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ORIGINAL ARTICLE

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Cytogenetic abnormalities of alveolar soft-part sarcomas using interphase fluorescent in situ hybridization: trisomy for chromosome 7 and monosomy for chromosomes 8 and 18 seem to be characteristic of the tumor

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Abstract Four alveolar soft-part sarcomas were investigated by means of standard immunohistochemistry and interphase cytogenetics to further characterize the immunophenotype and proliferative activity of this tumor. The main goal of this study was to explore the chromosomal changes of this rare soft-tissue sarcoma. One epithelial (KL1), three neurogenic [neuron specific enolase (NSE), PGP 9.5, and S100], and five myogenic (desmin, myoglobin, α-smooth muscle actin, α-sarcomeric actin, and MyoD1) markers were used for the immunophenotypical analysis. Proliferative activity was assessed using the Ki67 index. Twelve (peri)centromeric (1, 3, 4, 6, 7, 8, 10, 12, 15, 17, 18, and X) and one telomeric (17q25-qtel.) chromosomal probes were used for interphase cytogenetic analysis. Three of the cases showed cytoplasmic desmin and/or myoglobin, and one showed smooth muscle actin positivity. All of the four tumors had granular, cytoplasmic, possibly nonspecific MyoD1 and sarcomeric actin positivity. Two of the tumors were positive for vimentin, four gave focal and weak staining with neurogenic markers (four of four NSE, one of four S100, and four of four PGP 9.5), but none of them was positive with KL1. Alveolar soft-part sarcomas may show myogenic immunophenotype in a number of cases, which supports myogenic differentiation. Fluorescent in situ hybridization using alpha satellite chromosomal probes revealed significant alterations in all of the cases. Most frequent and repeated numerical changes, which seem to be characteristic of the neoplasm and may play an important part in its pathogenesis and/or progression, were trisomy 7, monosomy 8 and monosomy 18.

Keywords Alveolar soft-part sarcoma · FISH · Chromosome 1, 6, 7, 8, 18, 17q25-qtel. · Trisomy · Monosomy

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Introduction

Alveolar soft-part sarcoma (ASPS) is a rare, malignant soft tissue neoplasm of unknown origin, occurring mainly in children and young adults [12, 27]. Numerous attempts have been made to define its tissue or cell origin. Ultrastructural analyses revealed the presence of the highly specific crystalloids, consisting of characteristically arranged actin filaments [3, 13, 31]. This fact raised the possibility of its muscle origin. However, some authors regarded the neoplasm as neurogenic [13, 23, 48]. The immunohistochemical analysis has also led to controversial results. The majority of the authors support the muscle origin, but some others point out the absence or rare occurrence of muscle markers in these neoplasms [1, 8, 15, 16, 19, 30, 32, 36]. Still, another group of authors did not regard the desmin positivity as a safety sign of muscle differentiation [11, 20].

A limited number of studies describing some virtually characteristic chromosomal abnormalities have appeared in the medical literature and, so far, no particular gene or genes have been identified as responsible for the genesis of the tumor [4, 26, 43]. Some authors came to the conclusion that the loss of chromosome 17 or the involvement of the distal segment of the q arm of chromosome 17 may play a crucial role in the development of this neoplasm [18, 21, 41, 43, 46]. Complex chromosomal abnormalities were also found to add to the complexity of the issue, multiplying the number of genes and

Table 1 The clone and the type of labeling of the one telomeric and twelve centromeric probes used for the study. The last column demonstrates the applied developing system. *FISH* fluorescent in situ hybridization

Chromosome	Clone	Labeling	Developing system
1	PUC1.77	Digoxygenin	Antidigoxygenin/rhodamine
3	pα3.5	Biotin	Avidin/FITC
4	MCG4–01	Biotin	Avidin/FITC
6	MCG6–01	Biotin	Avidin/FITC
7	p7t1	Digoxygenin	Antidigoxygenin/rhodamine
8	pJM128	Biotin	Avidin/FITC
10	D10Z1	Digoxygenin	Antidigoxygenin/rhodamine
12	pα12H8	Biotin	Avidin/FITC
15	D15Z1	Digoxygenin	Antidigoxygenin/rhodamine
17	p17H8	Biotin	Avidin/FITC
17q25-qtel.		Digoxygenin	Antidigoxygenin/rhodamin
18	L1.84	Biotin	Avidin/FITC
X	pBamX5	Digoxygenin	Antidigoxygenin/rhodamine

Table 2 Chromosomal alterations compared with the normal genome. Chromosomal changes in tumor cases are given in percentage. Only the values of more than control mean+3×SD are shown. The values of 10% or above are highlighted. The empty boxes mean normosomy. The 8% disomy for chromosome X occurred in a male patient

Chromosome	Type of change	Control+3SD	Case 1	Case 2	Case 3	Case 4
1	Trisomy	1.4+1.05%	13%	19%	7%	4%
3	Trisomy	3.5+4.05%		13%	9%	
	Tetrasomy	0.2+0.6%			8%	4%
4	Monosomy	3.3+4.8%		27%		9%
	Trisomy	0.8+2.7%			12%	
6	Monosomy	3.3+6.6%	11%	11%	12%	
7	Monosomy	3.3+0.6%				13%
	Trisomy	0.7+1.05%	10%	13%	54%	30%
	Tetrasomy	0%	4%		9%	
8	Monosomy	2.7+3.45%	18%	12%	22%	23%
	Trisomy	0.5+0.6%		8%	7%	
	Tetrasomy	0%			7%	
10	Monosomy	2.9+3.15%		13%		
	Tetrasomy	0%			8%	
12	Monosomy	3.1+2.63%	12%		9%	
17q25-qtel.	Monosomy	0%	15%			
18	Monosomy	4.8+4.8%	18%	11%	19%	18%
X	Disomy	0%			8%	

chromosomal regions that may be involved in the tumorigenesis. Although some cytogenetic studies on ASPS using conventional G-banding have been published, the number of investigated metaphase cells was few. It is also questionable whether some metaphase cells could represent a tumor with low proliferative activity in which the great majority of the cells are in interphase. The aim of the present study was to utilize the chromosomal fluorescent in situ hybridization (FISH) on interphase nuclei, confirming the data obtained from conventional cytogenetic analyses, and describing new types of changes in this rare soft-tissue sarcoma.

Materials and methods

Light microscopy

The tissue blocks were fixed, processed, and stained according to the standard hamatoxylin and eosin (HE) histological protocol. Monoclonal and polyclonal antibodies were used to define the immunophenotype. The neuron specific enolase (NSE), S100, vimentin, α-smooth muscle actin (SMA), α-sarcomeric actin (SRCA), desmin, and myoglobin were purchased from Dako (Denmark), the KL1 and Ki67 were from Immunotech (France), the MyoD1 was from Novocastra (UK), and the PGP 9.5 was from Biogenesis (UK). In all of the cases, the recommended dilutions were applied. As a developing system and substrate, Vectastain Universal ABC kit and 3-amino-9-ethylcarbazole were used, respectively. In all of the immunohistochemical reactions, except with MyoD1, microwave pretreatment (700 W, three 5-min treatments) in standard citrate buffer was applied. Antigen retrieval for MyoD1 was pressure cooking in the same solution. Ki67 index was calculated by counting 1000 cells and expressing the number of positive nuclei as a percentage. For all of the immunoreactions, positive and negative slides were used to rule out the false results.

Probe preparation and interphase cytogenetics

For interphase cytogenetic analysis (IPC), cell nuclei were isolated from the formol-paraffin blocks of the tumors. Four sections (50-μm thick) were cut off the blocks and, following the conventional rehydration procedure, the slices were digested in 2 ml 0.5% pepsin (pH 1.5) solution at 37°C for 60–90 min. The digestion was checked microscopically, and the process was stopped with cold phosphate-buffered saline (PBS). Nuclear suspension was filtered and sedimented. The sediment was resuspended in 0.01% citrate buffer, treated in a microwave oven (700 W) for 5 min, and the aliquots were cytocentrifuged to glass slides. Twelve FISH probes specific for the (peri)centromeric regions of chromosomes 1, 3, 4, 6, 7, 8, 10, 12, 15, 17, 18, and X) and one telomeric probe specific for the 17q25-qtel. region (digoxigenin-labeled, purchased from the Oncor) were used (Table 1). DNA probes not giving a sufficiently strong hybridization signal (probe for chromosome 9, 11, 16, 20, and Y) and the cross-reacting probes (5/19, 13/21, and 14/22) were excluded from the study. The DNA probes were labeled by means of nick translation using biotin- (chromosomes 3,

4, 6, 8, 12, 17, and 18) or digoxygenin-conjugated dUTP (Boehringer) (chromosomes 1, 7, 10, 15, X). References of the probes, methods of probe labeling and ISH were published previously [33]. Probes with similar signal intensity were paired and cohybridized (double target ISH). In the course of ISH, 5 µl hybridization mixture containing 10 ng probe/60% formamide in 2× sodium saline citrate (SSC; Baker) were dropped onto the cell preparations and covered with plastic film. Samples and probes were denatured simultaneously at 90°C for 10 min followed by hybridization at 37°C in a wet chamber overnight. A post-hybridization wash was performed under the following conditions: three 5-min washes in 60% formamide/2×SSC (Baker) at pH 7, 37°C, and three 5-min washes in 2×SSC at room temperature. Biotin-labeled probes were detected by means of avidin-fluorescein isothiocyanate (FITC; Vector), and the digoxygenin-labeled probes were detected by means of anti-digoxygenin-rhodamine (Boehringer) developing systems, resulting in green and red fluorescence. Finally, the preparations were mounted with Vectashield mounting medium containing 0.02 µg/ml DAPI (4'-6'-diamidino-2-phenylindole) nuclear counterstain (Vector Lab). In all of the cases, the recommended quantities and dilutions were used.

Evaluation of the hybridization signals

A minimum of 100 cells per slide were examined and evaluated. Signals were counted only in intact and not overlapping nuclei. Peripheral blood mononuclear cells isolated from four healthy individuals served as controls to establish the normal distribution of the hybridization signals. Lymphoid cells isolated from formol–paraffin blocks of a reactive lymph node showed the same distribution of signals. Hybridization signals in the control (mean +3×SD) and the detected results in the tumor samples were compared, and the mono-, tri-, and tetrasomy values found in the tumor cases were accepted when these were more than the mean control +3×SD. Chromosomal numerical changes were given in percentage of the examined nuclei and were regarded as biologically relevant as they reached the 10% level. Only the values more than 10% were highlighted in Table 2.

Results

Patients, clinical data and the gross description

Four patients (two males: 25 years and 30 years of age and two females: 14 years and 63 years of age) with ASPS were involved in the study. The tumors localized to the volar surface of the right forearm, to the tongue, to the left calf, and to the left thigh, respectively (Table 3). All of the tumors appeared as a solid, relatively well-circumscribed, grayish–white mass with the largest diameter of 55, 33, 70, and 110 mm.

Light microscopy

All of the tumors show the same, characteristic pattern. The tumor cells have centrally or excentrically placed nuclei, only one prominent nuleolus, and a large, rounded or polygonal, eosinophilic cytoplasm. The cells arrange in alveolar spaces separated by fine, sinusoid-like channels. Periodic acid–Schiff (PAS)-D staining reveals enzyme-resistant, PAS-positive, typical crystalloid structures and/or delicate, "dust-like" granules varying from case to case (Fig. 1). The immunohistochemical profile of the tumors is summarized in Table 4. Two of the four tumors show strong, diffuse vimentin and focal, moderate to strong desmin reaction (cases 2 and 3; Fig. 2). Also, two cases are moderately positive for myoglobin (cases 1 and 2), and only one demonstrated SMA positivity. All of the four tumors are SRCA positive, showing a cytoplasmic granular pattern. (Fig. 3). The MyoD1 reveals a non-specific, granular cytoplasmic reaction in all of the four cases. In the case of the neurogenic markers, the staining is variably intense. In addition, four of four cases were NSE (moderate, partly granular) and PGP 9.5 (focal, sometimes granular) positive, since only one of four was S100 positive. All of the cases were KL1 negative and only two of four had limited proliferation activity at the periphery demonstrated by the Ki67 reaction (Table 4).

Table 3 Clinicopathological data of the four alveolar soft-part sarcomas

	Case 1	Case 2	Case 3	Case 4
Gender	Male	Female	Male	Female
Age	25 years	14 years	30 years	63 years
Localization	Forearm	Tongue	Calf	Thigh
Size	55 mm	33 mm	70 mm	110 mm

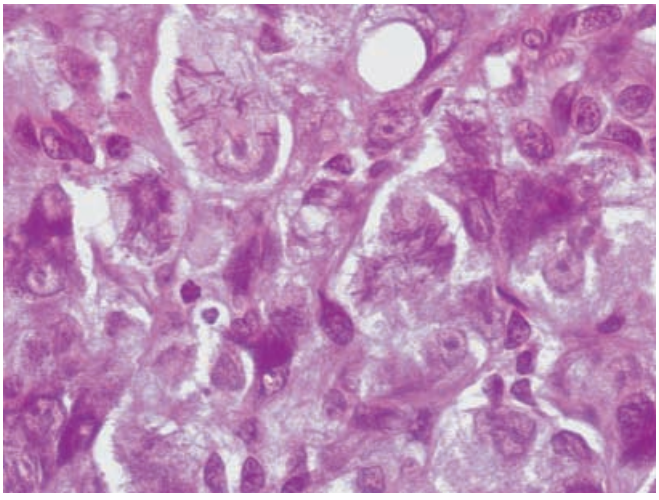

Fig. 1 Numerous diastase-resistant periodic acid–Schiff (PAS-positive crystalloid particles of variable size) could be seen in the cytoplasm of the tumor cells. Note the characteristic prominent nucleoli. Case 1; PAS-D; 400× original magnification

Interphase cytogenetics (FISH)

Each case shows significant chromosomal alterations compared with the normal genome. Aneusomy appears in the form of mono-, tri-, or tetrasomy, showing one, three, or four ISH signals, respectively. Data obtained are summarized in Table 2. All four of the cases show trisomy for chromosome 1 and, in two of them, the trisomic population exceeds 10% (13% and 19%, case 1 and case 2; Fig. 4) In case 1 (similar to case 4), the tetra-

Table 4 Immunprofile of the four alveolar soft-part sarcomas. See the muscle specific markers. *SRCA* α–sarcomeric actin; *SMA* α-smooth muscle actin; *NSE* neuron specific enolase

Antibody	Case 1	Case 2	Case 3	Case 4
Vimentin	–	+	+	–
Desmin	–	+ (Focal)	+ (Focal)	–
Myoglobin	+	+	–	–
α-Sarcomeric actin	+ (Granular)	+ (Granular)	+ (Granular)	+ (Granular)
α-Smooth muscle actin	–	+ (Focal)	–	–
MyoD1	–	–	–	–
Neuron specific enolase	+ (Focal)	+	+	+
S100	–	–	+ (Focal)	–
KL1	–	–	–	–
PGP 9.5	+ (Focal)	+ (Focal)	+ (Focal)	+ (Focal)
Ki67 (mib1)	Below 1%	8%	Below 1%	14%

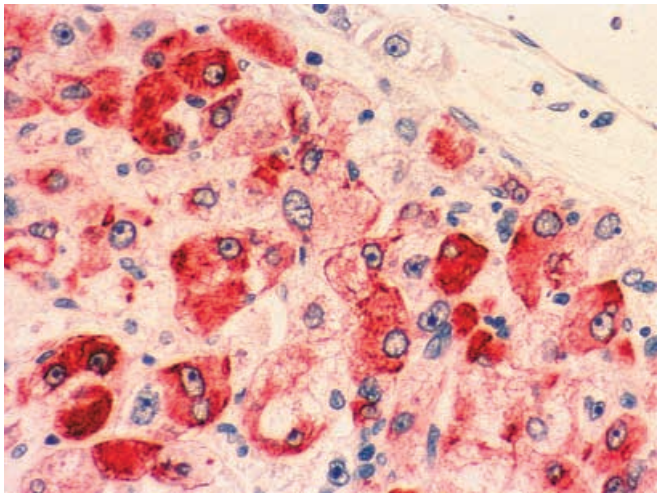

Fig. 2 Focal, strong intracytoplasmic immunoreaction of the tumor cells. Case 3; desmin; 200× original magnification

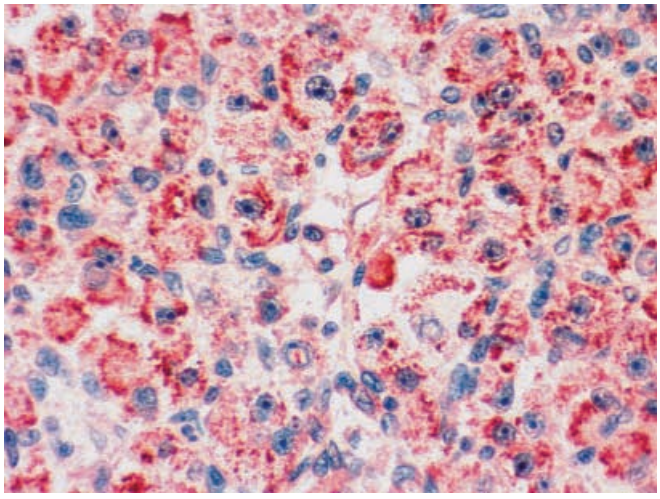

Fig. 3 Diffuse, granular intracytoplamic reaction in the cells arranged in typical alveolar pattern. Case 2; α-sarcomeric actin; 100× original magnification

somy for chromosome 1 reaches 6% which, together with the trisomic population, is 19%, showing a significant hypersomy for this chromosome. Case 2 and case 3 show 13% and 9% trisomy for chromosome 3, respectively. Case 3 also shows 8% tetrasomy, resulting in a 17% hypersomic cell population for this chromosome. Case 2 and case 3 exhibit 27% mono- and 12% trisomy for chromosome 4, respectively. In three cases (case 1, 2, and 3), 11%, 11%, and 12% monosomy can be registered for chromosome 6. In all of the cases, gains were characteristic for chromosome 7. Trisomy is found in 10%, 13%, 54%, and 30% in cases 1, 2, 3, and 4, respectively. (Fig. 4) In addition to the 54% trisomy in case 3, 9% tetrasomy can also be observed, increasing the hypersomic population up to 63%. Monosomy (13%) for chromosome 7 was also detected in case 4. Both chromosomal loss and gain are found for chromosome 8: monosomy can be seen in case 1 (18%), case 2 (12%), case 3 (22%), and case 4 (23%). However, chromosomal gain, namely 8% and 7% trisomy, is also found in case 2 and case 3, respectively. In addition to the 22% monosomy, 7% trisomy and 7% tetrasomy are observed in case 3, increasing the aneusomic population up to 36%. Changes for chromosome 10 are monosomy in 13% of the cells in case 2 and tetrasomy in 8% of the tumor cells in case 3. Monosomy for chromosome 12 in case 1 and case 3 is observed in 12% and 9% of the tumor cells, respectively. All of the other cases are disomic for these chromosomes. No changes can be detected using the centromeric probes for chromosomes 15 and 17, compared with the control cell population. Contrary to chromosomes 15 and 17, in the case of chromosome 18, monosomy can be found in all of the tumors (18%, 11%, 19%, and 18% in cases 1, 2, 3, and 4 respectively). One male tumor shows 8% disomy for chromosome X, which is the only change discordant to the gender of patients. Hybridization with the telomeric 17q probe (17q25-qtel.) results in loss of one signal in 15% of the tumor cells in case 1, but all of the other three tumors show two signals per cell (Fig. 4).

Discussion

Christopherson et al. described a unique, characteristic, and distinct soft tissue sarcoma in 1952. Since that time, this tumor, called ASPS, has been a permanent target of debates in relation to its cellular origin. ASPS accounts for less than 1% of all soft tissue sarcomas [27]. It oc-

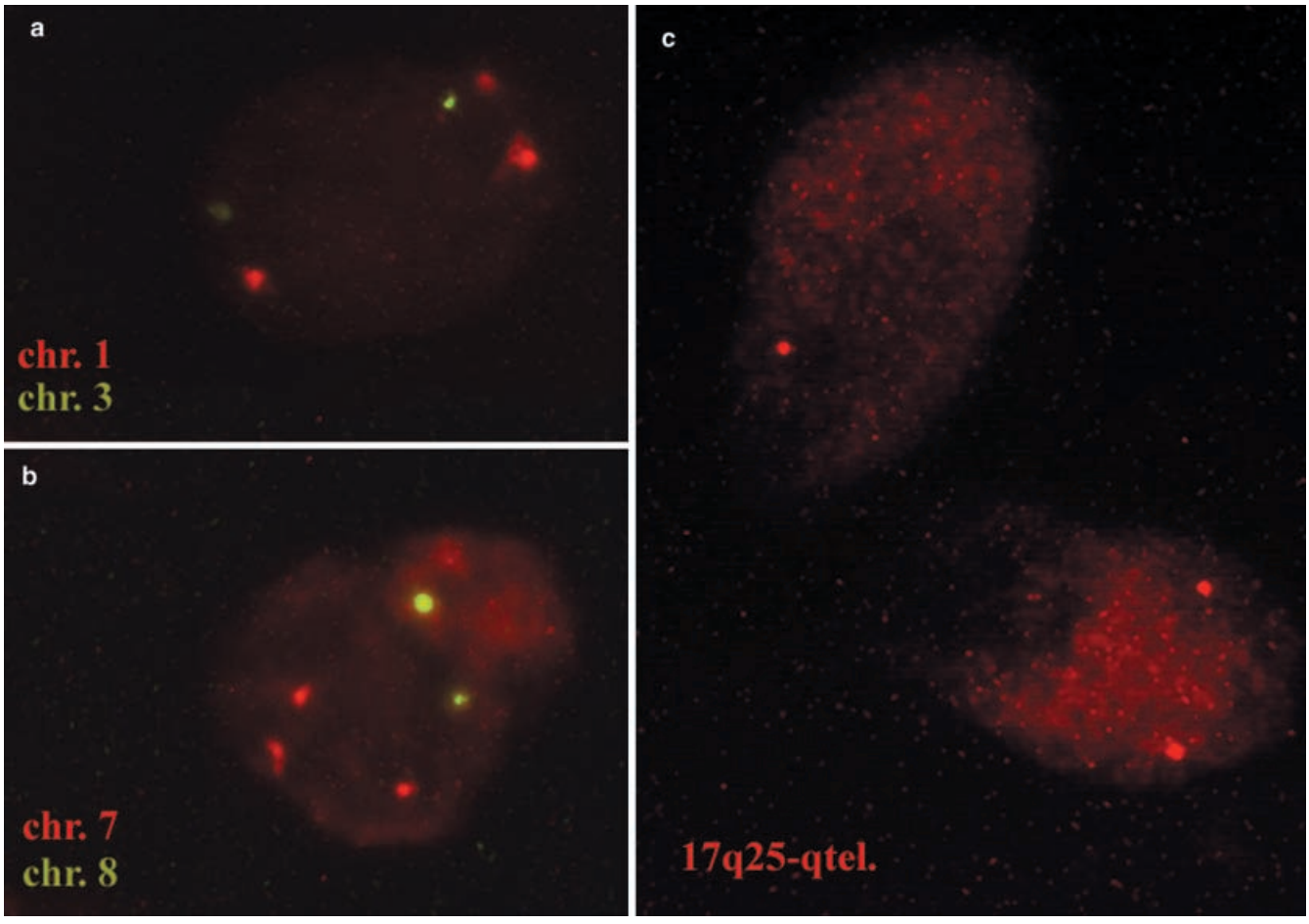

Fig. 4 Fluorescent in situ hybridization (FISH) on isolated cell nuclei. Three *red signals*, beside the normal two *green signals* for chromosome 3 (**a**) and chromosome 8 (**b**), represent trisomy for chromosome 1 (**a**) and chromosome 7 (**b**). Loss of one *red signal* in one of the nuclei demonstrates the deletion of a distal part of the long arm on chromosome 17 (**c**). Case 1(**a**, **c**) and case 2 (**b**), two color FISH with centromeric (chromosomes 1, 3, 7, and 8) and telomeric (17q25-qtel.) probes

curs mainly in the extremities, on the trunk, and in the head and neck regions, it grows slowly, and may metastasize to the lung, brain, and kidney sometimes a long time after the initial presentation [11, 28, 35]. Rare sites, such as the uterus, the mediastinum, and bones have also been described [14, 34, 38].

The majority of the pathologists dealing with this tumor tried to present evidence for its myogenic origin [3, 7, 13, 15, 16, 19, 27, 30, 31, 32, 36]. Some other "lines of differentiations" (paraganglioma, granular cell myoblastoma, neural neoplasm, and angioreninoma) – although only transiently – have also been raised in the literature [9, 23, 32]. In spite of the number of publications on ASPS, none of them served sufficient evidence for the real origin, resulting in its classification as sarcoma of uncertain origin. Our immunohistochemical findings do not help to decide the question. However, they point to the possibility of the myogenic differentiation, since about 75% of the cases investigated were desmin and/or myoglobin positive (case 1 was positive only for myoglobin, case 3 was positive only for desmin, and case 2 was positive for desmin, myoglobin, and SMA). Expression of some other muscle-specific markers, such as SRCA and MyoD1 is also of interest. Both showed fine granular pattern similarly to some fields of NSE and PGP 9.5 stained sections, which probably indicates a rather nonspecific reaction. In the case of MyoD1, which is an intranuclear myogenic regulatory protein, the data are controversial [10]. Rosai et al. reported on a case expressing strongly the MyoD1 antigen in the nuclei of the tumor cells, but other authors could not confirm this result [17, 40, 47]. These controversial results may be explained by the different fixation and/or antigen retrieval procedures. Using standard formol–paraffin blocks and pressure-cooking pretreatment, none of the present cases showed intranuclear positivity for this antibody. These and the former data point to the capability of ASPS to differentiate towards myogenic lineage in a certain number of cases. SRCA seems to be a reliable marker for skeletal muscle differentiation [42]. In all of our cases, this antigen was strongly expressed in the cytoplasm, exhibiting a granular pattern, which was similar to that of MyoD1 and some neurogenic markers. This may raise the possibility of a nonspecific reaction. Further investigations of SRCA, including Western-blot analysis, have to be performed to characterize this issue.

The focal positivity with the neurogenic markers (NSE, PGP 9.5, and S100) do not prove the neurogenic origin, since it is well known that a positive reaction can be detected in a significant portion of other, e.g., muscle tumors or even on normal striated muscle [5, 22, 29, 44]. Ultrastructural analysis of this neoplasm revealed a unique, characteristic, electron-dense crystalloid structure, consisting of actin filaments. However, there are "crystal deficient" cases in the literature [45]. There is only one report in which the ultrastructural and immunohistochemical analyses were combined. These results showed that the desmin and vimentin were coexpressed on the intermediate filaments of the tumor cells. Based on these data, the conclusion was that ASPS is a primitive myogenic tumor that does not show rhabdomyogenic or leiomyogenic differentiation [19].

Comparing our newly obtained data to those found in the literature of ASPS, both similarities and discrepancies were found. Until now, 13 cases of ASPS have been investigated by means of comparative genomic hybridization (CGH), which resulted in repeated changes, including gains on 1q, 8q, 12q, and 16p chromosome arms. Similar to the results above, gains were found to be common in our series but in the form of repeated trisomies. Altogether, eight further cases were examined by means of conventional karyotyping and one of them also by FISH using a painting probe for chromosome X and centromeric and telomeric probes for chromosome 17. In six of the eight cases, abnormalities of chromosome 17, mainly on the segment of q25 (e.g., addition, duplication, translocation from Xp11) were found, raising the possibility that this particular region may encrypt gene(s), that is/are important in the pathogenesis of this tumor. [6, 18, 21, 26, 41, 43, 46]. Subtle rearrangements cannot be detected with interphase FISH using the commercially available probes, but the loss of the chromosome or the distal segment (17q25-qtel.) can certainly be detected using this method. This may explain why, in the present study, the loss of the 17q25-qtel. segment was found in one case only (case 1). In addition, our findings do not exclude the possibility of other, smaller alterations in this region in the other three cases. Our results also do not rule out the possible role of genes at this segment in the development or progression of ASPS but rather point to the low chance of large deletions or loss of chromosome 17 in these tumors.

Moreover, trisomy 12, 5, and 8 (the later as a single clonal karyotypic abnormality) were found in three separate cases in the literature [4, 6, 43]. Only the later change, the trisomy 8, could be registered in 8% of the nuclei of case 2 from our series. This change cannot be regarded as specific for this sarcoma type, since it could be found in other soft tissue tumors, such as clear cell sarcoma, myxoid liposarcoma, Ewing's sarcoma, or congenital fibrosarcoma [4].

Among the variable chromosomal changes found in this study by means of interphase FISH, the virtually most significant were the trisomy for chromosome 7 (four of four cases) and monosomy for chromosomes 8 and 18 (four of four cases each). Monosomy for chromosome 6 and trisomy for chromosome 1 were less frequent but repeated changes in this tumor. Although these numerical changes occurred in relatively large cell populations, and they seem to be characteristic of the tumor, the significance of them is still unknown. These findings may suggest that important gene(s) located on chromosome 1, 6, 7, 8, and 18 may be involved in the pathogenesis and/or clonal evolution of ASPS. So far, trisomy 7, trisomy 1, and monosomy 6, 8, and 18 described above have not been observed in ASPS. At present, there is no established relationship between the individual numerical or structural chromosomal changes and the clinical progression or metastatic capability of the neoplasm, since the number of cases involved in molecular studies is few, and there are no relevant clinical follow-up data in such cases. Although the above alterations seem to be characteristic of ASPS, these are not specific, since other non-related tumors also demonstrated similar numerical abnormalities. For example, in a part of primary and metastatic malignant melanomas, besides the loss of 1p36, overrepresentation of chromosomes 7 and 1 were found [7, 37]. In other studies, trisomy 7 and trisomy 1 were repeated lesions in gastric, oesophageal, and colorectal or high-grade localized prostate adenocarcinomas and papillary renal cell carcinomas [2, 24, 25, 39]. Therefore, these alterations may probably indicate, rather, the progression or clonal evolution of ASPS. Monosomy for chromosome 18 has also been found in colorectal tumors. However, monosomy for chromosome 6 has not yet been mentioned as a significant numerical aberration found in any tumor [24].

Reviewing the data presented, it is also worthy to note that the examined cell (nuclear) population is mixed: the tumor cells are diluted by, for example, stromal and endothelial cells. Therefore, the changes detected certainly under represents the true frequency of the genetic aberrations. The method applied for studying ASPS published in the literature is mainly conventional karyotyping using metaphase spreads. Cell nuclei used here for FISH are in interphase. This population represents better the entire tumor relative to a minor, mitotic population in a tumor with a rather low proliferative activity (see Ki-67 index in Table 4). Thus, the interphase cytogenetic examination of a tumor completes the data of conventional cytogenetics. The results obtained by applying this wide panel of centromeric and telomeric probes can add to our knowledge about ASPS and provides new data for the possible pathogenesis.

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