

Myofibroblasts in Pulmonary and Brain Metastases of Alveolar Soft-Part Sarcoma: A Novel Target for Treatment?¹

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Abstract

Alveolar soft-part sarcoma (ASPS) is a rare neoplasm with chromosomal translocation that results in *ASPL-TFE3* fusion. It is a slow-growing lesion associated with a high incidence of pulmonary and brain metastases indicating poor survival. We demonstrated that the ASPS metastases include also stromal myofibroblasts. These cells proliferate, express smooth-muscle genes, and synthesize extracellular matrix proteins, all of which are characteristics of activated myofibroblasts. The tumor cells also exhibited stromal components such as transforming growth factor beta (TGF β)-dependent, hypoxia-regulated cytoglobin (stellate cell activation association protein, cytg/STAP) and prolyl 4-hydroxylase, a collagen cross-linking enzyme. The pulmonary ASPS myofibroblasts synthesize serum response factor (SRF), a repressor of Smad3-mediated TGF β signaling essential for myofibroblast differentiation and Smad3. The phosphorylated active Smad3 was found mostly in the tumor cells. The brain tumor cells express cytg/STAP, but in contrast to the lung metastases, they also express SRF, Smad3, and phospho-Smad3. Halofuginone, an inhibitor of myofibroblasts' activation and Smad3 phosphorylation, inhibited tumor development in xenografts derived from renal carcinoma cells harboring a reciprocal *ASPL-TFE3* fusion transcript. This inhibition was associated with the inhibition of TGF β /SRF signaling, with the inhibition of myofibroblasts' activation, and with the complete loss in TFE3 synthesis by the tumor cells. These results suggest that the myofibroblasts may serve as a novel target for treatment of ASPS metastases.

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Introduction

Alveolar soft-part sarcoma (ASPS) is a rare soft tissue neoplasm that can arise in any region of the body. In young adults, the neoplasm often develops in the upper or lower extremities; in young children and adolescents, the tumor commonly originates in the head and neck where the orbit or tongue are the most favorable sites [1,2]. The neoplasm is characterized cytogenetically by an unbalance recurrent chromosomal translocation resulting in a consistent der(17)t(X;17)(p11;q25). This translocation results in the fusion of the *ASPL* gene on chromosome 17 to the *TFE3* gene on chromosome X [1,3]. Alveolar soft-part sarcoma is typically a slow-growing lesion, but frequently, it is

Abbreviations: ASPS, alveolar soft-part sarcoma; ECM, extracellular matrix; TGF β , transforming growth factor beta; SRF, serum response factor; α SMA, α smooth-muscle actin; cygb/STAP, cytoglobin, stellate cell activation association protein; P4H, prolyl 4-hydroxylase

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associated with high incidence of pulmonary and brain metastases early in the course of the disease [4]. Treatment of ASPS patients includes surgical resection of the primary tumor and of the metastases when possible, the use of chemotherapy, and, in some cases, the use of radiotherapy [2,5]. The curative potential of surgery alone has remained unclear. Local recurrences have been reported to occur in up to 20% of patients with ASPS [6], although most of the patients operated with wide surgical margins did not develop local recurrence [7]. Few, if any, of the patients showed a clinical response to chemotherapy, and chemotherapy or immunotherapy for metastatic tumor, including cisplatin, ifosfamide, doxorubicin, carboplatin, methotrexate, interferon, and interleukin 2, regimens was without any clinical response [7,8]. Tumor size, bone involvement, and especially metastases at presentation indicate a significantly poorer prognosis [7]. Therefore, alternative treatment approaches are needed especially for patients with a metastatic disease.

Most solid tumors consist of a mixture of neoplastic and nonneoplastic cells with extracellular matrix (ECM) components. This cellular microenvironment directly modulates tissue architecture, cell morphology, and cell fate [9], and the ECM–stromal cell interaction contributes to the neoplastic phenotype [10]. The conversion of fibroblasts to myofibroblasts, as mediated by transforming growth factor beta (TGF β) and its inducible transcription factor the serum response factor (SRF), is the most prominent stromal reaction in a large number of epithelial lesions [11–13]. After binding of TGF β to its receptor, signaling to the nucleus occurs predominantly by the phosphorylation of cytoplasmic mediators of the Smad family [14]. The regulation of matrix proteins, in general, and of collagen type I gene expression, in particular, involved the Smad3 signaling pathway [15]. In addition to the major increase in ECM components, the fibroblasts that acquire an activated phenotype are characterized by expressing smooth muscle genes such as α smooth-muscle actin (α SMA) and transgelin [16]. Smad3 directly links TGF β signaling to an SRF-associated regulatory network in controlling muscle-specific gene transcription [17]. The myofibroblasts are associated with the tumor cells at all stages of cancer progression [18], and in various malignancies, tumor-dependent differentiation of fibroblasts toward myofibroblasts further promotes neoplastic progression [19–21]. It is well established that collagen type I, the major ECM component produced by myofibroblasts, not only functions as a scaffold for the tissue but also regulates the expression of genes associated with cellular signaling and metabolism, gene transcription, and translation, thus affecting fundamental cellular processes that are essential for tumor progression. For example, collagen type I was found to be a signal for invasion, and its intratumoral expression level has been associated with increased tumor invasiveness [22]. Stellate cell activation association protein, also known as cytoglobin (cygb/STAP) is another TGF β -regulated and collagen-related gene expressed by the myofibroblasts [23]. Although its specific role is yet to be elucidated, a potential role in the detoxification of reactive oxygen species in tumors and other pathologies has been suggested [24,25].

In this study, we demonstrated the presence of activated myofibroblasts and demonstrated the existence of the TGF β /SRF–dependent Smad3 pathway in human ASPS lung and brain metastases. In xenografts produced by cells harboring the reciprocal *ASPL-TFE3* fusion transcript, inhibition of the Smad3 phosphorylation by halofuginone, an inhibitor of fibroblast-to-myofibroblast transition [26–28], resulted in the reduction in tumor growth that was accompanied by the inhibition of myofibroblast activation and reduction in TFE3 expression by the tumor cells.

Patient and Methods

Patients

Brain metastases were obtained from 18- and 20-year-old male ASPS patients with thigh tumors and lung metastases. After the removal of the primary tumors, one of the patients was clinically stable with no therapy for 4 years, when a single brain metastasis was identified by magnetic resonance imaging and removed surgically. Additional brain metastasis appears during the next 2 years, despite cranial irradiation. The second patient developed brain metastasis soon after diagnosis, and tissue was obtained from a resection. The pulmonary biopsy was taken from a lower lobe of a 16-year-old female who underwent a thoracotomy to lessen tumor burden. The primary tumor at the left scapula developed to multiple pulmonary nodules bilaterally.

Materials

Fetal calf serum, Dulbecco's modified Eagle's medium, and trypsin-EDTA solution (0.02–0.25%) were obtained from Biochemical Industries (Bet-HaEmek, Israel). Sirius red F3B was obtained from BDH Laboratory Supplies (Poole, England). Halofuginone bromhydrate was obtained from Collgard Biopharmaceuticals Ltd (Tel Aviv, Israel). Monoclonal antibodies to α SMA were from Dako A/S (Glostrup, Denmark), and antibodies to cygb/STAP were a gift from N. Kawada from the Department of Hepatology, Osaka City University, Japan. Smad3 antibodies were from Abcam (Cambridge, UK); TFE3, mMET, phospho-Smad3, and SRF antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals and Experimental Design

The human renal cell carcinoma cell line FU-UR-1 (a generous gift from Dr Masako Ishiguro, Department of Pathology, Fukuoka University School of Medicine, Fukuoka, Japan) with the reciprocal *ASPL-TFE3* fusion transcript [29] was cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. All animal experiments were carried out according to the guidelines of the Volcani Center Institutional Committee for Care and Use of Laboratory Animals. Nude (CD1 *nu/nu*) male mice (Harlan Laboratories, Israel) were housed in cages (four mice per cage) under conditions of constant photoperiod (12-hour light/12-hour dark) with free access to food and water. Xenografts were established by implanting the FU-UR-1 cells in Matrigel (2×10^6 cells/ml) subcutaneously using a 27-gauge needle. Halofuginone (5 μ g per mouse) was administered intraperitoneally three times a week starting at the time that the tumor volume reached 40 mm³. During the experiments, tumor volume was determined with a caliper according to the following formula: length \times width \times depth \times 0.5236; and it is presented as the mean \pm SE.

Preparation of Sections, In Situ Hybridization, and Immunohistochemistry

At the end of the experiments, tumors were collected and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Serial 5- μ m sections were prepared from the xenograft tumors and from the ASPS brain and lung metastases. Samples were stained with Sirius red for collagen. *In situ* hybridization for collagen α_1 (I) was performed as previously described [26]. Immunohistochemistry was performed with anti-Cygb/STAP (1:500), monoclonal anti- α SMA antibodies (1:200), Smad3 (1:200), TFE3 (1:100), mMET (1:50), phospho-Smad3 (1:50), and SRF (1:500) antibodies. Peroxidase activity was revealed by using 3,3'-diaminobenzidine as chromogen.

Results

Alveolar Soft-Part Sarcoma Pulmonary and Brain Metastases

Tumor cells in both lung and brain metastases were large but uniform, with abundant granular-to-vacuolated cytoplasm and well-defined cell borders. The nuclei were round-to-polygonal and vesicular, often with prominent nucleoli. The nuclei were positive for TFE3, and in both the lung and the brain specimens, extensive cell division was observed (Figure 1). In both pulmonary and brain metastases, the stroma and the tumor cells were positive for proliferating cell nuclear antigen. No *MET* receptor tyrosine kinase expression was observed in the pulmonary and brain ASPS metastases (data not shown), although it was identified in few malignancies with TFE3 translocations and was significantly overexpressed in some primary ASPS tumors [30].

Stromal Components in ASPS Pulmonary Metastases

The cancer cells of the pulmonary metastasis were surrounded by myofibroblasts that express the collagen $\alpha_1(I)$ gene that resulted in

high levels of collagen synthesis (Figure 2). The collagen is organized in fibrils that encircle clusters of cancer cells, which resulted in fibrous septa. No expression of the collagen $\alpha_1(I)$ gene was observed either in the surrounding lung tissue or in the primary tumor (data not shown). The myofibroblasts also exhibited high levels of α SMA, a hallmark of activated myofibroblasts as found in ASPS tumors from other locations [31]. High levels of α SMA were also observed in the blood vessels in the periphery of the metastasis. The cancer cells also display characteristic components characteristic of the stroma. They synthesized cytg/STAP that is usually expressed by myofibroblasts from various origins [27,28] and prolyl 4-hydroxylase (P4H), one of the major enzymes required for collagen cross-linking and maturation (Figure 3). Both cytg/STAP and P4H were exclusively synthesized by the tumor cells and not by the myofibroblasts within the tumor or by the surrounding lung tissue.

Smad3 Signaling in ASPS Pulmonary Metastases

Serum response factor, which acts as a nuclear repressor of Smad3-mediated TGF β signaling, is essential for myofibroblast differentiation

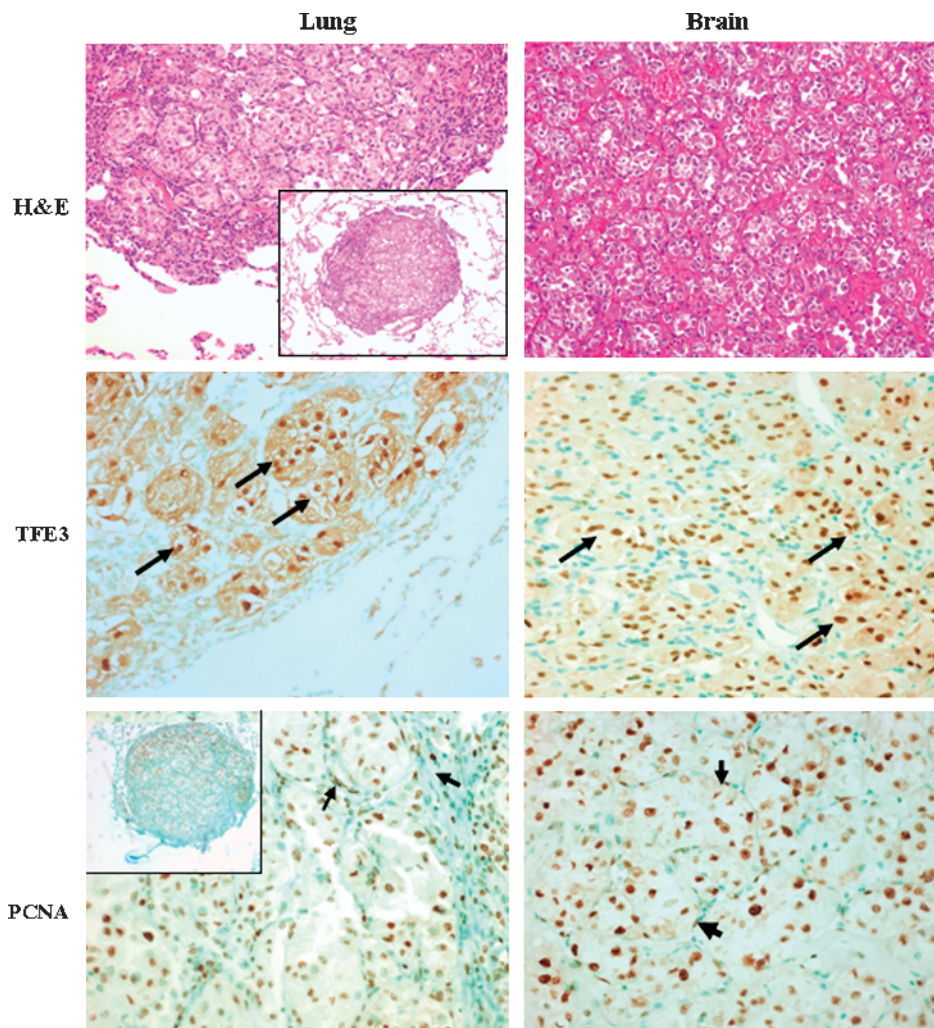


Figure 1. Pulmonary and brain ASPS metastases. Hematoxylin and eosin staining shows an organoid, pseudoalveolar large round to polyhedral eosinophilic cells displaying large nucleus with a uniform “small nest” pattern separated by fine fibrous septa (original magnification, $\times 100$; insert’s original magnification, $\times 40$). The pulmonary and brain tumor cells express high levels of TFE3 (arrows; original magnification, $\times 200$). Both the metastatic cells and the stroma cells (arrows) stain positive for proliferating cell nuclear antigen (original magnification, $\times 200$) suggesting that they are at their proliferative state.

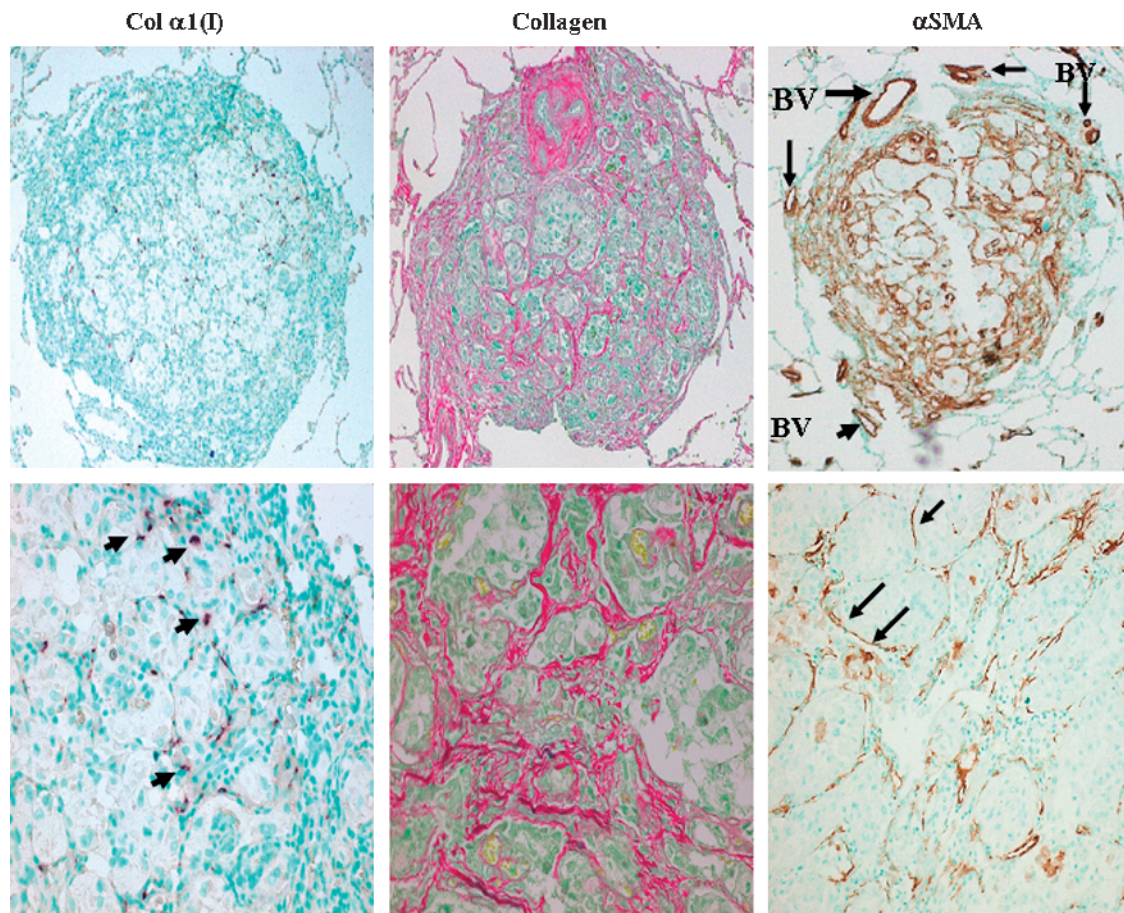


Figure 2. Pulmonary ASPS metastases contain activated myofibroblasts. The myofibroblasts surrounding clusters of cancer cells express the collagen $\alpha_1(I)$ gene (arrows) and synthesize large amounts of collagen (stained by Sirius red). These cells and the endothelial cells of the blood vessels (BV) at the periphery of the metastasis synthesize α SMA as determined by immunohistochemistry. Original magnifications: upper panel, $\times 40$; lower panel, $\times 400$.

[32]. Smad3, in turn, links TGF β signaling to SRF-associated regulatory network [17]. The stromal cells within the pulmonary metastasis, but not the tumor cells or the adjacent lung tissue, express SRF (Figure 4). Transforming growth factor beta possesses dual tumor-suppressive and oncogenic effects. During tumorigenesis, malignantly transformed cells often lose the response to the tumor-suppressive effects of TGF β , which, in turn, starts to act as an autocrine tumor-promoting factor by enhancing cancer invasion and metastasis. The stroma cells within the neoplastic tissue and the lung epithelial cells in the adjacent tissue stain positive for Smad3, whereas the phosphorylated active form of Smad3 was found mostly in the tumor cells, although some of the stromal cells exhibited phospho-Smad3 as well.

Alveolar Soft-Part Sarcoma Brain Metastases

The ASPS brain myofibroblasts, as detected by their ability to exhibit α SMA, synthesize much less collagen than the pulmonary ASPS cells, resulting in much thinner septa surrounding the tumor cells (Figure 5). Similar to the pulmonary tumor cells, the metastatic brain cancer cells express cytg/STAP, but in contrast to the lung metastases, they also express SRF, Smad, and the phosphorylated active form of Smad3. The same results were obtained from both ASPS patients' brain tumor specimens.

Inhibition of Tumor Growth Derived from Cells with Reciprocal ASPL-TFE3 Fusion Transcript

Xenografts were established in the nude mice through subcutaneous implantation of FU-UR-1 renal carcinoma cell line that contain the reciprocal *ASPL-TFE3* fusion transcript [29]. In the untreated mice, the tumors reached the volume of 277 mm³ at 33 days after implantation (Figure 6A). Halofuginone, an inhibitor of myofibroblast activation and of Smad3 phosphorylation [28,33,34], inhibited tumor growth and development and, at 33 days after cell implantation, had only grown to 38% of the volume of the control mice (Figure 6A). The inhibition in tumor volume by halofuginone was associated with the near complete reduction in TFE3 synthesis by the tumor cells and with the reduction in activated myofibroblasts as demonstrated by abrogation of collagen and α SMA synthesis (Figure 6B). Halofuginone also inhibited SRF synthesis, and in agreement with other studies [27,33,34], halofuginone inhibited the phosphorylation of Smad3 without affecting Smad3 levels.

Discussion

Alveolar soft-part sarcoma is a rare and uncommon tumor with a characteristic histopathology, and despite overall indolent clinical behavior, patients eventually develop distant metastases [31]. The ASPS lung and brain metastases consists of at least two cell types: the

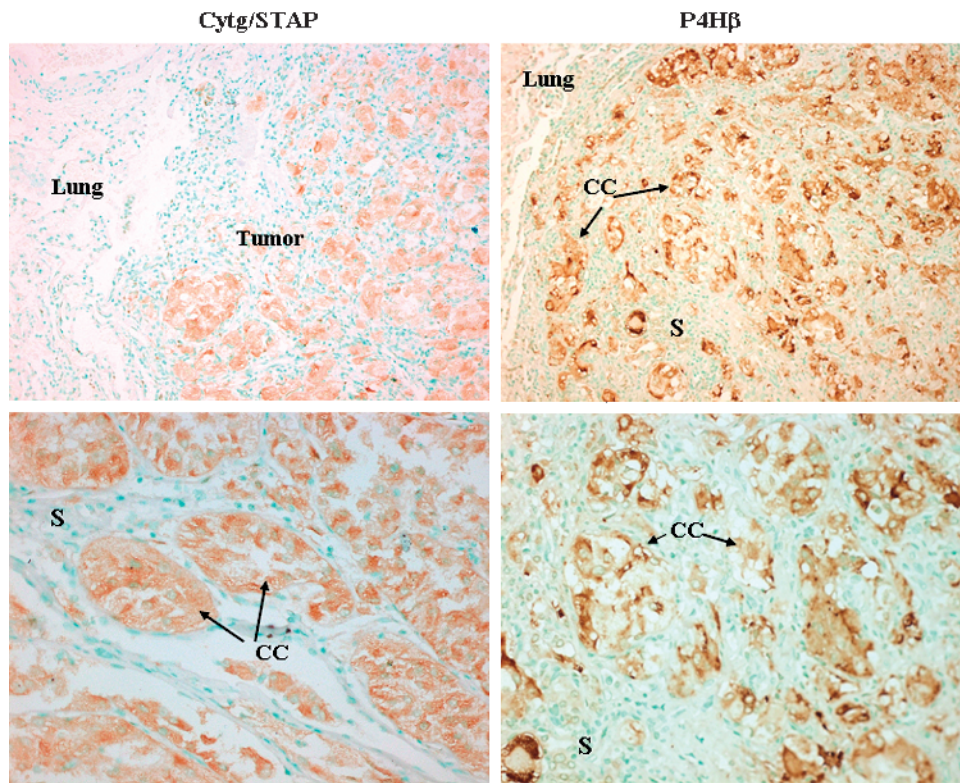


Figure 3. Pulmonary metastatic ASPS tumor cells express stromal components. The tumor cells, but not the stromal cells, synthesize cygb/STAP and the β subunit of prolyl 4-hydroxylase (P4H β) as demonstrated by immunohistochemistry (arrows). CC indicates cancer cells; S, stroma populated with myofibroblasts. Original magnifications: upper panel, $\times 100$; lower panel, $\times 400$.

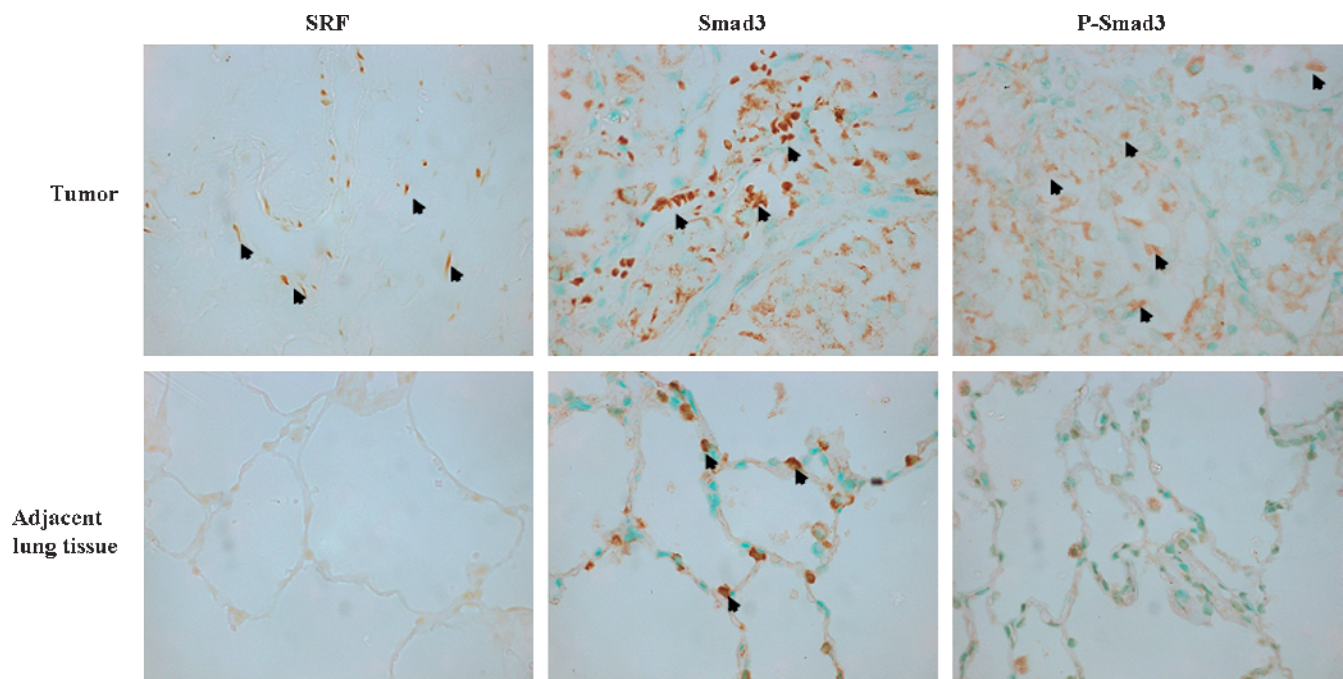


Figure 4. Smad3 signaling in pulmonary ASPS metastasis. The myofibroblasts in the pulmonary metastasis, but not in the surrounding lung tissue, exhibited SRF (arrows), whereas Smad3 is present both in the tumor stromal cells and lung epithelial cells. Phospho-Smad3 was demonstrated mostly in the tumor and some of the stroma cells but not by the lung epithelial cells. Original magnification, $\times 400$.

myofibroblasts and the ASPS tumor cells that express TFE3 (Figure 1) with a probable cross talk between the two. The ASPS metastatic myofibroblasts, like in other tumors [28,35], acquire muscle-specific phenotype as demonstrated by high levels of α SMA, synthesize high levels of ECM constituents especially collagen type I (Figure 2), and demonstrate proliferation capabilities (Figure 1), all of which are characteristic of activated myofibroblasts. The myofibroblasts within the pulmonary ASPS tumor exhibited SRF (Figure 4), which is a transcription factor that controls many mitogen-responsive and muscle-specific genes such as α SMA and *MyoD* through the regulatory elements-CARG boxes in their promoter [36,37]. In the brain, however, SRF is expressed by the tumor cells and not by the stromal cells, although the stroma cells synthesized high levels of α SMA (Figure 5), which may indicate a cross talk between the two cell types. The tumor cells are most likely involved in the collagen metabolism as well, which suggests again an intimate relationship between the two cell types. They express P4H, an enzyme required for collagen cross-linking (Figure 3), as was found in other tumors [38,39]. The brain and lung metastatic tumor cells also expressed *cygb*/STAP (Figures 3 and 5) that is involved in collagen metabolism as well [23]. It is interesting to note that the synthesis of these two proteins is under the control of hypoxia [24,40]. *Cygb*/STAP is probably involved in cellular oxygen homeostasis and supply and plays a role as an oxygen reservoir that is used under hypoxic conditions. In contrast to many tissues and tumors in which *cygb*/STAP is synthesized by the stroma cells [27,28,41], in ASPS lung and brain metastases, only the cancer cells exhibited high levels of *cytg*/STAP. Whether this localization of *cytg*/STAP is unique to ASPS is still to be determined.

The origin of ASPS is controversial, and based on reports that ASPS cells express muscle-specific genes such as α SMA, *MyoD1*,

and *myogenin*, it has been suggested that ASPS cells represent an unusual form of myogenic tumor [1,42,43]. Conversely, other studies could not detect muscle-specific genes in ASPS tumors, which do not support a myogenic origin [44–46]. Our findings suggest that the muscle-specific genes observed in ASPS may, in certain cases, be associated with the stroma fibroblast-to-myofibroblast transition and SRF function controlling muscle-specific gene transcription [17] rather than with an indication of its origin. These myofibroblasts that are unique mesenchymal cells with properties inherent to both muscle and nonmuscle cells express skeletal muscle structural and regulatory proteins. These include sarcomeric myosin heavy chain, α SMA, and *MyoD*, and despite the presence of such myogenic regulatory proteins, these cells do not terminally differentiate into skeletal muscle [47].

Recently, overexpression of the *MET* receptor tyrosine kinase gene was found in some ASPS primary tumors. It was demonstrated that ASPL-TFE3 mediated transcriptional up-regulation of the *MET* receptor tyrosine kinase, which results in its autophosphorylation and activation of downstream signaling in the presence of hepatocyte growth factor. Thus, *MET* signaling was suggested as a target for pharmacological intervention for ASPS patients [30]. The ASPS pulmonary and brain metastases were negative for *MET*, suggesting that different treatment strategies may be required for ASPS primary tumors and metastases.

The myofibroblasts together with ECM components provide the microenvironment that is pivotal for tumor cell growth, tumor invasion, and metastasis [18]. Thus, inhibition of fibroblast activation may become a viable approach for ASPS tumor treatment. Halofuginone, which at present is being evaluated in clinical trials [48], inhibits the fibroblast-to-myofibroblast transition of the tumor microenvironment [28,49] by inhibiting Smad3 phosphorylation downstream of the

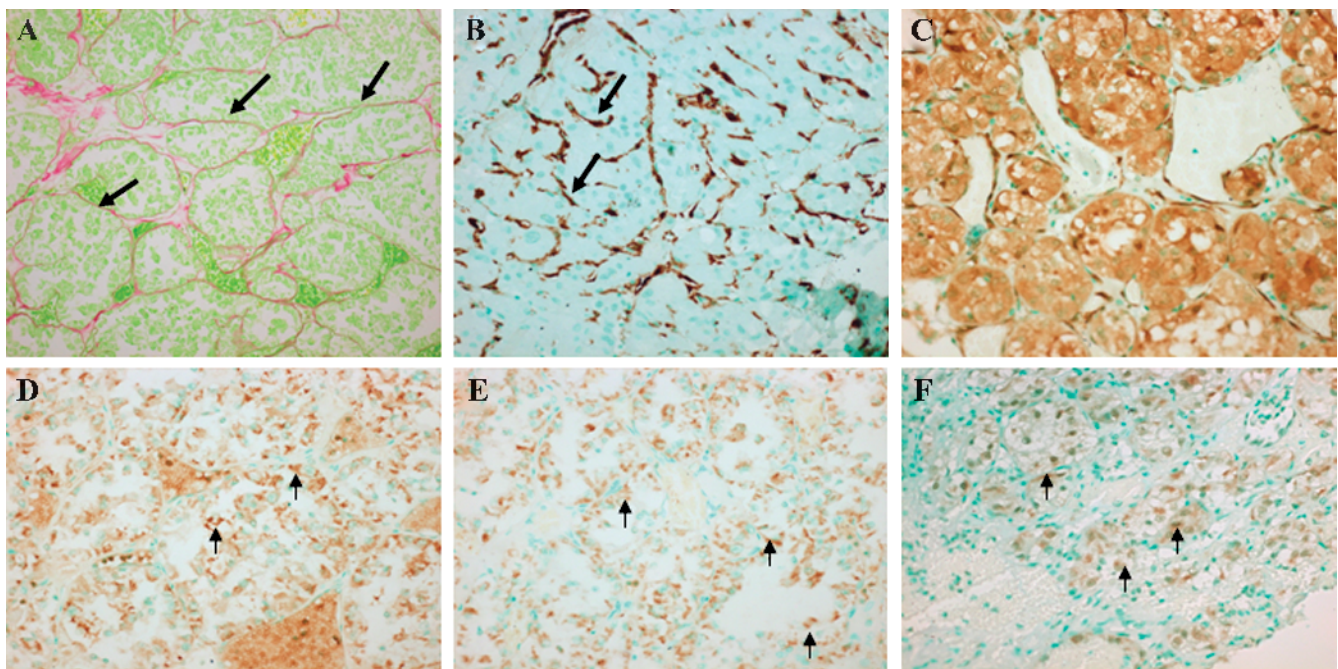


Figure 5. Stroma components and Smad signaling in the ASPS brain metastases. The tumor cells in the brain metastases are surrounded by fibrous septa (A; original magnification, $\times 100$) and populated by myofibroblasts displaying a large amount of α SMA (B; original magnification, $\times 100$). The tumor cells exhibited high levels of *cytg*/STAP (C; original magnification, $\times 100$), SRF (D; original magnification, $\times 400$) Smad3 (E; original magnification, $\times 400$), and phospho-Smad3 (F; original magnification, $\times 100$; insert's original magnification, $\times 400$).

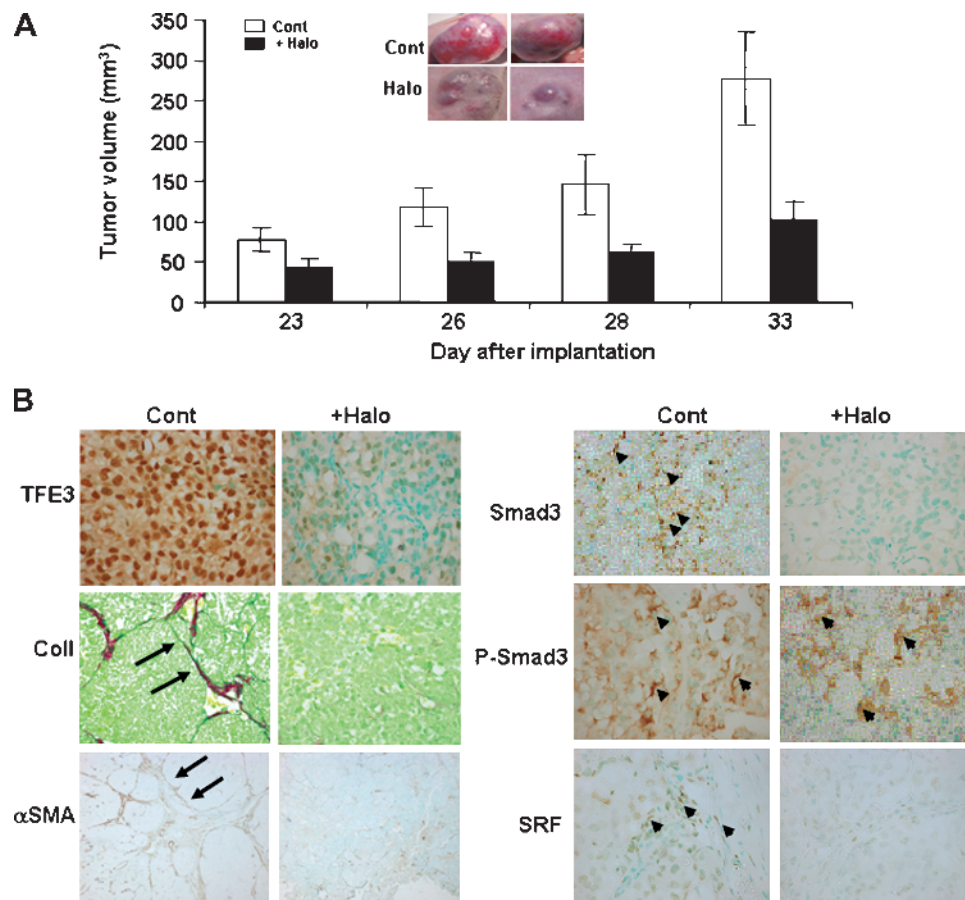


Figure 6. Inhibition of tumor growth in an *ASPL-TFE3* fusion transcript xenografts by halofuginone. (A) Halofuginone treatment inhibited tumor growth and development in xenografts derived from the FU-UR-1 cell with reciprocal *ASPL-TFE3* fusion transcript. Insert: Tumors of control and halofuginone-treated mice at 33 days after FU-UR-1 cell implantation. (B) Inhibition of TFE3, myofibroblasts' activation, and Smad3 signaling in FU-UR-1 xenograft by halofuginone. At day 33 after FU-UR-1 cell implantation, tumors from control and halofuginone-treated mice were taken for histopathology analysis. Almost all of the tumor cells of the untreated mice exhibit high levels of TFE3, which was abrogated by halofuginone treatment (original magnification, $\times 400$). Halofuginone treatment inhibited the collagen and α SMA synthesis observed in the control mice (arrows; original magnification, $\times 200$) suggesting inhibition of myofibroblasts' activation. Halofuginone also inhibited the SRF and phosphorylation of Smad3 without affecting Smad3 levels (arrows; original magnification, $\times 400$).

TGF β /SRF signaling [33,34]. Previously, we reported that targeting the fibroblast-to-myofibroblast transition with halofuginone may synergize with low doses of chemotherapy in achieving a significant antitumoral effect, avoiding the need of a high dose of chemotherapy and its toxicity without impairing treatment efficacy [28]. In xenografts that contain the reciprocal *ASPL-TFE3* fusion transcript, halofuginone inhibited myofibroblasts' activation as demonstrated by the abrogation of α SMA and collagen synthesis probably due to the TGF β /SRF-Smad3 signaling inhibition (Figure 6). This resulted in a major decrease in tumor growth and development (Figure 6A). The role of TGF β in tumor development is controversial and may be dependent on the origin of the tumor. Serum response factor, which is part of the TGF β signaling pathway, is implicated in cancer progression, specifically at the epithelial-mesenchymal transition [50,51]. Overexpression of SRF accelerates migration and invasion of tumor cells with subsequent acquisition of mesenchymal phenotype and activation of immediate early genes. We demonstrated that in ASPS metastasis, Smad3 downstream of TGF β and SRF were found in the tumor cells and in the myofibroblasts. Thus, functional antagonism of SRF or inhibition of Smad3 phosphorylation may provide a thera-

peutic approach by controlling tumor cell invasion and metastasis on one hand and fibroblast-to-myofibroblast transition on the other. Moreover, halofuginone treatment caused an almost complete inhibition in TFE3 synthesis by the tumor cells (Figure 6B). TFE3 has been found to bind Smad3 and Smad4 and to synergistically trans-activate the transcription of Smad7 in response to TGF β [52]. Thus, TFE3-Smad3 response elements may represent a common target for TGF β -induced gene expression, a feature that may be shared with various TFE3 fusions including ASPS.

In conclusion, our data demonstrated for the first time the existence of the TGF β /SRF-dependent Smad3 pathway and the presence of activated myofibroblasts in pulmonary and brain ASPS metastases that may serve as a novel target for treatment of metastatic lesions in ASPS.

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