Candidate Molecules as Tumor Suppressor for Human Uterine Mesenchymal Tumor

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ABSTRACT : Uterine leiomyosarcoma (Ut-LMS) develops more often in myometrium 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 factor(s) are not yet known. Importantly, a diagnostic-biomarker, which distinguishes malignant tumor Ut-LMS from benign tumor leiomyoma (LMA), is yet to be established. Accordingly, it is necessary to examine risk factor(s) associated with Ut-LMS, to establish a diagnosis and a clinical treatment method. The mice with a homozygous deficiency for proteasome β-ring subunit, low-molecular mass polypeptide (LMP)2/β1i spontaneously develop Ut-LMS, with a disease prevalence of ~37% by 12 months of age. In the recent study, we found LMP2/β1i expression to be absent in human Ut-LMS, but clearly present in other human uterine mesenchymal tumors including uterine LMA. Further analyses with clinical materials and the gene-modified mice have not clarified the biological significance of the TP53 and retinoblastoma (Rb) pathway in malignant myometrium transformation, thus implicating LMP2/β1i as an anti-tumorigenic candidate. This role of LMP2/$\square$ 1i as a tumor suppressor may lead to new therapeutic targets in human Ut-LMS. (191 words)

KEYWORDS: LMP2/β1i, p53, Rb, tumor suppressor, leiomyosarcoma, mesenchymal tumor,

I. INTRODUCTION

The uterus is made up of three special layered linings of tissue and muscle. The middle layer of the uterus is called the myometrium, and it is also known as the muscular uterine layer. It comprises of smooth muscles which is vital during childbirth to move the baby out of the womb. Uterine mesenchymal tumors, which develop in the myometrium, have been traditionally divided into benign leiomyoma (LMA) and malignant uterine leiomyosarcoma (Ut-LMS) based on cytological atypia, mitotic activity and other criteria. Ut-LMS is relatively rare mesenchymal tumor, having an estimated annual incidence of 0.64 per 100,000 women [1]. Ut-LMS accounts for 2%~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 human Ut-LMS is resistant to chemotherapy and radiotherapy, surgical intervention is virtually the only means of treatment [2-4]. Ut-LMS is an aggressive malignancy, with a 5-year survival of only 35% for tumors confined to the uterus [5]. However, developing an efficient adjuvant therapy is expected to improve the prognosis for human Ut-LMS. Uterine LMA may occur in as many as 70%~80% of women by the age of 50 years [6]. Distinguishing human uterine LMA from Ut-LMS is very difficult, and a diagnosis generally requires surgery and cytoscopy [7]. Diagnostic categories for uterine mesenchymal tumors and morphological criteria are used to assign cases [8,9]. The non standard subtypes of uterine mesenchymal tumors 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 molecular mechanisms by which uterine LMA and human Ut-LMS transform are not yet known, though tumors that have initiated and grown 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 human 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 human Ut-LMS. The TP53 tumor suppressor pathway is one of the most well characterized pathways in various malignant tumors. The retinoblastoma (Rb) pathway furthermore represents a second major tumor suppressor pathway deregulated in many malignant tumors. However, correlation between these pathways and human Ut-LMS tumorigenesis is unclearly understood unlike epithelial malignant tumors i.e. cancer.The ubiquitin-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 *β ring subunits* that contain three to seven protease active sites. Alternative *β-ring* forms denoted LMP2/β1i can be expressed in the myometrium in response to exposure to pro-inflammatory signals such as cytokines, in particular, interferon (IFN)-γ. Ut-LMS reportedly occurred in female LMP2/β1i-deficient mice at age 6 months or older, and the incidence at 12 months of age was about 37%. The determination of the malignant potential of mesenchymal tumors 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. The identification of a risk factor and/or biological candidate(s) associated with the development of human Ut-LMS, i.e. LMP2/β1i, would significantly contribute to the development of preventive and therapeutic treatments.

II. DEVELOPMENT OF UT-LMS IN LMP2/β1I-DEFFICIENT 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, and it is located in the nucleus and the cytoplasm [13,14]. The ubiquitin-proteasome degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression and immunological function [15]. IFN-γ induces the expression of large numbers of responsive genes, proteasome subunits, i.e., LMP2/β1i, LMP7/β5i and LMP10/β2i [16]. The individual expression of LMP2/β1i, LMP7/β5i and LMP10/β2i 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/β5i in controlling pathogenic immune responses and provided a therapeutic rationale for targeting LMP7/β5i in autoimmune disorders, especially rheumatoid arthritis [17].

Recent reports demonstrate LMP2/β1i as obligatory for tumor surveillance and a tissue- specific role for LMP2/β1i in protection from spontaneous uterus neoplasms [18,19]. Homozygous mice deficient in LMP2/β1i show tissue- and substrate- dependent abnormalities in the biological functions of the proteasome [18,20]. Ut-LMS reportedly occurred in female LMP2/β1i-deficient mice at age 6 months or older, and the incidence at 14 months of age was about 40% [19,20] (Figure 1). The disease prevalence in mice is similar to that of human Ut-LMS, which occurs after menopause [19]. Pathological studies of LMP2/β1i-deficient 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 myometrium cells arranged into bundles (Figure 1). The nuclei of the tumor cells varied in size and shape, furthermore, mitosis was frequent. The tumor consisted of uniform elongated myometrium cells arranged into bundles. In contrast, the myometrium cells of its parental mice, C57BL/6 mice were normal in appearance [19,20]. Whereas relatively few MIB1/ki-67-positive cells, the proliferating cells, were observed in the basal cell layer of the normal myometrium, most of the basal cells in LMP2/β1i-deficient mice vividly expressed MIB1/ki-67 [19] (Figure 1). This immunohistochemistry (IHC) study indicates abnormal proliferation of the LMP2/β1i-lacking cells in the basal layer. LMP2/β1i-deficient mice that have developed Ut-LMS undergo considerable body-weight loss, and then die by 14 months of age. They also potentially exhibit skeletal muscle metastasis from the Ut-LMS [21]. Therefore these research findings suggest that LMP2/β1i-deficient mice with Ut-LMS die as a result of tumor growth and metastasis. In general, it is not easy to distinguish uterine mesenchymal tumors from human Ut-LMS, however, in mice, because of such characteristic pathological findings, significant body-weight loss, and skeletal muscle metastasis, a tumor that develops in the uterus of an LMP2/β1i-deficient mouse can be considered malignant, i.e., an Ut-LMS [19-21].

III. DEFECTIVE LMP2/B1I EXPRESSION IN HUMAN UT-LMS

The non-standard subtypes of uterine mesenchymal tumors 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 [7-9]. IHC studies were performed to demonstrate the validity and reliability of LMP2/β1i as a diagnostic biomarker under the combination of other candidate molecules, for instance cyclin E and calponin h1 [22,23].

Of the 54 cases we examined with human Ut-LMS, 46 cases were negative for LMP2/β1i expression, 4

cases were focally positive, and 2 cases were partially positive [23]. Two human Ut-LMS cases were stained for LMP2/β1i. The expression levels of LMP2/β1i were also evaluated in skeletal muscle and rectum metastases from individual human Ut-LMS patients [23]. Histological findings were consistent with metastatic LMS for the skeletal muscle and rectum lesions. In western blotting and RT-PCR experiments, LMP2/β1i was expressed in normal myometrium, but not in human Ut-LMS, both strongly supportive of the IHC findings [22-24] (Figure 2).

IV. TUMOR SUPPRESSOR AND ONCOGENIC PATHWAY IN UT-LMS

The TP53 pathway: Molecular analyses have shown that many of the canonical tumor suppressor pathways, such as the TP53 and retinoblastoma (Rb) pathways are ablated in these tumors [25]. Furthermore, some sarcomas also harbor activating oncogenic mutations; such as expression of oncogenic K-ras. Together, disruption of these genes and pathways are thought to be a driving force in sarcomagenesis. The TP53 tumor suppressor pathway is one of the most well characterized pathways in cancers [26]. The *TP53* gene encodes a transcription factor required for the activation of numerous DNA damage-dependent checkpoint response and apoptotic genes [27,28], and thus its activities are often ablated in many cancers. In addition to loss of TP53 functions via inherited germline somatic mutations, the TP53 pathway is commonly disrupted by point mutations in the *TP53* gene during sporadic sarcomagenesis [29]. However, even though *TP53* gene alterations are widely regarded as having a significant impact on sarcomagenesis, many sarcomas retain wild type TP53, yet phenotypically display a loss of TP53 function. These findings suggest that changes in other components of the TP53 pathway; such as amplification of Mdm2, a negative regulator of the TP53 pathway, may result in TP53 inactivation [30,31]. Furthermore, both mice and humans with elevated levels of Mdm2 due to a high frequency single nucleotide polymorphism in the Mdm2 promoter (Mdm2SNP309) are more susceptible to sarcoma formation [32-34]. Additionally, deletion or silencing of $p19^{Arf}$ (p14Arf in human), an inhibitor of the MDM2-TP53 axis, often results in development of sarcomas. Together, these data indicate that while inactivation of the TP53 pathway is observed in the vast majority of human sarcomas, the mechanisms leading to disruption of the pathway can vary greatly.

People who inherit only one functional copy of the *TP53* gene will most likely develop tumors in early adulthood, a disease known as Li-Fraumeni syndrome. More than 50 percent of human tumors contain a mutation or deletion of the *TP53* gene [35]. To increase tumor incidence and better assess the role of systemic expression of p53 in responses to initiation of Ut-LMS tumorigenesis, LMP2/β1i-deficient mice were bred with Tp53-deficient mice to create $Lmp2^{-/-}Tp53^{-/-}$ double knockout mice. Ut-LMS incidence and death rates were similar in $Lmp2^{-/-}Tp53^{-/-}$ mice and closely matched those for control $Lmp2^{-/-}Tp53^{+/+}$ mice. The correlation between defective p53 function and Ut-LMS tumorigenesis is unclear. Although we previously demonstrated that the abnormal expression of ovarian steroid receptors, p53 and MIB1/ki-67 and mutations of p53 were frequently associated with human Ut-LMS, defective LMP2/β1i expression appears to be more characteristic of Ut-LMS than any of these factors [22-24].

The retinoblastoma (Rb) pathway: The Rb pathway represents a second major tumor suppressor pathway deregulated in many sarcomas. Individuals inheriting a germline *Rb* mutation typically develop cancers of the eye early in life [36-38]. However, in addition to retinal cancers, these children have a significantly higher propensity to develop sarcomas than the general population [39]. While inheritance of a germline *Rb* alterations increases sarcoma risk, there are also numerous examples of sporadic sarcomas harboring spontaneous Rb somatic mutations and deletions, particularly osteosarcomas and rhabdomyosarcomas [40]. Furthermore, $p16^{Ikn4a}$, a negative regulator of the CDK-cyclin complexes that phosphorylate and activate Rb, is often deleted in sarcomas [41,42].

Long term survivors of hereditary retinoblastoma are at risk of developing a variety of non-ocular second primary malignancies, the most common histological subtype of which is osteosarcoma. The reported risk varies widely, but a cumulative incidence of 1% for each year of life has been suggested as an approximate estimate. Earlier reports indicated that many of these malignancies are radiation or chemotherapy induced [43-46]. However, more recent studies [46-49] suggest that the development of second primary neoplasms in patients with hereditary retinoblastoma results partly from a genetic predisposition and partly from the potentiating effect of radiotherapy on tumorigenesis through mutation of the second *Rb* gene (RB1) allele, the first *RB1* allele being mutated in all patients with hereditary retinoblastoma [50]. A recent analysis of second primary neoplasms in retinoblastoma survivors revealed that 76% of the second tumors occurred in the head and neck region and that soft tissue sarcomas

were the single largest category, comprising 24% of the total [49]. The median age at diagnosis of a second tumor for the entire series was 16.4 years. The youngest patient to develop a soft tissue tumor was 4

years old and the oldest was 41. In previously reported cases of leiomyosarcoma occurring outside the head and neck region, the patients have been in the 4th to 6th decades. Importantly, in female patients, uterine leiomyosarcoma has not been reported after hereditary Rb, although simultaneously occurring benign Ut-LMS have been described [51].

Recent reports describe three patients who developed leiomyosarcomas, one involving the subcutaneous tissue of the thigh and the pelvic soft tissues and the other the urinary bladder, following hereditary retinoblastoma 36, 38 and 49 years earlier, respectively [51,52]. We also have reported a case of primary bladder LMS in a 45-year-old woman with a history of hereditary Rb. The cases we report, together with others described, suggest that with increasing duration of survival after hereditary retinoblastoma there is an increased risk of developing leiomyosarcoma in areas remote from the site of irradiation. Such patients thus require appropriate follow up. Over all research experiments including with gene-deficient mouse models and clinical research suggest that defective Rb expression does not take part in Ut-LMS onset.

V. CONCLUSION

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 human Ut-LMS, the female hormone, and hormone receptors has been unclear. A recent report showed the expression of *LMP2/β1i* mRNA and protein in luminal and glandular epitheliua, placenta villi, trophoblastic shells, and arterial endothelial cells [53-55]. These results implicate LMP2/β1i in the invasion of placental villi, degradation of the extracellular matrix, immune tolerance, glandular secretion, and angiogenesis [53-55]. However, these findings do not help to elucidate the regulatory role of LMP2/β1i in human Ut-LMS tumorigenesis. The LMP2/β1i-deficient mouse was the first animal model of spontaneous Ut-LMS to be established [19,22,23]. In the recent studies, LMP2/β1i is reported to negatively regulate human Ut-LMS independently of its role in the proteasome [56-59]. Moreover, several lines of evidence indicate that calcium binding protein, calponin h1 clearly affects LMP2/β1i-induced cellular morphological chances [58,59]. Further experiments are also required to elucidate the molecular mechanism of human Ut-LMS tumorigenesis involved biological significance of LMP2/β1i. 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 neuroendocrin tumor (LANT) are summarized [60-65]. 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 clinical treatments for the disease.

VI. FINAL CONSIDERATIONS

Human Ut-LMS is refractory to chemotherapy and has a poor prognosis. Defective LMP2/β1i expression is likely to be one of the risk factors in the development of human Ut-LMS as it is in the LMP2/β1i-deficient mouse. While mouse model can not completely predict the outcome of Ut-LMS, the molecular biological and cytological information obtained from LMP2/β1i-deficient mice and human clinical materials will contribute remarkably to the development of preventive methods, a potential diagnostic-biomarker, and new therapeutic approaches against human mesenchymal tumors, especially human Ut-LMS.

VII. ACKNOWLEDGEMENTS:

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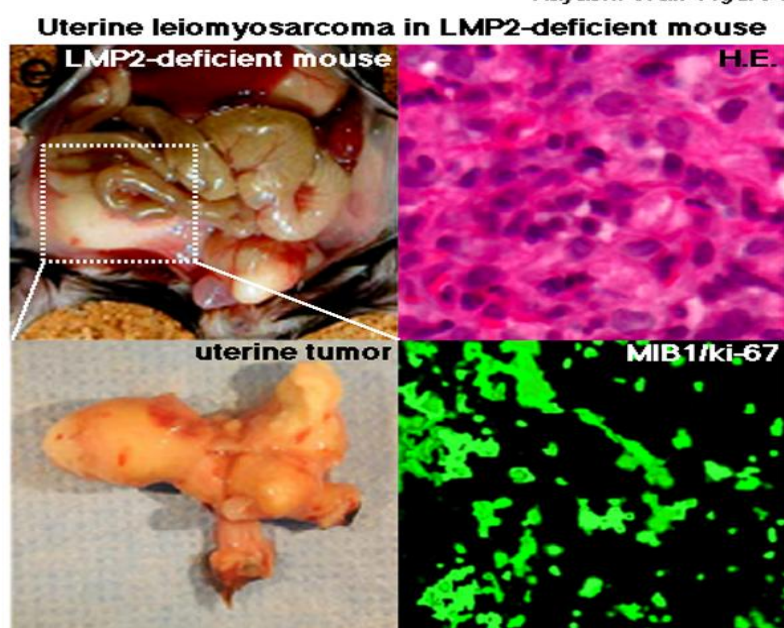[65] Avritscher R, Iyer1 RB, Ro J, Whitman G. Lipoleiomyoma of the Uterus. Am. J. Radiol. 2001; 177: 856

Figure 1. Homozygous mice deficient in LMP2/β1i, an interferon (IFN)-γ-inducible factor, show tissue-

and substrate-dependent abnormalities in the biological functions of the proteasome [19,20]. Ut-LMS reportedly occurred in female LMP2/β1i-deficient mice at age 6 months or older, and the incidence at 14 months of age was about 40% [19,20]
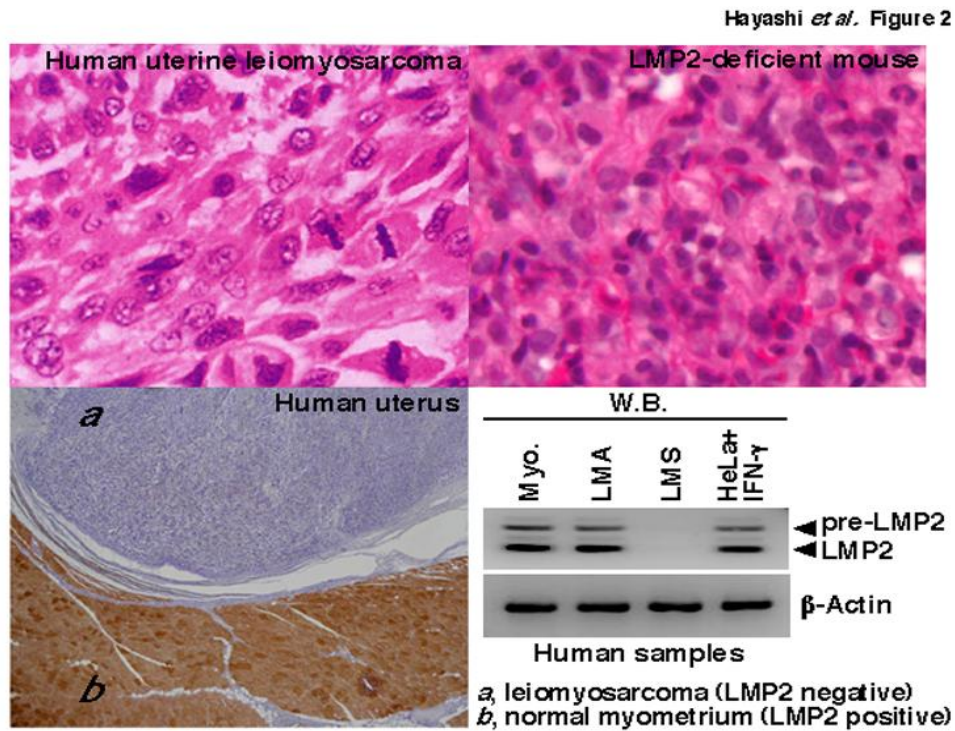
Figure 2. Defective LMP2/β1i expression in human uterine leiomyosarcoma. H.E. staining tissue sections of uterine leomyosarcoma of LMP2/β1i-deficient mouse and patient. Immunohistochmical staining tissue section of human uterine leiomyosarcoma with anti-human LMP2/β1i monoclonal antibody. Extracts of 50 μg were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The levels of LMP2 and β-actin were examined by western blot (W.B.) analysis with appropriate antibodies. Myo.; human myometrium, LMA; human leiomyoma, LMS; human leiomyosarcoma, HeLa+IFN-γ; HeLa cells treated by IFN-γ.