

# The somatic mutations in Interferon- $\gamma$ signal molecules in human uterine leiomyosarcoma

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## Abstract

Human uterine leiomyosarcoma (U-LMS) is neoplastic malignancy that typically arises in tissues of mesenchymal origin. The identification of novel molecular mechanism leading to human U-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)/ $\beta 1i$* , an interferon (IFN)- $\gamma$  inducible factor, spontaneously develop U-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- $\gamma$  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 expression of PSMB9/ $\beta 1i$  in human U-LMS that was traced to the IFN- $\gamma$  pathway and the specific effect of somatic mutations of JANUS KINASE (JAK) 1 molecule or promoter region on the locus coding *PSMB9/ $\beta 1i$*  gene. Understanding the molecular mechanisms of human U-LMS may lead to identification of new diagnostic candidates or therapeutic targets against human U-LMS.

Human uterine leiomyosarcoma (U-LMS) is neoplastic malignancy that typically arises in tissues of mesenchymal origin. The identification of novel molecular mechanism leading to human U-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 (Psmb9)/ $\beta 1i$* , an interferon (IFN)- $\gamma$  inducible factor, spontaneously develop U-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- $\gamma$  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 expression of PSMB9/ $\beta 1i$  in human U-LMS that was traced to the IFN- $\gamma$  pathway and the specific effect of somatic mutations of JANUS KINASE (JAK) 1 molecule or promoter region on the locus coding *PSMB9/ $\beta 1i$*  gene. Understanding the molecular mechanisms of human U-LMS may lead to identification of new diagnostic candidates or therapeutic targets against human U-LMS.

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 (U-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 U-LMS typically present with vaginal bleeding, pain, and a pelvic mass. Gynecological tumour, 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 U-LMS compared with normal myometrium. These low expressions of receptor were found to not correlate with the promotion of initial disease development or with the overall survival of patients with U-LMS.

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As U-LMS is resistant to chemotherapy and radiotherapy, and thus surgical intervention is virtually the only means of treatment for this disease, however, molecular targeting therapies against tumours have recently shown remarkable achievements [4-8]. It is noteworthy that, when adjusting for stage and mitotic count, LMS has a significantly worse prognosis than carcinosarcoma; developing an efficient adjuvant therapy is expected to improve the prognosis of the disease [9]. 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 U-LMS. To the best of our knowledge, little is known regarding the biology of U-LMS; therefore, the risk factors that promote the initial development of U-LMS and regulate their growth *in vivo* remain poorly understood.

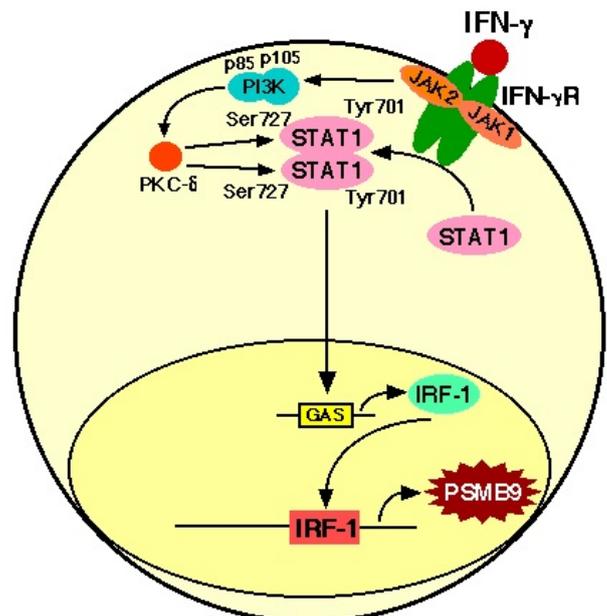
The mice with a targeted disruption of proteasome *beta* subunit 9 (PSMB9)/ $\beta$ 1i, which is interferon (IFN)- $\gamma$ -inducible proteasome subunit, exhibited a defect in tissue- and substrate- dependent proteasome function, and female PSMB9/ $\beta$ 1i-deficient mice shown to develop U-LMS, with a disease prevalence of 37% by 14 months of age [11,12]. Defective expression of PSMB9/ $\beta$ 1i is likely to be one of the risk factors for the development of human U-LMS, as it is in PSMB9/ $\beta$ 1i-deficient mice [12]. Recent report shows that stable PSMB9/ $\beta$ 1i 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- $\gamma$  pathway in the activation of shared-promoter of the transporter associated with antigen processing (TAP) 1 and PSMB9/ $\beta$ 1i have been established, it is demonstrated that the defective expression of PSMB9/ $\beta$ 1i was attributable to G871E somatic mutation in the ATP-binding region of JAK1 in SKN cell line, which is established from patient with U-LMS. It is furthermore likely that the PSMB9/ $\beta$ 1i expressions are down-regulated in human U-LMS tissues such like human U-LMS cell line. We demonstrate that there are serious mutational defects in the factors on the IFN- $\gamma$  pathway, which is the key cell-signaling pathway for PSMB9/ $\beta$ 1i expression and promoter region of *PSMB9/ $\beta$ 1i* gene, in human U-LMS. The somatic mutational defects in the IFN- $\gamma$  pathway may induce the initial development of U-LMS. Recent advances in our understanding of the biological characters of U-LMS have concentrated on the impaired IFN- $\gamma$  pathway. It is clear that somatic mutations in key regulatory genes 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 U-LMS may ultimately lead to novel therapies and improved outcome.

The effects of IFN- $\gamma$  on expression of PSMB9/ $\beta$ 1i was examined using five cell lines [13]. Expressions of PSMB9/ $\beta$ 1i were not markedly induced by IFN- $\gamma$  treatment in human U-LMS cell lines, although cervical epithelial adenocarcinoma cell lines and normal human uterus smooth muscle cells underwent strong induction of PSMB9/ $\beta$ 1i following IFN- $\gamma$  treatment [13]. Furthermore, the immunohistochemistry (IHC) experiments revealed a serious loss in the ability to induce expression of PSMB9/ $\beta$ 1i in human U-LMS tissues in comparison with normal myometrium tissues located in same tissue sections and other 4 mesenchymal tumour types. Of 58 U-LMS, 50 cases were negative for PSMB9/ $\beta$ 1i, 4 cases were focally positive, 2 cases were weakly positive, and 2 cases were positive. IHC analyses showed positivity for Ki-67/MIB1 and differential expression of ESTROGEN RECEPTOR (ER), PROGESTERONE RECEPTOR (PR), TUMOUR PROTEIN 53 (TP53), and CALPONIN h1. In addition, the expression level of PSMB9/ $\beta$ 1i was also examined in the skeletal muscle metastasis

from U-LMS, the histological diagnosis was consistent with metastatic LMS for skeletal muscle lesions. Pathological study of surgical samples showed presence of a mass measuring 3 cm at largest diameter in lumbar quadratus muscle without a fibrous capsule. All lymph nodes were negative. In western blotting and RT-PCR experiments, PSMB9/ $\beta$ 1i was expressed in normal myometrium, LMA, and IFN- $\gamma$ -treated HeLa cells, but not in human U-LMS. The both research experiments strongly supported the research findings obtained from IHC experiments.

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 U-LMS tissues (23 U-LMS tissue sections and normal tissue sections located in the same tissue) was performed to detect somatic mutations in the IFN- $\gamma$  pathway, JAK1, JAK2, STAT1 and promoter region of *PSMB9/ $\beta$ 1i* gene (Figure 1). As the catalytic domains of these factors 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 U-LMS tissues had serious mutations in the ATP binding region or kinase-specific active site of JAK1; furthermore, 43.5% (10/23) of U-LMS tissues had serious mutations in essential sites of the promoter region of *PSMB9/ $\beta$ 1i*

### Signaling pathway for PSMB9/ $\beta$ 1i expression



**Figure 1.** IFN- $\gamma$  treatment markedly increased the expression of PSMB9/ $\beta$ 1i, a subunit of the immunoproteasome, which alters the proteolytic specificity of proteasomes. After binding of IFN- $\gamma$  to the type II IFN receptor, which is constructed by two components, IFN- $\gamma$  receptor subunit 1 (IFNGR1) and IFN- $\gamma$  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) [32,33]. Tyrosine phosphorylated STAT1 forms homodimers that translocate to the nucleus and bind GAS (IFN- $\gamma$ -activated site) elements in the promoters of IFN- $\gamma$ -regulated genes [32,33]. IFN- $\gamma$  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- $\delta$  (PKC- $\delta$ ), which in turn regulates the phosphorylation of STAT1 on the Ser727. 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 [34,35].

*$\beta$ 1i* gene, which is required for transcriptional activation of *PSMB9/ $\beta$ 1i* gene (Table 1). No somatic mutation in essential sites, Tyr701 and Ser727, which are required for STAT1 transcriptional activation, was elucidated in uterine LMS. Nearly 21.7% (5/23) of U-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 U-LMS (Table 1). MOTIF Search profiling [14] 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 genes, resulted in impaired activations of tyrosine kinases or transcriptional factor [15].

In a recent report, a comparative genomic hybridization (CGH)-based analysis of 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 U-LMS. Other reports showed that among the most intriguing changes in genes were losses of JAK1 (1p31-p32) and *PSMB9/ $\beta$ 1i* (6p21.3) [16,17]. 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 LMS having a clear functional loss at JAK1 (1p31-p32) and *PSMB9/ $\beta$ 1i* (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 U-LMS.

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

U-LMS [4-8]. Although adjuvant pelvic irradiation appears to decrease the rate of local recurrence, adjuvant therapy does not appear to significantly improve survival. Furthermore, gynecological 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 U-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 U-LMS; however, molecular targeting therapies against tumours have recently shown remarkable achievements [18]. To improve the prognosis of human U-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 U-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 U-LMS [19-21]. However, in terms of the tumourigenesis of human U-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 U-LMS, such as B-cell Lymphoma-2 (BCL-2), BCL-2-Associated X protein (BAX), P16 Inhibits 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

**Table 1.** Somatic mutations in IFN- $\gamma$  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/ $\beta$ 1i* gene.

Mutations in the IFN- $\gamma$ pathway in human uterine leiomyosarcoma								
Gene Name	Locus	GenBank Accession	MIM ID	Tumor	Nucleotide	Amino Acid	Domain	Evolutionary conservation
JAK1	HUMPTKJAK1	M64174.1	*147795	ULMS	G2612A G2618A G2626A G2642T G2643A A2957C A2960C A2985T	G781E G873D G876R C881F C881 Stop Q986P Y987S R995S	ATP binding ATP binding ATP binding ATP binding ATP binding active site active site active site	p,c,m,r,g,d
JAK2	AF005216	AF005216.1	+147796	ULMS	ND <sup>2</sup>	ND	ND	p,c,b,m,r,g,d
STAT1	NM_007315	NM_007315	+600555	ULMS	A2104C T2128G T2078G A2148C	1702E S710A L693R R7168	NA <sup>3</sup> NA NA NA	c,b,m,r,g,d
PSMB9 <sup>1</sup>	X62741	X62741.1	*177045	ULMS	A209T A210G C213A C214T G215A A216G A217G G219 A G239 A		RF-E site RF-E site RF-E site RF-E site RF-E site RF-E site RF-E site RF-E site HSF site	

<sup>1</sup>LMP2 promoter region, NCBI Reference Sequence NT\_007592.15 Homo sapiens Chromosome 6<sup>2</sup>not detected <sup>3</sup>non-kinaseactivation region <sup>4</sup>Evolutionary conservation refers to the species in which an identical residue was observed in the homolog (p, pan troglodytes; c, canis lupus familiaris; b, bostaurus; m, musmusculus; r, rattusnorvegicus; g, gallusgallus; d, danio rerio)

of these factors directly correlates to the initiation and promotion of human U-LMS. PSMB9/ $\beta$ 1i-deficient mice were reported to be prone to the development of U-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/ $\beta$ 1i-deficient uterine neoplasms revealed common characteristic abnormalities of U-LMS. In addition, recent research reports show the loss in the IFN- $\gamma$ -inducible ability of PSMB9/ $\beta$ 1i expressions in SKN cell line and other primary U-LMS cells established from patients. The histopathological experiments demonstrated a serious loss in the ability to induce the expression of PSMB9/ $\beta$ 1i in human U-LMS tissues in comparison with normal myometrium tissues located in same tissue sections.

IFN- $\gamma$  treatment markedly increased the expression of PSMB9/ $\beta$ 1i, a subunit of the proteasome, which alters the proteolytic specificity of proteasomes. Sequence analysis demonstrated that the loss of IFN- $\gamma$  responsiveness in the human U-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- $\gamma$  signal pathway since IFN- $\gamma$  treatment could not strongly induce JAK1 kinase activity in human U-LMS cell lines. Genetic alterations in tyrosine kinases have previously been firmly implicated in tumorigenesis, but only a few serine/threonine kinases are known to be mutated in human cancers [22-25]. For instance, mice carrying homozygous deletion of *Phosphatase and Tensin Homolog Deleted from Chromosome 10 (Pten)* alleles developed widespread SMC hyperplasia and abdominal LMS [26], and JUN oncogene amplification and over-expression block adipocytic differentiation in highly aggressive sarcomas. 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 (tumour-specific) mutations in the IFN- $\gamma$  signal molecules. In a recent report, high-resolution genome wide array comparative genomic hybridization (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 U-LMS. Among the most intriguing genes, whose copy number sequence was revealed by CGH, were loss of JAK1 (1p31-p32) and PSMB9/ $\beta$ 1i (6p21.3) [16,17]. The discovery of these mutational defects in a key cell-signaling pathway may be an important development in the pathogenesis of human U-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- $\gamma$  signaling [27]. The growth of the original SKN cells, which had defective JAK1 activity, was unaffected by IFN- $\gamma$  treatment. In contrast, the growth of JAK1-transfected SKN cells, which had strong exogenous JAK1 activity, was prevented by IFN- $\gamma$  treatment. Interestingly, when PSMB9/ $\beta$ 1i-transfected SKN cells, which have marked the expression of PSMB9/ $\beta$ 1i, were analyzed, expression of exogenous PSMB9/ $\beta$ 1i resulted in cell growth inhibition. Conversely, the growth of PSMB9/ $\beta$ 1i-transfected SKN cells was unaffected by IFN- $\gamma$  signal pathway. Taken together, IFN- $\gamma$  response to cell growth inhibition may be attributable to the physiological significance of PSMB9/ $\beta$ 1i.

In conclusion, the down regulation of major histocompatibility complex (MHC) expression, including the *TAP1* and *PSMB9/ $\beta$ 1i* genes, is one of the biological mechanisms tumour cells use to evade host immunosurveillance [28-30]. Recently, the incidence of IFN- $\gamma$  unresponsiveness in human tumours was examined in several cancers, and revealed that approximately 33% of each group exhibited a reduction in IFN- $\gamma$  sensitivity [31]. Nevertheless, the expression of PSMB9/ $\beta$ 1i, rather than providing an escape from immune surveillance, seems to play an important role in the negative regulation of human U-LMS cell growth. Defective expression of PSMB9/ $\beta$ 1i is likely to be one of the risk factors for the development of human uterine neoplasm, as it is in the PSMB9/ $\beta$ 1i-deficient mouse. Thus, gene therapy with PSMB9/ $\beta$ 1i expression vectors may be a new treatment for U-LMS that exhibits a defect in the expression of PSMB9/ $\beta$ 1i. Because there is no effective therapy for unresectable U-LMS, our results may bring us to specific molecular therapies to treat this disease.

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