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Expression of p53, cyclin D1 and EGFR correlates with histological grade of adult soft tissue sarcomas: a study on tissue microarrays

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Soft tissue sarcomas (STSs) are rare heterogeneous tumors with variable clinical course and outcome. The management of STSs depends upon the accurate histopathological diagnosis and assessing their histological grade. Currently, core needle biopsies are becoming increasingly popular for diagnosing STSs but value of histological grading is limited from this type of specimens. To evaluate the immunohistochemical expression of p53, mdm-2, cyclin D1, p16, nm23, EGFR and Ki-67 labelling index in adult STSs patients and their association with histological grade of STSs, we analysed 101 primary untreated STSs of the limbs and trunk using the tissue microarray technique on formalin-fixed, paraffin-embedded tissue samples. The cases consisted of 15 G1, 28 G2 and 58 G2 sarcomas. Ki-67 labelling index (LI) was calculated from whole block sections for the possibility to select the most proliferative regions. The LI ranged from 1.26 to 75.5% (median 26.7%) and strongly correlated with the mitotic count ($p < 0.0001$), the histologic grade ($p < 0.0001$), extent of necrosis ($p = 0.0003$) and overexpression of p53 ($p = 0.0008$). Tissue microarrays were produced with three representative cores from each of the sarcoma cases and immunohistochemical staining for p53, mdm-2, cyclin D1, p16, nm23 and EGFR was performed. Nuclear accumulation of p53 was detected in 25 (24.8%) STSs. Overexpression of mdm-2 was seen in 63 cases (62.4%) and 29 STSs (28.7%) were cyclin D1 immunopositive. The aberrant immunoreactivity for p16 was found in 95 cases (94%) and negativity of p16 was found in 59 sarcomas (58.4%). A decreased expression of nm23 was noted in 42 cases (41.6%). Membranous positivity for EGFR was found in 45 (44.6%) sarcomas. p53 ($p = 0.005$), cyclin D1 ($p = 0.028$) and EGFR ($p = 0.018$) expression significantly correlated with the histological grade. This study supports previously published investigations indicating that expression of p53, cyclin D1, EGFR and Ki-67 labelling index may serve as prognostic markers for adult patients with STSs and may assist in establishment of the histological grade in STSs.

Key words: Immunohistochemistry, soft tissue sarcoma, Ki-67, p53, cyclin D1, EGFR, histological grade, tissue microarrays

Adult soft tissue sarcomas (STSs) are an exceptionally heterogeneous group of uncommon tumors constituting less than 1% of all malignant tumors and consist of more than 50 histopathologic types and subtypes [1]. STSs can arise from the nonepithelial extraskeletal tissue of mesenchymal origin anywhere in the body, exclusive of reticuloendothelial system and glia; the most common localisation is the deep soft parts of the limb or limb girdle but also the subcutaneous tissue, trunk wall, head and neck, and retroperitoneal, intraabdominal and pelvic areas [2]. A common feature of STSs is the potential to recur locally and/or to disseminate haematogenously, particularly to the lung [3]. Pre-treatment radiologic imaging is essential for determining the local extent of a tumor, planning biopsies and aiding in diagnosis. Currently, core-needle biopsy is the preferred biopsy procedure for diagnosing STSs [4]. Wide excisional surgery and often combined with radiotherapy is the primary treatment; the use of chemotherapy is controversial except for rare rhabdomyosarcoma and Ewing sarcoma/primitive neuroectodermal tumour (PNET) and adjuvant chemotherapy in general affects little the natural history of the disease [2, 4]. Complete surgical excision is the only curative treatment for the vast majority cases of STSs [5]. The overall 5-year survival rate of STSs is about 60% [3, 4, 5], but biological behaviour of STSs is highly variable. Certain histological types of sarcoma have an indolent clinical

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course with low metastatic potential, whereas other types characteristically pursue a very aggressive course. The key element of survival is control of both local recurrence as well as distant metastasis. The histopathological malignancy grade is an important prognostic factor, in primary extremity lesions 10-year disease specific survival for high-grade STSs is 55% compared with 90% for low-grade tumors [5]. Some soft tissue sarcomas demonstrate histotype-specific behaviour (e.g. well differentiated liposarcoma); whereas other histologic subtypes (e.g. leiomyosarcoma) show a broad spectrum of clinical behaviour [7]. A useful grading system separate tumors into those with a favourable outcome and those with a poor outcome, leaving as few cases as possible in the uninformative intermediate grade category. Ideally, the good prognosis group should encompass all tumours with a tendency for local recurrence but with a very low metastatic potential and amenable to surgery alone. The tumors in the poor outcome group should include sarcomas with a high risk of distant metastasis and for which adjuvant therapy may be advantageous. At present, the two most commonly accepted grading systems in use for STSs are those proposed by the French Federation of Cancer Centers Sarcoma Group (FNCLCC) [8] and the National Cancer Institute (NCI) grading system [9]. Both systems are effective for predicting the probability of distant recurrence and they are less valuable for predicting the likelihood of local recurrence. However, all grading schemes have limitations; some are related to the popular tendency toward use of core needle specimens for diagnosing of STSs [10] and histological grading is not suitable for small biopsy specimens.

To investigate the possible prognostic relevance, the immunohistochemical studies of Ki-67, p53, mdm-2, p16 , nm23 and cyclin D1 expressions were performed on the 101 STSs using tissue microarrays (TMA) and compared with the histological grade to find suitable markers that might enhance our ability to estimate malignancy grade on limited biopsy material.

Material and methods

Tissue samples. One hundred and one specimens of adult primary untreated malignant soft tissue tumors of the trunk and limbs received between 1998 and 2007 were retrieved from the files of the First Department of Pathological Anatomy, St. Anne's Faculty Hospital and Faculty of Medicine, Masaryk University, Brno. The samples were formaldehyde fixed and paraffin embedded. The sarcoma cases were classified according WHO histologic classification of soft tissue tumors [1] and histologic grading was performed using the updated version of the FNCLCC grading system [11]. Immunohistochemistry was used for diagnostic or confirmatory purposes if necessary.

Tissue microarray preparation. Representative areas without necrosis or fibrosis were selected from the tissue sections of each specimen. From corresponding regions of the original donor paraffin blocks 3 tissue cylinders per specimen with a diameter of 1.5 mm were obtained and brought into a recipient paraffin block using a manual tissue microarray instrument (the product of Department of Oncological and Experimental Pathology, the Masaryk Memorial Cancer Institute, Brno, the Czech Republic).

Immunohistochemistry. 4 μm thick sections of the tissue microarray block were cut and placed on charged poly-L-lysine pretreated glass slides. For Ki-67 whole block sections were used for the possibility to select the most proliferative regions with the maximum density of staining. Sections were deparaffinized in xylen and rehydrated in graded alcohols. Endogenous peroxidase was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.5, for 15 min. Heat-induced antigen retrieval consisted of incubating the slides in buffer (pH 6.0 or 9.0, Dako, Glostrup, Denmark A/S) in a pressure chamber Pascal (Dako, Glostrup, Denmark A/S) 20 min at 117°C or 97°C alternatively. Proteinase antigen retrievel included a 4-minute incubation in Proteinase K solution (Dako, Glostrup, Denmark A/S). The slides were incubated with primary antibodies and followed by the detection of the primary antibody by detections systems. Detection was performed using the labeled streptavidin-biotin complex or polymer-based immunoperoxidase method. The primary antibodies, dilutions, retrieval and detection systems used are detailed in Table 1. 3´,3´-diaminobenzidin was used as the chromogen for visualisation (DAB+, Dako, Glostrup, Denmark A/S) and Mayer's haematoxylin as the nuclear counterstaining.

The positive controls were colorectal cancer for p53, breast carcinoma for mdm-2, mantle-cell lymphoma for cyclin D1, squamous cell carcinoma for EGFR and soft tissue sarcoma for nm23.

The cases were classified as p53 and mdm2 positive if more than 10% of the tumor cells showed unambiguous nuclear

Table 1. Characteristics of antibodies used in the study

Antibody	Clone	Source	Dilution	Retrieval
Ki-67	SP 6	Lab Vision	1:3 000	Pressure Chamber, 117°C, pH 6
p53	DO-1	Novocastra	3:100	Pressure Chamber, 97°C, pH 6
mdm-2	1B10	Novocastra	1:300	Pressure Chamber, 97°C, pH 9
p16	6H12	Novocastra	3:100	Pressure Chamber, 97°C, pH 9
cyclin D1	SP4	Lab Vision	Prediluted	Pressure Chamber, 97°C, pH 9
nm-23	polyclonal	Abcam	Prediluted	Pressure Chamber, 97°C, pH 6
EGFR	2-18C9	Dako	Prediluted	Proteinase K

staining, in accordance with previous reports [12]. Each case was scored for p16 immunoreactivity according to formerly published criteria [12], i.e. tumors with expression in more than 80% of cells were regarded as normal, sarcomas with less than 20% of cells stained were classified as negative and cases with expression of p16 in 20 – 80% of cells were marked as abnormal. Only nuclear staining was considered. For the expression of cyclin D1, cases were classified as cyclin D1 positive if more than 5% of the tumor cells showed nuclear staining [13]. Expression of the EGFR was regarded as positive if more than 10% of tumor cells showed membrane positivity regardless of partial or complete membrane staining [14].

Negative controls were carried out by omitting the primary antibody for each staining series.Vimentin antibody (DAKO, Glostrup, Denmark A/S) was used in the each TMA section as control for tissue immunoreactivity.

Labelling index. Labelling index (LI) was calculated by counting the percentage of Ki-67 positive cell nuclei per at least 1000 tumor cells in whole block sections for the possibility to select the most proliferative regions with the maximum density of staining. Two to six representative successive fields were obtained at 400x magnification for each tumor. These were digitized as JPEG images using a digital camera system (BX45 Research Microscope, digital camera C5050-Z, Olympus C&S Ltd., Prague, the Czech Republic). The digital images were analyzed by counting Ki-67 positive and negative cell nuclei using freely available ImageJ software (The National Institutes of Health, Maryland, USA). An average number of 1172 cells (range, 1006 – 1803) were counted to determine LI. Neoplastic cells were considered Ki-67 positive if there was any staining of the karyoplasm or nucleoli, independent of staining intensity.

Statistical analysis. Statistical data analysis was performed with the Statistica software (StatSoft CR s r.o. 2007, STATISTICA Cz (data analysis software system), version 8.0. www.statsoft.cz, Prague, the Czech Republic). Associations between marker expression and histologic variables were studied applying $\chi 2$ test, Fisher exact test, Spearman correlation test and Mann-Whitney test. $P < 0.05$ was defined as the level of significance.

Results

Of 101 patients with adult STSs, 50 patients were male and 51 female. The median age at the time of diagnosis was 59 years (range, 18 – 91 years). Tumour locations included the extremity in 89 cases (88.12%), trunk in 9 cases (8.91%) and in the remaining 3 cases (2.97%) the tumor site was unknown.

The 101 tumors were classified into 8 main categories as follows: malignant fibrous histiocytoma (MFH) in 24 cases (23,8%), synovial sarcoma in 23 patients (22,8%), liposarcoma in 21 cases (20,8%), leiomyosarcoma in 8 cases (7,9%), myxofibrosarcoma in 7 (6,9%) cases, malignant peripheral nerve sheath tumor in 5 cases (4,9%), unclassified sarcoma in 6 cases (5,9%) and others in 7 patients (6,9%; 3 extraskeletal myxoid chondrosarcomas, 2 Ewing sarcomas/PNET, 1 rhabdomyosarcoma and 1 malignant solitary fibrous tumor). According to the FNCLCC grading system, 15 tumours (14.9%) were grade 1, 28 tumors (27.7%) were grade 2 and 58 tumors (57.4%) were grade 3.

The fraction of proliferating cells (LI) ranged from 1.26 to 75.49% (median 26.68%). The LI varied widely within each grade across the histological type, but the LI strongly correlated with the mitotic count ($r = 0.636$, $p < 0.0001$), the histologic grade ($r = 0.58$, $p < 0.0001$) and extent of necrosis ($r = 0.357$, $p = 0.0003$), evaluated as a part of the FNCLCC grading scheme on the original biopsy material. The LI also strongly correlated with the overexpression of p53 ($p = 0.0008$).

Positive immunostaining for p53 was observed in 25 tumors (24.8 %). In relation to the histologic grade, p53 positivity was absent in all grade 1 tumours, but was present in 4 (14.3%) grade 2 and 21 (36,2%) grade 3 sarcomas. The frequency of p53 positivity significantly increased with an increasing histologic grade ($p = 0.005$). Expression of mdm-2 was detected in 63 (62.4%) of the 101 cases. It was present in 11 (73.3%) grade 1, 19 (67.9%) grade 2 and 33 (56.9%) grade 3 tumors. No significant correlation was found between mdm-2 expression and histological grade and other variables. The expression of the cyclin D1 was found in 29 cases (28.7%). None of the grade 1 sarcomas were positive for cyclin D1, whereas 9 (32.1%) of the grade 2 and 20 (34.5%) of the grade 3 tumors showed positivity. The cyclin D1 overexpression showed a weak statistical association with tumor grade ($p = 0.028$). The nuclear positivity for p16 was usually accompanied by the cytoplasmic staining the intensity of which diminished in cases with abnormal or negative p16 immunoreactivity. Altered pattern of p16 expression was detected in 95 (94%) sarcomas and 59 (58.42%) STSs demonstrated complete negativity of p16. Negative immunostaining for p16 was found in 9 (60%) of the grade 1 tumors, in 13 (46.43%) of the grade 2 tumors and in 37 (63.79%) of the grade 3 lesions ($p > 0.05$). A decreased expressions level of nm23 was seen in 42 (41.58%) samples. In the group of grade 1 lesions 8 cases (53.33%), in grade 2 lesions 10 (35.71%) and in grade 3 lesions 24 (43.38%) cases were negative for nm23 ($p > 0.05$). Of 101 sarcoma cases, 45 (44.55%) revealed membranous immunoreactivity for EGFR. 1 (6.67%) grade 1 tumor was EGFR positive. In the group of grade 2 and grade 3 tumors the EGFR expression was found in 11 (39.29%) and 33 (56.9%) cases, respectively. EGFR immunopositivity strongly correlated with histological grade ($p = 0.018$), mitotic count ($p = 0.008$) and overexpression of cyclin D1 ($p = 0.007$). There were differences in the frequency of EGFR immunoreactivity among diverse histotypes of STTs. Excluding sporadic cases of STSs in our cohort, the highest frequency of EGFR positive cases was in the group of synovial sarcoma (19 of 23 samples, 82.61%), un-

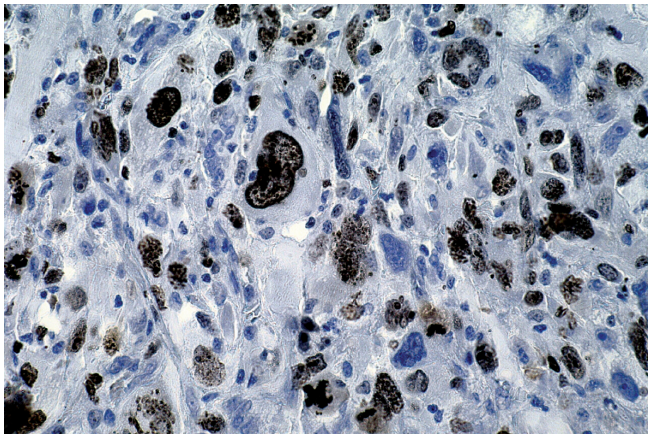

Figure 1. High Ki-67 labelling index in malignant fibrous histiocytoma (original magnification x400).

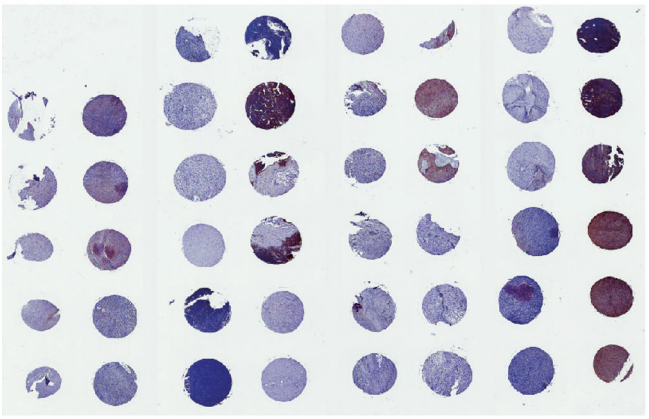

Figure 2. Low-magnification photograph of group of 15 soft tissue sarcomas, each with 3 samples in the tissue microarray. Immunostaining for EGFR.

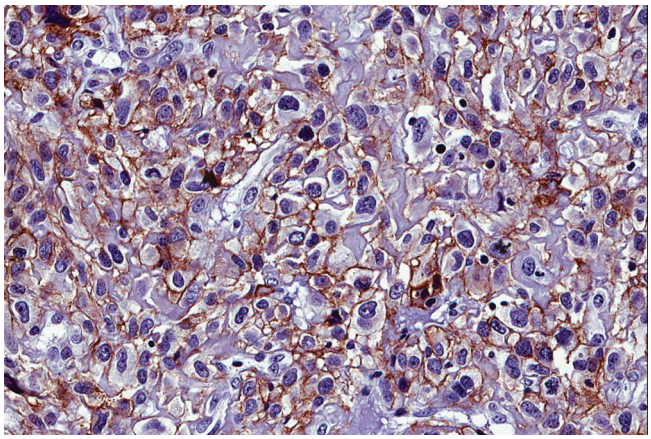

Figure 3. Membranous immunoreactivity for EGFR in the case of malignant fibrous histiocytoma (original magnification x200).

classified sarcoma (4 of 6 cases, 66.67%), malignant peripheral nerve sheath tumor (3 of 5 cases, 60%) and malignant fibrous histiocytoma (14 of 24 samples, 58.33%). The results of the analysis are summarized in Table 2.

Table 2. Correlation of expression of p53, mdm-2, p16, cyclin D1, nm-23 and EGFR with histological grade

	Grade 1 (n = 15)	2 (n = 28)	3 (n = 58)	P Value
p53	0 (0%)	4 (14.3%)	21 (36.2%)	0.005
mdm-2	11 (73.3%)	19 (67.9%)	33 (56.9%)	NS
p16	9 (60%)	13 (46.3%)	37 (63.8%)	NS
cyclin D1	0 (0%)	9 (32.1%)	20 (34.5%)	0.028
nm-23	7 (46.7%)	18 (64.3%)	34 (58.6%)	NS
EGFR	1 (6.7%)	11 (39.3%)	33 (56.9%)	0.018

NS, not significant

Discussion

A small number of molecular factors have been found to correlate with prognosis of patients with STSs. Ki-67 have been identified to be associated with prognosis in STSs in several independent studies [15, 16, 17]. The Ki-67 antigen is a nuclear protein associated with cellular proliferation and it is expressed in all active phases of the cell cycle except in quiescent cells of G0 phase [18]. Several studies have documented a significant correlation between Ki-67 expression and histologic grade of STSs [16], which is also demonstrated in the present study. Moreover, the LI strongly positively correlated with the number of mitotic figures, extent of necrosis and overexpression of p53 in this study.

Mutations or alterations in tumor suppressor genes and oncogenes and their products have been linked to the development of STSs and may also play a role in the prognosis of this disease [3, 4]. Two these genes particularly relevant to STSs comprise the retinoblastoma (*RB*) gene and the TP53 tumour suppressor gene. Immunohistochemical evaluation of overexpression of p53 in STSs has been associated with prognosis of STSs in several studies [19] but the published results are inhomogeneous and there are investigations that could not prove a correlation between p53 overexpression and prognosis [20]. Our results are comparable with the findings of Jensen et al [16] and Cordon-Cardo et al [21] who found p53 immunoreactivity in 24% and 26,5% STS patients, respectively. Expression of p53 was significantly more frequent in high-grade tumors, when none of grade 1 tumors was p53 positive, 14.3% grade 2 tumors were positive and 36.2% grade 3 tumors exhibited p53 nuclear accumulation.

The p53 function is controlled by mdm-2, which binds to p53 and prevents p53-dependent cell cycle arrest or apoptosis [22]. Overexpression of mdm-2 can lead to functional inactivation a wild-type p53 with the same effect as mutation of *TP53*

tumour suppressor gene [21]. We detected overexpression of mdm-2 in 62.4% of cases. There was no association between mdm-2 positivity and histological grade or other variables.

The $p16^{INK4A}$ protein is a cyclin-dependent kinase inhibitor that negatively regulates the complex between cyclin-dependent kinase 4 (CDK4) and cyclin D1, participating in phosphorylation of pRb. Action of $p16^{INK4A}$ thus prevents progression of the cell cycle through the G1 to S phase [23]. Inactivation of the $p16^{INK4A}$ is documented in a wide variety of human malignancies [24]. In our study, 58.4% of sarcomas were found to be negative for expression of $p16^{INK4A}$ and 94% of samples have reduced expression. Our results indicate that alteration of $p16^{INK4A}$ is a very frequent event in development of STSs, in accordance with the conclusion of Orlow et al [25]. We found no association between reduced expression of $p16^{INK4A}$, histological grade and other variables.

Cyclin D1 is a member of G1 cyclins which form complexes with cyclin-dependent kinases and promotes entry of the cell cycle into S phase [26]. Cyclin D1 gene amplifications or overexpression of cyclin D1 were observed in various cancers, including lung, breast, oesophageal and colorectal carcinomas as well as B-cell lymphomas [25, 26]. In that study, overexpression of cyclin D1 was seen in 28.7% of cases and cyclin D1 overexpression correlated positively with histological grade and expression of EGFR. Our results are in concordance with those of Kim et al [27] who also observed increased expression of cyclin D1 in 29% cases and association with the high histological grade. In his cohort of STSs high levels of cyclin D1 predicted poorer survival on multivariate analysis.

Product of *NM-23* gene is a protein with the putative tumor metastasis suppressor function and with controversial data in the literature about the role of *NM-23* in the prognosis of cancer patients. Royds et al [28] found no relationship between the expression of nm-23 and prognosis in sarcoma patients. D´Souza et al [29] documented even correlation between the nm-23 expression and histological grade in adult soft tissue sarcomas which is in the contradiction with the supposed metastasis suppressor function of nm-23. In our study, the expression of nm-23 was reduced in 41.6% of samples and there was found no correlation between the histological grade and nm-23 expression.

New therapeutic strategies of cancer treatment directed against growth factor receptors such as the epidermal growth factor receptor (EGFR) are currently in use in the management of various carcinomas [30]. Experimental results have given clues that blockage of EGFR mediated pathways might also be of therapeutical benefit in soft tissue sarcomas [31]. The frequency of EGFR immunoreactivity in soft tissue sarcomas varies greatly, ranging from 0.3% to 52.9% depending on the antibody used [14]. Some authors have suggested that EGFR overexpression is related to the histological grade and decreased overall survival [14, 32]. In our study 45 of 101 cases (44.6%) were EGFR positive and we found strong association between the EGFR expression and histological grade. Our study confirmed high incidence EGFR expression in STSs, especially in the cases of synovial sarcoma. Thus, EGFR targeted therapy appears deserving to be considered in patients with EGFR positive STSs.

To conclude, high Ki-67 labeling index and overexpression of p53, cyclin D1 and EGFR detected by immunohistochemistry correlated strongly with histological grade, indicating their valuable contributing to the assessment of tumor aggressiveness and to the establishing of histological grade in adult soft tissue sarcoma patients, especially in small tissue samples.

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