higher BMI and worse prognosis for patients with hormonal receptor (HR)-positive tumors. In addition, enhanced BMI in their study correlated with worse prognosis only for HR-positive and for human epidermal growth factor receptor 2 (HER2)-negative but not HER2-positive or triple-negative tumors, irrespective of menopausal status [7, 8].

Thus, the effect of BMI on breast cancer incidence and prognosis seems to be restricted to ER-positive breast cancers. However, it is not yet clear why prognosis correlates with BMI of premenopausal patients. In a study by Berclaz et al. [5], a positive association between BMI and prognosis was recognized for patients treated with chemotherapy but not with endocrine therapy. Similar findings were obtained in a study reported by Sparano et al. [7] in which adjuvant chemotherapy was administered to all patients. Furthermore, according to data from the ATAC (anastrozole, tamoxifen, alone, or in combination) trial, postmenopausal women with high BMI treated with anastrozole showed significantly more distant recurrences than those with a low BMI, possibly caused by ineffective suppression of estrogen production resulting from an increase in aromatase activity in patients with high BMI [9]. Nevertheless, in spite of these findings, it is currently still unknown how adjuvant treatments including chemotherapy and endocrine therapy affect the relationship between BMI and prognosis.

If the occurrence of tumors in patients with high BMI is the result of an increase in estrogen status, these tumors are likely to be highly estrogen dependent, thus resulting in a favorable prognosis. On the other hand, a poorer prognosis for patients with high BMI may indicate that BMI plays a significant part in breast cancer etiology and prognosis by mediation through various mechanisms. To determine the crucial role of BMI, the relationship between tumor biological characteristics and BMI of patients thus needs to be thoroughly analyzed, but this issue has not yet been specifically discussed in the literature. The purpose of the study presented here was to disclose the relationship between BMI at the time of diagnosis and biological characteristics, focusing specifically on ER, PR, and Ki67 expression levels in terms of different subtypes.

Materials and methods

Patients

The 525 cases of invasive breast cancers treated with mastectomy or breast-conserving surgery at Hyogo College of Medicine or Tokushima Breast Clinic between 2005 and 2012 were recruited consecutively. Histological diagnosis of breast cancer was confirmed in each case (493 invasive ductal carcinomas, 15 invasive lobular carcinomas, and 19 other types), and patients with noninvasive carcinoma were excluded. This study was approved by the Ethics Committee of Hyogo College of Medicine.

Immunohistochemical staining and classification of subtypes

For classification of subtypes, immunohistochemical staining of ER, PR, HER2, and Ki67 was performed. Formalin-fixed, paraffin-embedded tissues were cut and used for immunohistochemical analyses. Staining was followed by automated immunostaining with BOND-MAX (Leica Microsystems, Tokyo, Japan) for ER and PR, and with Autostainer (Dako, Tokyo, Japan) for HER2 and Ki67. The primary antibodies used for this study were ER (1D5), PR (PgR636), HER2 (Hercep Test), and Ki67 (MIB1), all from Dako (Glostrup, Denmark). Expression levels of these proteins were determined immunohistochemically as the percentage of positive cancer cells in the nuclei for ER, PR, and Ki67, and by membrane staining for HER2. When nuclear stained cells accounted for 1 % or more, they were deemed positive for ER and PR, and HER2 positivity was defined as HER2 (3+), or HER2 (2+) and fluorescence in situ hybridization (FISH) positive. Different areas of densely stained lesions were selected microscopically, and more than 500 cancer cells were counted to determine Ki67 expression levels.

The criteria reported by Cheang et al. [10] were used to separate ER-positive and HER2-negative breast cancers into luminal A and luminal B subtypes by using a cutoff point of 14 % Ki67. The subtypes were defined as follows: luminal A, ER+/HER2−, Ki67 $<$ 14 %; luminal B, ER+/HER2−, Ki67 $\geq$ 14 %; luminal/HER2, ER+/HER2+; HER2, ER−/HER2+; and triple negative (TN), ER−/HER2−.

Statistical analysis

The relationship between BMI, calculated as weight (kg) divided by height (m^2), and breast cancer characteristics was determined with the chi squared test. ER, PR, and Ki67 expression levels for each subtype were calculated with the Mann–Whitney test. Differences were considered statistically significant if the P value was less than 0.05. JMP10 (SAS Institute Japan, Tokyo, Japan) was used for all analyses.

Results

Correlation between BMI and clinicopathological characteristics of breast cancers

The BMI of the postmenopausal patients (mean, 23.3 kg/m^2; standard deviation, 3.8 kg/m^2) was significantly higher than

that of premenopausal patients (22.0, 4.1) ($P < 0.0001$; Table 1). We used the categorization by Kawai et al. [6] of BMI of Japanese breast cancer patients into quartiles (<21.2, ≥21.2 to <23.3, ≥23.3 to <25.8, and ≥25.8 kg/m^2) to set 23.3 kg/m^2 as the median cutoff and divide the patients into high- and low-BMI level groups (low, <23.3 kg/m^2; high, ≥23.3 kg/m^2). As shown in Table 1, there were significantly more postmenopausal ($n = 153$, 73.9 %) than premenopausal patients with high BMI ($n = 56$, 26.1 %) ($P = 0.0002$; Table 1).

Table 2 shows that a significantly higher percentage of premenopausal than postmenopausal patients with high BMI had lymph node metastases (46.4 % vs. 22.9 %, $P = 0.005$), whereas the correlation between large tumor size (37.0 % vs. 23.7 %) and high nuclear grade (grade 3, 39.0 vs. grade 1, 27.8 %) was marginally significant ($P = 0.05$ and $P = 0.07$, respectively). However, the two groups of postmenopausal patients showed no significant differences in tumor size, nuclear grade, or histological type (Table 3). More postmenopausal patients with high BMI tended to have PR-positive than PR-negative tumors (48.9 % vs. 37.8 %, $P = 0.05$), but there was no significant difference in HER2 positivity between the two BMI groups.

Correlation between BMI and breast cancer subtypes

We also divided breast cancers into five groups: luminal A (ER+/HER2− and Ki67 < 14 %, $n = 237$), luminal B (ER+/HER2− and Ki67 ≥ 14 %, $n = 150$), luminal/HER2 (ER+/HER2+, $n = 40$), HER2-positive (ER−/HER2+, $n = 37$), and TN (ER−/HER2−, $n = 61$). There were no statistically significant differences between BMI and breast cancer subtypes (Table 4) even when menopausal status was factored in.

Table 1 Relationship between body mass index (BMI) and menopausal status of breast cancer patients

	n	Premenopausal	n	Postmenopausal	P value
Age[a] (years) (SD)	187	44.5 (6.5)	338	65.2 (9.8)	
BMI[a] kg/m^2 (SD)	187	22.0 (4.1)	338	23.3 (3.8)	<0.0001
BMI[b]	Premenopausal		Postmenopausal		P value
Low	133 (41.8 %)		185 (58.2 %)		0.0002
High	54 (26.1 %)		153 (73.9 %)		

[a] Mean (standard deviation)

[b] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2

Table 2 Relationship between body mass index (BMI) and clinicopathological characteristics in premenopausal patients

Characteristics	Low BMI[a] (%)	High BMI[a] (%)	P value
Tumor size			
≤2.0 cm	87 (76.3)	27 (23.7)	0.05
<2.0 cm	46 (63.0)	27 (37.0)	
Lymph node metastasis			
Negative	101 (77.1)	30 (22.9)	0.005
Positive	32 (57.1)	26 (42.9)	
Nuclear grade			
1	83 (72.2)	32 (27.8)	0.07
2	21 (87.5)	3 (12.5)	
3	25 (61.0)	16 (39.0)	
Unknown	4 (44.4)	5 (55.6)	
Histological type[b]			
IDC	124 (71.7)	49 (28.3)	0.82
ILC	5 (62.5)	3 (37.5)	
Others	4 (66.7)	2 (33.3)	
Estrogen receptor			
Positive	112 (69.6)	49 (30.4)	0.24
Negative	21 (80.8)	5 (19.2)	
Progesterone receptor			
Positive	110 (69.2)	49 (30.8)	0.16
Negative	23 (82.1)	5 (17.9)	
HER2			
Positive	20 (62.5)	12 (37.5)	0.23
Negative	113 (72.9)	42 (27.1)	

[a] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2

[b] *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma

ER, PR, and Ki67 expression levels in tumors of patients with high BMI

Findings of the analysis of ER expression levels by subtype of ER-positive breast cancers of patients in the two BMI groups are shown in Table 5. There were no significant differences between high and low BMI patients with luminal A and luminal B cancers, but ER expression of luminal/HER2 tumors of patients with high BMI [mean ± standard deviation (%), 90.4 ± 21.1] was significantly higher ($P = 0.001$) than that of patients with low BMI (40.5 ± 33.2). There were no differences in PR expression levels between the two BMI groups for any of the subtypes (Table 6). Because luminal A and luminal B breast cancers were initially classified according to the Ki67 expression level, we also classified luminal subtype (luminal A and luminal B), and their Ki67 expression levels were analyzed in relationship to high or low BMI (Table 7). However, there were no significant differences of Ki67 expression levels between the two BMI groups for any of the subtypes.

Table 3 Relationship between body mass index (BMI) and clinicopathological characteristics in postmenopausal patients

Characteristics	Low BMI[a] (%)	High BMI[a] (%)	*P* value
Tumor size			
≤2.0 cm	102 (53.4)	89 (46.6)	0.57
<2.0 cm	83 (56.5)	64 (43.5)	
Lymph node metastasis			
Negative	130 (54.4)	109 (45.6)	0.84
Positive	55 (55.6)	44 (44.4)	
Nuclear grade			
1	103 (45.5)	86 (54.5)	0.32
2	40 (39.4)	26 (60.6)	
3	36 (52.0)	39 (48.0)	
Unknown	6 (75.0)	2 (25.0)	
Histological type[b]			
IDC	174 (54.7)	144 (45.3)	0.55
ILC	5 (71.4)	2 (28.6)	
Others	6 (46.2)	7 (53.8)	
Estrogen receptor			
Positive	140 (52.6)	126 (47.4)	0.13
Negative	45 (62.5)	27 (37.5)	
Progesterone receptor			
Positive	116 (51.1)	111 (48.9)	0.05
Negative	69 (62.2)	42 (37.8)	
HER2			
Positive	27 (60.0)	18 (40.0)	0.44
Negative	158 (53.9)	135 (46.1)	

[a] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2

[b] *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma

Discussion

The results of our study confirmed that higher BMI of premenopausal patients was significantly associated with lymph node metastasis and marginally associated with large tumor size as well as high nuclear grade. However, no such differences were detected in postmenopausal patients. In addition, we could not find any associations between BMI and breast cancer subtypes. Biglia et al. [11] found that postmenopausal patients with higher BMI (>25) showed a higher frequency of ER/PR-positive cancers than did those with lower BMI (87 % vs. 75 %), but no such difference was seen in premenopausal patients. Consistent with this observation, we found that the frequency of PR-positive tumors was marginally higher for postmenopausal patients but not for premenopausal patients with high BMI, which strongly suggests that a higher BMI influences tumor biology mediated through an increase in the production of estrogens.

Although high BMI was significantly more prevalent among postmenopausal (55.4 %) than premenopausal patients (30.3 %), the distribution of the subtypes was not significantly different for patients with either high or low BMI even when menopausal status was taken into consideration. Because ER, PR, and Ki67 expression levels of the subtypes did not differ between high and low BMI, except for ER expression levels in postmenopausal luminal/HER2 breast cancers, we hypothesize that the influence of high BMI on the biological characteristics of these tumor is limited. Alternatively, we confirmed that higher BMI was significantly associated with lymph node metastasis in premenopausal patients.

It has been reported that BMI of postmenopausal and/or premenopausal women is significantly associated with characteristics of the aggressive tumor phenotype, i.e., large tumor size, lymph node metastasis, and higher

Table 4 Relationship between body mass index (BMI) and breast cancer subtypes

Subtype	Low BMI[a]	High BMI[a]	*P* value
Premenopausal (*n*, %)			
Luminal A	63 (70.8)	26 (29.2)	0.34
Luminal B	38 (71.7)	15 (28.3)	
Luminal/HER2	11 (57.9)	8 (42.1)	
HER2	9 (69.2)	4 (30.8)	
TN	12 (92.3)	1 (7.7)	
Postmenopausal (*n*, %)			
Luminal A	69 (46.6)	79 (53.4)	0.12
Luminal B	59 (60.8)	38 (39.2)	
Luminal/HER2	12 (57.1)	9 (42.9)	
HER2	15 (62.5)	9 (37.5)	
TN	30 (62.5)	18 (37.5)	

Definitions of breast cancer subtypes are given in the "Materials and methods" section

[a] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2

Table 5 Estrogen receptor (ER) expression levels by body mass index (BMI) in ER-positive subsets

Subtype	Low BMI[a]		High BMI[a]		*P* value
Premenopausal					
Luminal A	63	73.9 ± 23.8	26	73.6 ± 23.9	0.88
Luminal B	38	72.1 ± 26.4	15	79.9 ± 15.0	0.5
Luminal/HER2	11	53.3 ± 34.4	8	54.4 ± 39.5	0.71
Postmenopausal					
Luminal A	69	88.2 ± 14.9	79	83.1 ± 19.8	0.06
Luminal B	59	71.2 ± 36.4	38	76.0 ± 30.4	0.74
Luminal/HER2	12	40.5 ± 33.2	9	90.4 ± 21.1	0.001

Definitions of breast cancer subtypes are given in the "Materials and methods" section

[a] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2; *n*, mean ± standard deviation (%)

Table 6 Progesterone receptor (PR) expression levels by body mass index (BMI) in ER-positive subsets

Subtype	Low BMI[a]		High BMI[a]		*P* value
Premenopausal					
Luminal A	63	60.3 ± 32.3	26	67.6 ± 31.5	0.19
Luminal B	38	45.4 ± 29.4	15	58.3 ± 34.2	0.14
Luminal/HER2	11	42.1 ± 35.4	8	26.4 ± 32.9	0.77
Postmenopausal					
Luminal A	69	43.5 ± 37.7	79	41.0 ± 33.3	0.75
Luminal B	59	29.3 ± 31.0	38	40.5 ± 35.1	0.15
Luminal/HER2	12	8.6 ± 15.3	9	17.3 ± 29.2	0.74

Definitions of breast cancer subtypes are given in the "Materials and methods" section

[a] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2; *n*, mean ± standard deviation (%)

Table 7 Ki67 expression levels by body mass index (BMI) in subtypes

Subtype	Low BMI[a]		High BMI[a]		*P* value
Premenopausal					
Luminal[b]	101	13.7 ± 13.7	41	16.6 ± 14.5	0.14
Luminal/HER2	8	22.8 ± 20.3	4	25.9 ± 14.1	0.73
HER2	6	40.5 ± 19.1	2	26.8 ± 18.7	0.31
TN	8	51.9 ± 38.1	1	23.2	0.69
Postmenopausal					
Luminal[b]	128	17.9 ± 18.2	117	13.8 ± 12.9	0.13
Luminal/HER2	7	12.7 ± 9.4	7	21.4 ± 10.5	0.17
HER2	8	36.3 ± 26.1	6	47.8 ± 19.5	0.36
TN	22	39.4 ± 26.0	11	44.8 ± 29.6	0.52

Definitions of breast cancer subtypes are given in the "Materials and methods" section

[a] BMI: low, <23.3 kg/m^2; high, ≥23.3 kg/m^2

[b] Includes luminal A and luminal B; *n*, mean % ± standard deviation

proliferation index [11, 12]. Thus, the characteristics of tumors with high BMI seem to be different from those of tumors of patients with low BMI, which are caused not only by estrogens but also by other factors. Fatty tissues secrete several cytokines including leptin, tumor necrosis factor-α, insulin-like growth factor (IGF), and interleukin-6 [13], which are likely to be involved in tumor biology-mediating crosstalk between estrogen and growth factor signaling. On the basis of the findings of these studies and of others that demonstrated that higher insulin levels and elevated C-peptide levels are associated with increased recurrence of or death from breast cancers [14, 15], the factors associated with obesity may explain, at least in part, the poorer prognosis for patients with high BMI.

The reason why there is an association between high BMI and aggressive characteristics in premenopausal but not in postmenopausal patients is currently not known. Because PR positivity of breast cancers of postmenopausal patients with high BMI was marginally higher, these cancers are likely to feature higher estrogen dependency and less aggressive phenotype. On the other hand, ER expression levels of luminal/HER2 cancers with low BMI were comparatively lower, which may indicate there is a difference in estrogen dependency between breast cancers of patients with high and low BMI.

Since the study by Berclaz et al. [5] established that an association between overall survival (OS) and obesity was restricted to patients treated by chemotherapy, it has been speculated that there is a difference in sensitivity to chemotherapy between tumors of patients with high and with low BMI. Sparano et al. [7] reported that disease-free survival (DFS) and OS were significantly poorer for HR-positive/HER2-negative/unknown cancer subsets of obese patients. High estrogen dependency and less sensitivity to chemotherapy may be one explanation of this difference [16]. However, as we found no differences in ER and PR expression levels between breast cancers with high and low BMI among premenopausal patients, we think differences in chemosensitivity between them are unlikely. Alternatively, the worse prognosis for breast cancers of premenopausal patients with high BMI could be at least partly explained by their aggressive phenotype (higher frequency of lymph node metastasis, large tumor size, and high nuclear grade).

In conclusion, we established that a difference exists between the clinicopathological characteristics of breast cancers of patients with high and low BMI. Because distribution by subtypes did not show significant differences, and ER, PR, and Ki67 expression levels were similar for tumors in premenopausal patients with high and low BMI, the influence of BMI on these biological characteristics seems to be limited. However, the fact that lymph node metastasis was significantly higher for premenopausal patients with high BMI leads us to think that differences in aggressive characteristics may in fact be influenced by BMI, mediated not through estrogens but through other factors. The main limitation of this study is that the conclusion was reached based on a study with a limited number of subjects, so that there is a clear need for verification studies comprising a large number of breast cancers as well as focusing on prognosis and drug sensitivity.

Conflict of interest Y. Miyoshi has received honoraria from Sanofi, AstraZeneca K.K., and GlaxoSmithKline K.K. T. Katagiri is a board member of Oncotherapy Science Co. Ltd. The other authors declare that they have no conflicts of interest in this article.

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The Transcription Factor Sp3 Regulates the Expression of a Metastasis-Related Marker of Sarcoma, Actin Filament-Associated Protein 1-Like 1 (AFAP1L1)

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Abstract

We previously identified actin filament-associated protein 1-like 1 (AFAP1L1) as a metastasis-predicting marker from the gene-expression profiles of 65 spindle cell sarcomas, and demonstrated the up-regulation of *AFAP1L1* expression to be an independent risk factor for distant metastasis in multivariate analyses. Little is known, however, about how the expression of *AFAP1L1* is regulated. Luciferase reporter assays showed tandem binding motives of a specificity protein (Sp) located at −85 to −75 relative to the transcriptional start site to be essential to the promoter activity. Overexpression of Sp1 and Sp3 proteins transactivated the proximal *AFAP1L1* promoter construct, and electrophoretic mobility shift assays showed that both Sp1 and Sp3 were able to bind to this region *in vitro*. Chromatin immunoprecipitation experiments, however, revealed that Sp3 is the major factor binding to the proximal promoter region of the *AFAP1L1* gene in AFAP1L1- positive cells. Treatment with mithramycin A, an inhibitor of proteins binding to GC-rich regions, prevented Sp3 from binding to the proximal promoter region of *AFAP1L1* and decreased its expression in a dose-dependent manner. Finally, knocking down Sp3 using small inhibitory RNA duplex (siRNA) reduced AFAP1L1 expression significantly, which was partially restored by expressing siRNA-resistant Sp3. These findings indicate a novel role for Sp3 in sarcomas as a driver for expression of the metastasis-related gene *AFAP1L1*.

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Introduction

Soft tissue sarcoma (STS) is a malignant neoplasm that can arise in fat, muscle, fibrous tissue, blood vessels, or other supporting tissue in any part of the body. STSs are divided into two groups based on morphology; small round cell sarcomas and spindle cell sarcomas. The former include rhabdomyosarcomas and extra-skeletal Ewing's tumors, against which chemotherapy and radiotherapy are effective at least in the initial stages, and therefore treatment other than surgery is usually the first choice. STSs in the latter group, such as leiomyosarcomas and malignant fibrous histiocytomas, however, are radio- and chemoresistant in most cases and therefore wide resection with proper surgical margins is the only way to control local tumors. In spite of proper treatment for local disease, approximately half of patients develop metastasis in distant organs, particularly in the lungs. Although recent studies have demonstrated a beneficial effect of chemotherapy, the improvement is far from satisfactory. Considering the associated side effects, it is desirable to identify high-risk patients, to whom additional treatments should be administered.

AFAP1L1 was previously identified as a metastasis-predicting marker from the gene-expression profiles of 65 spindle cell sarcomas by our group [1]. In univariate and multivariate analyses, higher expression of AFAP1L1 was found to contribute to the occurrence of distant metastases, along with patient age and tumor grade. Knocking down of the *AFAP1L1* gene in sarcoma cells reduced cell invasiveness and forced expression of *AFAP1L1* in immortalized human mesenchymal stem cells increased anchorage-independent cell growth as well as cell invasiveness. These results suggest that the molecular mechanism up-regulating the expression of *AFAP1L1* is a key to the progression of sarcomas. In this study, we explored the transcriptional regulation of *AFAP1L1* in order to find factors responsible for the up-regulation

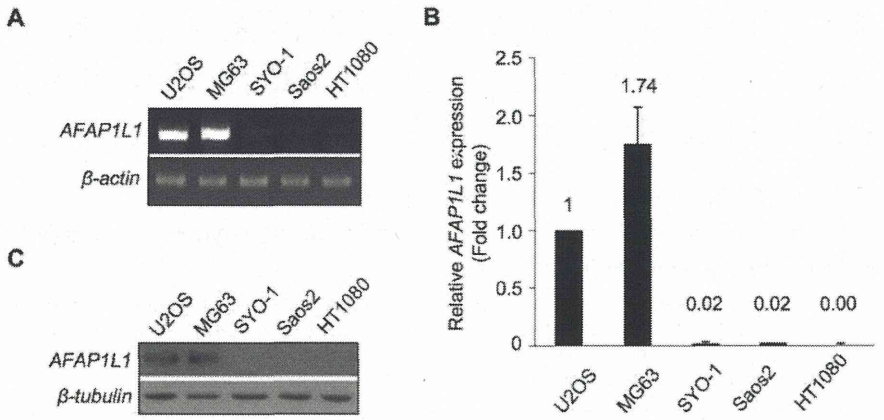

Figure 1. AFAP1L1 expression in sarcoma cell lines. (A) mRNA expression of the *AFAP1L1* gene in sarcoma cell lines. Reverse transcribed cDNA from each cell line was used as a template for PCR with primers specific for the *AFAP1L1* gene. The *β-actin* gene was used as a control. (B) Quantitative analysis of the gene expression of *AFAP1L1*. qPCR was performed with a Taqman probe and the primers listed in Table S1. Expression levels were calculated as fold changes relative to U2OS. (C) Protein expression of AFAP1L1. Total cell lysate from each cell line was used for Western blotting. β-tubulin was used as a control. Error bars indicate standard deviations.
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of AFAP1L1 expression, which will help us to understand how sarcoma cells gain the malignant phenotype.

Materials and Methods

Cell Lines, antibodies and reagents

Human osteosarcoma cell lines (U2OS, MG63, and Saos2) and a human fibrosarcoma cell line (HT1080) were obtained from American Type Culture Collection (ATCC, Manassas, VA). PC-3 (human prostate cancer) and 293T were also obtained from ATCC. SYO-1 (human synovial sarcoma cell line) [2] was provided by Dr. A. Kawai (National Cancer Center, Japan), and 293T was described elsewhere [3]. Informed consent was obtained from the patient with written consent, and the procedure was approved by the Ethics Committee of Graduate School of Medicine and Dentistry, Okayama University. Cells were cultured in DMEM (for U2OS, MG63, Saos2, 293T, HT1080 and SYO-1) or RPMI (for PC-3) supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin under 5% CO2 at 37°C. The anti-AFAP1L1 polyclonal antibody was produced in our laboratory as described previously [1]. The anti-Sp1 antibodies (1C6 and PEP2) and anti-Sp3 antibody (D-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-β-tubulin antibody was obtained from Thermo Fisher Scientific Inc. (Waltham, MA), and anti-acetylated H3K9 (06-942), from Millipore Corp (Billerica, MA). The anti-Flag antibody and mithramycin A were purchased from Sigma-Aldrich (St. Louis, MO).

Semiquantitative reverse-transcription (RT)-PCR and quantitative real-time RT-PCR (qPCR)

The procedures for extracting total RNA and RT-PCR have been described previously [4]. Sets of primers for RT-PCR and qPCR are listed in Table S1. To quantitate AFAP1L1 expression, qPCR was performed in triplicate using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) and a thermal cycler (ABI 7300 Real-Time PCR System, Applied Biosystems). qPCR for ChIP assays was done using SYBR GREEN reagent (Applied Biosystems) and a set of primers used in RT-PCR. Conditions for PCR and qPCR are available upon request.

Plasmid constructs

Information on the 5′ flanking regulatory region of the *AFAP1L1* gene was obtained from GenBank (NC_000005.9). A 2,325-bp DNA fragment from −2250 to +75 relative to the transcription start site (TSS) was amplified by PCR using a sense primer with a XhoI site and an antisense primer with a HindIII site. DNA synthesis was performed with Primestar DNA polymerase (Takara, Shiga, Japan). The product was digested by XhoI and HindIII and cloned into a luciferase reporter plasmid, PGV-basic (Toyo Ink, Tokyo, Japan), to obtain PGV-(−2250). Other reporter vectors harboring a shorter DNA fragment (−1039, −778, −688, −601, −410, −224, −71, −53 or −46 to +75) were generated by a PCR-based method using PGV-(−2250) as a template. The primers used to amplify each fragment are listed in Table S1. Plasmids harboring mutations in the Sp-binding site (SBS) or Ets-binding site (EBS) were created by PCR-based mutagenesis using PGV-(−224) as a template. Briefly, PCR was performed with pairs of primers containing mutations in SBS1 (−86 -GGGCGGGGCGG- −76 to G*TT*CGG*TT*CGG), SBS2 (−102 -GGGCGG- −97 to G*TT*CGG), EBS1 (−60 -ATCCT- −56 to AT*AA*T) and EBS2 (−121 -TTCCG- −117 to TT*AA*G). The PCR product was digested by DpnI (TOYOBO, Osaka, Japan), transformed to competent cells and propagated. pEVR2/Sp1 and pRC/Sp3 were kindly provided by Dr. G. Suske (Marburg University, Marburg, Germany). Because pRC/Sp3 lacks the N-terminal part of the *Sp3* gene [5] , a vector that includes a full-length version of the *Sp3* gene was created as described previously [4]. Briefly, a PCR-amplified EcoRI-NotI fragment of the N-terminal part of *Sp3* and a NotI-XhoI fragment from pRC/Sp3 were sequentially cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA), yielding pcDNA/Sp3(li-1), which contained a long isoform of the *Sp3* gene [5]. Using this vector as a template, another type of long isoform (li-2) [5] and two types of short isoform (si-1 and si-2) [5] were created by a PCR-based method and subcloned into pcDNA3.1(+) vectors, yielding pcDNA/Sp3(li-2), pcDNA/Sp3(si-1), and pcDNA/Sp3(si-2). Sequences of all the cDNAs were confirmed by sequencing. Plasmid vectors for Ets1, Ets2, ELK1, SAP1, PEA3 and dominant negative Ets (DN-Ets) were kindly provided by Dr. E. Hara (The Cancer

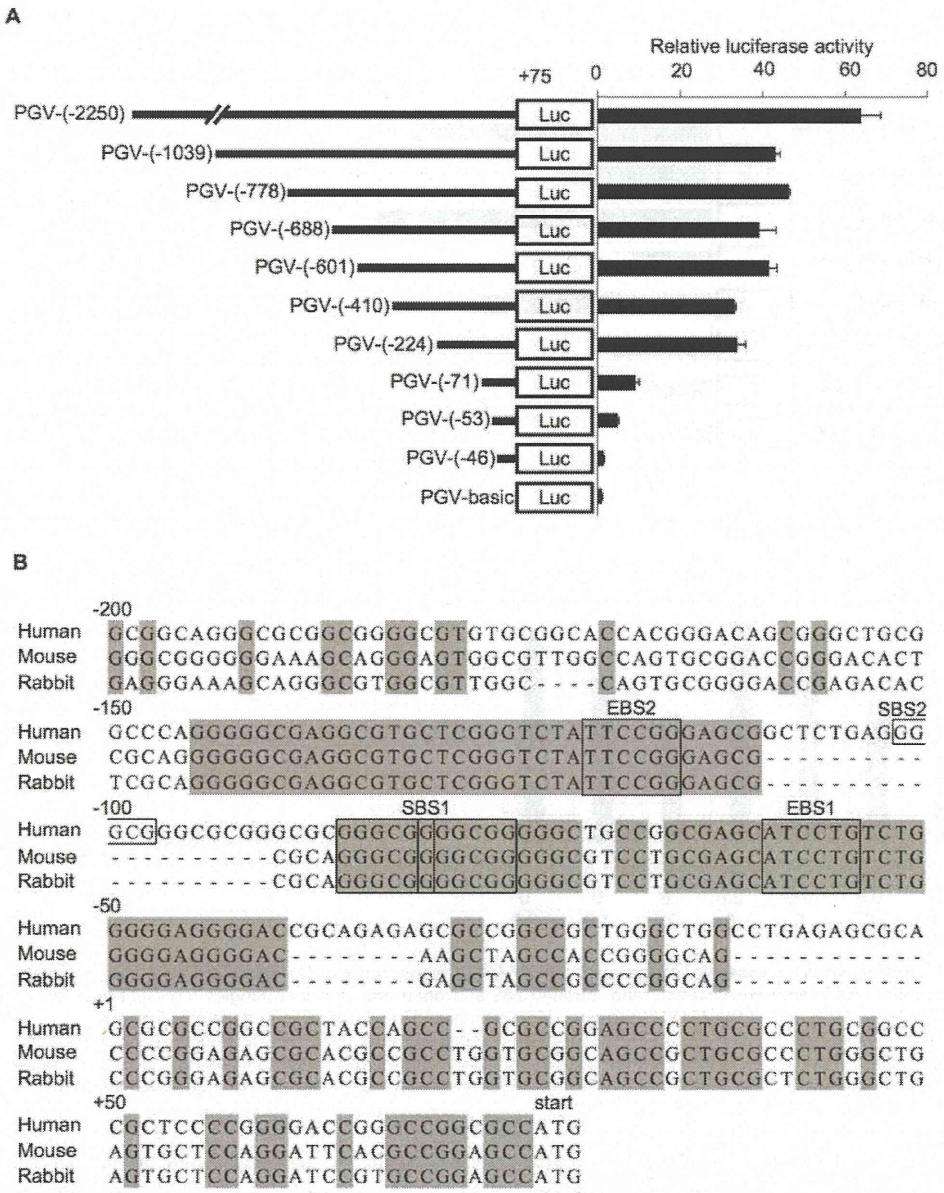

Figure 2. Identification of the core promoter region of the *AFAP1L1* gene. (A) Transcriptional activity of the 5′-flanking region of the *AFAP1L1* gene. Luciferase reporter assays were performed using a series of constructs carrying DNA fragments derived from the 5′-flanking region of the *AFAP1L1* gene. Numbers indicate the position relative to the transcriptional start site (TSS), and in all cases, the 3′ end of fragments was at the start codon, which was located 75 bases upstream of TSS. (B) Comparison of 5′-flanking region of the *AFAP1L1* gene among species. Human, mouse and rabbit sequences of the 5′-flanking region of the *AFAP1L1* gene are aligned, and conserved sequences are shown in gray boxes. EBS, Ets-binding site; SBS, Sp1-binding site.
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Institute of Japanese Foundation for Cancer Research, Tokyo, Japan).

Luciferase assays

Cells (2×10^4) in 24-well dishes were transfected with 0.5 μg of each reporter plasmid and 2 ng of pRL-TK control vector (Toyo Ink) using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. In the co-transfection experiments, the total amount of plasmid was adjusted with pcDNA3.1(+) to 2 μg. Cells were harvested at 24 h after transfection and luciferase assays were performed with the Dual Luciferase Assay Reporter System (Promega, Madison, WI) as described previously [4].

Electrophoretic Mobility Shift Assay (EMSA)

Single-stranded oligonucleotides (ONDs) corresponding to sense and antisense sequences of the wild-type or mutated SBS1 site were synthesized (Table S1), and mutated ONDs (25 pmol) were end-labeled at 37°C for 30 min in a 50-μl reaction mixture containing 1 μl of [γ-^{32}P]ATP and 10 units of T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA). Sense and antisense

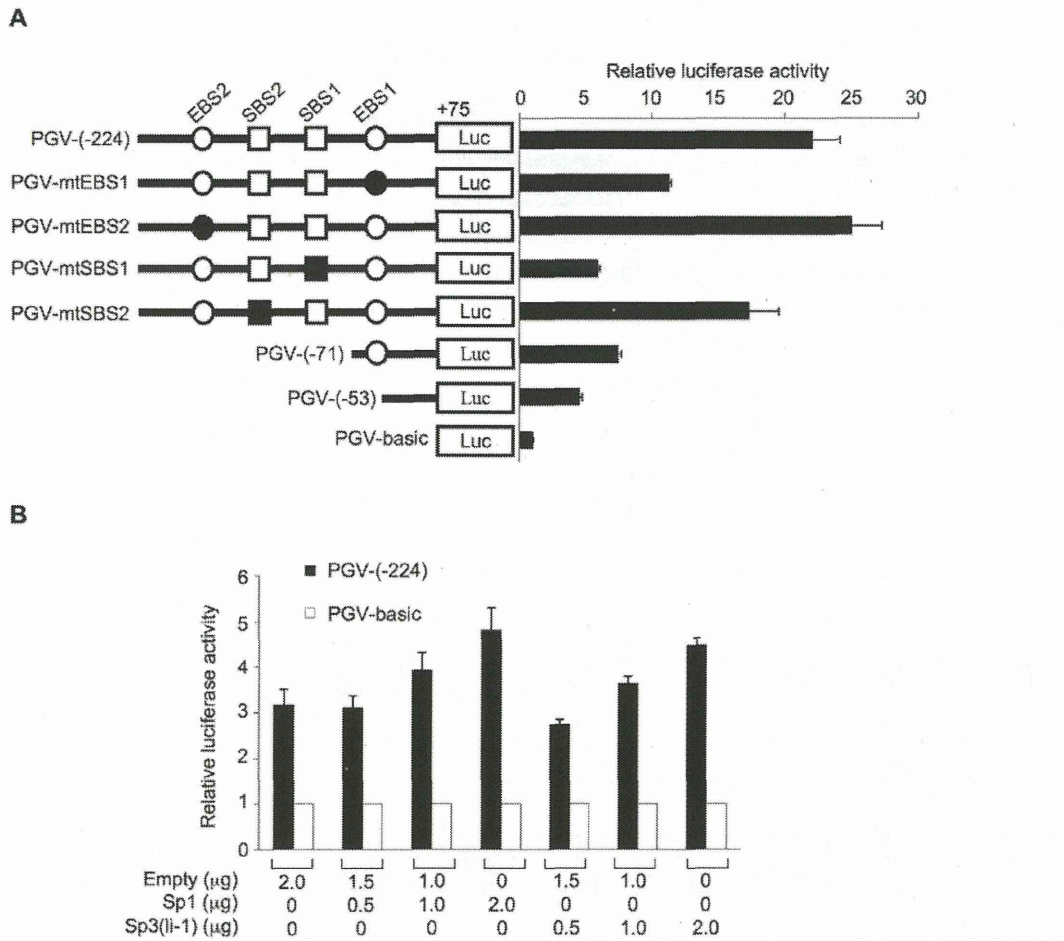

Figure 3. Identification of Sp1-binding sites as essential sequences for *AFAP1L1* transcription. (A) Identification of core domains for transcriptional activity. Open and closed circles represent wild-type and mutated EBS and open and closed rectangles represent wild-type and mutated SBS. PGV-vectors containing various segments of the *AFAP1L1* promoter were transfected into U2OS cells, and their luciferase activities were measured. (B) The effect of exogenous Sp1 and Sp3 on the transcriptional activity of the core promoter region of the *AFAP1L1* gene. The luciferase activity of the core promoter region (−224 to +75) was evaluated after Sp1 or Sp3-expressing vectors were co-transfected into U2OS cells. The total amount of transfected plasmid DNA was equalized by the addition of pcDNA3.1(+), an empty vector. Error bars indicate standard deviations.
doi:10.1371/journal.pone.0049709.g003

ONDs of each pair were mixed and annealed by heating at 98°C for 1 min and cooling off at room temperature for 1 h in a block incubator. Double-stranded ONDs, designated SBS1WT and SBS1MUT respectively, were purified with illustra ProbeQuant™ G-50 Micro Colum's (GE Healthcare, Little Chalfont, United Kingdom). Nuclear extracts were prepared from cells by using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific Inc.). The radio-labeled DNA probe was incubated for 15 minutes at room temperature with the reaction mixtures, containing nuclear extract of U2OS (12 μg), 2 μl of 10× binding buffer (Thermo Fisher Scientific Inc.), 1 μg of poly(dI-dC), 2.5% glycerol, 5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol and 0.5 mmol/L $ZnCl_2$. DNA-protein complexes were loaded on a 6% nondenaturing polyacrylamide gel and electrophoresed at 200 V for 70 min. In the supershift assay, nuclear extracts were mixed with the anti-Sp1 antibody (1C6), the anti-Sp3 antibody (D-20), or rabbit non-immunized control IgG (Dako, Tokyo, Japan) in the reaction mixture and incubated for 30 min on ice before the formation of DNA-protein complexes. In the competition experiment, excess amounts of unlabeled ONDs were added to the reaction mixtures before the incubation with the labeled DNA probe.

Chromatin Immunoprecipitation (ChIP) assay

Cells in a semi-confluent state in a 150-mm dish were fixed with formaldehyde at a final concentration of 1.0% for 10 min at room temperature to cross-link protein to DNA. Cells were washed with ice-cold PBS and lysed in 300 μl of lysis buffer (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate and 0.5% N-laurolysarcosine), then sonicated on ice. Triton-X 100 was added at a final concentration of 10% to dissolve the protein-DNA complexes. A soluble fraction was obtained after centrifugation at 20,000× g for 10 min at 4°C. Fifteen microliters of supernatant (one-twentieth of the total volume) was saved as an input, and the rest was divided into three and mixed with Dynabeads (Invtirogen) at 4°C overnight with rotation, which were pre-incubated with 2 μg of anti-Sp1 (PEP2) or -Sp3 (D-20) antibodies, or rabbit non-immune IgG at 4°C overnight. The next day, immunoprecipitated complexes were

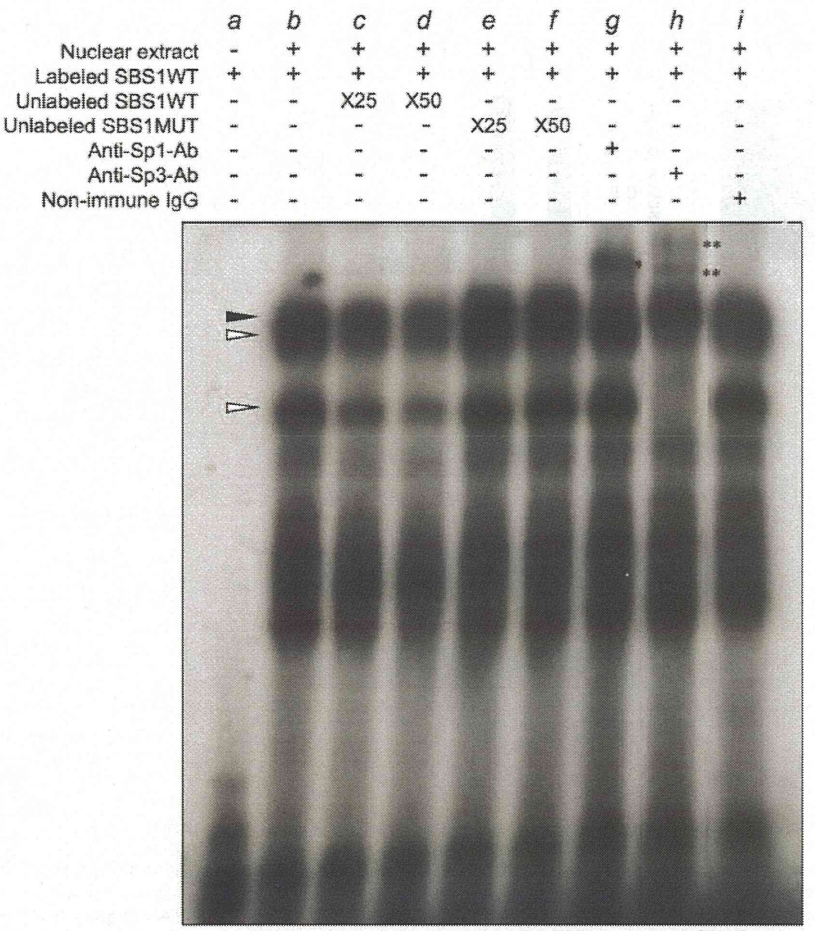

Figure 4. Binding of Sp transcription factors to the core-promoter region of the *AFAP1L1* gene *in vitro*. EMSA was performed to analyze the binding ability of putative transcription binding sites. Nuclear extracts were prepared from U2OS cells. Cold competitor experiments were conducted by the addition of 25- and 50-fold excess amounts of unlabeled SBS1WT or SBS1MUT to nuclear extracts before incubating with labeled SBS1WT (*lanes c–f*). Supershift experiments were conducted by the addition of anti-Sp1 or anti-Sp3 antibody to protein-OND complexes (lanes *g* and *h*). Non-immune IgG was used as a control (lane *i*). Open and closed arrowheads indicate the Sp3-OND and Sp1-OND complex, respectively. Single and double asterisks indicate bands supershifted by the addition of Sp1 or Sp3 antibody, respectively.
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washed with low salt, high salt, LiCl RIPA buffer and finally with TE (pH 8.0) buffer containing 50 mM NaCl. The complexes were eluted from Dynabeads by treatment with the elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS) and boiling at 65°C for 15 min. After centrifugation at 16,000× g for 15 min at room temperature, DNA-protein cross-links were reversed by incubating overnight at 65°C. Following RNaseA and proteinase K treatment, DNA was purified and precipitated with the phenol-chloroform method. PCR was performed to amplify a DNA fragment spanning from -136 to +142 including two SBSs and EBSs by KOD Plus polymerase (TOYOBO) and a set of primers listed in Table S1.

Western blot analyses

Western blotting was performed as described previously [1]. Membranes were probed with anti-AFAP1L1 (1:2000), anti-Sp1 (1C6, 1:1000), anti-Sp3 (1:1000), anti-β-tubulin (1:1000), anti-acetyl H3K9 (1:2500) and anti-Flag (1:2000) antibodies. An NE-PER Kit (Thermo Fisher Scientific Inc.) was used to prepare nuclear protein before the Western blotting.

Small inhibitory RNAs (siRNAs)

siRNA duplexes were transfected into cells (1.5×10^6 cells) using RNAiMAX (Invitrogen) at a concentration of 20 nM. RNA and protein were extracted 48 h and 72 h after transfection, respectively. To knock down the *Sp1* and *Sp3* genes, two different siRNAs were used (siSp1#1 and siSp1#2 for Sp1; siSp3#1 and siSp3#2 for Sp3). siSp1#1 and siSp3#1were purchased from Dharmacon (Thermo Fisher Scientific Inc.) and had been used in our previous study [6]. Luciferase GL2 siRNA (siGL2) and GL3 siRNA (siGL3) were also purchased from Dharmacon. siSp1#2, siSp3#2, and an siRNA sequence targeting Sp4 gene (siSp4) were synthesized by Sigma-Aldrich (Table S1).

siRNA-resistant Sp3 gene

A vector that harbors the *Sp3(li-1)* gene resistant to both siSp3#1 and siSp3#2 was generated by a mutagenesis-based method. Primers for mutagenesis were designed to harbor silent mutations at the third nucleotide of every codon in the target sequence (Table S1). pcDNA/Sp3(li-1) was sequentially mutated using the two sets of primers, and the construct was transferred to