

## Array-comparative Genomic Hybridization Analysis of Alveolar Soft Part Sarcoma

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**Background :** Alveolar soft part sarcomas (ASPSs) are rare, histologically distinctive soft tissue sarcomas of unknown origin. Although ASPSs are characterized by a specific alteration, der(17)t(X;17)(p11;q25), the entire spectrum of genetic events underlying the pathogenesis of ASPS is unclear. Using array-based comparative genomic hybridization (array-CGH), we examined the DNA copy number changes in ASPSs. **Methods :** Array-CGH, composed of 4,030 clones, was performed in two samples of fresh frozen tumor tissues from a 29-year-old male and a 16-year-old female. **Results :** We identified 16 commonly altered chromosomal regions involving 25 genes. Eleven altered regions were located on chromosome Xp (Xp22.33, Xp22.11, Xp11.3, Xp11.3-Xp11.23, Xp22.2, Xp22.12, Xp22.31, Xp22.32, Xp21.1, Xp21.3, and Xp11.4). Additional regions with an increased copy number were observed at 1q25.1, 7q35, 12p12.1, and 17p11.2. Loss was found in only one region of chromosome 22q11.23. Several genes located within the amplified region of Xp included *GYG2*, *ARSD*, *ARSE*, *ARSH*, *UBE1*, *USP11*, *PCTK1*, *ARAF*, *SYN1*, *TIMP1*, *XK*, *PDK3*, *PCYT1B*, *PHEX*, *ARX*, *RPS6KA3*, *TMSB4X*, *TMEM27*, *BMX*, and *KAL1*. **Conclusions :** This was the first application report of genome-wide copy number changes by BAC array-CGH in ASPSs. Our study showed unique genomic regions and new candidate genes that suggest a neural origin and are associated with tumor pathogenesis in ASPSs.

**Key Words :** Sarcoma; Alveolar soft part; Comparative genomic hybridization

Alveolar soft part sarcomas (ASPSs) are rare, malignant soft tissue tumors of unknown histogenesis that were first described and named by Christopherson *et al.*<sup>1</sup> in 1952. Most ASPSs occur in adolescents and young adults, with a predilection for females. Histologically, ASPSs have a distinctive appearance, usually consisting of the proliferation of large, polygonal cells arranged in a prominent alveolar pattern. Intracellular PAS-positive crystals are characteristic of ASPSs. Although the tumor exhibits a relatively indolent clinical course, the ultimate prognosis is poor with an unexpectedly high metastatic activity.

The histogenesis of ASPS is thought to have a myogenic, neural, or neuroendocrine origin.<sup>2-4</sup> However, the cellular origin of ASPS has not been determined; immunohistochemical analysis plays a limited role in the diagnosis of ASPSs. ASPSs are characterized by an unbalanced translocation [der(17)t(X;17)(p11;q25)]. This translocation results in a novel fusion of the ASPL

gene on chromosome 17 and the *TFE3* gene on X chromosome.<sup>5</sup> Consequently, a novel ASPL-*TFE3* fusion protein seems to act as an aberrant transcription factor. Nonetheless, the entire spectrum of genetic events underlying the pathogenesis of ASPS is unclear. Moreover, only a few molecular genetic studies have been performed on ASPSs, such as the DNA copy number change by comparative genomic hybridization (CGH),<sup>6</sup> a loss of heterozygosity (LOH) study of tumor-suppressor genes,<sup>7,8</sup> and oligomicroarrays for angiogenesis-promoting genes.<sup>9</sup>

Array-CGH is a powerful tool for the rapid and accurate detection of specific gene copy number changes, allowing the identification of novel candidate genes in tumorigenesis. This technique has been used in gene copy number profiling in various types of mesenchymal tumors to study their pathogenesis.<sup>10,11</sup> In order to identify genomic alterations further, we performed array-CGH on two cases of ASPSs confirmed by histologic and

immunohistochemical diagnoses.

## MATERIALS AND METHODS

### Tumor specimens

Two cases of histologically-defined ASPSs were selected from the Pusan National University Hospital database. No pre-operative radiotherapy or chemotherapy was administered in the selected cases. Tumors were collected immediately after surgery, cut into small pieces, frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$  until use for array CGH. Tissue samples were also fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at  $4\text{ }\mu\text{m}$ . The sectioned samples were stained with hematoxylin and eosin for routine histologic study.

### Immunohistochemistry

One paraffin block per case was selected for immunohistochemical analysis. Sections ( $5\text{ }\mu\text{m}$  thickness) were dewaxed and rehydrated using standard procedures and washed with PBS. For immunohistochemical staining, sections were subjected to standard microwave antigen retrieval pre-treatment. Immunohistochemical staining was performed using the avidin-biotin peroxidase complex method with aminoethylcarbazole as a chromogen and the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. The following monoclonal antibodies were used: anti-vimentin antibody (Dako, Glostrup, Denmark), anti-cytokeratin antibody (Santa Cruz Biotechnology, Inc., CA, USA), anti-EMA antibody (Dako), anti-S100 protein antibody (Dako), anti-NSE antibody (Santa Cruz Biotechnology, Inc.), anti-SMA antibody (Santa Cruz Biotechnology, Inc.), anti-desmin antibody (Dako), and CD56 (Dako).

### Array-CGH

Bacterial artificial chromosome (BAC) clones were selected from MacroGen's proprietary BAC library. The arrays were manufactured by Molecular Dynamics, (Sunnyvale, CA, USA) Generation III using 12 pin format. Each BAC clone was represented on an array as spots in triplicate. The array used in this study consisted of 4,030 human BACs, which were spaced approximately 2.3 Mb across the whole genome (MacroGen, Seoul, Korea, <http://www.macrogen.com>).

Genomic DNA was isolated from tumor tissue by standard phenol chloroform extraction. A pool of normal female or male genomic DNA (Promega, Madison, WI, USA) was used as a reference, depending on patient gender. Two  $\mu\text{g}$  of tumor DNA and reference DNA were digested with Dpn II overnight. After purification,  $20\text{ }\mu\text{L}$  of solution containing 500 ng of reference DNA or tumor DNA,  $20\text{ }\mu\text{L}$  of BioPrime<sup>®</sup> aCGH Labeling System random primer solution (Invitrogen, San Diego, CA, USA), and water were combined, incubated for 5 min at  $95^{\circ}\text{C}$  and subsequently cooled on ice. After the addition of  $5\text{ }\mu\text{L}$  of 10x dNTPs labeling mix (0.6 mM dCTP, 1.2 mM dATP, 1.2 mM dGTP, and 1.2 mM dTTP),  $3\text{ }\mu\text{L}$  of 1 mM Cy3-dCTP or Cy5-dCTP (Perkin-Elmer, Foster, CA, USA) and 40 U of BioPrime<sup>®</sup> aCGH Labeling System Klenow fragment (Invitrogen), the mixture was gently mixed and incubated for 16 h at  $37^{\circ}\text{C}$ . The addition of  $5\text{ }\mu\text{L}$  of BioPrime<sup>®</sup> aCGH Labeling System Stop Buffer (Invitrogen) ended the reaction. After labeling, unincorporated fluorescent nucleotides were removed by the use of the BioPrime<sup>®</sup> aCGH Purification Module (Invitrogen). In one tube, the Cy3-labeled sample and Cy5-labeled reference DNA were mixed together, and  $70\text{ }\mu\text{g}$  of human Cot I DNA (Invitrogen),  $20\text{ }\mu\text{L}$  of 3 M sodium acetate, and  $600\text{ }\mu\text{L}$  of cold 100% ethanol were precipitated. The pellet was resuspended in  $40\text{ }\mu\text{L}$  of a hybridization solution containing 50% formamide, 10% dextran sulfate,  $2\times\text{ SSC}$ , 4% SDS and  $200\text{ }\mu\text{g}$  yeast tRNA. The hybridization solution was denatured for 10 min at  $70^{\circ}\text{C}$  and was subsequently incubated for 1 h at  $37^{\circ}\text{C}$ , to allow blocking of repetitive sequence. Hybridization was performed in slide chambers for 48 h at  $37^{\circ}\text{C}$ .

### Scanning and analysis

After post-hybridization washes, arrays were rinsed, dried with spin, and scanned into two 16-bit TIFF image files using the GenePix 4200A two-color fluorescent scanner (Axon Instruments, Union City, CA, USA) and were quantitated using GenePix software (Axon Instruments). The median ratio of the three replicate spots for each clone was calculated. The log-transformed fluorescent ratios were calculated using background-subtracted median intensity values. Chromosomal aberrations were categorized as a gain when the normalized  $\log_2$  transformed fluorescent ratio was  $\geq 0.25$  and categorized as a loss when the ratio was  $\leq -0.25$ . MacroGen's MAC viewer, aCGH analysis software, MS Excel VBA, and Avadis 3.3 Prophetic were used for graphical illustrations and image analyses of array CGH data.

RESULTS

Clinical and pathologic findings

Clinical features of the two cases are summarized in Table 1. One patient was a 29-year-old man presenting with a lump in the posterior right calf (case 1). The other patient was a 16-year-old girl who presented with a mass in the right thigh (case 2). Neither patient had a history of previous illnesses or significant family histories.

On gross examination, both tumor masses were well-circumscribed and yellow-to-gray. No definite capsule was present. The

size of the tumors measured 8.5 × 4 × 4 cm and 14.5 × 11.5 × 9.5 cm in case 1 and case 2, respectively (Fig. 1A). The microscopic findings showed well-defined nests of uniformly large, round-to-polygonal cells, separated by thin-walled vascular channels (Fig. 1B). Typical crystalline material was noted on PAS stain with diastase digestion (Fig. 1C). Immunohistochemistry showed that the tumor cells were focally positive for NSE and vimentin, but were negative for cytokeratin, EMA, SMA, desmin, S100 protein, and CD56 in both cases. A final diagnosis of ASPS was made. Both patients had metastatic disease at the time of diagnosis. Case 1 had metastatic lesions in the right femur and scalp. Case 2 had metastatic nodules in the lung.

Table 1. Clinical characteristics of patients with ASPS

Case	Sex/age	Primary tumor site	Primary tumor size (cm)	Metastatic site
1	M/29	Right lower leg	8.5 × 4 × 4	Femur, scalp
2	F/16	Right thigh	14.5 × 11.5 × 9.5	Lung

ASPS, alveolar soft part sarcoma.

Table 2. Percentages of gains and losses of both ASPS cases

Case	Gain	Loss	Genes (gain)	Genes (loss)
1	174/4,030 (4.3%)	37/4,030 (1.0%)	139	30
2	204/4,030 (5.1%)	189/4,030 (4.7%)	164	132

ASPS, alveolar soft part sarcoma.

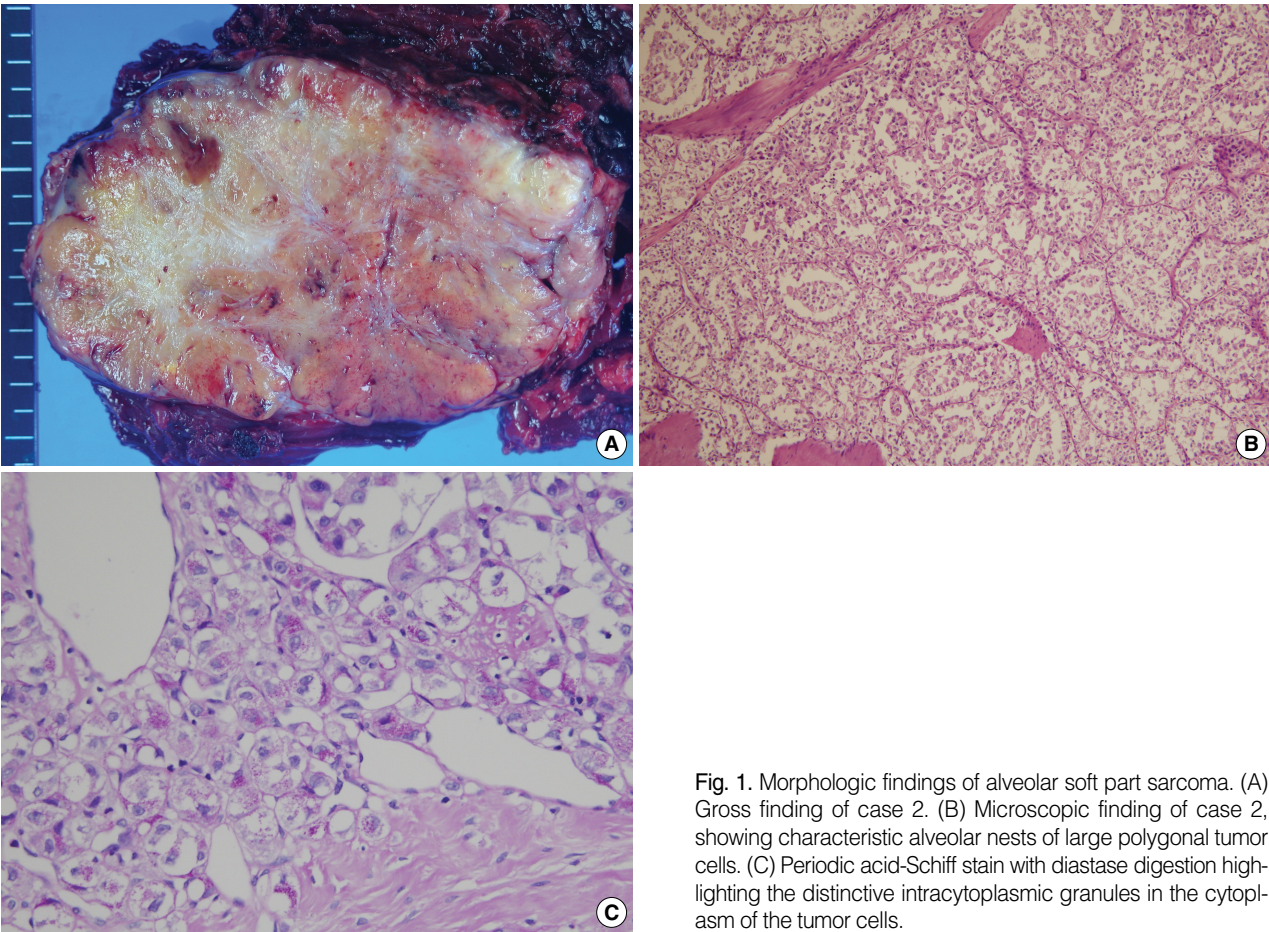


Fig. 1. Morphologic findings of alveolar soft part sarcoma. (A) Gross finding of case 2. (B) Microscopic finding of case 2, showing characteristic alveolar nests of large polygonal tumor cells. (C) Periodic acid-Schiff stain with diastase digestion highlighting the distinctive intracytoplasmic granules in the cytoplasm of the tumor cells.



Array CGH results

Each ASPS case had some gains and losses. Fig. 2 shows the entire genomic profile. Gains were more common than losses. The percentage of gains was 4.3% and 5.1%, respectively, and the percentage of losses was 1.0% and 4.7%, respectively. The average gains and losses of the ASPSs were 4.7% and 2.8%, res-

pectively (Table 2). Our data revealed 16 commonly altered chromosomal regions involving 25 genes. We identified 15 shared regions of gain, 11 of which were located on Xp. The most frequently gained regions were mapped to Xp22.33, Xp22.11, Xp11.3, Xp11.3-Xp11.23, and Xp22.2. Additional regions of gain were Xp22.12, Xp22.31, Xp22.32, Xp21.1, Xp21.1, and Xp11.4, 1q25.1, 7q35, 12p12.1 and 17p11.2 in cases 1 and 2,

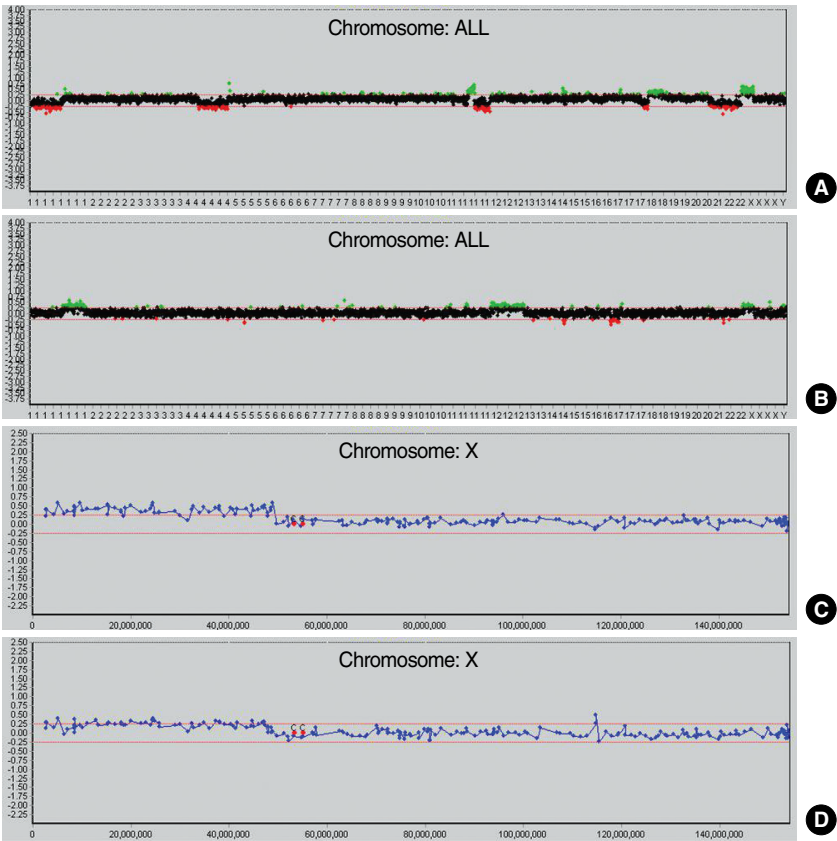


Fig. 2. Array CGH profiles and genome-wide frequencies of copy number alterations. Whole genome profiles are shown for two cases of ASPS (A, B). Each point represents a log<sub>2</sub> DNA copy number at 4,030 unique loci on chromosomes. Clones are ordered from chromosome 1 to 22, X and Y. The common region of gain is distinct at locus of chromosome Xp (C, D).

Table 3. Shared alteration in two ASPS cases

	Cyto-position	Genes	Gene ontology		Cyto-position	Genes	Gene ontology
Gain	Xp11.3	<i>UBE1</i>	DNA repair		Xp22.2	<i>TMEM27</i>	Membrane transport protein
	Xp11.3	<i>PCTK1</i>	Signal transduction		Xp22.2	<i>BMX</i>	Signal transduction
	Xp11.3	<i>USP11</i>	Cell cycle progression		Xp22.31	<i>KAL1</i>	Cell adhesion and motility
	Xp11.3-Xp11.23	<i>ARAF</i>	Signal transduction		Xp22.32		
	Xp11.3-Xp11.23	<i>SYN1</i>	Signal transduction		Xp22.33	<i>GYG2</i>	Glucose metabolism
	Xp11.3-Xp11.23	<i>TIMP1</i>	Cell proliferation		Xp22.33	<i>ARSD</i>	Development
	Xp11.4				Xp22.33	<i>ARSE</i>	Development
	Xp21.1	<i>XK</i>	Membrane transport protein		Xp22.33	<i>ARSH</i>	Cell signaling
	Xp21.3				1q25.1	<i>SERPINC1</i>	Cell adhesion
	Xp22.11	<i>PDK3</i>	Glucose metabolism		1q25.1	<i>RC3H1</i>	Cell adhesion
	Xp22.11	<i>PCYT1B</i>	Cell metabolism		7q35	<i>OR2A42</i>	Signal transduction
	Xp22.11	<i>PHEX</i>	Cell-cell signaling		7q35	<i>OR2A9P</i>	Signal transduction
	Xp22.11	<i>ARX</i>	Regulation of transcription		12p12.1		
	Xp22.12	<i>RPS6KA3</i>	Cell growth and differentiation		17p11.2		
	Xp22.2	<i>TMSB4X</i>	Cell proliferation	Loss	22q11.23	<i>GSTT1</i>	Cell metabolism

ASPS, alveolar soft part sarcoma.

respectively. Common loss was found in only one region (22q-11.23). The commonly altered regions, encoded genes, and cellular processes (by Gene Ontology) are represented in Table 3.

The gained genes were related to cell metabolism (*GYG2*, *PCYT1B*, and *PDK3*), cell signaling and signal transduction (*PCTK1*, *ARAF*, *SYN1*, *PHEX*, *BMX*, *ARSH*, *OR2A42*, and *OR2A9P*), cell proliferation (*TIMP1* and *TMSB4X*), cell adhesion (*KAL1*, *SERPINC1*, and *RC3H1*), development (*ARSD* and *ARSE*), membrane transport protein (*TMEM27* and *XK*), regulation of transcription (*ARX*), cell growth and differentiation (*RPS6KA3*), and DNA repair (*UBE1*). The gene encoded by the commonly lost BAC clone was *GSTT1* (cell metabolism).

## DISCUSSION

This is the first application report of genome-wide copy number changes by BAC array CGH in ASPS. Array-CGH proved to be very powerful for the detection of chromosomal rearrangement, although array CGH efficacy is limited to identification of copy number changes in the genome, and it cannot detect chromosomal translocations, mutations, or epigenetic gene expressions.<sup>10,11</sup>

ASPSs are rare malignant neoplasm of uncertain histogenesis. Several cytogenetic studies using diverse cytogenetic methods have been used to describe a number of chromosomal alterations in ASPSs. However, cytogenetic data on chromosomal aberrations on ASPS are limited.<sup>6,7,9,12-15</sup> Previous studies have shown complex chromosomal changes and have failed to reveal any common chromosomal abnormalities.

Sreekantaiah *et al.*<sup>12</sup> found deletions and translocations affecting chromosomes 1, 2, 3, 10, 11, 14, 15, and 16, and trisomy of chromosome 12 on G-banded karyotyping, while Cullinane *et al.*<sup>13</sup> described 1p- and 17q+ in one clone and trisomy 5 in another clone. Other studies have shown structural abnormalities of 17q25 in metaphase cells.<sup>14,16</sup> Another study was undertaken to analyze DNA copy number changes in cases of ASPSs, using CGH on formalin-fixed, paraffin-embedded tissue sections.<sup>6</sup> The study was the first to apply CGH to ASPS, and the investigators found DNA copy number changes in 4 of 13 cases. Gains were more common than losses. Gains observed in more than one case included 1q, 8q, 12q, and 16p. Polymerase chain reactions after tumor dissection were performed to search for possible LOH in chromosomes 1p, 9p, 17q, and p53 in primary and metastatic ASPSs. No LOH was found in any of the primary tumors, and only 1 (11%) of 9 metastases showed LOH for

D1S165 (at 1p36).<sup>7</sup> One study showed mutations of tumor-suppressor genes, including *p53*, *E-cadherin*, and *APC*, in some cases of ASPS.<sup>8</sup> A recent study reported, via angiogenesis oligo-microarray, that 18 angiogenesis-related genes, including *jag-1*, *midkine*, and *angiogenin*, were up-regulated in tumor tissue over adjacent normal tissues.<sup>9</sup>

Our array-CGH results showed that both cases of ASPS had some gains and losses. ASPSs showed a tendency to undergo more gains than losses, as in the previous CGH study.<sup>6</sup> The mostly frequently gained regions were mapped to Xp22.33, Xp22.11, Xp11.3, Xp11.3-Xp11.23, and Xp22.2. Previous studies have reported that the most frequent anomalies occurred in 1p, 9p, 17q, and 22q, and particularly suggested an important role for the long arm of chromosome 17-(most likely 17q25). A structural abnormality of 17q25 is likely to represent an unbalanced translocation, which cannot be visualized by CGH. Our results were not consistent with previous reports. However, it is of interest to note that 16 shared regions of gain were observed; 11 of which were located on Xp, and some of which, such as Xp11.3 and Xp11.3-Xp11.23, were present near the *TFE3* gene. Increased DNA copy number at Xp has rarely been detected in other malignancies. *UBE1*, ubiquitin-activating enzyme E1, mapped to this region, has been described in relation to the synovial sarcoma t(X;18)(p11.2;q11.2) breakpoint.<sup>17</sup> *USP11* participates in DNA damage repair functions within the BRCA2 pathway in relation to breast cancer.<sup>18</sup> *ARAF* is rarely mutated in human cancers, which suggests that alterations of the RAS pathway by *ARAF* gene mutations may not play an important role in the pathogenesis of human cancers.<sup>19</sup> *TIMP1* mRNA expression levels are best for distinguishing malignant from benign thyroid neoplasms<sup>20</sup> and are upregulated in squamous cell carcinoma of the lung.<sup>21,22</sup>

The cellular origin of ASPS is still undetermined. Cullinane *et al.*<sup>13</sup> reported that ASPSs are immunohistochemically-positive for NSE and S100 protein, but negative for desmin and muscle-specific antigen, and MyoD1 was not detected by Northern blotting. Their study suggested a neurogenic origin for this unusual tumor. However, a recent study<sup>3</sup> using a reverse transcription polymerase chain reaction showed the expression of MyoD1 and myogenin in some cases of ASPSs. According to these findings, it has been postulated that ASPSs are of skeletal muscle origin. *SYN1* (Xp11.3-Xp11.23) belongs to the synapsin family and consists of three neuronal-specific phosphoproteins associated with dynamic reorganization of the neuronal cytoskeleton.<sup>23</sup> The novel Aristaless-related homeobox gene, *ARX* (Xp22.11), is widely expressed in the brain and is thought to play

a key role in the regulation of brain development.<sup>24</sup> *KAL1* (Xp-22.31) affects neural cell adhesion and axonal migration, and is associated with human cancer.<sup>25</sup> These findings, along with the immunoreactivity for NSE demonstrated in our cases, might support a neurogenic origin for ASPSs rather than a myogenic origin. Recently, the influence of germline polymorphisms of *GSTT1* (22q11.23) in breast cancer susceptibility and survival has been studied, as well.<sup>26</sup>

In summary, array-CGH, a genome-wide, high resolution analysis of chromosomal alterations, performed on two cases of ASPS revealed unique chromosomal changes, particularly in Xp. These regions harbor several genes that may play a role in ASPSs. While these results provide support for the notion of a neural origin of ASPSs, the molecular mechanisms underlying the role of these genes in ASPS tumorigenesis are unknown. Further characterization of these candidate genes in relation to ASPS will lead to a clearer understanding of this malignant tumor.

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