Journal of Surgical Research **135,** 282–290 (2006)
doi:10.1016/j.jss.2006.01.023

Angiogenic Profile of Soft Tissue Sarcomas Based on Analysis of Circulating Factors and Microarray Gene Expression[1]

Sam S. Yoon, M.D.,* Neil H. Segal, M.D.,† Peter J. Park, Ph.D.,‡ Kara Y. Detwiller, B.S.,* Namali T. Fernando, B.S.,* Sandra W. Ryeom, Ph.D.,§ Murray F. Brennan, M.D.,† and Samuel Singer, M.D.†,[2]

*Department of Surgery, Massachusetts General Hospital, Division of Surgical Oncology, Harvard Medical School, Boston, Massachusetts; †Departments of Surgery and Pathology, Sarcoma Disease Management Team, Memorial Sloan-Kettering Cancer Center, New York, New York; ‡Harvard-Partners Center for Genetics and Genomics, §Division of Vascular Biology, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts

Submitted for publication December 20, 2005

Background. **Broader understanding of diverse angiogenic pathways in a particular cancer can lead to better utilization of anti-angiogenic therapies. The aim of this study was to develop profiles of angiogenesis-related gene and protein expression for various histologic subtypes of soft tissue sarcomas (STS) growing in different sites.**

Materials and methods. **Plasma levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoietin 2 (Ang2), and leptin were determined in 108 patients with primary STS. Gene expression patterns were analyzed in 38 STS samples and 13 normal tissues using oligonucleotide microarrays.**

Results. **VEGF and bFGF plasma levels were elevated 10–13 fold in STS patients compared to controls. VEGF levels were broadly elevated while bFGF levels were higher in patients with fibrosarcomas and leiomyosarcomas. Ang2 levels correlated with tumor size and were most elevated for tumors located in the trunk, while leptin levels were highest in patients with liposarcomas. Hierarchical clustering of microarray data based on angiogenesis-related gene expression demonstrated that histologic subtypes of STS often shared similar expression patterns, and these patterns were distinctly different from those of normal tissues. Matrix metalloproteinase 2, platelet-derived growth factor receptor, α and Notch 4 were among several genes that were up-regulated at least 7-fold in STS.**

Conclusions. **STS demonstrate significant heterogeneity in their angiogenic profiles based on size, histologic subtype, and location of tumor growth, which may have implications for anti-angiogenic strategies. Comparison of STS to normal tissues reveals a panel of upregulated genes that may be targets for future therapies.**

Key Words: **soft tissue sarcoma; angiogenesis; vascular endothelial growth factor; basic fibroblast growth factor; angiopoietin 2; leptin.**

INTRODUCTION

Soft tissue sarcomas (STS) are a heterogeneous group of tumors derived from cells usually of mesenchymal origin and are divided histologically into over 50 subtypes [1]. The biological behavior of STS range from low-grade tumors with a propensity for local recurrence to high-grade, aggressive tumors that metastasize early to distant sites. High-grade tumors metastasize to preferential locations such as the lung (for most histological subtypes) and the liver (for leiomyosarcomas) [2]. STS, as with other types of tumors, require angiogenesis, or the formation of new tumor blood vessels, to grow beyond a few millimeters in size [3]. This process is regulated by a balance between numerous pro-angiogenic and anti-angiogenic factors [4].

New tumor blood vessels are generally formed from the microvascular endothelial cells of the host organ or tissue [5], although a minority may be derived from

[1] This work was funded by NIH grants 5P01CA47179-145 (MFB, SS) and 5K12CA87723-03 (SSY), Kristan Ann Carr Fund (SSY, MFB, SS), and Sarcoma Foundation of America Brian J. Monaghan Memorial Research Award (SSY).

[2] To whom correspondence and reprint requests should be addressed at Department of Surgery, Sarcoma Disease Management Team, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. E-mail: singers@mskcc.org.

0022-4804/06 $32.00

bone marrow-derived endothelial cells [6]. STS grow in a variety of host organ environments including skeletal muscle, smooth muscle, and adipose tissue. A specific STS must create a positive angiogenic balance in a particular host organ environment to promote new blood vessel formation and subsequent tumor growth, and the angiogenic factors required for angiogenesis in one environment may be quite different from another environment. One gene expression profiling study of microvascular endothelial cells identified extensive differences in expression patterns between endothelial cells from different organs [7]. Also, the angiogenic pathways that maintain endothelium in normal tissues may be quite different from the pathologic angiogenic pathways that drive tumor angiogenesis.

Thus, the angiogenic pathways responsible for driving tumor angiogenesis in STS, given the variety of histological subtypes and locations of tumor growth, may be quite varied. Several studies have examined circulating angiogenic factors in patients with sarcomas [8–12]. These studies have demonstrated correlations between specific angiogenic factors and extent of disease [12], tumor size and grade [9], and risk of recurrence [8, 12]. However, these studies were too small to investigate differences in angiogenic factor levels based on histological subtype or site of tumor growth. Gene expression profiling has been used with sarcomas to differentiate between histological subtypes [13, 14], better classify equivocal histological subtypes [15], determine prognosis [16, 17], and determine response to chemotherapy [18]. Gene expression profiling specifically of angiogenesis-related genes has not previously been performed with sarcomas, but has been used with lung cancers to separate highly angiogenic from poorly angiogenic tumors [19].

Given anti-angiogenic agents are increasingly used in the treatment of various cancers [20] including STS [21], in this study we sought to develop an angiogenic profile of various histological subtypes of STS growing in different organ environments by measuring a panel of circulating angiogenic factors and by analyzing the expression of 200 angiogenesis-related genes using oligonucleotide microarrays.

PATIENTS AND METHODS

Study Population

For blood samples, 108 patients with primary or locally recurrent soft tissue sarcoma were enrolled at one institution (MSKCC) under an Institutional Review Board-approved protocol after informed consent. Patients with metastatic disease were excluded from this study. Thirty healthy age-matched and sex-matched controls were also enrolled. For tumor samples, 38 patients with STS were enrolled in an Institutional Review Board-approved protocol after informed consent. Clinical information regarding patients was obtained from a prospective sarcoma database as well as medical records. Patient demographic data at the time of blood and tissue collection included age and sex. Sarcoma characteristics of the primary tumor that were recorded included site, size, grade, depth, margin of resection (positive *versus* negative), and histological subtype. Any tumor distal to the shoulder or buttock was considered an extremity lesion. Buttock, shoulder, abdominal wall, and chest wall lesions were considered trunk tumors. Sarcoma tissues were examined by experienced sarcoma pathologists to determine histological subtype and grade (high *versus* low). Tumors adjacent or deep to the investing muscular fascia were considered deep tumors, while those above the investing muscular fascia were considered superficial tumors.

Enzyme-Linked Immunosorbent Assays

At the time of blood collection, all patients had either primary tumors or local recurrences *in situ*. Approximately 10 ml of blood was collected in EDTA- or heparin-containing Vacutainers. The samples were stored at 4°C before processing. Samples were centrifuged at $1000 \times g$ for 10 min followed by plasma collection. Plasma samples were stored at −80°C until analyses were performed. Plasma samples were measured for VEGF, bFGF, Ang2, and leptin using the following commercially available ELISA kits: Human VEGF Duoset, Quantikine FGF basic HS ELISA Kit, Human Ang2 Duoset, Human Leptin Duoset (all from R&D Systems, Minneapolis, MN). Manufacturer's protocols were followed, and samples were measured in duplicate. Mean values were used as the final concentration. ELISA plates were read using the Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA). Standard curves were generated using four parameter curves for all assays. Samples with angiogenic factor levels below the sensitivity of the VEGF and Leptin assays were assigned a value midway between 0 and the lower limit of detection of the assay (5.0 pg/ml for VEGF and 750 pg/ml for Leptin). No lower limit of detection was found for bFGF and Ang2 assays.

Tumor Specimens, RNA Isolation, and Gene Expression Analysis

Tissue specimens were obtained from patients with soft tissue sarcoma undergoing surgery under an Institutional Review Board-approved protocol. Tumor tissue was obtained from the periphery of the tumor and was embedded in OCT compound and frozen as a tissue block using liquid nitrogen. Tumor specimens were confirmed to be representative of tumor tissue by histological analysis. Cryopreserved tumor sections were homogenized under liquid nitrogen by mortar and pestle. Total RNA was extracted in Trizol reagent (Invitrogen, Carlsbad, CA) and purified using the Qiagen RNeasy kit (Valencia, CA). RNA quality was assessed with ethidium bromide agarose gel electrophoresis. cDNA was synthesized from 2 to 5 μg of RNA in the presence of oligo(dT)24-T7 (Genset Corp., La Jolla, CA). cRNA was prepared using biotinylated UTP and CTP and hybridized to HG-U133A oligonucleotide arrays (Affymetrix, Santa Clara, CA). Fluorescence was measured by laser confocal scanner (Agilent, Palo Alto, CA) and converted to signal intensity by means of Affymetrix Microarray Suite v4.0 software. For complete expression data, go to the GEO repository (www.ncbi.nlm.gov/geo/), accession number GSE2719.

Hierarchical Clustering Analysis Based on Angiogenesis-Related Genes

A unique list of 200 angiogenesis-related genes was comprised by reviewing the literature [22]. These 200 genes were represented on the HG-U133A Affymetrix GeneChip array by 295 probe sets Hierarchical clustering analysis was performed using Cluster 2.11 software (http://rana.lbl.gov/EisenSoftware.htm), using 1-r, where r is the Pearson correlation coefficient, to measure distance between genes and average linkage to measure cluster distance during partitioning [23]. Before analysis, the entire data matrix was log trans-

TABLE 1

Characteristics of Soft Tissue Sarcomas

Characteristic	*N* (%)
Site	
Retroperitoneal/intra-abdominal	42 (39%)
Extremity	36 (33%)
Trunk	30 (28%)
Size	
<=5 cm	18 (17%)
>5, ≤10 cm	30 (28%)
>10, ≤20 cm	39 (36%)
>20 cm	21 (19%)
Grade	
Low	37 (34%)
High	66 (61%)
Not determined	5 (5%)
Depth	
Superficial	6 (6%)
Deep	102 (94%)
Histology	
Liposarcoma	41 (38%)
Malignant fibrous histiocytoma	17 (16%)
Fibrosarcoma	11 (10%)
Leiomyosarcoma	9 (8%)
Desmoid	9 (8%)
Angiosarcoma	4 (4%)
Gastrointestinal stromal tumor	4 (4%)
Malignant peripheral nerve sheath tumor	4 (4%)
Synovial sarcoma	3 (3%)
Extraskeletal osteosarcoma	2 (2%)
Epithelioid sarcoma	1 (1%)
Ewing's/primitive neuroectodermal tumor	1 (1%)
Hemangiopericytoma	1 (1%)
Not specified	1 (1%)

formed and median polished (repeated application of median centering and setting the magnitude of the vector to be 1, alternating in the row and column directions).

Statistical Analysis

Levels of angiogenic factors between patients and controls were compared with the unpaired Student's *t*-test using GraphPad Instat 3.05 (GraphPad Software, Inc., San Diego, CA). Pearson correlation coefficients comparing tumor size with angiogenic factor levels were calculated using SPSS 11.0.1 (SPSS Inc., Chicago, IL). To identify up-regulated and down-regulated genes, the two-sample *t*-test was used to assess the statistical significance of each gene between patients and controls (statistical language R, www.r-project.org). The genes were ordered by their *P* values and their direction of change, and the top 10 were chosen from each category. These changes are statistically significant ($P < 0.01$) even after the conservative Bonferroni correction.

RESULTS

Circulating Angiogenic Factors in Patients and Healthy Controls

Blood samples were collected from 108 patients with primary or locally recurrent STS without clinical evidence of metastatic disease and from 30 healthy controls. Patients with metastatic disease were excluded from this study so that analysis based on site of tumor growth would not be confounded by patients with more than one site of growth. The mean age of STS patients and healthy controls was 54.6 and 52.9 years old, respectively. Forty-seven percent of patients were male and 53% were female in both groups. The primary characteristics of the STS are listed in Table 1. Tumors were roughly equally distributed in the abdomen, extremity, and trunk, and ranged in size from less than 5 cm to well over 20 cm, with a mean size of 13.8 cm. About two-thirds of tumors were high-grade, and the vast majority invaded or were deep to the muscular fascia. The most common histological subtypes were liposarcoma, malignant fibrous histiocytoma, and fibrosarcoma.

Mean plasma levels of VEGF, bFGF, and Ang2 were all significantly higher in patients compared to controls: VEGF levels were 10-fold higher in patients than controls, mean bFGF levels were 13-fold higher, and Ang2 levels were 40% higher (Table 2). Levels of these factors also demonstrated extreme variability in patients compared to controls (Fig. 1A–C). Leptin levels have been previously found to be much lower in healthy

TABLE 2

Circulating Levels of Angiogenic Factors in Sarcoma Patients and Controls

	VEGF (pg/ml) mean ± SEM	bFGF (pg/ml) mean ± SEM	Ang2 (pg/ml) mean ± SEM	Leptin (pg/ml) mean ± SEM
Sarcoma patients				
All patients (n = 108)	220.7 ± 21.3*	42.5 ± 5.6*	3685 ± 392*	14,990 ± 1120
Male patients (n = 51)	246.9 ± 27.6	51.5 ± 9.6	4684 ± 753	10,723 ± 1294**
Female patients (n = 57)	199.0 ± 32.6	34.4 ± 6.1	2792 ± 272	18,758 ± 1667
Controls				
All controls (n = 30)	20.3 ± 3.2	3.0 ± 0.3	2627 ± 320	12,696 ± 1996
Male controls (n = 14)	18.4 ± 5.1	3.1 ± 0.5	2360 ± 603	6247 ± 1331
Female controls (n = 16)	21.9 ± 4.0	2.8 ± 0.7	2861 ± 296	18,339 ± 2926

* *P* value < 0.05 compared to All controls.
** $P < 0.05$ compared to Male controls.

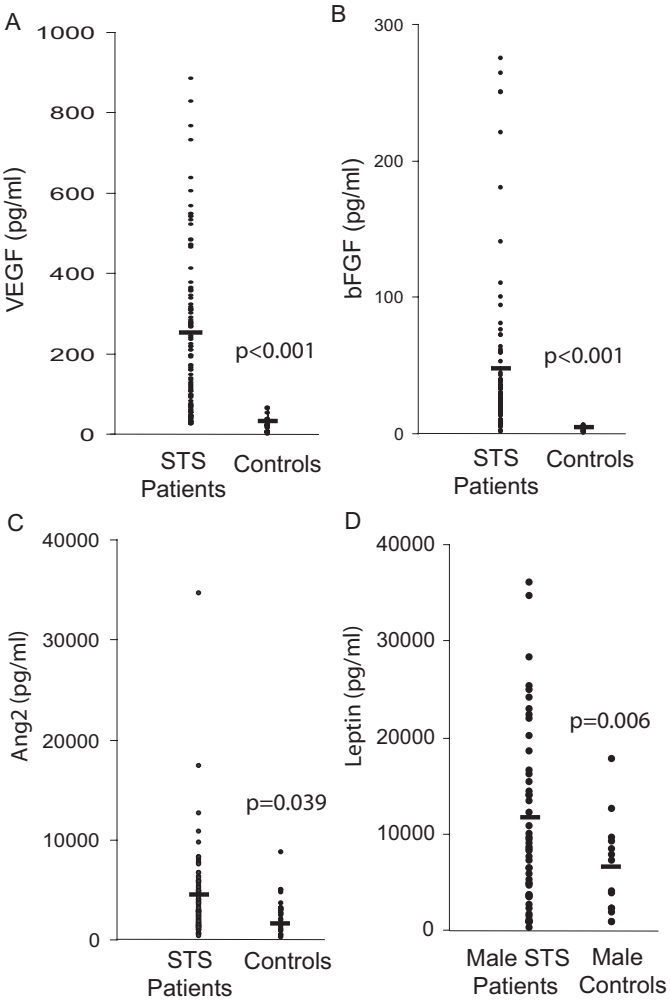

FIG. 1. Scatter plot of circulating plasma VEGF (A), bFGF (B), and Ang2 (C) levels in all STS patients (preoperative level) and all healthy controls. (D) Scatter plot of circulating plasma leptin levels in male STS patients and male controls. Bars represent mean.

men than in healthy women [24], and this was confirmed in our own healthy controls (Table 2). Leptin levels were significantly higher in male patients when compared to male controls, but no differences in leptin levels existed between female patients and female controls (Table 2, Fig. 1D).

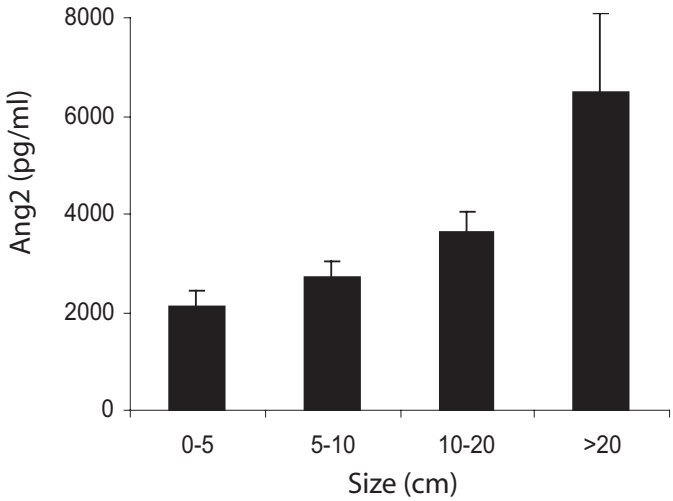

FIG. 2. Mean plasma levels of Ang2 according to tumor size. Bars represent SEM.

Subgroup Analysis of Circulating Angiogenic Factors

For a given patient, anywhere from one to all four angiogenic factors were elevated. Subgroup analyses were performed to determine if various tumor characteristics were associated with differences in circulating angiogenic factor levels. Table 3 shows the levels of angiogenic factors relative to tumor size. Ang2 levels increased significantly as tumor size increased (Fig. 2) with a Pearson correlation coefficient of 0.292 (P = 0.002). VEGF, leptin, and bFGF levels, however, did not correlate with tumor size with Pearson correlation coefficients of 0.131, −0.004, and −0.109, respectively ($P > 0.05$).

In analyzing angiogenic factor levels by histological subtype, VEGF levels were similarly elevated in all histological subtypes (Table 4). Basic FGF levels were highest in patients with fibrosarcomas and leiomyosarcomas. There were some differences in Ang2 levels according to histological subtype but these differences were largely explained by differences in mean tumor size among these histological subgroups. Patients with liposarcomas had higher levels of leptin than patients with other histological subtypes. In addition, well-differentiated liposarcomas secreted relatively more leptin

TABLE 3

Angiogenic Factor Levels According to Tumor Size

Tumor size	VEGF (pg/ml) mean ± SEM	bFGF (pg/ml) mean ± SEM	Ang2 (pg/ml) mean ± SEM	Leptin (pg/ml) mean ± SEM
≤5 cm (n = 18)	168.5 ± 35.9	42.42 ± 13.9	2121 ± 327.6	17,024 ± 3427
>5, ≤10 cm (n = 30)	233.3 ± 54.6	37.68 ± 9.9	2716 ± 310.6	15,363 ± 1901
>10, ≤20 cm (n = 39)	219.0 ± 27.8	49.90 ± 10.4	3617 ± 441.0	14,693 ± 1954
>20 cm (n = 21)	255.5 ± 52.9	35.46 ± 11.3	6469 ± 1635.1*	13,131 ± 2351

* P value < 0.05.

TABLE 4

Circulating Angiogenic Factor Levels According to Histologic Subtype

Histologic subtype	Size (cm) mean ± SEM	Male:Female	VEGF (pg/ml) mean ± SEM	bFGF (pg/ml) mean ± SEM	Ang-2 (pg/ml) mean ± SEM	Leptin (pg/ml) mean ± SEM
All liposarcomas (n = 41)	18.9 ± 1.8	21:20	244.9 ± 38.2	45.52 ± 9.7	4195 ± 830.7	16,306 ± 1848*
Well-differentiated liposarcoma (n = 25)	20.5 ± 2.3	13:12	259.5 ± 55.6	47.79 ± 14.0	4512 ± 1326.6	17,516 ± 2596
Other liposarcoma (n = 16)	16.3 ± 4.4	8:8	222.2 ± 47.0	42.34 ± 13.4	3699 ± 535.8	14,414 ± 2469
MFH (n = 17)	9.5 ± 1.3	8:9	242.4 ± 32.7	33.12 ± 8.7	2858 ± 534.6	13,604 ± 2739
Fibrosarcoma (n = 11)	11.0 ± 2.5	6:5	245.2 ± 121.6	60.17 ± 23.9*	4375 ± 1011.3	12,900 ± 3062
Leiomyosarcoma (n = 9)	10.4 ± 2.9	5:4	244.5 ± 72.7	58.64 ± 26.7*	2920 ± 657.3	7594 ± 2040
Desmoid (n = 9)	9.4 ± 1.6	1:8	205.8 ± 227.8	42.03 ± 26.4	2389 ± 476.7	21,015 ± 4605
Other (n = 21)	10.8 ± 1.8	10:11	143.9 ± 16.5	27.97 ± 5.1	3816 ± 906.7	15,093 ± 2833

* P value < 0.05.

than other types of liposarcomas, although this difference was not significant. Levels of leptin were also high in desmoid patients, reflecting the significant female predominance in this histological subtype.

Angiogenic factors were next compared according to site of tumor growth (Table 5) and several site-specific differences were found. Patients with trunk tumors had Ang2 levels that were significantly higher than patients with extremity tumors (P = 0.027) while abdominal tumor patients had intermediate levels. Patients with trunk tumors also had higher levels of leptin than those with abdominal tumors, but this was likely secondary to the high female:male ratio in this subgroup. No significant differences in VEGF and bFGF levels according to site of tumor growth were found.

Finally, subgroup analysis according to tumor grade was performed (data not shown). There were no significant differences in angiogenic factor levels in patients with low-grade and high-grade tumors. Patients with low-grade tumors tended to have higher bFGF levels but this difference was not significant. Stratification into deep and superficial tumors was not performed because there were only four tumors superficial to the muscular fascia, with the remaining 104 tumors invaded or were deep to the muscular fascia.

Angiogenesis-Related Gene Expression Using Oligonucleotide Microarrays

To further investigate the expression of angiogenic factors in STS, tumor samples were collected from 38 STS comprised of 10 liposarcomas, 9 malignant fibrous histiocytomas, 7 fibrosarcomas, 6 leiomyosarcomas, 4 synovial sarcomas, and 2 gastrointestinal stromal tumors. Thirteen normal tissue samples were used for comparison. Gene expression patterns in these tissues were analyzed on Affymetrix HG-U133A oligonucleotide microarrays. These arrays include 22,000 probe sets for 18,400 transcripts, including 14,500 well-characterized human genes.

The microarray data were first analyzed for gene expression of the circulating angiogenic factors analyzed in the first portion of this study: VEGF, bFGF, Ang2, and leptin (Table 6). When expression of these genes in STS samples was compared to expression in normal tissues, bFGF and Ang2 were found to be significantly higher in STS samples. This correlated with our findings of elevated circulating protein levels of bFGF and Ang2 in STS patients. The VEGF gene is represented by five probes sets on the HG-U133A microarray. These probe sets are not unique to the VEGF gene and share sequence homology to other known sequences. Thus only one of the five VEGF probe sets demonstrated significantly higher expression in STS samples compared to normal tissues. Leptin gene expression was not significantly different in STS and normal tissues.

Hierarchical clustering analysis using all probe sets present on the microarray was then used to group tumor and control tissues into groups with similar gene expression patterns (Fig. 3A). STS formed four main clus-

TABLE 5

Circulating Angiogenic Factor Levels According to Site

Tumor site	Size (cm) mean ± SEM	Male:Female	VEGF (pg/ml) mean ± SEM	bFGF (pg/ml) mean ± SEM	Ang-2 (pg/ml) mean ± SEM	Leptin (pg/ml) mean ± SEM
Abdomen (n = 42)	17.8 ± 1.8	24:18	241.5 ± 37.5	39.79 ± 8.3	4362 ± 826.6	10,906 ± 1714
Extremity (n = 36)	12.6 ± 1.3	18:18	237.3 ± 42.2	46.79 ± 10.3	3656 ± 502.2	16,765 ± 1640
Trunk (n = 30)	9.0 ± 1.2	9:21	175.1 ± 27.2	62.24 ± 27.0	5253 ± 804.8*	18,484 ± 2456

* P value < 0.05.

TABLE 6

HG-U133A Microarry Gene Expression

Gene	Probe set	Sarcoma samples (n = 38) mean ± SEM	Normal tissues (n = 13) mean ± SEM	P-value
VEGF	210512_s_at	1337.3 ± 166.5	2048.0 ± 351.1	NS
	210513_s_at	4920.1 ± 575.8	673.3 ± 150.8	<0.0001
	211527_x_at	421.3 ± 65.4	409.3 ± 119.6	NS
	212171_x_at	928.8 ± 101.8	1259.3 ± 261.1	NS
	211668_s_at	666.9 ± 113.9	2255.4 ± 2143.7	NS
bFGF	204421_s_at	176.4 ± 22.7	79.0 ± 14.2	0.0007
Ang2	205572_at	361.5 ± 96.2	62.1 ± 21.1	0.0042
Leptin	207092_at	22.2 ± 3.6	34.2 ± 14.6	NS

ters and normal tissues formed one main cluster in this analysis. Various categories of liposarcomas and leiomyosarcomas tended to group together: cluster 1 consisted of all four round cell liposarcomas and one pleomorphic liposarcoma and cluster 2 consisted of all six leiomyosarcomas and three dedifferentiated liposarcomas. Cluster 3 was comprised of all nine MFH along with two fibrosarcomas and two pleomorphic liposarcomas. Finally, cluster 4 consisted of all synovial sarcomas, five of the seven fibrosarcomas, and both GISTs. Of note, no normal tissue clustered in a STS group and no STS clustered in the normal tissue group.

To determine which tumors had similar patterns of angiogenesis-related gene expression, a unique set of 200 angiogenesis-related genes with 295 probes present on the Affymetrix HG-U133A oligonucleotide microarray was created. Unsupervised hierarchical clustering analysis was performed on STS and normal tissues using only these angiogenesis-related genes. This analysis clustered STS samples into five groups and all normal tissues into one group (Fig. 3B). Both gastrointestinal stromal tumors clustered together (cluster A). Cluster B was the largest group and was comprised of all malignant fibrous histiocytomas, two fibrosarcomas, and four liposarcomas. Cluster C contained all six leiomyosarcomas and one dedifferentiated liposarcoma. Cluster D was comprised of all four round cell liposarcomas and one pleomorphic liposarcoma. Finally, cluster E had all four synovial sarcomas and four fibrosarcomas. These data suggest that the expression patterns of angiogenesis-related genes for STS are significantly different compared to normal tissues. In addition, certain histological subtypes of STS, including malignant fibrous histiocytomas, leiomyosarcomas, round cell liposarcomas, and synovial sarcomas, have similar patterns of angiogenesis-related gene expression.

Next, a supervised analysis of STS and normal tissues was performed to determine which angiogenesis-related genes were differentially expressed between

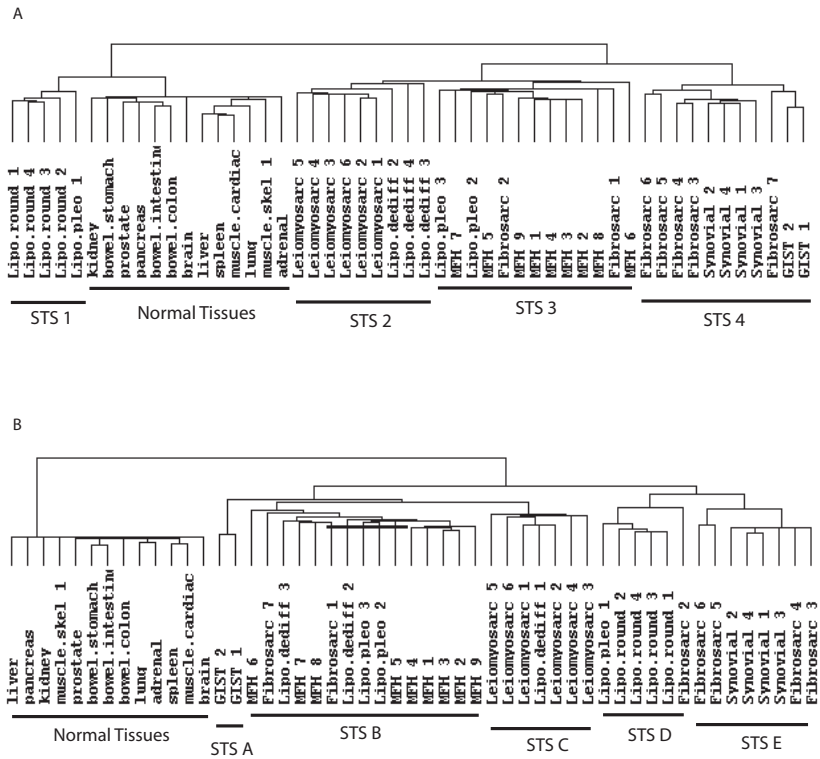

FIG. 3. Hierarchical clustering analysis of normal tissues and STS. Analysis is based on 295 probe sets representing 200 angiogenesis-related genes divides samples into one cluster of normal tissues and five clusters of STS (STS A–E). Lipo.round, round cell liposarcoma; Lipo.pleo, pleomorphic liposarcoma; Leiomyosarc, leiomyosarcoma; Lipo.dediff, dedifferentiated liposarcoma; MFH, malignant fibrous histiocytoma; Fibrosarc, fibrosarcoma; Synovial, synovial sarcoma; GIST, gastrointestinal stromal tumor.

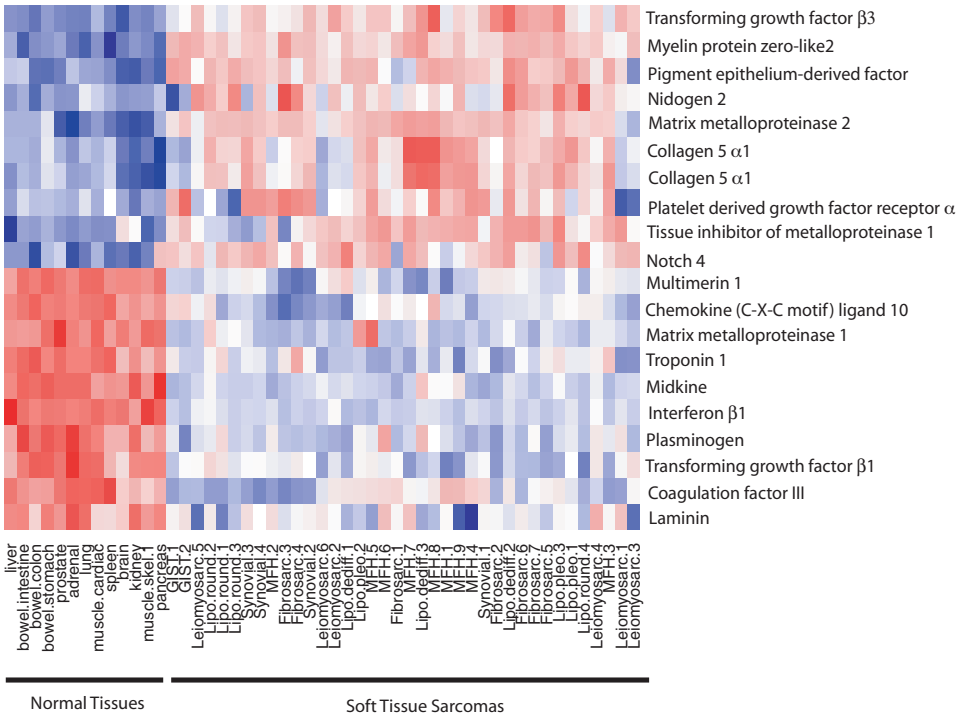

FIG. 4. Differentially expressed angiogenesis-related genes and heat map. The heat map demonstrates expression of the top 10 over-expressed and under-expressed angiogenesis-related probe sets in STS compared to normal tissues. Red designates high expression, white moderate expression, and blue low expression. (Color version of figure is available online.)

these two groups. Unique probe sets that were up-regulated and down-regulated at least seven-fold are shown in Fig. 4. New tumor blood vessel formation requires an exchange of regulatory signals between tumor cells and blood vessel cells (endothelial cell, pericytes, and smooth muscle cells). Several angiogenesis-related cytokines such as transforming growth factor (TGF) $\beta 3$ were up-regulated whereas other cytokines including interferon $\beta 1$ and TGF $\beta 1$ were down-regulated. Two cell surface receptors associated with blood vessel formation, platelet-derived growth factor α (PDGFRα) and Notch 4, were also highly up-regulated. To form new blood vessels, the basement membrane and extracellular matrix surrounding these vessels must be remodeled. Extracellular matrix proteins associated with angiogenesis such as nidogen 2 and collagen 5α1 demonstrated increased expression in STS. Matrix metalloproteinases (MMP) are proteases that digest components of the extracellular matrix and other extracellular proteins, and certain MMPs are up-regulated in virtually all tumors [25]. The MMP and tissue inhibitor of metalloproteinase (TIMP) family of genes represent three of the top 20 differentially expressed genes, with MMP 2 and TIMP 1 being over-expressed in STS in contrast to MMP1 which was underexpressed at low levels. Finally, the coagulation cascade is altered during new blood vessel formation, and several proteins involved in coagulation were found to be down-regulated including multimerin 1, plasminogen, and coagulation factor III.

DISCUSSION

VEGF and bFGF are two of the most well-characterized angiogenic factors, and levels of these proteins have been previously found to be significantly elevated in the blood of patients with STS [8–12]. In this study, VEGF and bFGF levels were on average 10 to 13 times higher in patients than controls, although there was large variability in patient levels. VEGF plasma levels were broadly elevated when examined based on histological subtype and location of tumor growth, while bFGF levels were highest for two specific subtypes: fibrosarcomas and leiomyosarcomas. Increased VEGF levels in a broad spectrum of tumor histology and location suggests that anti-VEGF therapies such as bevacizumab may be effective for most STS. However, it is still unclear whether elevated levels of circulating VEGF will correlate with response to anti-VEGF therapies.

Angiopoietins are another class of pro-angiogenic proteins with two family members, Ang1 and Ang2, that have differential effects. Ang2 competes with Ang1 in binding to the Tie2 receptor, which is selectively expressed on vascular endothelium [26], and has been found to be important in the vascular remodeling associated with tumor angiogenesis [27]. One model suggests that in early tumor angiogenesis, Ang2 acts on endothelial cells and causes blood vessel destabilization which is followed by VEGF-induced new blood vessel formation [28]. Several studies have demon-

strated increased Ang2 expression in tumors [29], but circulating levels of Ang2 have been found to be elevated in only a few cancers [30]. This study is the first to demonstrate that Ang2 levels are elevated in sarcoma patients and that Ang2 levels correlate with tumor size.

To our knowledge, this is also the first study to examine circulating levels of leptin in patients with STS. Leptin is a circulating hormone produced by adipose tissue and has been recently shown to have pro-angiogenic effects [31]. In this study, leptin levels were generally higher in patients with liposarcoma and highest in patients with well-differentiated liposarcoma. Since normal adipocytes produce leptin, this would imply that liposarcomas maintain leptin secretion during malignant transformation, and this may contribute to the formation of new blood vessels in these tumors.

Our analysis of angiogenesis-related gene expression in sarcomas revealed that STS have different angiogenesis-related gene expression patterns compared to normal tissues and that specific histological subtypes have similar angiogenesis-related gene expression patterns. Investigators continue to develop novel methods of using gene expression data to analyze the biological behavior of tumors. For example, Ramaswamy *et al.* analyzed gene expression data on 76 adenocarcinomas and identified an expression pattern for metastasis based on 128 genes [32]. As anti-angiogenic therapies become more broadly used in the treatment of sarcomas, perhaps further analysis of angiogenesis-related genes may help predict response to specific anti-angiogenic agents or even direct the use of one anti-angiogenic agent over another.

Given the majority of endothelial cells that comprise new tumor blood vessels arise from the organ or tissue in which the tumor is growing, it could be postulated that angiogenic factors that drive tumor angiogenesis may be more site-specific than histological subtype-specific. However, further hierarchical clustering analysis of our microarray data demonstrated that similarities in clusters were much more apparent based on histological subtype than based on site of tumor growth (data not shown). For example, round cell liposarcomas commonly originate in the retroperitoneum and in the extremity. All four round cell liposarcomas clustered together despite three of these tumors growing in the extremity and one growing in the retroperitoneum. Leiomyosarcomas arise in bowel and in the retroperitoneum, and all six leiomyosarcomas clustered together despite four arising in the retroperitoneum and two arising in bowel. Thus, we conclude that histological subtype is a more important determinant of angiogenesis-related gene expression than site of tumor growth.

Our microarray analysis identified several other angiogenesis-related genes that are differentially expressed in STS *versus* normal tissues. PDGFRα was highly up-regulated in the STS samples that we analyzed. PDGFRα has been found to be important not only in tumor angiogenesis [33] but also tumor progression [34]. Normal mesenchymal cells such as fibroblasts and smooth muscle cells expression PDGFRα, and activation of this receptor by its ligand, PDGF, is associated with increased proliferation in these cell types [34]. While PDGF itself was not found to be up-regulated in STS, activating mutations in PDGFRα, which allow the receptor to be ligand-independent, are found in some gastrointestinal stromal tumors [35] and other types of STS [36]. PDGFRα is a promising target for STS because inhibition of this receptor may inhibit sarcoma cell proliferation as well as angiogenesis, and several PDGFRα inhibitors, such as imatinib and SU11248, are currently in clinical use [37].

Notch 4 was one of the most highly up-regulated genes, and further analysis revealed that another member of the Notch family of receptors, Notch 1, was also highly up-regulated in STS as was the Notch ligand, Jagged (data not shown). Although Notch has been demonstrated by a number of groups to be critical for vascular development and patterning [38] and arterial differentiation [39], its role in tumor angiogenesis is less clear. This signaling pathway is important in cell differentiation and is often deregulated in human malignancies [40]. The up-regulation of Notch receptors and its ligand Jagged in STS suggests that this signaling pathway may be another potential therapeutic target for STS [41].

In conclusion, this study examines a panel of circulating angiogenic factors in patients with STS and the expression pattern of 200 angiogenesis-related genes in STS specimens. Levels of circulating angiogenic factors are highly variable in STS depending on tumor size, histological subtypes, and site of tumor growth. Histological subtypes of STS also have significantly different patterns of angiogenesis-related genes, and several genes and gene families are highly up-regulated in STS compared to normal tissues. Further investigation is needed to determine if angiogenic profiling will lead to advances in the diagnosis, prognosis, or treatment of STS or other cancers [42].

ACKNOWLEDGMENTS

We thank Rachel Jacobi and Beata Korytowski for technical assistance with blood and tissue samples and Monica Cha for expert database management.

REFERENCES

1. Singer S, Demetri GD, Baldini EH, Fletcher CD. Management of soft-tissue sarcomas: An overview and update. Lancet Oncol 2000;1:75.
2. Brennan MF, Lewis, JL. Diagnosis and management of soft tissue sarcoma. London: Martin Dunitz, 2002.

3. Folkman J. Tumor angiogenesis: Therapeutic implications. N Engl J Med 1971;285:1182.
4. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86: 353.
5. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401.
6. Peters BA, Diaz LA, Polyak K, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 2005;11:261.
7. Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci USA 2003;100:10623.
8. Feldman AL, Pak H, Yang JC, Alexander HR, Jr., Libutti SK. Serum endostatin levels are elevated in patients with soft tissue sarcoma. Cancer 2001;91:1525.
9. Graeven U, Andre N, Achilles E, Zornig C, Schmiegel W. Serum levels of vascular endothelial growth factor and basic fibroblast growth factor in patients with soft-tissue sarcoma. J Cancer Res Clin Oncol 1999;125:577.
10. Kuhnen C, Lehnhardt M, Tolnay E, Muehlberger T, Vogt PM, Muller KM. Patterns of expression and secretion of vascular endothelial growth factor in malignant soft-tissue tumours. J Cancer Res Clin Oncol 2000;126:219.
11. Linder C, Linder S, Munck-Wikland E, Strander H. Independent expression of serum vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in patients with carcinoma and sarcoma. Anticancer Res 1998;18:2063.
12. Yoon SS, Segal NH, Olshen AB, Brennan MF, Singer S. Circulating angiogenic factor levels correlate with extent of disease and risk of recurrence in patients with soft tissue sarcoma. Ann Oncol 2004;15:1261.
13. Nielsen TO, West RB, Linn SC, et al. Molecular characterisation of soft tissue tumours: A gene expression study. Lancet 2002;359:1301.
14. Segal NH, Pavlidis P, Antonescu CR, et al. Classification and subtype prediction of adult soft tissue sarcoma by functional genomics. Am J Pathol 2003;163:691.
15. Segal NH, Pavlidis P, Noble WS, et al. Classification of clear-cell sarcoma as a subtype of melanoma by genomic profiling. J Clin Oncol 2003;21:1775.
16. Ohali A, Avigad S, Zaizov R, et al. Prediction of high risk Ewing's sarcoma by gene expression profiling. Oncogene 2004; 23:8997.
17. Lee YF, John M, Falconer A, et al. A gene expression signature associated with metastatic outcome in human leiomyosarcomas. Cancer Res 2004;64:7201.
18. Ochi K, Daigo Y, Katagiri T, et al. Prediction of response to neoadjuvant chemotherapy for osteosarcoma by gene-expression profiles. Int J Oncol 2004;24:647.
19. Khatua S, Peterson KM, Brown KM, et al. Overexpression of the EGFR/FKBP12/HIF-2alpha pathway identified in childhood astrocytomas by angiogenesis gene profiling. Cancer Res 2003;63:1865.
20. McCarty MF, Liu W, Fan F, Parikh A, Reimuth N, Stoeltzing O, Ellis LM. Promises and pitfalls of anti-angiogenic therapy in clinical trials. Trends Mol Med 2003;9:53.
21. Heymach JV. Angiogenesis and antiangiogenic approaches to sarcomas. Curr Opin Oncol 2001;13:261.
22. Peale FV, Jr., Gerritsen ME. Gene profiling techniques and their application in angiogenesis and vascular development. J Pathol 2001;195:7.
23. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998;95:14863.
24. Wauters M, Van Gaal L. Gender differences in leptin levels and physiology: A role for leptin in human reproduction. J Gend Specif Med 1999;2:46.
25. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: Trials and tribulations. Science 2002;295:2387.
26. Dumont DJ, Gradwohl GJ, Fong GH, Auerbach R, Breitman ML. The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene 1993;8:1293.
27. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000;407:242.
28. Liekens S, De Clercq E, Neyts J. Angiogenesis: Regulators and clinical applications. Biochem Pharmacol 2001;61:253.
29. Tait CR, Jones PF. Angiopoietins in tumours: The angiogenic switch. J Pathol 2004;204:1.
30. Caine GJ, Blann AD, Stonelake PS, Ryan P, Lip GY. Plasma angiopoietin-1, angiopoietin-2 and Tie-2 in breast and prostate cancer: A comparison with VEGF and Flt-1. Eur J Clin Invest 2003;33:883.
31. Sierra-Honigmann MR, Nath AK, Murakami C, et al. Biological action of leptin as an angiogenic factor. Science 1998;281:1683.
32. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. Nat Genet 2003;33:49.
33. Tsutsumi N, Yonemitsu Y, Shikada Y, et al. Essential role of PDGFRalpha-p70S6K signaling in mesenchymal cells during therapeutic and tumor angiogenesis in vivo: Role of PDGFRalpha during angiogenesis. Circ Res 2004;94:1186.
34. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 1999;79:1283.
35. Heinrich MC, Corless CL, Duensing A, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. Science 2003; 299:708.
36. Ostman A, Heldin CH. Involvement of platelet-derived growth factor in disease: Development of specific antagonists. Adv Cancer Res 2001;80:1.
37. von Mehren M. New therapeutic strategies for soft tissue sarcomas. Curr Treat Options Oncol 2003;4:441.
38. Limbourg FP, Takeshita K, Radtke F, Bronson RT, Chin MT, Liao JK. Essential role of endothelial Notch1 in angiogenesis. Circulation 2005;111:1826.
39. Bicknell R, Harris AL. Novel angiogenic signaling pathways and vascular targets. Annu Rev Pharmacol Toxicol 2004;44: 219.
40. Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: A new approach to the development of cell fate modifying agents. Oncogene 2003;22:6598.