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Expression of CAVEOLIN 1 in uterine mesenchymal tumors: No relationship between malignancy and CAVEOLIN 1 expression

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ABSTRACT

Although most smooth muscle neoplasms detected in the human uterus are benign, uterine leiomyosarcoma (Ut-LMS) is extremely malignant with high rates of recurrence and metastasis. CAVEOLIN 1 (CAV1) levels in the epithelial cells of some carcinomas have been reported to increase during tumor progression. We herein evaluated the relationship between CAV1 expression and the pathological features of patients diagnosed with uterine mesenchymal tumors at several clinical facilities. No clinical link was observed between CAV1 expression and the malignancy of human uterine mesenchymal tumors. CAV1 expression was decreased in the normal myometrium, whereas it was strongly expressed in uterine mesenchymal tumors. However, the expression of CAV1 was not a potential biomarker to distinguish Ut-LMS from other types of uterine mesenchymal tumors. The perivascular expression of CAV1 was clearly observed in all types of uterine mesenchymal tumors and myometria. Therefore, the results of the present study suggest that CAV1 may not act as a potential biomarker of uterine malignant mesenchymal tumors.

1. Introduction

CAVEOLIN 1 (CAV1) is a protein that is encoded by the *CAV1* gene in humans [1]. The scaffolding protein encoded by this gene is the main component of the caveolae plasma membranes found in most cell types. This protein links integrin subunits to the tyrosine kinase FYN, an initiating step in coupling integrins to the RAS-ERK pathway and promoting cell cycle progression. CAV1 is a tumor suppressor gene candidate or a negative regulator of the RAS-p42/44 MAP kinase cascade. *CAV1* and *CAV2* genes are located next to each other on chromosome 7 and express co-localizing proteins that form a stable hetero-oligomeric complex. Using alternative initiation codons in the same reading frame, two isoforms (alpha and beta) were found to be encoded by a single transcript from the *CAV* gene [2]. Two of them (CAVEOLIN 1/CAV1 and CAVEOLIN 2/CAV2) are widely co-expressed in fully differentiated mesenchymal and endothelial normal tissues as well as in many solid tumors, whereas CAVEOLIN 3/CAV3 is primarily expressed in muscle cells [2,3]. Numerous disease processes have recently been suspected of being affected by the ablation or mutation of caveolins/CAVs that regulate many signaling molecules and signaling cascades [1].

Previous studies revealed that CAV1 levels in the epithelial cells of some carcinomas increased during tumor progression. Conversely, CAV1 expression in peritumoral stromal cells was found to decrease in advanced and metastatic cancers, which may also occur in sarcomas [3,4]. Uterine leiomyomas (LMA) are the most frequently detected mesenchymal tumors in the uterus. Most uterine mesenchymal tumors are readily classifiable as uterine LMA or uterine leiomyosarcoma (Ut-LMS) based on their gross and microscopic appearances [5]. Uterine mesenchymal tumors that

Abbreviations: CAV1, caveolin 1; Ut-LMS, uterine leiomyosarcoma; LMA, leiomyomas; LMP2, large multifunctional protease 2; IFN-γ, interferon-γ; STUMP, smooth muscle tumor of uncertain malignant potential.

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cannot be histologically diagnosed as unequivocally benign or malignant are currently termed cellular LMA, Bizarre LMA, mitotically active LMA, and a smooth muscle tumor of uncertain malignant potential (STUMP) [5–7]. Recent studies have suggested that immunohistochemical (IHC) staining for some proliferation markers such as ki-67, LMP2, CYCLIN B, and CALPONIN h1 may be useful for differentially diagnosing uterine mesenchymal tumors [5–7]. CAV1 has been shown to act as either a tumor suppressor or tumor promoter in different tumors [8–11]. However, few studies have examined the function of CAV1 in uterine mesenchymal tumors [12,13]. The aim of the present study was to investigate IHC for CAV1 expression in uterine mesenchymal tumors and evaluate the relationship between CAV1 expression and many prognostic findings such as tumor size, stage of the disease, histological features, age, survival rate, and overall and event-free survival.

2. Material and methods

2.1. Tissue collection

Fifty-six patients aged between 32 and 83 years who were diagnosed with smooth muscle tumors in the uterus were selected from pathological files. Serial sections were cut from at least 2 tissue blocks from each patient for hematoxylin and eosin staining and immunostaining. All tissues were used with the approval of the Ethical Committee of Shinshu University after obtaining written consent from each patient. The pathological diagnosis of uterine smooth muscle tumors was performed using established criteria with some modifications [14]. Briefly, usual leiomyoma (usual LMA) was defined as a tumor showing typical histological features with a mitotic index (MI) [obtained by counting the total number of mitotic figures (MFs) in 10 high-power fields (HPFs)] of <5 MF s per 10 HPFs. Cellular leiomyoma (cellular LMA) was defined as a tumor with significantly increased cellularity (>2000 myoma cells/HPF) and an MI < 5, but without cytologic atypia. Bizarre leiomyoma (BL) was defined as a tumor with either diffuse nuclear atypia and MI < 2 or with focal nuclear atypia and MI < 5 without coagulative tumor cell necrosis. A smooth muscle tumor of uncertain malignant potential (STUMP) was defined as a tumor with no mild atypia and MI < 10, but with coagulative tumor cell necrosis. Uterine leiomyosarcoma (Ut-LMS) was diagnosed in the presence of MI > 10 with either diffuse cytologic atypia, coagulative tumor cell necrosis, or both. Of the 70 smooth muscle tumors examined, 38 were diagnosed as LMA, 11 were smooth muscle tumor of uncertain malignant potential (STUMP), 3 were Bizarre leiomyoma, and 56 were Ut-LMS.

2.2. Immunohistochemistry (IHC)

IHC staining for LMP2, CAV1, VE-CADHERIN, ER, PR, TP53, and Ki-67 was performed on serial human Ut-LMS sections. Antibodies for ER(ER1D5), PR(PR10A), TP53(DO-1), and Ki-67(MIB-1) were purchased from Immunotech (Tassigny, Marseille, France). The antibody for CAV1 was purchased from GeneTex, Inc., (Irvine, CA). The antibody for VE-CADHERIN was purchased from eBioscience, Inc. (San Diego, CA). The antibody for ERG1 was purchased from Santa CruzBiotechnology, Inc. (Dallas, TX). The anti-human LMP2 antibody was produced by SIGMA-Aldrich Israel Ltd. (Plaut Street, Rehovot, Israel). IHC was performed using the avidin-biotin complex method as described previously. Briefly, one representative 5-μm-thick tissue section was cut from a paraffin-embedded sample of a radical hysterectomy specimen from each Ut-LMS patient. Normal myometrium portions in each specimen were used as positive controls. To examine the expression levels of target molecules, we performed immunofluorescence experiments for CAV1, ERG1, and VE-CADHERIN on paraffin-embedded human myometrium or Ut-LMS. The sections were then incubated with the appropriate antibodies at 4 °C overnight. We used a rabbit polyclonal antibody to CAV1 (1:200), a mouse monoclonal antibody to VE-CADHERIN (1:200), or a mouse monoclonal antibody to ERG1 (1:200) as the primary antibodies. These experiments were conducted at Shinshu University in accordance with institutional guidelines (approval no. M192).

2.3. Reverse transcription-polymerase chain reaction analysis (RT-PCR)

The expression of LMP2, CAV1, ERG1, and β-actin transcripts was examined using RT-PCR. Total RNA was prepared from human uterine LMS tissues and normal myometrium tissues using TRIzol reagent according to the manufacturer's protocol (Invitrogen Co., Carlsbad, CA, USA). RNA was reverse-transcribed with the Superscript II enzyme (Invitrogen Co.), and single stranded cDNA was used for amplification.

2.4. DNA transfection and western blotting

The transfection of pCEM9-LMP2wt or the empty pCEM9 vector was carried out with the FuGENE6 Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's recommendations with 5 μg of plasmid DNA and 5×10^5 SKN cells (from Dr. I. Ishiwata, Ishiwata Clinic, Tsuchiura, Ibaraki, Japan) or 5×10^5 HeLa cells (from Dr. Y. Adachi, Shinshu University, Matsumoto, Nagano, Japan) plated onto 6-well tissue culture dishes (Corning, New York, NY, USA) on the previous day.

Details of methods and any associated references are available in the Supplementary data.

3. Results

Surgery and chemotherapy for Ut-LMSs were the treatment modalities applied alone or in combination for all 56 patients according to their individual features. In this series, there were 49 LMA, 3 Bizarre leiomyoma, 56 Ut-LMS, and 56 myometria as normal tissue cases. The mean age of patients was average 56.3 years (ranging from 32 to 83 years). Patients with Ut-LMS (Average 55.7 years/32 to 83 years) were older than those with STUMPs (Average 61.2/33 to 83 years) and LMAs (Average 56.0 years/32 to 83 years). The mean mitoses count was average 68.9 in STUMPs and average 65.9 in LMSs. Thirty-five cases were alive, while the oldest Ut-LMS case died from a condition apparently unrelated to Ut-LMS. The mean overall survival times of STUMPS and Ut-LMSs were average 48.6 (8–97) months and average 32.6 (8–54) months, respectively. The expression of CAV1 was marked in 52 (93%) Ut-LMSs and 11 STUMPs (100%), and 3 Bizarre leiomyomas (100%), while perivascular CAV1 expression was detected in 52 Ut-LMSs (93%), 11 STUMPs (100%), and 3 Bizarre leiomyomas (100%) (Fig. 1 and Table 1). Perivascular CAV1 expression was also clearly detected in all types of LMAs including STUMPs (100%) and Ut-LMSs (100%) at same level as CAV1 expression in the normal myometrium (Fig. 1B, Table 1). The expression of ETS-related gene-1 (ERG1), which positively regulates *CAV1* gene activation, was also examined in all the samples tested [15]. Although tumors became more aggressive and invasive, no significant changes were observed in the expression of tumor CAV1 or perivascular CAV1 (Fig. 1 and Table 1). However, parallel changes were observed in CAV1 and ERG1 expression between uterine mesenchymal tumor types and normal myometria (Fig. 1 and Table 1). In western blotting (W.B.) and RT-PCR experiments, CAV1 and ERG1 were expressed in LMAs, Bizarre leiomyomas, and Ut-LMSs, but not in the normal

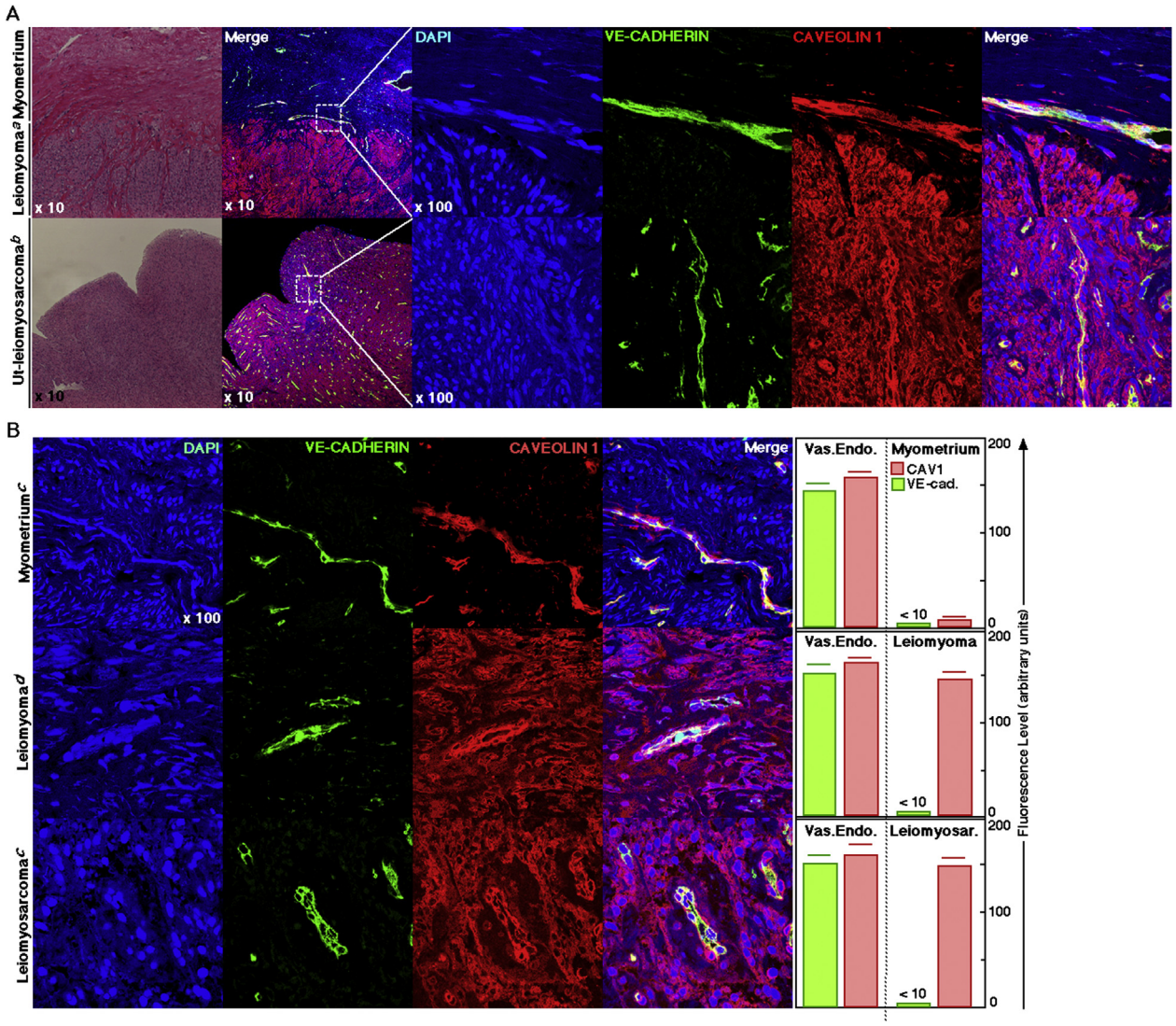

Fig. 1. Expression of CAVEOLIN 1 and VE-CADHERIN in human uterine leiomyoma (LMA), leiomyosarcoma (LMS), and normal myometrium tissues. (A) IHC experiments individually performed at several medical facilities revealed that the ability to induce CAVEOLIN 1 expression was markedly lower in human normal myometrium tissues than in human leiomyoma (LMA, patient UL6[a]) located in the same tissue section as well as in human uterine leiomyosarcoma tissues (patient #20[b]). (B) The perivascular expression of CAVEOLIN 1 was similar between normal myometria and all types of tumors. The immunohistochemistry (IHC) of CAVEOLIN 1 and VE-CADHERIN in normal myometrium (patient #13[c]), uterine leiomyoma (LMA, patient UL1[d]), and uterine LMS (patient #13[c]) tissues were each taken from the same sample. In all samples, 5-μm-thick sections of tissue were stained with the anti-hCAVEOLIN 1 antibody and anti-hVE-CADHERIN antibody and revealed by Alexa Fluor 488 conjugated anti-mouse IgG or the Alexa Fluor 546 anti-rabbit IgG antibody and DAPI (Vector Laboratories, Inc.) (magnification × 10 and × 100). Levels of immunofluorescence were calculated and graphically indicated. A quantitative analysis was performed using WinRoof Ver.6.30 (Mitani Co., Ltd. Fukui Japan). [a,b,c] The details of patients with LMA or LMS are shown in Supplementary data Tables S2 and S3. Normal total: 70 cases, LMA total: 49 cases, Bizarre Leiomyoma total: 3 cases, LMS total: 56 cases. Experiments were performed three times with similar results. [a,b,c,d,] The details of patients with LMA or LMS are shown in Supplementary data Tables S2 and S3.

Table 1
Expression of CAVEOLIN 1 and ERG1 in human uterine leiomyoma (LMA), Bizarre leiomyoma, leiomyosarcoma (LMS), and normal myometrium tissues. Normal total: 70 cases, LMA total: 49 cases, Bizarre Leiomyoma total: 3 cases, LMS total: 56 cases. Experiments were performed three times with similar results.

	Age (years)	n	CAVEOLIN 1 expression								ERG1 expression					
			Mesenchyma tumors				Perivascular SMCs				Mesenchy. Tumors			Perivascular SMCs		
			−	−/+[1]	focal+[2]	+++[3]	−	−/+[1]	focal+[2]	+++[3]	−	−/+[4]	+++[3]	−	−/+[4]	+++[3]
Normal (myometrium)	32~83	70	70							70	70					70
Leiomyoma	32~83	49				49				49			49			49
(Ordinaly leiomyoma)		(29)														
(Cellular leiomyoma)		(9)														
(Tumor of uncertain malignant potential)		(11)														
Bizarre Leiomyoma	44,49,55	3				3				3			3			3
Leiomyosarcoma	32~83	56			4	52			2	54		4	52		2	54

Results from IHC sudies, −/+[1]: partially positive (5%−10% of cells stained), focal+[2]: focal-positive (focal or sporadic staining with less than 5% of cells stained), +++[3]: diffuse-positive (homogeneous distribution with more than 90% of cells stained), −/+[4]: including focal+, -: negative (no stained cells with appropriate antibodies).

myometrium (Fig. 2B), which was strongly supportive of the IHC findings. A similar relationship was observed with survival time. In the Kaplan Meier survival analysis, no significant differences were noted between the overall survival of cases according to the status of perivascular and tumoral CAV1 expression ($p = 0.953$ and $p = 0.985$).

Ut-LMS was previously detected in female *Lmp2*-deficient mice at 6 months of age or older, and proteasome subunit LMP2 played a role as a tumor suppressor in human Ut-LMS [15–17]. In the present study, we investigated the relationship between CAV1, ERG1, and LMP2 in sarcomagenesis in human Ut-LMS. Differential responsiveness to genetically modified stable LMP2 expression in the SKN human Ut-LMS cell line was also examined to determine whether reintroducing LMP2 into an Ut-LMS cell line affected the expression of CAV1 and ERG1 for the development of human Ut-LMS. SKN cells were transfected with pCEM9 or pCEM9-LMP2wt, and SKN-CEM9 clones and SKN-LMP2 clones were selected in medium containing G418. The expression of CAV1 and ERG1 was markedly down-regulated by the presence of LMP2 in the SKN-transfectants clones (Fig. 3 and Supplementary data STable 1), suggesting that LMP2 may involve the function of CAV1 in the tumor suppression of human Ut-LMS.

4. Discussion

CAVEOLINs play a paradoxical role in the development of human disease. They have been implicated in both tumor suppression and oncogenesis [4]. The strong expression of CAVs has been shown to inhibit cancer-related pathways, such as growth factor signaling pathways. However, certain cancer cells that express caveolins are more aggressive and metastatic because of a potential for anchorage-independent growth. Previous studies demonstrated that CAV1 facilitated both ERK and AKT signaling in cancer cells from the kidney, colon, prostate, epidermis, muscle, and brain and promoted cell invasion, proliferation, angiogenesis, and multi-drug resistance [8–11,17–19]. CAV1-positive tumor cells have been

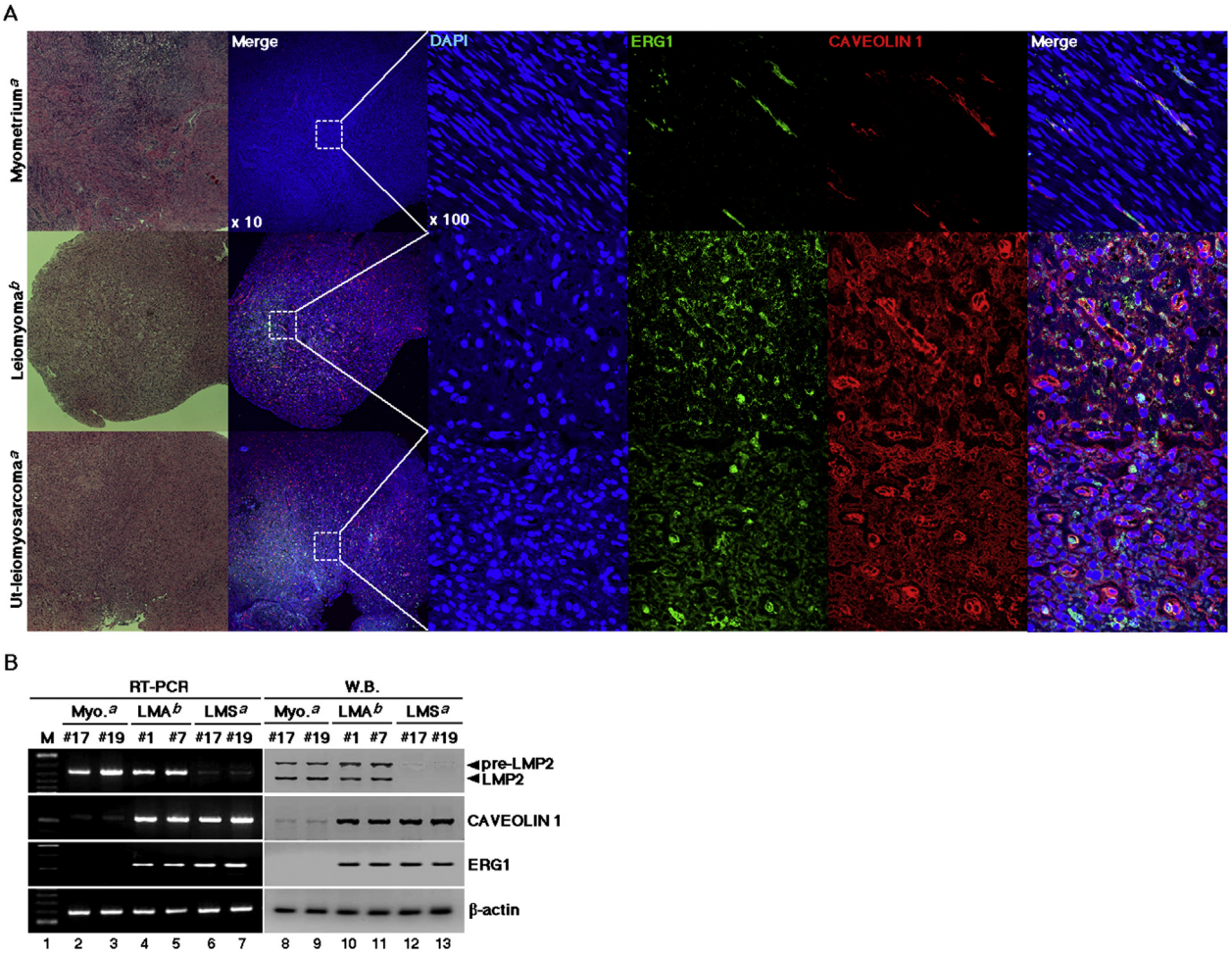

Fig. 2. Expression of CAVEOLIN 1 and ERG1 in human uterine leiomyoma (LMA), leiomyosarcoma (LMS), and normal myometrium tissues. (A) Immunohistochemistry (IHC) experiments individually performed at several medical facilities revealed that the ability to induce the expression of CAVEOLIN 1 and ERG1 was markedly lower in human normal myometrium tissues than in human leiomyoma located in the same tissue section as well as in human uterine leiomyosarcoma tissues. The perivascular expression of CAVEOLIN 1 and ERG1 was similar between normal myometria and all types of tumors. The IHC of CAVEOLIN 1 and ERG1 in normal myometrium (patient #15[a]), uterine leiomyoma (LMA, patient UL6[b]), and uterine LMS (patient #15[a]) tissues were each taken from the same sample. In all samples, 5-μm-thick sections of tissue were stained with the anti-hCAVEOLIN 1 antibody and anti-hERG1 antibody and revealed by Alexa Fluor 488 conjugated anti-mouse IgG or the Alexa Fluor 546 anti-rabbit IgG antibody and DAPI (Vector Laboratories, Inc.) (magnification ×10 and ×100). (B) Examinations of mRNA expression for *LMP2*, *CAVEOLIN 1*, *ERG1*, and *β-actin* in normal myometrium (Myo.), uterine LMA, and uterine LMS were performed by a reverse transcription-polymerase chain reaction (RT-PCR) with the appropriate primers indicated in the Materials and Methods section. The DNA products amplified by RT-PCR were loaded onto agarose gels. Cytosolic extracts were prepared from normal human myometrium (patient #17 and #19[a]), uterine leiomyoma (LMA, patient UL1, UL7[b]), and uterine LMS (patient #17 and #19[a]) tissues. Extracts of 50 mg were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The levels of LMP2, CAVEOLIN 1, ERG1 and β-actin were examined by a western blotting (W.B.) analysis with appropriate antibodies. [a,b,] The details of patients with LMA or LMS are shown in Supplementary data Tables S2 and S3.

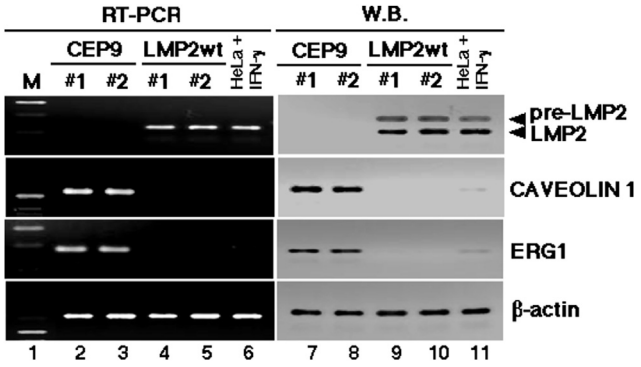

Fig. 3. Biological activity of LMP2 in the human uterine leiomyosarcoma cell line, SKN. (A) RT-PCR experiments revealed LMP2, CAVEOLIN 1, ERG1, and β-actin mRNA expression in SKN-CEP9 clones (SKN-CEP9#1 and SKN-CEP9#2), SKN-LMP2 clones (SKN-LMP2#1 and SKN-LMP2#2), and IFN-γ-treated HeLa cells. Western blotting (W.B.) revealed the expression of precursor-LMP2 (pre-LMP2), LMP2, CAVEOLIN 1, ERG1, and β-actin in SKN-transfectant clones, and IFN-γ-treated HeLa cells. The details of SKN-transfectant clones are shown in Supplementary data Table S1.

suggested to serve as tumor promoters by these signaling pathways [3,11–13,18–20].

The pathogenesis of Ut-LMS and leiomyoma should differ; however, we detected the expression of CAV1 in most LMAs, a limited number of STUMPs, and in most LMSs. Although LMSs became more aggressive and invasive, no significant changes were observed in the expression of CAV1 or perivascular CAV1 (Fig. 1 and Table 1). A malignant tumor is a multistep genetic and epigenetic disease that results from the progressive accumulation of mutations that either inactivate tumor suppressor genes and senescence genes or activate dominant proto-oncogenes [21]. Therefore, mice with a homozygous deficiency for *Cav1* do not present a higher or lower incidence of malignant tumors than that of wild type mice [22]. The role played by CAV1 in malignant tumors and progression has not been fully clarified. Although a fundamental mechanistic understanding to explain a fully CAV1-induced malignant phenotype is currently lacking, it is well documented that CAV1 cooperates with other proteins to promote malignant tumor dissemination by affecting the critical functions of cellular maintenance and homeostasis. CAV1 reportedly increases anchorage independence, invasion, and migration, and, as such, may increase metastatic potential in sarcoma.

We have been conducting a whole genome territory netting epigenetics analysis using each organization of normal myometrial, LMA and Ut-LMS. After the information obtained from the DNA methylation analysis was considered in a cluster analytical manner, the DNA methyl of normal myometrial, LMA and Ut-LMS was independently classified into a different cluster. CAV1 may interact with molecules/receptors, which specifically regulate the initial steps of cell transformation as well as their metastatic potential in Ut-LMS. It should be noted that not all the data reported below have been independently repeated in multiple model systems; therefore, not all have the same significance. However, certain cancer cells that express CAVs are more aggressive and metastatic because of the potential for anchorage-independent growth. Recent studies suggested that stromal and tumoral CAV1 may play an important role in promoting tumor progression and metastasis [23]. In other words, stromal CAV1 expression in association with the strong tumoral expression of CAV1 is closely related with poor outcomes in different malignancies [1,3,11–13,18–25]. Mice lacking *Caveolins* also have impaired angiogenic responses as well as abnormal responses to vasoconstrictive stimuli [26,27]. CAVs are thought to be crucially involved in the development of the tumoral microvasculature [28].

Evidence to suggest that more advanced or metastatic tumors have CAV1 activation is increasing. CAV1 is commonly up-regulated in several advanced epithelial tumors including prostate, kidney, breast and bladder carcinomas and brain tumors. İn the present study, we could not evaluate the expression of CAV1 in peritumoral uterine stroma, and perivascular CAV1 expression within tumors was not associated with the malignancy of uterine mesenchymal tumors. The role played by CAV1 in malignant mesenchymal tumor growth and progression has not yet been fully clarified. Further experiments are required with gene-modified mice and human clinical materials in order to determine whether CAV1 expression is associated with the malignancy of uterine mesenchymal tumors, especially Ut-LMS. Although CAV1 expression has been extensively studied in several carcinomas, there is little or no data on the expression and significance of CAV1 in uterine mesenchymal tumors [12,13]. Recent studies demonstrated that CAV1 was often expressed in LMAs, but was absent in normal myometrial cells [13]. We herein demonstrated that the expression status of tumoral and perivascular CAV1 was not significantly changed by tumor aggressiveness.

In conclusion, the results of the present study revealed that the altered expression of the CAV1 protein in uterine mesenchymal tumors may be a component for tumor dedifferentiation. Although our results need to be confirmed in larger series, they still suggest that CAV1 plays an important role during the development of uterine mesenchymal tumors, but may not mediate tumoral malignancy. We previously demonstrated the abnormal expression of ovarian steroid receptors; however, TP53 and ki-67 and mutations in TP53 were frequently associated with human Ut-LMS, and defective LMP2/β1i and CALPONIN h1 expression and strong CYCLIN B1 expression appeared to be more characteristic of human Ut-LMS than these factors [7,29–31] (Supplementary data Tables S2 to S4). Taken together, our results suggest that CAV1 does not act as a targeted molecule for a biomarker like previously examined molecular candidates against uterine malignant mesenchymal tumors, *i.e.* human Ut-LMS.

5. Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.046.

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