ASS1 as a Novel Tumor Suppressor Gene in Myxofibrosarcomas: Aberrant Loss via Epigenetic DNA Methylation Confers Aggressive Phenotypes, Negative Prognostic Impact, and Therapeutic Relevance

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Abstract

Purpose: The principal goals were to identify and validate targetable metabolic drivers relevant to myxofibrosarcoma pathogenesis using a published transcriptome.

Experimental Design: As the most significantly downregulated gene regulating amino acid metabolism, argininosuccinate synthetase (ASS1) was selected for further analysis by methylation-specific PCR, pyrosequencing, and immunohistochemistry of myxofibrosarcoma samples. The roles of ASS1 in tumorigenesis and the therapeutic relevance of the arginine-depriving agent pegylated arginine deiminase (ADI-PEG20) were elucidated in ASS1-deficient myxofibrosarcoma cell lines and xenografts with and without stable ASS1 reexpression.

Results: ASS1 promoter hypermethylation was detected in myxofibrosarcoma samples and cell lines and was strongly linked to ASS1 protein deficiency. The latter correlated with increased tumor grade and stage and independently predicted a worse survival. ASS1-deficient cell lines were auxotrophic for arginine and susceptible to ADI-PEG20 treatment, with dose-dependent reductions in cell viability and tumor growth attributable to cell-cycle arrest in the S-phase. ASS1 expression was restored in 2 of 3 ASS1-deficient myxofibrosarcoma cell lines by 5-aza-2′-deoxycytidine, abrogating the inhibitory effect of ADI-PEG20. Conditioned media following ASS1 reexpression attenuated HUVEC tube-forming capability, which was associated with suppression of MMP-9 and an antiangiogenic effect in corresponding myxofibrosarcoma xenografts. In addition to delayed wound closure and fewer invading cells in a Matrigel assay, ASS1 reexpression reduced tumor cell proliferation, induced G1-phase arrest, and downregulated cyclin E with corresponding growth inhibition in soft agar and xenograft assays.

Conclusions: Our findings highlight ASS1 as a novel tumor suppressor in myxofibrosarcomas, with loss of expression linked to promoter methylation, clinical aggressiveness, and sensitivity to ADI-PEG20. Clin Cancer Res; 19(11); 2861–72. ©2013 AACR.

Introduction

Myxofibrosarcomas are characterized by increased metastasis after relentless local recurrence (1–3). Such a recurrence has been shown to parallel the cytogenetic complexity and eventually lead to metastasis (4, 5). The overall survival rate in patients with a myxofibrosarcoma is approximately 75% at 5 years, and several series have provided conflicting data for clinicopathologic prognosticators (1, 3, 4, 6, 7). We and others have addressed the importance of clear margins, which predicted better local control and translated into

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Translational Relevance

In the prognostication and treatment of myxofibrosarcomas, the molecular markers and therapeutic options are inadequate. Through data mining of the myxofibrosarcoma transcriptome, we identified ASS1 gene as the most prominently downregulated candidate among those regulating amino acid metabolism. An ASS1 protein deficiency was validated in myxofibrosarcoma samples and was associated with gene methylation, adverse prognosticators, and worse outcomes. In vitro and in vivo, ASS1-deficient myxofibrosarcomas were susceptible to arginine-depriving ADI-PEG20 at therapeutic doses, compared with ASS1-reexpressing counterparts and fibroblasts. An ASS1 deficiency contributed to aggressive phenotypes, including tumor growth, migration/invasion, and angiogenesis, which were attenuated by forced ASS1 reexpression. We substantiate the clinical, biologic, and pharmacologic relevance of ASS1, highlighting its novel tumor suppressive role and therapeutic potential in myxofibrosarcomas.

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Survival benefits (1, 7). However, the molecular determinants of aggressiveness and therapeutic targets in myxofibrosarcomas remain elusive.

Various genetic, epigenetic, and posttranslational aberrations of metabolism-associated enzymes may alter signaling pathways in human cancers (8–12). Converging to adapt to core cell metabolism, these aberrations are essential for malignant transformation and sustained cell growth (8–12), making metabolic deregulation a cancer hallmark of renewed interest (8). Genome-wide approaches with derived targeted therapies have prompted efforts to characterize coordinately regulated gene expressions of specific pathways (13, 14). Because amino acids are building blocks in the anabolic process of cell proliferation, the development of compounds targeting the amino acid metabolism is a promising cancer therapy (15). A successful example is asparaginase, which depletes asparagine in the plasma of patients with acute lymphoblastic leukemia devoid of asparagine synthetase (16). To explore genes associated with tumorigenesis, we analyzed the published transcriptome of myxofibrosarcomas and identified arginosuccinate synthetase (ASS1) as a prominently downregulated gene regulating amino acid metabolism. ASS1 catalyzes the penultimate step of arginine biosynthesis (17). Tumors devoid of ASS1, such as hepatocellular carcinomas and melanomas, become auxotrophic for arginine (18, 19). This feature has been exploited as a biomarker to deprive plasma arginine using pegylated arginine deiminase (ADI-PEG20; refs. 20, 21).

Neither the functions of ASS1 in regulating tumorigenesis nor the clinical relevance and regulation of ASS1 expression were previously characterized in soft tissue sarcomas. We have shown that ASS1 is frequently methylated in myxofibrosarcoma clinical samples and cell lines, resulting in aberrant loss of ASS1 expression. The ASS1 deficiency causes in vitro and in vivo susceptibility to ADI-PEG20 and contributes to the novel tumor suppressor role of ASS1, with validation in tumor samples, cell lines, and xenograft models.

Materials and Methods

Analysis of published genomic and transcriptomic profiling datasets

The complete datasets of our published genomic profiling study for myxofibrosarcoma tissues and cell lines (4) were available in Gene Expression Omnibus (GEO, GSE35483). To identify genes critical in myxofibrosarcoma pathogenesis, we reappraised expression profiling datasets of myxofibrosarcomas versus nonneoplastic soft tissues from GSE21122 deposited in GEO. The raw CEL files of Affymetrix U133A microarray platform were imported into Nexus Expression 3 software (BioDiscovery) to analyze all probe sets without preselection or filtering. Supervised comparative analysis and functional profiling were conducted to identify significant differentially expressed genes, with special attention to amino acid biosynthesis in Gene Ontology (GO: 0008652). Those with $P$ less than 0.01 and log2-transformed expression fold change more than 2 were chosen for further analysis.

Cell culture, transfection, and stable clones

The derivation and maintenance of the OH931, NMFH-1, and NMFH-2 myxofibrosarcoma cell lines and CCD966SK dermal fibroblasts were previously reported (22–25). Culture methods of human umbilical venous endothelial cells (HUVEC) and the ASS1-expressing LPS510 cell line were described in Supplementary Method S1, along with the authentication of all cell lines by short-tandem repeat profiling.

Full-length pASS1-DDK-Myc expression plasmid and empty pCMV6 vector (Origene) were validated by sequencing. In a 24-well plate, 105 cells each of 3 myxofibrosarcoma cell lines (OH931, NMFH1, and NMFH2) were seeded and ASS1 expression by Western blotting. To transfect SFG gene (Clontech), 105 OH931 cells were incubated with Lipofectamine 2000 (Invitrogen) for 4 hours at 37°C to transfected various plasmids (1.5 μg). To standardize transfection efficiency, eGFP (Promega) were transfected in parallel. Afterwards, cells were cultured for further 24 hours at 37°C and lysed with Passive Lysis Buffer (Promega). Finally, myxofibrosarcoma cells stably expressing ASS1 or DDK-Myc tags alone were selected with neomycin. The transfected cells were analyzed for exogenous ASS1 expression by Western blotting. To transfect SFG plasmid carrying the GFP-FFLuc gene (Clontech), 105 OH931 cells were incubated with Lipofectamine 2000 (Invitrogen) for 4 hours at 37°C. GFP+ OH931 cells were sorted by flow cytometry to isolate successfully transfected cells. The methods to establish stable E2H2-knockdown NMFH-1 cells and ASS1-knockdown LPS510 cells were essentially as previously reported (4), with modifications detailed in Supplementary Method S2.
Quantification of transcripts of ASS1, EZH2, and angiogenesis-associated genes

Using the ABI StepOnePlus System, a real-time reverse-transcription PCR (RT-PCR) was carried out on cell samples to measure ASS1 and EZH2 mRNA abundances and explore mediators of ASS1 in inhibiting angiogenesis with an RT-PCR expression array (PAHS-024, SABioscience) essentially as previously reported (4) and detailed in Supplementary Method S3.

Tumor characteristics

Tissue procurement of myxofibrosarcomas was approved by the Institutional review board (100-0895B). The criteria for the diagnosis and parameter assessment were previously elaborated (1, 4, 26). Ninety formalin-fixed, primary myxofibrosarcoma samples (Table 1) receiving curative resection without neoadjuvant radiation or chemotherapy were analyzed for an ASS1 methylation-specific PCR and ASS1 immunostaining.

Evaluation of ASS1 gene methylation

Methylation-specific PCR was carried out as described (27) with pyrosequencing confirmation of all cell lines and selected tissue samples detailed in Supplementary Method S4.

Immunohistochemistry

Tissue microarray (TMA) sections were prepared for antigen retrieval as described (4, 26, 28), encompassing all cases subjected to methylation-specific PCR. The incubation with the antibody against ASS1 (1:1,000, DesigneRx) and the cut-off value of less than 5% cytoplasmic reactivity to define aberrant ASS1 loss were as previously reported (18). Whole sections from formalin-fixed xenografted specimens were stained with anti-CD31 (1:50, BD Pharmpingen) and antKi67 (1:200, Abcam).

Western blotting and enzyme-linked immunosorbent assays (ELISA) were as previously reported (4), with minor modifications detailed in Supplementary Methods S5 and S6.

Pharmacologic agents and in vitro ADI-PEG20 treatment

ADI-PEG20 and 3-Dznep (targeting EZH2-mediated H3K27 histone methyltransferation) were provided by DesigneRx and Dr. Marquez (National Cancer Institute; Frederick, MD), respectively. UNC0638 (targeting G9a-mediated histone methylation of H3K9), trichostatin (a histone deacetylase inhibitor), and 5-aza-2’-deoxycytidine were obtained from Sigma. Pharmacokinetic characterization of the arginine-depriving efficiency of ADI-PEG20 was described in Supplementary Method S7, apart from ADI-PEG20 treatment in various cell lines.

Functional assays

To elucidate functional alterations associated with ASS1 reexpression, ASS1 knockdown, and ADI-PEG20 treatment, various cancer phenotypes were evaluated using cell viability, bromodeoxyuridine (BrdUrd), electric cell-substrate impedance sensing (ECIS), cell-cycle kinetic, soft-agar, HUVEC tube formation, and wound healing and invasion assays, as previously reported (4) with modifications detailed in Supplementary Methods-S8-S14.

Animal xenografts

The protocol was approved (98121505) by the animal use committee. OH931 myxofibrosarcoma cells transfected with plasmids expressing GFP-FFLuc, ASS1, or the empty control were inoculated into severe combined immunodeficient (SCID) mice to analyze the in vivo effects of ADI-PEG20 treatment and ASS1 reexpression as described in Supplementary Method S15.

In vivo imaging system for monitoring ADI-PEG20 treatment of xenografts

The successfully transfected, GFP-FFLuc-bearing OH931 cells were validated using the IVIS camera system (Xenogen) to ascertain the presence of bioluminescence in complete media supplemented with 150 μg/mL α-luciferin. Real-time tumor growth and the therapeutic effects of ADI-PEG20 were monitored in such OH931

Table 1. Associations of ASS1 expression with clinicopathologic parameters

<table>
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</tr>
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*aStatistically significant.
*bForty-four cases with methylation and immunohistochemical data.
xenografts. Mice were anesthetized with 3% isofluorane after intraperitoneal injection of 150 mg/kg body weight α-luciferin. Ten minutes after injection of the α-luciferin, images were acquired for 10 seconds to 2 minutes. A photograph was taken for projection of the pseudocolor images representing the spatial distribution of photon counts. For bioluminescence imaging plots, photon flux was calculated for each mouse using a square region of interest, encompassing the head of the mouse in a supine position. This value was scaled to a comparable background value (from a luciferin-injected mouse without tumor cells) and normalized to the value obtained immediately after xenografting (day 0).

**Statistical analysis**

Associations of ASS1 immunoexpression with clinicopathologic factors and the ASS1 methylation status were evaluated using a χ² or Fisher exact test as appropriate. Follow-up data were available in 89 patients, and the median follow-up time was 30.6 (range, 2–229) months. The end points were metastasis-free and disease-specific survival. Univariate survival analyses were compared using the log-rank test. A multivariate model was carried out using the Cox regression, including parameters with univariate P < 0.05. Student t test was used to analyze the quantitative RT-PCR results, and functional and pharmacologic assays for cell line and xenograft samples.

**Results**

**ASS1 as a differentially downregulated gene regulating amino-acid biosynthesis**

From the dataset of 34 myxofibrosarcomas in the public transcriptome, we focused on 30 probes covering 22 genes regulating amino-acid biosynthesis. Among statistically significant genes, ASS1 was the top-ranking differentially expressed candidate (Fig. 1A and Supplementary Table S1), prompting us to characterize endogenous expressions of the ASS1 transcript and protein in OH931, NMFH-1, and NMFH-2 myxofibrosarcoma cell lines. Unlike CCD966SK dermal fibroblasts, the real-time RT-PCR assay showed either undetectable or barely detectable endogenous ASS1 mRNA in all 3 myxofibrosarcoma cell lines (Fig. 1B, top), reflective of complete depletion of the ASS1 protein in the Western blots (Fig. 1B, bottom).

**Epigenetic methylation underlying the ASS1 deficiency in myxofibrosarcoma cells**

We speculated on whether DNA deletion might account for depletion of ASS1. Reappraisal of our published genomic profiling data of myxofibrosarcomas did not reveal copy number imbalances at the probes spanning ASS1 at 9q34.11 in the majority of samples, except for one featuring a hemizygous deletion (Supplementary Fig. S1A). As deletion did not seem to be a predominant event underlying the ASS1 deficiency in myxofibrosarcomas, we next considered
whether this aberration resulted from epigenetic silencing similar to that seen in mesotheliomas and ovarian cancers (27, 29). Methylation-specific PCR showed ASS1 gene methylation in all 3 myxofibrosarcoma cell lines devoid of protein expression but not in ASS1-expressing CCD966Sk dermal fibroblasts (Fig. 1C), consistent with the pyrosequencing results (Supplementary Fig. S1B). Accordingly, myxofibrosarcoma cells were treated with 5-aza-2'-deoxycytidine, which restored ASS1 expression with comparable fold changes between transcripts and proteins in both OH931 and NMFH-2 cells, albeit being more prominent in NMFH-2 (Fig. 1D). However, 5-aza-2'-deoxycytidine failed to rescue ASS1 expression in NMFH-1 cells. This difference prompted us to speculate that deregulated modification of histone codes likely mediates ASS1 epigenetic silencing in this particular cell line. Regardless of the presence or absence of 5-aza-2'-deoxycytidine, neither separate nor combined treatment with trichostatin, 3-Dznep, and/or UNC0638 restored ASS1 expression. This result remained identical when short-hairpin RNA (shRNA) against EZH2 was applied as an alternative to 3-Dznep to suppress histone methyltransferase of H3K27 by downregulating polycomb recessive complex 2-associated EZH2 that has intrinsic methyltransferase activity (Supplementary Fig. S1C).

**Associations of ASS1 immunoeexpression with methylation status, clinicopathologic features, and patient survival**

TMA-based ASS1 immunohistochemistry was conducted on 90 primary myxofibrosarcomas linked to clinicopathologic characteristics listed in Table 1. Being identified in 40 (44%) cases, aberrant ASS1 loss was strongly related to hypermethylation of ASS1 gene in 25 of 44 cases (57%) with informative data from methylation-specific PCR ($P < 0.001$, Fig. 2A) and was significantly associated with increasing FNCLCC grades ($P = 0.005$, Fig. 2B) and American Joint Committee on Cancer AJCC stages ($P = 0.007$). The accuracy of methylation-specific PCR was substantiated in 10 selected tumor samples subjected to pyrosequencing (Supplementary Fig. S1B and Supplementary Table S2). The results indicated that a methylation-associated ASS1 deficiency contributed to myxofibrosarcoma progression. Furthermore, an aberrant ASS1 deficiency univariately predicted worse outcomes (Fig. 2C) for both disease-specific ($P = 0.0035$) and metastasis-free survival ($P = 0.0052$). In the multivariate comparison (Supplementary Table S3), this aberration also remained prognostically independent, along with higher grades for both disease-specific ($P = 0.0230$, HR: 10.416) and metastasis-free survival ($P = 0.0213$, HR: 4.237).

**ADI-PEG20 attenuated cell viability in ASS1-deficient myxofibrosarcoma cells**

Since hepatocellular carcinomas respond to ADI-PEG20 treatment because of auxotrophy for arginine in tumor cells devoid of ASS1, we investigated whether ADI-PEG20 holds promise as targeted therapy in ASS1-deficient myxofibrosarcomas (21). We characterized the pharmacokinetics of ADI-PEG20 in arginine deprivation, revealing a dose-dependent reduction in arginine in the culture medium of OH931 cells within a range of ADI-PEG20 concentrations up to 100 ng/mL (Supplementary Fig. S2A), at which point arginine was no longer detectable. Myxofibrosarcoma cells and fibroblasts were incubated with the indicated concentrations of ADI-PEG20 for 72 hours, drastically attenuating cell viability in all myxofibrosarcoma cells at 100 ng/mL. However, ASS1-expressing CCD966Sk fibroblasts showed resistance without cytotoxicity at a concentration of 500 ng/mL (Fig. 3A). We next evaluated how ADI-PEG20 affected myxofibrosarcoma cells engineered to stably reexpress ASS1 (Fig. 3B) and found almost complete reversion of the inhibition of cell viability, substantiating that this selective cytotoxicity to ADI-PEG20 was directly ascribed to the ASS1 deficiency in myxofibrosarcoma cells (Fig. 3C). Because the methylated ASS1 gene could be reacted with 5-aza-2'-deoxycytidine in OH931 and NMFH-2 cells, we further explored whether this demethylating treatment compromised the sensitivity of these cell lines to ADP-PEG20. After pretreatment with 1 μmol/L 5-aza-2'-deoxycytidine 4 days beforehand, incubation of ADP-PEG20 for a further 72 hours no longer diminished cell viability in either OH931 or NMFH-2 cells (Fig. 3D), providing an argument against combined therapy with demethylating and arginine-depriving drugs in myxofibrosarcomas.

**Therapeutic effect of ADI-PEG20 in OH931 xenografts**

We examined further the therapeutic efficacy of ADI-PEG20 in murine xenografts of OH931 cells transfected with GFP-FLuc. Compared with phosphate-buffered saline (PBS)-treated mice, ADI-PEG20 led to significantly smaller tumor volumes from day 14 posttreatment until day 28 at sacrifice (Fig. 4A). The kinetics of tumor growth inhibition with ADI-PEG20 treatment in mice were monitored by bioluminescence and quantified. Expressed as decreased photon flux, significantly attenuated bioluminescence signals were seen in the ADI-PEG20–treated group at a dose as low as 2.5 IU/kg (Fig. 4B). Upon sacrifice, obvious tumor shrinkage with regressing fibrosis was observed in ADI-PEG20–treated xenografts, in striking contrast to PBS controls (Supplementary Fig. S2B). On histologic examination (Fig. 4C), PBS controls displayed pleomorphic cells with dense collagenous matrix. Immunohistochemically (Fig. 4C), there were fewer Ki67-labeled cells ($P < 0.001$) in the ADI-PEG20–treated xenografts, and vice versa in the PBS controls, consistent with significantly less BrdUld uptake in all ASS1-reexpressing myxofibrosarcoma cells (Supplementary Fig. S2C). To elucidate the basis of the antiproliferative effect of ADI-PEG20, we analyzed cell-cycle profiles of ADI-PEG20–treated versus PBS–treated OH931 cells by flow...
cytometry, which revealed dose-dependent induction of the S-phase arrest by ADI-PEG20 (Supplementary Fig. S2C).

**ASS1 as a novel tumor suppressor of myxofibrosarcomas**

Apart from the therapeutic potential, the biologic effects of ASS1 on various cancer subtypes remain unclear. We used stable ASS1-reexpressing versus empty transfectants to decipher how ASS1 modulates cellular phenotypes in myxofibrosarcomas.

**ASS1 reexpression inhibited tumoral angiogenesis.** In tumorigenesis, it is compelling that an angiogenic switch is governed by counterbalancing molecules that induce or suppress angiogenesis (8, 30). Because myxofibrosarcomas are characterized by an extensive vascular network, we hypothesized that ASS1 is involved in regulating angiogenesis in myxofibrosarcomas. After incubation with conditioned media, the HUVEC tube-forming capability decreased significantly to 64%, 63%, and 70% for OH931, NMFH-1, and NMFH-2 ASS1-reexpressing transfectants, respectively.
compared with the corresponding controls (Fig. 5A). This result signified that ASS1 deficiency conferred a proangiogenic function in myxofibrosarcomas, prompting us to explore mediators of ASS1 in inhibiting angiogenesis with an RT-PCR expression array. According to the selection criteria described in Supplementary Method S3, mRNA

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Figure 3. ASS1 deficiency in myxofibrosarcomas accounts for susceptibility to ADI-PEG20. A, ADI-PEG20 (100 ng/mL) strongly decreased the viability of ASS1-deficient OH931, NMFH-1, and NMFH-2 myxofibrosarcoma cell lines, whereas CCD966SK fibroblasts showed no response. B, stable exogenous ASS1 reexpression was confirmed in OH931 (left), NMFH-1 (middle), and NMFH-2 (right) myxofibrosarcoma cell lines, showing the expected bands with anti-ASS1, anti-DDK, and anti-Myc. C, unlike the sensitivity in the parent lines and empty controls, ASS1 reexpression led to resistance to ADI-PEG20 in OH931 (top), NMFH-1 (middle), and NMFH-2 (bottom) cells. D, unlike the vehicle control, 5-aza-2′-deoxycytidine rescued ASS1 expression but abrogated the inhibitory effect of ADI-PEG20 on OH931 (top) and NMFH-2 (bottom) cells.
expressions of 6 candidate proangiogenic genes were significantly and consistently downregulated in ASS1-reexpressing transfectants; in contrast, there were no obviously upregulated genes (Supplementary Table S4). There was a concomitant downregulation of VEGFR1, CXCL-9, and matrix metalloproteinase (MMP)-9 proteins by Western blotting (Fig. 5B), respectively, encoded by FLT1, CXCL9, and MMP9 genes, from all the myxofibrosarcoma ASS1-reexpressing transfectants. However, FGFR3, TGF-α, and CXCL10 protein levels decreased inconsistently in 1 or 2 ASS1-reexpressing cell lines alone (Fig. 5B). Furthermore, ELISAs (Supplementary Fig. S3A) were conducted to quantify each protein in conditioned media from various transfectants of myxofibrosarcoma cell lines, whereas only MMP-9 was consistently and significantly downregulated in all ASS1-reexpressing cell lines. These findings indicated that ASS1 may modulate the angiogenic switch of myxofibrosarcomas through downregulating proangiogenic molecules, such as MMP-9.

ASS1 reexpression inhibited tumor growth by suppressing cell proliferation with the induction of G1 arrest. To decipher other biologic roles of ASS1 associated with its anti-tumor function, we compared the BrdUrd-incorporating rate of myxofibrosarcoma cells, which was significantly reduced in all ASS1-reexpressing myxofibrosarcoma cell line transfectants. Among these, we further evaluated NMFH-2 cells for real-time cell proliferation by using an ECIS assay, which revealed a significantly slower cell growth rate in ASS1-reexpressing transfectants (Supplementary Fig. S3B). These findings support the inhibition of ASS1 in cell proliferation, prompting us to investigate how ASS1 expression affects the cell-cycle redistribution and anchorage-independent and in vivo tumor growth. Using flow cytometry, we observed that ASS1-reexpressing transfectants of OH931 and NMFH-2 cells displayed a cell-cycle arrest at the G1 phase. Especially prominent in NMFH-2 cells was increased cells at the G1 phase with concomitant lower percentages of cells in the S and G2 phases (Supplementary Fig. S4). We conducted Western blotting to evaluate alterations in G1- and G1–S–associated cyclins and cyclin-dependent kinases, which revealed consistent downregulation of cyclin E expression across all myxofibrosarcoma cell lines upon ASS1 reexpression. However, this cell-cycle arrest was not significantly induced in NMFH-1 cells by ASS1 reexpression, probably because of an extremely high fraction of cells in the G1 phase in the empty control.

In a soft agar assay, significantly smaller and fewer cell colonies appeared in ASS1-reexpressing transfectants in all myxofibrosarcoma cell lines, substantiating the role of ASS1 in restraining anchorage-independent growth (Fig. 5C).

ASS1 reexpression inhibited cell migration and invasion. To investigate whether ASS1 is implicated in cell motility and metastasis, a wound-healing assay was conducted, showing significantly slower wound closure in ASS1-reexpressing transfectants of all myxofibrosarcoma cell lines (Supplementary Fig. S5A). This indicated that ASS1 might impair the migration of myxofibrosarcoma cells. Moreover, OH931 and NMFH-1 cells stably transfected with ASS1 or an empty vector were seeded in modified Boyden chambers to elucidate the capability for cell invasion. In 22 hours after seeding, significantly fewer invading tumor cells were counted for ASS1-reexpressing cells, suggesting an antinvasive attribute of ASS1 in myxofibrosarcomas (Supplementary Fig. S5B).

Stable ASS1 knockdown conferred proliferative and metastatic capabilities. As a surrogate for unavailable ASS1-expressing myxofibrosarcoma cells, LP5510 liposarcoma cells were stably silenced against endogenous ASS1 to cross-validate its tumor-suppressive function. In Supplementary Fig. S6, the proliferation, viability, migration, and invasion of tumor cells increased significantly in ASS1-knockdown LP5510 cells.
In vivo tumor growth-inhibiting function of ASS1

The role of ASS1 in tumor growth inhibition was also examined in OH931 xenografts with and without ectopic ASS1 reexpression. The ASS1-reexpressing group displayed a significantly smaller average tumor volume from day 11 onward, and this trend continued until sacrifice on day 28 (Fig. 5D, left). The excised xenograft tumor specimens were obviously shrunken and lighter in weight compared with the controls (Fig. 5D, right). On histologic examination (Fig. 5D, bottom), the control xenografts displayed spindle-shaped to pleomorphic cells in a fibromyxoid matrix, imparting a high-grade myxofibrosarcoma. However, ASS1-reexpressing xenografts showed reduced cellularity with few, if any, mitoses ($P < 0.001$) in a myxoid matrix.
reminiscent of a low-grade myxofibrosarcoma. Immunohistochemically (Fig. S5, bottom), ASS1-reexpressing xenografts displayed significantly fewer intratumoral vessels, as evidenced by staining with the CD31 endothelial marker.

Discussion

Soft tissue sarcomas are mesenchymal malignancies of great histologic and biologic heterogeneity, and there are inadequate molecular markers and therapeutic options for clinical management (13, 31, 32). By reappraising differentially expressed genes in a published transcriptome, we provided evidence for ASS1 as a novel molecular target of myxofibrosarcomas associated with amino-acid metabolism implicated in tumor progression and therapeutic strategies. For the first time among soft tissue sarcomas, ASS1, a rate-limiting enzyme that converts citrulline into arginine in the urea cycle (17), was found to have been aberrantly lost in a considerable percentage of myxofibrosarcomas. This protein deficiency correlated with ASS1 gene silencing secondary to promoter methylation and conferred preferential susceptibility to arginine-deprivation with ADI-PEG20. The antitumor efficacy of ADI-PEG20 was dose-dependent and substantiated in both in vitro and in vivo xenograft models, opening a new avenue in myxofibrosarcoma management for which complete resection remains the mainstay (7, 33). This is therapeutically relevant given the relentless recurrence and unsatisfactory chemoradiotherapeutic responses of myxofibrosarcomas (34). We also provide multiple lines of evidence reinforcing the novel tumor-suppressive role of ASS1 in myxofibrosarcoma pathogenesis, including its antiangiogenic, antiproliferative, and antimigratory/invasive properties.

Arginine is a nonessential amino acid in mammalian cells because of the ubiquitous presence of ASS1 (17). However, its expression differs in abundance in various tissues and is mostly regulated at the transcriptional level by extracellular hormones, nutrients, cytokines, and transcription factors (17). Compared with normal tissue counterparts, a wide spectrum of tumor types display remarkable variations in ASS1 expression levels, which are elevated in gastric and colorectal cancers but frequently downregulated in hepatocellular carcinomas, melanomas, and mesotheliomas, among others (18, 20, 21, 27). However, the mechanistic underpinning responsible for suppression or reexpression of ASS1 is yet to be elucidated, especially in cancer tissues. Until recently, DNA methylation was identified as an inactivating mechanism of ASS1 expression only in pleural mesotheliomas, lymphomas, and ovarian carcinomas (27, 29, 35). Nevertheless, data for ASS1 methylation in hepatocellular carcinomas or melanomas are limited, despite being 2 of the best-known neoplasms devoid of ASS1 expression, with auxotrophy for arginine and sensitivity to ADI-PEG20 (20). ASS1 was silenced by DNA methylation in all myxofibrosarcoma cell lines by methylation-specific PCR and pyrosequencing. This epigenetic aberration was also detected in over a half of successfully analyzed primary myxofibrosarcomas with a strong association with protein deficiency. Immunohistochemically, the ASS1 deficiency in primary myxofibrosarcomas correlated with increasing tumor grade and stage and independently predicted a worse outcome, especially metastasis-free and disease-specific survival. This clinical relevance prompted us to address the functional attributes of ASS1 associated with various cancer hallmarks.

In all myxofibrosarcoma cell lines, stable ASS1 reexpression led to decreased BrdUrd uptake, signifying attenuated DNA synthesis, which was reconciled with significantly reduced proliferation of ASS1-reexpressing NMFH-2 cells by real-time ECIS measurements. Furthermore, anchorage-independent growth was impaired in all ASS1-reexpressing transfectants with significantly fewer and smaller colonies in the soft-agar assays. These findings highlight the antiproliferative function of ASS1 in suppressing the growth of myxofibrosarcomas, which was associated with the induction of G1 arrest, most likely resulting from downregulation of cyclin E. Distant dissemination is the leading cause of mortality in sarcomas, and is a great hindrance to treatment (31, 32). In osteosarcomas of the bone, reduced ASS1 immunoexpression was reported to be a biomarker of pulmonary metastasis after neoadjuvant chemotherapy and curative resection, whereas there was no mechanistic elucidation of the ASS1 deficiency with enhanced tumor metastasis (36). Herein, we further reinforced the role of ASS1 in suppressing metastasis. In stable ASS1 transfectants of myxofibrosarcoma cell lines, we observed delayed closure in wound-healing assays and decreased tumor cell invasion using Matrigel assays. These results are consistent with the ASS1 deficiency as a harbinger of worse metastasis-free survival in myxofibrosarcomas. Moreover, we also validated the antiproliferative and antimetastatic properties of ASS1 using RNA interference in the ASS1-expressing LPS510 cell line.

In cancer cells, angiogenesis is necessary to deliver nutrients and oxygen and eliminate metabolic wastes (8). The angiogenic switch is characterized by deregulated predominance of proangiogenic over antiangiogenic factors (8, 30). ASS1 reexpression was substantiated to exhibit an antangiogenic effect in all 3 myxofibrosarcoma cell lines with significantly attenuated HUVEC tube-forming capability. The downregulating effect of ASS1 on MMP-9 expression was significant and consistent in 3 different myxofibrosarcoma cell lines, as validated by a real-time RT-PCR, Western blotting, and ELISA. Among the secreted MMPs, MMP-2 and MMP-9 have attracted particular attraction because of their frequent overexpression and association with aggressiveness in various cancer types (37). Also unique within their catalytic domains is the presence of fibronectin type II repeats that bind to stromal collagens where MMP-9 cleaves collagen IV, unmasking cryptic sites, and mobilizes the vascular endothelial growth factor (VEGF), which is important for angiogenesis (37, 38). Regardless of whether it is secreted from tumor cells or inflammatory and stromal cells in the tumor microenvironment, the classical role of activated
MMP-9 is to degrade the extracellular matrix to enhance tumor invasion and metastasis (37, 39). Therefore, the antimigratory and anti-invasive capabilities of ASS1 re-expression may be partly mediated by modulating MMP-9 expression and activity in myxofibrosarcomas.

As a pegylated arginine deiminase, ADI-PEG20 has a long half-life in the plasma to deprive arginine. Promising benefits have been obtained in clinical trials of advanced hepatocellular carcinomas and melanomas (20), and the number of other cancer types known to lack ASS1 expression is increasing (18). Among soft tissue sarcomas, in vitro and in vivo growth inhibition was first achieved by ADI-PEG20 treatment in myxofibrosarcoma at therapeutic doses. It attenuated cell proliferation, induced a S-phase arrest, and led to a profound loss of tumorigenicity in OH931 xenografts. This potent effect indicated the therapeutic potential of ADI-PEG20 in myxofibrosarcoma, warranting a systematic assessment of ASS1 immunoexpression for prospective clinical trials. However, 5-aza-2'-deoxycytidine pretreatment recovered ASS1 expression in 2 of 3 ASS1-methylated myxofibrosarcoma cells at the cost of losing the original susceptibility to ADI-PEG20. In contrast, in the ASS1-methylated NMFH-1 cells, 5-aza-2'-deoxycytidine pretreatment failed to regain ASS1 expression irrespective of combined or separate use of EZH2 knockdown and various inhibitors against formation of repressive histone codes. This finding in NMFH-1 myxofibrosarcoma cells was not in keeping with the series of ASS1-methylated T-cell lymphoma cell lines, in which the ASS1 enzyme was readily reexpressed by demethylating agents (35). Our results indicated that a combination therapy of ADI-PEG20 and demethylating agents appears without effect in myxofibrosarcoma, a subset of which may use additional mechanism(s) other than DNA methylation and/or deregulated histone modifications to abolish ASS1 expression.

In conclusion, ASS1 deficiency is associated with promoter methylation and has a negative impact on prognosis in the clinic. ASS1 has been substantiated in vitro and in vivo as a tumor suppressor in myxofibrosarcoma via antiangiogenic, antiproliferative, and antimigratory/anti-invasive properties. ASS1 deficiency renders ADI-PEG20 as a promising strategy in the management of myxofibrosarcomas.

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

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References

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ASS1 as a Novel Tumor Suppressor Gene in Myxofibrosarcomas: Aberrant Loss via Epigenetic DNA Methylation Confers Aggressive Phenotypes, Negative Prognostic Impact, and Therapeutic Relevance
