

# Biological Significance of the Proteasome Subunit LMP2/ $\beta$ 1i as a Tumor Suppressor in Human Uterine Leiomyosarcoma

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**Abstract:** Uterine leiomyosarcoma (Ut-LMS) develops more often in the muscle tissue layer of the uterine body than in the uterine cervix. The development of gynecologic tumors is often correlated with female hormone secretion; however, the development of Ut-LMS is not substantially correlated with hormonal conditions, and the risk factors are not yet known. Importantly, a diagnostic-biomarker which distinguishes malignant Ut-LMS from other uterine mesenchymal tumors including leiomyoma (LMA) is yet to be established. Accordingly, it is necessary to analyze risk factors associated with Ut-LMS, to establish a clinical treatment method. Proteasome subunit, low-molecular mass polypeptide(LMP2)/ $\beta$ 1i-deficient mice spontaneously develop Ut-LMS, with a disease prevalence of ~40% by 14 months of age. Recent experiments with human and mouse uterine tissues revealed defective LMP2/ $\beta$ 1i expression in human Ut-LMS that was traced to the interferon (IFN)- $\gamma$  pathway and a specific effect of Janus kinase (JAK)-1 somatic mutations on LMP2/ $\beta$ 1i transcriptional activation. Furthermore, analysis of a human Ut-LMS cell line clarified the biological significance of LMP2/ $\beta$ 1i in malignant myometrium transformation and the cell cycle, thus implicating LMP2/ $\beta$ 1i as an anti-tumorigenic candidate. Therefore, defective-LMP2/ $\beta$ 1i expression may be a risk factor for human Ut-LMS. LMP2/ $\beta$ 1i is a potential diagnostic-biomarker for Ut-LMS, and may be a targeted-molecule for a new clinical therapeutic approach.

**Keywords:** LMP2/ $\beta$ 1i, calponin h1, tumor-suppressor, leiomyosarcoma, leiomyoma, biomarker.

## INTRODUCTION

The uterus, the organ in which the embryo grows, is composed of three layers, the uterine endometrium which serves as a bed for the embryo; the myometrium of the wall which protects the embryo; and a serous membrane enveloping the uterus. In general, the term uterine tumor refers to an epithelial malignant tumor of the uterus, which is roughly classified as a tumor of the uterine cervix or the uterine body. Because of the prevalence of medical checkups, the rate of mortality from uterine cervix cancer is decreasing. In contrast, the mortality rate for cancer of the uterine body is increasing, and the disease is rarely detected at the initial stages. While most tumors of the uterine body

are adenocarcinomas (derived from the subintimal gland), tumors of the uterine cervix are classified into squamous cancer and adenocarcinoma. Uterine mesenchymal tumors, i.e. smooth muscle tumors (SMTs) which develop in the myometrium, have been traditionally divided into benign LMA and malignant Ut-LMS based on cytological atypia, mitotic activity and other criteria. Ut-LMS is relatively rare, having an estimated annual incidence of 0.64 per 100,000 women [1]. Ut-LMS accounts for 2% to 5% of tumors of the uterine body and develops more often in the muscle layer of the uterine body than in the uterine cervix. As Ut-LMS is resistant to chemotherapy and radiotherapy, surgical intervention is virtually the only means of treatment [2-4]. The prognosis for Ut-LMS is not good, and the five-year survival rate is approximately 35%. However, developing an efficient adjuvant therapy is expected to improve the prognosis for Ut-LMS. Uterine LMA may occur in as many as 70%~80% of women by

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the age of 50 years [5]. Distinguishing uterine LMA from Ut-LMS is very difficult, and a diagnosis generally requires surgery and cytoscopy [6]. Diagnostic categories for uterine SMTs and morphological criteria are used to assign cases [7, 8] (NOTE 1). The non-standard subtypes of uterine SMTs such as the epithelioid and myxoid types are classified in a different way using these features, so the establishment of a diagnostic method for the identification of non-standard smooth muscle differentiation is important [7, 8].

High estrogen levels are considered to significantly influence the development of tumours in the uterine body [9-11]. The molecular mechanisms by which uterine LMA and Ut-LMS develop are not yet known, though tumours that have developed in the myometrium for some reason gradually become larger due to the influence of the female hormone, estrogen, and generate tumors. However, no correlation between the development of Ut-LMS and hormonal conditions, and no obvious risk factors, have been found. Although cases accompanied by hypocalcaemia or eosinophilia have been reported, neither clinical abnormality is an initial risk factor for Ut-LMS.

The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle and the regulation of gene expression. In structure, the proteasome is a cylindrical complex containing a core of four stacked rings around a central pore, each ring composed of seven individual proteins. The inner two rings are made of seven  $\beta$  subunits that contain three to seven protease active sites. Alternative  $\beta$  forms denoted LMP2/ $\beta$ 1i can be expressed in the myometrium in response to exposure to pro-inflammatory signals such as cytokines, in particular, IFN- $\gamma$ . Ut-LMS reportedly occurred in female LMP2/ $\beta$ 1i-null mice at age 6 months or older, and the incidence at 14 months of age was about 40%. The determination of the malignant potential of smooth muscle neoplasm also represents a significant diagnostic conundrum with important therapeutic ramifications. However, the genetic changes underlying the neoplastic transformation of uterine smooth muscle cells have not been fully characterized. Moreover, diagnostic biomarkers that are able to distinguish between LMS and LMA have yet to be established. The identification of a risk factor and/or biological candidate(s) associated with the development of Ut-LMS, i.e. LMP2/ $\beta$ 1i, would significantly contribute to the development of preventive and therapeutic treatments.

## Defective LMP2 Expression in Human Ut-LMS

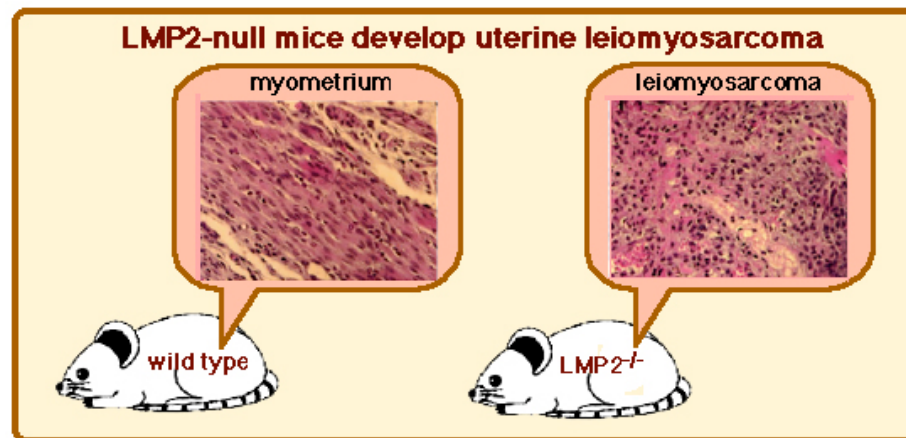
Cytoplasmic proteins are mostly degraded by a protease complex, which has many substrates consisting of twenty-eight 20 to 30-kDa subunits, referred to as the 20S proteasome, and is located in the nucleus and the cytoplasm [12, 13]. Proteasomal degradation is essential for many cellular processes, including the cell cycle, the regulation of gene expression and immunological function. Abnormal expression of proteasome subunits is correlated with tumor initiation and progression [14, 15]. IFN- $\gamma$  induces the expression of large numbers of responsive genes encoding proteasome subunits, i.e., low molecular weight protein (LMP2)/ $\beta$ 1i, LMP7/ $\beta$ 5i, and LMP10/multicatalytic endopeptidase complex-like (MECL)-1/ $\beta$ 2i [16]. The individual expression of the LMP2, LMP7, and LMP10(MECL-1) subunits is believed to contribute to the initiation and development of disorders. A recent study revealed a unique role for LMP7 in controlling pathogenic immune responses and provided a therapeutic rationale for targeting LMP7 in autoimmune disorders, especially rheumatoid arthritis [17]. Selective inhibition of LMP7 blocked production of interleukin-23 (IL-23) by activated monocytes and interferon- $\gamma$  and IL-2 by T cells. In mouse models of rheumatoid arthritis, LMP7-inhibitory treatment reversed signs of disease and resulted in reductions in cellular infiltration, cytokine production and autoantibody levels. Recent reports demonstrate LMP2/ $\beta$ 1i, as obligatory for tumor surveillance and a tissue-specific role for LMP2/ $\beta$ 1i in protection from spontaneous uterus neoplasms [18, 19]. The presentation of antigenic peptides by MHC class I molecules is important for tumor rejection by CTLs. Such antigenic peptides are generated as a result of the degradation of intracellular proteins by the proteasome pathway, a process that is influenced by the LMP2/ $\beta$ 1i subunit of the proteasome complex. LMP2/ $\beta$ 1i-null mice thus exhibit a defect in proteasome function. LMP2/ $\beta$ 1i-null mice are now shown to develop uterine neoplasms, with a disease prevalence of 36% by 12 months of age. This observation indicates that proteasome function is essential for MHC class I-mediated tumor rejection by CTLs.

Homozygous mice deficient in LMP2/ $\beta$ 1i show tissue- and substrate-dependent abnormalities in the biological functions of the proteasome [18, 19]. Ut-LMS reportedly occurred in female LMP2/ $\beta$ 1i-null mice at age 6 months or older, and the incidence at 14 months of age was about 40% [19, 20]. The disease prevalence in mice is similar to that of human Ut-LMS,

which occurs after menopause. Histological studies of LMP2/ $\beta$ 1i-null uterine tumors have revealed characteristic abnormalities of human Ut-LMS [19]. The tumors lacked lymphoid infiltrates, a sign of immune recognition, and consisted of uniform elongated smooth muscle cells arranged into bundles. The nuclei of the tumor cells varied in size and shape; furthermore, mitosis was frequent. The tumors lacked lymphoid infiltrates, a sign of immune recognition, and consisted of uniform elongated myometrium cells arranged into bundles. The nuclei of the tumor cells varied in size and shape. Furthermore, mitosis was frequent. In contrast, the myometrium cells of C57BL/6 mice were normal in appearance [19]. Whereas relatively few ki-67-positive cells, which were the proliferating cells, were observed in the basal cell layer of the normal myometrium, most of the basal cells in LMP2/ $\beta$ 1i-null mice vividly expressed ki-67 [19]. This immunological staining indicates abnormal proliferation of the LMP2/ $\beta$ 1i-lacking cells in the basal layer. LMP2/ $\beta$ 1i-null mice that have developed Ut-LMS undergo considerable weight loss, and then die by 14 months of age. They may also exhibit skeletal muscle metastasis from the Ut-LMS [21]. Therefore, it is likely that LMP2/ $\beta$ 1i-null mice with Ut-LMS die as a result of tumor growth and metastasis. In general, it is not easy to distinguish uterine LMA from Ut-LMS in humans, however, in mice, because of such characteristic pathological findings, significant weight loss, and possible skeletal muscle metastasis, a tumor that develops in the uterus of an LMP2/ $\beta$ 1i-null mouse can be considered malignant, i.e., a Ut-LMS [19, 20] (Figure 1).

Furthermore, immunohistochemistry (IHC) revealed a serious loss in the ability to induce LMP2/ $\beta$ 1i

expression in human Ut-LMS tissue in comparison with LMA or normal myometrium located in the same section [22, 23]. Of the 54 cases we examined with human Ut-LMS, 46 were negative for LMP2/ $\beta$ 1i expression, 4 were focally positive, and 2 were partially positive [23]. Two Ut-LMS cases were stained for LMP2/ $\beta$ 1i. LMP2/ $\beta$ 1i levels were also evaluated in skeletal muscle and rectum metastases from individual Ut-LMS patients [23]. Pathological examination of surgical samples showed the presence of a mass measuring 3 cm in its largest diameter in the lumbar quadratus muscle without a fibrous capsule. All lymph nodes were negative for human Ut-LMS metastases, and immunohistological studies showed positivity for ki-67 and negativity for LMP2/ $\beta$ 1i [23]. Histological findings were consistent with metastatic Ut-LMS for the skeletal muscle and rectum lesions [23]. In western blotting and RT-PCR experiments, LMP2/ $\beta$ 1i was expressed in normal myometrium, but not in human Ut-LMS, both strongly supportive of the IHC findings [22, 23]. To increase tumor incidence and better assess the role of systemic expression of TP53 in responses to initiation of Ut-LMS tumorigenesis, LMP2/ $\beta$ 1i-null mice were bred with TP53-null mice to create *Lmp2<sup>-/-</sup>Tp53<sup>-/-</sup>* double knockout mice. Uterine LMS incidence and death rates were similar in *Lmp2<sup>-/-</sup>Tp53<sup>-/-</sup>* mice and closely matched those for control *Lmp2<sup>-/-</sup>Tp53<sup>+/+</sup>* mice. The correlation between defective TP53 function and uterine LMS tumorigenesis is unclear. Although we previously demonstrated that the abnormal expression of ovarian steroid receptors, TP53 and ki-67 and mutations of TP53 were frequently associated with human Ut-LMS, defective LMP2/ $\beta$ 1i expression appears to be more characteristic of Ut-LMS than any of these factors [22-25] (Table 1).



**Figure 1:** Homozygous mice deficient in LMP2/ $\beta$ 1i, an interferon (IFN)- $\gamma$ -inducible factor, show tissue- and substrate-dependent abnormalities in the biological functions of the proteasome [18, 19]. Ut-LMS reportedly occurred in female LMP2/ $\beta$ 1i-null mice at age 6 months or older, and the incidence at 14 months of age was about 40%.

Table 1: Expression of ER, PR, p53, Ki-67, Calponin h1 and LMP2 in Human Uterine Leiomyosarcoma

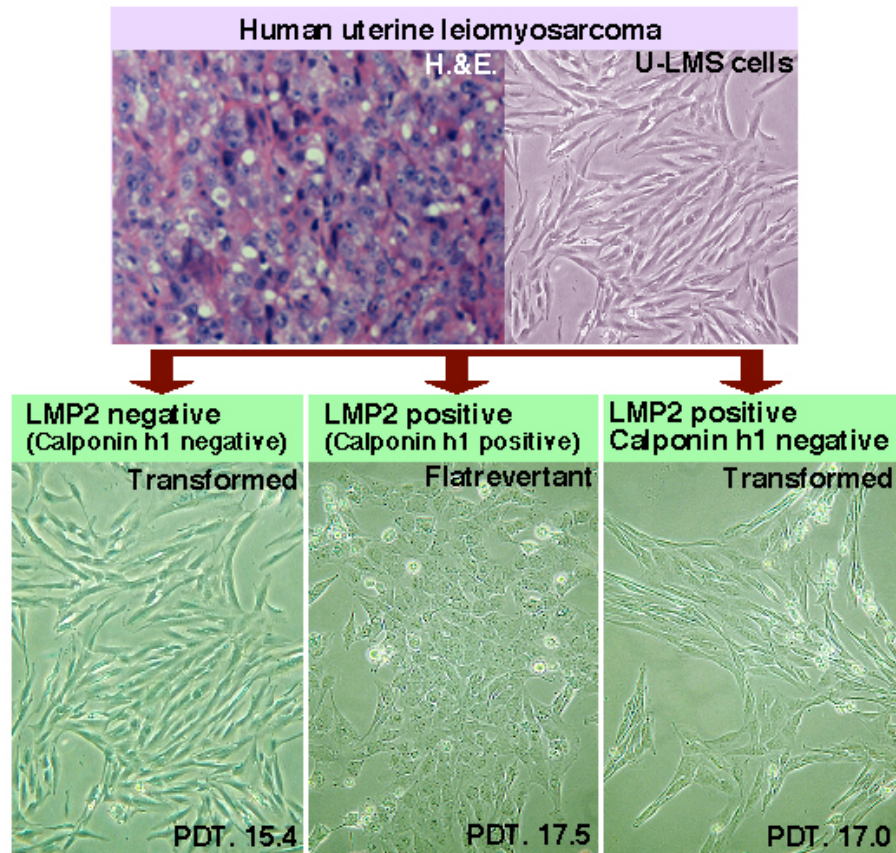
Patient No.	Age in yrs	Immunohistochemical							Somatic Mutation					Follow-Up (months)		
		TNM stage	MF	CCN	ER	PR	Ki-67	p53	LMP2	Cal.	p53	JAK1	JAK2		STAT1	LMP2-pro
1	37	T4N1M0	97	+	-	-	-	3000	+++	-	-	ND	ND	ND	ND	D(1)
2	58	T3N0M0	24	+	-	-	-	3500	+	+/-	-	ND	ND	ND	M	(23)
3	45	T2N0M0	32	+	+/-	+/-	+	2150	+++	-	-	M	M	M	M	D(24)
4	65	T1N0M0	30	+	+/-	+/-	+	1700	+++	-	-	M	M	ND	ND	D(20)
5	52	T1N0M0	107	+	-	+	+	2600	++	+	-	M	ND	ND	ND	D(13)
6	49	T1N0M0	46	+	-	-	-	4300	+	-	-	ND	ND	ND	ND	D(24)
7	55	T1N0M0	75	+	-	-	-	4000	+++	-	-	ND	ND	M	M	D(18)
8	43	T3N0M0	57	+	+	-	-	2000	-	+/-	+/-	ND	ND	ND	ND	D(10)
9	67	T1N0M0	13	+	-	-	+/-	1430	-	-	-	M	ND	ND	ND	A(34)
10	67	T1N0M0	37	+	-	-	-	2100	-	-	-	ND	ND	M	M	A(15)
11	51	T1N0M0	93	+	-	-	-	4500	-	-	-	ND	ND	M	ND	A(94)
12	48	T1N0M0	14	+	-	-	-	900	+++	+	+	ND	ND	ND	ND	A(58)
13	51	T1N0M0	22	+	+/-	+	+	450	+	-	-	M	ND	ND	M	A(34)
14	67	T1N0M0	64	+	-	+	+	1450	++	-	-	ND	ND	ND	ND	A(15)
15	52	T1N0M0	65	+	-	-	-	1780	++	-	-	M	ND	M	ND	D(23)
16	42	T3N0M0	73	+	-	-	-	2130	++	-	-	ND	ND	ND	M	A(21)
17	80	T1N0M0	98	+	-	-	-	1980	+++	-	-	M	ND	ND	ND	D(19)
18	56	T1N0M0	78	+	-	-	-	1860	++	-	-	ND	ND	ND	ND	A(11)
19	58	T1N0M0	40	+	-	-	-	1750	++	-	-	ND	ND	ND	ND	A(10)
20	65	T2N0M0	67	+	-	-	-	780	+++	-	-	M	ND	ND	M	A(12)

Er, estrogen receptor; PR, progesterone receptor; Cal., Calponin h1; Ki-67, positive cell number/10 high power fields; M, mutation; ND, not detected; D, died of disease; A, alive; MF, mitotic figure/10 high power fields; CCN, coagulative cell necrosis.

### The Biological Significance of LMP2 in Human Ut-LMS Tumorigenesis

The IFN- $\gamma$  pathway is important for control of tumor growth and invasion and has been implicated in several cancers. In recent studies, experiments with human and mouse uterine tissues revealed defective LMP2/ $\beta$ 1i expression in human Ut-LMS that was traced to the IFN- $\gamma$  pathway and the specific effect of somatic mutations in the catalytic domain of JAK-1, an IFN- $\gamma$  receptor-associated tyrosine kinase, on LMP2/ $\beta$ 1i transcriptional activation [22, 23, 25]. The differential responsiveness to genetically modified stable LMP2/ $\beta$ 1i expression in a SKN human Ut-LMS cell line was investigated to determine whether reintroducing LMP2/ $\beta$ 1i would affect tumorigenic properties for the development of human Ut-LMS and if the observed effect was due to the immunoproteosomal function of the protein. Recent reports demonstrated that LMP2 expression was markedly down-regulated in human uterine leiomyosarcoma (LMS) tissues in comparison with uterine leiomyoma (LMA) and normal myometrium tissues. Thus, LMP2 may be a potential diagnostic biomarker for distinguishing between LMS and LMA.

Further, we inferred that tumor growth and tumorigenesis were modulated by LMP2 expression, implicating LMP2 as an anti-oncogenic factor in uterine LMS. We isolated and expanded typical clones of each type and analyzed their growth properties, as well as the occurrence and expression of LMP2 in these typical clones. Growth rates, indicated as doubling time, were generally lower for the typical SKN-LMP2 (Flatrevertant cell type) clones than control SKN-CEM9 clones (Transform cell type) clones. FACS analysis demonstrated that the LMP2 expression possibly induced G1 arrest in the SKN-LMP2 (Flatrevertant cell type) clones. The efficiency of colony formation and size of the colonies in soft agar were greatly reduced for the F type clones. Tumor growth was clearly observed in mice inoculated with SKN-CEM9 (Transform cell type) clones; however, a reduction of tumor growth was observed in mice inoculated with the SKN-LMP2 (Flatrevertant cell type) clones. These experiments demonstrated that tumor growth and tumorigenesis were indeed modulated by LMP2 expression. The analysis of human Ut-LMS cells clarified the biological significance of LMP2/ $\beta$ 1i in



**Figure 2:** LMP2/ $\beta$ 1i molecules reportedly associate with cellular factor(s) to regulate cellular processes, such as the cell cycle and gene expression. Research experiments demonstrate that LMP2/ $\beta$ 1i-induced cellular morphological phenotypes are involved in the biological function of calponin h1.

malignant myometrium transformation including cellular morphological phenotype and the cell cycle, thus implicating LMP2/ $\beta$ 1i as an anti-tumorigenic candidate [22, 23, 25].

In addition, recent reports have shown an association between malignant transformation of the myometrium and reduced expression of calponin h1, a calcium-binding protein, which is specifically expressed in smooth muscle and binds calmodulin, actin, and tropomyosin [25-27]. Calponin h1 reportedly inhibits the ATPase activity of myosin and may play a role in smooth muscle contraction [28, 29]. Although calponin h1 may function as a tumor-suppressor in Ut-LMS, unlike mice lacking LMP2/ $\beta$ 1i, calponin h1-null mice do not exhibit Ut-LMS. The biological characterization of human Ut-LMS remains incomplete [25-27]. LMP2/ $\beta$ 1i molecules were reportedly associated with cellular factor(s) to regulate cellular processes, such as the cell cycle and gene expression. To examine the biological connection between LMP2/ $\beta$ 1i and calponin h1, cellular biological experiments were performed. The findings suggested that LMP2/ $\beta$ 1i-induced cellular morphological phenotypes are involved in the biological function of calponin h1 (Figure 2). Biological experiments on cell morphology following modified-gene expression suggest that single-unit LMP2/ $\beta$ 1i-mediated cellular factors other than calponin h1, prevent cell proliferation and tumorigenicity in Ut-LMS cells [22, 23, 25]. Elucidation of the mechanism by which LMP2/ $\beta$ 1i-induced biological events, including calponin h1 expression, are regulated may provide a great deal of information about the transformation of cellular phenotypes, the control of cell proliferative activity, and the pathogenesis of human uterine mesenchymal tumors at the molecular level. In conclusion, we have shown that LMP2/ $\beta$ 1i may be a tumor suppressor in human Ut-LMS. This role of LMP2/ $\beta$ 1i may lead to new therapeutic targets in human Ut-LMS.

## CONCLUSION

In the case of gynecological cancers, such as breast cancer, a female hormonal imbalance is often a risk factor for developing tumors [9-11]. As in the case of uterine LMA, however, a correlation between the development of human Ut-LMS, the female hormone, and hormone receptors has been unclearly understood. A recent report showed the expression of *Lmp2/ $\beta$ 1i* mRNA and protein in luminal and glandular epithelia, placenta villi, trophoblastic shells, and arterial endothelial cells [30-32]. These results implicate

LMP2/ $\beta$ 1i in the invasion of placental villi, degradation of the extracellular matrix, immune tolerance, glandular secretion, and angiogenesis [31]. Further study should help to elucidate the regulatory role of LMP2/ $\beta$ 1i in the implantation of embryos [24, 30, 31]. The LMP2/ $\beta$ 1i-null mouse was the first animal model of spontaneous Ut-LMS to be established. Defective LMP2/ $\beta$ 1i expression may be one of the causes of human Ut-LMS. The growth of cell lines that have JAK1 kinase activity has been demonstrated to be strongly inhibited by IFN- $\gamma$  treatment, whereas the growth of JAK1-deficient cell lines is unaffected [32]. Cellular effects of IFN- $\gamma$ , include inhibition of cellular proliferation and influences on apoptosis. Interestingly, in LMP2/ $\beta$ 1i-transfected human Ut-LMS cells, which have marked LMP2/ $\beta$ 1i expression, exogenous LMP2/ $\beta$ 1i expression resulted in cell growth inhibition [22]. Conversely, the growth of LMP2/ $\beta$ 1i-transfected human Ut-LMS cells was unaffected by IFN- $\gamma$  treatment [22]. Taken together, the effect of IFN- $\gamma$  on cell growth inhibition may be attributable to the inducibility of LMP2/ $\beta$ 1i. To demonstrate whether LMP2/ $\beta$ 1i is a potential biomarker for distinguishing human Ut-LMS from uterine LMA, we are investigating the reliability and characteristics of LMP2/ $\beta$ 1i as a diagnostic indicator with several clinical research facilities. The clinical research is yet to be concluded, and large-scale clinical studies need to be performed. Histologic and IHC characteristics of uterine mesenchymal tumors including cellular leiomyoma, bizarre leiomyoma mitotically active leiomyoma, and smooth muscle tumor of uncertain malignant potential (STUMP) are being examined at our clinical facilities. Clarification of the correlation between these factors and the development of human Ut-LMS and the identification of specific risk factors may lead to the development of new treatments for the disease. Human Ut-LMS is refractory to chemotherapy and has a poor prognosis. The molecular biological and cytological information obtained from research studies with LMP2/ $\beta$ 1i-null mice will contribute remarkably to the development of preventive methods, a potential diagnostic-biomarker, and new therapeutic approaches against human Ut-LMS.

(NOTE 1) The typical gross appearance is a large (>10cm), poorly circumscribed mass with a soft, fleshy consistency and a variegated cut surface that is grey-yellow to pink, with foci of hemorrhage and necrosis [7, 8]. The histologic classification of uterine sarcomas is based upon homology to normal cell types and includes human Ut-LMS (analogous to myometrium), stromal sarcoma (analogous to endometrial stroma),

and other heterologous cell types (i.e., osteosarcoma, liposarcoma). Microscopically, most human Ut-LMS are overtly malignant, with hypercellularity, coagulative tumor cell necrosis, abundant mitoses [ $>10$  to  $20$  mitotic figures (mf) per  $10$  high power fields (hpf)], atypical mitoses, cytologic atypia, and infiltrative borders. The mitotic rate is the most important determinant of malignancy, but is modified by the presence of necrosis and cytologic atypia. A diagnosis of human Ut-LMS may be made in the presence of tumor necrosis and any mitosis. In the absence of tumor necrosis, the diagnosis can be made with moderate to severe cytologic atypia and a mitotic index greater than  $10\text{mf}/10\text{hpf}$ . Without tumour necrosis and significant atypia, a high mitotic index is compatible with a benign clinical course, however, data is limited [7, 8].

### CONFLICT OF INTEREST

All authors report no conflict of interest.

### ACKNOWLEDGEMENTS

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### ABBREVIATIONS

LMP2	=	low-molecular mass polypeptide 2
Ut-LMS	=	uterine leiomyosarcoma
LMA	=	leiomyoma
SMT	=	smooth muscle tumor
JAK	=	Janus kinase
IFN- $\gamma$	=	interferon- $\gamma$
MECL-1	=	multicatalytic endopeptidase complex-like

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