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Immunohistochemical Detection of HER-2/*neu*, c-Kit (CD117) and Vascular Endothelial Growth Factor (VEGF) Overexpression in Soft Tissue Sarcomas

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Abstract. *Purpose: The aim of this study was to determine the incidence of HER-2/neu, VEGF and CD117 overexpression in soft tissue sarcomas (STS) and to study the effect of this overexpression, if present, on survival in patients with specific histological subtypes of STS. Materials and Methods: We conducted a retrospective observational study on patients diagnosed with STS during the period of 1986-2001. HER-2/neu overexpression was measured in these patients by immunohistochemistry (IHC) using the Hercep test developed by DAKO®. VEGF expression was detected by the avidin-biotin-complex method using Santa Cruz biotechnology (SC 7629). Immunohistochemical staining for c-kit was performed using a 1:250 dilution of the rabbit polyclonal antibody A4502 (IMPATH, CA) with the EnVision detection system. Results: Two hundred and seventy three patients were diagnosed as having STS between 1986 and 2001, however of these patients, only 90 (51 females and 49 males) had enough sample available for testing. Patients who overexpressed VEGF had a significantly shorter survival (23 vs. 52 months; p=0.01). There was no effect of overexpression of either CD117 or HER-2/neu on survival. Studying the individual histological subtypes we found that, in malignant fibrous histiocytoma, overexpression of either VEGF or CD117 increased survival (41.3 vs. 19.5 months, p=0.01; and 84.5 vs. 17 months, p=0.006 respectively). In leiomyosarcoma, VEGF overexpression significantly decreased survival (7.5 vs. 76 months, p=0.03), while CD117 overexpression significantly increased survival (70.9 vs. 46.3 months, p=0.03). Conclusion: VEGF overexpression is associated with an adverse outcome in STS. Whether this is true of any particular histological subtype is unclear and needs further investigation. Also, site-specific agents targeting these three bio-markers (alone or with conventional therapy) may have a therapeutic role and need to be elaborated in future clinical trials.*

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There are approximately 10,700 newly diagnosed soft tissue sarcomas (STS) per year in the United States (1). This represents about 1% of newly diagnosed malignant tumors and 15% of pediatric malignancies (2). The incidence of sarcomas has increased over recent decades (1). Obstacles to the success of contemporary treatments include the development of drug resistance by tumor cells and the insufficient tumor selectivity of treatments. Hence knowledge of the tumor biology of sarcomas is crucial to the development of new agents that will improve survival of these malignancies.

The HER-2/*neu* (also known as c-*erb*B-2) oncogene is the second member of the epidermal growth factor receptor family. It has been found to be overexpressed in many different types of human malignancies, notably breast, lung, ovarian, gastric, pancreatic, colorectal and cancers of the female genital tract (3-10). Overexpression of HER-2/*neu* in breast cancer has been associated with poor overall survival and has been shown to enhance malignancy and the metastatic phenotypes. HER-2/*neu* overexpression also seems to induce chemoresistance in certain experimental conditions (3). These findings suggest the HER-2/*neu* may serve as an excellent target for developing anticancer agents specific for HER-2/*neu*-overexpressing cancer cells and the benefit of the monoclonal antibody against HER-2/*neu* (trastuzumab) in the treatment of advanced female breast carcinoma has been demonstrated (11).

Vascular endothelial growth factor (VEGF) is an endothelial specific growth factor that can stimulate angiogenesis by increasing vascular permeability (12). Vascular endothelial growth factor (VEGF) expression has been detected at the mRNA and protein level in a number of malignancies like breast, renal cell, cervical, non-small cell carcinoma of the lung, ovarian, gastric and hepatocellular malignancies (13-19). Angiogenesis inhibition strategies have many advantages when they are compared to other approaches to anti-cancer therapy. Unlike most tumor cells, endothelial cells are readily accessible from the blood circulation and as genetically stable cells they are not likely to develop resistance to cytostatic therapy (20).

The c-kit proto-oncogene encodes a receptor tyrosine kinase that is crucial to hematopoiesis, melanogenesis and gametogeneis (21). This transmembrane receptor tyrosine kinase, KIT, is defined by the CD117 antigen, and is the product of the c-kit proto-oncogene. Activating mutations in the c-kit gene have been identified in the majority of gastrointestinal stromal tumors (GIST) and appear to be crucial to the pathogenesis of the disease (22). STI571 (imatinib) has been shown to be a selective inhibitor of the tyrosine kinase activity of CD117 (23).

Despite the recent emergence of these attractive molecular targets, there is not much data available regarding the association between these targets and soft tissue sarcomas. Hence, to evaluate the role of HER-2/*neu*, VEGF and CD117 overexpression in STS, we reviewed our 15-year multi-institutional experience in patients diagnosed with STS. The aim of this study was to determine the incidence of HER-2/*neu*, VEGF and CD117 overexpression in soft tissue sarcomas and to study the effect of this overexpression, if present, on survival in patients with specific histologic subtypes of STS.

Materials and Methods

We conducted a retrospective observational study on patients diagnosed with soft tissue sarcoma during the period of 1986-2001. An extensive chart review was performed and data regarding age, sex, smoking history, symptoms at presentation, site of malignancy, histology, stage at presentation and survival in months from the diagnosis was collected. Secondary data collected included distant metastatic sites, any other malignancies, treatment with surgery or chemotherapy and recurrence. These patients were then separated into groups based on survival, recurrence, initial site of the cancer, stage at presentation and histological type. These separate categories were used for analysis with the VEGF status.

The tissue specimens used to make the initial pathologic diagnosis of the type of malignancy were used to assess for the three clinicobiologic markers. HER-2/*neu* overexpression was measured in these patients by using immunohistochemistry (IHC). IHC staining was carried out on formalin-fixed, paraffin-embedded material, using the Hercep test developed by DAKO® (R & D Systems, Minneapolis, MN, USA). Immunostaining was classified as follows: 0=no staining; 1+=faint, incomplete membranous pattern; 2+=moderate, complete membranous pattern; 3+=strong membranous pattern (24). A trained pathologist (MK), who was blinded to the clinical history of the patient, interpreted these results. An IHC score of 2 + or greater was considered as HER-2/*neu* overexpression (25, 26). The pathologist was blinded to any knowledge of the clinical status/stage of the patient and other histopathological information.

Table I. *Distribution of the various histological types of sarcomas.*

No.	Histological type	No. of patients (%)	Overexpression of		
			HER-2/*neu* (%)	VEGF (%)	CD 117 (%)
1.	Malignant fibrous histiocytoma	15 (16.7)	0 (0)	4 (26.7)	2 (13.3)
2.	Leiomyosarcoma	15 (16.7)	0 (0)	4 (26.7)	7 (46.7)
3.	Carcinosarcomas	12 (13.3)	6 (50)	3 (25)	3 (25)
4.	Mullerian mixed tumor	9 (10)	1 (11.1)	1 (11.1)	0 (0)
5.	Dermatofibrosarcoma	7 (7.8)	0 (0)	1 (14.3)	2 (28.6)
6.	Nephroblastoma	5 (5.6)	0 (0)	0 (0)	0 (0)
7.	Sarcoma	4 (4.4)	0 (0)	1 (25)	0 (0)
8.	Myxoid liposarcoma	3 (3.3)	0 (0)	1 (33.3)	1 (33.3)
9.	Fibrosarcoma	2 (2.2)	0 (0)	0 (0)	0 (0)
10.	Stromal sarcoma	2 (2.2)	0 (0)	1 (50)	0 (0)
11.	Embryonal rhabdomyosarcoma	2 (2.2)	0 (0)	0 (0)	0 (0)
12.	Liposarcoma	2 (2.2)	0 (0)	0 (0)	1 (50)
13.	Phyllodes tumor	2 (2.2)	0 (0)	1 (50)	0 (0)
14.	Hepatoblastoma	2 (2.2)	0 (0)	0 (0)	1 (50)
14.	Others	10 (11.1)	1 (10)	2 (20)	3 (30)
	Total	90 (100)	8 (8.8)	19 (21.1)	20 (22.2)

VEGF expression was detected by the avidin-biotin-complex method using Santa Cruz biotechnology (SC 7629) (R & D Systems). The expression of VEGF was assessed according to the percentage of immunoreactive cells of a total of 1000 cells which was defined by Takahashi *et al.* (27). Immunoreactivity was graded as follows: more than 10% of the cells staining were graded as positive. No detectable staining or <10% of cells staining was graded as negative. The qualitative intensity of staining for VEGF was assessed using a scale between 0 and +++, with 0 representing no detectable stain and +++ representing strongest stain.

We performed c-kit (CD117) testing using the FDA approved immunohistochemical staining technique. The features of the immunoreaction were recorded on a semi-quantitative scale: the relative number of positive cells (0%, <10%, 10-50% and >50%) and the intensity of the reaction. C-kit results were reported as positive if they were >10% and negative if they were <10% as per the FDA guidelines. Immunohistochemical staining for c-kit was performed using a 1:250 dilution of the rabbit polyclonal antibody A4502 (IMPATH, CA, USA) with the EnVision detection system. The antigen retrieval method was not utilized. Appropriate positive and negative controls were used throughout the testing process.

Table II. *Survival based on overexpression of the clinicobiologic markers.*

		Mean survival (months)	*p*-value*
VEGF	Negative	52	0.01
	Positive	23	
CD 117	Negative	42	0.17
	Positive	64	
HER-2/*neu*	Negative	48	0.87
	Positive	34	

* *p*-value obtained using the Log rank test for survival function

The data was analyzed using SPSS 10 for Windows®. The Wilcoxon rank test was used to evaluate the effect of VEGF expression on age at diagnosis and the Chi square test for sex differences. Survival analysis was performed using the log rank sum test.

Results

Two hundred and seventy-three patients were diagnosed as having soft tissue sarcoma between 1986 and 2001. These included 164 females and 109 males with a mean age of 56 years (range: 1-93 years). However of these patients only 90 patients had enough samples for testing for the overexpression of all three markers. Hence, only these 90 patients were included for the purpose of this study. These 90 patients included 51 females and 49 males. The mean age at diagnosis was 56.9 years (range: 0-89 years).

The initial site of the sarcomas included gynecological sites namely, uterus, ovary and endocervix (24; 26.6%), soft tissue (22 patients; 24.4%), liver and gastrointestinal tract (10; 11.1%), skin (8; 8.9%), retroperitoneum (8; 8.9%), urinary system *i.e.* kidney and bladder (5; 5.6 %) and breast (2; 2.2%). Eleven patients (12.2%) developed the malignancy in other isolated sites. The details of the histological subtypes of the sarcomas are given in Table I.

Of these 90 patients, 69 (76.7%) underwent some form of surgical procedure to decrease the tumor load, while the remaining 21 (23.3%) were not thought to be surgical candidates. Thirty patients (33.3%) received chemotherapy while the remaining 60 (66.6%) did not receive any chemotherapeutic agent. Eight out of the 90 patients (8.8%) were not thought to be suitable candidates for either surgery or chemotherapy.

When the effects of overexpression of the three clinicobiologic markers (VEGF, CD 117 and HER-2/*neu*) were studied, it was found that patients who overexpressed VEGF had a significantly shorter survival as compared to those who did not (23 months *vs.* 52 months; $p=0.01$). There was no effect of overexpression of either CD117 or HER-2/*neu* on survival of the patients (Table II). We then further tried to correlate the overexpression of these three markers on survival in the most common individual histological subtype of STS. In MFH, overexpression of either VEGF or CD117 was associated with an increased survival (41.3 *vs.* 19.5 months, $p=0.01$; and 84.5 *vs.* 17 months, $p=0.006$, respectively). In LMS, VEGF overexpression was associated with a significantly decreased survival (7.5 *vs.* 76 months, $p=0.03$), while CD117 overexpression was associated with a significantly increased survival (70.9 *vs.* 46.3 months, $p=0.03$). In CS, however, there was no correlation between the overexpression of HER-2/*neu* and CD117 and patient survival although VEGF overexpression was associated with increased survival (24 *vs.* 14.8 months, $p=0.05$).

Discussion

Data studying the biology of STS is relatively sparse. One reason for this may be that these are a group of extremely rare, anatomically and histologically diverse malignant neoplasms (28). In this study we tried to evaluate the incidence of overexpression of three clinicobiologic markers, against which specific targeted therapies are available and to study the effect of this overexpression, if any, on survival in patients with STS.

In our study we found that VEGF was overexpressed in 19 out of 90 patients (21.1%) with STS. Chao *et al.* studied VEGF expression in selected paraffin-embedded tissue of surgical specimens from 79 patients with soft tissue sarcomas using immunohistochemical techniques, and found that the majority of high-grade soft tissue sarcomas in this study have high intensity VEGF expression. They also concluded that VEGF expression did not seem to be an independent predictor of clinical outcome (29). Similarly Iyoda and associates studied VEGF expression by immunohistochemical methods in thoracic sarcomas and concluded that strong VEGF expression is an independent prognostic factor in patients with thoracic sarcomas (30). Emoto and co-workers studied 21 patients with carcinosarcomas of the uterus using anti-VEGF antibodies and found that the tumors with lymph-vascular invasion showed a higher VEGF expression than the tumors without lymph-vascular invasion (31). Yudoh *et al.* studied 115 patients with soft tissue sarcomas and found that the tissue concentration of VEGF is an independent prognostic factor for the disease outcome of patients with soft tissue sarcoma (32). Our finding that overexpression of VEGF was associated with a significantly decreased survival (23 *vs.* 52 months, $p=0.01$) agrees with the above findings. In addition, when we evaluated individual subtypes of STS, we found that VEGF overexpression was associated with an adverse outcome in patients with LMS. The clinical relevance of this difference in survival in patients with LMS is unclear and may have appeared only because of the small number of patients in our study. This needs further validation in larger trials.

We found that 20% of the sarcomas overexpressed CD117, including 46.7% patients with LMS. Chen and co-workers however found c-kit reactivity in 87% of cases of granulocytic sarcomas (33). Similarly, Tamborini *et al.* found the presence of c-kit and SCF mRNA in 20 out of 23 cases of synovial sarcoma (34). These findings contrast with those of Hornick and Fletcher who performed IHC analysis for KIT in 365 soft tissue sarcomas. They found that most tumors evaluated were completely negative for KIT, including all cases of leiomyosarcoma (35). This difference could have been due to the difference in techniques used for the immunohistochemical assay of CD117. In this study, we used the same technique that was used in the large co-operative trials evaluating the efficacy of imatinib mesylate (Gleevec®) in gastrointestinal stromal tumors (GISTs).

Swisher *et al.* reported no HER-2/*neu* overexpression in their study of adenosarcomas and malignant mixed mesodermal tumors (36). In a study by Mark *et al.* using fluorescent *in situ* hybridization (FISH) to evaluate HER-2/*neu* overexpression in embryonal rhabdomyosarcoma, one case out of nine clearly showed HER-2/*neu* gene amplification (37). Both of these studies were relatively small and did not directly address the issue of HER-2/neu overexpression and its impact on survival or site predilection. Oliveira *et al.* found no overexpression of HER-2/neu in 162 cases of soft tissue sarcoma (38). In our study we found that 8 out of 90 specimens (8.8%) overexpressed HER-2/neu. Our findings agree with those of George and co-workers who found no evidence of c-erbB-2 protein overproduction in epithelioid sarcomas, hepatoblastomas, leiomyosarcomas, liposarcomas, rhabdomyosarcomas and synovial sarcomas (39). Similarly Duda *et al.* found HER-2/*neu* gene amplification in 5.7% of 105 specimens and demonstrated HER-2/*neu* overexpression in 37% of 94 specimens (40). These apparent differences in findings again may be explained by the different methods used for detection of HER-2/*neu* overexpression. In our study, we preferred IHC over FISH since it is a routine, relatively inexpensive analytic method and can be performed on permanent sections. It can be performed on the smallest of specimens, including cytology specimens, provided tumor cells are present. Interpretation is not influenced by non-tumor material present in the specimen. This method cannot detect normal low levels of the protein and experience has shown that, if definitive immunostaining is observed in paraffin sections, concordance exists with the FISH technique (24, 25).

In conclusion, the tumor biology of different histological subtypes of STS is different and this knowledge is critical while analyzing data on STS. We found that VEGF overexpression is associated with an adverse outcome in STS. Whether this is true of any particular histological subtype is unclear and needs to be studied further. Also, the role of angiogenesis inhibitors including thalidomide in STS needs to be elucidated in clinical trials. The incidence and role of c-kit overexpression in STS is unclear with current data showing conflicting evidence. Further larger trials need to be conducted in order to clarify this controversy. Carcinosarcomas seem to overexpress HER-2/*neu* more than any other subtype of STS. The role of trastuzumab (Herceptin®) alone or in conjunction with traditional therapy in this malignancy needs to be examined. Finally, our findings need to be validated in larger studies that include sizeable cohorts of patients with individual subtypes of STS.

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