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Detection of Somatic Mutations in Interferon-γ Signal Molecules in Human Uterine Leiomyosarcoma

Takuma Hayashi[1,9]*, Akiko Horiuchi[2], Kenji Sano[3], Nobuyoshi Hiraoka[4], Tomoyuki Ichimura[5], Osamu Ishiko[5], Yae Kanai[4,10], Nobuo Yaegashi[6], Hiroyuki Aburatani[7] and Ikuo Konishi[8]

[1]Department of Immunology and Infectious Disease, Shinshu University Graduate School of Medicine, Japan
[2]Horiuchi Ladies Clinic, Japan
[3]Department of Laboratory Medicine, Shinshu University Hospital, Japan
[4]Pathology Division, National Cancer Center Research Institute, Japan
[5]Department of Obstetrics and Gynecology, Osaka City University Graduate School of Medicine, Japan
[6]Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Japan
[7]Department of Obstetrics and Gynecology, Shinshu University, Japan
[8]Deaprtment of Obstetrics and Gynecology, Kyoto University Graduate School of Medicine, Japan
[9]Promoting Business using Advanced Technology, Japan Science and Technology Agency, Japan
[10]The International Human Epigenome Consortium (IHEC) and CREST, Japan Science and Technology Agency, Japan

***Corresponding author:** Takuma Hayashi, Department of Immunology and Infectious Disease, Shinshu University Graduate School of Medicine, 3-1-1, Asahi, Matsumoto, Nagano 390-8621, Japan, Tel: 81-263-37-2611; Fax: 81-263-2613; Email: yoyoyo224@hotmail.com

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Abstract

Human Uterine Leiomyosarcoma (Ut-LMS) is neoplastic malignancy that typically arises in tissues of mesenchymal origin. The identification of novel molecular mechanism leading to human Ut-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 Subunit Beta Type (PSMB) 9, an Interferon (IFN)-γ inducible factor, spontaneously develop Ut-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, tumor suppressive, and signaling pathways directly impact sarcomagenesis. The IFN-γ signaling pathway is important for control of tumor growth and invasion and has been implicated in several malignant tumors. In this study, experiments with human tissues revealed a defective PSMB9 expression in human Ut-LMS that was traced to the IFN-γ signaling pathway and the specific effect of somatic mutations of Janus Kinase (JAK) 1 molecule or *PSMB9* gene promoter region on the *PSMB9* gene transcriptional activation. Understanding the molecular mechanisms of human Ut-LMS may lead to identification of new diagnostic candidates or therapeutic targets in human Ut-LMS.

Keywords: PSMB9; Somatic mutation; Uterine leoimyosarcoma; Leiomyoma; Myometrium; IFN-γ

Abbreviations

Ut-LMS: Uterine Leiomyosarcoma; LMA: Leiomyomas; PSMB9: Proteasome Subunit Beta type 9; IFN-γ: Interferon-γ, JAK1: Janus Kinase; DTAT1: Signal Transducers and Activator of Transcription 1, STUMP: Smooth Muscle Tumor of Uncertain Malignant Potential

Introduction

Uterine mesenchymal tumors have been traditionally divided into benign tumor Leiomyomas (LMA) and malignant tumor Leiomyosarcomas (LMS) based on cytological atypia, mitotic activity and other criteria. Ut-LMS are relatively rare smooth muscle tumor, having an estimated annual incidence of 0.64 per 100,000 women [1-3]. Gynecological tumor, for instance endometrial carcinomas, are strongly promoted by female hormones, but the rate of hormone receptor expression is reported to be significantly less in human Ut-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 Ut-LMS. As human Ut-LMS is resistant to chemotherapy and radiotherapy, and thus surgical intervention is virtually the only means of treatment for this disease [4-6], however, molecular targeting therapies against tumors have recently shown remarkable achievements [7]. It is noteworthy that, when adjusting for stage and mitotic count, human Ut-LMS has a significantly worse prognosis than carcinosarcoma [8]; developing an efficient adjuvant therapy is expected to improve the prognosis of the disease [9]. Although typical presentations with hypercalcemia or eosinophilia have been reported, this clinical abnormality is not an initial risk factor for human Ut-LMS. To the best of our knowledge, little is known regarding the biology of human Ut-LMS; therefore, the risk factors that promote the initial development of human Ut-LMS and regulate their growth *in vivo* remain poorly understood.

The mice with a targeted disruption of Proteasome Subunit Beta type (PSMB) 9, which is IFN-γ -inducible proteasome beta subunit, exhibited a defect in tissue- and substrate- dependent proteasome function, and female *Psmb9*-deficient mice shown to develop Ut-LMS, with a disease prevalence of 37% by 14 months of age [10,11]. Defective expression of PSMB9 is likely to be one of the risk factors for the development of human Ut-LMS, as it is in *Psmb9*-deficient mice [11]. Recent report shows that stable expression of PSMB9

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contributes to cell proliferation, which directly correlates to the progressive deterioration with increasing stage of the tumor. As the importance and involvement of the interferon (IFN)-γ signal pathway in the transcriptional regulation of the *PSMB9* promoter have been established, the defective expression of PSMB9 was reportedly attributable to G871E mutation in the ATP-binding region of Janus Kinase (JAK) 1 in SKN cell line, which is established from patient with human Ut-LMS [12]. In this research, we demonstrate that there are serious mutational defects in the factors on the IFN-γ signaling pathway and *PSMB9* promoter region, in human Ut-LMS tissues. The somatic mutational defects in the IFN-γ signaling pathway may induce the initial development of human Ut-LMS. Recent advances in our understanding of the biology of human Ut-LMS have concentrated on the impaired IFN-γ signaling 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 tumor. Therefore, continued improvement of our knowledge of the molecular biology of human Ut-LMS may ultimately lead to novel therapies and improved outcome.

Materials and Methods Tissue Collection

A total of 56 patients aged between 32 and 83 years who were diagnosed as having smooth muscle tumors of the uterus were selected from pathological files. Serial sections were cut from at least 2 tissue blocks from each patient for Hematoxylin and Eosin (H.&E.) 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. 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 MFs per 10 HPFs. Cellular Leiomyoma (cellular LMA) was defined as a tumor with significantly increased cellularity (>2000 myoma cells / HPF) and a MI<5, but without cytologic atypia. Bizarre Leiomyoma (BL) was defined as a tumor either with diffuse nuclear atypia and a MI<2 or with focal nuclear atypia and a 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 a MI<10 but with coagulative tumor cell necrosis. Human Uterine Leiomyosarcoma (Ut-LMS) was diagnosed in the presence of a MI>10 with either diffuse cytologic atypia, coagulative tumor cell necrosis, or both. Of the 56 smooth muscle tumors, 49 were diagnosed as LMA, 3 were Bizarre leiomyoma, and 56 were human Ut-LMS. Of the 56 human Ut-LMS cases, 29 were histologically of the spindle-cell type and 9 were of the epithelioid type. The clinical stage of the human Ut-LMS patients was stage I in 7 cases, stage II or III in 20 cases, and stage IV in 5 cases. Protein expression studies with cervical epithelium and carcinoma tissues were performed using tissue arrays (Uterus cancer tissues, AccuMax Array, Seoul, ISU ABXIS Co., Ltd., Korea). Details about tissue sections are indicated in the manufacturer's literature (AccuMax Array).

Sequencing of the catalytic domains of JAK1, STAT1, JAK2, and *PSMB9* promoter region

To demonstrate whether the somatic mutations in the ATP-binding region and kinase activation domain of the JAK1 molecule, *PSMB9* promoter region, the Tyr701 and Ser727 amino acid of Signal Transducers and Activator of Transcription (STAT) 1 molecule, and the ATP-binding region and kinase activation domain of the JAK2 molecule playing in human Ut-LMS were identified, the isolating of gemonic DNA and direct sequencing was carried out. The gemonic DNA was extracted from the human Ut-LMS tissues and same patient-matched normal myometrium tissues using the standard protocol. The gemonic DNA was subjected to PCR procedure [12]. The restricted DNA fragments for the direct sequence analysis were amplified by PCR according to the published sequencing oligonuclrotide primers [12]. 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 *PSMB9* promoter region derived from the individual human uterine LMS tissue sections were registered at DDBJ (Accession: AB219242, DJ055380, DJ055379, DJ055378, DJ055377, DJ055376).

Result and Discussion

Defective PSMB9 expression of human Ut-LMS

The effects of IFN-γ on PSMB9 expression was examined using five cell lines [12]. PSMB9 expression were not markedly induced by IFN-γ treatment in human Ut-LMS cell lines, although cervical epithelial adenocarcinoma cell lines and normal human myometrium cells underwent strong induction of PSMB9 following IFN-γ treatment [12]. Furthermore, the Immunohistochemistry (IHC) experiments revealed a serious loss in the ability to induce PSMB9 expression in human Ut-LMS tissues in comparison with same patient-matched normal myometrium tissues located in same tissue sections and other 4 mesenchymal tumor types, usual leiomyoma, cellular leiomyoma, Bizarre leiomyoma, and STUMP [13]. Of 56 human Ut-LMS, 48 cases were negative for PSMB9, 4 cases were focally positive, 2 cases were weakly positive, and 2 cases were positive [13]. IHC analyses showed positivity for Ki-67/MIB1 and differential expression of ER, PR, TP53, and CALPONIN h1 [13]. In addition, PSMB9 expression level was also examined in the skeletal muscle metastasis from human Ut-LMS, the histological diagnosis was consistent with metastatic Ut-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. In western blotting experiments and RT-PCR experiments, PSMB9 was expressed in normal myometrium, LMA, but not in human Ut-LMS [13]. The both research experiments strongly supported the research findings obtained from IHC experiments.

Somatic mutations in IFN-γ signaling pathway in human Ut-LMS tissues

IFN-γ treatment markedly increased the expression of PSMB9, which alters the proteolytic specificity of proteasomes. After binding of IFN-γ to the type II IFN receptor, JAK1 and JAK2 are activated and phosphorylate STAT1 on the tyrosine residue at position 701 and the serine residue at position 727 [14,15] (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] (Figure 1). IFN-γ

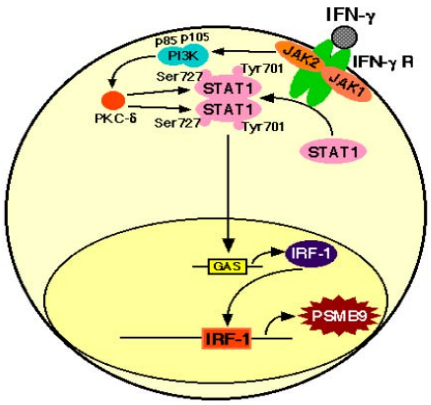

Figure 1: Signaling pathway for PSMB9 expression.
The interferon-γ signaling pathway and mutations in its components found in human uterine leiomyosarcoma. After binding of Interferon-γ (IFN- γ) to the type II IFN receptor, Janus Activated Kinase 1 (JAK1) and JAK2 are activated and phosphorylate signal 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-γ-activated site) elements, which are present in the promoters of IFN-γ-regulated genes. The IFN-γ-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-γ receptor subunit 1; IFNGR2, IFN-γ receptor subunit 2.

activated JAKs also regulate, through as yet unknown intermediates, activation of the catalytic subunit (p110) of Phosphoinositide 3-kinase (PI3K). The activation of PI3K ultimately results in downstream activation of Protein kinase C (PKC)-δ, which in turn regulates the phosphorylation of STAT1 on the Ser727. The phosphorylation of Ser727 is required for full transcriptional activation [16] (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 Ut-LMS cell lines. Sequence analysis demonstrated that the loss of IFN-γ responsiveness in the human Ut-LMS cell line was attributable to the inadequate kinase activity of JAK1 due to a G781E mutation in the ATP-binding region [14]. 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 malignant tumors [17-19]. For instance, mice carrying homozygous deletion of *Phosphatase and Tensin Homolog Deleted from Chromosome 10 (Pten)* alleles developed wide spread smooth muscle cell hyperplasia and abdominal LMS [18], and JUN oncogene amplification and overexpression block adipocytic differentiation in highly aggressive sarcomas [19]. In addition, JAK-STAT signal transduction is a crucial regulator of skeletal muscle regeneration, and targeting this pathway in mice relieves aspects of debilitating muscle wasting [20].

Most frequently, human Ut-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

Table 1: Somatic mutations in IFN−γ 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 LMP2 promoter.

Mutations in the IFN-γ signaling pathway in human uterine leiomyosarcoma								
Gene Name	Locus	GenBank Accession	MIM ID	Tumor	Nucle otide	Amino Acid	Domain	Evolutionary conservation[4]
JAK1	HUMPTKJAK1	M64174.1	*147795	Ut-LMS	G2612A	G781E	ATPbinging	p,c,m,r,g,d
					G2618A	G873D	ATPbinging	
					G2626A	G876R	ATPbinging	
					G2642T	C881F	ATPbinging	
					G2643A	C881 Stop	ATPbinging	
					A2957C	Q986P	Active Site	
					A2960C	Y987S	Active Site	
					A2985T	R995S	Active Site	
JAK2	AF005216	AF005216.1	+147796	Ut-LMS	ND[2]	ND	ND	p,c,b,m,r,g,d
STAT1	NM_007315	NM_007315	+600555	Ut-LMS	A2104C	I702L	NA[3]	c,b,m,r,g,d
					T2128G	S710A	NA	
					T2078G	L693R	NA	
					A2148C	R716S	NA	
PSMB9[1]	X62741	X62741.1	*177045	Ut-LMS	A209T		IRF-E site	p,c,b,m,r,d
					A210G		IRF-E site	
					C213A		IRF-E site	
					C214T		IRF-E site	
					G215A		IRF-E site	
					A216G		IRF-E site	
					A217G		IRF-E site	
					G219A		IRF-E site	
					G239A		HSF site	

[1]PSMB9 (proteasome beta subunit 9) promoter region, NCBI reference sequence NT_007592.15 Homo sapiens Chromosome 6; [2]ND: Not dected;[3]NA: Non-kinase activation region; [4]Evolutionary conservation refers to the species in which an identical residue was observed in the homolong(p: pan troglodytes; c: canis lupus familiaris; b: bos taurus; m: musculus; r: rattus norvegius; g: gallus d:danio rerio); Ut-LMS: human uterine leiomyosarcoma

remains unclear. Therefore, the examination of human Ut-LMS tissues (23 Ut-LMS tissue sections and normal tissue sections located in the same tissue) was performed to detect somatic mutations in the IFN-γ signaling cascade, JAK1, JAK2, STAT1 and *PSMB9* promoter region. As the catalytic domains of these genes are most likely to harbour mutations that activate the gene product, we focused on stretches containing the kinase domains, transcriptional activation domains and enhancer/promoter region. Over all, nearly 43.5% (10/23) of human Ut-LMS tissues had serious mutations in the ATP binding region or kinase specific active site of JAK1; furthermore, 43.5% (10/23) of human Ut-LMS tissues had serious mutations in essential sites of the *PSMB9* promoter region, which is required for *PSMB9* gene transcriptional activation (Table 1). No somatic mutation in essential sites, Tyr701 and Ser727, which are required for STAT1 transcriptional activation, was elucidated in human Ut-LMS. Nearly 21.7% (5/23) of human Ut-LMS tissues unexpectedly had mutations in the STAT1 intermolecular region, which is not yet reported to be important for biological function as transcriptional activation (Table 1). No somatic mutation in the ATP-binding region and kinase-active site of JAK2 was detected in human Ut-LMS (Table 1). In a recent report, high-resolution genomewide array comparative genomic hybridization (CGH) analysis of human Ut-LMS cases gave gene-level information about the amplified and deleted regions that may play a role in the development and progression of human Ut-LMS. Among the most intriguing genes, whose copy number sequence was revealed by CGH analysis, were loss of *JAK1* (1p31-p32) and *PSMB9* (6p21.3) [21,22]. The discovery of these mutational defects in a key cell-signaling pathway may be an important development in the pathogenesis of human Ut-LMS.

Conclusion

Defective PSMB9 expression is likely to be one of the risk factors for the development of human uterine neoplasm, as it is in the *Psmb9*-deficient mouse. Thus, gene therapy with PSMB9 expression vectors may be a new treatment for human Ut-LMS that exhibits a defect in PSMB9 expression. Because there is no effective therapy for unresectable human Ut-LMS, our results may bring us to specific molecular therapies to treat this disease.

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