulated, may disorganize the homeostasis in normal chondrocytes and contribute to the malignant transformation. Further study with larger sized probe sets may elucidate the closer relationship between miRNAs and chromosomal abnormalities in chondrosarcoma.

It has been proposed that miRNA profiling in cancer could be used not only as diagnostic or prognostic indicator,[23] but also as therapeutic target for cancer therapy.[24] The information about miRNA profiling in the current study may provide a basis for future chondrosarcoma diagnosis and therapy.

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Supporting Information

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