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Expression of c-Met receptor and hepatocyte growth factor/scatter factor in synovial sarcoma and epithelioid sarcoma

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Abstract Overexpression of c-Met receptor/hepatocyte growth factor (scatter factor) system (c-Met/HGF/SF) as a physiologically paracrine cellular signaling system is thought to be involved in the progression of malignant tumours. In 26 synovial sarcomas and epithelioid sarcomas, c-Met and HGF/SF expression was analysed immunohistochemically. There were 10 biphasic synovial sarcomas, 7 of which showed moderate to strong c-Met expression in epithelial areas compared with the fibrous component, with corresponding expression of HGF/SF. Six of 9 monophasic fibrous synovial sarcomas showed only very faint c-Met and corresponding HGF/SF expression. In 7 epithelioid sarcomas strong expression of c-Met and HGF/SF was observed within epithelioid tumour cells. Non-radioactive in situ hybridization demonstrated the synthesis of c-Met receptor in tumor cells by detecting c-met-mRNA. This analysis shows that in synovial sarcomas and epithelioid sarcomas, tumour entities with epithelial and mesenchymal structures, c-Met and HGF/SF overexpression can be detected, indicating a role of this signaling system in these subtypes of sarcoma, and especially in the more epithelioid tumour phenotype. An autocrine interaction between overexpressed c-Met receptor and HGF/SF may be hypothesized.

Key words c-Met receptor · Hepatocyte growth factor/scatter factor · Synovial sarcoma · Epithelioid sarcoma

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Introduction

The membrane tyrosine kinase receptor c-Met together with its ligand hepatocyte growth factor/scatter factor (HGF/SF) forms a paracrine cellular signaling system [4, 6, 14, 26]. Physiologically, the plasminogen-like protein HGF/SF is found predominantly in cells of mesenchymal origin, such as fibroblasts, smooth vascular muscle cells, endothelial cells and activated macrophages [26]. Scatter factor (SF) is identical to the hepatocyte growth factor (HGF) [34], which was originally discovered as a mitogen for hepatocytes and believed to play a part in the regeneration of liver cells [1]. After synthesis in mesenchymal tissue, HGF/SF develops a paracrine effect on the adjoining epithelium, influencing mainly cell growth (mitogen), motility (motogen) and morphogenesis (morphogen, for example by forming tubular structures) in epithelial cells [21, 26, 31, 33]. Each of these functions may be mediated by the c-Met receptor, inducing activations in the subsequent intracellular signal transduction pathway. The corresponding receptor c-Met is a transmembrane tyrosine kinase, encoded by the c-met proto-oncogene and allegedly expressed mainly in epithelial cells [3, 4, 26]. Apart from this, low levels of c-Met receptor are found in normal fibroblasts [6, 25].

Overexpression of both HGF/SF and c-Met has already been recorded in the tissue of various epithelial tumours, so that it seems this system may have some significance in the progression of carcinoma [6, 8, 13, 15, 20]. HGF-SF and c-Met expression has also been observed in various sarcomas [12, 14, 25, 29], suggesting a potential role for this signaling system in these tumours. For example, it has been possible to induce malignant transformation in mouse fibroblasts by overexpression of c-Met and HGF/SF [25]. Here, the autocrine interaction between HGF/SF and c-Met receptor seems to be a basic requirement for mitogenesis and malignant transformation of the fibroblasts.

Synovial sarcoma and epithelioid sarcoma are characterized by the immunohistochemical demonstration of both epithelial and mesenchymal intermediate filaments

[11, 17]. Next to epithelial tumour areas, spindle-shaped, more sarcomatous regions can be found. Especially in the biphasic variant of synovial sarcoma, these tumour components can often clearly be distinguished close by.

Owing to the "biphasic" nature of these sarcomas with both mesenchymal and epithelial tumour architecture, this study was performed to investigate whether and how the expression of c-Met and its ligand HGF/SF might represent a mesenchymal-epithelial signaling system in synovial sarcoma and epithelioid sarcoma.

Materials and methods

Primary tumour tissue was obtained in 6 patients with an epithelioid sarcoma through resection; a lung metastasis was removed in 1 patient. Tissue of the primary synovial sarcomas was obtained through resection in 19 patients with this diagnosis. For 2 patients additional material obtained from a tumor relapse was examined, and for 1 patient additional tissue was available from a soft tissue metastasis.

Histomorphological examination and diagnosis was performed on haematoxylin/eosin-stained sections with additional immunohistochemical detection of intermediate filaments cytokeratin/vimentin according to the proposed criteria [11, 17]. Of the synovial sarcomas, 10 were classified as biphasic and 9 as monophasic fibrous; 2 cases were graded as G I, 7 cases as G II, 10 cases as G III. Epithelioid sarcomas were graded as G II in 3 cases and as G III in 4 cases.

Specimens were analysed for c-Met-expression with a rabbit polyclonal antibody against a peptide corresponding to amino acids 1366–1390 of the human Met gene product (Santa Cruz, Calif.). Immunohistochemical staining for the hepatocyte growth factor/scatter factor was carried out using a goat polyclonal antibody raised against recombinant human HGF (rhHGF, R & D Systems, Minn.).

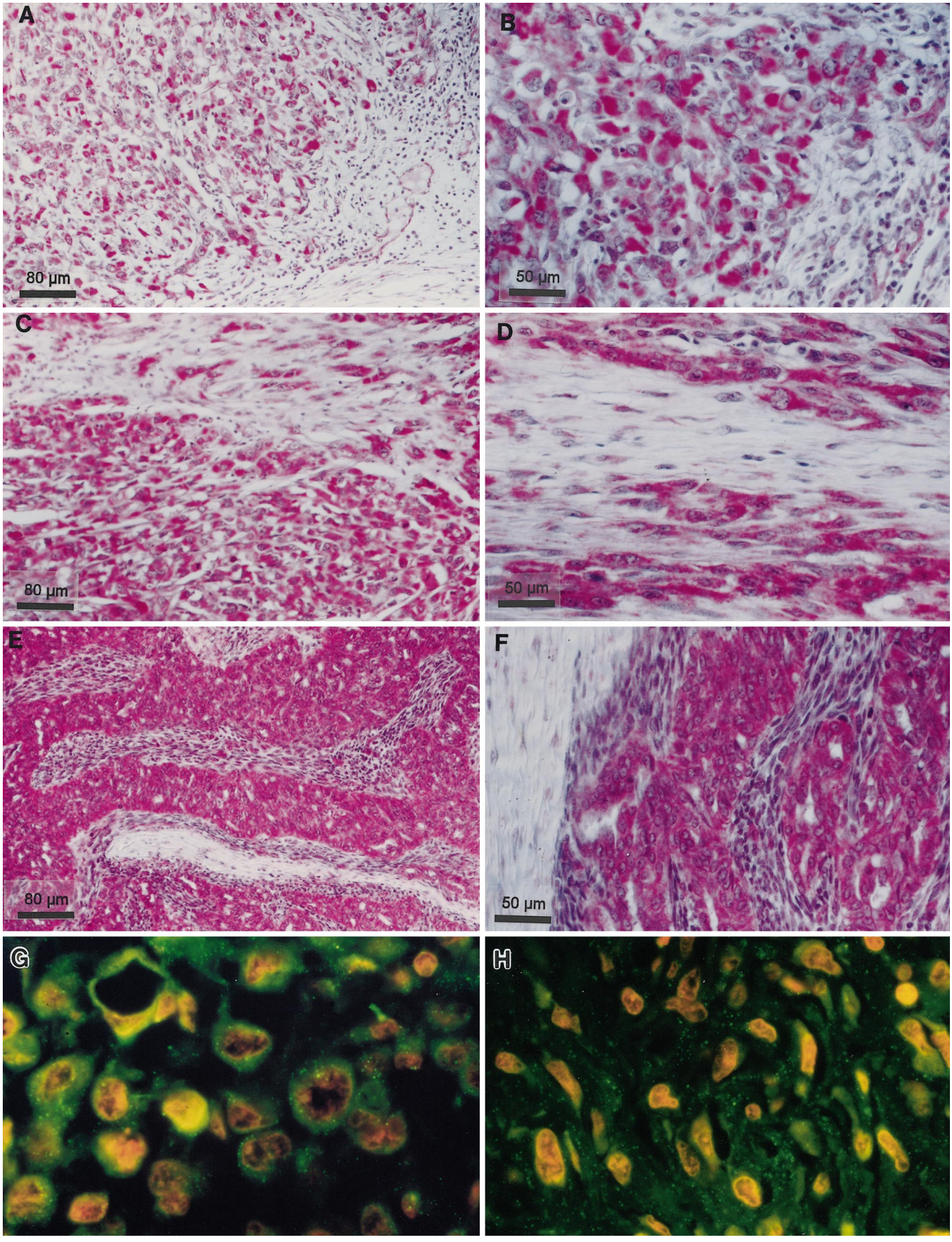
Immunostaining was performed using the alkaline-phosphatase anti-alkaline phosphatase method (APAAP) [5]. Sections 4 μm were cut consecutively from formalin-fixed and paraffin-embedded tissue samples, mounted on poly-L-lysin coated slides and dried overnight at 58°C. Paraffin sections were dewaxed by xylene, rehydrated with graded concentrations of ethanol and finally washed in Tris-Buffer (pH 7.6) for 10 min. c-Met required proteinase K predigestion in a working solution of 0.4 mg/ml (DAKO, Hamburg, Germany) for 10 min. at room temperature. The following steps were finalized by an automated staining system (DAKO TechMate 500, DAKO Hamburg, Germany).

Sections were incubated with the primary antibody solution for 25 min at room temperature, using as working dilutions of the antibodies 1:300 for c-Met, 1:150 for HGF/SF. Slides were rinsed once in buffer (Puffer Kit, DAKO). For c-Met immunostaining, sections were incubated with the secondary antibody, a mouse anti-rabbit serum, in a 1:150 concentration (DAKO) for 25 min at room temperature. For HGF/SF immunostaining sections were incubated with rabbit anti-goat serum in a 1:200 concentration (DAKO). Following this, the sections for HGF/SF were incubated with mouse anti-rabbit serum as described above for c-Met immunostaining. Slides were rinsed in buffer (Puffer Kit, DAKO). Immunoreaction was demonstrated with the APAAP Kit (DAKO) according to the specifications of the manufacturer. The secondary antibody was an alkaline phosphatase-labelled monoclonal calf antibody, and the bridging antibody a monoclonal anti-calf mouse antibody. Sections were incubated with the chromogen alkaline-phosphatase substrate (Fast Red, DAKO) for 20 min at room temperature. Finally, sections were counterstained with Mayer's haematoxylin (DAKO) for 1 min, dehydrated in graded ethanol, and coverslipped. Negative controls used all reagents except the primary antibody. Intensity of c-Met and HGF/SF staining was evaluated semiquantitatively: absent, faint < 25% positive tumour cells, moderate > 25% < 50% tumour cells positive, strong > 50% of tumour cells staining positive.

The cDNA-probe for human c-Met was a 500-bp fragment of clone pMet 5 purchased from ATCC [7] subcloned into the EcoRI-*Hind*III site of vector pVC19. The probes were labelled by digoxigenin using the random primed oligo labelling method with the Dig-Labelling Kit (Boehringer Mannheim, Germany).

We used a slightly modified in situ hybridization protocol described by Wiethege [35]. Paraffin sections 4 μm thick were placed on glass microscope slides, which had been previously immersed in 2% TESPA (3-aminopropyltrietoxysilane, Sigma, Germany) in acetone for 5 s and twice in acetone for 1 min each. Slides were dewaxed in xylene and rehydrated in decreasing ethanol concentrations (99 vol.%, 90 vol.%, 70 vol.%, 50 vol.%) in deionized water. After washing in phosphate-buffered saline (PBS, pH 7.4) and dehydration in graded series of ethanol (30 vol.%, 70 vol.%, 90 vol.% and 100 vol.%), samples were air-dried and treated by sequential incubation as follows: 0.2 N HCl (20 min.), double-destilled water (5 min), 0.125 mg/ml pronase (Serva, Germany; 10 min), 0.02 M glycin (Merck, Darmstadt, Germany) (30 s), twice PBS (30 s). Specimens were postfixed in 4% paraformaldehyde/PBS for 20 min and washed in PBS (5 min). After incubation in 0.1 M triethanolamine (pH 8.0) (Merck,Germany) containing freshly added 0.25 vol.% acetic anhydride for 10 min and dehydration in serial alcohols (30 vol.%, 70 vol.%, 90 vol.% and 99 vol.%) the sections were air-dried. All steps were performed at room temperature, and all solutions were treated with diethylpirocarbonate (Sigma, Germany). For prehybridization, samples were covered with 300 μl of prehybridization buffer solution containing 50% deionized formamide (Fluka, Germany), 0.3 M NaCl, 10 mM Tris pH 7.5, 10 mM $NaHPO_4$ pH 6.8, 5 mM EDTA, 0.1 × Denhardt's solution, 10 mM dithiotreitol (Sigma, Germany), 0.25 mg/ml yeast tRNA (Sigma,Germany), 12.5% dextransulphate (Pharmacia Biotech, Sweden) and 0.5 mg/ml salmon sperm DNA (Sigma,Germany), and incubated in a humid chamber for 2 h at 42°C. For hybridization, prehybridization mix was removed and slides were covered with 30 μl of hybridization solution, containing 1 μg digoxigenin labelled cDNA probe per ml. The slides were each mounted with a coverslip, sealed with rubber cement and incubated in a humid chamber for 18 h at 42°C. After removal of the coverslips, slides were washed twice in 50% formamide/2 × SSC (standard saline citrate)/1% mercapto-ethanol for 20 min at 44°C and in 2 × SSC and then 0.1 × SSC for 20 min at 51°C. Finally, specimens were rinsed for 3 min in PBS and covered with 30 μl of fluorescein-iso-thiocyanate (Boehringer Mannheim) -conjugated anti-digoxigenin antibody diluted 1:20 in 5% normal sheep serum in PBS. Sections were coverslipped and incubated in a light-protected humid chamber for 2 h at 37°C. After being washed twice for 10 min in PBS, specimens were counter-

Fig. 1A, B Immunohistochemical localization of *c-Met receptor* ▶ in epithelioid sarcoma **A** Strong c-Met immunostaining seen in polygonal epithelioid tumour cells **B** Endothelial cells of microvessels and some fibroblasts near the tumour cells, stained weakly positive with anti-c-Met antibody. **C–F** Immunohistochemical localization of hepatocyte growth factor/scatter factor (HGF/SF) in epithelioid sarcoma and synovial sarcoma. **C** Strong immunoreaction in polygonal tumour cells of epithelioid sarcoma. **D** Strong HGF/SF staining in more spindle-shaped cells of epithelioid sarcoma. Weak expression of HGF/SF in normal fibrocytes (*central region*). **E** Biphasic pattern of synovial sarcoma with strong expression of HGF/SF in the more epithelial areas of the tumour corresponding to c-Met expression. **F** Only weak staining for HGF/SF in the spindle cells of synovial sarcoma, in contrast to the epithelial pattern. **G–H** Detection of c-Met transcripts with nonradioactive in situ hybridization in epithelioid sarcoma. 625×. **G** Tumour cells labelled positive for c-Met mRNA and some endothelial cells of microvessels within the tumour. **H** Detection of c-Met transcripts in more spindle-shaped tumour cells


A
80 µm
B
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stained with 30 µl of propidium iodide in PBS (500 ng/ml; Boehringer Mannheim, Germany) for 5 min. After removing excess propidium iodide by washing PBS for 10 min, slides were dehydrated by series of alcohol (70 vol%, 90 vol%, 99 vol%) and air-dried. Finally, specimens were mounted in a glycerol/PBS solution, Citifluor (Plano, Germany) [16].

For negative controls, sections were hybridized with a digoxigenin-labelled pBR322 DNA probe (Boehringer Mannheim).

We used an epiluminated fluorescence microscope (Leitz, Germany) for the evaluation.

Results

Expression of c-Met and HGF/SF was established as the dark purple reaction product obtained by the APAAP technique. In all cases of c-Met positivity, intracytoplasmatic expression of c-Met was detected, including the cell membrane. In the 7 cases of epithelioid sarcoma a strong immunoreactivity for c-Met was found both in polygonal epithelioid tumour cells and in more spindle-shaped cells (Fig. 1A, B). Six of 9 monophasic fibrous synovial sarcomas showed slight to moderate inhomogeneously distributed staining; 3 cases did not label positive with anti-c-Met-antibody. There was a heterogeneous staining pattern in 7 of 10 synovial sarcomas with biphasic structure: a moderate to strong expression of c-Met was found in the epithelial areas, in contrast to the more fibrous part. A comparable staining reaction was observed in primary tumours and in cases of relapse tumour/metastasis. Adenoid structures (sweat glands and squamous epithelium of the epidermis) at the border of tumour tissue stained positive for c-Met. The stratum granulosum in particular showed a distinct membrane-bound reaction. Endothelial cells, smooth vascular cells, skeletal muscle cells and fibroblasts also labelled positive with anti-c-Met-antibody (Fig. 1B). Corresponding cells from normal tissue generally exhibited only a weak reaction compared with the tumour cells.

Corresponding to the c-Met-expression, a strong immunoreaction for HGF/SF was observed within the tumour cells of epithelioid sarcoma (Fig. 1C, D). The monophasic fibrous synovial sarcomas exhibited slight to moderate staining with anti-HGF/SF-antibody, and in 3 cases there was only quite faint staining (which was negative with anti-c-Met antibody). As found for c-Met, the tumour cells of the epithelial pattern in biphasic sarcomas labelled strongly positive for HGF/SF, in contrast to the fibrous areas (Fig. 1E,F). Fibroblasts and fibrocytes in normal tissue bordering on the tumour stained weakly positive for HGF/SF, as expected from the described mesenchymal derivation of the protein (Fig. 1D, F).

Twelve resected specimens of tumour tissue were analysed for c-Met transcripts. The tumour cells labelled strongly for c-Met mRNA (Fig. 1G, H). Detectable signals were also found in fibroblasts, skeletal muscle cells bordering on a sarcoma, and vascular endothelial cells within the tumour (Fig. 1G).

No detectable signals were found in control cases labelled with pBR322 DNA.

Discussion

The cytokine HGF/SF and the corresponding tyrosine kinase receptor c-Met are of major importance in a number of biological processes, such as embryogenesis (organogenesis) and wound healing [19, 28, 31]. There have been many reports on the possible influence of the c-Met/HGF/SF signaling system in oncogenesis and progression, including metastatic spread, of epithelial tumours. Increased expression and effects of HGF/SF seem to be involved in cell dissociation (scattering) during invasion and metastatic spread [27]. Pisters et al. observed a high c-Met expression in 84% of investigated carcinomas of the prostate, as against 18% of benign hyperplastic prostate alterations [23]. c-Met was also detected in all lymph node and bone metastases of these carcinomas of the prostate. A strong increase in c-Met expression was recorded in renal cell carcinoma [22], carcinoma of the breast [15], and the pancreas [10] and thyroid [9] and gastric [13, 18] tumours. In a number of investigations, the influence of c-Met overexpression on the development of sarcomas has been underlined [6, 25, 29]. A nonphysiological increase in HGF/SF and c-Met expression seems to play a decisive part in the pathogenesis of Kaposi's sarcoma [24].

Sarcomas are derived from mesenchymal cells with a variety of differentiation characteristics. Here, synovial sarcoma and epithelioid sarcoma seem to be special cases, since in these lesions both epithelial and mesenchymal cell features can be observed at the immunohistochemical and ultrastructural levels. Cells of synovial sarcoma reveal epithelial characteristics such as microvilli, tight junctions, desmosomes and structures of basement membrane, and at the ultrastructural level epithelioid sarcomas show specialized cell contacts [17].

Since synovial sarcoma and epithelioid sarcoma reveal both mesenchymal and epithelial areas, these tumours are a good model for the analysis of a possible varying distribution of the proteins of the c-Met/HGF/SF system in the adjacent tumour components.

We consider that as c-Met and HGF/SF are overexpressed in synovial sarcoma and epithelioid sarcoma, this may indicate a role in the progression of these tumour entities. The biological significance of c-Met- and HGF/SF overexpression as a phenotypic phenomenon is unclear and remains to be investigated. In a mouse model in vivo, the tumorigenic potency of HGF/SF overexpression has already been proven [32]. An autocrine mode of action between overexpressed c-Met receptor and HGF/SF has already been proposed by others [2, 6, 12, 29], and must be considerd in the cases of synovial sarcoma and epithelioid sarcoma, as we can detect the two proteins in the same tumour cells. In biphasic synovial sarcomas, a higher expression of c-Met and HGF/SF is observed in the epithelial area of the tumour than in the more fibrous part; this ligand/receptor pair seemingly is thus activated more in the epithelial than in the mesenchymal component.

Like other authors [6, 25], we found that intracytoplasmic expression of the c-Met receptor in fibroblasts, endothelial cells and striated muscle fibres was common in our own material, in connective tissue areas at the border of tumour tissue. Using in situ hybridization, protein synthesis in the respective cells could be verified by demonstrating mRNA. This is in accordance with the known physiological – albeit low – c-Met expression in fibroblasts [10, 25]. Whether this is merely an activation of the relevant connective tissue cells near the tumour process, like the activation of smooth vessel wall myocytes near inflammatory processes with concurrent c-Met expression, remains to be discussed.

c-Met expression seems to be a phenotypic feature of tumour cells with more epithelial differentiation rather than sarcomatous cells, a fact that may be explained by the physiological presence of c-Met expression during the differentiation process towards epithelium-like cells.

Surprisingly, the reported predominant synthesis of HGF/SF in mesenchymal cells could not be demonstrated in our sarcoma cases with epithelial structures: in contrast, fibrous sarcoma components had a markedly lower HGF/SF expression than epithelial tumour cells.

However, overexpression of this ligand receptor system does not seem to be mandatory in sarcoma progression, since most purely monophasic-fibrous synovial sarcomas revealed only a faint immunohistochemical reaction.

HGF/SF concentration in the tumour tissue has already been reported to be a prognostic factor in breast and non-small cell lung tumours [30, 36].

Further studies will be needed to show whether the immunohistochemical demonstration of c-Met and/or HGF/SF in tumour biopsy samples of sarcoma is of prognostic value, comparable for example, to grading, tumour size and surgical procedure. Apart from this, blocking antibodies against HGF/SF producing tumour cells or c-Met receptor may offer future therapeutical concepts [24].

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