BIOPATHOLOGY OF TUMOR HETEROGENEITY IN SYNOVIAL SARCOMA



JOSÉ MANUEL LOPES

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BIOPATHOLOGY OF TUMOR HETEROGENEITY IN SYNOVIAL SARCOMA

BIOPATOLOGIA DA HETEROGENEIDADE TUMORAL NO SARCOMA SINOVIAL

JOSÉ MANUEL PEDROSA BAPTISTA LOPES

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I. Lopes JM, Bjerkehagen B, Sobrinho-Simões M, Nesland JM: The ultrastructural spectrum of synovial sarcomas: A study of the epithelial type differentiation of primary tumors, recurrences, and metastases. **Ultrastruct Pathol** 17: 137-151, 1993.

II. Lopes JM, Bjerkehagen B, Holm R, Bruland Ø, Sobrinho-Simões M, Nesland JM: Immunohistochemical profile of synovial sarcoma with emphasis on the epithelial-type differentiation. A study of 49 primary tumours, recurrences and metastases. **Pathol Res Pract** 190: 168-177, 1994.

III. Lopes JM, Bjerkehagen B, Holm R, Bruland Ø, Sobrinho-Simões M, Nesland JM: The proliferative activity of synovial sarcoma. An immunohistochemical evaluation of Ki-67 labeling indices of 52 primary and recurrent tumors. **Ultrastruct Pathol** 19: 101-106, 1995(em publicação).

IV. Lopes JM, Hannisdal H, Bjerkehagen B, Bruland ØS, Danielsen HE, Pettersen EO, Sobrinho-Simões M, Nesland JM: Synovial sarcoma. DNA ploidy and proliferation (PCNA and Ki-67) markers in the evaluation of prognosis (enviado para publicação).

V. Lopes JM, Bruland ØS, Bjerkehagen B, Silva MC, Holm R, Pettersen EO, Solheim ØP, Sobrinho-Simões M, Nesland JM: Synovial sarcoma. Immunohistochemical expression of P-glycoprotein and glutathione S transferase-pi and clinical drug resistance (enviado para publicação).

Em cumprimento do disposto no referido Decreto-Lei declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com activa colaboração dos outros autores.

À ISABEL, à RITA e ao FRANCISCO

NOTA EXPLICATIVA

A presente Dissertação está escrita em Inglês na sua quase totalidade, exceptuando o Sumário e Conclusões, pelo facto de o Doutor Jahn Nesland ter sido o seu co-orientador.

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[Tumors are "heterogeneous" in several ways. There is the heterogeneity among cancers in different individuals who nominally have the same type of disease. It is this heterogeneity which fuels the search for prognostic indicators and for methods to individualize therapy. A second type of heterogeneity is that seen within the same patient over the course of time. The biological, as well as the clinical, characteristics of an "early", preinvasive tumor are not the same as exhibited by the same cancer when it has disseminated. This type of heterogeneity is acknowledged by Fould's concept of "progression".

Heterogeneity is also seen within a single tumor at any one time. Histological examination of tumor samples reveals considerable differences in the morphology of cancer cells in different areas of the same lesion. Host infiltrating and connective tissue are not evenly distributed. Areas of necrosis may be present. Depending upon tumor size, marked disturbances in vasculature can occur, leading to focal differences in oxygen tension, pH, substrate supply, and waste drainage. Related in part to this structural heterogeneity is heterogeneity in growth compartments. The cells within a tumor may be cycling or noncycling, quiescent or reproductively dead. If cycling, they may be at any stage in the cycle. Insofar as stage of cell cycle may influence cellular properties such as membrane biochemistry, antigen expression, sensitivity to immune killing, drug cytotoxicity, and ability to metastasize, tumors will be heterogeneous in regard to those properties.]

Gloria H Heppner and Bonnie E Miller, 1983 [29]

INTRODUCTION

The major steps in our understanding of synovial sarcoma (SS) are presented in the following historical background in a chronological order.

1852 - Chassignac (quoted by Moberger et al [53]) describes tumors arising from joint capsules, tendon sheaths and serous bursae.
1894 - Herdie (quoted by Lejars et al [41]) uses the term "sarcoma synovial du genou" to describe a tumor in an adult female.
1910 - Lejars and Rubens-Duval [41] report the first adequately described case of SS under the name of "synovial endothelioma".
1927 - Smith [71] introduces the term "synovioma".
1936 - Knox [35] introduces the term " synovial sarcoma".
1944 - Murray et al [54] describe in vitro cultures of human SS and demonstrate that the two apparently morphological distinct cell types of SS are not generically separate.
1951 - Tillotson et al [72] describe "synovial fibrosarcoma" as a distinct type of SS.
1952 - King [34] suggests that SS is derived from connective tissue cells and not from special synovial cells.
1960 - Vincent [78] suggests that SS arises from specialized mesenchymal cells near or distant to a joint, bursa or tendon sheath and that it rarely enters a joint cavity.
1960 - Luse [43] reports the first ultrastructural description of SS and favors a distinct similarity of tumor cells with normal synovial lining.
1965 - Enzinger [15] characterizes 3 histological subtypes of SS: "pseudoglandular", "fibrosarcoma", and "endothelioid".
1966 - Ghadially and Roy [22] report experimentally produced SS.
1968 - Moberger et al [53] describe 3 different types of SS: "synovioblastic", "synoviocytic", and "fibrosarcoma".
1971 - Gabbiani et al [20] report an electron microscopic study of a typical SS and show little resemblance of tumor cells with synovial membrane.
1976 - Fernandez and Hernandez [18] describe the "poorly differentiated" SS.

1977 - Mackenzie [44] questions the existence of "monophasic" SS as an histological entity.
1981 - Krall et al [36] describe the ultrastructural features supporting the recognition of "monophasic" SS.
1981 - Hajdu et al [25] report SS strains that are transplantable into nude mice.
1982 - Miettinen et al [47] describe the presence of keratin proteins in SS.
1982 - Farris and Reed [17] report examples of "monophasic glandular" SS.
1987 - Turc-Carel et al [75] report a characteristic chromosomal translocation, t (X;18) (p11.2;q11.2) in SS.

Present situation

SS is a rare malignant soft tissue tumor that represents 5 to 10% of all soft tissue sarcomas [14]. SS is most prevalent in adolescents and young adults [14]. However, it can occur in any age, from newborns to elderly patients up to the ninth decade [6,9,14,53,60,67,72,80]. There is a tendency for male predominance [14]. SS occurs predominantly in the extremities being especially frequent in the thigh and knee regions [14]. Unusual locations include head and neck region [14,26,65,69] (soft palate [45], tongue [30,52], tonsil [13], maxillofacial region [55,69], scapular region [42], esophagus [1,3], pharyngeal [32] , laryngeal [51], and nasopharynx [77] region, and orbit [62]), abdominal wall[2,6,19], mediastinum [79], retroperitoneum [16,70], heart [68], large vessels [23,48], vagina [58], intra-osseous [64], bursa [10], and intra-articular [46,63] .

At present, there is general consensus regarding the existence of 3 morphological subtypes of SS [14,17,18,36]: "biphasic", "monophasic" ("fibrous" and "epithelial" variants), and "poorly differentiated". However, the pathogenic relationship between these subtypes of SS remains unclear [11,12,21,44]. In addition, the relationship between cell proliferation and cell differentiation in different subtypes of SS is still unsettled . This applies particularly to progression of SS.

Three main reasons raised our interest in SS. Firstly, the characteristic epithelial differentiation present in many SS is rather curious in a well established malignant soft tissue tumor [8,20,33,47,50,59,74]. Secondly, the vivid controversy on the histogenesis of "synovial sarcoma", since the synovial origin has been ruled out [20,21,39,49].

Thirdly, persistent guarded prognosis of SS in spite of some therapy improvements reported in the last years [4,5,7,19,24,27,28,31,37,38,40,56,57,61,66,73,74,76,80,81].

Despite the huge amount of information available in the literature, several questions on the biopathology of this peculiar neoplastic entity remain to be clarified.

Aims

1. To investigate the relationship between biphasic (BSS) and monophasic (MSS) variants of synovial sarcoma (SS).
Questions:
a) Does this subdivision (BSS and MSS) of synovial sarcoma reflect a different histogenesis ?
b) Is MSS (of the fibrous type) the exact counterpart of the spindle cell component of BSS or a poorly differentiated variant of BSS ?

2. To investigate the relationship between cells in solid/glandular component and the surrounding spindle cells of BSS.
Questions:
a) Are the solid/glandular and spindle cell components of BSS the result of a divergent (epithelial and mesenchymal) differentiation or do they reflect the result of an evolution and transformation (transition) between them?
b) What is the relationship between cell differentiation and cell proliferation in both components of BSS ?

3. To investigate the clinicopathologic features that influence prognosis in SS.
Questions:
a) What is the role of the treatment regimes in the control of local and distant disease in SS ?
b) Are there any clinical or morphological (tumor and/or host) features, apart from TNM staging, that can be used to predict the relapse-free survival and/or overall survival of the patients ?

c) Does the ploidy status and/or the growth fraction of the tumors influence the prognosis of the patients ?

4. To investigate the relationship between the immunohistochemical expression of drug resistance markers (P-glycoprotein and glutathione S transferase-pi) and clinical drug resistance of SS.
Questions:
a) Is there any relationship between the expression of both drug resistance markers and proliferation and/or differentiation of SS ?
b) What is the relationship between drug resistance and clinical progression of SS (and survival of the patients harboring SS)?
c) Does the expression of both drug resistance markers predict the response to chemotherapy of untreated primary SS and/or is this expression influenced by chemotherapy ?

The five studies that are reproduced as papers I to V of the present Thesis substantiate our attempts to find answers to the aforementioned questions.

Since each paper has its own Introduction, Material and Methods, Discussion/ Conclusions, and References, we decided to simplify as much as possible the structure of this Introduction as well as those of References (pp. 16-24; 102-106) and Conclusions (pp. 98-102).

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Paper I

The Ultrastructural Spectrum of Synovial Sarcomas: A Study of the Epithelial Type Differentiation of Primary Tumors, Recurrences, and Metastases

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Synovial sarcomas (SS) are malignant soft tissue tumors of unknown origin. Their classification as carcinomas (monophasic synovial sarcomas, MSS) or carcinosarcomas (biphasic synovial sarcomas, BSS) still raises controversy. In an attempt to settle this controversy, an ultrastructural study was undertaken of 25 primary SS (12 BSS and 13 MSS), 5 recurrences (3 BSS and 2 MSS), and 2 metastases (2 BSS) based upon precise selection of different aspects of BSS and MSS on numerous semithin sections from each case. Ultrastructural markers of epithelial type differentiation of neoplastic cells were found in every type of cellular component of SS regardless of the tumoral pattern (biphasic or not). No major differences were found between MSS and the nonglandular areas of BSS (not even regarding the presence of abortive glandular lumina). Cytoarchitectural transitions were frequently observed; these included spindle to epithelioid cell types and fascicular to solid (MSS and BSS) or fascicular to glandular (BSS) patterns. These findings support the assumption that spindle cells of SS are neoplastic and may evolve to glandular cells in SS. Based on the cytogenetic data pointing to a common pathogenesis of both phenotypes (BSS and MSS), SS may represent true carcinomas of soft tissues with a biphasic and/or monophasic pattern depending on the degree of differentiation.

KEY WORDS: carcinoma of soft tissue, carcinosarcoma of soft tissue, synovial sarcoma, ultrastructure.

INTRODUCTION

Synovial sarcomas (SS) are soft tissue tumors of unknown origin[1] that, based on ultrastructural and immunohistochemical evidence, most investigators consider carcinosarcomas or carcinomas of soft tissues.[2-7] It is the presence of biphasia (clear-cut alternating areas of epithelial and spindle cells) that is used for the diagnosis of biphasic synovial sarcoma (BSS).[1] Monophasic synovial sarcoma (MSS) lacks distinct biphasia and consists only of epithelial or spindle/epithelioid cells.[1-5,8-16]

Although many investigators have described immunohistochemical and/or ultra-

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structural features supporting epithelial type differentiation of spindle cells of BSS and spindle/epithelioid cells of MSS,[2-6,8-10,12-16] the relationship between both subtypes is still unsettled. Some believe that MSS is the phenotypic counterpart of the spindle (monophasic fibrous variant) and epithelial (monophasic epithelial variant) components of BSS.[8,9,11] Others claim that fibrous MSS represents a poorly differentiated variant of BSS and not the spindle cell component of BSS.[5]

The use of terms such as *epithelial* and *epithelioid* (ie, *epithelial-like*) to describe cells of SS still raises controversy. Dardick et al[5] used the term *epithelioid* to describe the appearance of MSS cells, whereas Dickersin[15] used the same term to describe cells both in the interglandular zones of BSS and in MSS. Finally, despite numerous descriptions of cytoarchitectural transitions in BSS,[2,6,8-10,13,15,16] no tumor cells transitional between spindle and epithelial components have been noted in BSS according to Dardick et al.[5]

To clarify the aforementioned controversies, we made a specifically oriented ultrastructural study of the different components of SS in a series of 12 cases of BSS and 13 cases of MSS.

MATERIALS AND METHODS

Twenty-five cases of primary SS were selected from the files of the ultrastructural units of the Pathology Department of the Norwegian Radium Hospital, Norway (18 cases) and the Medical School of Porto, Portugal (7 cases). In 6 cases, material from recurrences (5 tumors) and metastases (2 tumors) was also available for examination.

In every case, extensive sampling (4 to 15 paraffin blocks per primary tumor and per recurrence or metastasis) was available for routine histologic evaluation. The cases were classified, on the basis of light microscopic and immunohistochemical features according to the criteria established by Enzinger and Weiss,[1] BSS (n = 12) and MSS (n = 13). Clinicopathologic features of the cases are given in Table 1. All the MSS were of the fibrous type.

TABLE 1 Clinicopathologic Features of SS

Case number	Patient age (years)	Patient sex	Location of tumor
Biphasic			
1	48	Female	Thigh
2*†	58	Female	Trunk
3*	38	Female	Arm
4	18	Male	Leg
5	81	Male	Thigh
6	17	Male	Leg
7	55	Female	Knee
8*	61	Male	Abdominal wall
9†	28	Male	Thigh
10	20	Female	Pharynx
11	13	Male	Knee
12	26	Female	Thigh
Monophasic			
13	15	Male	Leg
14	28	Female	Leg
15*	31	Male	Mediastinum
16	42	Male	Thigh
17	39	Male	Retroperitonium
18*	53	Male	Retroperitonium
19	48	Female	Arm
20	43	Male	Thigh
21	32	Male	Knee
22	25	Female	Thigh
23	19	Female	Arm
24	35	Female	Thigh
25	17	Male	Hand

*Cases with recurrences available for ultrastructural study.

†Cases with metastases available for ultrastructural study.

Ultrastructural study was performed on material fixed in phosphate-buffered 2.5% glutaraldehyde (13 primary tumors and 7 recurrences or metastases) or in buffered 10% formaldehyde (7 primary tumors). The specimens were postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanols, and embedded in an Epon-Araldite mixture. In the remaining 5 primary tumors ultrastructural study was made of small fragments retrieved from paraffin blocks according to the method described by Johannessen.[17] Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead ci-

trate, and examined under the transmission electron microscope.

Semithin sections (8 to 10 blocks per tumor), cut with glass knives and stained with toluidine blue, were thoroughly scrutinized in every case for light microscopic orientation of the different areas of the tumors that were afterward specifically studied at the ultrastructural level.

For the sake of simplicity, the cases were divided according to their histologic classification into BSS, MSS, and recurrence/metastasis.

BSS (n = 12)

In 6 tumors we were able to select from the semithin sections areas showing distinct biphasia (glandular or solid pattern and spindle cell pattern, Fig. 1). In the remaining 6 tumors only spindle and epithelioid cell components, without clear-cut biphasia but showing a solid or fascicular pattern, were observed (Fig. 2). All the tumors consisted of epithelioid and spindle cells. The epithelioid cells were columnar, polygonal, or round and had more abundant cytoplasm than the spindle cells. Transitions between spindle and epithelioid cells could be found in 10 of the tumors (Figs. 2B and 3). We also selected for ultrastructural study areas showing an architectural transition between a glandular or solid pattern and patternless foci (Fig. 3).

MSS (n = 13)

In 7 tumors there was a clear predominance of spindle cells arranged in a fasicular pattern (Fig. 4A). In the remaining 6 tumors a haphazard mixture of spindle and epithelioid cells predominated (Fig. 4B). Clusters of epithelioid cells (Fig. 5A) and transitions between spindle and epithelioid cells could be found in 6 cases (Figs. 4B and 5B) and were also specifically selected for ultrastructural study.

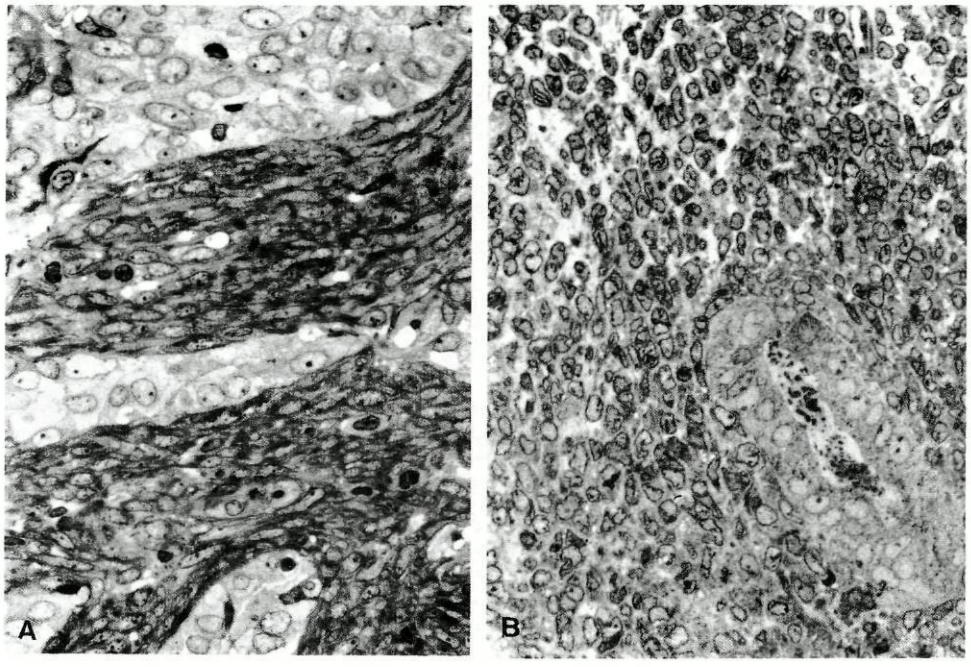


FIG. 1 *BSS: Solid* (A) *and glandular* (B) *areas alternating with spindle* (A) *and epithelioid* (B) *cells. Toluidine blue,* ×*560.*

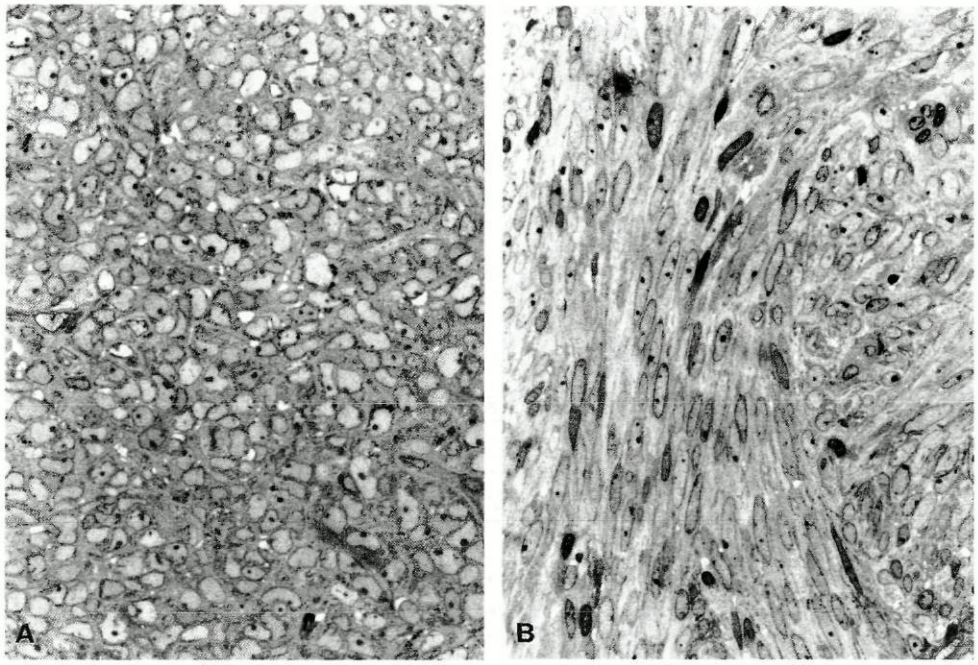


FIG. 2 *BSS: Solid epithelioid* (A) *and fascicular spindle/epithelioid* (B) *areas without distinct biphasia. Toluidine blue,* ×*560.*

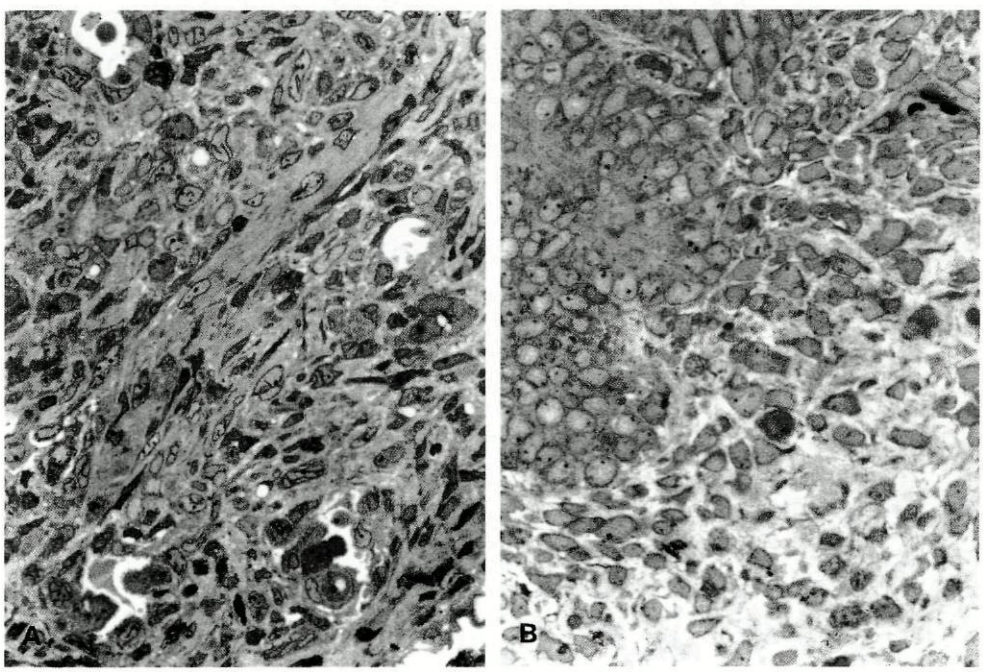


FIG. 3 *BSS: Cytoarchitectural transitions of glandular* (A) *and solid* (B) *areas to surrounding spindle/epithelioid cells. Toluidine blue,* ×*560.*

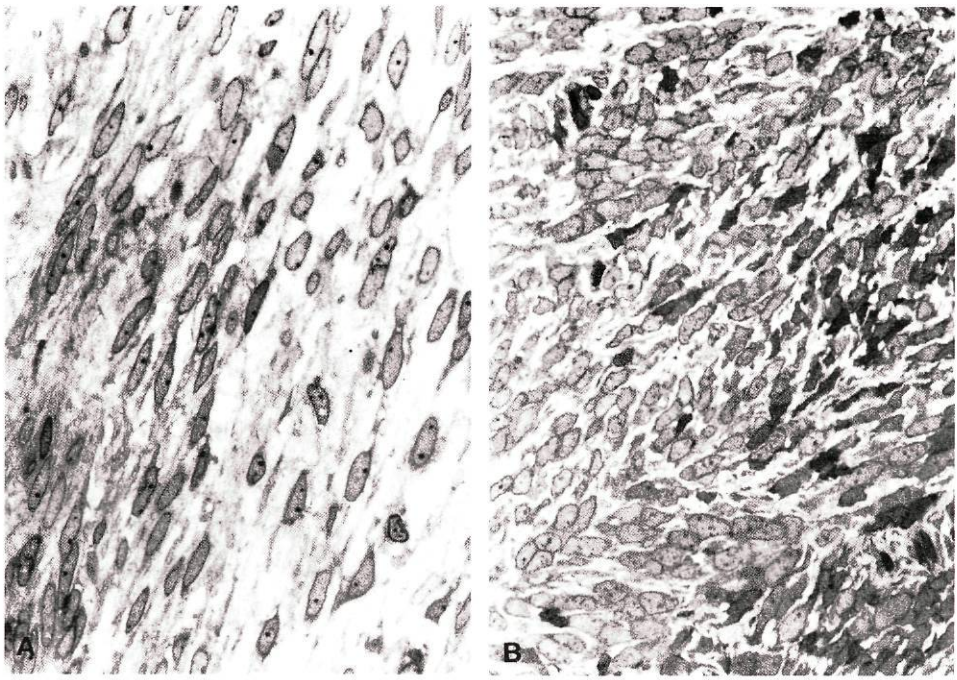


FIG. 4 *MSS: Spindle* (A) *and spindle/epithelioid* (B) *cells with fascicular pattern. Toluidine blue,* ×*560.*

Recurrences (n = 5) and Metastases (n = 2)

Semithin sections of recurrent and/or metastatic tumors showed neoplastic cell shape components and architecture similar to those of the corresponding primary tumors. Spindle cells were the only cell component present in 1 recurrence of MSS; all the other tumors showed both spindle and epithelioid cells.

RESULTS

BSS

Electron microscopy confirmed a clear-cut biphasic pattern with prominent glandular and/or solid foci in 6 cases. The glandular structures were lined by columnar, polygonal, cuboid, or sometimes flattened cells that rested on a continuous basal lamina (Figs. 6A and 7). The neoplastic cells were well to moderately equipped with organelles. Golgi complexes and aggregates of intermediate filaments were commonly present. Cilia and ciliary bodies and bundles of tonofilaments were found in 3 tumors (Fig. 7B). The luminal surface of the cells had short to long microvilli projecting into the lumen, which contained dense and amorphous material or cellular debris (Figs. 7A and 8A, inset) The nuclei were oval with prominent nucleoli. The cells were connected by different types of well-differentiated junctions (ie, tight junctions and desmosomes) and intermediate junctions. Intracellular lumina were often present, whereas intracellular lumina were rarely observed (Fig. 7A).

Nests of epithelioid cells showing minor degrees of glandular differentiation were also seen in areas classified by light microscopy as biphasic. These nests rested also on a continuous basal lamina.

Intercellular lumina with microvilli were found in the spindle/epithelioid cell components between solid and glandular areas of 1 tumor with clear-cut biphasia (Fig. 9A).

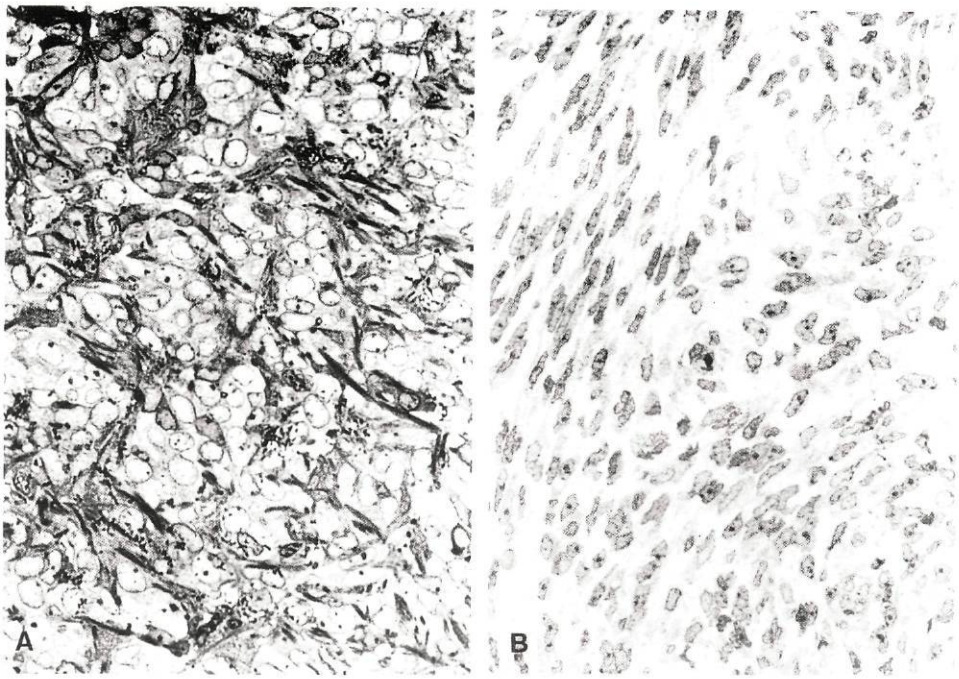


FIG. 5 *MSS: Clusters of epithelioid cells* (A) *and transitions between spindle and epithelioid cells* (B). *Toluidine blue, ×560.*

The cells composing the other 6 BSS (without apparent biphasia) were of spindle or epithelioid type (Fig. 10A). Both types of cells showed less equipped cytoplasm than the cells lining the glandular lumina. Aggregates of intermediate filaments, Golgi complexes, and cilia were usually less conspicuous than those in the cells of glandular or solid areas of SS with biphasia. Bundles of tonofilaments were never observed. Lipid inclusions and lysosomes were frequently found. Oval nuclei with prominent nucleoli were present in epithelioid cells (Fig. 10A). Intercellular spaces with filopodia and abortivelike microvilli were found in 6 cases (Fig. 11).

Most of the tumors showed intercellular junctions of the paired subplasmalemmal density (PSD) type[18] and, rarely, of the desmosome type connecting spindle and epithelioid cells. A discontinuous basal lamina was found around some spindle and epithelioid cells in most of the cases. In 1 case, a continuous basal lamina was seen around some epithelioid cell clusters without glandular differentiation (Fig. 6A).

Stroma between neoplastic cells was almost virtual in most glandular and solid areas (Figs. 7A and 10A). Moderately abundant stroma with bundles of collagen fibrils of the banded type (Figs. 6A, 7B, and 8A; long spacing type in 3 tumors) within a loose or floccular matrix predominated in spindle/epithelioid (Fascicular) areas without biphasia. In 3 cases an electron-dense (basal lamina–like) matrix was seen around some cells.

All sorts of cytoarchitectural transitions could be found in most BSS; these included spindle to epithelioid cell types and fascicular to solid or glandular patterns (Figs. 6B and 8A). The interface between some alternating glandular or solid areas and fascicular areas showed focal absence of a basal lamina and, thus, a distinct contiguity between cell components (Fig. 6B). Transitions of cytologic features within

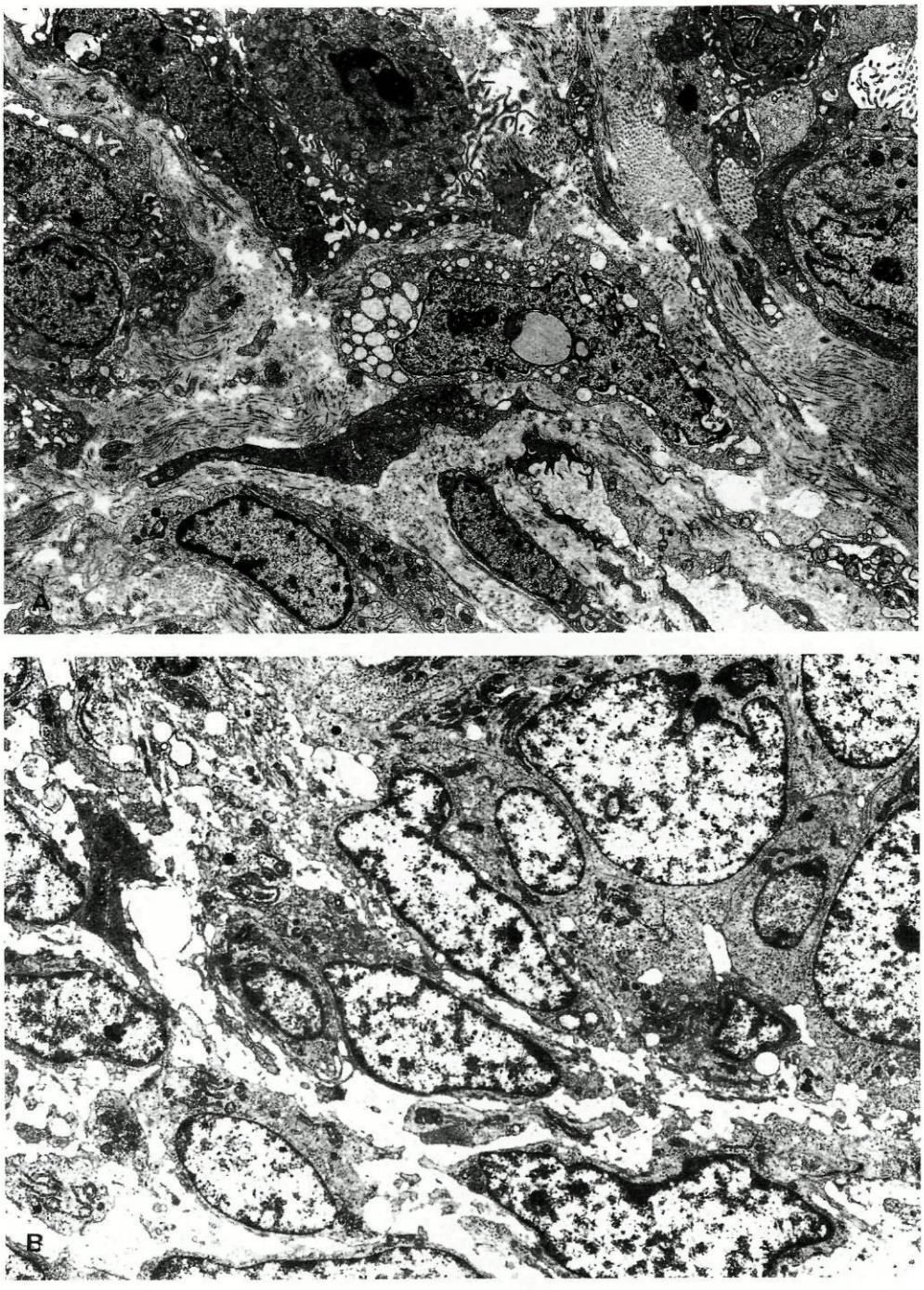


FIG. 6 *BSS:* (A) *Glandular* (upper right) *and solid areas surrounded by a continuous basal lamina. Uranyl acetate and lead citrate,* ×*3,630.* (B) *Solid area* (upper right) *with transition to surrounding epithelioid cells. No basal lamina is present. Uranyl acetate and lead citrate,* ×*3,630.*

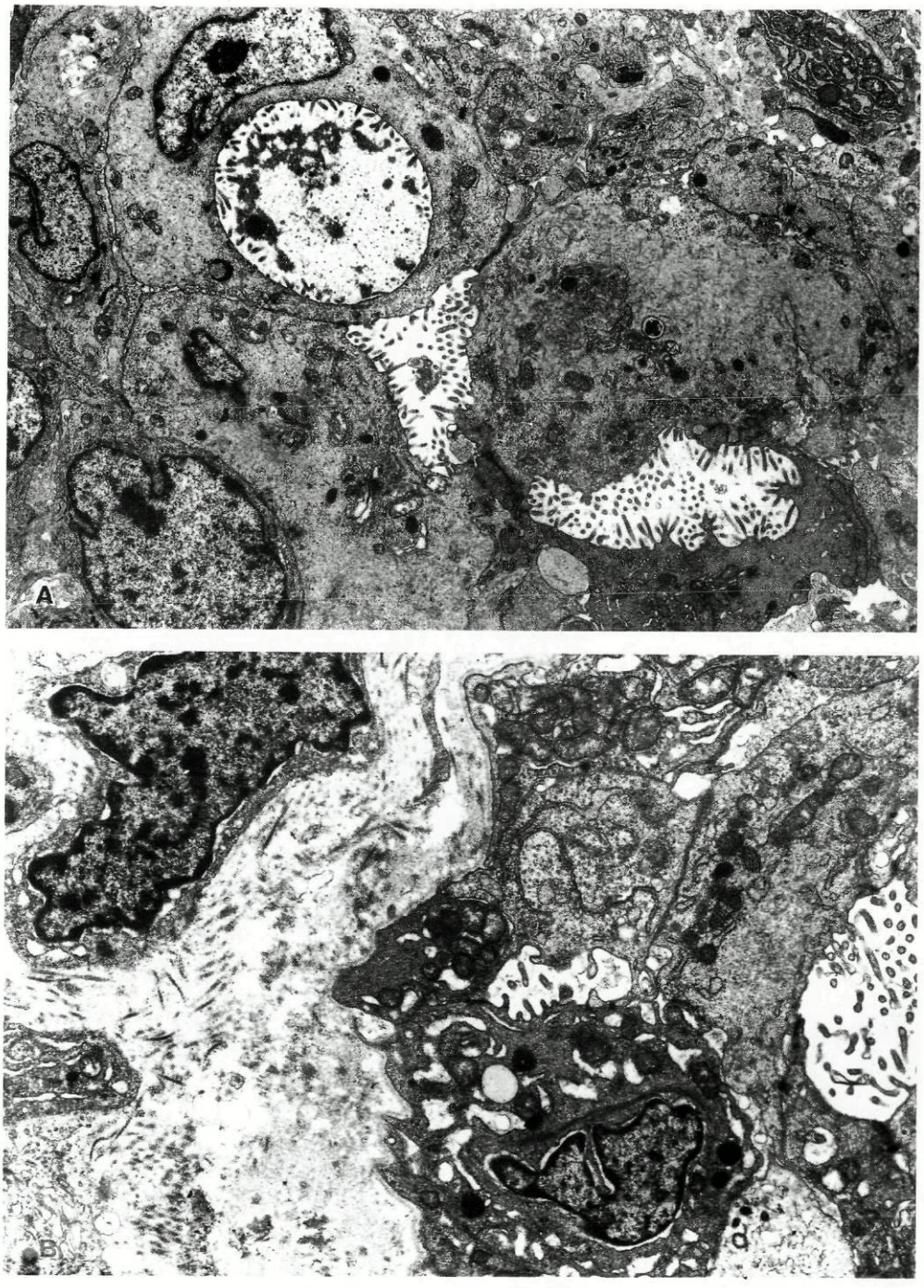


FIG. 7 *BSS:* (A) *Glandular area with an apparently intracytoplasmic lumen. Uranyl acetate and lead citrate,* ×*4,840.* (B) *Periphery of glandular area surrounded by a distinct basal lamina. Bundles of tonofilaments are present* (arrows). *Note the cilium in the spindle cell* (lower left). *Uranyl acetate and lead citrate,* ×*8,580.*

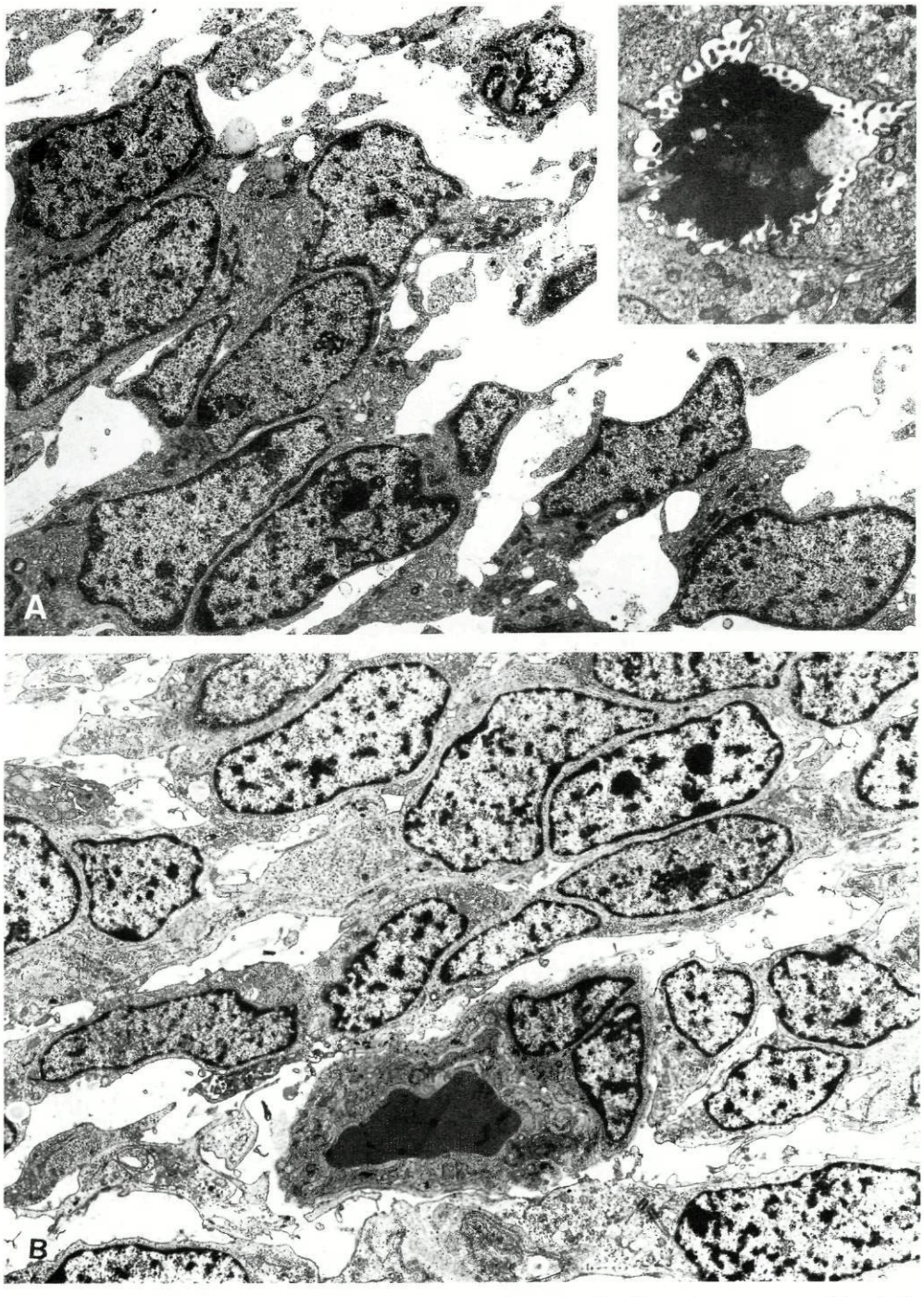


FIG. 8 (A) *BSS: Spindle and epithelioid transitional cells. Uranyl acetate and lead citrate, ×3,600.* (Inset) *The tumor had distinct glandular areas with intercellular lumens. Uranyl acetate and lead citrate, ×6,600.* (B) *MSS: Spindle and epithelioid transitional cells. Scattered filopodia are present. Uranyl acetate and lead citrate, ×3,630.*

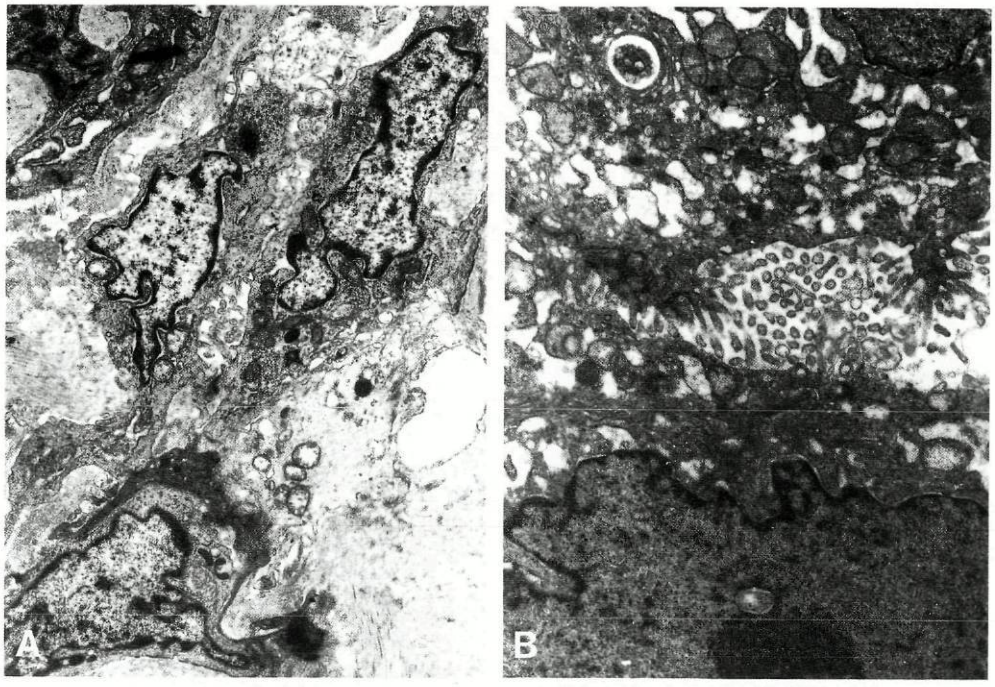


FIG. 9 (A) *BSS: Spindle cell component with glandular differentiation. Uranyl acetate and lead citrate, ×6,940.* (B) *MSS: Intercellular lumen lined with microvilli from neighboring epithelioid cells. Uranyl acetate and lead citrate, ×8,840.*

each pattern could also be observed whether biphasia was present or not.

MSS

In 6 tumors the cell exhibited a spectrum of shapes ranging from a long spindle appearance to a more round or polygonal epithelioid appearance (Figs. 8B and 10B). In the remaining 7 cases there was a predominance of spindle cells.

Epithelioid cells were better equipped with organelles (Fig. 10B) than spindle cells, especially with cilia-related structures, aggregates of intermediate filaments, and Golgi complexes. Nuclei were fusiform or oval and displayed prominent nucleoli in epithelioid cells (Fig. 10B). Intercellular junctions of the PSD type were observed in most of the cases, and a discontinuous basal lamina was more frequently found surrounding epithelioid cells (Fig. 12) than spindle cells. In 1 case a well-developed glandular structure with microvilli and desmosomes was seen in an area composed of epithelioid cells (Fig. 9B). A continuous basal lamina surrounding clusters of epithelioid cells was observed in this case.

Stroma was almost absent, particularly in predominantly epithelioid cell-rich areas (Fig. 10B). It was moderately abundant and contained dense (basal lamina-like) material or banded collagen fibrils within a loose or floccular matrix in the spindle cell-rich areas (Figs. 8B and 12). Collagen fibrils of the long spacing type were found in the matrix of 8 tumors.

Comparison of BSS and MSS

If one excludes the glandular areas that by definition are pathognomonic of BSS, the ultrastructural findings of MSS were simi-

lar to those of the nonglandular (interglandular areas and large solid or fascicular areas without biphasia) areas of BSS, namely regarding the features of epithelial type differentiation (Table 2) and the matrix. Major differences were the presence of more numerous cilia-related structures and less prominence of intermediate filament aggregates in the cytoplasm and discontinuous basal lamina around the neoplastic cells of MSS. Long spacing type collagen fibrils were more frequently found in the stroma of MSS than in BSS. A tumor of each group showed glandular differentiation within apparently nonglandular solid areas in the semithin sections (Fig. 9).

Comparison of Primary and Recurrent/Metastatic SS

Ultrastructural study of recurrences was performed in 5 cases (3 BSS and 2 MSS) and of metastases in 2 cases (BSS; Table 1). No major differences were found in the cellular components and architecture in comparison to the respective primary tumors, except for the greater prominence of nucleoli and abundance of polyribosomes in recurrent and metastatic tumor cells.

DISCUSSION

SS is a relatively frequent soft tissue tumor of unknown histogenesis that is most prevalent in adolescents and young adults.[1] The tumor raises particular interest because of its frequent double phenotypic histologic pattern, which often includes glandular foci.

Biphasic and monophasic (fibrous or epithelial) types are extremes of the histologic spectrum of SS according to most investigators. Recent data, mainly based on immunohistochemical and ultrastructural

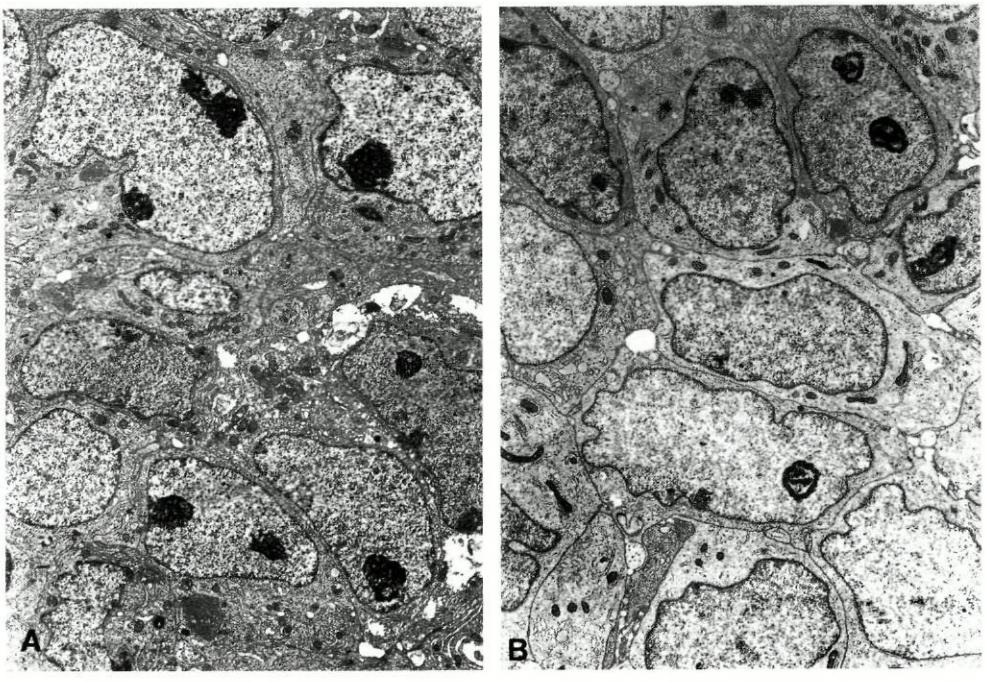


FIG. 10 (A) *BSS: Epithelioid cells without glandular structures. Uranyl acetate and lead citrate, ×3,510.* (B) *MSS: Epithelioid cells in a monophasic tumor. Note the similarity to the cells in* (A). *Uranyl acetate and lead citrate, ×3,520.*

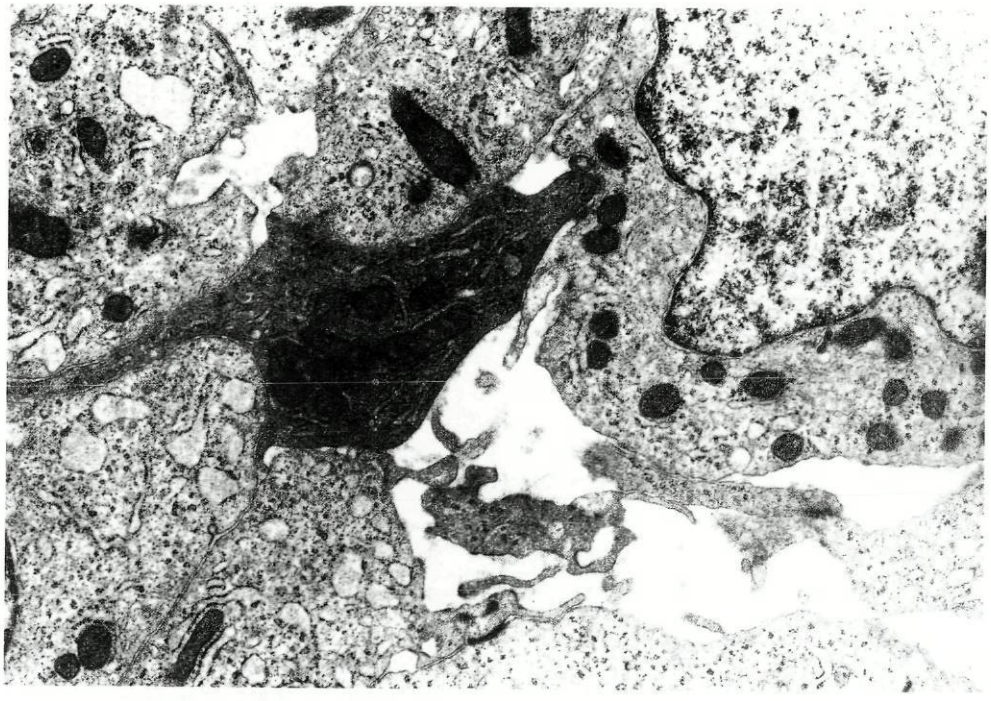


FIG. 11 *BSS: Abortive glandular structure lined by epithelioid cells. Uranyl acetate and leàd citrate, × 11,400.*

studies, support the epithelial (monophasic) or epitheliomesenchymal (biphasic) nature of SS.[2-7]

Monophasic subtypes are difficult to diagnose,[4-6,8-16] and some investigators have even questioned their true existence.[19] Although poorly differentiated SS are recognized as a distinct subgroup because of their ominous prognosis,[1,20] no well-defined criteria for the histologic grading of SS have been established so far.

To address the problem of neoplastic differentiation in SS, we followed in this study the criteria of Enzinger and Weiss[1] as a basis for the comparison between ultrastructural features of glandular and nonglandular areas of BSS as well as between nonglandular areas of BSS and MSS. To achieve this goal and to avoid misrepresentations at the ultrastructural level of the different histologic aspects, we carefully selected from each case the most representative semithin sections. These sections were afterward used to precisely orient the sites most appropriate for ultrastructural study.

Our results clearly show the existence of a spectrum varying from epithelial type differentiation (abortive glandular structures) to true epithelial differentiation (well-developed glandular structures) in all cellular components of SS. In this context, PSD type junctions[18] and discontinuous basal lamina observed in spindle and epithelioid components, both in primary and in recurrent SS (biphasic or monophasic patterns), should be considered putative signs of primitive epithelial type differentiation. Although rarely, we have also observed desmosomes and discrete glandular structures in the nonglandular areas of both tumor subtypes. This further supports the epithelial nature of these tumors, regardless of the predominant morphologic appearance of SS.

Another argument in favor of the epithe-

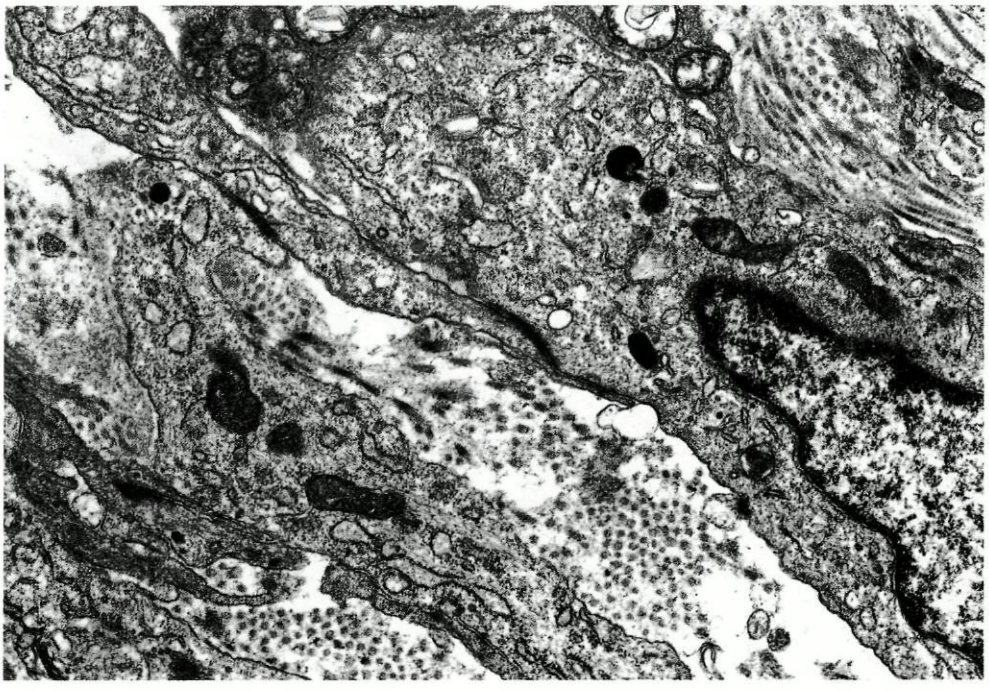


FIG. 12 *MSS: PSD between and discontinuous basal lamina around spindle and epithelioid cells. Uranyl acetate and lead citrate, × 11,400.*

TABLE 2 Ultrastructural Features Suggestive of Epithelial Type Differentiation in Spindle and Epithelioid Cell Components of Fibrous MSS and in Interglandular and/or Solid Areas without Biphasia of BSS

	Number of cases	
Characteristic	MSS (n = 15)	BSS excluding glandular areas (n = 17)
Intercellular spaces	7	10
Microvilli	1	1
Intercellular junctions		
PSD	13	14
Desmosomes	1	3
Tight junctions	1	2
Basal lamina		
Continuous	1	1
Discontinuous	6	12
Intermediate filament aggregates	5	11

lial differentiation of SS is the frequent demonstration of epithelial markers in the neoplastic cells, again regardless of their histologic appearance. Although epithelial membrane antigen and cytokeratins of the simple epithelial type keratins (8, 18, and 19) are being increasingly described in several other types of soft tissue tumors,[21,22] the same does not hold true for the stratified epithelial type cytokeratins, which, like others,[6,21] we have observed in all the cellular components of SS.

Transitions between cellular components of glandular or solid areas and nonglandular or solid areas could be found in our series, as has been reported previously.[2,6,8-10,13,15,16] We also found all sorts of transitions between spindle and epithelioid cell components of SS. Epithelial type differentiation was more prominent in epithelioid cells than in spindle cells. This difference was more evident in tumors displaying a biphasic pattern than in those displaying a monophasic pattern, but we repeatedly found unequivocal signs of epithelial type differentiation in areas of SS exclusively or almost exclusively composed of spindle cells.

Taking all this together, we believe that there is enough evidence to support the neoplastic nature of spindle cells of both BSS and MSS and, furthermore, in contrast to Dardick et al,[5] that spindle cells are able to transform and evolve into glandular cells of SS. We concur with Dickersin[15] that the coexistence of biphasic and monophasic patterns in the same tumor reflects different degrees of differentiation (transitions) of SS and not a combination of tumors with different natures (carcinosarcoma and carcinoma), as proposed by Dardick et al.[5]

There is growing evidence that neoplastic cell shapes and the degree of neoplastic differentiation are dependent on cell–cell and cell–matrix interactions. This could explain why carcinomas growing in the superficial mesenchyma (eg, the so-called pseudosarcomatous carcinomas of mucosa and skin) display a predominantly spindle cell architecture. It remains to be seen whether the mesenchyma where SS occur plays a role in the acquisition of the epithelioid (or true epithelial) architecture of neoplastic cells.

Nomenclature and criteria for the histologic grading of SS still raises controversy. In the most recent series, SS have been considered carcinomas or carcinosarcomas of soft tissues depending on the type of differentiation.[3-11,13] Cytogenetic data[23,24] point, however, to a common pathogenesis for both phenotypes (BSS and MSS). Based on this and on our own results, we suggest that SS represent true carcinomas of soft tissues with biphasic and/or monophasic patterns depending on the degree of differentiation.

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Paper II

Path. Res. Pract. 190, 168–177 (1994)

Immunohistochemical Profile of Synovial Sarcoma with Emphasis on the Epithelial-type Differentiation

A Study of 49 Primary Tumours, Recurrences and Metastases

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SUMMARY

The relationship between biphasic (BSS) and monophasic (MSS) subtypes of synovial sarcoma (SS) as well as the relationship between cells of solid/glandular areas and the spindle cells of BSS remain controversial. In order to further evaluate the immunohistochemical phenotype of SS we studied 34 primary tumours (15 BSS; 19 MSS), 7 recurrences (4 from primary BSS; 3 from primary MSS) and 8 metastases (7 BSS; one MSS), using several antibodies (EMA, CEA, keratins 1, 4, 5/6, 7, 8, 13, 18, 19, 20, vimentin, collagen IV and laminin) that work in paraffin-embedded material. Spindle cells outside solid/glandular areas of BSS and in MSS showed immunoreactivity for keratins 5/6, 7, 8, 18 and 19. The transition of solid/glandular areas to surrounding spindle cells also showed keratin staining and failed to show a distinct separation regarding the immunoreactivity for laminin and collagen IV. Peripheral cells of solid/glandular areas were immunoreactive for vimentin. No major differences were observed between immunophenotypical cell profiles of BSS and MSS, apart from the exclusive immunostaining of solid/glandular areas of BSS for keratin 13 and CEA. Downgrading of keratin and extracellular matrix antigens immunoreactivity was observed when primary tumours were compared to recurrent and/or metastatic tumours of both subtypes (MSS and BSS). We conclude that SS should be regarded as 'carcinomas of soft tissues with an immunohistochemical phenotype depending on the degree of epithelial differentiation: spindle cells (MSS and BSS) predominantly expressing simple keratins, and poorly differentiated (solid/glandular) as well as well-differentiated (glandular) areas (BSS) expressing, in addition, complex epithelial-type keratins.

Introduction

Synovial sarcomas (SS) are soft tissue tumours of unknown origin[9]. Based on ultrastructural and immunohistochemical studies, most authors consider them either as *carcinosarcomas* or as *carcinomas* depending on the presence or absence of biphasia[6,15,19,20].

Several immunohistochemical studies of SS have been published in the last ten years[1,3–6,10,15,17–21,23,24,26,27,29]. Most of these studies have clearly shown the epithelial differentiation of SS. However, individual keratins were only evaluated in a few series[17,21,27] and in a limited number of precisely classified SS. The limited number of cases studied to date contributes to explain why some

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controversies still persist regarding the pattern of immunoreactivity of SS.

The most vivid controversy resides on the relationship between biphasic (BSS) and monophasic (MSS) subtypes of SS and also on the relationship between cells in solid/glandular areas and the surrounding spindle cells of BSS[6,7].

Immunohistochemistry studies of BSS on record describe the almost constant expression of epithelial-type (keratins and/or EMA) and mesenchymal-type (vimentin) markers in solid/glandular and spindle cell components, respectively. MSS show lower and variable degrees of expression of the aforementioned markers[1, 3–6, 19, 15, 17–21, 23, 24, 26, 27, 19]. CEA immunoreactivity of solid/glandular component is an infrequently reported finding; to the best of our knowledge such immunoreactivity has usually been detected in BSS co-expressing keratins and EMA, as well as in rare MSS also co-expressing keratins and EMA[5,23,27]. Although S100 protein reactivity was described in some BSS and MSS cases, they also usually co-expressed keratins and EMA[9,11,23]. Other markers (e.g., desmin, smooth-muscle actin, Factor VIII) used in the differential diagnosis with other soft tissue tumours were consistently negative in all reported series[5,6,23,24,26,27]. Vimentin reactivity is frequently described in spindle cell component of BSS and MSS, and also occasionally in cells of solid/glandular component of few cases[23,24,26,27]. Few studies of collagen IV and laminin expression depicted continuous staining along solid/glandular component of BSS[22,23,27] and also, in one series[23], between groups of cells of the spindle cell component of BSS and MSS.

Solid/glandular component of BSS of the largest series showed co-expression of keratins and EMA[6,23]. Spindle cell component of BSS is variably described as non-reactive[6,17,18] or focally reactive in most cases for one or both of these markers[4,5,11,15,23,24,26,27,29].

Apart from these discrepancies it also remains to be clarified if there is any relationship between the expression of individual keratins and other immunohistochemical markers of SS.

The availability of commercial antibodies for individual keratins and the improvement in the processes of unmasking keratin epitopes led us to study the keratin profile together with several other immunohistochemical markers of the neoplastic cells and the matrix of a large series of paraffin-embedded SS, in an attempt to settle at least some of the aforementioned controversies.

Material and Methods

Biphasic (BSS) and monophasic (MSS) synovial sarcomas of 36 patients were selected from the files of the Department of Pathology, The Norwegian Radium Hospital (25 cases) and Porto Medical School (11 cases).

Thirty-four primary tumours (15 BSS; 19 MSS), 7 recurrences (4 from BSS; 3 from MSS) and 8 metastases (7 from primary BSS and one from primary MSS) were available for examination. In 2 patients with primary BSS only recurrence or metastases were available for immunohistochemical study.

In every tumour, several blocks (4–15 paraffin-blocks per primary tumour and per recurrence and/or metastasis) were available for routine histological examination. The cases were classified according to the light microscopic, immunohistochemical and electron microscopic criteria of Enzinger and Weiss[9]. The clinico-pathological features of the cases are summarized in Table 1. All MSS were of the fibrous type. One recurrence and one metastasis from BSS showed distinct biphasia, whereas all the other recurrent and metastatic tumours displayed monophasia.

Table 1. Clinico-pathologic features of synovial sarcomas

Biphasic	Age (years)	Sex	Location
*1	48	F	Thigh
*2†°	58	F	Trunk
*3°°	38	F	Arm
*4°	18	M	Leg
*5	81	M	Thigh
6	32	M	Thigh
*7	17	M	Leg
*8	55	F	Knee
*9†	61	M	Abdominal wall
*10†	28	M	Thigh
11†°	33	F	Trunk
12°°	38	M	Tongue
13	20	M	Foot
14	45	M	Thigh
15	14	M	Knee
16	35	M	Knee
17	46	F	Foot
Monophasic			
*18	15	M	Leg
*19	28	F	Leg
*20	31	M	Mediastinum
*21†	42	M	Thigh
*22	17	M	Hand
23	7	M	Thigh
24	40	F	Pharynx
25††	30	M	Foot
*26	39	M	Retroperitoneum
*27	53	M	Retroperitoneum
28	40	F	Leg
29°	61	F	Foot
*30	48	F	Arm
*31	43	M	Thigh
*32	32	M	Knee
33	28	F	Thigh
*34	25	F	Thigh
*35	19	F	Arm
*36	35	F	Thigh

Cases with ultrastructural study* and cases with recurrences † and/or metastases ° available for study. Cases 9 and 12 without primary tumours available for immunohistochemistry. F = female; M = male.

Immunohistochemistry

Consecutive sections were stained using the avidin-biotin-peroxidase complex (ABC) method[12]. Deparaffinized sections were redhydrated, washed with phosphat-buffered saline (PBS) pH 7.4 and treated with enzyme (Table 2). The sections were then treated with 0.3 % hydrogen peroxide (H_2O_2) in methanol for 30 minutes to block endogenous peroxidase. To unmask the epitopes of keratins 7 and 18, the sections were microwaved in an „antigen retrieval" solution[25]. The sections were then incubated

Table 2. List of primary antibodies*

Antibody against	Source	Catalog no	Dilution	Enzyme treatment
Cytokeratin 1	Enzo Diagnostic	C34940	1:75	Trypsin
Cytokeratin 4	Boehringer Mannheim Biochemica	1273370	1:100	Trypsin
Cytokeratin 5/6	Boehringer Mannheim Biochemica	1273396	1:10	Pronase
Cytokeratin 7	Sigma Immunochemicals	C6417	1:100	
Cytokeratin 8	Boehringer Mannheim Biochemica	1238817	1:25	Trypsin
Cytokeratin 13	Boehringer Mannheim Biochemica	1009460	1:50	Pepsin
Cytokeratin 18	BioGenex Laboratories	Mu143-uc	1:10	
Cytokeratin 19	Boehringer Mannheim Biochemica	1238825	1:50	Trypsin
Cytokeratin 20	Progen Biotechnik GMBH	61026	1:100	Pronase
Cytokeratin AE1/AE3	ICN Immuno Biologicals	69–140; 69–142	1:200	Trypsin
CEA	Dr. O. Børmer, Norw. Radium Hosp.		1:320	
EMA	Dakopatts A/S	M613	1:20	
Collagen IV	Dakopatts A/S	M785	1:100	Pepsin
Desmin	Dakopatts A/S	M760	1:100	
S100**	Dakopatts A/S	Z311	1:700	
Vimentin	Boehringer Mannheim Biochemica	8143318	1:100	
Muscle actin	Enzo Diagnostics	30931	1:10	
Laminin**	Serotec	AHP239	1:100	Pepsin

CEA = carcinoembryonic antigen, EMA = epithelial membrane antigen. – * unless otherwise specified all primary antibodies were monoclonal; ** polyclonal antiserum produced in rabbit.

for 20 minutes with normal serum from the species in which the secondary antibody was made to eliminate nonspecific staining. Excess normal serum was blotted from the slides before incubation with primary antibodies (Table 2) for 18–22 hours at 4 °C. The sections were then incubated with 1:200 dilution of biotin-labeled secondary antibody for 30 minutes and ABC (10 µg/ml of avidin and 2.4 µg/ml of biotin-labeled peroxidase) for 60 minutes (Vector, Burlingame, CA). Tissue was stained for 5 minutes with 0.05 % 3'3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.6, containing 0.01 % H_2O_2 and then counterstained with haematoxylin, dehydrated and monted in Diatex. For the two antibodies epithelial membrane antigen (EMA) and cytokeratin AE1/AE3 an alkaline phosphatase method was applied. After deparaffinization, washing and enzyme treatment (Table 2) the sections were incubated with primary antibodies (Table 2) for 30 minutes at room temperature. The sections were then incubated with alkaline phosphatase rabbit anti-mouse IgG (Dakopatts, Denmark) 1/15 dilution for 30 minutes and alkaline phosphatase swine anti-rabbit IgG (Dakopatts). Tissue was stained for 45 minutes at 37 °C with a chromogen mixture including fast red TR and naphthol as phosphate. The sections were counterstained with haematoxylin and mounted in glycerine-gelatine. All the dilutions of normal sera, antisera, biotin-labeled secondary antibodies and ABC were made with PBS containing 5 % bovine serum albumin as the diluent. All series included positive controls. Negative controls included substitution of polyclonal primary antiserum with normal rabbit serum, and mouse myeloma proteins of the same subclass and concentration as the monoclonal antibodies.

Immunoreaction was considered positive whenever staining was observed independently of the intensity of the staining. A semi-quantitative method was used to evaluate the immunoreactive patterns of the tumours: (-negative; rare-less than 10 % of positive cells; focal-10 to 30 % of positive cells; extensive – more than 30 % of positive cells).

Results

Primary BSS (n = 15)

Solid/glandular areas showed extensive or focal cytoplasmic reactivity in all tumours for EMA, AE1/AE3, keratins 8 and 19 (Table 3). Most of the tumours showed focal or rare solid/glandular areas immunoreactive for keratins 5/6 and 7 (Figs. 1 and 2) and, in six cases, for keratins 13 and 18 (Figs. 3 and 4). Although most of these antibodies depicted variable immunoreactive patterns within the same tumour, and from tumour to tumour, they were usually more frequently co-expressed and intense in the cuboidal, columnar of flattened cells lining the glan-

Fig. 1. BSS: Keratin 5/6 staining of cuboidal glandular cells. ABC, ×250. – Fig. 2. BSS: Keratin 7 staining of solid/glandular areas. ABC, ×250. – Fig. 3. BSS: Keratin 13 staining of flattened glandular cells. ABC, ×250. – Fig. 4. BSS: Keratin 18 staining of cuboidal and columnar glandular cells. ABC, ×250. – Fig. 5. BSS: Keratin 19 staining of glandular cells and of a few spindle cells. ABC, ×250. – Fig. 6. BSS: EMA staining of glandular cells, lumen content, and transitional cells to spindle component. APAAP, ×250. – Fig. 7. BSS: Vimentin staining of peripheral solid/glandular cells and of alternating spindle cells. ABC, ×250. – Fig. 8. BSS: Laminin continuous staining at the periphery of solid/glandular areas and around focal spindle cells. ABC, ×250. – Fig. 9. BSS: Collagen IV continuous staining at the periphery of solid/glandular areas and around focal spindle cells. ABC, ×250. ▶

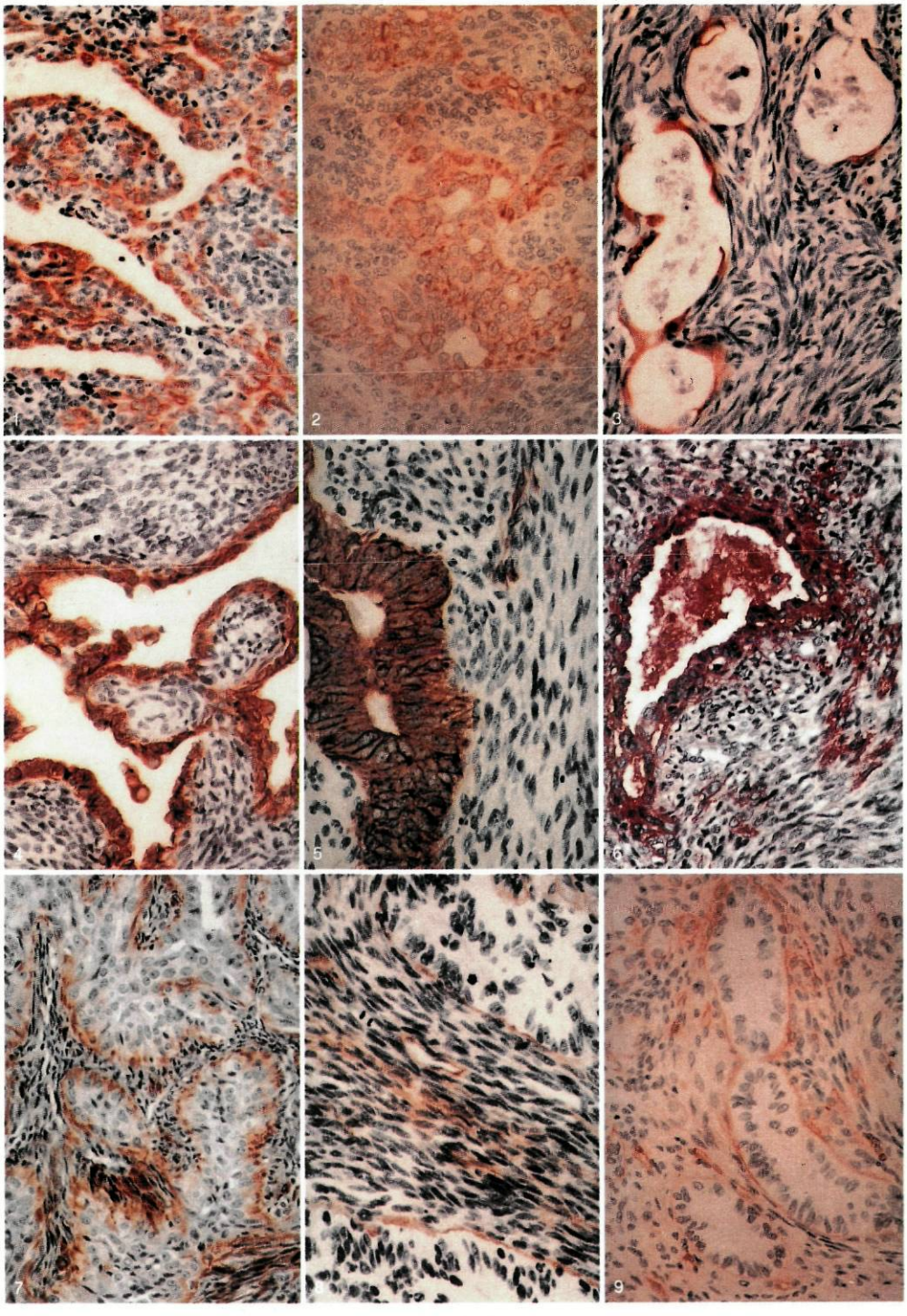

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Table 3. Immunohistochemistry of biphasic and monophasic synovial sarcomas

	Tumours positive, no (%)		
	Biphasic (n = 15)		Monophasic (n = 19)
	Glandular/solid component	Spindle component	
EMA	15 (100%)	11 (73.3%)	13 (68.4%)
AE1/AE3	15 (100%)	9 (60.0%)	10 (52.6%)
CEA	3 (33.3%)	0 (0%)	0 (0%)
CK1	0 (0%)	0 (0%)	0 (0%)
CK4	0 (0%)	0 (0%)	0 (0%)
CK5/6	10 (66.6%)	2 (13.3%)	6 (31.6%)
CK7	9 (60.0%)	6 (40.0%)	4 (21.1%)
CK8	15 (100%)	4 (26.6%)	5 (26.3%)
CK13	6 (40.0%)	0 (0%)	0 (0%)
CK18	6 (40.0%)	1 (7.0%)	1 (5.0%)
CK19	15 (100%)	12 (80.0%)	11 (57.9%)
CK20	0 (0%)	0 (0%)	0 (0%)
VIM	6 (40.0%)	15 (100%)	19 (100%)
COL IV	6 (40.0%)	7 (46.7%)	13 (68.4%)
LAM	8 (53.3%)	5 (33.3%)	6 (31.6%)
S100	5 (33.3%)	3 (20.0%)	4 (21.1%)

dular spaces. Keratin staining was usually cytoplasmatic but sometimes underlined the cell membrane of the reactive cells (Fig. 5). EMA immunoreactivity was more intense than the keratin staining and frequently found at the apical surface of cells lining the lumen of glandular areas of BSS as well as within the lumen itself (Fig. 6). In three cases immunoreactive for all the aforementioned antibodies, solid/glandular component also showed focal cytoplasmic CEA positivity. Focal or extensive S100 protein positivity was observed in solid/glandular component of one third of the cases. Vimentin was rarely or focally positive, particularly in the peripheral cells of solid/glandular component of six cases (Fig. 7). Laminin depicted a continuous outline, around focal solid/glandular areas (Fig. 8), in more cases (8/15) than collagen IV (6/15) (Fig.9). In five tumours, all of them co-expressing keratins and EMA in solid/glandular component, laminin was co-expressed with collagen IV. The other antibodies tested were negative in the solid/glandular component.

Spindle cells showed focal or rare positivity in most cases for keratin 19 (12/15) (Fig. 5), EMA (11/15) (Fig. 6) and AE1/AE3 (9/15), and less often for keratins 7 (6/15) and 8 (4/15) (Fig. 10) – Table 3. Rare or focal spindle cell immunoreaction was observed in few cases for keratins 5/6 (2/15) and 18 (1/15). Focal S100 protein staining was seen in spindle cells of three cases, two of them also immunoreactive in solid/glandular component. Immunoreactivity for vimentin was focally (11 tumours) or extensively (four tumours) present in spindle cells of the tumours (Fig. 11). Collagen IV and laminin depicted a discrete pericellular pattern around spindle cells in focal areas of seven and five cases, respectively; these cases co-expressed keratins and EMA. Co-expression of collagen IV and laminin was seen in the spindle cell component of four BSS. Spindle cell immunoreactive patterns were variable within the same tumour and from tumour to tumour; however, plump spindle (epithelioid) cells showed more frequent and intense positivity for keratins and EMA than thin spindle cells. Spindle cells were negative for all the other antibodies tested.

In nine case, the cells at the transition between solid/glandular and spindle cell components showed immunoreactivity for keratins 8 (Fig. 10) and 19 (Fig. 12), EMA (Fig. 6) and AE1/AE3. Immunostaining for laminin and collagen IV was weak or absent in these transitional areas.

Primary MSS (n = 19)

Most MSS showed focal or extensive immunoreaction for EMA (Fig. 13), AE1/AE3, or keratin 19 (Fig. 14) (Table 3). Co-expression of these antibodies was observed in six cases. Less than one third of the tumours showed rare or focal positivity for keratins 5/6 (Fig. 15), 7 or 8. Only one tumour, which co-expressed all the aforementioned individual keratins, showed rare immunoreactive cells for keratin 18. Although variable immunoreactive patterns were obtained with antibodies for individual keratins, EMA and AE1/AE3, epithelioid cells tended to show more frequent and intense immunoreactivity than spindle cells. Vimentin staining was observed in all the cases, usually showing an extensive (five cases) or focal (eight cases) immunoreactivity. Vimentin immunoreactivity was frequent and more intense in spindle cells than epithelioid cells. Focal immunoreactivity for S100 protein was present in four cases with variable expression of individual keratins, EMA and AE1/AE3. The antibody for collagen IV depicted discrete pericellular pattern around spindle or epithelioid cells (Fig. 16) in focal areas of more cases (13/19) than the antibody against laminin (6/19). Co-expression of collagen IV and laminin was observed in five tumours. Co-expression of individual keratins and/or EMA was observed in eleven and four cases immunoreactive for collagen IV and laminin, respectively. Negative staining was observed with all the other antibodies tested.

Recurrent BSS (n = 4) and MSS (n = 3)

Recurrences from BSS showed histological biphasia only in one tumour with focal or extensive immunoreaction for EMA and AE1/AE3, respectively, both in solid/glandular and spindle cell components. The same pattern of co-expression (EMA and AE1/AE3) was observed in two other recurrences of BSS displaying monophasia, one of them also showing focal positivity for keratin 19. Vimentin was focally (one tumour) or extensively (one tumour) positive in half of recurrent BSS.

Recurrent MSS showed focal positivity for EMA in the three tumours, two of them with co-expression of keratins 8, 19 and vimentin.

Negative staining was observed with all the other antibodies tested in recurrent BSS and MSS.

Metastatic BSS (n = 7) and MSS (n = 1)

Metastases from BSS showed biphasia in one tumour with focal co-expression of EMA and AE1/AE3, in

Table 4. Primary *versus* recurrent biphasic and monophasic synovial sarcomas*

	Tumours positive, no								
	EMA	AE1/AE3	CK5/6	CK7	CK8	CK13	CK18	CK19	VIM
Primary tumours (n = 5)									
Biphasic (n = 3)									
Solid/glandular	3	3	0	1	3	0	0	3	1
Spindle	2	1	2	1	1	0	0	3	3
Monophasic (n = 2)	2	1	0	0	2	0	0	2	2
Recurrences (n = 6)	5	3	0	0	2	0	0	3	4

CK = cytokeratin, EMA = epithelial membrane antigen, VIM = vimentin. – * In one of the recurrences of the overall series (Case 9 of Table 1) material from the primary was not available. In another case (Case 25 of Table 1) two recurrences were studied by immunohistochemistry.

solid/glandular areas, all the other antibodies being negative in this case. Half of the remaining metastatic BSS showed focal or rare immunoreactivity for keratin 19, EMA and vimentin. A focal AE1/AE3 or keratin 8 immunostaining pattern was observed in two tumours and in one tumour, respectively. Co-expression of keratin 19 and EMA and/or AE1/AE3 was observed in four cases. The antibody for collagen IV disclosed a pericellular outline in focal areas of two tumours without biphasia.

The metastatic MSS only showed focal vimentin positivity.

No immunoreactivity was observed for all the other antibodies tested in metastatic BSS and MSS.

Biphasic versus Monophasic Primary SS

EMA, AE1/AE3, keratin 8 and 19 were observed in all primary BSS and in most of MSS (except keratin 8 in MSS – 26.3 %). Cytoplasmic reactivity for EMA was observed both in solid/glandular areas of BSS and spindle cell component of BSS and in MSS. Keratins 5/6 and 7 were positive in most BSS in comparison to their presence in about one third and one fifth, respectively, of MSS. Keratins 13 and 18 were observed in 40 % of BSS, whereas only one MSS showed immunoreaction for one of these antibodies (keratin 18). Vimentin was detected in all the cases of both subtypes. S100 protein was detected in 40 % of BSS and in one fifth of MSS. CEA was detected only in BSS (3 of the cases).

Comparison between the immunoreactivity of the cells outside solid/glandular component of BSS and MSS (Table 3) showed identical percentage of tumours positive for EMA, AE1/AE3, keratin 8 and 18, and S100 protein. No immunoreactivity was found for keratin 13 and CEA. Keratins 19 and 7 were more frequently detected in BSS than MSS, the inverse occurring for keratin 5/6 (Table 3). Antibodies for laminin and collagen IV showed similar patterns; immunoreactivity for collagen IV was more frequently found in MSS than in the spindle cell component of BSS. A similar percentage of cases co-expressing epithelial-type (individual keratins and EMA) and extracellular matrix (laminin and collagen IV) markers was observed when comparing solid/glandular and spindle cell components of BSS with MSS.

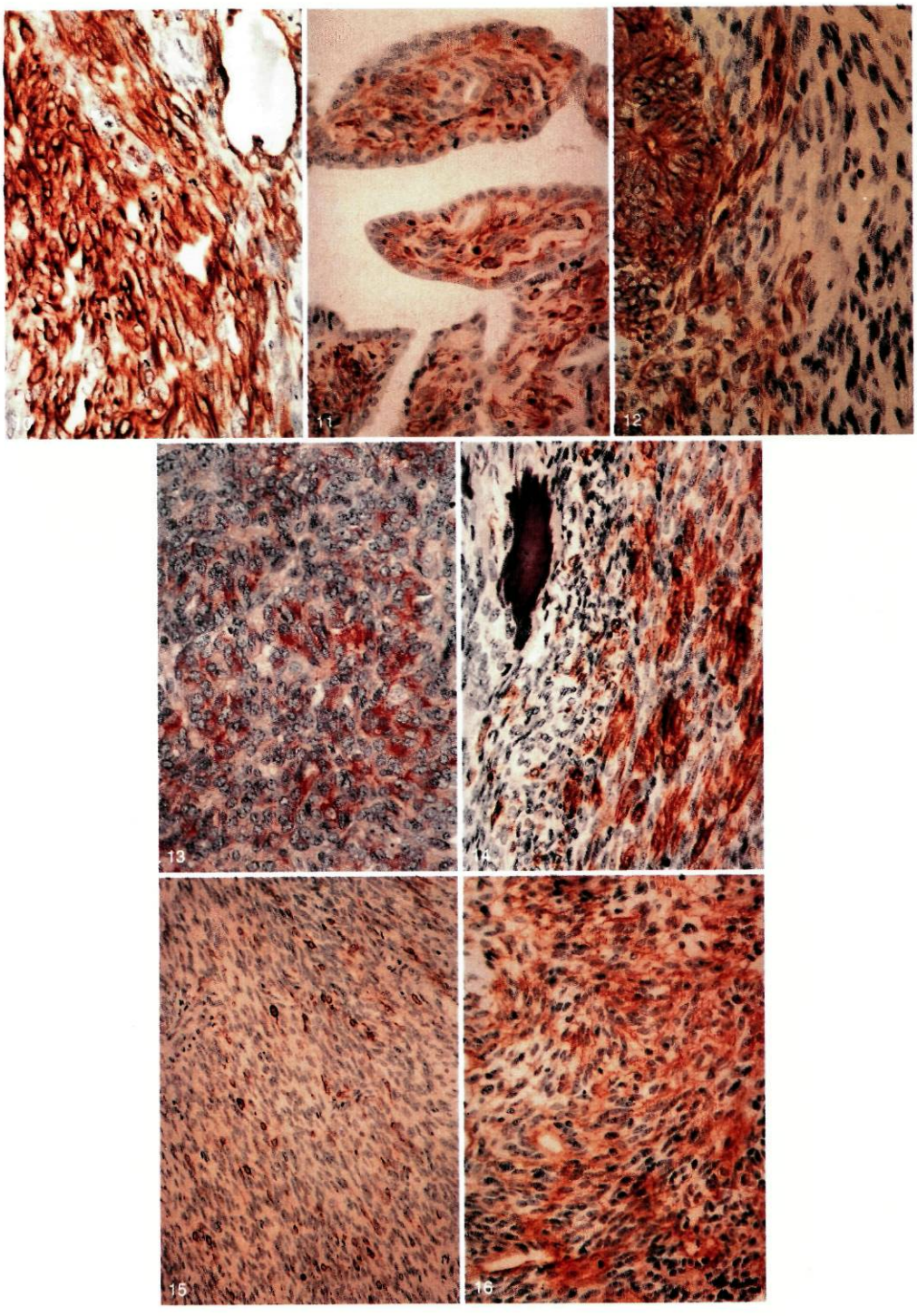
Primary versus Recurrent and Metastatic SS

Comparison between immunoreactions of primary tumours and the corresponding recurrences (two of them from one MSS) and metastases (two of them from one BSS) was possible in 8 patients (5 BSS; 3 MSS) – Table 1. The immunohistochemical study of recurrences (Table 4) and metastases (Table 5) showed less immunostained lesions and lower degree of immunoreactivity for all the antibod-

Table 5. Primary *versus* metastatic biphasic and monophasic synovial sarcoma*

	Tumours positive, no								
	EMA	AE1/AE3	CK5/6	CK7	CK8	CK13	CK18	CK19	VIM
Primary tumours (n = 5)									
Biphasic (n = 4)									
Solid/glandular	4	4	1	1	4	1	0	4	1
Spindle	3	3	2	2	1	0	0	4	4
Monophasic (n = 1)	1	1	1	1	1	0	1	1	1
Metastases (n = 6)	3	3	0	0	0	0	0	3	3

CK = cytokeratin, EMA = epithelial membrane antigen, VIM = vimentin. – * The primary tumour of Case 12 (two metastases studied by immunohistochemistry) was not available for study. In another case (Case 3) two metastases were studied by immunohistochemistry.



ies tested in comparison to the corresponding primary tumours, namely for EMA and AE1/AE3. Keratin 8 staining in two recurrences (from primary MSS) and keratin 19 staining in three recurrences (from one primary MSS and one primary BSS) and in three metastases (from primary BSS) were the only individual keratins detected in secondary SS (Tables 4 and 5), whereas they were positive in all primary tumours. None of the recurrences showed keratins 5/6, 7 and 13 positivity, whereas they were variably present in the corresponding primary tumours (Tables 4 and 5). None of the recurrences or metastases showed laminin or collagen IV immunoreactivity, whereas they were positive in four (BSS) and five (4 BSS; 1 MSS) primary tumours, respectively. We have not found any negative primary tumour regarding the different antigens in which immunoreactivity could be disclosed in recurrent or metastatic tissue.

Discussion

In a recent study we described the ultrastructural spectrum of SS and suggested, based on our results, that these tumours represent true carcinomas of soft tissues with biphasic and/or monophasic pattern depending on the degree of differentiation[16].

The results of the present study also support the epithelial-type differentiation of SS. The absence of staining for smooth-muscle actin and desmin observed in our cases also corroborates (indirectly) this assumption. Our finding of S100 protein immunoreactivity, both in solid/glandular component of BSS and spindle cell component of MSS and BSS, like it had been previously described[9,11,23], shows once more that the immunoreactivity for this protein should not be used as a definitive marker in the differential diagnosis of soft tissue tumours.

The potential value of keratin subtyping was recently stressed in two studies of soft tissue sarcomas[17,27]. Keratins 8 and 18 cannot be used as markers of epithelial differentiation in soft-tissue sarcomas, because they occur in all sorts of soft tissue tumours, whereas keratins 19 and 7 "were found to have a more limited distribution and to be restricted virtually to SS" in a recent study of spindle cell sarcomas[17].

The keratin profile we have observed in spindle cell component (positive for keratins 5/6, 7, 8, 18 and 19) outside solid/glandular areas of BSS and in MSS, support the epithelial-type differentiation of both types of SS. Furthermore, the transitional cells in the interface of solid/glandular areas to the spindle cell components also showed keratin positivity; in fact a transitional immunoreactive spectrum was observed in focal areas at this interface of nine BSS. No distinct separation was observed between the spindle cells and focal solid/glandular areas as depicted by the absence or weak immunostaining for laminin and collagen IV, thus supporting the existence of a phenotypical cell transition in BSS. Vimentin positivity of peripheral cells in solid/glandular areas of six BSS further corroborates this assumption.

No major differences were observed between immunophenotypical cells profiles of BSS and MSS, apart from the exclusive immunostaining of solid/glandular areas of BSS for keratin 13 and CEA. Keratin 13 reactivity of solid/glandular areas should be regarded as an expression of (secondary) differentiation-specific keratin phenotype of the non-cornified epithelial-type differentiation in SS[2,14]. This finding is an accordance with our own previous ultrastructural study[16]. It also fits other findings at the electron microscopy and immunohistochemical level[1,3–6,10,15,17–21,23,24,26,27,29]. The absence of cornified-type epithelial keratins, which was searched in our study with the antibody for keratin 1, is in keeping with the rarely reported epidermoid-type differentiation of SS[9]. Furthermore, a similar percentage of cases co-expressing epithelial-type and extracellular matrix markers was observed when comparing solid/glandular and spindle cell components of BSS with MSS.

We believe that partial epithelial differentiation of SS can lead to a complex keratin pattern namely of the hyperproliferation-status-type (keratins 5 and 6)[2,14]. We could confirm this complex keratin pattern both in MSS and BSS but not the hyperproliferation-status, since keratin 6 could not be separately evaluated from keratin 5 (the antibody used recognizes both keratins).

In our study, spindle cells of MSS and BSS predominantly expressed simple epithelial-type keratins, whereas both the poorly-differentiated (solid) glandular and the well-differentiated glandular areas (BSS) expressed, in addition, complex epithelial-type keratins.

Moreover, our previous ultrastructural findings[16] of discrete glandular structures within the nonglandular areas (in the semithin sections) of both tumour subtypes further supports the epithelial differentiation of spindle cell component of SS.

With regard to the extracellular matrix markers there is not yet enough information available. In the few studies on record that we are aware of the antibodies for collagen IV and laminin depict an immunoreactive pattern (continuous outline around solid/glandular component) of BSS[22,23,27] similar to our observations. Focal staining for collagen IV and/or laminin was detected between groups of cells of spindle cell component of BSS and in MSS, as

◀ Fig. 10. BSS: Keratin 8 staining of glandular and spindle cells. ABC, ×250. – Fig. 11. BSS: Vimentin staining of spindle cells. Note the absence of immunoreactivity of cells lining papillar formations. ABC, ×250. – Fig. 12. BSS: Keratin 19 staining at the transition of solid/glandular to spindle cell components. ABC, ×250. – Fig. 13. MSS: EMA staining of spindle/epithelioid cells. APAAP, ×250. – Fig. 14. MSS: Keratin 19 staining of spindle cells. ABC, ×250. – Fig. 15. MSS: Keratin 5/6 staining of spindle cells. ABC, ×250. – Fig. 16. MSS: Collagen IV staining around spindle and epithelioid cells. ABC, ×250.

also described by others[23,27]. Poorly defined borders between solid/glandular and spindle cell component of BSS, similar to our own findings, were described in one series[27].

Altogether these immunohistochemical studies show a lower degree of epithelial-type differentiation of MSS than BSS. They also point to the epithelial-type differentiation of the spindle cell component surrounding solid/glandular areas of BSS, as well as of the spindle cells of MSS. Finally, they show that transitional areas of BSS (solid/glandular to spindle cell component) express a complex immunophenotypical pattern of epithelial and mesenchymal-type markers[4,5,23,24,27].

A systematic comparison between immunoreactive profiles of primary and metastatic SS was previously performed only in one study[23] and showed a decrease of EMA and keratin immunoreactivity in metastatic MSS. The few metastatic BSS evaluated showed no major differences in comparison to the corresponding primary tumours[23] at variance with our finding of a downgrading of immunoreactivity in metastases. So far, there are no reported studies of systematic comparison between immunoreactive patterns of primary and recurrent SS that can serve as a basis for comparison with our own results.

Decreased keratin and extracellular matrix immunoreactivity was detected in our series when comparing primary to recurrent and recurrent to metastatic MSS and BSS, suggesting the down-regulation of both keratin and extracellular matrix expression in advanced SS.

The results obtained in metastatic and recurrent SS further support the concept that we are dealing with a single entity that is able to present all the intermediate steps (without discontinuity) between BSS with glandular areas and MSS exclusively composed of spindle (epithelial) cells.

Biphasic tumours are seen quite frequently in so-called carcinosarcomas, spindle cell carcinomas and metaplastic carcinomas[30]. Biphasic and monophasic variants of spindle-cell carcinomas are peculiar neoplasms that occur mainly in the upper aerodigestive tract[8,28]. Most of them are associated to surface epithelial changes varying from dysplasia to carcinoma "in situ". By light microscopy they exhibit epidermoid or glandular (*carcinomatous*) and/or spindle-cell (*sarcomatoid*) components. It is the immunohistochemical and/or ultrastructural evidence of epithelial differentiation in the sarcomatoid component that supports the name of *spindle-cell carcinomas* or *sarcomatoid carcinomas* for these tumours[8,28].

Alternatively, when the sarcomatoid component of biphasic tumours show specific mesenchymal differentiation (e.g. neoplastic bone, cartilage, or striated muscle), they are classified as *carcinosarcomas*[8,13,28].

Other authors use the term carcinosarcoma in a broader context, i.e., without requiring the demonstration of signs of specific mesenchymal differentiation. For these authors the detection of vimentin and the absence of epithelial markers in spindle cell areas allow their designation as bona fide sarcomatous areas of carcinosarcomas (for review see Wick and Swanson[30]).

We think we have found unequivocal evidence to claim that most if not all of the so-called SS display signs of epithelial differentiation as it had been previously described namely in BSS by Miettinen and Virtanen[18]. We have therefore no doubts that these tumours represent, from a phenotypic standpoint carcinomas.

This recognition raises by itself some major problems both at the histogenetic level – one has to admit epithelial differentiation in mesenchymal soft tissue and to face ones inability to explain why such tumours are so often located near large joints – and at the clinico-pathologic level, namely regarding the potential confusion that the term *"carcinoma of soft tissue"* will create to many oncologists and surgeons.

These problems outweigh in our opinion the controversy that may arise between defenders of the designations carcinoma and carcinosarcoma of soft tissues. We favour the former because, like Wick and Swanson[30], we think that "the bulk of available information on the nature of carcinosarcoma makes the continued use of this term indefensible". Like them, we consider that "most if not all carcinomatous and sarcomatous phenotypes, are actually examples of sarcomatoid carcinomas with varying degrees of divergent differentiation".

It remains to be found out where to draw the boundaries between SS and two distinct clinicopathologic entities, the so-called epithelioid sarcomas and malignant nerve sheath tumour with glandular differentiation, in which signs of epithelial differentiation are also observed.

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Paper III

Proliferative Activity of Synovial Sarcoma: An Immunohistochemical Evaluation of Ki-67 Labeling Indices of 52 Primary and Recurrent Tumors.

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The relationship between cell differentiation and proliferation in biphasic synovial sarcoma (BSS) and the connection between the two components of these tumors and monophasic synovial sarcomas (MSS) remains unknown. In this study we specifically compared the Ki-67 labeling indices (Ki-67-LI) in the solid/glandular component and spindle cell component of primary BSS (n=8) as well as the values thus obtained in primary BSS outside clear-cut biphasic areas(n=7) and in primary MSS (n=23).We have also compared the Ki-67 labeling indices of primary tumors (7 BSS; 7 MSS) and their respective first recurrences.We observed that the cells of solid/glandular component of BSS proliferate significantly faster (p=0.007) than those of spindle cell areas.No significantly different Ki-67-LI were observed in the comparison of the solid/glandular areas of BSS with the spindle/epithelioid component of BSS without clear-cut biphasia and MSS. No significant differences of Ki-67-LI were observed in the comparison of primary tumors of both subtypes with their respective first recurrence. The implications of the low proliferative activity of the spindle cell component of BSS exhibiting clear-cut biphasia, which was also confirmed in a concurrent study using anti-PCNA/Cyclin PC10, remains to be clarified.

Key words: Synovial sarcoma; immunohistochemistry; proliferative activity; differentiation; PCNA; Ki-67.

Despite the huge amount of information collected in recent years on the putative histogenesis and clinicopathologic features of synovial sarcomas (SS) (for a review see Fletcher and McKee[1]) many important questions regarding this tumor remain unsettled, such as the relationship between cell differentiation and cell proliferation in the so-called biphasic synovial sarcomas (BSS) and the connection between the two components of these tumors and the so-called monophasic synovial sarcomas (MSS).

Until recently, most of the methods used to evaluate cell proliferation were hampered by technical restraints (e.g. the need for frozen sections) or did not allow evaluation of subpopulations of cells within a single tumor (e.g. flow cytometry)[2-5].This situation has now improved due to the availability of reliable antibodies for cell cycle related antigen epitopes retained in paraffin embedded material.

In the present study we have addressed the possible link between cell differentiation and cell proliferation in SS using an antibody for Ki-67. We have specifically compared the labeling indices in the solid/glandular component and spindle cell component of BSS as well as the values thus obtained with those obtained in BSS outside clear-cut biphasic areas and in MSS. We have also compared the labeling indices of primary tumors and their respective first recurrences.

MATERIAL AND METHODS

Surgical specimens of 38 primary SS diagnosed at the Norwegian Radium Hospital (28 patients) and Porto Medical School (10 patients) were studied. In every case the material was obtained prior to chemotherapy

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and radiotherapy. In 14 cases, material from the first (local or distant) recurrence was available for study. All but one of these recurrent cases had been exposed to radiotherapy and/or chemotherapy protocols following initial surgery.

The 38 patients ranged in age from 7 to 81 years (mean, 35.4). There were 21 men (mean age, 32.5) and 17 women (mean age, 39.3).

The tumors were located in the lower extremities in 26 patients, the upper extremities in 4, the trunk in 6 and the parapharyngeal region in 2 patients.

In every case, several blocks (4-15 formalin-fixed paraffin-embedded fragments per tumor) were available for routine histological examination. The cases were classified according to the criteria of Enzinger and Weiss[6].

All 23 primary MSS were of the fibrous type. In 7 primary BSS only spindle or epithelioid cell areas (outside the areas of clear-cut biphasia in which the diagnosis of BSS was based) were available for immunohistochemistry, whereas in the remaining 8 primary BSS there were clear-cut biphasic areas in the histological sections selected for the immunohistochemical examination. For simplicity the former group of BSS were designated as BSS without clear-cut biphasia.

Seven of the 14 cases in which material from the first recurrence was available had still a biphasic pattern and the other 7 were MSS. Nine of the recurrences were local (4 BSS and 5 MSS) and the remaining 5 were in the lungs (3 BSS and 2 MSS).

Immunohistochemistry

Sections were stained using the avidin-biotin peroxidase complex (ABC) method[7]. Deparaffinized sections were treated with 0.3% hydrogenperoxide (H_2O_2) in methanol for 30 minutes to block endogenous peroxidase. To unmask the epitopes of Ki-67 the sections were microwaved in <1% lead solution[8] and 10 mM citrate buffer pH 6.0 [9]. The sections were then incubated for 20 minutes with normal serum from the species in which the secondary antibody was made. This was done to minimize nonspecific staining. Excess normal serum was blotted from slides before incubation with polyclonal Ki-67 antibody diluted 1:100 (A 047, Dako, Denmark) for 18-22 hours at 4°C. The sections were then incubated with 1:200 dilution of biotin-labeled secondary antibody for 30 minutes and ABC (10 µg/ml of avidin and 2.4 µg/ml of biotin-labeled peroxidase) for 60 minutes (Vector, Burlingame, CA). The tissue sections were stained for 5 minutes with 0.05% 3'3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.6, containing 0.01% H_2O_2 and then counterstained with haematoxylin, dehydrated, and mounted in Diatex. All the dilutions of normal sera, antiserum, biotin-labeled secondary antibodies and ABC were made with PBS containing 5% bovine serum albumin. All series included positive controls. Negative controls included substitution of polyclonal antibody with normal rabbit IgG. All control examinations were satisfactory .

Immunoreaction was considered positive whenever nuclear staining was observed, independently of the intensity of staining. Areas with more numerous stained nuclei were selected and used for counting at random. The labeling indices were determined in every case (both in primary tumors and in recurrent tumors) by counting the number of positive tumor cell nuclei among a total of 1,000 cells. In addition, in every BSS with clear-cut biphasia we performed also the evaluation of the immunoreactivity in 500 cells of the solid/glandular areas and in 500 cells of the surrounding spindle cell areas. The labeling indices were expressed in percentage of positive cells.

Statistical analysis

The results are expressed in percentage or in mean ± standard deviation. The statistical analysis was performed using the paired and the unpaired Student's two sided t-test. The results were considered significantly different if $p<0.05$.

RESULTS

Primary BSS (n=15)

The mean Ki-67 labeling index (Ki-67-LI) was significantly higher ($p=0.007$) in solid/glandular areas (16.9) than in the respective spindle cell areas (3.9) of the same 8 tumors (Fig. 1 and 2). The mean Ki-67-LI of solid/glandular areas of the aforementioned 8 BSS with clear-cut biphasia (16.9) was not significantly different from that of spindle/epithelioid areas of 7 BSS without clear-cut biphasic pattern (22.4) (Table 1).

Primary MSS (n=23)

The mean Ki-67-LI in 23 primary MSS (Fig.3) was 16.5 (Table 1).

BSS *versus* MSS primary tumors

The mean Ki-67-LI of 23 primary MSS (16.5) was not significantly different from that observed in solid/glandular areas of BSS with clear-cut biphasia (16.9) and in spindle/epithelioid areas of BSS without clear-cut biphasic areas (22.4) (Table 1).

Primary *versus* first recurrence of BSS and MSS

The mean Ki-67-LI of the first recurrences of 7 BSS (24.2) was not significantly different from that observed in the respective 7 primary tumors (21.9)(Table

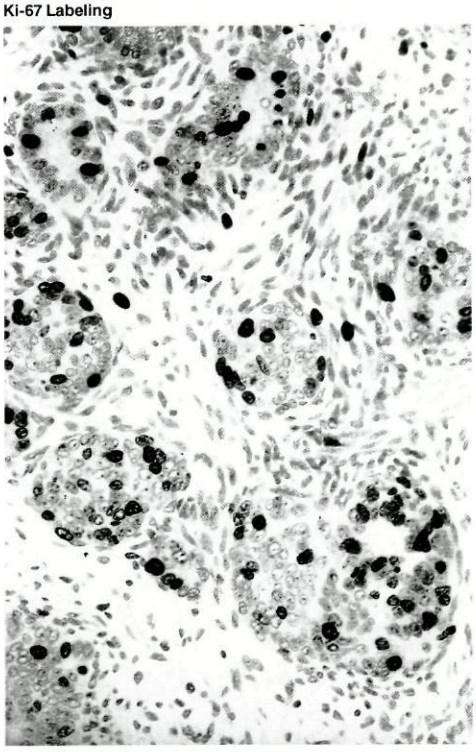


Fig 1 *Biphasic synovial sarcoma displaying Ki-67 immunoreactivity of solid/glandular and surrounding spindle components. ABC, 280 x.*

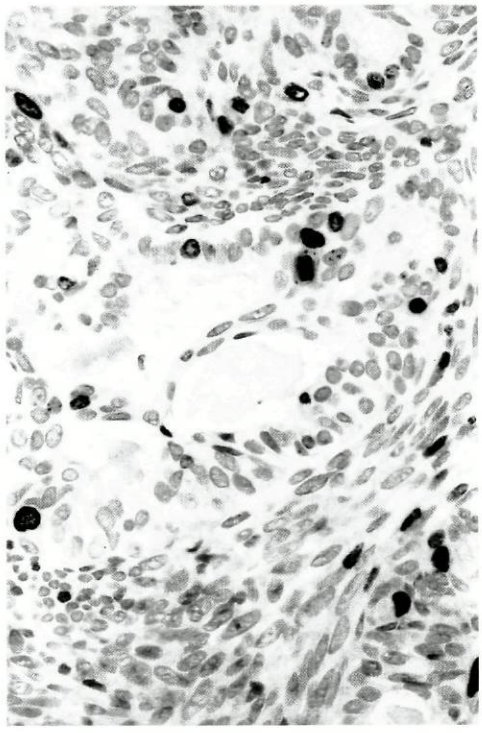


Fig 2 *Biphasic synovial sarcoma displaying Ki-67 immunoreactivity of glandular and surrounding spindle components. ABC, 280 x.*

2). The mean Ki-67-LI of the first recurrences of 7 MSS (26.1) was not significantly different from that observed in the respective 7 primary tumors (19.7) (Table 2).

Similar results were obtained if local recurrences and lung metastases were considered separately (data not shown).

DISCUSSION

The main objective of the present study was to clarify the relationship between cell proliferation and differentiation of SS. The availability of antibodies that recognize Ki-67 working in paraffin-embedded material, led us to compare the labeling indices (LI) of the two components (solid/glandular and spindle cell) of BSS as well as the values thus obtained with those observed in areas of spindle/epithelioid cells of BSS without clear-cut biphasia and in MSS. We have also compared the mean Ki-67-LI of primary tumors and their respective first recurrences.

There are still controversial results regarding the use of antibodies for cell cycle related antigens (e.g., PCNA and Ki-67) in the identification of proliferative cell populations[2-5,10-12]. Several explanations have been proposed for the described discrepancies in sensitivity between different commercial antibodies for PCNA and Ki-67 [13-16]. It is also known that the results using anti-PCNA antibodies depend largely on the fixation of the material [10,14,17-19]. Although some authors claim that PCNA antibodies yield unreliable proliferation indices in formalin-fixed-paraffin-embedded tissues[14,18], Oda *et al* [20]reported recently that PCNA score, using routinely processed material, was a prognostic factor in SS. So far, there are no reports on Ki-67 studies of SS in routinely processed material.

As Swanson and Brooks [21] and Ueda *et al* [22] had already observed searching for Ki-67 in frozen sections, and Oda *et al*[20] for PCNA in paraffin sections, we have found a striking variation in Ki-67 score from tumor to tumor and even within each subset of SS. We have also observed a marked variation in Ki-67 im-

TABLE 1- Ki-67 Labeling Indices (LI) of Primary BSS and MSS

	Ki-67-LI mean ± SD (range)
BSS with clear-cut biphasia (n=8)	
Solid/glandular areas	16.9±0.8 (6.8-39.8)
Spindle cell areas	3.9±2.5 (0.4-8.0)
BSS without clear-cut biphasia* (n=7)	
Spindle/epitheloid cell areas	22.4±19.9 (2.3-50.6)
MSS (n=23)	16.5±17.3 (0.1-53.8)

*In these cases classical biphasia observed in other areas were not available for immunohistochemical study.

munoreactivity from area to area within each tumor, as Oda *et al* [20] and Garcia *et al* [23] have shown for PCNA.

We report, to the best of our knowledge for the first time, that the two components (solid/glandular and spindle cell) of BSS display significantly different proliferative activities. We observed, moreover, that this difference relied upon a higher proliferative activity in solid/glandular areas than that in spindle cell areas. These results differ from those obtained by Oda *et al* [20] who stated that "PCNA expression did not appear to distinguish epithelial component from spindle cell areas in the biphasic type" of SS. This discrepancy may depend not only on the different antigens identified, but also methodological differences. In fact, we have separately evaluated after histological assessment the labeling indices of the two components of BSS in areas displaying clear-cut biphasia, regardless of the intensity and extent of nuclear staining. In contrast to our study, Oda *et al* [20] used an image analysis computer system for the assessment of the percentage of the positive nuclear areas. Since Oda *et al* [20] did not provide the results of the differential score evaluation of the two components of BSS a direct comparison cannot be made. Furthermore, in our study the significant difference in the proliferative activity of the two components of BSS was confirmed using anti-PCNA/Cyclin PC10 (Lopes JM *et al*, unpublished results).

Our study of Ki-67 immunoreactivity shows mainly the low proliferative activity of the spindle cell areas of BSS, compared with solid/glandular areas of the same tumors. No significantly different Ki-67 scores were observed in the comparison of the solid/glandular areas of BSS with the spindle/epithelioid component of

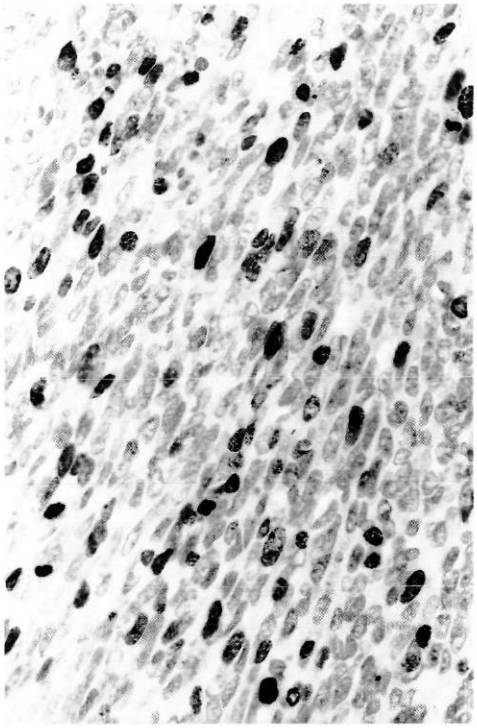


Fig 3 *Monophasic synovial sarcoma displaying Ki-67 immunoreactivity of spindle/epithelioid cells. ABC, 560 x.*

BSS without clear-cut biphasia and MSS. Our study shows also the absence of significant differences of Ki-67-LI when comparing primary SS of both subtypes with their respective local and distant recurrences.

The histogenetic relationship between the solid/glandular and the spindle cell components of BSS remains controversial [24,25]. The same is true for the relationship between MSS and the two components of BSS [25,26].

TABLE 2- Ki-67 labeling indices of primary BSS and MSS and their respective first local or distant recurrences

	Primary tumors Ki-67-LI mean±SD (range)	First recurrences Ki-67-LI mean±SD (range)
BSS (n=7)	21.9 ±13.6 (8.7-46.8)	24.2±15.6 (3.6-40.0)
MSS (n=7)	19.7±15.2 (0.1-43.5)	26.1±17.1 (6.3-50.3)

In two previous studies we have reported ultrastructural and immunohistochemical evidence supporting the concept that synovial sarcoma is a single entity displaying a variable phenotype, depending on the degree of epithelial differentiation[27,28]. Extremes of this spectrum were found when comparing solid/glandular ("strong" epithelial differentiation) with alternating spindle ("weak" epithelial differentiation) cell components of BSS. MSS disclosed variable degrees of epithelial phenotype. Moreover, we have observed, as also later described by Guarino *et al* [29], transitional areas of BSS (solid/glandular to spindle cell components) expressing a complex epithelial-mesenchymal phenotype.

The similar proliferative activity observed in solid/glandular areas of BSS, spindle/epithelioid cell areas of BSS without clear-cut biphasia and MSS fits with the close relationship between both subtypes of SS.

The implications of the low proliferative activity of the spindle cell component of BSS exhibiting clear-cut biphasia remains to be clarified. Assuming that the spindle cell component of BSS shares a common origin with the solid/glandular component, it is possible that such spindle cells may represent a sort of terminal differentiation stage of the neoplastic cell population of BSS. Our results fit also with the possibility that the cells of solid/glandular areas of clear-cut biphasic BSS proliferate faster than those of the spindle cell areas regardless of the degree of differentiation. Finally, one may consider the possibility that most of spindle cells of BSS are stromal cells rather than true neoplastic cells. We do not concur with the latter possibility not only because of the aforementioned ultrastructural and immunohistochemical evidence [27,28] but also because we have observed the same trend towards low proliferative activity of the spindle cell component of BSS in clear-cut biphasic areas of metastatic tumors.

We observed similar proliferative activities in primary and recurrent SS of both subtypes. This result should be interpreted with care because we cannot estimate the degree of proliferative modulation played by therapy in almost every recurrent SS, in contrast to the absence of any chemotherapy or radiotherapy prior to surgery in primary tumors.

The evaluation of the putative usefulness of Ki-67-LI for prognostic purposes is beyond the scope of the present study since the criteria for enrollment of the cases was the availability of material from primary and, whenever possible, recurrent tumors, regardless of the staging of the tumor and clinical evidence of recurrences.

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Paper IV

Synovial sarcoma. DNA ploidy and proliferation (PCNA and Ki-67) markers in the evaluation of prognosis.

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Background: Controversy still exists regarding the validity of parameters commonly used in the evaluation of prognosis of patients with synovial sarcoma (SS).
Patients and methods: Forty-nine cases of previously untreated primary SS (23 females and 26 males, ranging in age from 7 to 81, with 31 tumors located in the lower extremity, 8 at the upper extremity, and 10 at the trunchus), without regional lymph-node or distant metastases were studied. We investigated the relationship between (flow and image) DNA cytometry, proliferation activity, clinicopathologic parameters, and relapse-free and overall survival of the patients. The prognostic value of gender, age, duration of symptoms, location, compartmentalization, size, adequacy of surgical margins, residual tumor, adjuvant therapy, histologic subtype, extent of necrosis, glandular differentiation, calcification, and extent of hemangiopericytic areas, mitotic rate, amount of mast cells, blood vessel invasion, histologic (UICC and NCI) grades, ploidy, percentage of cells in S and S+G2 phases, PCNA and Ki-67 labeling indices (LI), and TNM (UICC) stage of the tumors, were evaluated by univariate and multivariate (Cox hazard model) analyses.
Results: Short duration of symptoms (<12 months), biphasic SS, scarcity of mast cells (<10/10 HPF), high mitotic rate (≥ 10/10 HPF), high histologic grade (grade 3), high PCNA-LI (≥20%), high Ki-67-LI (≥10%), aneuploidy, and advanced TNM stage (stage III) were features associated with significantly shorter relapse-free and overall 5-year survival rates in the univariate analyses. Scarcity of mast cells, high mitotic rate, or high PCNA-LI were significant predictors of poor survival, in addition to TNM stage, in the multivariate analyses. The amount of mast cells was inversely correlated with mitotic rate and PCNA-LI.
Conclusion: Scarcity of mast cells, high mitotic rate, or high PCNA-LI, are factors associated with poor prognosis, in addition to advanced TNM stage, in patients with localized SS.

Key words: synovial sarcoma, mast cells, mitotic rate, PCNA, staging, prognosis, Ki-67, DNA flow cytometry, DNA image cytometry, soft tissue tumor.

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INTRODUCTION

Synovial sarcoma (SS) is a well-defined entity that has been extensively described in the literature. Reported 5-year survival rates of SS range from 30.0% to 82.6% [1-20]. Many clinical and morphological features have been applied in the evaluation of the prognosis such as: gender, age at diagnosis, clinical symptoms, location of tumor, staging of the neoplastic disease, treatment modalities, tumor size, histologic subtype, extent of glandular differentiation, mitotic rate, histologic grading, presence of rhabdoid cells, amount of mast cells , extent of calcification, ploidy score, and PCNA score . However, there is still contradictory and insufficient evidence concerning the validity of most of these parameters [1-6,10-47].

In this study, we have performed a retrospective evaluation regarding the influence on the survival of 49 patients with primary SS of various clinicopathologic features with particular emphasis on the analysis of cytometric and proliferative parameters.

PATIENTS AND METHODS

Forty-nine cases of primary synovial sarcoma (SS) treated by surgery were selected from the files of the Department of Pathology, The Norwegian Radium Hospital (36 cases) and Porto Medical School (13 cases). None of the patients presented with regional lymph-node metastases or distant metastases. Preoperative radiotherapy or chemotherapy was not given to any patient.

Clinical features

Clinical data were collected from the patients' medical records. The surgical margins were classified according to the criteria of Enneking et al [48]. Margins obtained at the second operation were recorded, whenever the primary tumors were treated by two sequential surgical procedures separated by less than one month, in an attempt to achieve adequate surgical margins.

Follow up was done by clinical examination every third month for the first year, with subsequently longer intervals for the following years. Chest X-rays and blood profile, including liver function tests, were performed at each visit. CT-scans of the lung was performed whenever chest X-rays indicated metastases or were inconclusive.

Pathologic features

All available material for histologic study was examined without knowledge of the clinical course of the patients, using formalin-fixed paraffin-embedded 5µm sections (4-15 per tumor) stained with hematoxylin and eosin. Representative sections from the surgical margins of each tumor were also histologically evaluated.

The cases were classified, according to the criteria of Enzinger and Weiss [38], in biphasic (BSS, n=19) and monophasic (MSS, n=30) subtypes of SS. Whenever necessary, immunohistochemical and ultrastructural examinations were performed to confirm the diagnosis.

In 42 cases, formalin-fixed paraffin-embedded fragments were available for the following analyses performed in consecutive sections of one representative block from each tumor: flow cytometry (FCM) in 42 cases, image cytometry (ICM) in 41 cases, PCNA immunohistochemistry and acidified toluidine blue (pH, 3.0) in 40 cases, and Ki-67 in 37 cases, depending on the representation of tumor tissue available in each section of the paraffin blocks that had been selected.

Mitotic rate was determined by counting the most mitotic areas in ten successive high power fields (a high power field-HPF-, x 400 measured 0.15 mm2) selected at random. The amount of mast cells was evaluated in ten successive HPF cells chosen at random after selecting the area displaying the highest number of mast cells. The percentage of extent of tumor necrosis (based on gross and microscopical findings), as well as that of solid/glandular areas, were evaluated in all the sections and classified as follows: 0-none; <15; 15-50;>50. Extent of calcification and extent of hemangiopericytic areas were classified in four degrees: 0 (none);+ (<15%);++(15-30%);+++(>30%). Histologic grade of malignancy was independently evaluated according to UICC [49] and NCI [50] grading systems. Briefly, UICC grade (1-3) was based on the estimation of cellularity, cellular pleomorphism (e.g., nuclear atypia), mitotic activity, and necrosis. NCI grading was based on the gross and microscopical evaluation of necrosis: <15% = grade 2, >15%= grade 3. Blood vessel invasion was classified as absent or present. Residual tumor at the surgical margins (R0- no residual tumor; R1- microscopical residual tumor; R2- macroscopical residual tumor) and TNM staging (I to III) of the tumors were classified according to the UICC system[49].

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were stained using the avidin-biotin peroxidase complex (ABC) method [51]. Deparaffinized sections were treated with 0.3% hydrogenperoxide (H_2O_2) in methanol for 30 minutes to block endogenous peroxidase. To unmask the epitopes of PCNA and Ki-67 the sections were microwaved in <1% lead solution [52] and 10 mM citrate buffer pH 6.0 [53]. The sections were then incubated for 20 minutes with normal serum from the species in which the secondary antibody was made. This was done to minimize nonspecific staining. Excess normal serum was blotted from slides before incubation with monoclonal PCNA antibody diluted 1:25 (NCL-PCNA, Novocastra Laboratory Ltd. UK) and polyclonal Ki-67 antibody diluted 1:100 (A 047, Dako, Denmark) for 18-22 hours at 4°C. The sections were then incubated with 1:200 dilution of biotin-labeled secondary antibody for 30 minutes and ABC (10 µg/ml of avidin and 2.4 µg/ml of biotin labeled peroxidase) for 60 minutes (Vector, Burlingame, CA). The tissue sections were stained for 5 minutes with 0.05% 3'3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.6, containing 0.01% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All dilutions of normal sera, antiserum, biotin-labeled secondary antibodies and ABC were made with PBS containing 5% bovine serum albumin. All series included positive controls. Negative controls included substitution of polyclonal antibody with normal rabbit IgG, whereas negative control for the monoclonal antibody was performed using mouse myeloma protein of the same subclass and concentration. All control examinations were satisfactory.

Immunoreaction for PCNA and Ki-67 was considered positive whenever nuclear staining was observed, independently of the intensity of the staining. Areas with more numerously stained nuclei were selected and used for counting at random. The labeling indices were determined in every case by counting the number of positive tumor cell nuclei among a total of 1,000 cells. The labeling indices were expressed in percentage of positive cells.

DNA Cytometry

To minimize the amount of non-tumor tissue, all areas with tumor were outlined on blocks corres-

ponding to the best sections, and most of the non-tumor tissue was removed. From each block one 100-µm section for flow cytometry (FCM) and two 50-µm sections for image cytometry (ICM) were cut. In the end, a 5-µm section was cut for evaluation of representativity of the section and for the estimation of the amount of tumor tissue present. After deparaffinization with xylol, the tissues were rehydrated in graded ethanols, rinsed in phosphate buffered saline (PBS) and incubated for 30 minutes with protease (Sigma No. 24.) at 37°C. The protease activity was stopped by adding 4 ml cold PBS, and thereafter the specimens were rinsed twice in 4 ml PBS before centrifugation at 1250 X [54]. The specimens were prepared in series to control interspecimen variation of the disintegration index.

Flow cytometry

Cell suspensions were prepared by the method described by Hedley [54], with minor modifications[55,56], stained with ethidium bromide. DNA FCM was performed by the use of a laboratory built flow cytometer [57]. Briefly the instrument was based on a Nikon Diaphot invertoscope equipped with excitation light ranges of 510-560 nm and a beam splitter at 580 nm. The output signals were sorted by a multichannel analyzer (Nuclear Data ND 66) into histograms with 256 channels.

Image cytometry

The isolated cells were post-fixed in buffered 4% formalin for at least 12 h in room temperature and centrifuged (Hettich, Tuttingen, FRG) on polylysin-coated slides at 1250 X. Feulgen Schiff staining was applied (5 N HCL, 60 minutes, 22°C) with Pararosanilin-Schiff. Hydrolysis curves were made to ensure reproducible staining. Images of the nuclei obtained with a Zeiss photomicroscope III equipped with a plan 40/0.95 objective lens and a 546-nm green filter were transferred to a MINI-IPS image processing unit (Kontron, Munich, FRG) using a Grundig FA 76 camera with a pasecon tube with a fixed gain level. The image processing unit was equipped with 4 megabytes of image memory, array processor and digitizer unit controlled by a Z80 host processor. Images were stored as 512x512 pixels with 256 gray levels. The software was written in FORTRAN using Kontron's sub-routine library for access to the array processor. The Integrated Optical Density of each nucleus was calculated on the basis of measurements of optical density and area. Background optical density was measured and corrected for each image, ensuring stable and reproducible measurements.

At least 300 nuclei per specimen were measured. Normal human lymphocytes were used as external staining controls of the normal diploid (2c) DNA content due to the scarcity of lymphoid cells observed in SS. All nuclei were randomly selected from multiple areas of the slide. Only nuclei which were structurally intact without overlapping, and to which no nuclear fragments were attached were measured. The reproducibility of the ICM method was verified by reanalyzing one third of the cases.

Ploidy definition

FCM. The histograms were analyzed with reference to the DNA indices of distinguishable stemlines[58]. Histograms with only one modal peak were regarded as diploid (Coefficient of Variation-CV: 2.8-9.4%, mean 5.1%). A tumor was considered to be non-diploid if several peaks were distinguishable. The first peak of the histogram was always regarded as being diploid. The further ranges for DNA indices (DIs) were defined as follows: Tetraploid range - $1.80<DI<2.20$; Aneuploid range - $1.10<DI<1.80$ and $DI \geq 2.20$; Multiploid - more than one non-diploid stemline. A tetraploid stemline was recorded if the number of cells within a tetraploid peak exceeded the number of cells between the diploid and the tetraploid range. The percentage of cells in S and G2 phases was analyzed from DNA histograms by use of the MULTICYCLE® computer program (Phoenix Flow System Inc). Since cell nuclei, and not the whole cells, were measured, mitotic figures were washed out during preparation and were not included in the measurements.

ICM. A tumor was considered to be in the diploid range if only one peak was present (CV: 4.5-14.9.%, mean 10.7%). If more than one peak could be identified, the first peak of the histogram was considered to be in the diploid range and served for calculation of the ploidy and the coefficient of variation. When more than 10% of the nuclei were found in the tetraploid region ($2 \times 2c \pm 2 \times CV$) the tumor was considered to be in the tetraploid range. Polyploid tumors had a significant peak in the multiple of 2c. A tumor was classified as aneuploid either if the DNA content of 4 or more cells exceeded the 5c value according to Böcking [59] or if a prominent peak was identified between 2c and 4c.

Statistics

The Mann-Whitney rank-sum test was used to compare the distributions of continuous variables. This non-parametric method was chosen as the values for the variables did not follow a normal distribution.

For two-way frequency tables, Fisher's exact test (2-tailed) was used. The inter-relationship between continuous variables was estimated with the Spearman correlation coefficient [60].

The probabilities of surviving were calculated with the Kaplan-Meier product-limit method. The differences between the survival curves were tested by the log-rank test [61].

The Cox proportional hazards model was used to analyze the simultaneous importance of several prognostic factors [62]. The proportional assumption in the Cox model was examined with plots [60]. The Wald test was used for significance test of the regression coefficients [60].

The following strategy was followed due to many candidate variables available:

For continuous variables, cut-offs, reported by other investigators, were tested in univariate survival analyses. The other continuous variables were grouped into quartiles and univariate survival analyses were performed. Finally, a two-grouping was selected from these analyses. Only significant variables/cutoffs were included in the final regression analysis.

The statistical software of BMDP (PC-90) was used[60].

RESULTS

Clinical features

The series comprised 23 females and 26 males. The median age was 32 years (range, 7-81 years). The sites of the primary tumors were as follows: lower extremity (n=31), upper extremity (n=8), and trunchus (n=10). The tight (n=12) was the most frequent single location. Nine of the tumors were intracompartmental and 40 were extracompartmental tumors. None of the tumors was restricted to the subcutaneous compartment.

The most common initial symptom was the presence of a palpable painless mass (25 patients), followed by pain (15 patients). The median duration of symptoms was 10 months (range, 1-96 months). Initial presentation and duration of symptoms was unknown in 9 cases.

The median tumor size was 6 cm (range, 1-25 cm). Twenty-four tumors were 5 cm or smaller than 5 cm. All patients were initially treated by surgery. Adequate margins (wide, n=14; and amputation, n=8) were achieved in 22 cases and inadequate margins (intralesional, n=1; and marginal, n=24) in 25 cases. In two cases the surgical margins were unknown.

Twenty-three patients received some form of adjuvant therapy: post-operative radiotherapy, n=17; adjuvant chemotherapy, n=5; and radio- and chemotherapy, n=1.

Local recurrences were diagnosed in 15 patients and distant recurrences (lung metastases) in 29 patients, including 11 patients with prior local recurrence. Synchronous (local and lung) recurrences were diagnosed in one patient. The relapse-free survival rate (to local recurrence or metastasis) was 34% at 5 years (95% Confidence Interval-CI, 20-48%). The local recurrence-free (metastatic relapses treated as censored observations) and the metastasis-free survival rates (local relapses treated as censored observations) were 69% (95% CI, 55-83%) and 60% (95%CI, 46-74%) at 5 years, respectively.

The median follow-up time after initial treatment was 51 months (range, 6-276 months). Twenty patients were alive with no evidence of disease, 5 were alive with disease, 22 were dead due to the tumor, and 2 were lost for follow-up. The overall 5-year survival after initial treatment was 64% (95%CI, 50-78%).

Light microscopic features

Seven of the BSS were of the classical type and 12 showed focal biphasia. More than 50% of solid/glandular areas were observed in 5 BSS. All MSS were of the fibrous variant. None of the cases showed presence of rhabdoid cells. Median mitotic rate was 4/10 HPF (range, 0-67/10 HPF). Fifteen cases showed mitotic rates higher than 10/10 HPF, and in 8 of them it was higher than 15/10 HPF.

Necrosis was present in 26 cases and occupied more than 15% of the tumor volume in 22 cases. Focal (n=10) or moderate (n=3) calcification was present in 13 cases. Focal (n=3), moderate (n=3) or extensive (n=7) hemangiopericytic areas were observed in 13 cases. The median amount of mast cells (n=40) was 11/10 HPF (range, 0-467/10 HPF). Usually, an accumulation of mast cells were observed at the periphery of the tumor, and only in some cases mast cells were scattered throughout the tumor. The amount of mast cells was higher than 10/10 HPF in 22 cases, and higher than 20/10 HPF in 17 of them. Tumor cells in blood vessels were observed in 7 cases. Focal (n=7) or extensive (n=1) areas of tumor tissue were present at the surgical margins in 8 tumors.

The grades of malignancy obtained with the UICC and NCI systems were concordant (grade 2, n=19; grade 3, n=18) in 37 cases. The 12 discordant cases comprised 2 tumors grade 1 by UICC and grade 2 by NCI, 4 grade 2 by UICC and grade 3 by NCI, and 6 grade 3 by UICC and grade 2 by NCI. The mitotic count was low (<10/10 HPF) in the six cases in which

the UICC grade was lower than the NCI grade, and high (≥ 15/10 HPF) in the other 6 discordant cases.

Immunohistochemistry

In all but one case immunoreactivity for PCNA and Ki-67 was relatively homogeneous; BSS showed, with both markers, a higher number of immunoreactive cells in solid/glandular areas than in the surrounding spindle cells (Figs. 1-2). The median PCNA-LI (n=40) was 14.5% (range, 0.1-48.8%). Eleven cases showed PCNA-LI higher than 20%. The median Ki-67-LI (n=37) was 10.7% (range, 0.1-53.8%). Twenty cases showed Ki-67-LI higher than 10%.

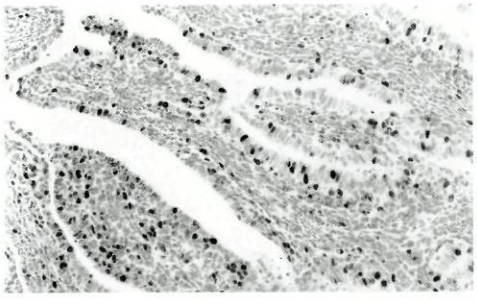


Fig. 1- *Biphasic synovial sarcoma displaying prominent PCNA immunoreactivity of the cells lining clefts and glandular spaces. ABC, 280 x*

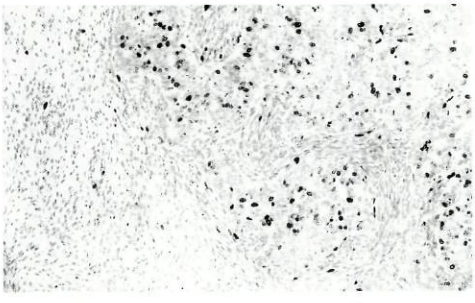


Fig. 2- *Biphasic synovial sarcoma displaying Ki-67 immunoreactivity almost exclusively in cells of solid/glandular areas. ABC, 280x*

DNA cytometry

Non-evaluable histograms (due to bad suspensions) were obtained in two cases, one by ICM and another by FCM. Ploidy descriptors (diploid, n= 30; and aneuploid, n=4) were concordant for the two methods in 34 cases. The 6 discordant cases comprised: 4 tumors that were diploid by ICM and aneuploid by FCM, one that was tetraploid by ICM and diploid by FCM, and one that was aneuploid by ICM and tetraploid by FCM.

The 4 cases that were aneuploid by FCM and diploid by ICM had DNA indices in the near-diploid range (Fig 3). In the case tetraploid by FCM, the ICM histogram depicted a 5c exceeding rate (Fig. 4). The case which was diploid by FCM, showed a tetraploid population higher than 10% by ICM. Whenever aneuploidy was observed by any of the two methods it was used as such for survival evaluation. The calculations of percentages of cells in S and G2 phases of the cell cycle were not possible in five cases due to overlapping of G2 peaks of diploid and aneuploid cells, or due to small G2 peaks. Median percentage of cells in S phase (n=36) was 9.1% (range, 1.4-40.4%) and the median percentage of cells in G2 phase (n=36) was 13.8% (range, 7.8-66.7%).

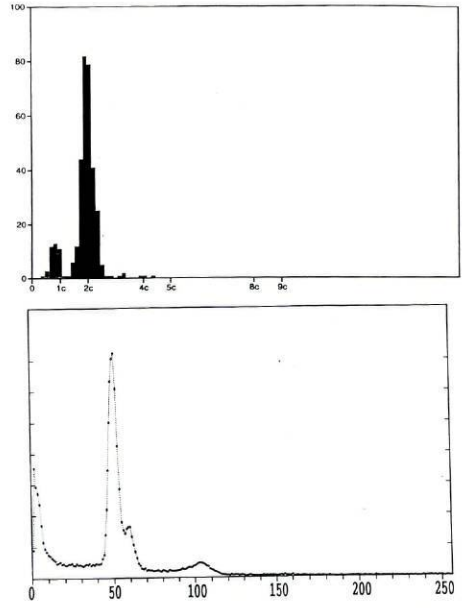


Fig. 3- *Diploid DNA histogram by ICM (above) and aneuploid (near-diploid range) by FCM (below) of a synovial sarcoma.*

Univariate analysis

The results of the univariate analysis of clinicopathologic features in relation to survival are summarized in Table 1.

Gender of the patients, compartmentalization and location, dimension, extent of calcification and of hemangiopericytic areas, percentage of glandular areas, blood vessel invasion, adequacy of surgical margins, and residual tumor were not significant predictors of survival (relapse-free and overall survival) (Table 1).

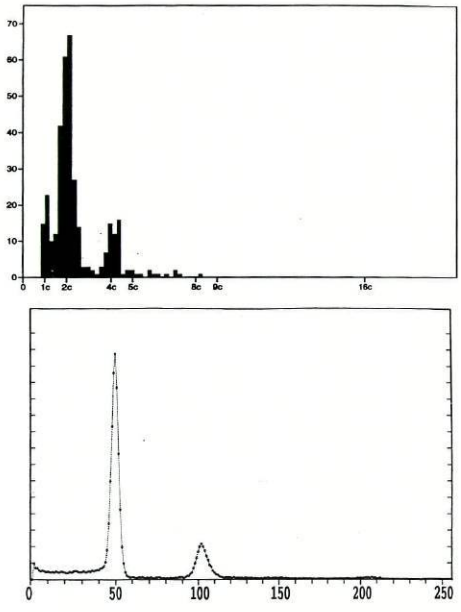


Fig. 4- *Aneuploid DNA histogram by ICM (above) and tetraploid by FCM (below) of a synovial sarcoma.*

Young age of the patients (p=0.04), duration of symptoms (p<0.01), UICC grade (p<0.0001), NCI grade (p=0.06), histologic subtype (p=0.03), mitotic rate (p<0.001), amount of mast cells (p<0.0001), necrosis (p<0.01), PCNA-LI (p=0.04), Ki-67-LI (p<0.0001), ploidy (p=0.04), and TNM stage (p<0.0001) were significant predictors of relapse-free survival (to local recurrence or metastasis) (Table 1).

Duration of symptoms (p<0.05), UICC grade (p<0.0001) , NCI grade (p=0.04), histologic subtype (p=0.04) , mitotic rate (p=0.01), amount of mast cells (p<0.001), necrosis (p<0.02), PCNA-LI (p<0.001), Ki-67-LI (p=0.005), ploidy (p=0.007) and TNM stage (p=0.0001) were significant predictors of overall survival (Table 1).

Short duration of symptoms (<12 months), biphasic type (BSS), scarcity of mast cells (<10/10 HPF), presence of necrosis, high mitotic rate (>10/10 HPF), high histologic grade (grade 3), high PCNA-LI (≥20%), high Ki-67-LI (≥10%), aneuploidy, and advanced TNM stage (stage III) were features associated with significantly shorter relapse-free and significantly shorter overall 5-year survival rates. Young age (younger than 30 years) was a significant predictor of relapse-free survival but not of overall survival. The five-year survival rates of the patients for each of these parameters are given in Table 1.

Multivariate analysis

The results of the multivariate analysis are summarized in Table 2. Amount of mast cells , mitotic rate, or PCNA-LI were significant predictors of survival in addition to TNM stage (Fig 5). Only one of them at each time provided significant additional information to TNM staging. Curiously, the additional prognostic information provided by the three parameters seemed to be nearly the same.

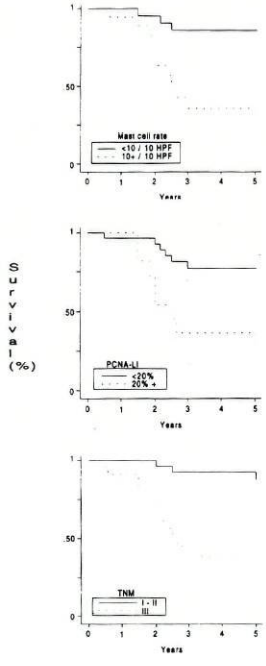


Fig. 5- *Survival curves of patients with synovial sarcoma stratified by amount of mast cells, PCNA labeling index, and TNM staging.*

Correlation between mast cell count, mitotic rate, and PCNA-LI

Correlation between the significant features found in the multivariate analysis are summarized in Table 3. The amount of mast cells was significantly correlated with mitotic rate (p=0.003) and PCNA-LI (p=0.04). Tumors with mast cell count less than 10/10 HPF had frequently higher mitotic rates (≥10/10 HPF) and high PCNA-LI (≥ 20%). In addition, the median mitotic rate and the median PCNA-LI were significantly higher (p=0.003 and p=0.03, respectively) in tumors containing few mast cells compared to tumors containing numerous mast cells. The Spearman rank correlation coefficient for mitotic rate and amount of mast cells was -0.49 and between PCNA-LI and amount of mast cells -0.42.

Table 1 **Univariate survival analysis of clinicopathologic features in patients with localized synovial sarcoma.**

FEATURES	GROUPS	#	NO.	RELAPSE-FREE	OVERALL SURVIVAL	
				Logrank p-value	5-year (%)	Logrank p-value
Gender	Male		26	0.56		0.38
	Female		23			
Age (years)	<30		23	0.04		0.28
	≥30		26			
Duration of symptoms (months)	<12	#	24	<0.01	49	<0.05
	≥12		16		75	
Location	Extremities		39	0.06		0.09
	Truncal		10			
Compartment	Compartmental		9	0.48		0.88
	Extracompmental		4			
Dimension	≤5cm		24	0.11		0.88
	>5cm		25			
Surgical Margin	Inadequate		25	0.21		0.65
	Adequate		22			
Residual tumor	RO		41	0.34		0.26
	R1+R2		8			
Adjuvant Therapy*	No		26	0.77		0.67
	Yes		23			
Subtype	BSS	#	19	0.03	56	0.04
	MSS		30		70	
Glandular differentiation	<15		42	0.67		0.80
	≥15		7			
Necrosis	Absent		23	0.01	81	0.02
	Present	#	21		47	
Mitotic rate (10 HPF)	<10		34	<0.0001	83	<0.0001
	≥10	#	15		22	
Mast cell rate (10 HPF)	<10	#	18	<0.0001	24	<0.001
	≥10		22		86	
Calcification	Absent		36	0.47		0.24
	Present		13			
Hemangiopericytic areas	Absent		36	0.17		0.58
	Present		13			
Vessel invasion	Absent		42	0.78		0.90
	Present		7			
UICC grade	1+2		25	<0.0001	91	<0.0001
	3	#	24		29	
NCI grade	2		27	0.06	76	0.04
	3	#	22		46	
Ploidy (FCM/ICM)	Euploid		32	0.04	72	0.007
	Aneuploid	#	10		40	
S-phase (%)	<10		9	0.95		0.07
	≥10		27			
S+G2 phase (%)	<20		28	0.18		0.25
	≥20		8			
PCNA-LI	<20		29	0.04	77	<0.001
	≥20	#	11		24	
Ki-67-LI	<10		17	<0.0001	88	0.005
	≥10	#	20		44	
TNM	I-II		25	<0.0001	87	0.0001
	III	#	24		31	

Parameters associated with short interval-free and overall survival

* Postoperative radiotherapy and adjuvant chemotherapy were grouped together

BSS: Biphasic Synovial Sarcoma; MSS: Monophasic Synovial Sarcoma

HPF: High Power Field; FCM: Flow Cytometry; ICM: Image Cytometry

PCNA-LI: Proliferating Cell Nuclear Antigen Labeling Index

Ki-67-LI: Ki-67 Labelling Index

Table 2 Multivariate survival analysis (Cox model) in patients with localized synovial sarcoma using 3 alternative models.

Prognostic factors	Regression coefficient	Relative death risk	95% CI	Wald test p-values
Group 1, n=40				
TNM: III vs I-II	1.3	3.5	1.2-10.5	<0.01
Mast cell rate*: <10 vs 10+	1.5	4.5	1.5-13.6	<0.001
or				
Group 2, n=49				
TNM: III vs I-II	1.3	3.5	1.2-10.7	<0.05
Mitotic rate*: 10+ vs <10	1.1	3.0	1.1- 8.2	<0.05
or				
Group 3, n=40				
TNM: III vs I-II	2.1	8.4	2.6-26.7	<0.001
PCNA-LI: 20%+ vs <20%	2.1	7.9	2.7-23.0	<0.001

CI: Confidence interval

* Per 10 high power fields

PCNA-LI: Proliferating Cell Nuclear Antigen Labeling Index

Table 3 Relationship between the amount of mast cells, mitotic rate and PCNA-LI in synovial sarcomas.

	Mast cell rate		P-value
	<10/10 HPF (n=18)	≥10/10 HPF (n=22)	
Mitotic rate			
<10/10 HPF	7	19	0.003*
≥10/10 HPF	11	3	
Median (%)	12.5	2.5	0.003**
PCNA-LI			
<20%	10	19	0.04*
≥20%	8	3	
Median (%)	18.1	7.5	0.03**

HPF = High Power Field; PCNA-LI = Proliferating Cell Nuclear Antigen Labeling Index

* = Fisher's exact test (2-tailed) ** = Mann-Whitney rank-sum test

DISCUSSION

Synovial sarcoma (SS) is a rare soft tissue tumor usually of high grade malignancy. Since 1980 all but one series on record dealing with survival rates are based upon less than one hundred cases[1-19]. The five-year survival rates reported are summarized in Table 4. The 64% 5-year survival rate observed in the present series of localized SS is within the range of the series previously reported [1-19].

It is well established that staging is the most important parameter in the evaluation of soft tissue sarcomas. It gives valuable information, both guiding choice of treatment and prognosis. Tumor size, local spread, metastases, and histologic grade have all been used in different staging systems of soft tissue sarcomas [48,49,63-66].

By using the TNM staging system[69] we found, like in other studies of soft tissue sarcomas including SS [17,63,66], that stage is a powerful prognosticator of relapse-free and overall survival. Actually, in our series of localized SS, patients with tumors at TNM stage I-II at presentation had a much better prognosis than patients with tumors at TNM stage III.

Table 4 5-year survival in 19 series of synovial sarcoma

	Series	Number of patients	5-year overall survival (%)
1981	Buck *et al*[1]	33	36.0
1982	Wright *et al*[2]	185	38.0
1982	Varela-Duran *et al*[3]	26	82.6
1983	Tsuneyoshi *et al*[4]	17	51.0
1984	Zito *et al*[5]	48	50.0
1984	Rapjal *et al*[6]	52	43.5
1987	Collin *et al*[7]	63	56.0
1988	Tsujimoto *et al*[8]	26	44.9
1989	Lack *et al*[9]	50	55.0
1990	Goulouh *et al*[10]	36	64.0
1991	Henderson *et al*[11]	20	43.0
1992	Brodsky *et al*[12]	95	59.0
1992	Hashimoto *et al*[13]	73	52.0
1992	Santavirta[14]	31	55.0
1992	Ivanov-Dutescu *et al*[15]	46	30.0
1993	Lee *et al*[16]	18	78.4
1993	Oda *et al*[17]	56	54.0
1993	Ladenstein *et al*[18]	31	73.5
1993	Fetch *et al*[19]	27	50.0

Favorable clinical features previously reported include feminine gender [1,2,6,22,23], young age [1,2,17,20,23,25], duration of symptoms [23,25,26], location of the tumors in the extremities [1,2,6,17,18,28,29], and a small size (less than 5 cm) of the tumors [2,5,6,12,17-20,24,25,28,30-32]. Out of the five aforementioned features only duration of symptoms (more than 12 months) turned out to be a favorable factor for relapse-free (to local or metastatic recurrence) and overall survival in the univariate analysis of our series. Patients of young age (less than 30 years) had a longer relapse-free period but not longer overall survival. Our results are therefore consistent with those of others that did not find a significant association between age[5,10-12,19,21,22,24], gender [5,10-12,20,21,24], tumor location[11,12,22,23] and tumor size [10,11,33], and survival of patients with SS.

In the present series, compartmental location of the tumor, adequacy of surgical margins, and adjuvant treatment (radiotherapy or chemotherapy) did not predict significantly the relapse-free and overall survival of patients with SS. Also, the residual tumor at the surgical margins, positive only in 8 cases (16.3%), turned out not a prognostic marker for overall survival. The 5- year relapse-free rate of our series (34%) is within the range of previously reported results (21.2-44.4%) regarding the control of local and distant disease in SS [6,15,16,23,44]. Recently, Ladenstein et al [18] reported 74.2% 5- year event-free survival in a multicenter trial including 31 pediatric patients with SS.

The prognostic significance of histologic subtyping of SS remains controversial. Several authors conclude that there are no significant differences between the survival of patients with the two most common subtypes of SS (BSS and MSS) [10-13,18,20,24,27-34,36]. On the other hand, some groups have found BSS to carry a more favorable prognosis [1,5,21,25,35] and others have found that MSS appears to be the less aggressive variant [4,22,28].

Although we found in the univariate analysis that the BSS subtype indicates a shorter relapse-free and overall survival, we observed, like others [10-13,18,20,24,27-34,36], that histologic subtype was not a significant prognostic factor in the multivariate analysis. In agreement with others [2,10,17,21,24,37] and in contrast to Cagle et al[34], we observed no relationship between the extent

of glandular differentiation and the overall survival.

Both calcification [3] and poor differentiation [37] in SS are among the morphological features reported to be related to the prognosis. Calcification is frequently seen in SS [38]. Extensive calcification was reported by Varela-Duran and Enzinger to be associated with a good prognosis [3]. However, in our series, no relationship between calcification and survival of the patients was observed . On the other hand, none of the cases in our series showed more than moderate extent of calcification.

Hemangiopericytic pattern is one of the features described in the poorly differentiated SS and is thought to be associated with an unfavorable prognosis [38]. None of the cases in our series was classified as poorly differentiated and the extent of hemangiopericytic pattern was not predictive for the overall survival.

We have found, like other authors in several series of different types of soft tissue sarcomas [67,68] and Oda et al [17] in SS specifically, that a large amount of mast cells is a favorable prognostic feature. The prognostic significance of this feature was maintained in the multivariate analysis in our study.

The frequent presence of perivascular and interstitial mast cells has been described as a characteristic feature of some benign (e.g., schwannomas, hemangiomas and cellular dermatofibromas) and malignant (e.g., neurofibrosarcomas, synovial sarcomas and malignant fibrous histiocytomas) soft tissue tumors[38,67,69,70]. It has also been described in other neoplasms such as breast carcinoma and cervical carcinoma [71,72].

It is interesting that, as we observed in the present study and as has been reported previously in several types of soft tissue tumors, mast cells are preferentially located in the peripheral areas of the neoplastic masses displaying active tumor growth.

The putative role of mast cells in tumor growth remains to be clarified. There are experimental studies favoring an involvement of mast cells in tumor suppression[73,74]. In contrast to these studies, Roche[75] reported that mast cells enhance tumor proliferation of transplanted sarcomas in mice.

We observed that the amount of mast cells showed a significant inverse correlation with proliferation markers (mitotic rate and PCNA-LI). Moreover the amount of mast cells was a significant predictor of the interval-free of disease and the overall survival of the patients. Thus, mast cells may be involved in suppression of tumor growth.

Extent of necrosis has been reported as an important prognostic factor in series composed of different types of soft tissue sarcomas [9,13,50,66,76,77] and also in SS [10,13,17,24,36]. Despite having observed a significant relationship between necrosis and overall survival in the univariate analysis we, like Oda et al [17,36], failed to confirm such relationship at the multivariate analysis.

The importance of the mitotic rate as a prognostic factor reported in the present series after multivariate analysis corroborates Enzinger statement [38] on the existence of "an inverse correlation between survival and mitotic activity" in SS, and concurs also with the reports of other groups [5,10,11,17,19,20,34,36].

Histologic grading of soft tissue sarcomas is a matter of great controversy [8,9,50,66,78-87]. There are several grading systems using different criteria and variable thresholds for each of them [49,50,66,78,80,81,83]. Our results are consistent with those of Moberger et al [22] and with the results recently reported by Pappo et al [31], showing that grading of SS is of prognostic value. We compared the prognostic strengths of the UICC[49] and the NCI [50] grading systems, and the former system appeared to be better in our study. By using the UICC grading system we showed that the mitotic rate, which is not used in the NCI system for SS, is important in the grading of SS. The results we have obtained using the UICC system comply with the existence of a spectrum (from 1 to 3, in a 3 grade scale) of malignancy in SS, as outlined by Enzinger[66]. At variance with this, with the NCI grading system SS are always high grade tumors (grades 2-3), depending on the extent of gross and microscopical necrosis (15% threshold). Moreover, in our multivariate analysis, necrosis did not significantly predict the survival probability, thus contributing to weaken the potential strength of the NCI grading system.

We observed blood vessel invasion in 7 cases. This feature was not associated with a statistically significant difference on the survival, like it was described by Röser et al in SS[24] and at variance to the results reported by other groups in series composed by different types of soft tissue sarcomas [9,77,87-89].

The prognostic values of DNA cytometry parameters (ploidy and percentage of tumor cells in S and S+G2 phases) have been studied in several series of different histologic types of soft tissue sarcomas [20,36,39,77,89-110]. In our series, less than 1/4 of the cases were aneuploid. This percentage is smaller than that reported by other authors . In fact, taking together the data reported in several series dealing with SS, the overall percentage of aneuploid SS is 40% (43 aneuploid SS out of 116 SS)[20,36,89,94,103,111,112].

No association between prognosis and ploidy has been observed in several studies [36,97-110], whereas in other reports [20,39,89-96,110-112] aneuploidy has been found to be an unfavorable prognostic factor for patients

with soft tissue sarcomas in general. In our univariate analysis we observed, as Oda et al[36], an association between aneuploidy and an unfavorable prognosis. However, at variance with the findings of El-Naggar et al [20] and Åkerman et al [39], this association was lost in the multivariate analysis, as was also described by Oda et al [36]. Moreover, the evaluation of the percentage of tumor cells in S and S+G2 phases did provide any significant additional prognostic information (Oda et al[36], present study).

In our series, DNA ploidy was studied by image (ICM) and flow (FCM) cytometry. The concordance rate of ploidy descriptors observed in the present series fits with other reports [91,99,112-116]. In these studies, examples of discordance between ploidy descriptors (diploid by FCM and aneuploid by ICM and vice-versa) have been reported. Several explanations have been advanced to explain such discordances: inherent differences in the two methods, problems at the histogram interpretation level, differences in tissue sampling, and intratumor heterogeneity. In addition, the possible effect of formalin fixation as a limiting factor in cytometry evaluation should be considered as it has been recently pointed on by Zalupski et al in a series of soft tissue sarcomas studied by FCM [119].

Few studies on the prognostic value of PCNA expression in soft tissue sarcomas have been reported to date. High PCNA expression was found to be associated with a poor prognosis in a series of hemangiopericytomas [101] and in a series of different types of soft tissue sarcoma [68]. No significant association between PCNA expression and survival was observed in a series of different types of soft tissue sarcomas [96] and in a series of malignant fibrous histiocytoma (MFH)[118]. High PCNA expression was associated with a better prognosis in a series of rhabdomyosarcomas [120].

PCNA labeling index remained a statistically significant prognostic factor in our multivariate analysis, as it was reported by Oda et al[36].

There are few reported studies on the prognostic value of Ki-67 expression in soft tissue sarcomas. In one study dealing with MFH, the expression of Ki-67 was not significantly associated with prognosis[120]. In other studies, high Ki-67 levels were found to be an unfavorable prognostic factor for patients with soft tissue sarcomas [68,121,122].

To the best of our knowledge this is the first report on the prognostic value of Ki-67 in SS. In the univariate analysis, high Ki-67-LI was associated with an unfavorable prognosis. However, this association did not attain the threshold of statistical significance in the multivariate analysis.

CONCLUSION

Besides TNM stage (UICC), our study revealed several factors significantly associated with the survival of patients with localized SS in the univariate analysis.

Amount of mast cells, mitotic rate, or PCNA-LI, remained as significant prognosticators, in addition to staging, at the multivariate analysis level. Further studies are needed to clarify the putative relationship between mast cells abundance and proliferation markers.

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Paper V

Synovial sarcoma. Immunohistochemical expression of P-glycoprotein and glutathione S tranferase-pi and clinical drug resistance.

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Purpose: To study the role of the expression of P-glycoprotein (Pgp), and glutathione S transferase-pi (GST-pi) in predicting the response to chemotherapy, relapse-free interval, and survival of patients with synovial sarcoma (SS).
Patients and methods: Thirty-seven cases of primary SS, without regional lymph node or distant metastases, were studied. There were 17 females and 20 males, ranging in age from 7 to 81 years (median, 31 years), with tumors located in the lower extremity (n=24), upper extremity (n= 5), and trunchus (n= 8). The cases were retrospectively studied without knowledge of clinical course to compare the immunohistochemical expression of Pgp and GST-pi, flow cytometry parameters (ploidy and % of cells in S+G2 phases), and PCNA and Ki-67 labeling of primary tumors before any therapy, with that observed in local recurrences and metastases after chemotherapy. The relationship of the aforementioned parameters with clinicopathological features (gender, age, and histo-blood group of the patients, size, location, histological subtype, TNM stage, and clinical response to chemotherapy of the tumors) was also evaluated.
Results: Pgp and GST-pi was expressed in 29.7% and 40.5% of the cases, respectively. In 48.6% of the tumors there was expression of at least one of the drug resistance markers. The markers were co-expressed in 25.0% of the tumors. The prevalence of Pgp expression was lower, but not significantly, in stage I-II (17.6%) than in stage III (40.0%) tumors and also in cases without (16.7%) than in cases with clinical progression (36.0%). No such differences were observed for GST-pi expression. Pgp and GST-pi expressions were significantly associated with biphasic SS and were particularly noticeable in solid/glandular areas of biphasic SS. The expression of the drug resistance markers was not significantly associated wit gender, age, and histo-blood group of the patients, dimension, location, and proliferative activity of the tumors; it was not also significantly related to relapse-free interval and survival of the patients. The expression of Pgp and GST-pi was not significantly associated either to response to chemotherapy or influenced by chemotherapy.
Conclusion: Pgp and GST-pi expressions are not good predictors of the response to chemotherapy in patients with localized SS. Other drug resistance mechanisms may be active in SS.

INTRODUCTION

Despite improvements in the multimodality treatment, the prognosis of patients with synovial sarcoma (SS) remains poor, mainly due to hematogenous spread of tumor cells to the lungs, with subclinical metastases at the time of diagnosis. Local control implies that adequate surgical margins can be obtained, either as wide or compartmental excision, or by combining primary surgery with postoperative radiotherapy[1-14]. Local recurrences and/or metastases of SS are most frequently seen within first 2 years following primary treatment, but can also appear after longer periods; up to 35 years[15,16]. In most of the series published since the 1980s, the 5-year survival rate is 50% on average[4-10,17-27]. Recently, improved outcome with five-year survival rate of 73.5% was obtained in a series of pediatric patients with SS[28].

Although different single-agent and combined chemotherapy regimes have been associated with positive responses in the treatment of local recurrences[28-31], the clinical benefit of chemotherapy in the treatment of SS metastases remains controversial [30,32-37]. Responses are usually of short duration and rarely complete [30,36].

It seems evident that a wide variety of mechanisms are involved in clinical drug resistance[38]. Several resistance markers such as P-glycoprotein (Pgp) and glutathione S transferase-pi (GST-pi) have been studied in various cancers, including soft tissue sarcomas[39-81]. However, the results reported regarding their value in predicting the clinical response to chemotherapy have been conflicting[38,41,43,48-51,53,54,56,65,67,76-79,81,82].

To the best of our knowledge, present study is the first describing the immunohistochemical expression of Pgp and GST-pi in SS using commercially available antibodies and routinely formalin-fixed paraffin-embedded tissues. Our objectives were to compare the expression of Pgp and GST-pi in primary SS prior to therapy with that observed in local recurrences and/or metastases of matched tumors following chemotherapy. Furthermore, to evaluate the potential role of these markers in predicting the response to chemotherapy, relapse-free interval, and overall survival of patients with SS.

PATIENTS AND METHODS

Thirty-seven cases of primary synovial sarcoma (SS) without regional lymph-node metastases and distant metastases, and no prior radio- and chemotherapy were selected from the files of the Department of Pathology, The Norwegian Radium Hospital (28 cases) and Porto Medical School (9 cases).

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Clinical features

Pertinent clinical data were collected from the medical records of the patients.

The surgical margins were classified according to the criteria of Enneking[83]. The cases were classified according to the TNM staging system of UICC[84]. All responses to treatment were classified based on the clinical and radiological findings according to standard criteria reported by Miller et al [85]. In our study, complete responses (CR)- no detectable measurable disease - and partial responses (PR)- 50% or more tumor volume reduction lasting for more than one month - were grouped separately. Stable disease (SD) and mixed responses (MD) were grouped together with progression (P). This last group (SD + MD+ P) will be designated in the present study as the non-responding (NR) group.

Follow-up was done by clinical examination every third month for the first year with subsequently longer intervals for the following years. Chest X-rays were performed at each visit. CT-scans of the lungs were performed whenever chest X-rays indicated metastases or were inconclusive.

Pathological features

All available material for each patient in the study was examined without knowledge of the clinical course. The histological examination was performed on formalin-fixed paraffin-embedded 5 µm sections (4-15 per tumor) stained with hematoxylin and eosin.

The cases were classified, according to the criteria of Enzinger and Weiss[15], in biphasic (BSS, n=15) and monophasic (MSS, n=22) SS subtypes. Whenever necessary, immunohistochemical and ultrastructural examinations were performed to confirm the diagnosis.

In fourteen (8 BSS and 6 MSS) out of the 37 cases, 9 local recurrences (from 5 primary BSS and 4 primary MSS) and 12 lung metastases (from 8 primary BSS and 3 primary MSS) were also available for study. Two consecutive metastases were from the same patient with BSS. These 14 cases will be designated as matched tumors in present study.

Immunohistochemistry

Sections for immunohistochemistry were stained using the avidin-biotin-peroxidase complex (ABC) method [86]. Deparaffinized sections were treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes to block endogenous peroxidase. To unmask the epitopes of Pgp (JSB-1) and Ki-67 the sections were microwaved in 10 mM citrate buffer pH 6.0 [87], whereas microwaving in <1% lead solution [88] was used for unmasking the epitopes of PCNA. The sections were then incubated for 20 minutes with normal serum from the species in which the secondary antibody was made. This was done to eliminate nonspecific staining. Excess normal serum was blotted from the slides before incubation with monoclonal antibodies JSB-1 diluted 1:20 (MON 9011, Monosan, Netherlands) and PCNA diluted 1:25 (NCL-PCNA, Novocastra Laboratory Ltd. UK) and polyclonal antibodies GST-pi diluted 1:100 (A8000, Oncor, MD) and Ki-67 diluted 1:100 (A 047, Dako, Denmark) for 18-22 hours at 4° C. The sections were then incubated in 1:200 dilution of biotin-labeled secondary antibody for 30 minutes and ABC (10 µg/ml of avidin and 2.4 µg/ml of biotin-labeled peroxidase) for 60 minutes (Vector, Burlingame, CA). Tissue was stained for 5 minutes with 0.05% 3'3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.6, containing 0.01% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted in Diatex. All the dilutions of normal sera, antibodies, biotin-labeled secondary antibodies and ABC were made with phosphate-buffered saline, pH 7.4 containing 5% bovine serum albumin. All series included positive controls. Negative controls included substitution of polyclonal antibody with normal rabbit IgG, whereas negative controls for the monoclonal antibody were performed using mouse myeloma protein of the same subclass and concentration as the monoclonal antibody. All controls gave satisfactory results.

Immunoreaction for PCNA and Ki-67 was considered positive whenever nuclear staining was observed, independently of the intensity of the staining. Areas with more numerously stained nuclei were selected and used for counting at random. The labeling indices were determined in every case by counting the number of positive tumor cell nuclei among a total of 1,000 cells. The labeling indices were expressed in percentage of positive cells. This evaluation was performed in 14 cases with matched tumors: 14 primary tumors (8 BSS and 6 MSS), 9 local recurrences, and 12 lung metastases.

Immunoreaction for Pgp was considered positive whenever plasma membrane and/or paranuclear dot (Golgi-like) staining was observed. Intensity of Pgp staining was graded as weak (+1) and strong (+2).

Immunoreaction for GST-pi was considered positive whenever cytoplasmic staining was observed.

The extent of Pgp and GST-pi positive staining was evaluated as the percentage of stained tumor cells :focal (<10%), moderate (10-30%), and diffuse (>30%).

DNA flow cytometry

To minimize the amount of non-tumor tissue, all areas with tumor were outlined on blocks corresponding to the best sections, and most of the non-tumor tissue was removed. From each block one 100-µm section for flow cytometry (FCM) was cut. In the end, a 5-µm section was cut for evaluation of representativity of the section and for the estimation of the amount of tissue present. After deparaffinization with xylol, the tissues were rehydrated in graded ethanols, rinsed in phosphate buffered saline (PBS) and incubated for 30 minutes with protease (Sigma No. 24.) at 37° C. The protease activity was stopped by adding 4 ml cold PBS, and thereafter the specimens were rinsed twice in 4 ml PBS before centrifugation at 1250 x [89]. The specimens were prepared in series to control interspecimen

variation of the disintegration index.
Cell suspensions were prepared by the method described by Hedley [89], with minor modifications [90,91], stained with ethidium bromide. DNA FCM was performed by the use of a laboratory built flow cytometer [92]. Briefly the instrument was based on a Nikon Diaphot invertoscope equipped with excitation light ranges of 510-560 nm and a beam splitter at 580 nm. The output signals were sorted by multichannel analyzer (Nuclear Data ND 66) into histograms with 256 channels.

Ploidy definition

The histograms were analyzed with reference to the DNA indices of distinguishable stemlines [93]. Histograms with only one modal peak were regarded as diploid (Coefficient of Variation-CV: 4.2-8.5%, mean 5.5%). A tumor was considered to be non-diploid if several peaks were distinguishable. The first peak of the histogram was always regarded as being diploid. The further ranges for DNA indices (DIs) were defined as follows: Tetraploid range - 1.80<DI<2.20; Aneuploid range - 1.10<DI<1.80 and DI 2.20; Multiploid - more than one non-diploid stemline. A tetraploid stemline was recorded if the number of cells within a tetraploid peak exceeded the number of cells between the diploid and the tetraploid range. The percentage of cells in S and G2 phases was analyzed from DNA histograms by use of the MULTICYCLE® computer program (Phoenix Flow System Inc.). Since cell nuclei, and not the whole cells, were measured, mitotic figures were washed out during preparation and were not included in the measurements.

The FCM study was performed in consecutive sections of the same blocks of the 14 cases with matched tumors studied by PCNA, Ki-67, Pgp and GST-pi: 14 primary tumors (8 BSS and 6 MSS), 9 local recurrences, and 12 lung metastases.

Statistics

The Wilcoxon's two-sided rank sum test was used to test the probability of nondifference between matched variables or paired observations such as PCNA-LI, Ki-67-LI, and %S+G2.
To evaluate which characteristics at diagnosis predicted the outcome , the patients were stratified according to gender, age, location of the tumor, tumor stage, tumor dimension, Pgp and GTS-pi expression of neoplastic cells (positive and negative), and blood type.
The response rates in terms of relapse interval and survival at 5 years were compared by the chi-square test and Fisher's exact test.
The duration of relapse-free survival and overall survival was estimated by Kaplan-Meier product-limit method. Log-rank analysis of survival curves were used to determine whether the characteristics at diagnosis could be used to predict relapse and death before and after adjustment for tumor stage which is admittedly the most significant prognostic factor in SS.
Two sided statistical tests were used in all analyses. The statistical software of BMDP (PC-92)[94] was used.

RESULTS

Clinico-pathological features of the patients are shown in Table 1.

Seventeen females and 20 males were included in the series . The median age was 31 years (range, 7-81 years). ABH(O) histo-blood group types of 30 patients were as follows: 13 group A (7 BSS and 6 MSS), 2 group B (1 BSS and 1 MSS), and 15 group H(O) (5 BSS and 10 MSS). The ABH(O) histo-blood group of 7 patients were unknown.

The primary tumors occurred in the following locations: lower extremity (n=24), upper extremity (n=5), and trunchus (n=8). The thigh (n=11) was the most frequent single location. Eight of the tumors were intracompartmental and 29 were extracompartmental. The median tumor size was 6 cm (range, 1-25 cm). Twenty-two tumors were 5 cm or smaller.

Initial treatment included surgery in all patients (Table 2). Adequate surgical margins (wide, n=11; and amputation, n=6) were achieved in 17 cases and inadequate margins (intralesional, n=1; and marginal, n=19) in 20 cases.

Fifteen patients received postoperative radiotherapy. Adjuvant chemotherapy was given to 6 patients (Table 2). Doxorubicin (Adriamycin ®) monotherapy was administered to 4 patients, CAOS-regimen to one patient [95], and aggressive combination chemotherapy, according to the "Ewing protocol SSG-IV" [96], to one patient.

Local recurrences were diagnosed in 11 patients, whereas lung metastases occurred in 24 patients, including 10 patients who had previously presented a local recurrence as first sign of relapse (Table 2). The relapse-free survival was 31.4% at 5 years (95% confidence interval-CI, 18.4-44.4%).

The median follow-up time after initial treatment was 81 months (range, 6-188 months). Sixteen patients were alive without evidence of disease, one patient was alive with disease, 19 patients died due to tumor regrowth, and one was lost from follow-up (Table 2). The overall 5-year survival rate after initial treatment was 56.3% (95% CI, 43.3-69.3%).

Treatment of local recurrences and metastases consisted of surgery as the only modality in 2 cases, surgery and radiotherapy in one case, chemotherapy alone in 10 cases, chemotherapy and surgery in 3 cases, and a combination of chemotherapy, surgery and radiotherapy in 9 cases (Table 2).

Radiotherapy was used treating local recurrences in 5 patients. Total lung-field radiotherapy was given in 7 patients with multiple metastases to the lungs.

Various chemotherapy regimes were used in the treatment of recurrences. Repeated cycles of CYVADIC [97] were given to 15 patients; seven of these patients were, later in the course of the disease, treated with ifosfamide (Holoxane ®) monotherapy. Four patients

Table 1 Characteristics at diagnosis of 37 patients with synovial sarcoma, according to tumor histology

Characteristic	All	(37)	BSS	(15)	MSS	(22)
	No	%	No	%	No	%
Gender						
Males	20	54.1	8	53.3	12	54.5
Females	17	45.9	7	46.7	10	45.5
Age						
≤ 20	8	21.6	2	13.3	6	27.3
21-50	21	56.8	9	60.0	12	54.5
> 50	8	21.6	4	26.7	4	18.2
Tumor stage						
I, II	17	45.9	5	33.3	12	54.5
III	20	54.1	10	66.7	10	45.5
Dimension						
≤ 5cm	22	59.5	9	60.0	13	59.1
> 5cm	15	40.5	6	40.0	9	40.9
P-Glycoprotein*						
Positive	11	29.7	9	60.0	2	9.1
Negative	26	70.3	6	40.0	20	90.9
GST-pi						
Positive	15	40.5	9	60.0	6	27.3
Negative	22	59.5	6	40.0	16	72.7
Pgp/GST-pi**						
Positive (at least one of the markers)	18	48.6	11	73.3	7	31.8
None positive	19	51.4	4	26.7	15	68.2
Histo-blood type						
A	13	43.3	7	53.8	6	35.3
B	2	6.7	1	7.7	1	5.9
H (O)	15	50.0	5	38.5	10	58.8

BSS: Biphasic Synovial Sarcoma; MSS: Monophasic Synovial Sarcoma

GST-pi: Glutathione S Transferase-pi; Pgp: P-glycoprotein;

Fisher test: *(p=0.002), and **(p=0.02)

were treated with a combination of etoposide, as 72 hours continous infusion, and ifosfamide [98]. Three patients received low dose doxorubicin (Adriamicin®) weekly.

The clinical responses to chemotherapy treatment of recurrences were complete (CR) in 3 patients and partial (PR) in 6 patients (Table 2). NR (grouped as mixed responses, stable disease, or progression) was observed in 13 patients. One patient treated only surgically with lung metastasectomy is still disease-free after 76 months of follow-up. The overall 5-year survival rate of patients with stage I-II (100%) was higher and significantly different ($p<0.0001$) from that of patients with TNM stage III (17.6%) (Fig.1). The outcome of the patients was as follows: 14 patients were apparently free of disease and 22 patients had signs of disease (Table 2).

Proliferation markers (PCNA-LI, Ki-67-LI, and %S+G2) of matched tumors (n=14)

In all but one case, the immunoreactivities for PCNA and Ki-67 of the fourteen primary tumors were relatively homogenous, but BSS showed (with both markers) a higher number of positive cells in solid/glandular than in the surrounding spindle cells of clear-cut biphasic areas.

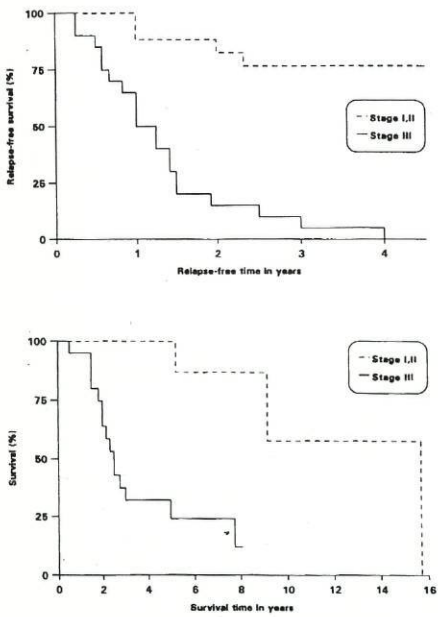


Fig.1- *Relapse-free and overall survival curves of patients with synovial sarcoma stratified according to TNM staging.*

Table 2 Summary of the treatments of primary tumors and recurrences, response to chemotherapy, outcome, and survival of patients with synovial sarcoma according to tumor stage and Pgp/GST-pi immunoreactivity

Treatment and Outcome	Tumor Stage I, II					Tumor stage III				
	Total	Pgp		GST-pi		Total	Pgp		GST-pi	
		+	-	+	-		+	-	+	-
Primary treatment (no)										
S only	9	1	8	4	5	8	2	6	4	4
S+C	1	0	1	1	0	4	2	2	1	3
S+R	6	2	4	3	3	8	4	4	2	6
S+C+R	1	0	1	0	1	0	0	0	0	0
Recurrence (no)										
Local	1	0	1	0	1	0	0	0	0	0
Metastases	0	0	0	0	0	14	7	7	7	7
Local + metastases	4	1	3	3	1	6	1	5	0	6
None	12	2	10	5	7	0	0	0	0	0
Recurrence treatment (no)										
C only	0	0	0	0	0	10	3	7	4	6
S only	1	0	1	0	1	1	1	0	1	0
C+S	1	0	1	1	0	2	2	0	1	1
S+R	1	0	1	0	1	0	0	0	0	0
C+S+R	2	1	1	2	0	7	2	5	1	6
Response to chemotherapy (no)										
Complete	0	0	0	0	0	3	0	3	0	3
Partial	1	1	0	1	0	5	2	3	1	4
None	2	0	2	2	1	11	5	6	5	6
Outcome (no)*										
No evidence of disease	13	2	11	5	8	1	1	0	1	0
With disease	4	1	3	3	1	18	7	11	5	13
Relapse-free survival (yr)										
Median	6.0	NR	6.2	5.7	NR	1.0	1.0	1.0	1.0	1.1
Range	1.0-13.0	1.0-5.5	1.0-13.0	1.0-13.0	1.0-10.2	0.3-4.0	0.3-4.0	0.3-3.0	0.6-4.0	0.3-3.0
Overall survival*										
Number of patients alive	13	2	11	5	8	4	3	1	2	2
Number of patients dead	4	1	3	3	1	15	5	10	4	11
Survival (yr)										
Median	10.0	NR	10.4	8.7	9.2	2.4	2.2	2.4	2.0	2.5
Range	3.1-15.7	5.3-5.5	3.1-15.7	5.3-15.7	3.1-10.2	0.5-8.1	1.5-8.1	0.5-7.8	1.5-8.1	0.5-7.8

Pgp: P-glycoprotein; Transferase-pi; GST-pi: Glutathione S; *: one patient was lost to follow-up; NR: not reached

The median PCNA-LI of primary tumors was 17.8 (range, 0.1-35.9). The PCNA-LI of BSS did not differ significantly from that of MSS primary tumors. The median PCNA-LI of local recurrences was 18.0 (range, 5.7-40.5). The median PCNA-LI of metastases was 18.8 (range, 1.3-37.7).

The median Ki-67-LI of primary tumors was 15.2 (range, 0.1-46.8). The Ki-67-LI of BSS did not differ significantly from that of MSS primary tumors. The median Ki-67-LI of local recurrences was 23.6 (range, 7.9-56.7). The median Ki-67-LI of metastases was 22.6 (range, 6.0-47.3).

DNA ploidy of the fourteen primary tumors was as follows: 4 aneuploid (all BSS), and 10 diploid (4 BSS and 6 MSS). Change in ploidy descriptor (aneuploid to diploid), in the comparison of primary tumors with the respective local recurrence, was observed in one BSS. There was also a change in ploidy descriptor (aneuploid to diploid) from the primary tumor to the respective metastasis in one BSS.

Median %S+G2 of primary tumors was 13.9 (range, 7.8-66.7). The %S+G2 of BSS was not significantly different from that of MSS primary tumors. The median %S+G2 of local recurrences was 17.7 (range, 10.9-50.9). The median %S+G2 of metastases (n=11) was 20.2 (range, 10.0-39.7). Calculation of %S+G2 was not possible in one metastasis due to overlap of aneuploid and diploid populations.

Proliferation markers (PCNA-LI, Ki-67-LI, and %S+G2) varied in both directions (increase and decrease), in most of the cases when comparing their values in the primary tumors to those observed with the respective local recurrences and metastases (Table 3).

No significant statistical difference between the values of the proliferation parameters (PCNA, Ki-67, and %S+G2) in primary tumors and in the respective local recurrences and metastases was observed.

Table 3 **Variation of PCNA-LI, Ki-67-LI, %S+G2, Pgp, GST-pi in recurrences (n=9) and metastases (n=11)**

	Recurrences (n=9)			Metastses (n=11) #		
	No change	Increase	Decrease	No change	Increase	Decrease
PCNA-LI	0	5	4	0	7	4
Ki-67-LI	0	5	4	1	8	2
%S+G2*	0	6	3	0	7	3
Pgp	8	0	1	10	1	0
GST-pi	5	2	2	4	5	2

PCNA-LI: Proliferating cell nuclear antigen labelling index; Ki-67-LI: Ki-67 labelling index;

%S+G2: Percentage of cells in S and G2-phases; Pgp: P-glycoprotein; GST-pi: Glutathiona S Transferase-pi

* In one case the calculation of %S+G2 was not possible in one metastasis due to overlap of G2 peaks of diploid and aneuploid cells

\# In one case two metastasis were from the same tumor

P-glycoprotein (Pgp)

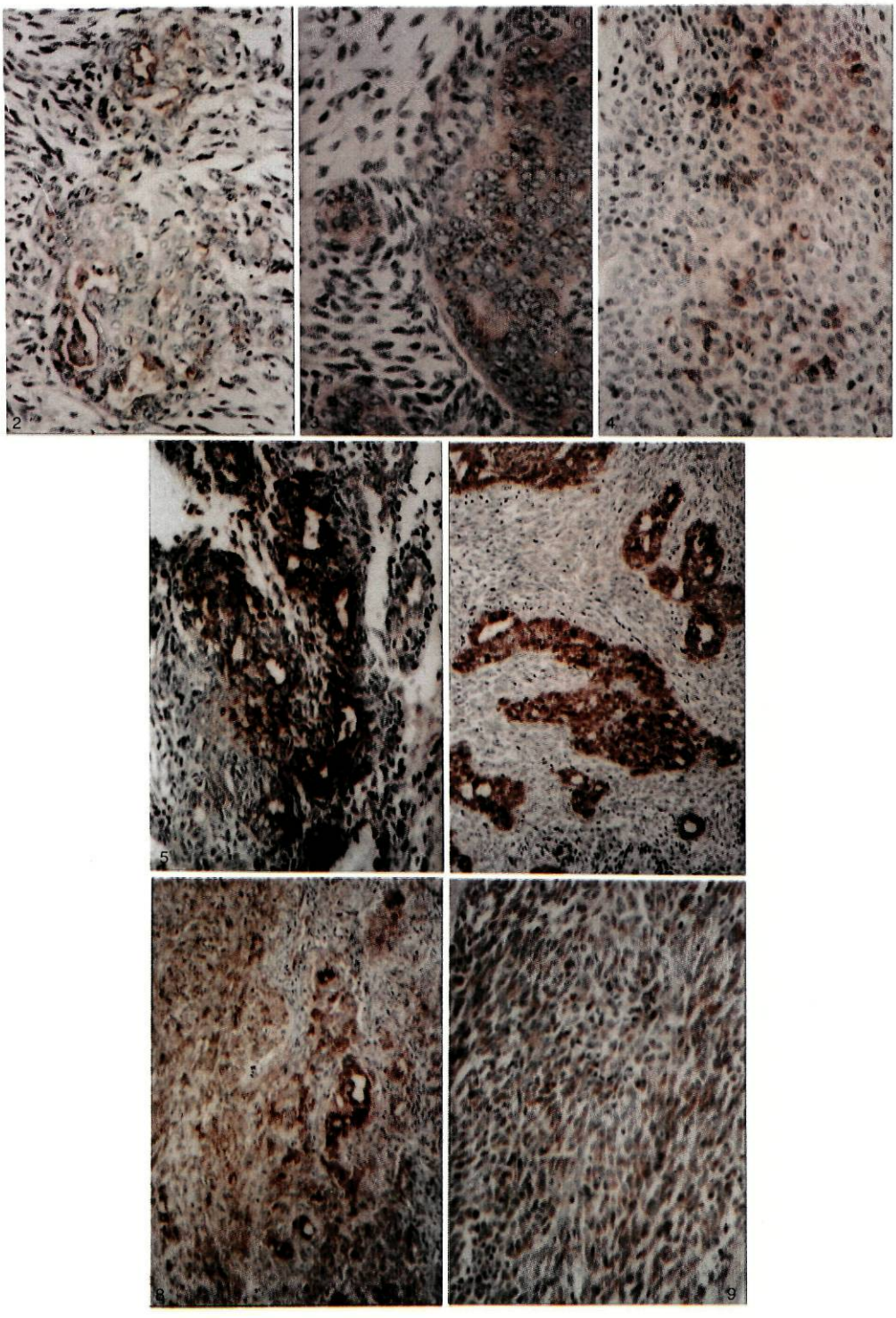
Primary tumors (n=37)

Membrane immunoreactivity for Pgp (Fig.2) was observed in 11 cases (9 BSS and 2 MSS) (Table 1), three of them showing also paranuclear dot (Golgi-like) staining (Fig.3). Pgp immunostaining of the tumors was always focal (10%). The staining intensity was weak in two cases (1 BSS and 1 MSS). Pgp positivity of BSS was always observed in cells of solid/glandular component (Figs. 2 and 3) and also in scattered cells of the spindle cell component in one of the positive cases. Pgp positivity of MSS was observed in epithelioid cells (Fig.4). Diffuse weak cytoplasmic staining was observed, in addition to membrane staining, in 6 cases. Pgp positivity was significantly associated (p=0.002) with BSS subtype.

Out of 9 patients with known histo-blood group and Pgp positive tumors, 5 were group A (4 BSS and 1 MSS), 3 were group H(O) (2 BSS and 1 MSS), and 1 was group B (1 BSS). Paranuclear dot (Golgi-like) staining (Fig.3) was observed only in positive tumors of 3

►

Fig. 2- Primary biphasic synovial sarcoma displaying discrete Pgp immunoreactivity at the apical membrane of glandular cells. ABC, 80 x. - Fig. 3- Primary biphasic synovial sarcoma displaying para-nuclear Pgp immunoreactivity in the cells of solid/glandular component. ABC, 80 x. - Fig. 4- Primary monophasic synovial sarcoma displaying Pgp immunoreactivity in some epithelioid cells. ABC, 80 x. - Fig. 5- Metastatic biphasic synovial sarcoma displaying strong immunoreactivity in the glandular cell component. ABC, 80 x. - Fig. 7- Primary biphasic synovial sarcoma displaying prominent GST-pi immunoreactivity in the solid/glandular component. ABC, 80 x. - Fig. 8- Primary biphasic synovial sarcoma displaying stronger GST-pi immunoreactivity in solid/glandular cells than in the surrounding spindle cells. ABC, 80 x. - Fig. 9- Primary monophasic synovial sarcoma displaying GST-pi immunoreactivity in most of the cells. ABC, 80 x


2
4
5
8
9

patients of histo-blood group A. There was no significant association between Pgp positivity of the tumors and histo-blood group phenotype of the patients.
Pgp expression was not significantly associated with age and gender of the patients, dimension and location of the tumors, and clinical response to chemotherapy.

Primary tumors versus local recurrences (n=9)
The comparison between Pgp immunoreactivity of primary tumors and the respective recurrences is summarized in Table 3.
Eight cases (5 BSS and 3 MSS) maintained the same pattern of immunoreaction (positive or negative) in the recurrences as in the respective primary tumors. The remaining case was a MSS that was positive in the primary tumor and negative in the respective recurrence.

Primary tumors versus metastases (n=11)
The comparison between Pgp immunoreactivity of primary tumors and the respective metastases is summarized in Table 3.
Seven BSS (one of them with two metastases) and 3 MSS primary tumors maintained a similar immunoreaction (positive or negative) pattern in the respective metastases. The remaining case was one BSS that was negative in the primary tumor and showed focal and strong positivity in the metastasis (Fig.5).

Pgp, staging of the tumors, clinical progression and survival
The prevalence of Pgp immunoreactivity was lower in stage I-II (17.6%) than in stage III (40.0%) tumors (Table 2). The prevalence of Pgp immunoreactivity was also lower in cases that did not develop recurrences or metastases (16.7%) than in cases with progression of the neoplastic disease (36.0%). These differences do not attain the threshold of statistical significance. Pgp expression did not significantly influence relapse-free survival nor overall survival of the patients (Fig.6). The results were similar, both by analyzing the primary tumors alone and by evaluating recurrences and metastases.

Glutathione S transferase-pi (GST-pi)

Primary tumors (n=37)
Cytoplasmic immunoreactivity for GST-pi was observed in 15 primary tumors (9 BSS and 6 MSS) (Table 1). Positivity of the tumor was focal in 4 (2 BSS and 2 MSS), moderate in 7 (5 BSS and 2 MSS), and diffuse in 4 cases (2 BSS and 2 MSS).
In 3 BSS the solid/glandular component showed more extensive positivity than the spindle cell component (Fig.7), in 5 cases both components showed similar extent of positivity, and in one case the spindle cell component was GST-pi negative. Usually the positivity of the solid/glandular component was stronger than that of the spindle cell component (Fig.8).

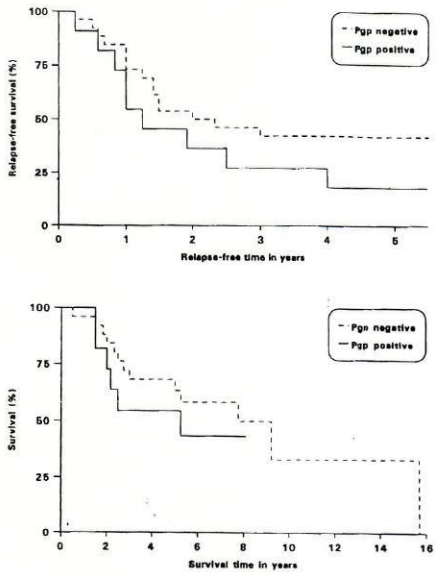


Fig. 6- *Relapse-free and overall survival curves of patients with synovial sarcoma stratified according to Pgp expression.*

The intensity of the immunoreaction appeared to be weaker in MSS than that observed in solid/glandular component of BSS, and similar (Fig.9) to that observed in spindle cell component of BSS (Fig. 8).
The GST-pi expression of primary tumors was not significantly associated with any subtype of SS, dimension, location of the tumor, gender or age of the patients, and clinical response to chemotherapy.

Primary tumors versus local recurrences (n=9)
The comparison between GST-pi immunoreactivity of primary tumors and the respective recurrences is summarized in Table 3.
Four BSS and one primary MSS showed a similar (positive or negative) pattern of GST-pi immunoreaction in the respective local recurrences. Two GST-pi positive primary MSS were negative in the respective local recurrences. The remaining two cases (1 BSS and 1 MSS) were negative in the primary tumors and showed positive GST-pi immunoreaction in the respective recurrences.

Primary tumors versus metastases (n=11)
The comparison of GST-pi expression of primary tumors with the respective metastases is summarized in Table 3.
Metastases of 4 BSS and 2 MSS showed a similar (positive or negative) immunoreaction, but two metastases from BSS showed increased extent of immunostaining (from moderate to diffuse), in comparison to the respective primary tumors. Metastases of two other (focal and moderate) positive BSS showed nega-

tive immunoreaction. Metastases from two BSS and one MSS negative primary tumors showed focal, diffuse, and moderate positive immunostaining, respectively.

GST-pi, staging of the tumors, clinical progression and survival

The prevalence of GST-pi immunoreactivity was similar in stage I-II (47.1%) and in stage III (35.0%) tumors as well as in cases without (41.7%) or with recurrences or metastases (40.0%)(Table 2). GST-pi expression was not significantly associated with relapse-free survival or overall survival (Fig.10) of the patients.

Pgp and GST-pi coexpression

Eighteen primary tumors (11 BSS and 7 MSS) were immunoreactive for at least one marker (PGP or GTS) (Table 1). Co-expression of both markers was observed in 9 cases (7 BSS and 2 MSS). The relapse-free survival and overall survival of these 9 patients did not significantly differ from those of the other patients.

Drug resistance (Pgp and GST-pi) versus proliferation (PCNA, Ki-67, and %S+G2) markers

The comparison between Pgp and GST-pi expressions and PCNA-LI, Ki-67-LI, and %S+G2 of primary tumors with those of the respective recurrences and metastases is summarized in Table 3.

The variation between primary tumors and the respective local recurrences and metastases was less common regarding Pgp than GST-pi expression. Such a variation displayed also a stronger tendency for increase in GST-pi expression than Pgp expression (Table 3). There was no significant statistical association between Pgp and GST-pi expressions and the values of proliferative markers (PCNA, Ki-67, and %S+G2) in the primary tumors and in the respective local recurrences and metastases.

Pgp and GST-pi before and after chemotherapy

Clinicopathologic features and sequence of treatment of the 14 cases with matched tumors is shown in Table 4.

Expression of Pgp and GST-pi before and after chemotherapy of eleven cases is shown in Table 5. There were frequent changes of Pgp and GST-pi immunoreactivity in both directions (positive to negative, and vice-versa) when comparing tumors before and after chemotherapy. Increase of Pgp and GST-pi immunoreactivity were observed in one and 3 cases, and decrease in one and 3 cases, respectively.

DISCUSSION

At present, no generally accepted chemotherapy regime exists in the treatment of SS, neither for metastatic disease nor in the adjuvant setting. With few

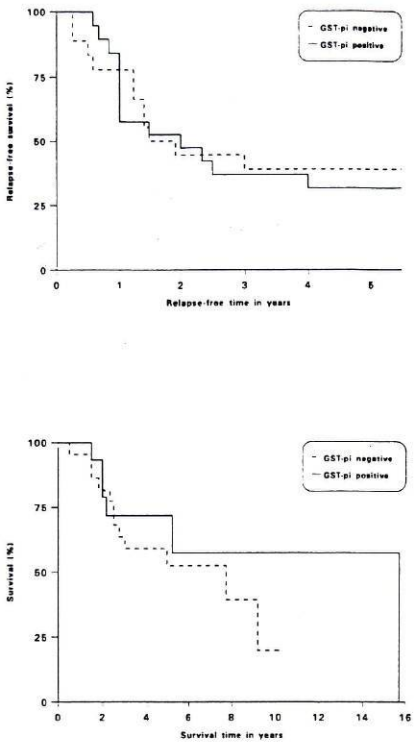


Fig. 10- *Relapse-free and overall survival curves of patients with synovial sarcoma stratified according to GST-pi expression.*

exceptions, low rates of complete responses have been reported [5,7-13,25,28,30-37]. The tumor cell drug resistance may be related to many host and tumor factors, besides differences in pharmacokinetics of the different drugs [38,39,82,99-112].

Cancer cells use several pathways to overcome the cytotoxic effects of chemotherapeutic agents [38]. Expression of P-glycoprotein (Pgp), glutathione S tranferases (GST), topoisomerase II, thymidylate synthase, metallothionein, dihydrofolate reductase, and O6-alkylguanine-DNA alkyltranferase are examples of normal cell proteins that have been reported in several cancer models and in clinical drug resistance [38].

Pgp (also known as multidrug resistance-mdr1) is a pleiotropic protein highly conserved across species and is present in many normal tissues and tumors [39-56,113,114]. There is an extensive heterogeneity of Pgp expression in normal organs and tumors from different individuals, probably reflecting, at least to some extent, a tissue specific regulation [39-56,113,114].

It seems that Pgp is a membrane transporter with broad specificity. However, little is known about the substrates for Pgp transport in vivo [99,102,103,105,108,115,116]. Although it has been linked to drug resistance the role of Pgp in clinical resistance remains unknown [99,102,103,105,108,115,116].

Table 4 Clinicopathological features, treatment sequency, and outcome of matched cases of synovial sarcoma (n=14)

No	Age (years)	Sex	Location	Subtype	Treatment sequency	LR-F	MR-F (months)	Follow-up
1.	58	F	Trunk	BSS	S*+R→S*→C→C→S*→R	12	18	33 DT
2.	38	F	Arm	BSS	S*+R→C→S*→C→S*→C →C→S→C→S→S→S		30	97 AWD
3.	22	F	Trunk	BSS	S*+C→S*+R→S→C→R→C→S*	17	72	93 DT
4.	17	M	Leg	BSS	S*→S*		48	76 NED
5.	25	M	Thigh	BSS	S*+R→C→S*→R→C		10	26 DT
6.	33	F	Trunk	BSS	S*→S*+R→C→S*+C→S→R+C	8	15	36 DT
7.	36	F	Foot	BSS	S*+R→S*→C→S*→C→C	24	36	63 DT
8.	63	M	Foot	BSS	S*→S*→C→S*→C→R	12	60	63 DT
9.	31	M	Trunk	MSS	S*+C→S*+R→C→R→C	15	21	30 DT
10.	42	M	Thigh	MSS	S*+R→C→R→S*+C+R		6	28 DT
11.	7	M	Thigh	MSS	S*+R+C→S*	28		104 NED
12.	43	M	Foot	MSS	S*→S*→S*→C→R→C	156	174	188 DT
13.	61	M	Foot	MSS	S*→S→S*→R	12	80	110 DT
14.	19	F	Arm	MSS	S*→S*+R→C	36	58	60 DT

F: female; **M:** male; **BSS:** biphasic subtype; **MSS:** monophasic subtype. *: surgical specimens studied by cytometry and immunohistochemistry; **NED:** no evidence of disease; **AWD:** alive with disease; **DT:** dead of tumor; **LR-F:** local relapse-free interval; **MR-F:** metastase-free interval

Table 5 Pgp and GST-pi immunoreactivity in matched cases of synovial sarcoma before and after chemotherapy

Case No	Subtype	Pretreatment Pgp	Pretreatment GST-pi	Posttreatment Pgp	Posttreatment GST-pi	Clinical response	Outcome
1	BSS	-	-	-	D	PR	DT
2	BSS	F+1	D	F+1	D	PR	AWD
3	BSS	-	-	-	-	PR	DT
5	BSS	F+2	F	F+2	-	NR	DT
6	BSS	-	-	F+2	F	PR	DT
7	BSS	-	M	-	D	NR	DT
8	BSS	F+2	M	F+2	D	PR	DT
9	MSS	F+1	M	-	-	NR	DT
10	MSS	-	-	-	-	CR	DT
12	MSS	-	F	-	-	NR	DT
14	MSS	-	-	-	-	NR	DT

Pgp: P-glycoprotein; **GST-pi:** Glutathione S Transferase-pi; **BSS:** Biphasic Synovial Sarcoma; **MSS:** Monophasic Synovial Sarcoma; **F+1/F+2:** Focal and weak/Focal and strong, respectively; **M:** Moderate; **D:** Diffuse; **CR:** Complete Response; **PR:** Partial Response; **NR:** No Response; **DT:** Dead due to the Tumor; **AWD:** Alive With Disease

GST-pi is a cytosolic protein. It belongs to a family of glutathione S transferases that serves as detoxification enzymes and intracellular binding proteins [82,106,107,110,112]. GST-pi is present in many human tissues and tumors [57-77,80,82,106,107,110,112,116]. Increased levels of GST-pi have been associated with resistance to a wide range of chemotherapeutic drugs [38,82,112].

In this study, expression of one or both markers (Pgp and GST-pi) was observed in almost half of the SS cases prior to treatment. Our results are consistent with those described in other human cancers unexposed to chemotherapy [42,43,51,67,76,104,108]. In the latter cases it has also been shown that the normal tissue counterparts of the cancerous tissues expressed low levels of both markers (Pgp and GST-pi)[42,43,51,67,76,104,108]

Variations in specificity and sensitivity of immunohistochemical Pgp detection have been described for some antibodies [118]. According to Gala et al [118], immunohistochemistry using JSB-1 is the most useful method being even more sensitive than Western blotting to detect single Pgp positive cells.

In our study of Pgp expression we used the monoclonal antibody (Mab) JSB-1 that recognizes a highly conserved intracellular epitope of Pgp [119]. It is likely that JSB-1 is as specific for the detection of Pgp (mdr1) as the antibody (Mab C494) used by Chan et al [46,48,49]. Both antibodies work in formalin-fixed paraffin-embedded tissues.

We used, as others [40,51], the ABC immunohistochemical method [86] whereas Chan et al used a modified immunoperoxidase "sandwich" staining method with three layers of secondary antibodies [120]. We do not know whether the aforementioned methodological differences between our study and that of Chan et al [46,48,49] may explain the existence of discrepant results regarding the putative usefulness of Pgp expression in predicting the clinical behavior of some types of sarcomas other than SS [53,54,56,79].

In some cases we observed cells with cytoplasmic immunoreactivity but, as Chan et al [46,48,49], we only considered cases showing membrane and/or Golgi-like staining as Pgp positive. We still dispute the significance of Pgp cytoplasmic immunoreactivity. It probably represent protein in transit from the Golgi apparatus to the plasma membrane [121].

Weinstein et al [121] reported that histo-blood group type of patients predicted the subcellular localization of Pgp based on the association between Golgi staining and histo-blood group A. In our study Golgi-like staining was observed only in tumors from patients with histo-blood group A.

It has been reported that some lots of commercially available antibodies for Pgp detection (including JSB-1) contained contaminant antibodies to blood group A carbohydrate determinants, and Finstad et al [122] suggested that this could explain some of the false-positive results obtained by using commercial lots. In our study we did not observe a significant association between Pgp expression and histo-blood group of the patients.

We observed that immunoreactivity for Pgp and GST-pi was significantly associated with the BSS subtype and was more intense in solid/glandular areas of this subtype of SS. These findings suggest an association between expression of Pgp and GST-pi and epithelial-type differentiation of SS. This suggestion is reinforced by our finding of Pgp immunoreactivity in epithelioid cells of MSS.

We did not find a significant association between the expression of Pgp and GST-pi and survival of SS. No significant association was also found between immunoreactivity of both markers and age and gender of the patients, dimension, location, and clinical progression of the tumors.

In all but two cases the immunoreactivity for Pgp did not change from primary tumors to local recur-

rences and metastases. In contrast to this, the GST-pi immunoreactivity varied extensively from primary tumors to recurrences and metastases. Notably, GST-pi immunoreactivity showed a tendency for increase with clinical progression of SS.

We did not observe any significant difference when comparing the proliferative activity of primary synovial sarcomas with the respective local recurrences and metastases. Many local recurrences and metastases seemed to have a higher proliferative activity (%S+G2, and PCNA and Ki-67 labeling) than the respective primary tumors, but in several cases the values were lower. These results should be interpreted carefully because we cannot estimate the degree of chemotherapy modulation occurring in treated patients.

Pgp and GST-pi protein expressions have been linked with resistance to several drugs, including adriamycin and etoposide [38]. Pgp has also been linked to vincristin [38] and GST-pi to cyclophosphamide resistance [38]. These anticancer agents were commonly used in patients included in the present study.

We did not find a clear influence of chemotherapy in the Pgp immunoreactivity, like Toffoly et al had already pointed out in 4 SS [76]. In most of the cases we observed a tendency for increased expression of GST-pi in local recurrences and metastases after chemotherapy, but in some cases the GST-pi immunoreaction decreased.

Furthermore, we did not observe, like other authors in various sarcomas [53,54,56,79], a significant association between Pgp positivity of primary tumors and poor clinical response to chemotherapy and survival. This is in contrast to observations reported in studies concerning other types of tumors, soft tissue sarcomas included [44,46,48,49,52,77]. However, we did find a (not significant) tendency for a higher prevalence of Pgp immunoreactivity in stage III than in stage I-II tumors and in cases that developed recurrences and metastases than in those that remained localized.

Conclusions

We observed a frequent expression of Pgp and GST-pi in untreated SS. These proteins were co-expressed in 25% of the cases.

Pgp and GST-pi expressions were significantly associated with BSS subtype and were more intense in solid/glandular areas. It remains to be explained why these markers have a stronger expression in the more epithelial differentiated components of synovial sarcomas. Golgi-like staining was exclusively found in tumors from patients with histo-blood group A but no significant association was observed between that expression and the histo-blood group of the patients.

We did not observe a significant association between the expression of Pgp and GST-pi with gender, and age of the patients, and dimension, location, and proliferative activity of the tumors. The expression of Pgp and GST-pi was not also significantly related to the survival curves of patients with SS.

Our results raise the possibility that Pgp and GST-pi immunoreactivity observed in SS represent an expression of intrinsic drug resistance phenotype; this possibility is difficult to reconciliate based on our results due to the absence of a significant association between expression of both markers and response to chemotherapy. The expressions of Pgp and GST-pi were not significantly influenced by the drugs used in the patients of our series. Our findings suggest that other drug resistance mechanisms are active in SS.

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CONCLUSIONS

For the sake of simplicity we will follow in this chapter the sequence of the Aims/Questions as outlined in the Introduction of this Thesis.

1. The histogenesis of epithelial-type differentiation in soft tissue tumors remains unexplained [12,20-23,39,49]. The same holds true for the co-expression of epithelial (e.g., keratins) and mesenchymal-type (e.g., vimentin) intermediate filaments which occurs in many cancers (e.g., carcinomas and soft tissue sarcomas), despite being considered "normal" in normal human fetal and adult tissues [1,4,31].

Most primary MSS and BSS express ultrastructural and immunohistochemical features of epithelial-type differentiation, in addition to mesenchymal-type features (Papers I and II).
Our ultrastructural results clearly show the existence of a spectrum varying from epithelial type differentiation (abortive glandular structures) to true epithelial differentiation (well-developed glandular structures) in all cellular components of both subtypes of SS (Paper I). The MSS epithelial and mesenchymal-type differentiation is similar to that of spindle cell component of BSS (Papers I and II). Apart from the exclusive immunostaining for keratin 13 and CEA of solid/glandular areas of BSS, which by definition are pathognomonic areas of BSS, no major differences were observed between the phenotypic epithelial-type differentiation of BSS and MSS (Papers I and II). Vimentin positivity was present in peripheral cells of solid/glandular component of BSS, in addition to being systematically present in spindle cell component of BSS as well as in MSS (Paper II).
BSS shows a tendency for depicting a monophasic morphology with clinical progression, and the immunohistochemical features of local recurrences and metastases suggest a down-regulation of the epithelial-type differentiation in both variants of advanced SS (Paper II).
The co-expression of mesenchymal-epithelial features in SS fits with an origin from a pluripotential mesenchymal stem cell that may differentiate, in variable proportions, into cells with mesenchymal and/or epithelial phenotypes. It is possible that the frequent expression of keratin genes in SS is modulated during the progression process of this peculiar soft tissue tumor.

Assuming that BSS and MSS share a common origin, we conclude that SS represents a sort of carcinoma of soft tissues, with a spectrum of (ultrastructural and immunohistochemical) features, ranging from simple epithelial-type (MSS and spindle cell component of BSS) to more complex epithelial-type (solid/glandular component of BSS) phenotype, depending on the degree of epithelial differentiation. From a phenotypic standpoint, our results comply with the possibility that MSS and BSS share a common histogenesis, and that MSS is similar to the spindle cell component of BSS.

2. Epithelial-mesenchymal interactions occur during embriogenesis of several organs and in many cancers [14,16,40]. Cell-matrix interactions play an important role in the regulation of cell differentiation (e.g., cytoskeleton phenotype, cell shape, and cell polarity) and tissue differentiation, and in the regulation of cell proliferation in normal tissues as well as in many cancerous tissues [7,8,17,19,21,28,43].

BSS shows light microscopical, ultrastructural, and immunohistochemical cytoarchitectural transitions between cells of solid/glandular component and the surrounding spindle cells (Papers I and II). Such transitions were also observed between the different (spindle and epithelioid) cell components of MSS (Papers I and II). BSS and MSS show similar co-expression of epithelial (e.g., individual keratins and EMA) and extracellular matrix markers (i.e., laminin and collagen IV) (Paper II). Transitions of cell shape and cell polarity, as well as of extracellular matrix, occurs at the solid/glandular to spindle cell component interface of BSS (Paper II).The cells at the transition between solid/glandular and spindle cell components of BSS show immunoreactivity for keratins (e.g., keratins 8 and 19), EMA, and vimentin (Paper II). The interface between solid/glandular areas and spindle cell areas of BSS shows focal absence of basal lamina (Paper I) and weak or absent immunostaining for laminin and collagen IV (Paper II), and thus, demonstrate the existence of a true contiguity between the two cell components of BSS.

Solid/glandular component of BSS displays similar proliferative activity (e.g., PCNA and Ki-67 labeling indices) to that of MSS, and higher proliferative activity than that of the spindle cell component of BSS (Papers III, IV, and V).

During clinical progression (from primary tumors to local recurrences and metastases) there is an inverse correlation between the expression of epithelial and extracellular matrix markers, and proliferation activity, in both subtypes of SS (Papers II, III, and IV).

We conclude that MSS and BSS co-express epithelial and mesenchymal features, and that proliferation activity is higher in the more epithelial differentiated areas of both subtypes of SS. Our results support the possibility of a phenotypic transformation and evolution between the cells of both (solid/glandular and spindle) cell components during progression of BSS.

3. The prognosis of patients with SS remains poor (50% five-year survival rate in average) despite improvements in the multimodality treatment ([3,5,6,11,15,18,20,24,25,27,32,34,35,37-39,41,43] - see point 4). There is still controversy regarding the validity of most of the parameters used in evaluating the prognosis of patients with SS [3,5,6,11,15,18,20,24,25,27,32, 34,35,37-39,41,43] .

Good control of local disease is related to lower rates of local recurrence (Paper IV). Most of the chemotherapeutic regimes used for the control of metastatic SS are associated with poor clinical response, mainly due to clinical drug resistance (Papers IV and V - see point 4).
Apart from TNM stage of the tumors, short duration of symptoms (less than 12 months), low mast cell content (<10/10 HPF), aneuploidy, and high proliferative activity (i.e., mitotic rate ≥ 10/10 HPF; PCNA labeling index ≥ 20%; and Ki-67 labeling index ≥ 10%) are features linked with poor prognosis of patients with SS on an univariate analysis (Paper IV). Patients with BSS have worse prognosis, but not independently from the TNM stage , than patients with MSS (Paper IV).

Out of the several aforementioned parameters, only the amount of mast cells, mitotic rate, or PCNA labeling, have significant prognostic meaning, in addition to TNM stage of SS, at the level of multivariate analysis (Paper IV) . Mast cell content is inversely correlated with proliferation markers and is probably involved in "suppression" of SS (Paper IV).

The results obtained in the clinicopathologic study described in Paper IV led us to explore the putative meaning of drug resistance in the relatively poor outcome of patients with SS.

4. Drug resistance of tumor cells may be related to many host and tumor factors (e.g., cell differentiation and proliferation, Pgp and GST-pi), besides differences in pharmakokinetics of different drugs used for the treatment of cancer [2,13,29,36].

Expression of at least one of two drug resistance markers (Pgp and GST-pi) is frequent (48.6%) and higher than their co-expression (25.0%) in both subtypes of SS before chemotherapy of the tumors (Paper V). Both markers of drug resistance were more frequently expressed in BSS (60% for Pgp and GST-pi), and more intense in solid/glandular component, which is the component with the highest proliferative activity, than in MSS (9.1% for Pgp, and 27.3% for GST-pi) (Paper V).

BSS shows a tendency for carrying worse prognosis than MSS, but this behavior is not independent of the stage of the tumors (Paper IV). Prevalence of Pgp expression is lower, but not significantly, in TNM stage I-II than in stage III tumors and also in cases without than in cases with clinical progression of both subtypes of SS (Paper V). Such differences do not occur with GST-pi expression (Paper V).

Expression of both drug resistance markers is neither correlated with shorter relapse-free interval nor with poor survival of patients with SS (Paper V). Pgp and GST-pi expressions neither predict the response to chemotherapy nor are influenced by chemotherapy (Paper V).

We conclude that Pgp and GST-pi are more frequently expressed in the cell components of both subtypes of SS that show an higher degree of epithelial differentiation (i.e., solid/glandular component of BSS and epithelioid cell component of MSS) and also a higher proliferative activity (i.e., high PCNA and Ki-67 labeling indices), probably reflecting an expression of the intrinsic drug resistance phenotype of SS. However, the expression of both markers is not associated with

the response to chemotherapy and clinical progression; this finding suggests that other drug resistance mechanisms may be active in SS.

Taking together the results of Papers IV and V we think there is evidence enough to claim that the poor prognosis of SS is mainly due to presence of subclinical metastases and intrinsic drug resistance at the time of diagnosis. New systemic therapeutic strategies are needed to overcome drug resistance in treating patients with SS. Criteria to select patients for therapy regimes should include the evaluation of the amount of mast cells and of proliferation rates as prognostic parameters, in addition to TNM stage.

UNSOLVED QUESTIONS/FUTURE PROSPECTS

We still do not know the mechanisms involved in the epithelial differentiation of SS, nor the mechanisms of the dual (epithelial-mesenchymal) phenotype characteristic of this soft tissue tumor. Another question that remains unanswered is why do the epithelial differentiated areas of SS show an higher degree of proliferation and a higher expression of drug resistance markers than spindle cells ? If one moves further in depth the main unsolved problem can be summarized as follows: Which are the genetic mechanisms involved in the oncogenesis of both histologic subtypes of SS ? Is SS a monoclonal or a polyclonal tumor? In the affirmative case, does this apply only to BSS or also to MSS? (In other words: Is there a solid genetic basis underneath the existence of the two strikingly different histologic subtypes of SS ?).

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SUMÁRIO E CONCLUSÕES

O sarcoma sinovial (SS) é uma neoplasia maligna rara de mau prognóstico que ocorre nos tecidos moles de adolescentes e adultos jovens. Pode apresentar três subtipos histológicos: "bifásico" (SSB), "monofásico" (SSM), e, menos frequentemente, "pouco-diferenciado" (SSPD). O SSB caracteriza-se pela co-existência de um componente sólido/glandular envolvido por células fusiformes (padrão bimórfico). O SSM caracteriza-se pela presença exclusiva de células epitelióides e/ou fusiformes (padrão monomórfico). O SSPD caracteriza-se pela presença de esboço de áreas bimórficas ocasionais e um padrão hemangiopericítico.

Numa tentativa de esclarecer a biopatologia da heterogeneidade do SS, efectuámos a avaliação fenotípica (diferenciação e proliferação), prognóstica e de resposta à terapêutica, de uma série de sarcomas sinoviais utilizando parâmetros de diversa índole e informações detalhadas sobre a evolução clínica dos casos. As diferentes vertentes do nosso estudo foram objecto dos seguintes trabalhos:

1. "The ultrastructural spectrum of synovial sarcomas: A study of the epithelial type differentiation of primary tumors, recurrences, and metastases".

Realizámos o estudo ultrastrutural de 25 tumores primários (12 SSB e 13 SSM), 5 recidivas locais (3 SSB e 2 SSM) e 2 metástases (2 SSB), para avaliar a expressão de marcadores de diferenciação de tipo epitelial (componentes intracelulares, junções intercelulares, e componentes da matriz extacelular) dos vários subtipos de SS.

Ambos os subtipos de SS (SSB e SSM) expressam marcadores de diferenciação de tipo epitelial. Observa-se um espectro de diferenciação epitelial, desde estruturas glandulares abortivas até estruturas glandulares bem diferenciadas, em todos os componentes celulares de ambos os subtipos de SS. As recidivas locais e metástases do SSB tendem a apresentar um aspecto morfológico monomórfico.

2. "Immunohistochemical profile of synovial sarcoma with emphasis on the epithelial-type differentiation. A study of 49 primary tumours, recurrences, and metastases".

Realizámos o estudo imuno-histoquímico de 34 tumores primários (15 SSB e 19 SSM), 7 recidivas locais (4 SSB e 3 SSM) e 8 metástases (7 SSB e 1 SSM) para avaliar a expressão de marcadores de diferenciação de tipo epitelial (queratinas) e mesenquimatoso (vimentina), e de componentes da matriz extracelular (laminina e colagénio IV).
Para além da expressão de queratina 13 e antigénio carcino-embrionário no componente sólido/glandular do SSB, não se observaram diferenças consideráveis entre a expressão dos marcadores epiteliais em SSB e SSM. A expressão de marcadores de diferenciação epitelial e mesenquimatosa e dos componentes da matriz extracelular no SSM é idêntica à observada no componente de células fusiformes do SSB. A co-expressão de marcadores de diferenciação epitelial e mesenquimatosa, e a escassez ou mesmo ausência de elementos da matriz extracelular nas áreas de transição sólido/glandular - células fusiformes traduz a existência de uma verdadeira contiguidade entre ambos os componentes do SSB. As recidivas locais e metástases de ambos os subtipos de SS apresentam uma diminuição da expressão de marcadores epiteliais, quando comparadas com os respectivos tumores primários.

3. "The proliferative activity of synovial sarcoma. An immunohistochemical evalua-tion of Ki-67 labeling indices of 52 primary and recurrent tumors".

Realizámos o estudo imuno-histoquímico da expressão de Ki-67 em 38 tumores primários (15 SSB e 23 SSM), 9 recidivas locais (4 SSB e 5 SSM) e 5 metástases (3 SSB e 2 SSM) para avaliar os índices de actividade proliferativa dos vários componentes celulares do SS.
O componente sólido/glandular do SSB exprime índices de actividade proliferativa significativamente superiores às dos observados no componente de células fusiformes do SSB. Tal diferença não se observa quando se comparam entre si os índices de actividade proliferativa dos componentes sólido/glandular do SSB, os do SSM e os das áreas monomórficas (epitelióides/fusocelulares) do SSB. De

igual modo, a comparação dos índices de actividade proliferativa de SS primários com os das recidivas não revelou diferenças significativas.

4. "Synovial sarcoma. DNA ploidy and proliferation (PCNA and Ki-67) markers in the evaluation of prognosis".

Realizámos o estudo de 49 casos (19 SSB e 30 SSM) de SS localizado, sem terapêutica prévia, para avaliar a relação entre parâmetros clinico-patológicos, de citometria (fluxo e estática) do ADN, e de actividade proliferativa (índices mitótico, PCNA, e Ki-67) com a duração do intervalo livre de recidivas e a sobrevida global dos doentes.
A análise univariada dos vários parâmetros estudados revelou que a duração curta dos sintomas (<12 meses), subtipo bifásico, escassez de mastócitos (< 10/10 campos de grande ampliação), presença de necrose, índice mitótico elevado (≥10/10 campos de grande ampliação), grau histológico de malignidade elevado (grau 3), índices PCNA e Ki-67 elevados (≥20% e ≥10%, respectivamente), aneuploidia, e estadiamento TNM avançado (estadio III) são factores de mau prognóstico (intervalo livre de recidiva e sobrevida global mais curtos).
Na análise multivariada dos parâmetros referidos, apenas a quantidade de mastócitos do tumor e o índice PCNA fornecem informação prognóstica adicional à do estadiamento TNM.
Obervou-se uma correlação inversa entre a quantidade de mastócitos e a actividade proliferativa do SS.

5. "Synovial sarcoma. Immunohistochemical expression of P-glycoprotein and glutathione S transferase-pi and clinical drug resistance".

Realizámos o estudo imuno-histoquímico de 37 casos (15 SSB e 22 SSM) de SS primários, antes e depois da instituição de quimioterapia, para comparar a expressão de marcadores de resistência à quimioterapia (Pgp e GST-pi) com parâmetros de citometria de fluxo (ploidia e % de células em fase S+G2) e índices de actividade proliferativa (PCNA e Ki-67). Avaliámos ainda a relação dos parâmetros atrás referidos com os seguintes parâmetros clinico-patológicos: sexo, idade, histo-grupo sanguíneo, dimensão, localização, subtipo histológico, estadia-

mento TNM, resposta clínica à quimioterapia, intervalo livre de recidiva e sobrevida global dos doentes.
Observámos expressão de Pgp e GST-pi em 29,7% e 40.5% dos casos, respectivamente, e co-expressão de ambos os marcadores em 25,0% dos casos.
A expressão de Pgp e GST-pi é significativamente mais frequente no componente sólido/glandular do SSB. Não se observou associação significativa entre a expressão de qualquer dos marcadores de resistência à quimioterapia e os restantes parâmetros estudados.

Conclusões

1. A co-expressão de características epiteliais e mesenquimatosas, em ambos os subtipos de SS, coaduna-se com uma provável origem comum em células mesenquimatosas primitivas (células "stem") com potencial de diferenciação epitelial e/ou mesenquimatosa durante o processo de progressão neoplásica do SS.

2. O SS representa uma forma de carcinoma das partes moles com um espectro morfológico de diferenciação que varia desde um fenótipo epitelial simples (SSM e componente fusocelular do SSB) até um fenótipo epitelial complexo (componente sólido/glandular do SSB).

3. Há uma relação inversa entre diferenciação epitelial e actividade proliferativa nos vários componentes celulares do SS. É provável a existência de uma evolução fenotípica entre as células do componente sólido/glandular e do componente fusiforme do SSB durante a progressão neoplásica.

4. Embora a expressão de Pgp e GST-pi em células do SS possa representar um fenótipo de resistência intrínseca à terapêutica, tal fenótipo não é um bom indicador para prever os resultados da quimioterapia nem a morbilidade e/ou mortalidade dos doentes.

5. O mau prognóstico dos doentes com SS resulta, em grande parte, da ineficácia em ultrapassar a resistência à terapêutica, associada à existência de metástases subclínicas na altura do diagnóstico. Os critérios de elegibilidade para regimes terapêuticos (alternativos) deverão incluir, para além do estadiamento TNM, a avaliação não só de parâmetros de resposta do hospedeiro (ex: quantidade de mastócitos) como do tumor (ex: actividade proliferativa) em doentes com SS.

Solicitou-se à JNICT um subsídio para custear parte das despesas com a publicação da presente Dissertação

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