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Advances in Brief

Expression of Insulin-like Growth Factor-1 Receptor in Synovial Sarcoma: Association with an Aggressive Phenotype[1]

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Abstract

It is well known that insulin-like growth factor-1 receptor (IGF-1R) plays a crucial role in proliferation and survival of transformed cells. Overexpression of IGF-1R in certain tumors has been reported, but there is still little known about its importance *in vivo*. Here, we evaluated the IGF-1R levels in 35 human synovial sarcoma tumors by Western blot and reverse transcriptase-PCR. In 18 of these, IGF-1R was detectable by Western blot, whereas 17 were nondetectable. There was a significant association between the amount of receptor proteins and mRNA transcripts. Furthermore, we found that the IGF-1R Western blot-positive tumors were associated with a high incidence of lung metastases. Eleven of 18 (61%) developed metastases in the IGF-1R detectable group, compared to 3 of 17 (18%) in the nondetectable group ($P = 0.01$). Moreover, in the detectable group of IGF-1R, 12 of 18 (67%) exhibited a high tumor cell proliferative rate, compared to 5 of 16 (31%) in the nondetectable group ($P = 0.04$). On the other hand, no association was found between the IGF-1R and type of fusion gene transcript (*SYT-SSX1* or *SYT-SSX2*). Our results suggest that expression of IGF-1R can underlie an aggressive phenotype in synovial sarcoma.

Introduction

Synovial sarcoma, which accounts for 5–10% of all soft tissue sarcomas, is primarily located in the extremities and can occur at any age, including childhood, but it is most commonly found in young adults (1). Surgery is the main treatment, often supplemented with radiotherapy. Synovial sarcomas primarily metastasize to the lung, ultimately leading to death of the patient. Both thoracic surgery and chemotherapy have been shown to be of limited value. Although excellent local control has been reported from sarcoma centers, long-term survival rates have, at best, been reported to ~50% (2). Characteristic for synovial sarcoma is the translocation t(X;18)(p11.2; q11.2) (SYT-SSX; Ref. 3), resulting in one of three types of fusion transcripts, *SYT-SSX1*, *SYT-SSX2* (4), or *SYT-SSX4* (5). Recently, it has been reported that type of fusion transcript may be of prognostic significance (6, 7).

IGF-1R[3] is a plasma membrane-bound heterotetrameric receptor, composed of two α-subunits (M_r 130,000) and two β-subunits (M_r 90,000 each), linked by disulfide bonds with intrinsic tyrosine kinase activity (8). Evidence has been provided that the IGF-1R plays a crucial role in tumor cell growth in several different ways, such as mediating mitogenesis and maintaining a transformed phenotype, as well as protecting tumor cells from apoptosis (9–11). Overexpression of the IGF-1R reduces growth factor requirements and the susceptibility to apoptosis (12). Conversely, impairment of the IGF-1R function by antisense strategies, antibodies, or dominant negative mutants causes large-scale apoptosis of tumor cells, abrogation of tumor growth, and metastasis (13).

IGF-1R is highly expressed in a number of different tumor tissues, such as breast cancer, osteosarcoma, and melanoma (12, 14–16). Studies on breast cancer have suggested that overexpression of IGF-1R is associated with local tumor recurrence (12). Few studies on IGF-1R and the insulin-like growth factor-1 pathway have been performed on soft tissue sarcomas. In this study, we determined the IGF-1R levels by Western blot and RT-PCR in 35 synovial sarcoma tumors. Furthermore, we investigated whether there was an association between IGF-1R and incidence of lung metastasis.

Materials and Methods

Patients. From 1988 to 1998, 35 patients with histologically verified localized synovial sarcoma were referred to Scandinavian Sarcoma Group centers. There were 17 males and 18 females, with a mean age at diagnosis of 40 years. RT-PCR confirmed that all cases carried SYT-SSX fusion transcripts. All patients underwent surgery with a curative intent. Adjuvant radiotherapy was administered to 17 patients, and 6 patients received chemotherapy. The mean follow-up period was 46 months (range, 2–111 months). Fourteen patients developed lung metastases during the follow-up period. Twenty-nine of the total number of cases were included in another study focusing on the association between type of SYT-SSX fusion transcript and clinical outcome (6).

Cell Line. A Ewing's sarcoma cell line, RD-ES (HTB-166), was obtained from the American Type Culture Collection (Manassas, VA) and used as control. Cells were cultured routinely in RPMI 1640 with 10% FCS and maintained at 37°C in a humidified 5% CO_2 atmosphere.

RT-PCR: Quantification of IGF-1R mRNA Levels. Total RNA was isolated from the tumor specimens and the control cell line according to the manufacturer's protocol (Qiagen, Hilden, Germany). Five hundred ng of total RNA were reverse-transcribed using random hexadeoxynucleotide primer (Pharmacia, Uppsala, Sweden). The PCR using IGF-1R primers (5′-GCCCGAAGGTCTGTGAGGAAGAA-3′ and 5′-GGTACCGGTGCCAGGTTATGA-3′) and β-actin primers (5′-CATGCCATCCTGCTGGAC-3′ and 5′-CACGGAGTACTTGCGCTCAGGAGG-3′) was performed at 94°C for 30 s, 60°C for 30°C s, and 72°C for 30 s for 28, 30, and 32 cycles, followed by a final elongation for 10 min. PCR products were electrophoresed on 2% agarose and NuSieve gel, visualized by ethidium bromide staining, and quantified by densitometry (MultiAnalyst; Bio-Rad, Hercules, CA). The kinetics of the various reactions were evaluated using the control cell line cDNA to determine the optimal cycle numbers, ensuring that the PCR productions of IGF-1R as well as the internal control β-actin remained exponential (data no shown).

Because the quantity of amplified β-actin product is assumed to be proportional to the amount of initial mRNA template, a relative amount of IGF-1R product could be determined by normalizing IGF-1R amount to β-actin. Finally, the relative value of IGF-1R expression in synovial sarcoma specimens was normalized to the level of the positive control cell line (15, 17).

Detection of *SYT-SSX* Fusion Gene Transcripts. A primer SSX-A 5′-CACTTGCTATGCACCTGATG-3′ was used for reverse transcription. The cDNA was amplified by PCR with SYT primer 5′-AGACCAACACGCCTG-

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[3] The abbreviations used are: IGF-R, insulin-like growth factor-1 receptor; RT-PCR, reverse transcriptase-PCR.

GACCA-3′ and SSX primer 5′-TGCTATGCACCTGATGACGA-3′. After the amplification, the PCR products were directly sequenced by cycle sequencing with dye-labeled terminators (BigDye Terminators; Perkin-Elmer, Norwalk, CT) and analyzed on an ABI Prism 377XL DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA) to discriminate the two fusion types.

Western Blot. Tumor samples were washed twice with cold PBS, and 100–200 mg of tumor tissues were homogenized on ice by lysis buffer. After centrifugation at 600 × *g* for 10 min at 4°C, the supernatants, containing the cellular proteins, were used for analysis. The protein concentrations were assessed by dye-binding Bio-Rad protein assay. For all specimens, the same amount of total protein (450 μg) was loaded on the SDS-polyacrylamide gel and analyzed using a 4% stacking gel and a 7.5% separating gel. Coomassie Brilliant Blue staining was used to check the amount and quantity of tumor proteins. Western blot using a rabbit polyclonal against the IGF-1R α-subunit (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as described elsewhere (18). The density of IGF-1R bands was assessed using the Multi-Analyst software.

Ki-67 Analysis. Immunostaining with Ki-67 antibody (MIB-1; Immunotech, Marseilles, France) on corresponding samples was performed according standard avidin-biotin complex technique (Elite Standard Kit; Vector Laboratories, Burlingame, CA). The percentages of Ki-67-positive nuclei were determined as described previously (19). Specimens with a Ki-67 index of <10% were considered to have a low proliferation rate, and specimens with a Ki-67 index of ≥10% were regarded as highly proliferative (19).

Results

Analysis of IGF-1R Expression in Synovial Sarcoma by Western Blot and RT-PCR. There were considerable differences in the signal intensity among the cases analyzed by Western blot (Fig. 1*A*). Corresponding samples were stained with Coomassie Brilliant Blue to ensure that the quality and amounts of the fractionated proteins did not differ between the cases. As shown in Fig. 1*B*, the electrophoretic pattern and the intensity at the protein bands were essentially similar. The IGF-1R signals detected by Western blot in all 35 cases were quantified by densitometry. The obtained values are presented in Table 1. Eighteen cases were found to have detectable signals for the IGF-1R protein.

IGF-1R gene expression was analyzed by RT-PCR. Fig. 2 shows the IGF-1R PCR products from 6 of the 35 cases and the control cell line. Using densitometry, we determined the relative amount of transcripts for all cases (Table 1). The value for the control cell line (RD-ES) was set at 1.00. The relative values ranged from 0.01 to 1.26 among the cases. The association between the amount of IGF-1R proteins obtained by densitometry of Western blot signals and IGF-1R transcripts obtained by RT-PCR is shown in Fig. 3*A*. There was a significant association noted ($r = 0.68$, $P = 0.03$). Nineteen of the 35 (54%) cases had RT-PCR values of ≥0.50, which is in good concordance with the 18 cases with positive Western blot.

IGF-1R Association with Metastatic Disease, Tumor Cell Proliferation, and Fusion Transcript. In accordance with the Western blot results, the patients were divided into IGF-1R detectable and nondetectable groups (Table 1). In the detectable group, 11 of 18 (61%) developed lung metastases, as opposed to 3 of 17 (18%) in the

Table 1 *IGF-1R correlation with lung metastasis, fusion gene type, and Ki-67 in synovial sarcomas*

	Metastasis		SYT-SSX		IGF-1R	
Patient no.	Site	Time after diagnosis (months)	fusion type	Ki-67[a]	Western blot value	RT-PCR value
1			1	0	Negative	0.01
2			2	0	Negative	0.07
3	Lung	41	1	0	Negative	0.11
4			1	1	Negative	0.14
5			2	0	Negative	0.16
6			2		Negative	0.21
7			2	0	Negative	0.25
8			2	0	Negative	0.30
9			1	0	Negative	0.39
10			2	0	Negative	0.42
11			2	0	Negative	0.42
12			1	1	Negative	0.42
13			2	1	Negative	0.46
14	Lung	7	1	0	Negative	0.47
15	Lung	37	1	1	Negative	0.51
16			2	1	Negative	0.67
17			2	0	Negative	0.77
18	Lung	15	2	1	0.07	0.42
19	Lung	62	2	0	0.05	0.44
20	Lung	0	1	1	0.19	0.50
21	Lung	0	2	0	0.38	0.52
22	Lung	0	2	1	0.28	0.58
23	Lung	77	1	1	0.1	0.58
24			2	1	0.27	0.61
25	Lung	5	1	1	0.14	0.62
26			2	0	0.12	0.63
27	Lung	23	1	1	0.03	0.67
28	Lung	15	1	1	0.29	0.82
29	Lung	36	1	0	0.55	0.87
30			2	0	0.09	0.92
31			2	0	0.43	0.94
32			1	1	0.35	1.06
33	Lung	16	2	1	0.13	1.22
34			2	1	1.14	1.22
35			1	1	0.41	1.26

[a] 0, Ki-67 index < 10% (low); 1, Ki-67 index ≥ 10% (high).

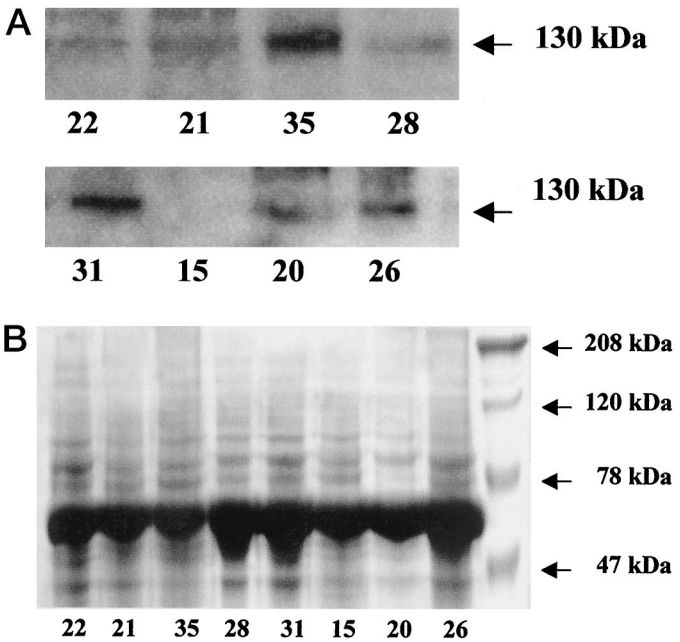

Fig. 1. *A*, IGF-1R protein was analyzed by Western blot, using an antibody against the α-subunit of IGF-1R. The same amount of total protein was loaded for all samples. *B*, protein samples were stained with Coomassie Brilliant Blue. The same amount of total protein was loaded on the gel.

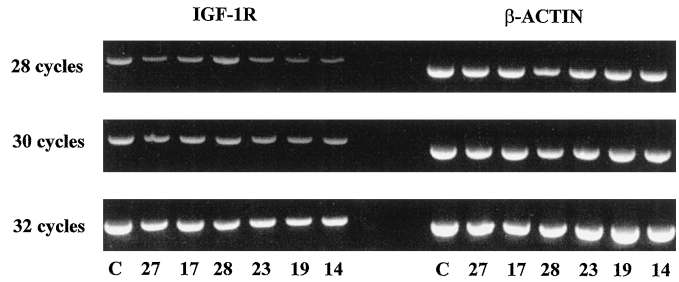

Fig. 2. *IGF-1R* gene expression in six samples and the control cell line. Total mRNA were reverse-transcribed and then amplified with IGF-1R and β-actin primers for 28, 30, and 32 cycles.

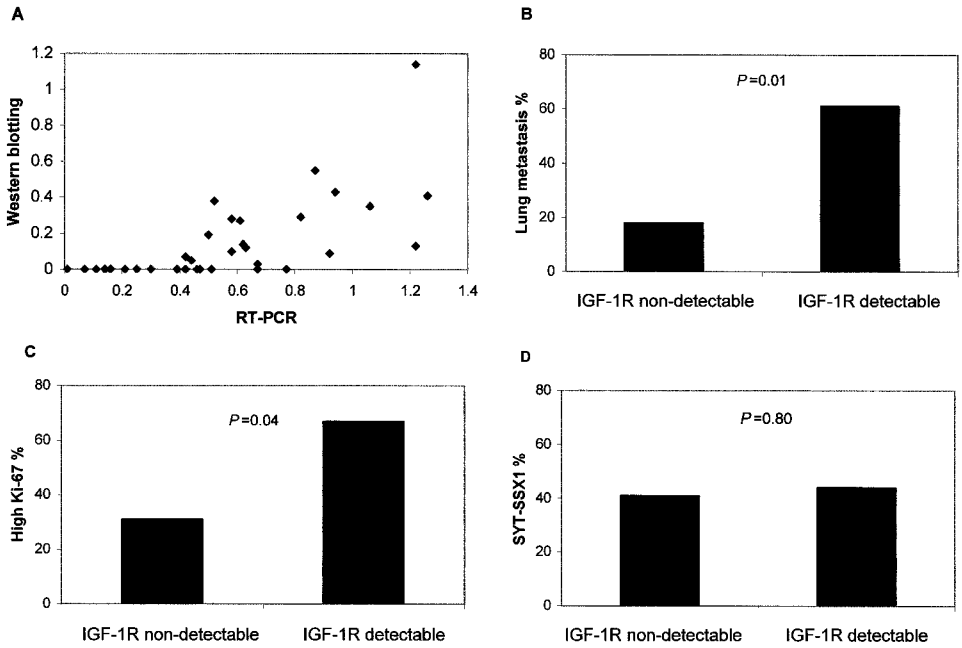

Fig. 3. *A*, association of Western blot and RT-PCR data. *B*, association of IGF-1R with lung metastases. *C*, association of IGF-1R with high-level Ki-67. *D*, association of IGF-1R with the *SYT-SSX1* fusion gene.

nondetectable group (χ^2 test, $P = 0.01$; Fig. 3*B*). We also found in the IGF-1R detectable group that 12 of 18 (67%) exhibited high Ki-67 values, compared with 5 of 16 (31%) in the nondetectable group ($P = 0.04$; Fig. 3*C*). In contrast, there was no association between IGF-1R and the fusion gene transcript *SYT-SSX1* ($P = 0.80$; Fig. 3*D*).

Discussion

To date, there have been few studies on the role of the IGF-1R pathway in sarcoma, and most of these have been undertaken on bone tumors, *i.e.*, osteosarcoma and Ewing's sarcoma (14, 15). Burrow *et al.* (14) reported that ~50% of osteosarcoma tumors express high levels of IGF-1R. In a study by Scotlandi *et al.* (20), it was shown that the IGF-1R-mediated loop was the only growth factor circuit that was constantly present in several Ewing's sarcoma cell lines and clinical samples. They also showed that the IGF-1R-neutralizing antibody αIR-3 significantly inhibited the ability of Ewing's sarcoma cells to grow in soft agar as well as their mobility. IGF-1R and insulin-like growth factor-1 have been found to be expressed in some soft tissue sarcomas, *e.g.*, malignant fibrous histiocytoma and liposarcoma (15). However, to our knowledge, no studies have shown any association between IGF-1R and the malignancy potential in sarcomas.

Here, we determined the level of IGF-1R in 35 synovial sarcoma samples. IGF-1R expression was independently evaluated by Western blot and RT-PCR. Analysis with Western blot showed that 51% of the cases expressed the receptor protein. There was a significant concordance between the protein and mRNA levels.

Invasion and metastasis are the most life-threatening aspects of malignant disease. In synovial sarcoma, metastatic lesions develop in ~50% of the cases and often appear several years after the initial diagnosis. The principal site of metastasis is the lung (1). Our results show that 61% of the patients whose tumors were expressing IGF-1R developed lung metastases, compared to 18% in the IGF-1R nondetectable group. These findings strongly suggest that synovial sarcomas expressing IGF-1R have an aggressive phenotype. Similar results demonstrated for breast cancer suggest that IGF-1R is prognostic. In a series of patients with breast cancers, Turner *et al.* (12) found high levels of IGF-1R in 52% of the tumors. These cases relapsed more frequently within 4 years after diagnosis compared to cases with low IGF-1R expression. However, the role of this receptor in regulation of invasion/metastasis has not been fully elucidated. The process of metastasis involves multiple sequential interactions between the disseminating tumor cells and host tissues. Animal models have demonstrated that overexpression of IGF-1R results in an increase of metastatic potential. H-59 is an example of a highly metastatic variant of the Lewis lung carcinoma. When these tumor cells were transfected with an IGF-1R antisense RNA plasmid, they lost their ability to metastasize to lung or liver (21). In another model using the M-27 carcinoma cells, an increase of the expression of the IGF-1R was found to enhance invasiveness and increase liver-colonizing potential (22). Similar results have been reported by Burfeind *et al.* (23), using a rat prostate cancer model. Recently, functional impairment of the IGF-1R in two different estrogen receptor-negative breast cancer cell lines was shown to suppress adhesion and invasive ability, resulting in fewer distant metastases *in vivo* (24).

Here, we also found that IGF-1R was associated with a high rate of tumor cell proliferation. No relationship was found between the IGF-1R and type of fusion transcript (*SYT-SSX* or *SYT-SSX2*). *SYT-SSX1* has recently been implicated as a marker for poor clinical outcome (6, 7). Therefore, it seems likely that IGF-1R and *SYT-SSX1* fusion transcript affect the biological behavior of synovial sarcoma through different and independent pathways. An approach to the mechanisms underlying the aggressive phenotype in synovial sarcomas expressing IGF-1R makes an interesting issue for further research.

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