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The Identification of Somatic Mutations in Interferon-g Signal Molecules in Human Uterine Leiomyosarcoma

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Abstract

Human uterine leiomyosarcoma (LMS) is neoplastic malignancy that typically arises in tissues of mesenchymal origin. The identification of novel molecular mechanism leading to human uterine LMS formation and the establishment of new therapies has been hampered by several critical points. We earlier reported that mice with a homozygous deficiency for proteasome *beta* subunit 9 (PSMB9)/b1i, an interferon (IFN)-g inducible factor, spontaneously develop uterine LMS. The use of research findings of the experiment with mouse model has been successful in increasing our knowledge and understanding of how alterations, in relevant oncogenic, tumour suppressive, and signaling pathways directly impact sarcomagenesis. The IFN-g pathway is important for control of tumour growth and invasion and, has been implicated in several malignant tumours. In this study, experiments with human tissues revealed a defective PSMB9/b1i expression in human uterine LMS that was traced to the IFN-g pathway and the specific effect of somatic mutations of Janus kinase (JAK1) molecule or promoter region on the transcriptional activation of *PSMB9/b1i* gene. Understanding the molecular mechanisms of human uterine LMS may lead to identification of new diagnostic candidates or therapeutic targets in human uterine LMS.

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Introduction

Uterine mesenchymal tumours have been traditionally divided into benign tumour leiomyomas (LMA) and malignant tumour leiomyosarcomas (LMS) based on cytological atypia, mitotic activity and other criteria. Uterine LMS, which are some of the most common neoplasms of the female genital tract, are relatively rare uterine mesenchymal tumour, having an estimated annual incidence of 0.64 per 100,000 women (1). They account for approximately one-third of uterine sarcomas, of only 53% for tumours confined to the uterus (2,3). Generally, patients with uterine LMS typically present with vaginal bleeding, pain, and a pelvic mass. Gynecological cancer, for instance breast cancer and endometrial carcinomas, are strongly promoted by female hormones, but the rate of hormone receptor expression is reported to be significantly less in human uterine LMS compared with normal myometrium. These low receptor expressions were found to not correlate with the promotion of initial disease development or with the overall survival of patients with uterine LMS.

As uterine LMS is resistant to chemotherapy and radiotherapy, and thus surgical intervention is virtually the only means of treatment for this disease (4,5,6), however, molecular targeting therapies against tumours have recently shown remarkable achievements (7,8). It is noteworthy that, when adjusting for stage and mitotic count, uterine LMS has a significantly worse prognosis than carcinosarcoma (9); developing an efficient

adjuvant therapy is expected to improve the prognosis of the disease. A trend towards prolonged disease-free survival is seen in patients with matrix metalloproteinase (MMP)-2-negative tumours (10). Although typical presentations with hypercalcemia or eosinophilia have been reported, this clinical abnormality is not an initial risk factor for uterine LMS. To the best of our knowledge, little is known regarding the biology of uterine LMS; therefore, the risk factors that promote the initial development of uterine LMS and regulate their growth *in vivo* remain poorly understood.

The mice with a targeted disruption of proteasome *beta* subunit 9 (PSMB9)/b1i, which is interferon (IFN)-g -inducible proteasome subunit, exhibited a defect in tissue- and substrate- dependent proteasome function, and female PSMB9-deficient mice shown to develop uterine LMS, with a disease prevalence of 37% by 14 months of age (11,12). Defective PSMB9/b1i expression is likely to be one of the risk factors for the development of human uterine LMS, as it is in PSMB9/b1i-deficient mice (12). Recent report shows that stable PSMB9/b1i expression contributes to cell proliferation, which directly correlates to the progressive deterioration with increasing stage and grade of the tumour. As the importance and involvement of the IFN-g signal pathway in the transcriptional regulation of the transporter associated with antigen processing (TAP1) and PSMB9/b1i promoter have been established, it is demonstrated that the defective PSMB9/b1i expression was attributable to G871E somatic mutation in the ATP-binding region of JAK1 molecule in SKN cell line, which is established from patient with uterine LMS. It is

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furthermore likely that the expressions of PSMB9/b1i are down-regulated in human uterine LMS tissues such like human uterine LMS cell line. We demonstrate that there are serious mutational defects in the factors on the IFN-γ signal pathway, which is the key cell-signaling pathway for PSMB9/b1i expression and promoter region of *PSMB9/b1i* gene, in human uterine LMS tissues. The somatic mutational defects in the IFN-γ signal molecules may induce the initial development of human uterine LMS. Recent advances in our understanding of the biological characters of uterine LMS have concentrated on the impaired IFN-γ signal pathway. It is clear that somatic mutations in key regulatory factors (tumour suppressors and proto-oncogenes) alter the behavior of cells and can potentially lead to the unregulated growth seen in malignant tumour. Therefore, continued improvement of our knowledge of the molecular biology of uterine LMS may ultimately lead to novel therapies and improved outcome.

Result and Discussion

Defective PSMB9/b1i Expression of Human Uterine LMS

The effects of IFN-γ on expression of PSMB9/b1i was examined using five cell lines (13). The expression of PSMB9/b1i were not markedly induced by IFN-γ treatment in human uterine LMS cell lines, although cervical epithelial adenocarcinoma cell lines and normal human uterus smooth muscle cells underwent strong induction of PSMB9/b1i following IFN-γ treatment (13). Furthermore, the immunohistochemistry (IHC) experiments revealed a serious loss in the ability to induce expression of PSMB9/b1i in human uterine LMS tissues in comparison with normal myometrium tissues located in same tissue sections and other 4 mesenchymal tumour types (Figure 1). Of 58 uterine LMS, 50 cases (86.2%) were negative for PSMB9/b1i, 4 cases (6.9%) were focally positive, 2 cases (3.4%) were weakly positive, and 2 cases (3.4%) were positive (Figure 1B). IHC analyses showed positivity for Ki-67/MIB1 and differential expression of estrogen receptor (ER), progesterone receptor (PR), tumour protein (TP53), and CALPONIN h1 (Supplementary Table 1). In addition, PSMB9/b1i expression level was also examined in the skeletal muscle metastasis from uterine LMS, the histological diagnosis was consistent with metastatic LMS for skeletal muscle lesions. Pathological examination of surgical samples showed presence of a mass measuring 3 cm at largest diameter in lumbar quadrate muscle without a fibrous capsule. All lymphnodes were negative. In western blotting experiments and RT-PCR experiments, PSMB9/b1i was expressed in normal myometrium, LMA, and IFN-g-treated HeLa cells, but not in human uterine LMS (Figure 1C). The both research experiments strongly supported the research findings obtained from IHC experiments.

Somatic Mutations in IFN-γ Signal Pathway in Human Uterine LMS Tissues

IFN-γ treatment markedly increased the expression of PSMB9/b1i, a subunit of the immunoproteasome, which alters the proteolytic specificity of proteasomes. After binding of IFN-γ to the type II IFN receptor, which is constructed by two components, IFN-γ receptor subunit 1 (IFNGR1) and IFN-γ receptor subunit 2 (IFNGR2), Janus-activated kinase 1 (JAK1) and JAK2 are activated and phosphorylate the signal transducer and activator of transcription 1(STAT1) on the tyrosine residue at position 701 (Tyr701) and the serine residue at position 727 (Ser727) (14,15) (Supplementary Figure 1). Tyrosine phosphorylated STAT1 forms homodimers that translocate to the nucleus and bind GAS (IFN-γ-activated site) elements in the promoters of IFN-γ-regulated genes (14,15) (Supplementary Figure 1). The phosphorylation of Ser727 is not essential for the translocation of STAT1 to the nucleus or for the binding of STAT1 to enhancer/promoter region of targeted DNA, but it is required for full transcriptional activation (16,17) (Supplementary Figure 1).

The defect was localized to JAK1 activation, which acts upstream in the IFN-γ signal pathway since IFN-γ treatment could not strongly induce JAK1 kinase activity in human uterine LMS cell lines. Sequence analysis demonstrated that the loss of IFN-γ responsiveness in the human uterine LMS cell line was attributable to the inadequate kinase activity of JAK1 due to a G781E somatic mutation in the ATP-binding region (13). Genetic alterations in tyrosine kinases have previously been firmly implicated in tumourigenesis, but only a few serine/threonine kinases are known to be mutated in human cancers (18,19,20,21,22). For instance, mice carrying homozygous deletion of *Pten* alleles developed wide spread smooth muscle cell hyperplasia and abdominal leiomyosarcomas (21), and JUN proto-oncogene amplification and over-expression block adipocytic differentiation in highly aggressive sarcomas (22).

Most frequently, LMS have appeared in the uterus, retroperitoneum or extremities, and although histologically indistinguishable, they have different clinical courses and chemotherapeutic responses. The molecular basis for these differences remains unclear. Therefore, the examination of human uterine LMS tissues (23 LMS tissue sections and normal tissue sections located in the same tissue) was performed to detect somatic mutations in the IFN-γ signaling molecules cascade, JAK1, JAK2, STAT1 and promoter region of *PSMB9/b1i* gene. As the catalytic domains of these molecules are most likely to harbour mutations that activate the gene product, we focused on stretches (exons) containing the kinase domains, transcriptional activation domains and enhancer/promoter region. Over all, nearly 43.5% (10/23) of uterine LMS tissues had serious mutations in the ATP binding region or kinase-specific active site of JAK1; furthermore, 43.5% (10/23) of uterine LMS tissues had serious mutations in essential sites of the promoter region of *PSMB9/b1i* gene, which is required for transcriptional activation of *PSMB9/b1i* gene (Table 1, 2). No somatic mutation in essential sites, Tyr701 and Ser727, which are required for transcriptional activation of STAT1, was elucidated in human uterine LMS. Nearly 21.7% (5/23) of human uterine LMS tissues unexpectedly had mutations in the STAT1 intermolecular region, which is not yet reported to be important for biological function as transcriptional activation. No somatic mutation in the ATP-binding region and kinase-active site of JAK2 was detected in human uterine LMS (Table 1, 2). MOTIF Search profiling (23) and NCBI's Conserved Domain Database and Search Service, v2.17 analysis also revealed that somatic mutations, which were identified in the catalytic domains of these molecules, resulted in impaired activations of tyrosine kinases or transcriptional factor (24).

In a recent report, a comparative genomic hybridization (CGH)-based analysis of human LMS using a high resolution genome-wide array gave gene-level information about the amplified and deleted regions that may play a role in the development and progression of human uterine LMS. Other reports showed that among the most intriguing changes in genes were losses of JAK1 (1p31-p32) and PSMB9/b1i (6p21.3) (25,26). It has also been demonstrated that a correlation exists between the development of malignant tumours and ethnic background, so we conducted CGH experiments with tissue samples obtained from Japanese patients in order to obtain gene-level information. Our results showed that human uterine LMS having a clear functional loss at JAK1 (1p31-p32) and PSMB9/b1i (6p21.3) also harbored one nonsense mutation and one deletion, suggesting a possible homozygous loss of function. The discovery of these mutational defects in a key cell-signaling pathway may be important in understanding the pathogenesis of human uterine LMS.

Gene Analyses of Human Uterine Lyomyosarcoma

Uterine LMS are relatively rare mesenchymal tumours, having an estimated annual incidence of 0.64 per 100 000 women. They account for approximately

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Mutations in JAK1 kinase, PSMB9 promoter region, and STAT1 in human uterine leiomyosarcoma

Patient #	JAK1 kinase	PSMB9 promoter region	STAT1(701Y,727S)[5]	JAK2 kinase	PSMB9[7]
#1	wt	wt	wt	wt	Neg.
#2	wt	A210G, C214T(IRF-E)[3]	wt	wt	P.Posi
#3	Q986P(active) R995S(active)[1]	C214T, G219A(IRF-E)	(S710A)[6]	wt	Neg.
#4	G876R(ATP)[2]	wt	wt	wt	Neg.
#5	C881F (ATP)	wt	wt	wt	P.Posi.
#6	wt	wt	wt	wt	Neg.
#7	wt	A216G(IRF-E)	(L693R)[6]	wt	Neg.
#8	wt	wt	wt	wt	F.Posi.
#9	Y987S(active)	wt	wt	wt	Neg.
#10	wt	A217G(IRF-E)	(R716S)[6]	wt	Neg.
#11	wt	wt	(I702L)[6]	wt	Neg.
#12	wt	wt	wt	wt	F.Posi.
#13	Y987S(active)	A216G(IRF-E)	wt	wt	Neg.
#14	wt	wt	wt	wt	Neg.
#15	G871E(ATP)	wt	(I702L)[6]	wt	Neg.
#16	wt	G239A(HSF)[4]	wt	wt	Neg.
#17	C881F(ATP)	wt	wt	wt	Neg.
#18	wt	wt	wt	wt	Neg.
#19	wt	wt	wt	wt	Neg.
#20	G873D(ATP)	A210G(IRF-E)	wt	wt	Neg.
#21	C881Stop(TGC-TGA)	G209T(IRF-E)	wt	wt	Neg.
#22	Q986P(active)	G215A(IRF-E)	wt	wt	F.Posi.
#23	wt	C213A(IRF-E)	wt	wt	Neg.

[1] Kinase activation site of JAK1
[2] ATP binding region of JAK1
[3] Interferon-γ regulatory factor-enhanced site
[4] Heat Shock Factor binding site
[5] Tyr701 or Ser727 phosphorylation of STAT1
[6] Mutation is not located in major functional regions of STAT1
[7] The results of LMP2 expression are immunohistochemical, Neg. = Negative, P.Posi. = Partial Positive, F.Posi. = Focally Positive

Table 1: Identification of somatic (tumour-specific) mutations in the cataclytic domains of JAK1, JAK2 kinases, STAT1 or activation region of the promoter region of PSMB9/b1i gene in human uterine leiomyosarcoma (total 23 cases). The gemonic DNA was extracted from the human uterine leiomyosarcoma tissues and normal uterine smooth muscle tissues using the protocol indicated in the materials and methods section of this manuscript. The restricted DNA fragments for the direct sequence analysis were amplified by PCR procedure with the appropriate primers for the ATP-binding region and kinase activation domain of the JAK1 molecule, the promoter region of PSMB9/b1i gene, the Tyr701 and Ser727 aminoacid of STAT1 molecule, and the ATP-binding region and kinase activation domain of the JAK2 molecule. Information of human uterine leiomyosarcomas and the primer sets was indicated in Supplementary Table 1 and the materials and methods section of Supplementary material.

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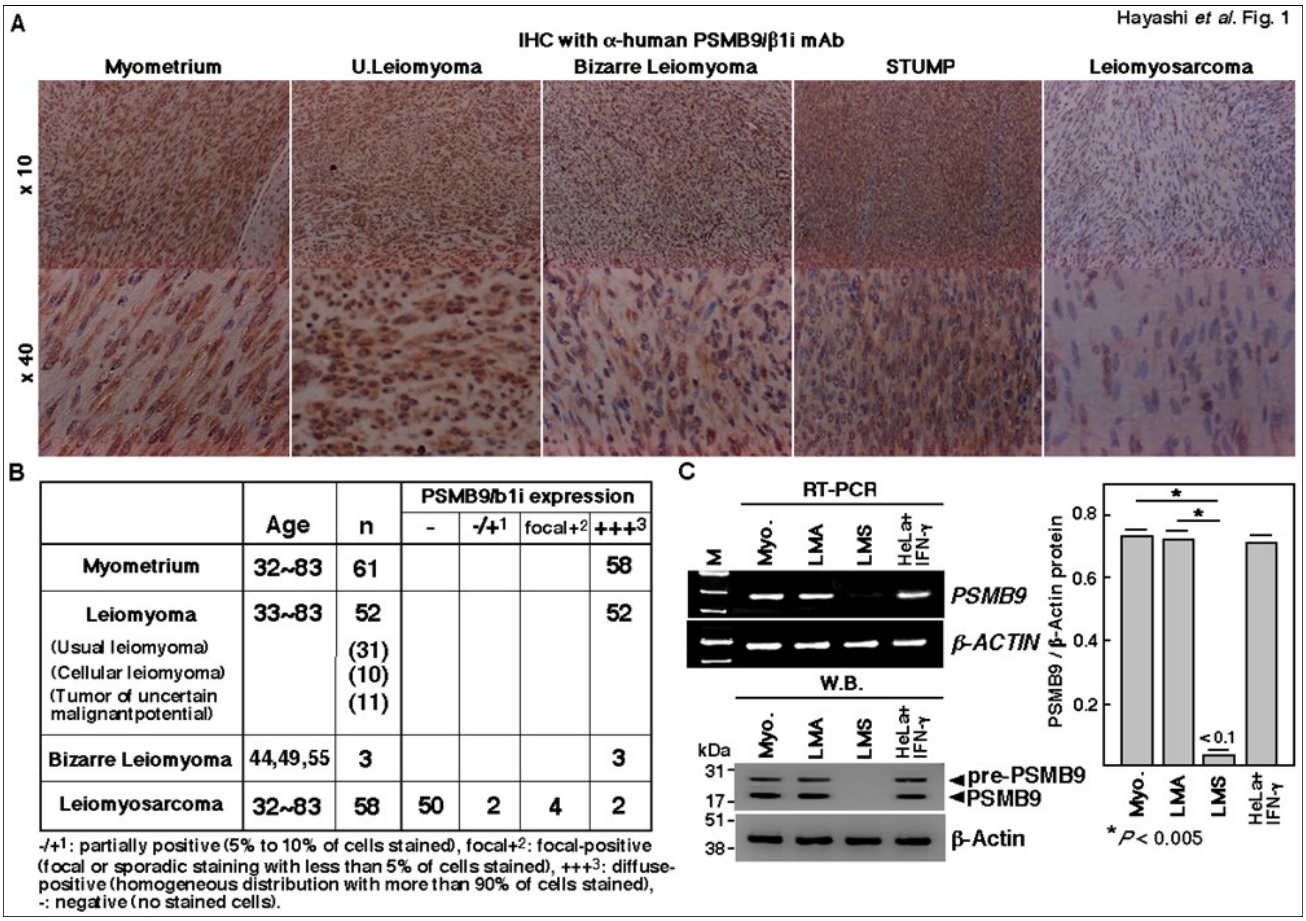
Mutations in the IFN-γ pathway in human uterine leiomyosarcoma

Gene Name	Locus	GenBank Accession	MIM ID	Tumor	Nucleotide	Amino Acid	Domain	Evolutionary[4] conservation
JAK1	HUMPTKJAK1	M64174.1	*147795	ULMS	G2612A G2618A G2626A G2642T G2643A A2957C A2960C A2985T	G781E G873D G876R C881F C881Stop Q986P Y987S R995S	ATP binging ATP binding ATP binging ATP binging ATP binding active site active site active site	p,c,m,r,g,d
JAK2	AF005216	AF005216.1	+147796	ULMS	ND[2]	ND	ND	p,c,b,m,r,g,d
STAT1	NM_007315	NM_007315	+600555	ULMS	A2104C T2128G T2078G A2148C	I702L S710A L693R R716S	NA[3] NA NA NA	c,b,m,r,g,d
PSMB9[1]	X62741	X62741.1	*177045	ULMS	A209T A210G C213A C214T G215A A216G A217G G219A G239A		IRF-E site IRF-E site IRF-E site IRF-E site IRF-E site IRF-E site IRF-E site IRF-E site HSF site	p,c,b,m,r,d

[1]LMP2 promoter region, NCBI Reference Sequence NT_007592.15 Homo sapiens Chromosome 6 [2]not detected [3]non-kinase activation region [4]Evolutionary conservation refers to the species in which an identical residue was observed in the homolog (p, pan troglodytes; c, canis lupus familiaris; b, bos taurus; m, mus musculus; r, rattus norvegicus; g, gallus gallus; d, danio rerio)

Table 2: Somatic mutations in IFN-g signaling pathway in human uterine leiomyosarcoma. The data of somatic mutations in table 1 was shown separately with respect to each gene, JAK1, JAK2, STAT1 and activation region of the promoter of PSMB9/b1i gene.



	Age	n	PSMB9/b1i expression			
			-	-/+[1]	focal+[2]	+++[3]
Myometrium	32~83	61				58
Leiomyoma	33~83	52				52
(Usual leiomyoma)		(31)				
(Cellular leiomyoma)		(10)				
(Tumor of uncertain malignantpotential)		(11)				
Bizarre Leiomyoma	44,49,55	3				3
Leiomyosarcoma	32~83	58	50	2	4	2

-/+[1]: partially positive (5% to 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), -: negative (no stained cells).

Figure 1: Differential expression of PSMB9/b1i expression in human normal myometrium and several mesenchymal tumour types. (A) Immunohistchemistry of PSMB9/b1i in normal myometrium, usual leiomyoma, Bizarre leiomyoma, smooth muscle tumour of uncertain malignant potential (STUMP) and uterine leiomyosarcoma tissues located in same tissue. For all samples, 5-mm sections of tissues specimens were stained with anti- PSMB9/b1i antibody revealed by peroxidase-cojugated anti-rabbit IgG antibody. (B) IHC experiments individually performed at several medical facilities revealed a marked loss in the ability to induce PSMB9/b1i expression in human uterine LMS tissues compared to that in normal human myometrium located in the same tissue section, as well as to that in LMA tissues. Normal total: 58 cases, LMA total: 50 cases, Bizarre Leiomyoma total: 3 cases, LMS total: 56 cases. The experiments were performed three times with similar results. (C) Examinations of mRNA expression for *PSMB9/b1i* and *b-ACTIN* in normal human myometrium (Myo.), uterine usual leiomyoma (LMA), uterine leiomyosarcoma (LMS) and IFN-g-treated HeLa cells by reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed with the appropriate primers indicated in the materials and methods section of this manuscript. The DNA products amplified by RT-PCR were loaded in agarose gel. The expression levels of PSMB9/b1i and b-ACTIN were examined by western blotting with appropriate antibodies. The cytosolic extracts were prepared from normal human myometrium, uterine usual leiomyoma (LMA) and uterine leiomyosarcoma (LMS), and IFN-g-treated HeLa cells, and 50 mg of cytosolic extracts were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). PSMB9/b1i levels were significantly decreased in human uterine LMS compared with normal human myometrium and human usual LMA. Student's *t*-test. $*P < 0.005$. (n = 8 per group).

one-third of uterine sarcomas and 1.3% of all uterine malignancies. They are the disease with extremely poor prognosis, considering aggressive malignancies with a 5-year survival rate of only 50% for tumours confined to the uterus. At present, surgical intervention is virtually the only means of treatment for uterine LMS (4,5,6,7,8). Although adjuvant pelvic irradiation appears to decrease the rate of local recurrence, adjuvant therapy does not appear to significantly improve survival. Furthermore, gynaecological cancer, for instance breast cancer and endometrial carcinomas, are strongly promoted by female hormones, but the rate of estrogen receptor and progesterone receptor expression is reported to be significantly less in human uterine LMS compared with normal myometrium. These low receptor expressions were found to not correlate with the promotion of initial disease development or with the overall survival of patients with human uterine LMS; however, molecular targeting therapies against tumours have recently shown remarkable achievements (27). To improve the prognosis of human uterine LMS, research experiments were performed to identify the key role of pro- or anti-oncogenic factors that have an important function in their pathogenesis and that could serve as molecular targets for tumour treatment. For this purpose, several research facilities conducted a microarray procedure between human uterine LMS and normal myometrium and showed that several known pro-oncogenic factors, such as brain-specific polypeptide PEP-19 and a transmembrane tyrosine kinase receptor, c-kit, may be associated with the pathogenesis of human uterine LMS (28,29,30). However, in terms of the tumourigenesis of human uterine LMS, merely comparing the expression of potential pro-oncogenic factors between normal and malignant tissues is not sufficient because the results obtained may be the consequence of malignant transformation and, therefore, not necessarily the cause. In addition, dysregulation of apoptotic mechanisms has also been implicated in many human malignancies. Although the significant differential expression of apoptotic and cell cycle regulatory factors in human uterine LMS, such as B-cell Lymphoma-2 (BCL-2), BCL-2-Associated X protein (BAX), P16 Inhibts CDK4 (P16/INK4a), P21 Cyclin-Dependent Kinase Inhibitor 1 (P21/CIP1), P27 Kinase Inhibitor Protein 1 (P27/KIP1), Cellular v-KIT Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (c-KIT), Mitogen-Inducible Gene-2 (MIG-2), MDM2, Tumour Protein 53 (TP53), have all been reported and compared to normal myometrium, there exists no scientific evidence to show that abnormal expression of these factors directly correlates to the initiation and promotion of human uterine LMS. PSMB9/b1i-deficient mice were reported to be prone to the development of uterine LMS, but not in their parental mice, C57BL/6 mice (12). The percentage of mice with overt tumours increased with age after six months, with a cumulative prevalence of disease in female mice of 37% by 14 months of age and no apparent plateau at this late observation time. Histopathological examinations of PSMB9/b1i-deficient uterine neoplasms revealed common characteristic abnormalities of uterine LMS. In addition, recent research reports show the loss in the IFN-g-inducible ability of PSMB9/b1i expressions in SKN cell line and other primary human uterine LMS cells established from patients. The histopathological experiments demonstrated a serious loss in the ability to induce the expression of PSMB9/b1i in human uterine LMS tissues in comparison with normal myometrium tissues located in same tissue sections (Fig. 1).

IFN-g treatment markedly increased the expression of PSMB9/b1i, a subunit of the proteasome, which alters the proteolytic specificity of proteasomes. Sequence analysis demonstrated that the loss of IFN- g responsiveness in the human uterine LMS cell line was attributable to the inadequate kinase activity of JAK1 due to a G781E somatic mutation in the ATP-binding region (13). The defect was localized to JAK1 activation, which acts upstream in the IFN-g signal pathway since IFN-g treatment could not strongly induce JAK1 kinase

activity in human uterine LMS cell lines. Genetic alterations in tyrosine kinases have previously been firmly implicated in tumourigenesis, but only a few serine/threonine kinases are known to be mutated in human cancers (31,32,33,34). For instance, mice carring homozygous deletion of *Phosphatase and Tensin Homolog Deleted from Chromosome 10 (Pten)* alleles developed widespread smooth muscle cell hyperplasia and abdominal LMS (35), and JUN oncogene amplification and over-expression block adipocytic differentiation in highly aggressive sarcomas (22). Most frequently, LMS have appeared in the uterus, retroperitoneum or extremities, and although histologically indistinguishable, they have different clinical courses and chemotherapeutic responses. The molecular basis for these differences remains unclear, therefore, the examination of human uterine LMS tissues (23 LMS tissue sections and normal tissue sections located in the same tissue) was performed to detect somatic mutations in the IFN-g signal molecules. In a recent report, high-resolution genomewide array comparative genomic hybrodization (CGH) analysis of LMS cases gave gene-level information about the amplified and deleted regions that may play a role in the development and progression of human uterine LMS. Among the most intriguing genes, whose copy number sequence was revealed by CGH, were loss of JAK1 (1p31-p32) and PSMB9/b1i (6p21.3) (25,26). The discovery of these mutational defects in a key cell-signaling pathway may be an important development in the pathogenesis of human uterine LMS.

The growth of JAK1-deficient cell lines reportedly is unaffected; similarly, the cell cycle distribution pattern of freshly explanted tumour cells derived from JAK1-deficient tumours shows no response to IFN-g signaling (36). The growth of the original SKN cells, which had defective JAK1 activity, was unaffected by IFN-g treatment. In contrast, the growth of JAK1-transfected SKN cells, which had strong exogenous JAK1 activity, was prevented by IFN-g treatment. Interestingly, when PSMB9/b1i-transfected SKN cells, which have marked the expression of PSMB9/b1i, were analyzed, expression of exogenous PSMB9/b1i resulted in cell growth inhibition. Conversely, the growth of PSMB9/b1i-transfected SKN cells was unaffected by IFN-g signal pathway. Taken together, IFN-g response to cell growth inhibition may be attributable to the physiological significance of PSMB9/b1i.

The down regulation of major histocompatibility complex (MHC) expression, including the *TAP1* and *PSMB9/b1i* genes, is one of the biological mechanisms tumour cells use to evade host immunosurveillance (37,38,39). Recently, the incidence of IFN-g unresponsiveness in human tumours was examined in several malignant tumours, and revealed that approximately 33% of each group exhibited a reduction in IFN-g sensitivity (40). Nevertheless, the expression of PSMB9/b1i, rather than providing an escape from immune surveillance, seems to play an important role in the negative regulation of human uterine LMS cell growth. Defective expression of PSMB9/b1i is likely to be one of the risk factors for the development of human uterine neoplasm, as it is in the PSMB9/b1i-deficient mouse. Thus, gene therapy with PSMB9/b1i expression vectors may be a new treatment for human uterine LMS that exhibits a defect in the expression of PSMB9/b1i. Because there is no effective therapy for unresectable human uterine LMS, our results may bring us to specific molecular therapies to treat this disease.

Materials and Methods

Tissue Collection

A total of 51 patients aged between 32 and 83 years who were diagnosed with smooth muscle tumours 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 human uterine mesenchymal tumours was performed using established criteria with some modifications (41,42). Briefly, usual leiomyoma (usual LMA) was defined as a tumour 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 MFs per 10 HPFs. Cellular leiomyoma (cellular LMA) was defined as a tumour with significantly increased cellularity (>2000 myoma cells/HPF) and a MI<5, but without cytologic atypia. Bizarre leiomyoma (BL) was defined as a tumour either with diffuse nuclear atypia and a MI<2 or with focal nuclear atypia and a MI<5 without coagulative tumour cell necrosis. A tumour of uncertain malignant potential (UMP) was defined as a tumour with no mild atypia and a MI<10, but with coagulative tumour cell necrosis. Leiomyosarcoma (LMS) was diagnosed in the presence of a MI>10 with either diffuse cytologic atypia, coagulative tumour cell necrosis, or both. Of the 113 uterine mesenchymal tumours, 52 cases were diagnosed as uterine LMA, 3 cases were Bizarre LMA, and 58 cases were uterine LMS. Protein expression studies with cervical epithelium and carcinoma tissues were performed using tissue arrays (Uterus cancer tissues, AccuMax Array, Seoul, Korea). Details regarding tissue sections are indicated in the manufacturer's literature (AccuMax Array).

Immunohitochemistry (IHC)

Immunohistochemical staining for PSMB9/b1i, Estrogen Receptor (ER), Progesterone Receptor (PR), TP53, and Ki-67/MIB1 was performed on the serial human uterine LMS sections. Antibodies for ER(ER1D5), PR(PR10A), TP53(DO-1), and Ki-67(MIB-1) were purchased from Immunotech (Marseille, France). Anti-human PSMB9 antibody was produced by SIGMA-Aldrich collaboration Laboratory (SIGMA-Aldrich, Japan Science and Technology Agency (JST) and Shinshu University). IHC was performed using the avidin-biotin complex method previously described. Briefly, one representative 5-mm tissue section was cut from a paraffin-embedded sample of the radical hysterectomy specimen from patients with uterine LMS. Sections were deparaffinized and rehydrated in graded alcohols and then incubated with normal mouse serum for 20 min. Sections were incubated at room temperature for 1 h with primary antibody. Afterwards, sections were incubated with a biotinylated secondary antibody (Dako, Carpinteria, CA, USA) and then exposed to a streptavidin complex (Dako). Complete reaction was revealed by 3, 3¢-diaminobenzidine, and the slide was counterstained with hematoxylin. Normal USM portions in the specimens were used as positive controls. Negative controls consisted of tissue sections also incubated with normal rabbit IgG instead of the primary antibody. These studies are registered, at Shinshu University in accordance with local guidelines (approval no. M192)

Reverse Transcription-Polymerase Chain Reaction Analysis (RT-PCR)

The expressions *PSMB9/b1i* and *b-ACTIN* transcripts were examined using RT-PCR. Total RNA was prepared from human uetine LMS tissues and normal myometrim tissues using TRIzol reagent according to the manufacturer's protocol (Invitrogen Co., CA). The RNA was reverse-transcribed with Superscript II enzyme (Invitrogen), the single strand cDNA was used to amplify. *PSMB9/b1i* and *b-ACTIN* transcripts using PCR analysis with the appropriate primer sets following a program of 35 cycles of 94°C for 30s, 60°C for 30s and 72°C for 1.5min with additional 5min for extention of transcripts (13,43,44). The primers used for PCR are as follows: *PSMB9/b1i*; 5'-GGGATAGA ACTGGAGGAACC-3' and 5'-AGATGACACCCCCGCTTGAG-3', *b2-MICROGLOBULIN*; 5'-T GAAGCTGACAGCATTCG-3' and 5'-TGCGGCATCTTCAAACCTCC-3', *b-ACTIN*; 5'-TCCGG AGACGGGGTCA-3' and 5'-CCTGCTTGCTGATCCA-3'. These studies are registered, at Shinshu University in accordance with local guidelines (approval no. 4737, no.

150 and no. M192)

Western Blotting

Equal amounts of proteins (20 mg) were size fractionated on 7.5% SDS–polyacrylamide gel electrophoresis and transferred onto a poly-vinylidene difluoride membrane (PVDF). The blots were allowed to air dry and then placed in blocking buffer (1% BSA in 10 mM Tris buffer with 100 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature. The blots were then incubated with specific primary antibodies for 1 h at room temperature. All the primary antibodies were mouse monoclonal or rabbit polyclonal, obtained from several industries, and were used at different final dilutions (1:1000~1:500) in the blocking buffer. These antibodies were raised using the following proteins as immunogens: PSMB9/b1i (23.4 kDa protein), PSMB8/b5i (30.3 kDa protein), b-ACTIN (41.7 kDa protein). The blots were washed three times for 30 min each with wash buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH 7.5) and then incubated with alkaliphosphatase conjugated goat-anti-mouse IgG antibody or anti-rabbit IgG antibody (Promega, Madison, WI) diluted in 5% non -fat milk in wash buffer. The PVDF membranes were washed with wash buffer three times for 30 min, and Western Blue Stabilized Substrate (Promega Co. Madison, WI) was added and incubated as previously reported (45,46).

Sequencing of the Catalytic Domains of JAK1, STAT1, JAK2, and *PSMB9/b1i* Promoter Region

To demonstrate whether the somatic mutations in the ATP-binding region and kinase activation domain of the JAK1 molecule, promoter region of *PSMB9/b1i* gene, the Tyr701 and Ser727 aminoacid of STAT1 molecule, and the ATP-binding region and kinase activation domain of the JAK2 molecule playing in human uterine LMS were identified, the isolating of gemonic DNA and direct sequencing was carried out. The gemonic DNA was extracted from the human uterine LMS tissues and normal USM tissues using the standard protocol. The gemonic DNA was subjected to PCR procedure. The restricted DNA fragments for the direct sequence analysis were amplified by PCR according to the published sequencing oligonuclrotide primers. Polymerase chain reaction products were directly sequenced using DYEnamic Terminator Cycle sequencing Kit (Amersham-Biosciences, Piscataway, NJ, USA) by ABI Prism 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The sequences of mutant JAK1, STAT1, and promoter region of *PSMB9/b1i* gene derived from the individual human uterine LMS tissue sections were registered at DDBJ (Accession: AB219242, DJ055380, DJ055379, DJ055378, DJ055377, DJ055376). Details of direct sequencing are indicated in Supplementary material.

Acknowledgments

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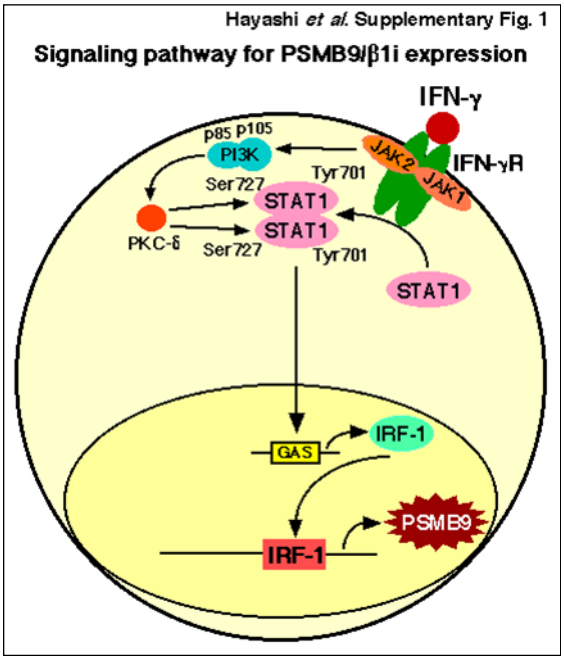
Supplementary Material

Sequencing of the Catalytic Domains of the JAK1, STAT1, JAK2 genes and the promoter region of PSMB9/b1i gene. To determine whether somatic mutations exist in the ATP-binding region or kinase activation domain of JAK 1 and JAK2, in the promoter region of PSMB9/b1i gene at Tyr701 or Ser727 of STAT1, or in the ATP-binding region and kinase activation domain of JAK2 in human uterine LMS, genomic DNA was isolated and direct sequencing was carried out. Genomic DNA was extracted from



Supplemental Fig. 1 The interferon-g signaling pathway and mutations in its components found in human uterine leiomyosarcoma. After binding of interferon-g (IFN-g) to the type II IFN receptor, Janus activated kinase 1 (JAK1) and JAK2 are activated and phosphorylate signal transducer and activator of transcription 1 (STAT1) on the tyrosine residue at position 701 (Tyr701). The tyrosine-phosphorylated form of STAT1 forms homodimers that translocate to the nucleus and bind GAS (IFN-g-activated site) elements, which are present in the promoters of IFN-γ-regulated genes. The IFN-g-activated JAKs also regulate, through as-yet-unknown intermediates, activation of the catalytic subunit (p110) of phosphatidylinositol 3-kinase (PI3K). The activation of PI3K ultimately results in downstream activation of protein kinase C-δ (PKC-δ), which in turn regulates phosphorylation of STAT1 on the serine residue at position 727 (Ser727). The phosphorylation of Ser727 is not essential for the translocation of STAT1 to the nucleus or for the binding of STAT1 to DNA, but it is required for full transcriptional activation. IFNGR1; IFN-g receptor subunit 1, IFNGR2; IFN-g receptor subunit 2.

Hayashi *et al.* Supplementary Table 1

Expression of ER, PR, Ki-67, p53, PSMB9, Calponin h1 and Senescence staining in human uterine leiomyosarcoma

Patient No.	Age in yrs	TNM stage	MF	Immunohistochemical staining							Somatic mutation					Follow-up (months)	Senescence	
				CCN	ER	PR	Ki-67	TP53	PSMB9	CAL.	JAK1	JAK2	STAT1	PSMB9pro.	p53		β-gal.	PML
1	37	T4N1M0	97	+	−	−	3000	+++	−	−	ND	ND	ND	ND	SM	D(1)	−	−
2	58	T3N0M0	24	+	−	−	3500	+	+/−	−	ND	ND	ND	SM	SM	D(23)	+/−	+/−
3	45	T2N0M0	32	+	+/−	+/−	2150	+++	−	−	SM	ND	SM	SM	SM	D(24)	−	−
4	65	T1N0M0	30	+	+/−	+/−	1700	+++	−	−	SM	ND	ND	ND	SM	D(20)	−	−
5	52	T1N0M0	107	+	−	+	2600	++	+	−	SM	ND	ND	ND	ND	D(13)	+	+/−
6	49	T1N0M0	46	+	−	−	4300	+	−	−	ND	ND	ND	ND	ND	D(24)	−	−
7	55	T1N0M0	75	+	−	−	4000	+++	−	−	ND	ND	SM	SM	ND	D(18)	−	−
8	43	T3N0M0	57	+	+	−	2000	−	+/−	+/−	ND	ND	ND	ND	ND	D(10)	+/−	+/−
9	67	T1N0M0	13	+	−	+/−	1430	−	−	−	SM	ND	ND	ND	ND	A(34)	−	−
10	67	T1N0M0	37	+	−	−	2100	−	−	−	ND	ND	SM	SM	ND	A(15)	−	−
11	51	T1N0M0	93	+	−	−	4500	−	−	−	ND	ND	SM	ND	ND	A(94)	−	−
12	48	T1N0M0	14	+	−	−	900	+++	+	+	ND	ND	ND	ND	ND	A(58)	+/−	+/−
13	51	T1N0M0	22	+	+/−	+	450	+	−	−	SM	ND	ND	SM	ND	A(34)	−	−
14	67	T1N0M0	64	+	−	+	1450	++	−	−	ND	ND	ND	ND	ND	A(15)	−	−
15	52	T1N0M0	65	+	−	−	1780	++	−	−	SM	ND	SM	ND	ND	D(23)	−	−
16	42	T3N0M0	73	+	−	−	2130	++	−	−	ND	ND	ND	SM	ND	A(21)	−	−
17	80	T1N0M0	98	+	−	−	1980	+++	−	−	SM	ND	ND	ND	ND	D(19)	−	−
18	56	T1N0M0	78	+	−	−	1860	++	−	−	ND	ND	ND	ND	ND	A(11)	−	−
19	58	T1N0M0	40	+	−	−	1750	++	−	−	ND	ND	ND	ND	ND	A(10)	−	−
20	65	T2N0M0	67	+	−	−	780	+++	−	−	SM	ND	ND	SM	ND	A(12)	−	−
21	45	T1N0M0	52	+	−	−	1045	++	−	−	SM	ND	ND	SM	ND	A(13)	−	−
22	57	T2N0M0	62	+	−	−	980	++	+/−	−	SM	ND	ND	SM	ND	A(11)	+/−	+/−
23	54	T1N1M0	54	+	−	−	860	+++	−	−	ND	ND	ND	SM	ND	A(02)	−	−

ER; estrogen receptor, PR; progesterone receptor, TP53; tumor protein p53, CAL.; CALPONIN h1 (Ref.31,32), Ki-67; positive cell number/10 high power fields, SM; somatic mutation, ND; not detected, D,;died of disease, A; alive, MF; mitotic figure/10 high power fields, CCN; coagulative cell necrosis, β-gal.; β-galactosidase, PML; α-promyelocytic leukaemia.

Supplemental Table 1 Expression of ER, PR, Ki-67, TP53, PSMB9/b1i, and CALPONIN h1, and somatic mutations of cathartic domains of JAK1, JAK2, STAT1, and the promoter region of *PSMB9/b1i* gene in human uterine leiomyosarcoma. Immunohistochemistry of ER, PR, Ki-67, TP53, PSMB9/b1i, and CALPONIN h1 in normal uterine smooth muscle and uterine leiomyosarcoma tissues located in same tissue section was performed. The isolating of gemonic DNA of uterine leiomyosarcoma tissues and direct sequencing for cathartic domains of JAK1, JAK2, STAT1, and the promoter region of *PSMB9/b1i* gene in human uterine leiomyosarcoma was carried out.

consecutive paraffin-embedded human uterine LMS tissue and normal myometrium tissue sections using the microwave-based DNA extraction method for PCR amplification(1). To avoid contamination of normal myometrium or inflammatory cells, the tumour areas were confirmed using a hematoxylin and eosin-stained glass slide as a template. The tumour tissues were scraped by razor-micro dissection from paraffin-embedded consecutive tissue sections. The genomic DNA was subjected to PCR, and restricted DNA fragments for direct sequencing analysis were amplified using published oligonucleotide primers. PCR products were directly sequenced using a DYEnamic Terminator Cycle Sequencing Kit (Amersham-Biosciences, Piscataway, NJ) with an ABI Prism 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA). The sequences of mutant JAK1, STAT1, and the promoter region of PSMB9/b1i gene derived from individual uterine LMS tissue sections are registered in the DDBJ (Accession: AB219242, DJ055380, DJ055379, DJ055378, DJ055377, DJ055376).

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Primer Sets for Direct Sequence Analysis.

JAK1:(F, 5′-caccaaatctttaaaccggaccccagcctt-3′, R, 5′-tacgatggggcttccctgataacagcacat-3′),
(F, 5′-atggcttt ctgtgctaaaatgaggagctcc-3′, R, 5′-tccatcctgctcggtcttggggtctcgaat-3′),
(F, 5′-attcgagaccccaagaccgagcagga tgga-3′, R, 5′-tccactggattccaagattcccagtcacca-3′),
(F, 5′-tggtgactgggaatcttggaatccagtgga-3′, R, 5′-ggcg gctcatgaggtctcccaagctgggga-3′),
(F, 5′-tccccagcttgggagacctcatgagccacc-3′, R, 5′-ccgtaatggggatgccggg gtcactgagct-3′), and
(F, 5′-agctcagtgaccccggcatccccattacgg-3′, R, 5′-cagatcagctatgtggttacctccactctc-3′)

JAK2:(F, 5′-cagattatgggtaatgattaaaggctccca-3′, R, 5′-cacagcatttctccaacatctgacaaccaaacc-3′),
(F, 5′-ga cagtctgctaattccagctactagaa-3′, R, 5′-gcctctccctctgggcattggcataagtcc-3′), and
(F, 5′-atgaagcaaccgtgttga agtagacattag-3′, R, 5′-cccacgtggactataaccatgactataagacc-3′), Primer sets for the nested-PCR: (F, 5′-gaa actatttgagtttccctgtatcatttag-3′, R, 5′-ctacaagcactccttaaaatgttgtagaaag-3′),
(F, 5′-gtaatttgccttgaaaactggt atttcc-3′, R, 5′-gcataagtccagatcgttaagacattgtac-3′), and
(F, 5′-gaagtagacattaggaaatcatctagacg-3′, R, 5′-cactgttactgtaaatatagaaatggcaaac-3′)

STAT1: (Ser727 F, 5′-cacttattgagagctacacacaggccagcc-3′, R, 5′-ggctggggacatgagaatcccatgagctgt-3′) and (Tyr701 F, 5′-tgctgataggcagtaacacggggatctcaa-3′, R, 5′-aggaggctaagctgtct agaaacacagtag-3′) Primer sets for the nested-PCR:

(Ser727 F, 5′-ttgagagctacacacaggccagccgtggta-3′, R, 5′-gggacatgagaatcccat gagctgtacttt-3′) and (Tyr701 F, 5′-tgctgataggcagtaacacggggatctcaa-3′, R, 5′-gtctagaaacacagtagaacttt aatcccc-3′)

The promoter region of PSMB9/b1i gene: (F, 5′-cgagaagctcagccatttaggggaaagcga-3′, R, 5′-cgcccgcagc atccctgcaaggcaccgctc-3′). Primer sets for the nested-PCR: (F, 5′-aagcgaaatcgaaagcggccgcctgctcac-3′, R, 5′-ctctcctcgccgcctggggcactggtttcc-3′)

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