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Research Article

Molecular Cancer Therapeutics

Reduced Argininosuccinate Synthetase Is a Predictive Biomarker for the Development of Pulmonary Metastasis in Patients with Osteosarcoma

Eisuke Kobayashi[1,4], Mari Masuda[1], Robert Nakayama[2,4], Hitoshi Ichikawa[2], Reiko Satow[1], Miki Shitashige[1], Kazufumi Honda[1], Umio Yamaguchi[1,5], Ayako Shoji[6], Naobumi Tochigi[3], Hideo Morioka[4], Yoshiaki Toyama[4], Setsuo Hirohashi[1], Akira Kawai[5], and Tesshi Yamada[1]

Abstract

Pulmonary metastasis is the most significant prognostic determinant for osteosarcoma, but methods for its prediction and treatment have not been established. Using oligonucleotide microarrays, we compared the global gene expression of biopsy samples between seven osteosarcoma patients who developed pulmonary metastasis within 4 years after neoadjuvant chemotherapy and curative resection, and 12 patients who did not relapse. We identified argininosuccinate synthetase (*ASS*) as a gene differentially expressed with the highest statistical significance (Welch's t test, $P = 2.2 \times 10^{-5}$). Immunohistochemical analysis of an independent cohort of 62 osteosarcoma cases confirmed that reduced expression of ASS protein was significantly correlated with the development of pulmonary metastasis after surgery (log-rank test, $P < 0.05$). Cox regression analysis revealed that ASS was the sole significant predictive factor ($P = 0.039$; hazard ratio, 0.319; 95% confidence interval, 0.108-0.945). ASS is one of the enzymes required for the production of a nonessential amino acid, arginine. We showed that osteosarcoma cells lacking ASS expression were auxotrophic for arginine and underwent G_0-G_1 arrest in arginine-free medium, suggesting that an arginine deprivation therapy could be effective in patients with osteosarcoma. Recently, phase I and II clinical trials in patients with melanoma and hepatocellular carcinoma have shown the safety and efficacy of plasma arginine depletion by stabilized arginine deiminase. Our data indicate that in patients with osteosarcoma, reduced expression of ASS is not only a novel predictive biomarker for the development of metastasis, but also a potential target for pharmacologic intervention. *Mol Cancer Ther; 9(3); 535–44. ©2010 AACR.*

Introduction

Although rare (200–300 newly diagnosed cases per year in Japan), osteosarcoma is the most frequent primary malignant bone tumor, developing mainly in the metaphysis of long bones of children and young adults. The introduction of preoperative high-dose combined chemotherapy in the last three decades has significantly improved the disease-free 5-year survival rate of young (ages <40 years) patients with osteosarcoma of the extremities to approximately 50% to 80% (1). However, a significant proportion of osteosarcoma patients develop metastasis even after curative resection of the primary tumor (1, 2). The lung is the most common organ to which osteosarcoma metastasizes first. Solitary metastasis can be treated by lung resection, but suitable management of patients with multiple pulmonary metastases has not been established. Furthermore, metastatic osteosarcoma often develops resistance to chemotherapeutic agents that were initially effective for treatment of the primary tumor. Osteosarcoma patients with lung metastasis have a poor prognosis, with an overall survival rate of <30% (3). Development of lung metastasis is the most significant determinant of poor prognosis in osteosarcoma, followed by poor response to neoadjuvant chemotherapy (1). Because such high-risk patients may derive some benefit from modification (intensification) of their preoperative and postoperative therapeutics, the development of a reliable diagnostic method that can stratify osteosarcoma patients according to their likelihood of developing lung metastasis would be highly valuable.

Various prognostic clinicopathologic factors have been reported, including patient age, tumor size, histologic subtype, and site of origin (1). The proximal and axial location of osteosarcoma significantly affects the outcome

Authors' Affiliations: [1]Chemotherapy, [2]Genetics, and [3]Pathology Divisions, National Cancer Center Research Institute; [4]Department of Orthopaedic Surgery, Keio University; [5]Orthopaedic Division, National Cancer Center Hospital; and [6]BioBusiness Group, Mitsui Knowledge Industry, Tokyo, Japan

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Microarray analysis: Microarray data of this study have been submitted to the Gene Expression Omnibus database (accession number GSE14827).

Corresponding Author: Mari Masuda, Chemotherapy Division, National Cancer Centre Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; Phone: 81-3-3542-2511; Fax: 81-3-3547-6045. E-mail: mamasuda@ncc.go.jp

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of patients (1). osteosarcoma originating in the pelvis and spine generally has a poor outcome. The prognosis of osteosarcoma patients older than 40 years is generally poor, probably because of their lower tolerance to high-dose chemotherapy and higher rate of axial tumor origin (1, 4). Through various experimental and assumption-based approaches, P-glycoprotein (multidrug resistance-1; ref. 5), ezrin (6), vascular endothelial growth factor (7, 8), matrix metalloproteinases (MMP; ref. 9), chemokine CXC motif receptor-4 (10), and other molecules have been shown to correlate significantly with outcome in osteosarcoma patients (11), but the clinical significance of these molecular biomarkers has not been established, and the molecular mechanisms behind the aggressive behavior of osteosarcoma still remain obscure.

Recently, Man and colleagues performed a microarray analysis of 34 cases of pediatric osteosarcoma and identified gene expression profiles that can predict response to chemotherapy (12). To clarify the alterations in gene expression associated with pulmonary metastasis, we have carefully selected cases with similar clinicopathologic backgrounds but demonstrating distinctly different outcomes, and did a microarray analysis under the assumption that osteosarcoma developing in older patients and/or in the trunk may have a different genetic background and different molecular mechanisms of progression. Here, we report that reduced expression of argininosuccinate synthetase (ASS) is a novel predictive biomarker for osteosarcoma patients with an unfavorable prognosis. Experimentally, osteosarcoma cells lacking ASS expression showed high sensitivity to arginine depletion. Our data seem to suggest a new therapeutic option for osteosarcoma patients with an unfavorable prognosis.

Materials and Methods

Patients and Tumor Samples

All tumor samples in this study were obtained by diagnostic incisional biopsy from primary sites of osteosarcoma before neoadjuvant chemotherapy at the National Cancer Center Hospital (Tokyo, Japan) between March 1996 and September 2007. We did not include patients older than 40 y and have primary tumors located outside the extremities. Each fresh tumor sample was cut into two pieces, one of which was immediately cryopreserved in liquid nitrogen, and the other fixed with formalin. The diagnosis of osteosarcoma and histologic subtypes were determined by certified pathologists. Only osteosarcoma samples with the osteoblastic, chondroblastic, fibroblastic, and telangiectatic histologic subtypes were included. The response to chemotherapy was classified as good if the extent of tumor necrosis was 90% or greater.

All patients provided written informed consent authorizing the collection and use of their samples for research purposes. The study protocol for obtaining clinical information and collecting samples was approved by the Institutional Review Board of the National Cancer Center (Tokyo, Japan).

Microarray Analysis

Total RNA was isolated using the IsoGen lysis buffer (Nippon Gene) and purified with a RNeasy Mini kit (Qiagen) in accordance with the manufacturer's protocol. We used a GeneChip Human Genome U133 Plus 2.0 array (Affymetrix) containing 54,613 probe sets. Target cRNA preparation, hybridization to the microarray, washing, staining, and scanning were done in accordance with the manufacturer's instructions (13). The relative expression values of the probe sets were calculated using the Array Assist 5.0 software package (Stratagene).

Real-time Reverse Transcription-PCR

For cDNA synthesis, 1 μg of total RNA was reverse transcribed by random priming with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene-specific Taqman primers and probes were designed by Applied Biosystems. Amplification data measured as an increase in reporter fluorescence were collected using the PRISM 7000 Sequence Detection system (Applied Biosystems). The mRNA expression level relative to the internal control (*ACTB*, β-actin gene) was calculated by the comparative threshold cycle (C_T) method (14).

Immunohistochemistry

Human anti-ASS monoclonal antibody was purchased from BD Bioscience. Formalin-fixed, paraffin-embedded tissue sections (4 μm thick) were stained using a DAKO streptavidin-avidin-biotin complex kit (DAKO Corp.; ref. 15).

Cell Lines

The human osteosarcoma cell lines U-2, MNNG/HOS, and MG-63 were purchased from the American Tissue Culture Collection. NOS-1 and HuO-9N2 were purchased from Riken BRC Cell Bank. Arginine-containing and arginine-free media were prepared by the Cell Science & Technology Institute (Miyagi, Japan) and were supplemented with 10% dialyzed fetal bovine serum (Invitrogen).

A plasmid containing human ASS cDNA (pAS4/1/9) was obtained from the American Tissue Culture Collection. The ASS cDNA was subcloned into the EcoRV site of pcDNA3.1 (Invitrogen). Colony formation assay was done as previously described (16), and the areas occupied by colonies were quantified using the Image J software package (v1.41, NIH).

Western Blot Analysis

Anti–β-actin mouse monoclonal antibody (AC-74) was purchased from Sigma-Aldrich. Protein samples were subjected to SDS- PAGE and transferred to Immobilon-P membranes (Millipore). After an overnight incubation with primary antibodies at 4°C and with relevant secondary antibodies at room temperature for 1 h, blots were detected using enhanced chemiluminescence Western blotting detection reagents (GE Healthcare UK; ref. 17).

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Table 1. Clinicopathologic characteristics of osteosarcoma patients analyzed using microarrays

Development of plumonary recurrence	Present (*n* = 7)	Abscent (*n* = 12)	
Gender			0.938*
Male	5	7	
Female	2	5	
Age			0.233
Mean (SD)	16 (5.3)	14 (4.0)	
Site of origin			0.317*
Femur, proximal	1	1	
Femur, distal	3	5	
Tibia, proximal	2	3	
Tibia, distal	1	2	
Other	0	1	
Histologic subtype			0.976*
Osteoblastic	6	9	
Others	1	3	
Metastasis at diagnosis			0.976*
Absent	6	9	
Present	1	3	
Neoajuvant chcemotherapy regimen			0.347
MTX+DOX/CDDP	5	5	
IFO+DOX/CDDP	2	5	
Others	0	2	
Response to neoadjuvant chemotherapy			0.667*
Good (necrosis ≥90%)	2	6	
Poor (necrosis <90%)	5	6	
Duration to the development of pulmonary metastasis (mo)			
Mean (SD)	28 (11.6)	NA	
Disease status			<0.001*
CDF	0	9	
NED	3	1	
DOD	4	2	
Follow-up period (mo)			0.290
Mean (SD), mo	62 (29.0)	72 (31.0)	
mRNA expression (microarray-based arbitrary unit), mean (SD)			
VEGF	1,151 (661)	2,519 (1,709)	0.056
MMP2	2,645 (1,812)	3,019 (2,158)	0.837
MMP9	6,521 (5,284)	7,981 (6,199)	0.711
CXCR4	989 (300)	844 (456)	0.536
TP53	108 (50)	74 (39)	0.120
ABCB1 (MDR1)	42 (10)	36 (5)	0.167
ERBB2 (Her2)	199 (96)	215 (69)	0.331
BIRC5 (Survivin)	424 (216)	432 (163)	0.711
VIL2 (Ezrin)	574 (213)	598 (321)	0.837
WT1	45 (9.0)	42 (5.9)	0.612
LRP5	48 (11)	51 (12)	0.482
FAS	303 (266)	316 (322)	0.837
ASS	86 (41)	541 (379)	<0.001

NOTE: Wilcoxon test was applied to assess differences in values.
Abbreviations: MTX, methotrexate; DOX, doxorubicin; CDDP, cisplatin; IFO ifosfamideof disease; NA, not applicable; CDF, chronic disease free; NED, no evidence of disease; DOD, deed of disease.
*Calculated by χ^2 test.

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Fluorescence-Activated Cell Sorting

Cells were harvested using trypsinization and centrifuged at 1,000 rpm for 5 min. A CycleTEST PLUS DNA Reagent kit (Becton Dickinson) was used to stain the cells. DNA content was analyzed using a cell sorter (FACSCalibur, Becton Dickinson).

Statistical Analysis

Estimates of overall and metastasis-free survival were computed using the Kaplan-Meier method. Overall survival was calculated from the day of diagnosis until the end of follow-up or death. Metastasis-free survival was calculated from the day of diagnosis until the detection

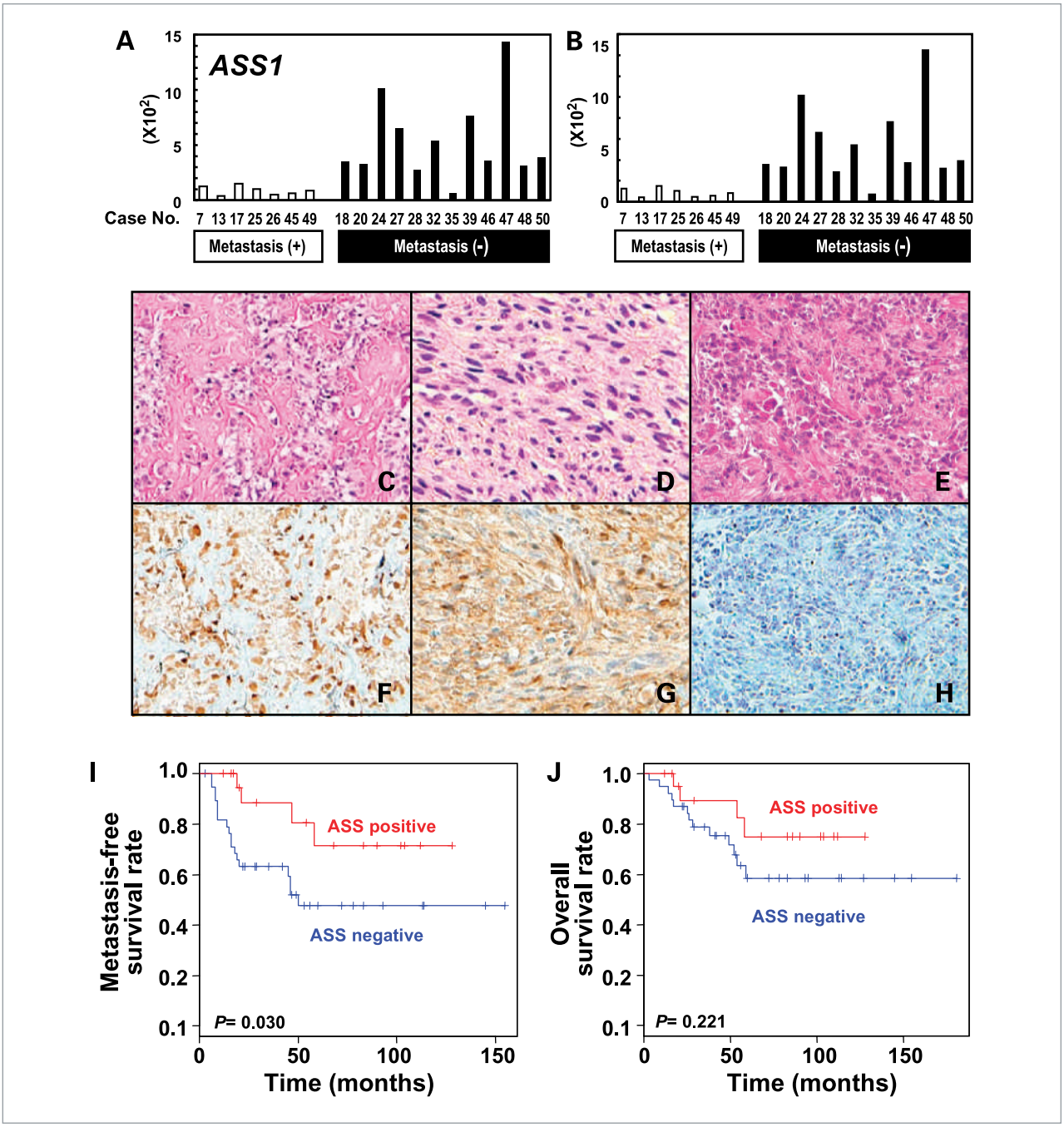

Figure 1. Downregulation of ASS in osteosarcoma patients who developed pulmonary metastasis. A and B, relative expression of ASS mRNA in osteosarcoma patients who did [metastasis (+)] and did not [metastasis (–)] develop pulmonary metastasis, determined by microarray and quantitative real-time reverse transcription-PCR (B). C to H, H&E (C–E) and immunoperoxidase (F–H) staining of ASS-positive (C, D, F, and G) and ASS-negative (E and H) osteosarcoma. I and J, Kaplan-Meier analysis of metastasis-free survival (I) and overall survival (J) of patients with ASS-positive and ASS-negative osteosarcoma.

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Table 2. Clinicopathologic data of 62 osteosarcoma patients examined by immunohistochemistry

Variables	No. of patients	%
All	62	100
Gender		
Male	41	66
Female	21	34
Age		
<10	7	11
10–19	37	60
20–29	12	19
30–39	6	10
Location		
Lower extremity	55	89
Upper extremity	7	11
Histogical subtype		
Osteoblastic	35	56
Chondroblastic	10	16
Fibroblastic	3	5
Telangiectatic	3	5
Not determined	11	18
Metastasis at diagnosis		
Absent	53	85
Present	9	15
Response to neoadjuvant chemotherapy		
Good	21	34
Poor	35	56
NA	6	10
Development of pulmonary recurrence		
Yes	23	37
No	39	63
ASS protein expression		
Positive	22	35
Negative	39	63
Not evaluable	1	2

Abbreviation: NA, not available.

of new pulmonary lesions. Analyses such as the log-rank test, χ^2 test, and Cox proportional hazards regression model were done using the R statistical package version 2.7.0.[7] Differences at $P < 0.05$ were considered significant.

Results

Downregulation of ASS in Osteosarcoma Patients Developing Pulmonary Metastasis

We compared the gene expression profiles of biopsy samples obtained from 7 osteosarcoma patients who later developed lung metastasis within 4 years after neoadjuvant chemotherapy and subsequent surgical resection, and 12 patients who did not. The latter included three patients who had lung metastases at the time of diagnosis but did not relapse after lung resection. We carefully matched the distribution of gender, age, primary sites, histologic subtypes, and chemotherapeutic regimens between the two groups (Table 1). All of the 19 patients were <40 years of age and the biopsy samples were obtained from their primary lesions (not recurrent or metastatic tumors) in the upper or lower extremities before chemotherapy.

Genes that are reportedly correlated with the prognosis or metastasis of osteosarcoma, such as *VEGF* (7, 8), *MMP2/9* (9), *CXCR4* (10), *TP53* (18), *ABCB1* (5), *ERBB2* (19), *BIRC5* (20), *VIL2* (6), *WT1* (21), *LRP5* (22), and *FAS* (23), did not show significant differential expression (Table 1). Supplementary Tables S1 and S2 list 102 differentially expressed genes showing a fold change of >2.0 and a *P* value of <0.05. It is noteworthy that only 7 genes were upregulated in osteosarcoma patients who developed lung metastasis, whereas the remaining 95 genes were downregulated. Among these genes, *ASS* attracted our interest. The expression of *ASS* was downregulated 6.3-fold, with the highest statistical significance ($P = 2.2 \times 10^{-5}$), in osteosarcoma patients who developed lung metastasis (Supplementary Table S1; Fig. 1A). The microarray data were confirmed by real-time reverse transcription-PCR (Fig. 1B).

Validation by Immunohistochemistry

ASS protein expression was assessed immunohistochemically (Fig. 1C–H) in an independent cohort comprising 62 osteosarcoma patients (Table 2). The cohort included 41 males and 21 females. The average age at diagnosis was 18 years (7–38 years) and the mean follow-up period was 54 months (3–181 months). Of these 62 patients, 23 developed pulmonary metastasis during the study period. No patients developed metastasis in other organs without having a pulmonary lesion.

ASS expression was positive in 22 (Fig. 1F and G) and was negative in 39 specimens (Fig. 1H). Metastasis-free survival of patients with ASS-negative osteosarcoma was significantly worse than that of patients with ASS-positive osteosarcoma ($P = 0.030$, log-rank test; Fig. 1I). The estimated metastasis-free survival rate was 88.5% at 2 years and 71.5% at 5 years after treatment in ASS-positive patients, compared with 63.2% and 47.7%, respectively, in ASS-negative patients. There was no significant intergroup difference in overall survival ($P = 0.221$), but there was a trend of favorable survival probability for osteosarcoma with ASS expression (Fig. 1J). This was probably due to the relatively small cohort size. Cox regression analysis revealed that age, gender, primary tumor site (upper or lower extremity and proximal or distal location), histologic subtype, response to chemotherapy, and presence of metastasis at diagnosis were not significantly correlated with metastasis-free survival

[7] http://www.r-project.org/

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Table 3. Univariate Cox regression analysis of metastasis-free survival

Variable	Hazard ratio	95% confidence interval		Z value	*P*
		Lower	Upper		
Age (y)					
<10 or ≥10	1.92	0.448	8.21	0.878	0.380
<20 or ≥20	1.94	0.836	4.512	1.55	0.120
<30 or ≥30	1.59	0.471	5.356	0.746	0.460
Gender					
Male or female	0.433	0.160	1.173	−1.64	0.100
Original site					
Lower or upper	1.14	0.338	3.824	0.207	0.840
Proximal or distal	0.93	0.401	2.154	−0.171	0.860
Histologic subtype					
Osteoblastic or others	1.240	0.450	3.423	0.416	0.680
Response to neoadjuvant chemotherapy					
Poor or good	0.627	0.24	1.638	−0.954	0.340
Metastasis at diagnosis					
Abscent or present	0.657	0.153	2.813	−0.565	0.570
ASS protein expression					
Negative or positive	0.319	0.108	0.945	−2.06	0.039*

*P value of < 0.05 was considered significant.

(Table 3). Only ASS expression was significantly correlated with metastasis-free survival ($P = 0.039$).

Overexpression of ASS Causes Growth Suppression of Osteosarcoma Cells

To examine the functional effect of ASS downregulation on osteosarcoma cell proliferation, four osteosarcoma cell lines (U2, NOS-1, MNNG/HOS, and MG63) were transfected with an expression plasmid containing human ASS cDNA (pcDNA3.1/ASS) or a control empty vector (pcDNA3.1). For all the cell lines, those transfected with pcDNA3.1/ASS formed significantly fewer colonies than those transfected with pcDNA3.1 (Fig. 2), indicating a growth-suppressive effect of ASS on osteosarcoma cells.

Cell Growth Inhibition of Osteosarcoma Showing Low ASS Expression Due to Arginine Deprivation

ASS is an essential enzyme for the production of arginine. U2 cells, which express a high amount of ASS (Fig. 3A), grew equally well in arginine-containing and arginine-free medium. Fluorescence-activated cell sorting analysis of U2 cells revealed no significant difference between those cultured in arginine-containing medium and those grown in arginine-free medium. On the other hand, the four cell lines with relatively low ASS expression (MNNG/HOS, NOS-1, HuO9N2, and MG63; Fig. 3A) showed no cell growth at all when cultured with arginine-free medium (Fig. 3B). An increase in the proportion of cells in the G_1 phase and a decrease of those in the G_2-M phase were observed in the four cell lines with relatively low ASS expression when the cells were cultured in arginine-free medium (Fig. 3C). These results indicated that arginine deprivation induced G_1 arrest in osteosarcoma cells with low ASS expression.

Discussion

Control of pulmonary metastasis is essential for improving the prognosis of osteosarcoma, and there is an urgent need to clarify the molecular mechanisms behind the process of metastasis, which could lead to the discovery of novel therapeutic approaches for osteosarcoma. Although several molecules associated with the metastatic potential of osteosarcoma have been identified by assumption- and microarray-based analyses, the lack of consistent results in reports to date precludes any definitive assessment of those molecules (11). This is likely due to the limited number of osteosarcoma patients as well as their heterogeneous characteristics such as age, tumor site, histologic subtype, and treatment history before sample collection. To identify more accurate predictive markers for patients at high risk of lung metastasis, appropriate patient selection is vital. We therefore assessed the expression profiles of a cohort of osteosarcoma patients ages <40 years whose tumors were located in the limbs (Table 1). The specimens were obtained by diagnostic biopsy from the primary sites of osteosarcoma to unify the sample conditions, and this sample cohort was composed of all four major histologic subtypes of osteosarcoma to minimize any selection bias. Our microarray

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analysis revealed that expression of ASS was significantly downregulated in osteosarcoma patients with postoperative metastasis (Fig. 1A and B). Moreover, we found a remarkable correlation between ASS expression and metastasis-free survival in osteosarcoma patients (Table 3), indicating that the loss of ASS could serve as a predictive biomarker for the postsurgical pulmonary recurrence of osteosarcoma. The expression of various genes has been reported to correlate with the outcome of osteosarcoma patients, but none of these showed a significant correlation with the development of pulmonary metastasis in our microarray analysis (Table 1), probably because of the different criteria used for the selection of cases for the analysis. However, four ASS-positive patients in the validation cohort developed pulmonary metastases (Fig. 1I), indicating that some unknown factor(s) other than ASS is also involved in the process of pulmonary metastasis.

ASS is a rate-limiting enzyme in the biosynthesis of arginine, converting citrulline to argininosuccinate, the immediate precursor of arginine (24, 25). ASS has three major functions in mammalian organisms: (*a*) ammonia detoxication through the urea cycle in the liver, (*b*) arginine production in the kidney proximal tubule, and (*c*) arginine synthesis for the production of nitric oxide in various cells (24, 25). Previous studies by others have shown that ASS deficiency is frequently evident in several human cancers, including melanoma, hapatocellular

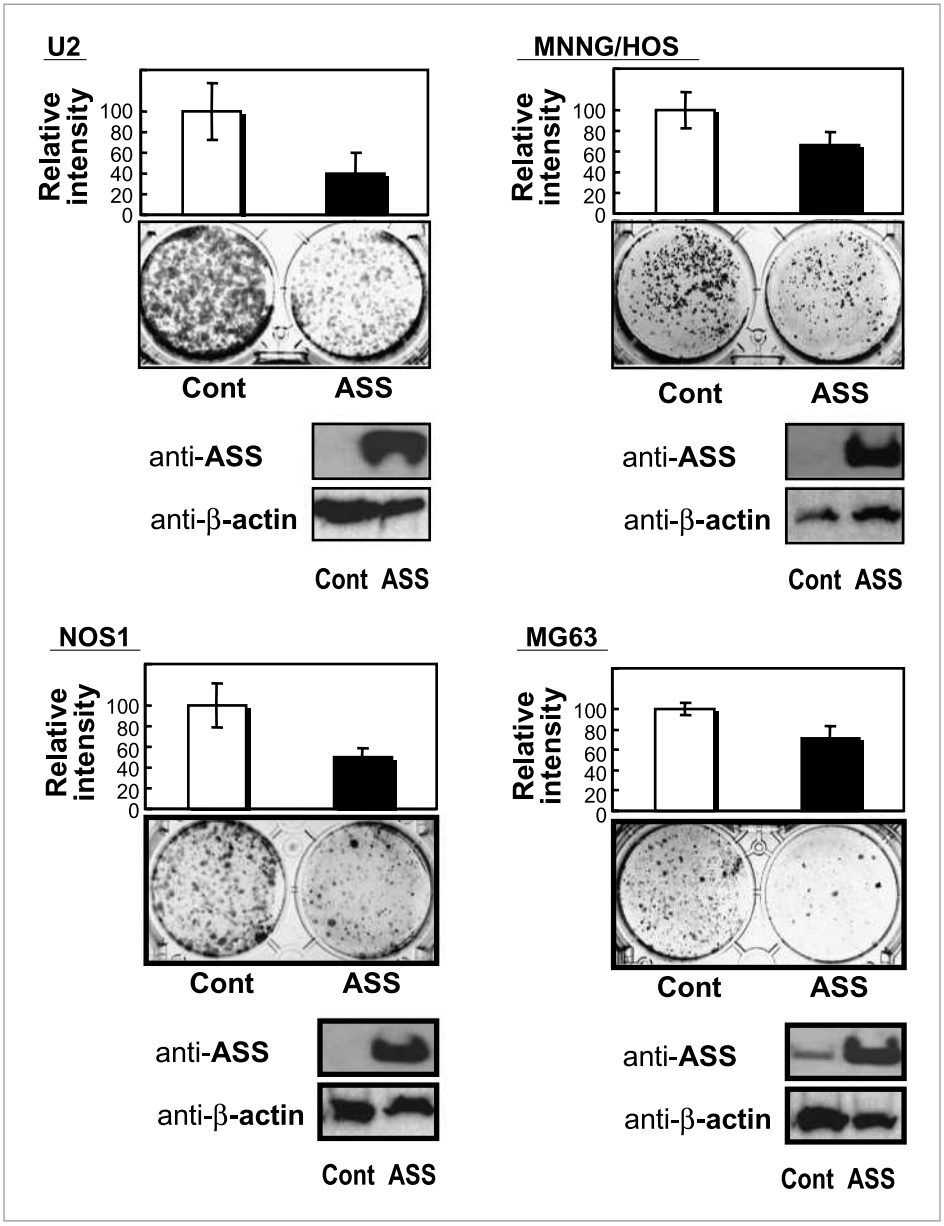

Figure 2. Effects of ASS expression on osteosarcoma cell growth. Colony formation of four osteosarcoma cell lines transfected with empty pcDNA3.1 vector (cont) and pcDNA3.1/ASS (ASS). Transfectants were cultured in the presence of G418 for 10 d, and then stained. Two days after transfection, cell lysates were subjected to immunoblotting with antibodies against ASS and β-actin (loading control).

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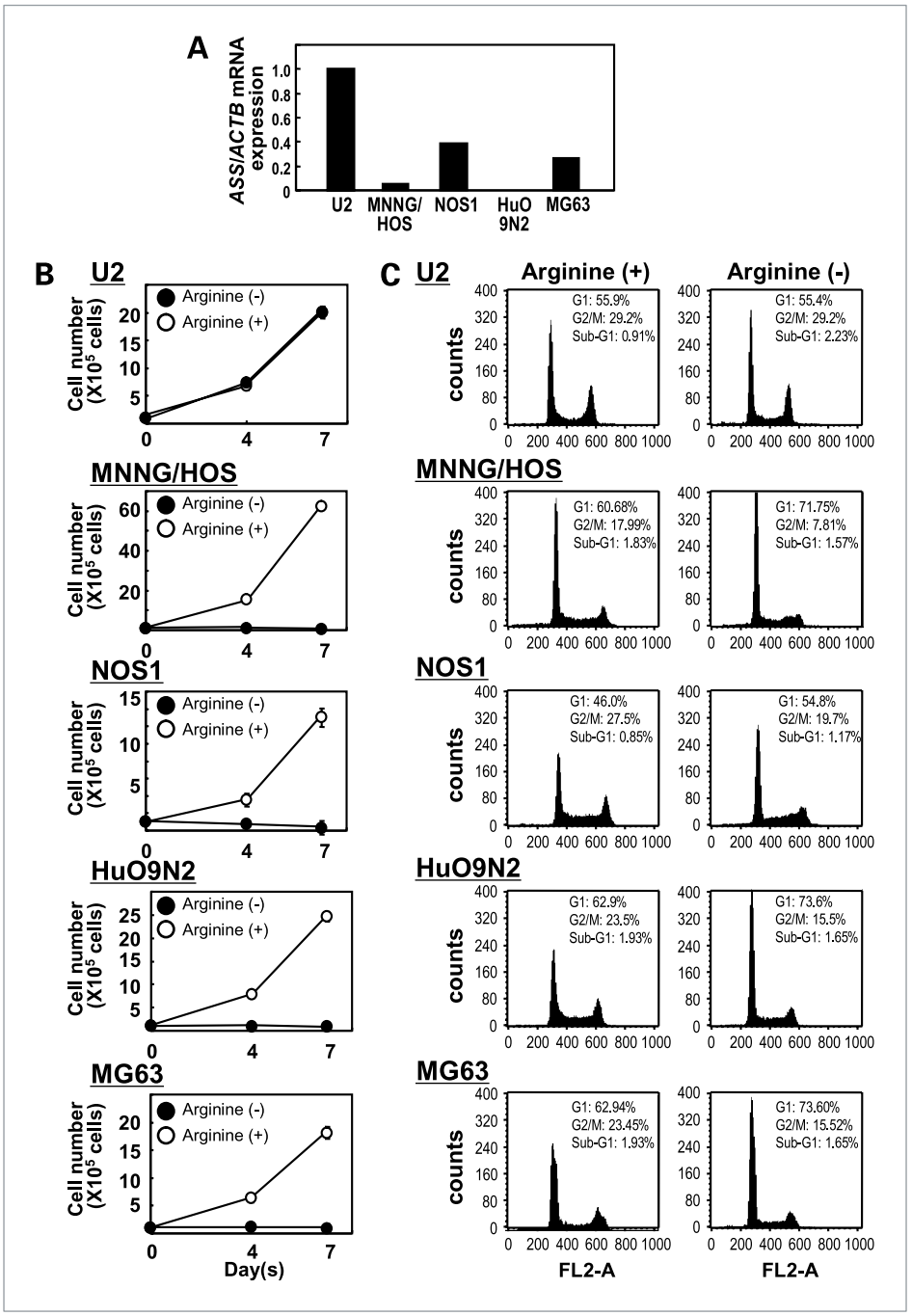

Figure 3. Effect of arginine deprivation on growth of osteosarcoma cells. A, relative *ASS* mRNA expression of five osteosarcoma cell lines determined by real-time reverse transcription-PCR. B, osteosarcoma cells were plated at 1.0×10^5 per well into six-well microplates and cultured in arginine-containing [arginine (+)] or arginine-free [arginine (–)] medium. The numbers of cells were then counted by trypan blue dye exclusion using a hemocytometer. C, osteosarcoma cells were cultured in arginine-containing (left) or arginine-free (right) medium for 3 d, and subjected to cell sorter analysis. The percentages of cells in the sub-G_1, G_1, and G_2-M phase are indicated.

carcinoma (HCC), and prostate carcinoma (26), and ASS deficiency is significantly associated with the lymphatic dissemination of esophageal carcinoma (27). However, no studies have clarified the mechanisms by which lack of ASS confers malignant phenotypes on tumor cells or how the *ASS* gene is downregulated. In the present study, we showed that the restoration of ASS expression in osteosarcoma cell lines suppressed their growth (Fig. 2). Considering that the lack of ASS expression is frequently observed in cells of several other cancers, such as melanoma and HCC, ASS may regulate normal cellular functions, thereby working as a tumor suppressor. Alternatively, the gain of arginine from the microenvironment or the circulation might confer some growth advantage on ASS-negative tumor cells, instead of generating arginine on their own. Further work is needed to clarify the role of ASS in the inhibition of tumor cell growth. In an attempt to investigate the molecular mechanism

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of *ASS* gene silencing, we tried to restore ASS expression by treating the osteosarcoma cells with a methyltransferase inhibitor, 5-aza-2′-deoxycytidine. However, we observed no effects on the restoration of ASS in these cells, indicating that the promoter methylation of the *ASS* gene is not responsible for the silencing of ASS (data not shown).

Because tumors not expressing ASS are auxotrophic for arginine, arginine deprivation has been reported to be an effective anticancer treatment for ASS-deficient tumors, as exemplified by HCC, melanoma, and renal cell carcinoma, both *in vitro* and *in vivo*, and also by malignant mesothelioma, retinoblastoma, and pancreatic cancer *in vitro* (28–36). It is therefore plausible that ASS deficiency could become a therapeutic target for osteosarcoma, besides being a predictive biomarker for postsurgical pulmonary metastasis. The effect of arginine deprivation has not been established in osteosarcoma (37). We showed that four osteosarcoma cell lines with low levels of ASS expression failed to grow in arginine-free medium, whereas ASS-positive cells were able to grow in medium with or without arginine. Furthermore, osteosarcoma cells that did not proliferate in arginine-free medium underwent G_0-G_1 arrest (Fig. 3B and C). In such cells, the sub–G_0-G_1 population was barely detectable, indicating that arginine deprivation for 3 days did not cause apoptosis. This finding may not contradict a previous observation by Gong et al. (37), who showed that arginine deiminase (ADI) induced apoptosis in cultured cells only at high concentration. ADI seems to have a variety of pharmacologic activities besides arginine depletion (31, 37–41).

Based on our present findings, we propose a new therapeutic approach for the management of osteosarcoma patients who are at high risk of lung metastasis. Before starting neoadjuvant chemotherapy, diagnostic biopsy specimens from osteosarcoma patients should be screened for ASS by immunohistochemical assay. Then, for those lacking ASS or expressing ASS at reduced levels, systemic arginine deprivation is recommended to reduce the risk of developing pulmonary metastasis. Given that ~50% of osteosarcoma patients are resistant to current chemotherapy, this approach could be a promising strategy for eradicating tumor cells in osteosarcoma patients with a higher recurrence potential, thus improving their prognosis. Encouraging results of arginine deprivation therapy with the use of ADI have recently been shown both *in vitro* and *in vivo* (28–30). Phase I and II trials of ADI-PEG20, a derivative of ADI with a prolonged half-life, have shown a partial response with tolerable adverse effects in patients with melanoma and HCC (34, 35). Future clinical trials are warranted to establish the clinical potential of systemic arginine deprivation therapy for osteosarcoma patients.

Our data indicate that ASS could serve as not only a novel predictive biomarker for metastasis development, but also become a potential target for pharmacologic intervention. Elucidation of the molecular mechanisms by which a reduced level of ASS increases the chances of pulmonary metastasis will be necessary.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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