

# A novel diagnostic marker: Proteasome LMP2/ $\beta$ 1i-differential expression in human uterus mesenchymal tumors

Takuma Hayashi<sup>1,7,8</sup>, Akiko Horiuchi.<sup>2</sup>, Nobuo Yaegashi.<sup>3</sup>, Tanri Shiozawa.<sup>4</sup>,  
Susumu Tonegawa.<sup>5</sup>, Ikuo Konishi<sup>6</sup>

<sup>1</sup>Dept. of Immunology and Infectious Disease, Shinshu University Graduate School of Medicine, Matsumoto, Nagano 390-8621, Japan

<sup>2</sup>Horiuchi Ladies Clinic, Matsumoto, Nagano 390-0821, Japan

<sup>3</sup>Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Miyagi 980-8574 Japan

<sup>4</sup>Department of Obstetrics and Gynecology, Shinshu University Graduate School of Medicine, Miyagi 390-8621 Japan

<sup>5</sup>Picower Institution and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139-4307 USA

<sup>6</sup>Dept. of Obstetrics and Gynecology, Kyoto University Graduate School of Medicine, Kyoto-city, Kyoto 606-8507, Japan

<sup>7</sup>Promoting Business using Advanced Technology, Japan Science and Technology Agency (JST), Chiyoda-ku, Tokyo 102-8666, Japan

<sup>8</sup>Sigma-Aldrich Collaboration Laboratory

## Email address

yoyoyo224@hotmail.com (T. Hayashi)

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**Abstract:** Uterine leiomyosarcoma (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 uterine LMS is not substantially correlated with hormonal conditions, and the risk factors are not yet known. Importantly, a diagnostic-biomarker, which distinguishes malignant LMS from benign tumor leiomyoma (LMA), is yet to be established. Accordingly, it is necessary to analyze risk factors associated with uterine LMS, to establish a treatment method. Proteasome LMP2/ $\beta$ 1i-deficient mice spontaneously develop uterine LMS, with a disease prevalence of ~40% by 14 months of age. We found LMP2/ $\beta$ 1i expression to be absent in human LMS, but present in human LMA. Therefore, defective-LMP2/ $\beta$ 1i expression may be one of the risk factors for LMS. LMP2/ $\beta$ 1i is a potential diagnostic-biomarker under the combination of candidate molecules for uterine mesenchymal tumors, especially uterine LMS, and may be a targeted-molecule for a new therapeutic approach. (160 words)

**Keywords:** LMP2/ $\beta$  1i, Uterine Leiomyosarcoma, Uterine Leiomyoma, Biomarker

## 1. 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 screening, uterine cervix cancer is decreasing in incidence, and usually detected at a very early stage. In contrast, cancer of the uterine body is increasing in incidence, and rarely detected at the initial stages. While most tumors of the uterine body are adeno-

carcinomas (derived from the subintimal gland), tumors of the uterine cervix are classified into squamous cancer and adenocarcinoma. Smooth muscle tumors (SMTs) which develop in the myometrium have been traditionally divided into benign leiomyoma (LMA) and malignant leiomyosarcoma (LMS) based on cytological atypia, mitotic activity and other criteria. Uterine LMS is relatively rare, having an estimated annual incidence of 0.64 per 100,000 women [1]. Uterine 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 uterine LMS is resistant to chemotherapy and radiotherapy, surgical intervention is virtually the only means of treatment [2-4]. The prognosis for uterine LMS is not good, and the five-year

survival rate is approximately 35% [5]. However, developing an efficient adjuvant therapy is expected to improve this. Uterine LMA may occur in as many as 70% ~ 80% of women by the age of 50 years [6]. Distinguishing uterine LMA from uterine LMS is very difficult, and a diagnosis generally requires surgery and cytology [7]. Diagnostic categories for uterine SMTs and morphological criteria are used to assign cases [8,9] (Attention 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 [8,9].

High estrogen levels are considered to significantly influence the development of tumors in the uterine body [10-12]. The mechanisms by which uterine LMA and LMS develop are not yet known, though tumors 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 uterine 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 uterine LMS. The identification of a risk factor associated with the development of uterine LMS would significantly contribute to the development of preventive and therapeutic treatments.

## 2. Development of Uterine Leiomyosarcoma in LMP2/ $\beta$ 1i-Deficient Mice

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 [13,14]. The proteasomal degradation is essential for many cellular processes, including the cell cycle, the regulation of gene expression and immunological function [15]. Interferon (IFN)- $\gamma$  induces the expression of large numbers of responsive genes, proteasome subunits, i.e., low-molecular mass polypeptide (LMP)2/ $\beta$ 1i, LMP7/ $\beta$ 5i, and LMP10/ $\beta$ 2i [16]. The individual expression of LMP2/ $\beta$ 1i, LMP7/ $\beta$ 5i and LMP10/ $\beta$ 2i subunits in various cell types or tissues is believed to contribute to the initiation and development of disorders. A recent study revealed a unique role for LMP7/ $\beta$ 5i in controlling pathogenic immune responses and provided a therapeutic rationale for targeting LMP7/ $\beta$ 5i in autoimmune disorders, especially rheumatoid arthritis [17].

Homozygous mice deficient in LMP2/ $\beta$ 1i show tissue- and substrate-dependent abnormalities in the biological functions of the proteasome [18]. Here we identify LMP2/ $\beta$ 1i, as obligatory for tumor surveillance and demonstrate a tissue-specific role for LMP2/ $\beta$ 1i in protection from spontaneous uterus neoplasms. In short, uterine LMS reportedly occurred in female LMP2/ $\beta$  1i-deficient mice at

age 6 months or older, and the incidence at 14 months of age was about 40% [19,20]. The curve indicating the incidence in mice is similar to that indicating the incidence of human uterine LMS, which occurs after menopause. Histological studies of LMP2/ $\beta$ 1i-lacking uterine tumors have revealed characteristic abnormalities of uterine LMS, and the tumors lacked lymphoid infiltrates, a sign of immune recognition, and consisted of uniform elongated myometrium cells arranged into bundles [19]. 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. Whereas relatively few ki-67/MIB1-positive cells, the proliferating cells of solid tumors, were observed in the basal cell layer of the normal myometrium, most of the basal cells vividly expressed ki-67/MIB1 in LMP2/ $\beta$ 1i-deficient mice [19]. This immunohistochemistry (IHC) study indicates abnormal proliferation of the LMP2/ $\beta$ 1i-lacking cells in the basal layer. LMP2/ $\beta$ 1i-deficient mice that have developed uterine LMS undergo considerable weight loss, and then die by 14 months of age [19,20]. The LMP2/ $\beta$ 1i-deficient mice also exhibit skeletal muscle metastasis from uterine LMS. Therefore it is likely that LMP2/ $\beta$ 1i-deficient mice with uterine LMS die as a result of the tumor mass and metastasis [19,20]. In general, it is not easy to distinguish uterine LMA from LMS, however, in mice, because of such characteristic pathological findings, significant weight loss, and skeletal muscle metastasis, a tumor that develops in the uterus of an LMP2/ $\beta$ 1i-deficient mouse can be considered malignant, i.e., a uterine LMS [19,20].

## 3. Defective LMP2/ $\beta$ 1i Expression in Human Uterine Leiomyosarcoma

IHC studies were performed to demonstrate the validity and reliability of LMP2/ $\beta$ 1i as a diagnostic biomarker under the combination of other candidate molecules, for instance cyclin B1, cyclin E and calponin h1, which reportedly function as anti-tumorigenic factor in human uterine LMS [21-25] (Table 1). IHC experiments revealed a serious loss in the ability to induce LMP2/ $\beta$ 1i and calponin h1 expression in human uterine LMS tissue in comparison with LMA or normal myometrium located in the same section [21-25]. Of the 54 cases we examined with uterine LMS, 46 were negative for LMP2/ $\beta$ 1i expression, 4 were focally positive, and 2 were partially positive [21,22,24]. Two LMS cases were stained for LMP2/ $\beta$ 1i. LMP2/ $\beta$ 1i levels were also evaluated in skeletal muscle and rectum metastases from individual uterine LMS patients. 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 LMS metastases, and IHC analyses showed positivity for ki-67/MIB1 and negativity for LMP2/ $\beta$ 1i. Histological findings were consistent with metastatic LMS for the skeletal muscle and rectum lesions. In western blot-

ting and RT-PCR experiments, LMP2/ $\beta$ 1i was expressed in normal myometrium, but not in human uterine LMS, both strongly supportive of the IHC findings [21,22]. Although we have previously demonstrated that the abnormal expression of the ovarian steroid receptors, TP53 and ki-67/MIB1 and mutations of TP53 were frequently associated with uterine LMS, defective LMP2/ $\beta$ 1i expression appears to be more characteristic of uterine LMS than these factors [21-25] (Table 1).

#### 4. LMP2/ $\beta$ 1i Differential Expressions in Human Uterine Mesenchymal Tumors

In the case of gynecological cancers, such as breast cancer, a female hormonal imbalance is often a risk factor for developing tumors [10-12]. As in the case of uterine LMA, however, a correlation between the development of uterine LMS, the female hormone, and hormone receptors has yet to be elucidated. A recent report showed the expression of Lmp2 mRNA and protein in luminal and glandular epithelia, placenta villi, trophoblastic shells, and arterial endothelial cells [26-27]. These results implicate LMP2/ $\beta$ 1i in the invasion of placental villi, degradation of the extracellular matrix, immune tolerance, glandular secretion, and angiogenesis [26-27]. The present study should help to elucidate the regulatory role of LMP2/ $\beta$ 1i in the implantation of embryos [26-27]. Unfortunately, it is unclear whether defective LMP2/ $\beta$ 1i expression is involved in the onset of uterine LMS. Risk factors for its development however, have not been identified because of the absence of a suitable animal model. The LMP2/ $\beta$ 1i-deficient mouse was the first animal model of spontaneous uterine LMS to be established [19,20]. Defective LMP2/ $\beta$ 1i expression may be one of the causes of uterine LMS. To demonstrate whether LMP2/ $\beta$ 1i is a potential biomarker for distinguishing uterine LMS from 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. To demonstrate whether LMP2/ $\beta$ 1i is a potential biomarker for distinguishing human uterine LMS from uterine LMA under the combination with other candidate molecules, especially cyclin B1, cyclin E and calponin h1 which are identified as potential diagnostic candidates [21,22,24,25,30-37], we are investigating the reliability and characteristics of LMP2/ $\beta$ 1i as a diagnostic indicator with several clinical research facilities [22-25,30-32] (Table 1). The clinical research is yet to be concluded, and large-scale clinical studies need to be performed with additional clinical research facilities. Histologic and IHC characteristics of uterine mesenchymal tumors including mitotically active leiomyoma, bizarre leiomyoma, lipoleiomyoma, uterine smooth muscle tumors of uncertain malignant potential (STUMP), leiomyomatoid angiomatous neuroendocrine tumor (LANT) are summarized [32-37] (Table 1). Clarifi-

cation of the correlation between these factors and the development of uterine LMS and the identification of specific risk factors may lead to the development of new treatments for the disease. Uterine LMS is refractory to chemotherapy and has a poor prognosis. The molecular biological and cytological information obtained from LMP2/ $\beta$ 1i-deficient mice will contribute remarkably to the development of preventive methods, a potential diagnostic-biomarker, and new therapeutic approaches against uterine LMS.

#### 5. Conclusion

Human uterine LMS is refractory to chemotherapy and has a poor prognosis. Defective LMP2/ $\beta$ 1i expression is likely to be one of the risk factors in the development of human uterine LMS as it is in the LMP2/ $\beta$ 1i-deficient mouse. LMP2/ $\beta$ 1i might function as an anti-tumorigenic factor in human uterine LMS. The molecular biological and cytological information obtained from LMP2/ $\beta$ 1i-deficient mice will contribute remarkably to the development of preventive methods, a potential diagnostic biomarker, and new therapeutic approaches against human mesenchymal tumors, especially human uterine LMS.

#### Attention 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 [8,9]. The histologic classification of uterine sarcomas is based upon homology to normal cell types and include human uterine LMS (analogous to myometrium), stromal sarcoma (analogous to endometrial stroma), and other heterologous cell types (i.e., osteosarcoma, liposarcoma). Microscopically, most human uterine 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. Mitotic rate is the most important determinant of malignancy, but is modified by the presence of necrosis and cytologic atypia. The diagnosis of uterine LMS may be made in the presence of tumor necrosis and any mitoses. 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 tumor necrosis and significant atypia, a high mitotic index is compatible with a benign clinical course; however, data is limited [8,9].

#### Conflict Interest

All authors report no conflict of interest.

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**Table 1.** Classification of human uterine mesenchymal tumors

Tumor type	Atypia	Mitotic activity	Necrosis	Protein expression*													Cliical comments
				Cyt	Des	MSA	SMA	Vim	ER/P R	End	EGF	CyB	CyE	LMP 2	Cal	Ki67	
Endometrial stromal tumors.																	
Endometrial stromal nodule	minimal	infrequent	-/inconspicuous	+	-	+	+	+	+++	+	*	+	-	++	++	-	Absence of myometrial infiltration
Endometrial stromal sarcoma		infrequent	-/inconspicuous	-/+	-	+	+	+	+++	+	+	+	-	-/+	++	-/+	
Undifferentiated endometrial sarcoma	marked	Frequent (atypical MF)	+	-/+	foc	*	*	-	-	+	+	+	+	-/+	+	+	Lack specific differentiation
Smooth muscle tumors																	
Leiomyoma, NOS		<5 MF/10HPF		foc	+	+	+	*	+++	-/+	-/+	+	-	++	++	-/+	Well-circumscribed
Mitotically active leiomyoma		>5 MF/10HPF		*	+	+	+	*	+++	-/+	-/+	+	-	++	++	-/+	Pseudocapsul
Cellular leiomyoma		infrequent		*	+	+	+	*	+++	-/+	-/+	+	-	++	++	-/+	Increased cellularity
Hemorrhagic cellular leiomyoma		infrequent		*	+	+	+	*	+++	-/+	-/+	+	-	++	++	-/+	Hormone induced changes
Epithelioid leiomyoma		<5 MF/10HPF		*	+	+	+	*	+++	-/+	-/+	*	-	++	++	-/+	Epithelial-like cells
Myxoid leiomyoma		<5 MF/10HPF		*	*	*	*	*	*	-/+	-/+	*	+	-/+	+	+	Myxoid material

		F																
Atypical leiomyoma	moderate	<10 MF/10HP F	-	-	+	+	+	*	+++	+	-/+	+	+	-/+	-/+	+	Separates tumor cells	
Lipoleiomyoma STUMP#	-	infrequent	-	*	+	+	+	*	+++	*	-/+	*	+	-/+	*	-/+	Scattered adipocytes	
	-	infrequent	-	*	+	+	+	*	*	*	-/+	*	-/+	-/+	*	-/+		
	-/+	>10 MF/10HP F	+/-uncertain	*	+	+	+	*	*	*	-/+	*	-/+	-/+	*	-/+		
	Marked	borderline	-	*	+	+	+	*	*	*	-/+	*	-/+	-/+	*	-/+		
	-	infrequent	+/-difficult classify	*	+	+	+	*	*	*	-/+	*	-/+	-/+	*	-/+		
Leiomyosarcoma	moderate	>10 MF/10HP F	+	+	+	*	-/+	-	-	+	-/+	++	+++	-	-	++	Infiltrative	
Leiomyosarcoma epithelioid variant	moderate	>5 MF/10HP F	+	+	+	*	-/+	-	-	+	-/+	++	+++	-	-	++	Infiltrative, >50% epithelioid cells	
Leiomyosarcoma myxoid variant	moderate	Any MF	+	+	+	*	-/+	-	-	+	-/+	++	++	-	-	++	Infiltrative, myxoid extracellular matrix	
Leiomyomatoid tumor																		
LANT#	absent	frequent	+	+	-	-	+	+	*	*	+	+	++	-	-	-/+	NOTE1	

\*insufficient data or not applicable.

Cyt., cytokeratin; Des., Desmin; MSA, muscle specific actin; SMA, smooth muscle actin; Vim., vimentin; ER/PR, estrogen receptor/progesterone receptor; End., Endoglin, CD105/TGFβ receptor (stem cell marker); EGF, EGFR, epidermal growth factor receptor; CyB, cyclin B1; CyE, cyclin E, LMP2, low-molecular mass polypeptide; Cal., calponin h1; CD56, neural cell adhesion molecule (N-CAM); WT-1, wilms tumor 1; NOS, not otherwise specified; MF, magnification factor; HPF, high power field; Foc., focal; STUMP, smooth muscle tumors of uncertain malignant potential. Protein expression\*, estimated-protein expressions by immunoblot analysis, immunohistochemistry (IHC) and/or RT-PCR (quantitative-PCR), +/-, partial expression; +, expression; ++, medium expression; +++, high expression; -, no evidence of expression; ER/PR(ref.24), LMP2(ref.22,23), cyclin E(ref.24,32), calponin h1(ref.29,30,31), Ki-67(ref.24,33). STUMP#(ref.33,34). Cyclin E, LMP2, calponin h1 are potential bio-marker for human uterine mesenchymal tumours. LANT#, leiomyomatoid angiomatous neuroendocrin tumor (LANT) is described as a dimorphic neurosecretory tumor with a leiomyomatous vascular component (ref.35,36). NOTE1, Low-grade neuroendocrine tumor possibly related to null cell adenoma.