Histopathology 2009, **55**, 750–755. DOI: 10.1111/j.1365-2559.2009.03436.x

Validation of potential therapeutic targets in alveolar soft part sarcoma: an immunohistochemical study utilizing tissue microarray

Alexander J Lazar,[1,2] Guy Lahat,[2,3] Sarah E Myers,[2,4] Kerrington D Smith,[2,3] Changye Zou,[2,3] Wei-Lien Wang,[1] Dolores Lopez-Terrada[5] & Dina Lev[2,4]

Departments of [1]Pathology, [3]Surgical Oncology, [4]Cancer Biology and the [2]Sarcoma Research Center, The University of Texas M. D. Anderson Cancer Center, and [5]Department of Pathology, Texas Children's Hospital and Baylor College of Medicine, Houston, TX, USA

Date of submission 22 June 2009
Accepted for publication 26 August 2009

Lazar A J, Lahat G, Myers S E, Smith K D, Zou C, Wang W-Lien, Lopez-Terrada D & Lev D (2009) *Histopathology* **55**, 750–755

Validation of potential therapeutic targets in alveolar soft part sarcoma: an immunohistochemical study utilizing tissue microarray

Aims: The molecular signature of alveolar soft part sarcoma (ASPS) is a specific der(17)t(X;17)(p11.2;q25) translocation, resulting in a chimeric transcription factor (ASPSCR1–TFE3). When this disease is no longer amenable to surgical curative intervention, uniformly efficacious therapies are lacking. The aim of this study was to evaluate the expression of potential molecular therapeutic targets in a cohort of ASPS tumour samples.
Methods and results: Immunohistochemical analysis for hepatocyte growth factor, c-Met, phosphorylated c-Met, phosphorylated AKT, phosphorylated MEK, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), p53 and vimentin was performed on an ASPS tissue microarray, yielding complete data from 26 tumours. Activation of c-Met and its downstream effectors was noted, whereas only limited EGFR expression was seen. VEGF was expressed to varying degrees. Only one sample exhibited strong nuclear p53 expression, while 10 expressed low levels. Vimentin expression was negative in the vast majority of samples (96%).
Conclusions: There is a crucial need for better anti-ASPS therapies. Activated c-Met and the phosphorylation of its downstream effectors validate an intact signalling cascade probably induced by the ASPSCR1–TFE3 chimeric transcription factor. The angiogenic phenotype of these tumours is supported by increased angiogenic factor expression. Combination therapies targeting both tumour cells and angiogenesis merit further investigation.

Keywords: alveolar soft part sarcoma, angiogenesis, ASPSCR1-TFE3, c-Met, HGF, VEGF

Abbreviations: ASPS, alveolar soft part sarcoma; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; TMA, tissue microarray; UTMDACC, University of Texas M. D. Anderson Cancer Center; VEGF, vascular endothelial growth factor receptor.

Introduction

Alveolar soft part sarcoma (ASPS) is an uncommon soft tissue sarcoma of uncertain differentiation first described in 1952.[1,2] It presents in younger patients, often in the extremities.[3,4] Despite relatively high rates of metastasis, patients often experience prolonged survival in the metastatic setting relative to other

Addrerss for correspondence: A J Lazar, MD, PhD, The University of Texas M. D. Anderson Cancer Center, Sarcoma Research Center, 1515 Holcombe Blvd—Unit 85, Houston, TX 77030-4009, USA. e-mail: alazar@mdanderson.com

sarcomas, but most will ultimately succumb to disease.[5,6] Whereas surgery, even in the setting of metastatic disease, can improve outcomes, traditional cytotoxic chemotherapy and/or radiotherapy confer no significant survival advantage; there exists a need for novel therapeutic interventions to improve disease outcome.[5,7] The approach to cancer therapy has recently undergone shifts from non-specific cytotoxic agents towards utilization of molecularly targeted treatments. This personalized tumour-specific approach requires identification of tumour type-related 'oncogenic addiction/s'; i.e. molecular aberrations essential for tumour maintenance and progression.[8,9] To make progress in ASPS management it is crucial to identify commonly expressed deregulated targets that are amenable to pharmaceutical inhibition. Traditional pathology techniques such as immunohistochemistry are integral to this discovery and validation process.

The hallmark of ASPS is a chromosomal rearrangement at 17q25 and Xp11.2 engendering an *ASPSCR1–TFE3* fusion gene responsible for an aberrant transcription factor presumably enabling pathogenesis.[10] This aberrant chimeric transcription factor retains the N-terminal DNA binding domain encoded by *TFE3* while the *ASPSCR1* encoded portion probably provides domain(s) modulating gene expression.[10] The presence of this 'super-activated' transcription factor may induce the expression of numerous molecules contributing to ASPS progression and metastasis. Several such proteins have been identified (e.g. c-Met) that might serve as unique anti-ASPS therapeutic targets. Histologically, ASPS has a distinctive angiogenic phenotype with prominent capillary vessels surrounding individual, often pseudoalveolar, tumour nests. Gene expression profiling of ASPS demonstrates an array of potentially therapeutically targetable angiogenic factors,[11,12] a promising anticancer strategy that has established efficacy in several tumour systems.[13,14]

These initial insights into ASPS biology are essential in developing novel therapeutics. To amplify these observations, it is important to validate the expression of candidate, clinically relevant ASPS therapeutic targets. Although target presence does not guarantee therapeutic efficacy, lack of target expression strongly implies minimal therapeutic effect. A major limitation in ASPS research is the rarity of this tumour, making access to human specimens difficult. We have recently constructed a focused ASPS tissue microarray (TMA); utilizing this unique bioresource, the expression of potential targets in ASPS was evaluated.

Materials and methods

ASPS TMA

Institutional review board approval was obtained from The University of Texas M. D. Anderson Cancer Center (UTMDACC). Eighty-two ASPS patients seen at UTMDACC between 1986 and 2005 were identified; 33 had formalin-fixed paraffin-embedded tumour available (17 primary and 16 metastatic ASPS). These materials were used to construct a TMA as previously described.[11] Sections (4 μm) were cut, and standard haematoxylin and eosin-stained slides were examined to verify viable tumour presence.

IMMUNOHISTOCHEMISTRY

Commercially available antibodies against hepatocyte growth factor (HGF) (affinity-purified polyclonal sf21, dilution 1:5; R&D Systems, Minneapolis, MN, USA), c-Met (polyclonal c-28, dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated (Tyr1234/Tyr1235) c-Met (affinity-purified polyclonal, dilution 1:10; R&D Systems), phosphorylated/Ser221 MEK (clone 166F8, dilution 1:50; Cell Signaling Technology, Beverly, MA, USA), phosphorylated/Ser473 AKT (clone D9E, dilution 1:50; Cell Signaling), epidermal growth factor receptor (EGFR) (clone 31G7, dilution1:50; Zymed, San Francisco, CA, USA), vascular endothelial growth factor receptor (VEGF) (clone A-20, dilution 1:50; Santa Cruz), p53 (clone DO7, dilution 1:100; DakoCytomation, Carpinteria, CA, USA), TFE3 (affinity-purified goat polyclonal; P-16; Santa Cruz Biotechnology) and vimentin (monoclonal V9(1), dilution 1:900; Dako) were used for immunohistochemistry. Briefly, 4-μm thick unstained slides cut from the ASPS TMA were steamed for 30 min in citrate buffer pH 6.0 and incubated overnight at 4°C with primary antibody. Appropriate secondary antibodies were used for detection. Positive and negative controls were performed in parallel. Labelling intensity was graded as none (0), weak (1), moderate (2) or strong (3), and the percentage of positive tumour cells was estimated.

Results

Of the 33 tumours on the ASPS TMA, there were 26 in which all 10 of the markers could be evaluated; these form the core of this analysis (Figure 1, Table 1). As noted previously, all samples showed nuclear TFE3 reactivity suggesting the presence of a chimeric protein, and 16 of 18 cases with evaluable RNA showed evidence of a fusion transcript.[11] The expression

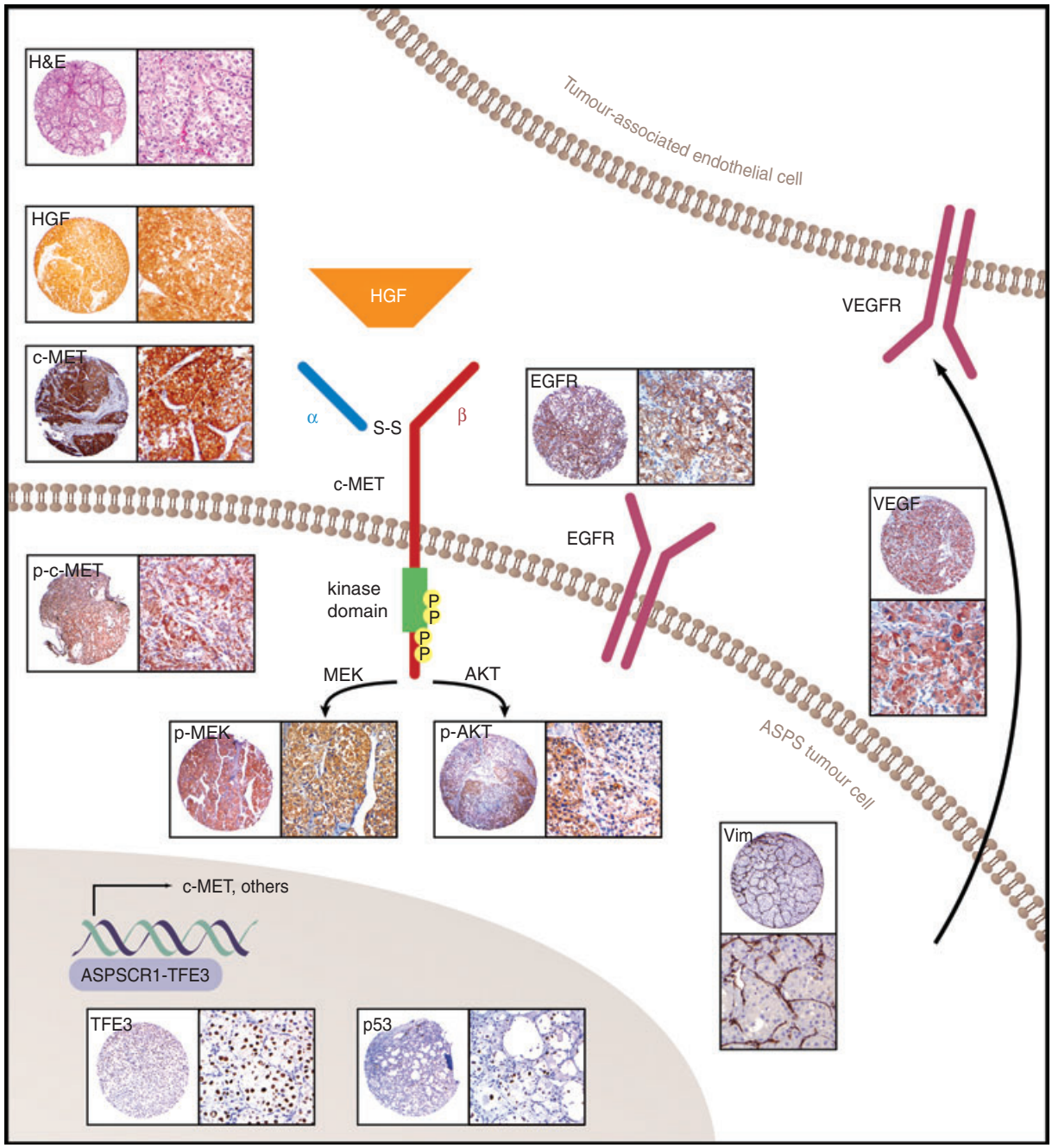

Figure 1. Simplified depiction of the hepatocyte growth factor/c-Met pathway with representative examples of staining of tissue microarray samples. The c-Met receptor is formed by combining a and b chains and when ligand is bound, the cytoplasmic domain is phosphorylated both at sites within the catalytic kinase domain, which phosphorylates other cytoplasmic proteins, and just distal or c-terminal to this domain. H&E, TFE3, p53, vimentin (Vim), epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) are also illustrated. Although other proangiogenic factors are also expressed by alveolar soft part sarcoma cells, VEGF may function by binding to its receptors (VEGFR) on tumour-associated endothelial cells. Many of the illustrated proteins or the pathways in which they play a role are potential therapeutic targets.

pattern was relatively uniform in the tumour cells of the paired 1-mm TMA samples; therefore, only intensity readings are noted in Table 1. Expression of HGF was strong in the samples, with all showing at least moderate intensity. c-Met demonstrated reactivity in all samples and 19 samples (73%) had at least moderate intensity. Phosphorylated or activated c-Met was present in all samples, with eight (30%) being moderate or

Table 1. Tabulation of tissue microarray alveolar soft part sarcoma cases including c-Met pathway, vimentin, TFE3 and presence of the ASPSCR1–TFE3 fusion transcript

Tumour	HGF	cMET	p-cMet	p-MEK	p-AKT	VEGF	EGFR	p53	Vimentin	TFE3	Fusion
P	3	3	3	3	2	3	0	0	0	+	NA
M	3	3	2	3	2	3	0	0	0	+	NA
M	3	3	2	3	1	3	0	1	0	+	NA
M	3	3	2	2	1	2	0	0	0	+	NA
P	3	2	2	2	1	2	2	0	0	+	1
P	3	2	2	2	1	2	2	1	2	+	1
M	3	2	2	2	1	2	0	1	0	+	1
P	3	2	2	2	1	3	0	0	0	+	2
M	3	2	1	1	1	1	1	1	0	+	NA
M	3	2	1	2	1	3	0	2	0	+	2
P	3	2	1	1	1	3	0	0	0	+	2
P	3	2	1	1	1	2	0	0	0	+	1
M	3	2	1	1	1	3	0	0	0	+	NA
M	3	1	1	1	1	2	0	1	0	+	2
M	3	1	1	1	1	3	0	0	0	+	2
M	3	1	1	1	1	2	0	0	0	+	1
M	3	1	1	1	0	1	0	0	0	+	NA
P	3	1	1	1	0	2	0	0	0	+	1
M	2	2	1	2	1	2	0	1	0	+	NA
R	2	2	1	2	1	2	0	1	0	+	NA
M	2	2	1	2	1	2	0	1	0	+	NA
M	2	2	1	2	1	2	0	1	0	+	NA
P	2	2	1	1	1	2	2	0	0	+	NA
M	2	2	1	1	1	3	0	0	0	+	NA
M	2	1	1	1	1	1	2	1	0	+	2
M	2	1	1	1	1	2	1	0	0	+	2

The characterized c-Met pathway constituents are colour coded for intensity and sorted based on strength of HGF, cMET, and p-cMET intensity. Tumour, tumour status; P, primary; M, metastasis; R, local recurrence; Fusion, fusion transcript type documented by reverse transcriptase-polymerase chain reaction if available; NA, not available, usually because of poor RNA quality in older paraffin blocks. ■ – negative, ■ – low, ■ – moderate, ■ – high.

strong and the remainder showing weak reactivity. In contrast, only six (23%) samples exhibited EGFR reactivity (moderate/weak = 6). All samples expressed phosphorylated MEK (moderate/strong = 12); phosphorylated AKT expression (moderate/weak) was observed in 24 samples (92%). VEGF was expressed by all ASPS samples to varying levels (moderate/strong = 23, weak = 3). Nuclear p53 immunoreactivity was

observed in 11 samples (42%); however, in the vast majority of cases ($n = 10$) only weak reactivity was seen. Vimentin was essentially negative in all but one case (Figure 1). The delicate capillary vasculature served as an internal positive control for vimentin reactivity.

Discussion

As ASPS is a very rare malignancy, current knowledge of potential therapeutic targets and their expression pattern in ASPS is limited. Data presented here identified several overexpressed potential drug targets in a cohort of human specimens demonstrating the utility of a focused TMA for such investigations.

MET, a gene encoding for the tyrosine kinase receptor and proto-oncogene c-Met, was recently identified as an ASPSCR1–TFE3 transcriptional target.[15] The promoter of this gene contains a TFE3 binding site; studies demonstrate that transcription of this gene is up-regulated by TFE3, ultimately producing functional protein.[15] c-Met is activated by HGF, resulting in autophosphorylation of the receptor and downstream signalling that promotes angiogenesis, proliferation, survival, cell motility and invasiveness in various systems.[16] Among the specific downstream pathways activated are the MEK/ERK and AKT pathways. Activity of these two proteins and c-Met can be assessed by their degree of phosphorylation. Study of a small number of ASPS samples indicated increased c-Met expression and activation.[15,17] Overall, our results support the presence of an intact signalling cascade downstream of the c-MET receptor. Immunohistochemistry revealed that the relevant ligand (HGF) is present, and c-Met itself is present and phosphorylated at expected sites for protein activation as well as the downstream effectors AKT and MEK. A Phase II clinical trial is currently accruing ASPS patients and others to evaluate the effect of ARQ 197, a novel c-Met inhibitor. Furthermore, it is possible that the ERK and AKT pathway activation seen is a result of stimulation by factors other than activated c-Met receptor. Currently, several AKT and MEK inhibitors are available, and targeting multiple activated pathways might result in superior anti-ASPS effects.

There is a strong rationale for targeting ASPS tumour-associated vasculature due to their highly angiogenic tumour phenotype. VEGF is the most widely investigated angiogenic factor and a target for several anti-cancer therapies. Overexpression of *VEGF* mRNA was identified in gene expression profiling of seven ASPS samples and was further confirmed via immunohistochemistry in this cohort of patients.[12] Our current study has further validated this finding, demonstrating VEGF overexpression in the evaluated samples. A recent case report suggested the efficacy of the anti-VEGF antibody, bevacizumab, in a patient with metastatic ASPS.[18] Taking all data together, further investigation of anti-VEGF therapies for ASPS treatment appears warranted. A recently described ASPS mouse xenograft model has also shown promise for anti-angiogenic therapy.[7] Furthermore, a growing number of combined inhibitors such as XL184 target multiple tyrosine kinase receptors, including c-Met and VEGFR; blockade of both overexpressed pathways in ASPS merits attention.

Lastly, TMA can be utilized to evaluate multiple markers potentially important in ASPS inception, differentiation and progression. For example, in the current study we evaluated the expression of p53 and vimentin. Mutations in p53 are a common molecular event in many soft tissue sarcoma types.[19,20] Increased nuclear expression is considered a marker for mutated/dysfunctional p53.[21] Our results demonstrate that p53 aberrations are apparently uncommon in ASPS; only one sample expressed relatively high nuclear p53 levels. Lack of vimentin immunoreactivity has previously been reported in ASPS; however, this is the largest single cohort where this marker has been explored.[22] ASPS do not bear resemblance to or express markers of either epithelial neoplasia or recognizable mesenchymal tissue. This lack of correlation with recognizable mesenchymal tissue is noted in other translocation-associated sarcomas – desmoplastic small round cell tumour, synovial sarcoma and Ewing's sarcoma – and warrants further elucidation of the potential ASPS cell of origin or its line of differentiation.[23]

The major limitation of ASPS research is the lack of cell lines and animal models to facilitate preclinical validation of potential therapeutic targets. Thus, treatment protocols for ASPS are largely empirical, since supportive preclinical studies are lacking. Cohorts of human ASPS samples are useful to demonstrate the expression and potential activation of therapeutic targets, an important first step towards establishing novel and sorely needed rational therapeutic approaches.

References

1. Christopherson WM, Foote FW Jr, Stewart FW. Alveolar soft-part sarcomas; structurally characteristic tumors of uncertain histogenesis. *Cancer* 1952; **5;** 100–111.
2. Folpe AL, Deyrup AT. Alveolar soft-part sarcoma: a review and update. *J. Clin. Pathol.* 2006; **59;** 1127–1132.

3. Portera CA Jr, Ho V, Patel SR *et al.* Alveolar soft part sarcoma: clinical course and patterns of metastasis in 70 patients treated at a single institution. *Cancer* 2001; **91;** 585–591.
4. Ogose A, Yazawa Y, Ueda T *et al.* Alveolar soft part sarcoma in Japan: multi-institutional study of 57 patients from the Japanese Musculoskeletal Oncology Group. *Oncology* 2003; **65;** 7–13.
5. Lieberman PH, Brennan MF, Kimmel M, Erlandson RA, Garin-Chesa P, Flehinger BY. Alveolar soft-part sarcoma. A clinicopathologic study of half a century. *Cancer* 1989; **63;** 1–13.
6. Lahat G, Tuvin D, Wei C *et al.* New perspectives for staging and prognosis in soft tissue sarcoma. *Ann. Surg. Oncol.* 2008; **15;** 2739–2748.
7. Vistica DT, Hollingshead M, Borgel SD *et al.* Therapeutic vulnerability of an *in vivo* model of alveolar soft part sarcoma (ASPS) to antiangiogenic therapy. *J. Pediatr. Hematol. Oncol.* 2009; **31;** 561–570.
8. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 2009; **136;** 823–837.
9. Weinstein IB, Joe A. Oncogene addiction. *Cancer Res.* 2008; **68;** 3077–3080; discussion 3080.
10. Ladanyi M, Lui MY, Antonescu CR *et al.* The der(17)t(X;17)(p11;q25) of human alveolar soft part sarcoma fuses the TFE3 transcription factor gene to ASPL, a novel gene at 17q25. *Oncogene* 2001; **20;** 48–57.
11. Lazar AJ, Das P, Tuvin D *et al.* Angiogenesis-promoting gene patterns in alveolar soft part sarcoma. *Clin. Cancer Res.* 2007; **13;** 7314–7321.
12. Stockwin LH, Vistica DT, Kenney S *et al.* Gene expression profiling of alveolar soft-part sarcoma (ASPS). *BMC Cancer* 2009; **9;** 22.
13. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. *Mol. Cancer Ther.* 2008; **7;** 3670–3684.
14. Goh PP, Sze DM, Roufogalis BD. Molecular and cellular regulators of cancer angiogenesis. *Curr. Cancer Drug Targets* 2007; **7;** 743–758.
15. Tsuda M, Davis IJ, Argani P *et al.* TFE3 fusions activate MET signaling by transcriptional up-regulation, defining another class of tumors as candidates for therapeutic MET inhibition. *Cancer Res.* 2007; **67;** 919–929.
16. Gentile A, Trusolino L, Comoglio PM. The Met tyrosine kinase receptor in development and cancer. *Cancer Metastasis Rev.* 2008; **27;** 85–94.
17. Jun HJ, Lee J, Lim DH *et al.* Expression of MET in alveolar soft part sarcoma. *Med. Oncol.* 2009; (Epub ahead of print).
18. Banihani MN, Al Manasra AR. Spontaneous regression in alveolar soft part sarcoma: case report and literature review. *World J. Surg. Oncol.* 2009; **7;** 53.
19. Taubert H, Meye A, Wurl P. Soft tissue sarcomas and p53 mutations. *Mol. Med.* 1998; **4;** 365–372.
20. Lahat G, Lazar A, Lev D. Sarcoma epidemiology and etiology: potential environmental and genetic factors. *Surg. Clin. North Am.* 2008; **88;** 451–481.
21. Das P, Kotilingam D, Korchin B *et al.* High prevalence of p53 exon 4 mutations in soft tissue sarcoma. *Cancer* 2007; **109;** 2323–2333.
22. Auerbach HE, Brooks JJ. Alveolar soft part sarcoma. A clinicopathologic and immunohistochemical study. *Cancer* 1987; **60;** 66–73.
23. Lazar A, Abruzzo LV, Pollock RE, Lee S, Czerniak B. Molecular diagnosis of sarcomas: chromosomal translocations in sarcomas. *Arch. Pathol. Lab. Med.* 2006; **130;** 1199–1207.

