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ARTICLE

# Her-2 Oncogene Amplification, Chromosome 17 and DNA Ploidy Status in Synovial Sarcoma

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**The treatment options for synovial sarcoma (SS) are very limited, though this type of sarcoma seems to be more heterogeneous than it has been traditionally considered. The present study investigates the Her-2 oncogene status of 20 cases of SS, to determine whether Her-2 amplification can be considered as a prognostic factor. Her-2 oncogene amplification was determined on smears (frozen material was used from our tumor bank in each case), using fluorescence in situ hybridization technique (dual color FISH with centromeric probe for chromosome 17 and specific probe for Her-2 oncogene). Moreover, protein expression was assessed by immunohistochemistry, and DNA ploidy status was measured using image analysis. We had 5 biphasic and 15 monophasic SSs, patients' age ranged from 13 to 68 years (mean, 39.8 years). Tumor size was larger than 5 cm in each case. Follow-up time ranged from 6 to 78 months (mean, 38.5 months). For statistical analysis the chi-square test was used. Her-2 oncogene amplification was found in three cases (15.0%) of 20 SSs. These cases proved to be 2+ positive by immunohistochemistry, but massive amplification, characteristic of a subset of breast carcinomas, was not observed. Her-2 oncogene amplification was significantly associated with a lower risk of developing metastasis (P<0.05) (none of the 3 amplified cases had metastases), while no association was found with recurrence. Six cases proved to be aneuploid and 14 were diploid, but no association was found between Her-2 amplification status and ploidy, and between ploidy status and metastasis or recurrence. Our results emphasize and confirm that Her-2 oncogene amplification is a rare event in SS, but the small subset of SS with Her-2 amplification has a better overall prognosis. Furthermore, this may open a theoretically new treatment possibility with Trastuzumab for Her-2-amplified cases of SS.** (Pathology Oncology Research Vol 11, No 3, 133–138)

*Key words:* synovial sarcoma, Her-2 oncogene, FISH, DNA ploidy, immunohistochemistry, prognostic factor

## *Introduction*

Synovial sarcoma is a unique soft tissue neoplasm, characterized by a better prognosis compared to other high-grade sarcomas, with a 5-year overall survival rate of 50% to 80% depending on the patient's age, the tumor size, extent of poorly differentiated areas, and the resectability of the tumor.[1-4] It appears in two major forms, biphasic and monophasic, and is cytogenetically characterized by the t(X;18)(p11, q11) translocation (fusion proteins are SYT-SSX1 and SYT-SSX2) found in more than 90% of cases.[5] Many studies have attempted to identify nuclear markers of prognostic significance, including Her-2 oncogene status. Several recent reports have examined Her-2 status in synovial sarcoma, but it appears that the expression rate and gene amplification in synovial sarcoma is controversial. Furthermore, the role of Her-2 oncogene and DNA ploidy as prognostic factors in synovial sarcoma also seems to be controversial based on previous studies. For these reasons and to further investigate the possibility of Her-2 expression and amplification in synovial sarcoma, we examined a cohort of 20 patients by immunohistochemistry, fluorescence in situ hybridization (FISH), and DNA cytometry.

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### *Materials and methods*

*Tumor samples*

Fresh and formalin-fixed, paraffin-embedded tissue samples of synovial sarcoma were obtained from the Department of Pathology, St John's Hospital, Budapest, and from the Department of Human and Experimental Tumor Pathology, National Institute of Oncology, Budapest. In all cases, the primary tumor was available for study. Surgically resected tumor tissues were partly stored at –80°C for FISH, and partly fixed in buffered formalin and embedded in paraffin. Clinical history by chart review was obtained after approval had been obtained from the institutional review board. The diagnosis of synovial sarcoma was confirmed as defined by World Health Organization criteria: immunohistochemical expression of at least one epithelial marker (AE1/AE3, Cam 5.2 or EMA), and/or the presence of t(X;18). Clinical data are summarized in *Table 1*. We evaluated 20 synovial sarcomas (5 biphasic and 15 monophasic ones).

*Immunohistochemistry*

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, and exposed to the primary antibody using the EnVision+ system (Dako, Carpinteria, CA). Primary anti–HER-2/neu antibody (rabbit polyclon-

***Table 1.*** **Clinical data, Her-2 status by FISH and immunohistochemistry, and DNA ploidy index of 20 synovial sarcomas**

| | *Sex ; Age* | *Diagnosis* | *Localization* | *Her-2 copy number* | *Her-2 protein* | *DI* | *Recurrence, metastasis* |
|---|---|---|---|---|---|---|---|
| 1. | M ; 26 | biphasic synovial sc. | l. leg | 2 | – | 1.03 | local recurrence, metastases; died |
| 2. | M ; 58 | monophasic synovial sc. | r. thigh | 2 | focal + | 1.03 | metastases, lung |
| 3. | F; 29 | monophasic synovial sc. | r. thigh | 2 | focal + | 0.99 | metastases, lung |
| 4. | F ; 52 | monophasic synovial sc. | r. leg | 2 | – | 0.89 | metastases, lung |
| 5. | F ; 68 | monophasic synovial sc. | r. leg | 2 | – | 1.01 | local recurrence |
| 6. | M; 67 | biphasic synovial sc. | r. popliteal region | 2 | focal + | 1.05 | metastases, lung; died |
| 7. | F; 41 | monophasic synovial sc. | l. thigh | 2 | – | 0.98 | local recurrence, metastases; died |
| 8. | M; 48 | monophasic synovial sc. | r. fore arm | 2 | – | 0.96 | local recurrence |
| 9. | M; 42 | monophasic synovial sc. | l. fore arm | **>10%; 5.4 amplification** | **++** | 0.98 | no |
| 10. | M; 29 | monophasic synovial sc. | pelvis | 2 | – | 0.98 | local recurrence, metastases; died |
| 11. | F ; 13 | biphasic synovial sc. | r. hip | **>10%; 4.8 amplification** | **++** | 1.23 | no |
| 12. | M ; 41 | monophasic synovial sc. | r. fore arm | 2 | focal + | 1.01 | metastases, lung; died |
| 13. | M , 37 | biphasic synovial sc. | r. leg | **3, chr 17 trisomy** | – | 1.74 | no |
| 14. | M ; 40 | monophasic synovial sc. | r. leg | 2 | – | 1.06 | local recurrence |
| 15. | M ; 33 | biphasic synovial sc. | r. thigh | **>10%; 5.8 amplification** | **++** | 1.00 | local recurrence |
| 16. | F ; 28 | monophasic synovial sc. | l. thigh | 2 | – | 1.19 | local recurrence |
| 17. | F ; 35 | monophasic synovial sc. | l. hip | **4, chr 17 tetrasomy** | – | 2.12 | local recurrence, metastases, lung |
| 18. | M ; 41 | monophasic synovial sc. | retroperit. | 2 | – | 1.09 | no |
| 19. | F; 41 | monophasic synovial sc. | r. thigh | 2 | – | 1.08 | no |
| 20. | F; 25 | monophasic synovial sc. | r. foot | 2 | – | 1.16 | local recurrence |

Her-2 copy number: determined by FISH; Her-2 protein: determined by immunohistochemistry. DI: DNA index

al, catalog number A0485; Herceptest, Dako) was applied at a dilution of 1:2000 for 60 minutes at room temperature. Before exposure to the primary antibody, sections were microwave-pretreated in EDTA, pH 8.0, to retrieve antigenicity, and incubated with endogenous peroxidase-blocking solution for 10 minutes at room temperature. Positive control, constituted by a breast carcinoma showing more than 80% positive staining for HER2/neu, as well as negative control, in which the primary antibody was omitted, were stained in parallel. All cases were examined for both cytoplasmic and membrane immunoreactivity. Results of immunohistochemistry for Her-2 were scored according to the manufacturer's instructions. Briefly, this scoring is as follows: 0, no membrane staining; 1+, cytoplasmic and/or faint partial membrane staining; 2+, weak complete membrane staining in >10% of cells; 3+, intense complete membrane staining in >10% of cells.

*Interphase fluorescence in situ hybridization for Her-2*

FISH for Her-2 gene amplification was performed on smears, obtained from our tumor bank. We used the dual color chromosome 17q12(Her-2/NEU)/alphasatellite17 cocktail, direct labeled probe (Qbiogene, France). Five µl hybridization mixture containing 5 µg probe/60% formamide in 2x sodium saline citrate (SSC) were dropped onto the cell preparations and covered with plastic film. Samples and probes were denatured simultaneously at 75°C for 5 min, followed by hybridization at 37°C in a wet chamber overnight. A post-hybridization wash was performed under the following conditions: three 5-min washes in 60% formamide/2x SSC at pH 7, 37°C, and three 5-min washes in 2x SSC at room temperature. The preparations were mounted with Vectashield mounting medium containing 0.02 µg/ml DAPI (4'-6'-diamidino-2-phenylindole) nuclear counterstain (Vector Laboratories, Burlingame, CA). In all of the cases, the recommended quantities and dilutions were used. Slides were evaluated using dual-band and triple-band filtered fluorescence microscope (Olympus BX40). One hundred randomly selected tumor nuclei per smear were captured on an applied image analysis system and evaluated. A tumor was considered positive for Her2/neu gene amplification if the quotient of the average number of Her-2 signals and signals of alphasatellite17 per nucleus was greater than two.

*DNA image analysis*

We analyzed the nuclear DNA content by DNA image cytometry. Smears were post-fixed in 4% buffered formalin. We stained the samples by a stoichiometric method according to Feulgen using Schiff reagent (Merck, Darmstadt, Germany). Sample preparation, fixation and staining were performed according to the consensus report of the ESAP task force on the standardization of diagnostic DNA image cytometry.[6] DNA image data processing was carried out using a regular microscope with an image-sensing scanner interfaced to a general purpose computer with the appropriate software (CYDOK R®, Fa. Hilgers, Königswinter, Germany). This system complies with the methodological conditions needed for precise DNA image cytometry. Details of the system concerning software corrections for shading and glare errors, for stability over time and densitometric linearity, are described by Kindermann and Hilgers.[7] We used a 40 objective and an interference filter (565±10 nm half value width for parafuchsine). At least 100 diagnostic cells (but in many cases 200–300 cells) were analyzed for each smear. The integrated optical density of Feulgen-stained reference cells (e.g. lymphocytes or granulocytes) was used as a known internal standard for the normal diploid content (2c) to rescale the IOD-values to c-values. The coefficient of variation (CV) of the reference cells was between 3% and 5%. The reference cells were nontumor cells found in the samples. Having measured 30 reference cells and at least 100 diagnostic cells, the computer automatically generated image analysis histograms. To define aneuploidy, we used the stem line interpretation according to Haroske et al.[6] This classical aneuploidy interpretation refers to the modal value (the most frequent value) of a cell population, i.e. the channel of the aneuploid peak divided by the channel of the normal/diploid peak, thus defining the site of a stem line. A DNA index (DI) of 1.0 corresponds to a stem line at 2.0 and a DI of 2.5 to a stem line of 5c.

*Statistics*

For statistical analysis the chi-square test was used. Differences were considered statistically significant when P value was <0.05.

## *Results*

*Clinicopathologic data*

Patients' age ranged from 13 to 68 years (mean, 39.8 years). Tumor size was larger than 5 cm in each case. Follow-up time ranged from 16 to 78 months (mean, 48.5 months). All patients received doxorubicin monotherapy (75 mg/m$^2$ on the first day, repeated every 21 days in 6 cycles). In 10 cases local recurrence developed, in 9 cases there was metastasis in lung, while both metastasis and recurrence occurred in 4 cases. Three patients died due to the tumor (*Table 1*).

*Expression of HER-2 protein in synovial sarcoma by immunohistochemistry*

Immunohistochemical results are summarized in *Table 1*. We had focal weak (1+) cytoplasmic positivity in 4 of 20 cases, while stronger (2+) positivity (both cytoplasmic and membranous) was observed in 3 of 20 cases (*Figure 1*).

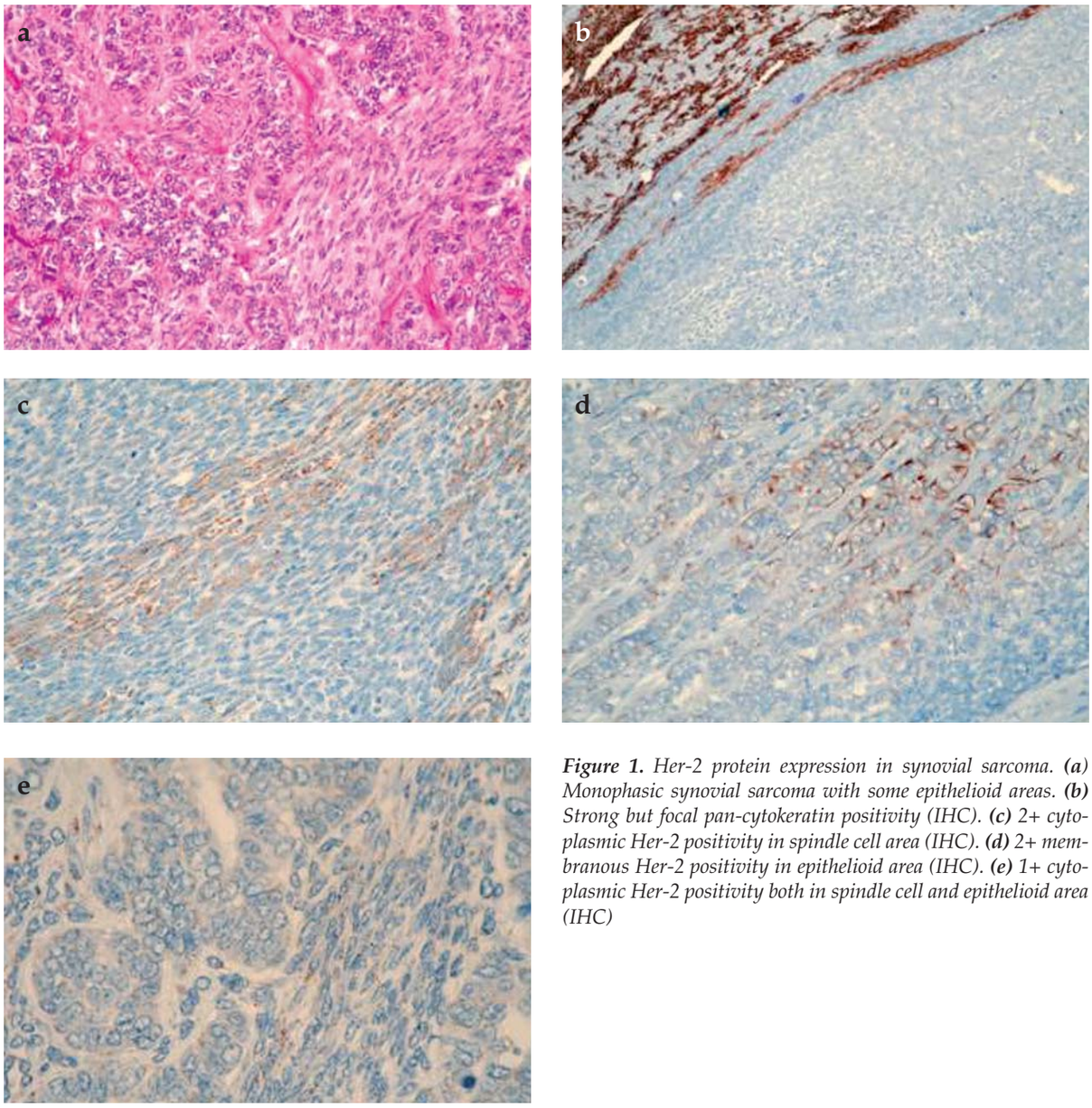


***Figure 1.*** *Her-2 protein expression in synovial sarcoma.* ***(a)*** *Monophasic synovial sarcoma with some epithelioid areas.* ***(b)*** *Strong but focal pan-cytokeratin positivity (IHC).* ***(c)*** *2+ cytoplasmic Her-2 positivity in spindle cell area (IHC).* ***(d)*** *2+ membranous Her-2 positivity in epithelioid area (IHC).* ***(e)*** *1+ cytoplasmic Her-2 positivity both in spindle cell and epithelioid area (IHC)*

Strong (3+) positivity was not observed. No difference was seen in the quality of staining between epithelioid regions and areas demonstrating spindle cell morphology. Altogether, the 7 positive cases represented 35% of all SSs.

*HER-2 amplification in synovial sarcoma by fluorescence in situ hybridization*

By FISH analysis Her-2 gene amplification was found in 3 cases (15.0%). The amplification rate was two to three-fold in each case, involving more than 10% of evaluated cells (results are summarized in *Table 1*) (*Figure 2*). Massive amplification was not observed. The Her-2 oncogene-amplified cases were the same ones that were 2+ positive by immunohistochemistry, while there was no amplification in the 1+ cases. We found chromosome 17 trisomy in one, and polysomy in another case, not affecting the status of Her-2 protein expression (*Table 1*).

*DNA cytometry*

We found 6 aneuploid cases of 20 synovial sarcomas (30.0%), two biphasic and four monophasic ones (*Table 1*). One of the aneuploid cases was hypodiploid with a

DNA index of 0.89. We had 11 monophasic and 3 biphasic diploid synovial sarcomas. Among the three amplified cases one was aneuploid, while two were diploid.

*Correlations of Her-2 status, DNA ploidy, and prognosis*

Her-2 oncogene amplification was significantly associated with a lower risk of developing metastasis ($P<0.05$); none of the 3 amplified cases had metastases. However, no association with recurrence was found. Moreover, no association was found between Her-2 amplification status and ploidy, and between ploidy status and the occurrence of metastasis or recurrence either.

## *Discussion*

Synovial sarcoma is a fairly aggressive soft tissue tumor. Its incidence is up to 10% of sarcomas, with a peak in adolescents and young adults. Synovial sarcoma was traditionally considered to be a high-grade neoplasm, but recent investigations have suggested that different factors influence prognosis, including morphological and cytogenetic features, DNA ploidy status, apoptotic index, p53 expression, and treatment strategies.[2,5,8] Considering the metastatic potential, the constellation of certain parameters, such as patients' age less than 25 years, tumor size less than 5 cm, and absence of poorly differentiated areas, has been associated with a low risk of developing metastasis. In contrast, cases with patients'age higher than 25 years, tumor size larger than 5 cm, and presence of any poorly differentiated area have a high risk of metastasis.[1]

Since in all of our 20 synovial sarcoma cases the tumor size was larger than 5 cm, and the mean patients' age was 39.8 years, though there was no poorly differentiated area in any case, this group seems to be ideal for testing whether Her-2 oncogene amplification can be considered as a prognostic factor. Her-2 gene amplification and protein overexpression have been proposed as prognostic factors for survival and predictors of response to chemotherapy and endocrine therapy in patients with breast cancer. Recently some studies focused on the Her-2 status of mesenchymal tumors, especially in synovial sarcomas. By cDNA microarray analysis, Allander et al[9] observed a high expression of the ERBB2 gene in synovial sarcoma compared with other fibrocytic sarcomas. They found 2 to 3+ Her-2 immunostaining in all five of biphasic synovial sarcomas and three of nine monophasic ones with epithelioid areas. Her-2 overexpression or gene amplification as a prognostic factor was not investigated. Barbashina et al[8] found 1-2+ Her-2 expression in 10 of 19 synovial sarcomas, but the staining was predominantly cytoplasmic. FISH analysis of eight positive cases showed no evidence of Her-2 gene amplification, and no definite correlation was shown between the overexpression of Her-2 and clinical outcome. Thomas et al[10] found Her-2 overexpression by immunohistochemistry in 52.6% of cases (all with a membranous pattern), and the presence of mRNA by Q-PCR in 73.3% of cases in synovial sarcoma. There was no evidence of gene amplification by chromogenic in situ hybridization (CISH). They concluded that Her-2 is expressed in the majority biphasic synovial sarcomas, albeit at low levels, and that treatment with tyrosine kinase inhibitors may represent an appropriate alternative therapy for these patients. Nuciforo et al[11] found overexpression at mRNA level (31% of synovial sarcomas had Her-2 mRNA levels above the mean), and Her-2 mRNA and protein overexpression was associated with a more favorable clinical course.

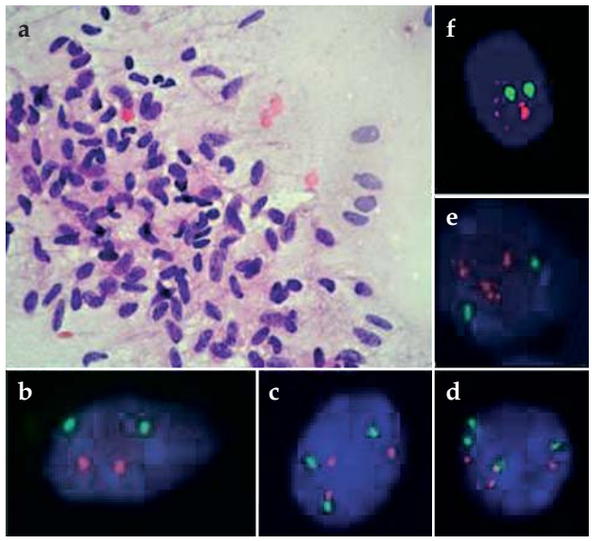


***Figure 2.*** *FISH of Her-2 and chromosome 17 in synovial sarcoma.* ***(a)*** *Smear of SS, gained from our tumor bank.* ***(b)*** *Normal diploid status of chromosome 17 and Her-2 (no amplification).* ***(c-d)*** *Tri- and tetrasomy of chromosome 17 (green signals) and corresponding three and four red signals of Her-2 ( no amplification).* ***(e-f)*** *2 to 3-fold amplification of Her-2 in synovial sarcoma.* ***(b-f)*** *FISH technique, green signals correspond to chromosome 17 and red signals correspond to Her-2.*

Our results are in harmony with the results of Nuciforo et al,[11] because Her-2 oncogene amplification was significantly associated with lower risk of developing metastasis, which means a better overall prognosis. However, no association was found with recurrence, in contrast to the result of Nuciforo et al.[11] Taken together, our results underline the fact that there is indeed a moderate level of Her-2 gene amplification in a subset of synovial sarcomas, and this amplification/protein overexpression may serve as a prognostic factor. Since we are the first who found gene amplification by FISH, the controversy between our results and those of others should be explained. First, the amplification in our three cases was weak (2 to 3-fold, and we could

not observe massive amplification, characteristic of a subset of breast carcinomas), and it occurred only in a portion of the tumor cells. However, the main difference is in the methodology, which may explain the discrepancies. Barbashina et al[8] and Thomas et al[10] used 4-μm-thick tissue sections for FISH and CISH respectively, while we used smears. In the case of 4-μm-thick tissue sections, the nuclei are cut, in contrast to smears where the nuclei are intact. Theoretically (and in practice too), the cut nuclei may not contain the whole number of signals, so the result (the small number of Her-2 signals) may be false. Moreover, the evaluation of FISH and CISH results on tissue sections is much more difficult than on smears (especially if the evaluation is automated).

It is also interesting that our amplified cases showed 2+ positivity by immunohistochemistry, while no amplification was observed in the 1+ cases. This emphasizes that stronger (at least 2+) positivity may indicate true amplification, which can be proved by FISH or other molecular methods. We found both cytoplasmic and membranous positivity by immunohistochemistry both in biphasic and monophasic synovial sarcomas, and even in a monophasic synovial sarcoma with epithelioid areas, so we cannot confirm the results of Thomas et al[10] who observed mainly membranous positivity and almost exclusively in biphasic tumors.

While in epithelial neoplasms abnormal expression of Her-2 often correlates with a high histologic grade and aggressive behavior of the tumor, in mesenchymal tumors the true incidence and the biologic role of Her-2 overexpression remain largely unknown.[9] Although the overexpression of Her-2 is generally associated with a less favorable prognosis (in breast, bladder, colon, and nasopharyngeal carcinomas),[12-15] it is interesting that in some tumors the overexpression is linked to a more favorable outcome (thyroid carcinomas and osteosarcomas),[16,17] and it seems that the same is true for synovial sarcoma.

El-Naggar et al[18] found that ploidy status (aneuploidy) significantly correlated with reduced patients' survival in synovial sarcoma. In our smaller series, however, no association was found between ploidy status and metastases or recurrences, nor between Her-2 amplification status and ploidy.

Summarizing, our results emphasize and confirm that Her-2 oncogene amplification is a rare event in SS, but the small subset of SS with Her-2 amplification has a better overall prognosis. Moreover, this may offer a theoretically new treatment possibility with Trastuzumab for Her-2-amplified cases of SS.

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