High-Resolution Array CGH and Gene Expression Profiling of Alveolar Soft Part Sarcoma

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Abstract

**Purpose:** Alveolar soft part sarcoma (ASPS) is a soft tissue sarcoma with poor prognosis, and little molecular evidence exists for its origin, initiation, and progression. The aim of this study was to elucidate candidate molecular pathways involved in tumor pathogenesis.

**Experimental Design:** We employed high-throughput array comparative genomic hybridization (aCGH) and cDNA-Mediated Annealing, Selection, Ligation, and Extension Assay to profile the genomic and expression signatures of primary and metastatic ASPS from 17 tumors derived from 11 patients. We used an integrative bioinformatics approach to elucidate the molecular pathways associated with ASPS progression. FISH was performed to validate the presence of the t(X;17)(p11.2;q25) ASPL–TFE3 fusion and, hence, confirm the aCGH observations.

**Results:** FISH analysis identified the ASPL–TFE3 fusion in all cases. aCGH revealed a higher number of numerical aberrations in metastatic tumors relative to primaries, but failed to identify consistent alterations in either group. Gene expression analysis highlighted 1,063 genes that were differentially expressed between the two groups. Gene set enrichment analysis identified 16 enriched gene sets (P < 0.1) associated with differentially expressed genes. Notable among these were several stem cell gene expression signatures and pathways related to differentiation. In particular, the paired box transcription factor PAX6 was upregulated in the primary tumors, along with several genes whose mouse orthologs have previously been implicated in Pax6 DNA binding during neural stem cell differentiation.

**Conclusion:** In addition to suggesting a tentative neural line of differentiation for ASPS, these results implicate transcriptional deregulation from fusion genes in the pathogenesis of ASPS. Clin Cancer Res; 20(6); 1521–30. ©2014 AACR.

Introduction

Alveolar soft-part sarcoma (ASPS) is a rare mesenchymal tumor of uncertain histogenesis. The tumor occurs primarily in adolescent and young adults with a female predilection, and accounts for 0.4% to 1% of all soft tissue sarcomas (1). Commonly, the tumor involves the deep soft tissues of the extremities, buttocks, abdominal, or thoracic wall in adults, and the head and neck region in children. ASPS has also been reported in tissues where skeletal muscle is not usually found, such as the stomach, pituitary gland, sacral bone, breast, lung, retroperitoneum, and female genital tract (1–3). ASPS is a malignant tumor with a slow, indolent clinical course, with survival rates of 77% at 2 years, 60% at 5 years, 35% at 10 years, and only 15% at 20 years (1). It is not particularly responsive to adjuvant chemotherapy, although there may be some role for adjuvant radiotherapy in reducing the risk of local recurrence (1). Vascular invasion is common with subsequent hematogenous metastases to lung and brain, and although prolonged survival is possible in such patients, the longer-term disease-specific mortality is high. Although tumor size has been correlated to the survival in the past (4), no specific clinical or histopathologic features exist currently that are reliably predictive of disease course in ASPS.

Since its description by Christopherson in 1952, the histogenesis of ASPS has remained controversial. Histologically, ASPS exhibits variable architecture ranging from alveolar to solid patterns composed of nonspecific, uniform round cells with central nuclei, diastase periodic acid Schiff (DPAS)–positive granular cytoplasmic crystals, and characteristic rectangular/thornoid cytoplasmic crystals seen...
with the electron microscope. On the basis of the morphology, this entity has been designated in the past as both malignant organoid granular cell myoblastoma and malignant nonchromaffin paraganglioma, inferring myogenic and neurogenic differentiation, respectively.

The current singular defining molecular feature of ASPS is the nonreciprocal translocation t(X;17)(p11;q25), resulting in the fusion of the nuclear transcription factor gene TFE3 to the ASPL gene (5). Although this translocation is not specific to ASPS (occurring in a subset of renal cell carcinoma, RCC; ref. 6), it is consistently and uniformly present in all ASPS cases. In this study, we employed high-throughput technology to profile the genomic and gene expression signatures of primary and metastatic ASPS. Moreover, we used an integrative bioinformatics approach to elucidate the molecular pathways associated with the progression of ASPS.

Materials and Methods

Case selection

Paraffin blocks from surgical specimens covering a 13-year period (1994–2007) were obtained from the archives of Massachusetts General Hospital, Brigham and Women’s Hospital, and Boston Children’s Hospital (Boston, MA), in accordance with the regulations for excess tissue use stipulated by the institutional review board at each hospital. Altogether, 17 tumors from 11 patients were used for this study. Archival material corresponding to both the primary tumor sites as well as from metastases or re-excisions was available for four of the cases. All cases were fixed in 10% neutral-buffered formalin and routinely embedded in paraffin. Pertinent clinical data are summarized in Table 1. Diagnosis was confirmed by retrospective review of hematoxylin and eosin (H&E)–stained sections by study pathologist E. Chen. World Health Organization diagnostic criteria were used for assigning histopathology diagnoses (7). Where necessary, periodic acid–Schiff (PAS) staining, TFE3 immunohistochemistry, and electron microscopy were utilized as ancillary aids to diagnosis (Fig. 1).

Immunohistochemistry

We used the P-16 polyclonal antibody to TFE3 (Santa Cruz# sc-5958; Santa Cruz Biotechnology), which binds to the C-terminal portion of the TFE3 protein downstream of the region encoded by exon 6 to help confirm the diagnosis of ASPS (6). Representative 5-μm formalin-fixed, paraffin-embedded (FFPE) sections from each case were mounted onto positively charged slides and, subsequently, processed for immunohistochemical (IHC) staining using a standard protocol. Briefly, the sections were deparaffinized in xylene, rehydrated using graded ethanol concentrations, and then subjected to antigen retrieval by boiling in citrate buffer at pH 6.0 for 10 minutes. Following quenching with peroxidase and blocking with avidin, sections were incubated overnight with a 1:500 dilution of the polyclonal antibody to TFE3 in PBS. Detection of antibody binding was achieved using a biotinylated secondary antibody and horseradish peroxidase–conjugated streptavidin (Dako) and 3,3′,5′-diaminobenzidine as chromogen.

FISH

Loci corresponding to the TFE3 and ASPL genes were visualized by dual color, single fusion FISH design. Fluorescent probes were generated by nick translation of bacterial artificial chromosome (BAC) clones RP11-634L102 (labeled SpectrumOrange) and CTD-2311N12 (Spectrum-Green) BACs (obtained from CHORI; www.chori.org), which map to 17q25 and Xp11.2, respectively. RP11-634L102 includes almost the entire ASPL gene, whereas the latter includes the entire TFE3 locus (Supplementary Fig. S1).

Four-micrometer FFPE sections were mounted on standard glass slides and baked at 60°C overnight. Following deparaffinization in xylene, the sections were dehydrated in ethanol and air dried. The sections were subsequently treated in 1 mol/L sodium isothiocyanate in 2× sodium chloride–sodium citrate (SSC) for 20 minutes at 75°C, and rinsed in 2× SSC for 5 minutes at room temperature, before digestion in 0.25 mg/mL proteinase K (Roche) in 2× SSC at 45°C for 20 minutes. The slides were rinsed in 2× SSC, then dehydrated in ethanol series, and air dried. The probe and target DNA (tissue sections) were codenatured at 80°C for 10 minutes (Hybrite; Abbott Molecular Inc.) and allowed to hybridize for 16 hours at 37°C. Post-hybridization washes were carried out in 2× SSC at 70°C for 10 minutes, then at room temperature for 5 minutes. The sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Abbott Molecular Inc.), and signals were visualized on an Olympus
### Table 1. Summary of clinical features in the ASPS study cohort

<table>
<thead>
<tr>
<th>Patient/site</th>
<th>Age/sex</th>
<th>Outcome</th>
<th>Survival</th>
<th>Treatment</th>
<th>Diagnosis</th>
<th>FISH$^a$</th>
<th>ACGH alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1MT (lung)</td>
<td>30F</td>
<td>Dead</td>
<td>84</td>
<td>Systemic chemotherapy</td>
<td>TFE3 +</td>
<td>NA</td>
<td>None observed</td>
</tr>
<tr>
<td>2MT (lung)</td>
<td>36M</td>
<td>Awd</td>
<td>132</td>
<td>Clinical trial: Gleevec</td>
<td>EM, DPAS+</td>
<td>46%; multiple fusion signals</td>
<td></td>
</tr>
<tr>
<td>3MT (adrenal)</td>
<td>41F</td>
<td>Dead</td>
<td>72</td>
<td>None</td>
<td>EM</td>
<td>22%</td>
<td>NA</td>
</tr>
<tr>
<td>4MT (lung)</td>
<td>27M</td>
<td>Lost to fu</td>
<td>72</td>
<td>Systemic chemotherapy</td>
<td>TFE3 +</td>
<td>20%</td>
<td>Loss of 1 chrX; breakpoint at Xp11.2 (gain to pter); copy loss of 19q and 22q</td>
</tr>
<tr>
<td>5MT (pancreas)</td>
<td>47M</td>
<td>Awd</td>
<td>156</td>
<td>None</td>
<td>TFE3 +</td>
<td>72%</td>
<td>Loss of 1 chrX; breakpoint at Xp11.2 (gain to pter); loss of 17q25 band; loss of 1p and 18p</td>
</tr>
<tr>
<td>6P (thigh)$^b$</td>
<td>29M</td>
<td>Dead</td>
<td>60</td>
<td>None</td>
<td>EM</td>
<td>17%</td>
<td>Loss of 1 chrX; breakpoint at Xp11.2 (gain to pter); no additional alterations</td>
</tr>
<tr>
<td>6MT1 (colon)$^b$</td>
<td>None</td>
<td>TFE3 +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6MT2 (abdomen)$^b$</td>
<td>None</td>
<td>TFE3 +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7P (retro-orbital)$^b$</td>
<td>27M</td>
<td>Awd</td>
<td>60</td>
<td>None</td>
<td>Morphology$^c$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>7MT (brain)$^b$</td>
<td>None</td>
<td>TFE3 +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8MT (brain)</td>
<td>28M</td>
<td>Dead</td>
<td>16</td>
<td>Systemic chemotherapy</td>
<td>Morphology$^c$</td>
<td>NA</td>
<td>One copy loss of 1q and 22q; BRUNOL4 loss (18q12.2); breakpoint at Xp11.2 (gain to pter)</td>
</tr>
<tr>
<td>9P (thigh)$^b$</td>
<td>28F</td>
<td>Dead</td>
<td>36</td>
<td>None</td>
<td>EM</td>
<td>80%</td>
<td>Multiple alterations</td>
</tr>
<tr>
<td>9MT (brain)$^b$</td>
<td>None</td>
<td>Radiotherapy</td>
<td></td>
<td></td>
<td>Morphology$^c$</td>
<td>48%</td>
<td>None observed</td>
</tr>
<tr>
<td>10P (back)</td>
<td>20M</td>
<td>Ned</td>
<td>24</td>
<td>None</td>
<td>DPAS+</td>
<td>80%</td>
<td>One copy loss of 21q; loss of 1 chrX; breakpoint at Xp11.2 (gain to pter)</td>
</tr>
<tr>
<td>11MT1 (intestine)$^b$</td>
<td>27F</td>
<td>Awd</td>
<td>168</td>
<td>Intestinal chemotherapy</td>
<td>EM</td>
<td>56%</td>
<td>Breakpoint at Xp11.2 not identified; multiple alterations</td>
</tr>
<tr>
<td>11MT2 (femur)$^b$</td>
<td>None</td>
<td>Radiotherapy</td>
<td></td>
<td></td>
<td>Morphology$^c$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12P (buttock)</td>
<td>26F</td>
<td>Awd</td>
<td>72</td>
<td>None</td>
<td>EM</td>
<td>46%</td>
<td>Breakpoint at Xp11.2; no additional alterations</td>
</tr>
<tr>
<td>13P (neck)</td>
<td>47F</td>
<td>Awd</td>
<td>96</td>
<td>None</td>
<td>EM</td>
<td>35%; multiple</td>
<td>11 alterations; breakpoint at Xp11.2 but loss from fusion signals</td>
</tr>
</tbody>
</table>

Abbreviations: Awd, alive with disease; chr, chromosome; F, female; fu, follow up; M, male; MT, metastases; NA, not available; Ned, no evidence of disease; P, primary.

$^a$Percentage of tumor nuclei with fusion.

$^b$Paired samples.

$^c$Similar to primary tumor.

$^d$In months.

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Molecular Profiling of Alveolar Soft Part Sarcoma

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BX51 fluorescence microscope (Olympus). Individual images were captured using an Applied Imaging system running CytoVision Genus version 3.9 (Applied Imaging).

Tumor regions were identified on each section by pathologists (E. Chen and R. Flavin) from corresponding H&E sections of the FFPE block. Analysis was limited to the intact, nontruncated and nonoverlapping tumor cell nuclei. A total of 50 nuclei per section were sampled from two separate regions, and FISH signals were scored for the number of TFE3 (green), ASPL (red), and fusion signals in each nucleus. Signals were deemed to be fused when the red and green signals were separated by less than three signal diameters. The FISH criteria used to evaluate the ASPL–TFE3 rearrangement were as follows: (i) in female patients, visualization of two green TFE3 (another normal Xp11 signal) and one red ASPL separate signals in addition to a fusion signal; (ii) in male patients, visualization of one green and one red ASPL signal in addition to a fusion signal (Fig. 1 and Supplementary Fig. S1).

Oligonucleotide human genome 500k aCGH

Normal female genomic DNA was obtained from Promega. Genomic DNA was extracted from 15 samples of ASPS tumors (5 primary and 10 metastases) using the QIamp DNA FFPE Tissue Kit (Qiagen). The Genomic DNA ULS labeling kit for FFPE Samples (Agilent) was used to chemically label 1 μg of genomic DNA with either ULS-Cy5 (tumor) or ULS-Cy3 dye (normal/reference DNA), according to the manufacturer’s protocol (Agilent Technologies, Inc.). An equimolar mixture of labeled normal and tumor DNA was applied to the Agilent Human Genome CGH 2×400k Microarray, which features 411,056 in situ synthesized 60-mer oligonucleotide probes that span coding and noncoding sequences with an average spatial resolution of 5.3 kb. Hybridization was carried out at 65°C for 40 hours, in a rotating oven at 20 rpm. The arrays were washed according to the manufacturer’s protocol, and then scanned using an Agilent DNA microarray scanner. CGH Analytics software version 3.4 (Agilent Technologies) was used to analyze the aCGH data.

The data quality of each microarray was assessed using the Quality Metrics report generated by the Agilent CGH analytics software (v.3.4). Copy-number aberrations were detected using the aberration detection method (ADM-1) algorithm, based on computing significance scores for all genomic intervals. Aberrations were assigned as either gain (ratio ≥ 0.5), loss (≤–1.0), or amplification (≥2.0). A contiguous change of imbalance level within a chromosome was considered to be indicative of an unbalanced translocation event. For this study, the threshold was optimized to a value of 6.0 to reduce inherent sample noise contribution in the analysis, without negating true consistent imbalances along each chromosome. Only aberrations spanning a minimum of 10 consecutive oligonucleotide probes were marked as significant, so that only imbalances spanning greater than approximately 50 kb will be included. Further details about these principles are available at the Agilent website (www.agilent.com/chem/goCGH). Array data have been published in compliance with MIAME 2.0 guidelines and deposited in the publicly available National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (GSE49327).
cDNA-mediated annealing, selection, ligation, and extension assay

Thirteen cases were amenable to global gene expression analysis (five primary tumors and eight metastases). Two samples were matched primary and metastatic tumors; two were paired cases of metastatic lesions from different sites. For each sample, H&E sections were viewed by a pathologist (R. Flavin) to ensure at least 80% tumor content. Five consecutive 20-μm sections were collected for analysis. Total RNA was extracted, cleaned up, and eluted using the RNeasy MiniKit and RNeasy MinElute Cleanup Kit, respectively (Qiagen), according to the manufacturer’s protocol. The total RNA yield per sample ranged from 191 ng to 2 μg. cDNA-mediated annealing, selection, ligation, and extension (DASL) expression assay (Illumina) was performed as per manufacturer’s instructions on a panel comprising 24,526 probes for a total of 18,631 genes (Human Ref-8 Expression; BeadChip).

Data were quantile normalized and a two-sample t test was used to detect differential expression of every gene across five primary and eight metastatic sarcomas using Broad Institute’s GenePattern platform. We obtained gene sets representing key biologic processes from GO, disease pathways from Molecular Signature Database (MSigDB), and gene expression signatures from the literature from GenesigDB were used for gene set enrichment analysis. The enrichment of each gene set among the top differentially expressed genes was tested with two-sided hypergeometric test in R. Finally, we used the Ingenuity Pathway Analysis tool to construct a network of direct and indirect interactions among the differentially expressed genes that contain PAX6-specific putative regulatory binding sites in their promoter regions.

Clinicopathologic correlation

We examined whether the presence of simple or complex chromosomal abnormalities was associated with clinicopathologic features of the ASPS samples (simple chromosomal abnormalities was defined as the presence of two or less gross abnormalities, including the breakpoint at Xp11.2). Two by two group comparison was performed using the Fischer exact test. In addition, we examined whether the presence of simple or complex chromosomal abnormalities was associated with patient survival. Kaplan–Meier survival analysis was performed using the log-rank test. Statistical analysis was performed with both Analyse-It Software Ltd. and MedCalc Software Ltd.

Results

Differential gene expression analysis and pathway analysis

On the basis of the t test for differential gene expression across primary and metastatic sarcomas, 1,063 genes were found to be significant at the nominal P value level of 0.05, of which 323 genes with a t test score greater than 3 were most differentially expressed; the full set of data may be downloaded from NCBI GEO (GSE49327). Among these top differentially expressed genes, 207 were upregulated in primary and 116 in metastases. The differential expression heat map is shown in Fig. 2. Gene set enrichment analysis identified 16 gene sets as enriched (P < 0.1), both among genes that are upregulated in primary and metastatic sarcomas (Supplementary Fig. S2). We observed that several stem cell gene expression signatures and pathways related to differentiation are enriched among the top differentially expressed genes (Fig. 3 and Supplementary Fig. S2). In particular, we noted distinct upregulation of the paired box transcription factor PAX6 and PAX6 target genes in primary sarcomas (Figs. 3 and 4). Interestingly, one of the targets, the LIM/homeobox protein Lhx6, is an important transcription regulator that is known to control differentiation and development of neural cells (8). By observing the known direct and indirect interactions among the differentially expressed genes that have putative binding sites in their promoters, we noted interesting subnetworks of hub nodes and GeneGO processes. Moreover, indirect connections between TFE3 and PAX6 via genes involved in the TGF-β signaling pathway could be inferred (Supplementary Fig. S3).

Oligonucleotide human genome 500k aCGH

Genomic aberrations in ASPS were analyzed using the Agilent 2× 500k high-resolution aCGH platform with
from the TFE3 locus to pter, we actually observed the opposite, which was a large segmental loss of the p-arm from TFE3 to pter. Not only did this sample show multiple fusion signals with FISH analysis, but like sample 2, this also had a segmental loss on chromosome 17 from 17p12 to pter, among numerous other alterations. Otherwise, we did not observe any consistent genomic imbalances when comparing primary and metastatic tumors, and no high-level amplifications were found in any of the tumors.

**FISH**

Dual-color, single-fusion FISH was performed on 4-μm tissue sections to validate the presence of the t(X;17)(p11.2; q25) ASPL–TFE3 fusion and, hence, to confirm the aCGH observations. For each sample, a total of 50 interphase nuclei were analyzed using the RP11-634I102 (SpectrumOrange) and CTD-2311N12 (SpectrumGreen) home-brew probes. A baseline for false-positive fusion signals of 1% was established by probing synovial sarcoma, which harbors a t(X;18) rearrangement. Of the 15 tumors analyzed, two samples failed to show signals and showed perinuclear chromatin halos, consistent with severe degradation of DNA. The remaining 13 samples consistently exhibited the characteristic ASPL–TFE3 fusion (Fig. 1), according to the expected gender-specific pattern. It is noteworthy, however, that the fusion was not uniformly present in all cells within the analyzed tissue regions; the vast majority of the samples contained less than 50% fusion-positive cells, with two samples exceeding 70% fusion-positive cells (Table 1). Genomic heterogeneity was further indicated by the presence of additional fusion signals in two of the samples (2M and 14P), as also observed on aCGH. In these sections, the majority of the cells showed the characteristic pattern, with few interspersed populations of cells that displayed two fusion signals, in addition to separately localized red and green signals. No consistent differences were observed between the primary, recurrent, and metastatic tumors with regard to the pattern or number of fusion-positive nuclei.

**Clinicopathologic features**

There was no statistically significant relationship between the presence of simple or complex chromosomal abnormalities and the clinicopathologic features of age, sex, site of disease, patient status, whether adjuvant therapy was received, and type of therapy (Supplementary Table S1). Genomic imbalance did not exhibit a statistically significant relationship with overall patient survival (P = 0.13 by log-rank test).

**Discussion**

In this study, we employed high-throughput approaches to profile the genomic and expression signatures of primary and metastatic ASPS. Moreover, we used an integrative bioinformatics approach to elucidate the molecular pathways associated with the progression of ASPS. To our
knowledge, this is the most comprehensive and largest cohort of ASPS cases analyzed to date.

Gene expression profiling identified a number of key gene sets in ASPS pathogenesis, a number of which were previously identified in three previous microarray studies, thereby providing cross-validation for our current analysis (10–12). These included the hypoxia, hypoxia-inducible factor (HIF), and angiogenesis pathways, suggesting an active role for these gene sets in ASPS pathogenesis and further supporting the rationale for the use of antiangiogenic inhibitors in therapeutic regimens for the disease. Interestingly, the study by Kobos and colleagues examined the functional properties of the ASPSCR1–TFE3 fusion oncoprotein, defined its target promoters on a genome-wide basis, and performed a high-throughput RNA interference screen to identify which of its transcriptional targets contribute to cancer cell proliferation (12). HIF1α was observed to be a shared target between both this study and our own analysis. Although HIF1α has a central role in the adaptation of tumor cells to hypoxia by activating the transcription of targeting genes, its dysregulation is seen in numerous other types of neoplasias (13). Although this is a comprehensive study, the overall biologic question addressed is significantly different from the one being addressed in our study. Specifically, the study design by Kobos and colleagues questions the mechanism by which the primary tumors arise, thus profiling the molecular signature of the early events in ASPS oncogenesis. In contrast, our study specifically interrogates the molecular mechanisms in the critical events of metastasis.

Recurrent regions of copy-number abnormalities in the genomic DNA of tumors can identify genes that drive disease pathogenesis. In this study, we therefore focused on identifying recurrent regions of gain or loss in primary and metastatic samples using a unique high-throughput approach. Until now, there has only been one metaphase CGH study on ASPS, which revealed gains of 1q, 8q, and 16q, including the gain of Xp11pter (14). In this study, high-resolution aCGH revealed a higher number of numerical aberrations in metastatic tumors relative to primaries, but failed to identify any consistent alterations in either group. Indeed, our analysis identified gains and losses not previously observed in ASPS, such as the imbalances seen in sample 2M (Table 1). Multiple and complex abnormalities involving deletions, translocations, trisomy 12, and loss of chromosome 17, obtained after chemotherapy, have been described (15). Given that we saw multiple fusion signals in sample 2M, it is likely that this tumor exhibits aneuploidy as a result of increasing chromosomal instability during the metastatic process. The only shared alteration that was observed in two metastatic samples was a 1 copy loss of chromosome 22. As expected from the unbalanced rearrangement involving the X chromosome, the most frequent chromosomal aberration detected was a large segmental gain on Xp, with the breakpoint localizing at the TFE3 gene and extending telomeric to it. Genomic loss at 17q25 was...
also observed frequently, though the amplitude of the alteration showed variation between samples. This seemed to correlate with the percentage of fusion-positive cells in the tumor samples (data not shown). As previously reported, this is the hallmark translocation of ASPS. Ladanyi and colleagues showed that this unbalanced translocation results in the fusion of \textit{TFE3} on Xp11.2, a member of the basic helix–loop–helix family of transcription factors, to \textit{ASPL} on 17q25. The resulting fusion gene encodes chimeric \textit{ASPL–TFE3} RNA transcripts. Two mutually exclusive types of fusion products, designated type 1 and type 2, are observed (5). \textit{ASPL} exons 1–7 are fused to \textit{TFE3} exon 6 in type 1 and exon 5 in type 2 transcripts (16). Interestingly, \textit{TFE3} is also found to be rearranged by specific reciprocal translocations involving Xp11.2 in a subset of pediatric RCCs, of which one variant translocation is a balanced t(X;17)(p11.2;q25), the breakpoints of which are cytogenetically identical to the translocation that is characteristic of ASPS (6).

A remarkable observation was that the low prevalence of the t(X;17)(p11.2;q25) rearrangement in tumor samples was belied by the uniform nuclear staining that was observed with \textit{TFE3} IHC staining. Discrepancy may arise from the fact that we were comparing a quantitative (FISH) method with a semiquantitative (IHC) method. Although the enumeration process in FISH is based on quantifying discrete signals, interpretation of IHC staining is generally made in a qualitative and subjective manner, where a positive result might refer to the presence of staining in any part of the studied tissue. Another reason for this discrepancy stems from a limitation of detecting rearrangements in FFPE tissue sections. Part of the cell can be lost during the sectioning process, leading to artificially induced loss of a significant percentage of nuclei. This truncation effect can generate a high false-negative rate of cells losing signals due to section artifact when using a 5-μm section.

Finally, it is noteworthy that the IHC and the FISH assays for a given tumor were not always performed on the same FFPE blocks. Specifically, the IHC assays were routinely performed on the samples as a part of the diagnostic workup and, in some cases, the particular blocks were not available for the retrospective FISH assays. Given the heterogeneous nature of the sarcomas, a part of the discrepancy may be attributable to the variation in the tumor makeup.

Figure 5. Composite analysis of the break at Xp11.23 and subsequent imbalance in three primary and four metastatic tumors. Blue horizontal line represents the location of the break at \textit{TFE3}. Aberrations above the ADM-1 threshold of 10.0 are represented by the colored regions deviating to the left (relative loss to control) or right (relative gain to control). Imbalances in the primary and metastatic tumors are color coded as yellow and blue, respectively.

Also observed frequently, though the amplitude of the alteration showed variation between samples. This seemed to correlate with the percentage of fusion-positive cells in the tumor samples (data not shown). As previously reported, this is the hallmark translocation of ASPS. Ladanyi and colleagues showed that this unbalanced translocation results in the fusion of \textit{TFE3} on Xp11.2, a member of the basic helix–loop–helix family of transcription factors, to \textit{ASPL} on 17q25. The resulting fusion gene encodes chimeric \textit{ASPL–TFE3} RNA transcripts. Two mutually exclusive types of fusion products, designated type 1 and type 2, are observed (5). \textit{ASPL} exons 1–7 are fused to \textit{TFE3} exon 6 in type 1 and exon 5 in type 2 transcripts (16). Interestingly, \textit{TFE3} is also found to be rearranged by specific reciprocal translocations involving Xp11.2 in a subset of pediatric RCCs, of which one variant translocation is a balanced t(X;17)(p11.2;q25), the breakpoints of which are cytogenetically identical to the translocation that is characteristic of ASPS (6).
antibodies (including MyoD1, desmin, and sarcomeric actin) in ASPS, which supported the hypothesis that ASPS is of muscular origin (19, 22). Moreover, recent gene expression profiling studies have identified a number of differentially expressed muscle-related transcripts in patients with ASPS, adding further weight to the case for a muscle cell progenitor as origin of the disease (11, 21).

However, many other IHC- and PCR-based studies have failed to support these findings (2, 20, 23). In this study, several stem cell gene expression signatures and pathways related to differentiation are enriched among the top differentially expressed genes. Furthermore, we noted distinct upregulation of the paired box transcription factor Pax6 in primary sarcomas, along with several genes whose mouse orthologs have previously been implicated in Pax6 DNA binding during neural stem cell differentiation (Figs. 3 and 4). Pax6 has important functions in the development of the eye, nose, pancreas, and central nervous system. Interestingly, Pax6 is necessary and sufficient for human neuroectoderm specification through repression of pluripotent genes and activation of neural genes (24). Furthermore, one of the Pax6 targets, the LIM/homeobox protein Lhx6, is an important transcription regulator that is known to control the differentiation and development of neural cells (8). Indeed, Pax6 as a putative tumor suppressor may have a role in the pathogenesis of many cancers (including breast, oral, lung, gastric, bladder, and brain) through promoter hypermethylation or through the sonic hedgehog–Gli signaling pathway (25–31). It is noteworthy, however, that many of the translocation-associated sarcomas use aberrant transcription factors in their pathogenesis, and tend to be difficult to classify in terms of their cell of origin, even when dominant patterns are found in terms of lines of differentiation. The signature reported herein, however, does bear a strong resemblance to the molecular mechanism observed in neural stem cells.

In conclusion, our findings seem to indicate that, in addition to suggesting a tentative neural line of differentiation for ASPS, transcriptional deregulation from fusion genes may have a critical role in the pathogenesis of ASPS, rather than extensive chromosomal instability.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


