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ORIGINAL ARTICLE

Exploring the Histogenesis and Diagnostic Strategy Using Immunoassay and RT-PCR in Alveolar Soft Part Sarcoma

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Abstract Alveolar soft part sarcoma (ASPS) is a rare soft tissue sarcoma, but it's easily misdiagnosed in rare locations. The derivation of ASPS is still uncertain, therefore we conducted this study to explore the histogenesis of ASPS by analyzing stem cell markers (ALDH1, CD29, CD133 and Nestin). Protein TFE3 and fusion gene ASPS-TFE3 were tested in paraffin to explore diagnostic strategy and molecular pathological features. In this study, nine cases of ASPS were immunostained with stem cell surface markers (ALDH1, CD29, CD133 and Nestin) and protein TFE3. Seven cases of ASPS mRNA were successfully extracted from nine paraffin-embedded tissues. The expression of fusion gene ASPL-TFE3 was examined by reverse transcriptase-polymerase chain reaction. The immunohistochemical staining of nine patients showed that CD29 and Nestin were negative in all nine cases (0/9). CD133 was weakly positive in one cases (1/9) and ALDH1 was weakly positive in one cases (1/9). TFE3 was positive in nine cases (9/9). Seven paraffin tissues could be successfully extracted with mRNA in nine cases. The results of Reverse Transcription Polymerase Chain Reaction (RT-PCR) showed that ASPL-TFE3 fusion transcripts could be tested in the seven cases (four cases being type 2 and three cases being type 1). The positive rate of CD133 and ALDH1 were less than 1% and the expression of CD29 and Nestin were negative in ASPS. Immunohistochemistry results indicated that the histogenesis of ASPS maybe not derive from mesenchymal stem cells. Immunohistochemistry staining showed that TFE3 protein expression was highly sensitive in ASPS. Furthermore, RT-PCR results showed that fusion gene ASPL-TFE3 (ASPL-TFE3 type 1 and ASPL-TFE3 type 2) was expressed in ASPS, which could provide information for clinical molecular pathological diagnosis and improve the diagnosis rate of rare atypical ASPS.

Keywords Alveolar soft part sarcoma · Stem cell markers · Immunohistochemistry · Fusion gene

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Introduction

Alveolar soft part sarcoma (ASPS) is a rare malignant soft tissue tumor that was initially described and named by Christopherson et al. in 1952 [1]. This tumor accounts for about 1% of all soft tissue sarcomas. Most ASPSs occur in adolescents young adults between 15 and 35 years of age [2]. It mostly presents as a slow growing mass in the deep soft

Table 1 Primary antibodies used in the study

Specificity	Dilution	Pretreatment	Immunostaining	Clone	Manufacturer
ALDH1	1:200	PCA-CB	Envision	EP1933Y	Abcam, Cambridge, UK
CD29	1:600	PCA-CB	Envision	EP1041Y	Abcam, Cambridge, UK
CD133	1:200	PCA-CB	Envision	05-PA1021	ARP, Waltham, America
Nestin	1:300	PCA-CB	Envision	SP103	Abcam, Cambridge, UK
TFE3	1:50	PCA-EDTA	Envision	EPR11591	Abcam, Cambridge, UK

PCA-CB, pressure cooker heating in citrate buffer (0.01 M, pH 6.0)

PCA-EDTA, pressure cooker heating in EDTA (pH 9.0)

tissue of the extremities, while usually metastasizes at presentation, frequently to lung, node, bone and brain [3–5].

Since ASPS has been reported, the histogenesis is still in dispute. The reported possible sources of ASPS include epithelial origin [6], neurogenic origin [7] and myogenous origin [8–10]. In recent years, many immunohistochemical studies showed that ASPS could express a variety of striated muscle related markers such as Actin, Desmin [9], Myogenin, MyoD1 [11, 12], so more scholars thought that ASPS origined from striated muscle, while the marked positive expression rate is weak and still a lack of convincing evidence. So the histogenesis of ASPS still need more exploration.

More recent studies reported that many soft tissue sarcomas originated from mesenchymal stem cells (MSCs) [13–15]. Alveolar soft part sarcoma belongs to soft tissue sarcoma. Immunohistochemical staining was usually used to explore the histogenesis of tumors. In this study, we performed an immunohistochemical study with a number of stem cell markers (ALDH1, CD29, CD133 and Nestin) to explore if the histogenesis of ASPS is derived from MSCs.

Typical ASPS has a clear morphological characteristics, while some ASPS grow in rare locations and the pathological morphological of partial ASPS is not typical which could cause misdiagnosed [16, 17].

Molecular studies found that the chromosome translocation of t (X; 17) in ASPS resulted in the formation of fusion gene ASPL-TFE3. These studies thought that the ASPL-TFE3 fusion gene derived from ASPL gene which located on chromosome 17 and TFE3 gene located on X chromosome [18–20]. In 2001, Ladanyi et al. detected the expression of fusion gene ASPL-TFE3 using reverse transcriptase-polymerase chain reaction (RT-PCR) for the first time, which not only has important diagnostic value but also further suggested the nosogenesis of ASPS [21, 22].

We detected the immunohistochemical expression of TFE3 and the expression of ASPL-TFE3 fusion gene to explore the molecular pathological features and diagnostic strategy of ASPS.

Materials and Methods

Materials

This study consisted of nine cases of ASPS which were collected from the Department of Pathology, Shihezi University School of Medicine, Xinjiang China between 1977 and 2011. This study was approved by the institutional ethics committee at the First Affiliated Hospital of Shihezi University School of Medicine. Each tumor was diagnosed according to the 4th edition WHO Classification of Tumors of Soft and Bone. All the cases were reviewed based on histopathological with HE staining and immunohistochemical analysis. A total of 9 formalin-fixed paraffin-embedded samples of primary ASPS from 9 patients were prepared for immunohistochemistry staining. Clinical information was obtained from the medical records.

Immunohistochemistry

The paraffin-embedded sections used for immunohistochemical staining were 4 um tissues obtained from formalin-fixed paraffin-embedded tissue blocks of ASPS. Microwave heat was used to enhance immunoreactivity. The antibodies and

Table 2 Primer sequences used in the study

Names of Primers	Sequences	Size
Actin forward	5-GAGCGGGAAATCGTCCGTGACATT-3	234 bp
Actin reverse	5-GATGGAGTTGAAGGTAGTTTCGTG-3	
ASPL forward	5-AAAGAAGTCCAAGTCGGGCCA-3	310 bp, 190 bp
TFE3 exon4 rev	5-CGTTTGATGTTGGGCAGCTCA-3	
N-ASPL forward	5-CGGGCCAGGATCCCCAGCAG-3	243 bp, 138 bp
N-TFE3 reverse	5-TGATGGCTGGTGTGGCCACG-3	

Table 3 The Clinicopathological data and molecular features for 9 tumors

Case	Age	Sex	Size (cm)	Site	β-actin	PCR for ASPL-TFE3	ASPL-TFE3 Type
1	36	F	8.6 × 5 × 4	Right thigh	+	+	2
2	34	M	3.5 × 3 × 1.5	Left thigh	+	+	2
3	17	F	5 × 4 × 2	Left upper arm	+	+	1
4	7	F	NA	Groin	−	−	/
5	11	F	7 × 6 × 4	Right orbit	−	−	/
6	36	M	NA	Left thigh	+	+	2
7	26	F	5.4 × 4.3 × 3	Brain	+	+	1
8	18	F	4 × 3.8 × 1.8	Right breast	+	+	2
9	24	M	NA	Left thigh	+	+	1

F female, *M* man, *NA* not available

positive controls for primary antibodies used in our study are summarized in Table 1. The immunohistochemical procedures was as following: EnVisions two-step immunohistochemical kit (Dako System, Glostrup, Denmark) were used to detect specific target proteins. The slides were first deparaffinized, dehydrated in graded ethanol concentrations, and slides were baked at 67 °C for 2 h and deparaffinized with xylene and rehydrated using graded alcohols. Heat-induced antigen retrieval was performed for TFE3 in EDTA (pH 9.0) and for stem cell markers in citrate buffer (pH 6.0). Then quenched with 3% hydrogen peroxide. The sections were incubated with the primary antibodies at 4 °C overnight. The samples were then washed with PBS and subsequently incubated with secondary antibodies in 37 °C. 3.3′-diaminobenzidine peroxidase substrate kit (Dako System, Glostrup, Denmark) was used as chromogen. Slides were then counterstained with hematoxylin and dehydrated. PBS was used to replace the primary antibody as negative control with the same procedures.

Reverse Transcription Polymerase Chain Reaction

Total mRNA was extracted from nine ASPS paraffin using TRIzol reagent according to standard protocols [23]. Seven cases were extracted successfully. One-step RT-PCR was performed in a volume of 25 mL, according to the manufacturer's

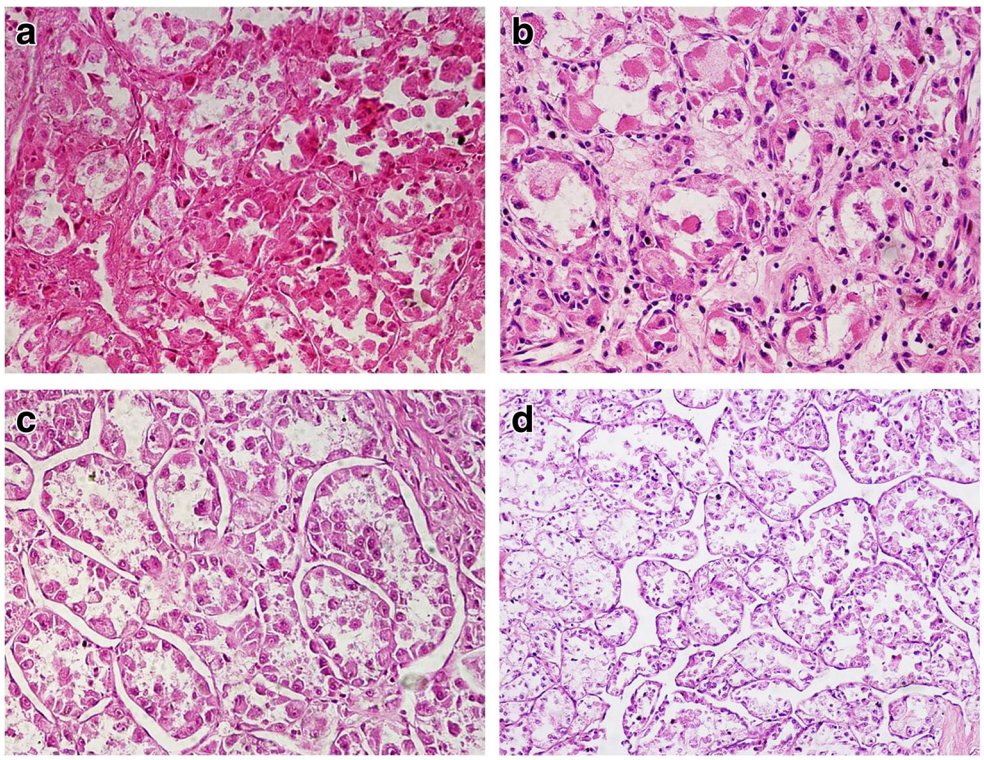

Fig. 1 Hematoxylin-eosin staining of ASPS showed most tumor cells arranged in alveolar or tubular structure. The tumor cells were separated into well-defined nests by thin vascular septa. These cells had round vesicular nuclei with prominent nucleoli and possess apparent eosinophilic cytoplasm. Magnification, ×200

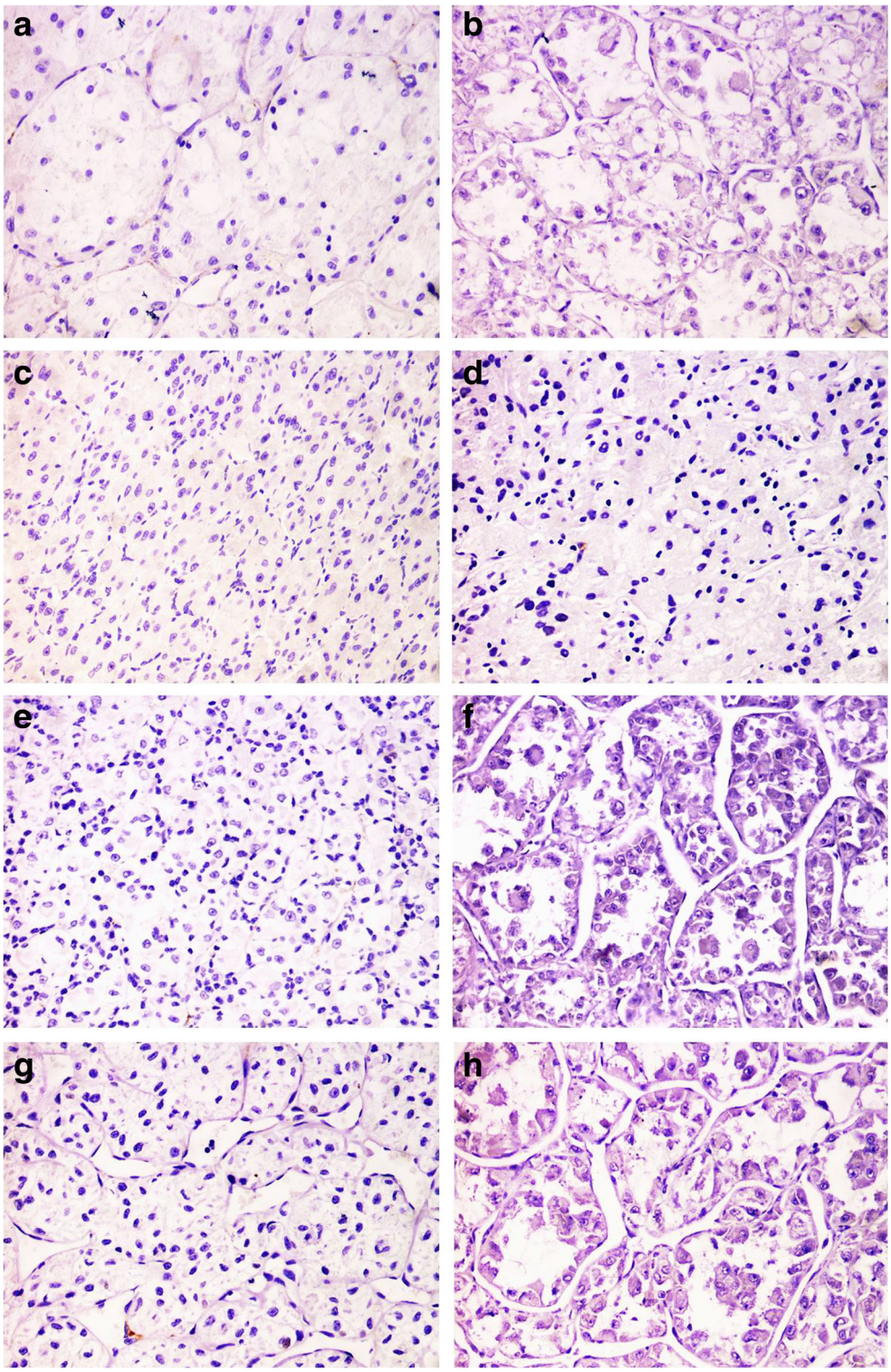

Fig. 2 Immunohistochemical staining of nestin, CD133, CD29 and ALDH1 expression in ASPS. The tumor cells show negative reactivity for nestin (**a** and **b**) and CD133 (**c** and **d**). CD29 was negative expressed in tumor cells (**e** and **f**). ALDH1 was weakly expressed in one case and the other cases were expressed negatively (**g** and **h**). Magnification, ×200

instructions. The information of primers used was showed in Table 2. The ASPL forward primers and TFE3 exon4 reverse primers were used in first-round PCR and N-ASPL primers and N-TFE3 exon4 primers were used in the nested procedure. Actin forward primers and Actin reverse primers were used to amplify β-actin gene.

Results

Clinical and Pathological Findings

The clinicopathological data and histopathologic characteristics of the 9 primary tumors are presented in Table 3 and Fig. 1. Six cases

of the patients were females, and three cases were males. The age of patients ranged from 7 to 36 years (median 18 years). The samples include eight primary tumors and one metastatic tumor. These tumor locations were limbs (5 cases), breast (1 case), groin (1 case), orbit (1 case) and brain (1 metastatic case). All patients underwent complete surgical excision. The minimum diameter of tumor is about 1 cm and the maximum diameter is about 9 cm (average diameter of 5 cm).

The clinical data of the nine cases showed that the most common symptoms of ASPS cases were painless and slowly growing mass. Some patients felt mild tenderness or numbness.

Immunohistochemical Findings

Stem Cell Markers Expression in ASPS

The stem cell surface markers were measured in nine cases. The immunohistochemistry of the 9 cases of ASPS showed that negative immunostaining for Nestin (Fig. 2a, b, Table 4). One case showed weakly positive for CD133, while the other eight cases was negative for CD133 (Fig. 2c, d, Table 4). The expression of CD29 was blood sinus tissue positive in four case, while it was negative expressed in all the 9 tumor areas (Fig. 2e, f, Table 4). ALDH1 was weakly expressed in one case, positive in blood sinus tissue in one case and negative expressed in the other 7 cases (Fig. 2g, h, Table 4). While TFE3 was positive in 9 cases (9/9) (Fig. 3, Table 4).

Reverse Transcription Polymerase Chain Reaction

The mRNA was successfully extracted from 7 cases of ASPS paraffin. The other 2 paraffin tissues were not enough to be extracted with mRNA. The results of RT-PCR showed that fusion gene ASPL-TFE3 type 1 (134 bp) was tested in 3 cases and fusion gene ASPL-TFE3 type 2 (243 bp) could be tested in other 4 cases (Fig. 4).

Table 4 Immunohistochemical findings

Case	CD29	CD133	Nestin	ALDH1	TFE3
1	–	–	–	–	+
2	–	–	–	–	+
3	Blood sinus +	Weak +	Blood sinus +	Weak +	+
4	Blood sinus +	–	–	–	+
5	–	–	–	–	+
6	–	–	–	–	+
7	Blood sinus +	–	–	–	+
8	Blood sinus +	–	–	Blood sinus +	+
9	–	–	–	–	+

Discussion

ASPS is a relatively rare and malignant tumor of the soft tissue [9]. It has a strong predilection for adolescents and young adults 15 to 35 years old, with a female predominance [24, 25]. ASPS grows slowly, but it is easy to relapse and metastasis [26]. Since first presented by Christopherson in 1952, the histogenesis of ASPS was still uncertain, so the biological behavior and pathogenesis was still not clear. In recent years, many methods were used to investigate the histogenesis of ASPS, including ultrastructure, cytochemistry, immune ultrastructure, biochemistry, immunohistochemistry, molecular genetics examination and so on. Up to now, there have been several hypotheses about the origins: (1) granulosa cells of skeletal muscle cells; (2) neuroendocrine; (3) juxtaglomerular cell; (4) muscle [6–10]. While the evidences of these hypothesis were not convincing. More scholars support the ASPS come from muscle source. They found that ASPS could express a variety of striated muscle related immunohistochemical markers. While the positive rate of muscle related markers, such as Actin, Desmin, Myogenin, MyoD1 were weak, the results are still no conclusive evidence. The histogenesis of ASPS should be further explored.

ASPS belongs to soft tissue sarcoma. Many studies suggested that soft tissue sarcoma could express differential stem cell-associated proteins and indicated that the histogenesis of some soft tissue sarcoma might be derived from mesenchymal stem cells, such as rhabdomyosarcomas, synovial sarcoma and so on [13–15]. MSCs, considered as multipotent cells, have been demonstrated reside in the connective tissues of numerous organs and have the self-renewal capability and potential for several differentiated cell types [27]. Recent studies have shown that MSCs can reside in the perivascular region in multiple organs, such as adipose tissue, brain and umbilical cord [28]. So we made an assumption that whether the histogenesis of ASPS was from mesenchymal stem cells as other soft tissue sarcomas. In the present study, stem cells surface markers (ALDH1, CD29, CD133 and Nestin) were tested by immunohistochemistry. Immunohistochemical findings showed that almost all MSCs markers expression were negative in the nine cases. This result failed to certificate that ASPS derived from MSCs. The histogenesis and pathogenesis of ASPS still need to been explored in future.

ASPS is feasible to be recognized in morphology. But ASPS growing in rare locations or with atypical pathological morphological could cause misdiagnosed and delay the early treatment of patients. It's important to find other useful ways to improve the diagnosis rate. Over the past years, further deep researches on the diagnostics and molecular pathogenesis of the specific chromosome translocation in soft tissue sarcoma have progressed, however studies about the cytogenetic changes and molecular

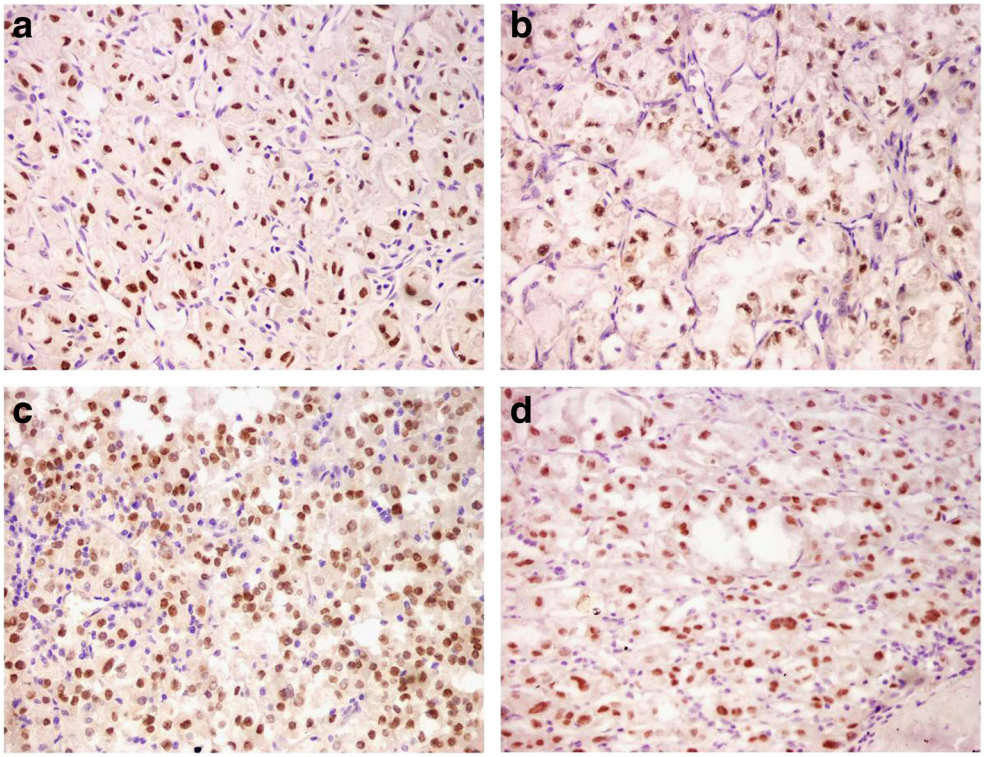

Fig. 3 Immunohistochemical staining of TFE3 in ASPS. The tumor cells showed strong nuclear immunostaining for TFE3 (**a**, **b**, **c** and **d**). Magnification, ×200

mechanism are less. From 1995, Jannie [29] and Heimann [30] et al. have successively found and domenstrated that special chromosome change der(17)t(x;17) (p11.2;q25) with fusion gene ASPL-TFE3 exist in ASPS. After that, Tamás Tornóczky [31] et al. have also demonstrated that in six of eight ASPS, the abnormalities of chromosome 17 mainly on the segment of q25 (e.g., addition, duplication, translocaton from Xp11) by FISH. In addition, they have found that alterations of trisomy for choromosome 7 and monosomy for chromosomes 8 and 18 may involve in the progresssion or clonal evolution of ASPS in 2001. These findings may indicate that gene(s) on chromosome 1, 6, 7, 8 and 18 are related with the pathogenesis of ASPS and these regions may encrypt gene(s) which are important in the pathogenesisi of ASPS. At the same year, Ladanyi et al. detected the expression of fusion gene ASPL-TFE3 by RT-PCR for the first time, which put out in the process of the occurrence of ASPS, chromosome loss and/or get known or unknown genes may have synergy with ASPS-TFE3 fusion gene protein, which indicate that the occurrence of ASPS besides chromosome translocation fusion gene transcription, also involves other aberrations caused by the changes of known or unknown gene structure and function and other ways [21, 22]. In the present study, we

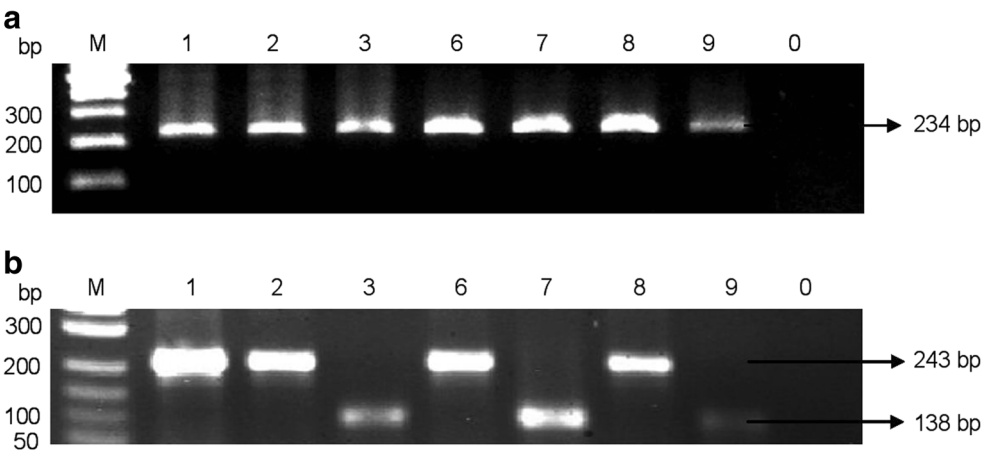

Fig. 4 ASPS-TFE3 fusion gene were detected in paraffin-embedded ASPS. A. Case 1 to 3 and 6 to 9 were extracted successfully from tumors. β-actin gene was detected by nested RT-PCR. B. ASPS-TFE3 fusion gene was detected by semi-nested PCR, the products of 138 bp correspond to ASPS-TFE3 type 1 and 243 bp correspond to ASPL-TFE3 type 2. M_1, 100-bp DNA ladder; M_2, 50-bp DNA ladder; 0, negative control

extracted ASPS mRNA from seven cases of paraffin and tested the expression of fusion gene ASPL-TFE3 using RT-PCR. At the same time, immunohistochemistry of TFE3 was tested in the nine cases. The results showed that 4 cases of ASPS could be tested fusion gene ASPL-TFE3 type 2, other 3 cases could be tested fusion gene ASPL-TFE3 type 1. The TFE3 antibody has shown a high sensitivity in the diagnosis of ASPS.

In summary, we explored the histogenesis of ASPS and found that ASPS maybe not derive from MSCs and the histogenesis need to been further explored. TFE3 immunostaining and fusion gene ASPL-TFE3 expression maybe provide information for clinical molecular pathological diagnosis and improve the diagnostic rate of ASPS.

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Compliance with Ethical Standards

Competing Interests The authors have declared that no competing interests exist.

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