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Original Articles

Establishment and characterization of a novel orthotopic mouse model for human uterine sarcoma with different metastatic potentials

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ABSTRACT

Uterine sarcomas are rare and aggressive gynecologic tumors with a poor prognosis because of recurrence and metastasis. However, the mechanisms of uterine sarcoma metastasis are largely unknown. To investigate this mechanism, we developed a novel uterine sarcoma tissue-derived orthotopic and metastatic model in KSN nude mice using a green fluorescent protein stably expressed uterine sarcoma cell line, MES-SA. Histological analysis showed that all orthotopic primary tumors were undifferentiated sarcoma. Primary tumors were characterized by high ^{18}F-fluorodeoxyglucose uptake with a positive correlation to the number of pulmonary metastases. In addition, we generated uterine sarcoma cell sublines with high or low metastatic potentials by serial in vivo selection. Microarray analysis between orthotopic tumors with high and low metastatic potentials revealed differential expression of genes related to cell proliferation and migration (*TNNT1*, *COL1A2*, and *ZIC1*). Our model would be useful to compensate for the limited clinical cases of uterine sarcoma and to investigate the molecular mechanisms of metastatic uterine sarcoma.

Introduction

Uterine sarcomas are a rare gynecologic malignancy accounting for 3–7% of uterine tumors [1]. Classifications of uterine sarcoma include carcinosarcoma, leiomyosarcoma (LMS), endometrial stromal sarcoma, adenosarcoma, and high-grade undifferentiated sarcoma. Carcinosarcoma is considered by some to be a high-risk dedifferentiated variant of endometrial adenocarcinoma [2]. The prognosis of patients that have undergone a hysterectomy is poor, even at the early stage. The 5-year survival rates are about 50–55% and 8–12% for stage I and advanced stages, respectively [1]. The main reason why uterine sarcoma has a poor prognosis is the high risk of recurrence and metastasis, especially to the lung (about 50% of patients). Although a clinical protocol is desired for improvement of treatment effects in uterine sarcomas, the development of efficient therapies has been hampered by the infrequent occurrence of the disease. Thus, there is great interest in studying uterine sarcoma biology in animal models, especially metastasis.

To elucidate the mechanisms of uterine sarcoma development and metastasis, several studies have reported knockout and transgenic mouse models [3]. Deletion of p53 in the reproductive tract [4] or low molecular mass polypeptide-2 results in the spontaneous development of uterine LMS in female mice [5]. In addition, uterine LMS develops in T antigens of SV40 early region or Cripto-1 transgenic female mice [6,7]. However, metastases to other organs have not been observed in these mouse models. Therefore, an orthotopic preclinical animal model with primary tumors and a pulmonary metastatic potential would be necessary for further analyses of the mechanisms of uterine sarcoma metastasis as well as drug development.

The MES-SA cell line was established from a human uterine sarcoma derived from a 56-year-old female patient [8]. Subcutaneously injected MES-SA cells in nude mice develop into tumors that maintain histological features nearly identical to those of the primary human uterine sarcoma. However, no metastatic potential has been observed in an orthotopic model using MES-SA cells [8–11].

Abbreviations: COL1A2, collagen, type 1, α2; FDG, fluorodeoxyglucose; GFP, green fluorescent protein; GLUT, glucose transporter; HCC, hepatocellular carcinoma; LMS, leiomyosarcoma; PET, positron emission tomography; TNNT1, troponin T type 1; ZIC1, Zic family member 1.

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In this study, we established an orthotopic model using MES-SA cells that stably express green fluorescent protein (MES-SA/GFP cells) with spontaneous metastasis to the lung. Histological analysis showed that all tumors were undifferentiated sarcomas with high expression of glucose transporter (GLUT) 1 and GLUT3. In addition, ^{18}F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) imaging detected primary tumor in this animal model. In addition, ^{18}F-FDG uptake in primary tumors had a significant positive correlation to the number of pulmonary metastases. To assess the genetic properties of the metastasis of human uterine sarcoma, several variant sublines of cells with different metastatic potentials were isolated by serial in vivo selection. Comprehensive gene expression analyses of the orthotopic tumors with different metastatic potentials showed that 467 and 235 genes were up- or down-regulated by ≥2-fold, respectively, in tumors with a high metastatic potential compared with those exhibiting a low metastatic potential.

This metastasis model will provide not only a screening system to identify anti-sarcoma drugs but also the means to investigate the molecular mechanisms of uterine sarcoma metastasis. Moreover, analyses of this preclinical animal model by various imaging modalities such as PET and bioluminescence will provide valuable biological information.

Materials and methods

Cell culture and mice

MES-SA cells (a human uterine sarcoma cell line) were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and gentamycin. Six-week-old, female KSN/Slc nude mice were obtained from Japan SLC (Hamamatsu, Japan). Nude mice were reared in standard laboratory cages with free access to food and water for at least 1 week before experiments.

Establishment of stable GFP-expressing uterine sarcoma cell lines

Establishment of human uterine sarcoma cells stably expressing GFP (MES-SA/GFP cells) was performed using the PiggyBac Transposon Vector System according to the manufacturer's instructions. Briefly, the PiggyBac Dual Promoter Vector (PB513B-1) and Super PiggyBac Transposase Expression Vector were transfected into MES-SA cells using lipofectamine LTX. The cells were then cultured in medium containing puromycin. The medium was replaced every 2 days. At 1 week after transfection, the cells were selected by limiting dilution cloning in medium containing puromycin. Finally, we established several cell lines of which two were used in further experiments.

Orthotopic uterine sarcoma model

All animal procedures and care were approved by the Regulations for Animal Research at the University of Fukui. Animal studies were performed in accordance with NIH Animal Research Advisory Committee guidelines. For subcutaneous transplantation, MES-SA/GFP cells were harvested with an EDTA solution (0.8 mM EDTA, 137 mM NaCl, 6.7 mM $NaHCO_3$, 5.6 mM dextrose, and 5.4 mM KCl). Collected cells were washed twice with D-PBS (−) and resuspended in D-PBS (−) at 5×10^6 cells/100 μL. Resuspended cells were kept on ice until the following procedures. Suspended cells were subcutaneously injected into female nude mice anesthetized with isoflurane. When the heterotopic xenografted tumor reached 15 mm in diameter, the tumor was extirpated from the mouse and cut into 2-mm pieces on ice. Immediately, a piece of tumor was orthotopically transplanted into the uterocervical junction via a small incision in another female mouse. Primary uterine sarcoma was extirpated from the mouse before the tumor reached 20 mm in diameter. The tumor was then used for in vivo passaging or further analyses. To isolate cell sublines with different metastatic potentials from MES-SA/GFP cells, we performed serial in vivo passaging (three to seven passages) of primary tumors from MES-SA/GFP tumor-bearing KSN mice with high or low metastatic potentials. Tumor-bearing mice with high or low metastatic potentials, which had the highest or lowest numbers of pulmonary metastasis, respectively, were used for in vivo passaging. The tumor volume was calculated by the formula: $V = \pi/6 \times L \times W^2$ (V, volume; L, length; W, width). Primary uterine sarcoma and pulmonary metastases were examined for fluorescent signals under a Leica MZ10F stereomicroscope (Leica Microsystems, Wetzlar, Germany).

Generation of primary MES-SA/GFP cell lines from orthotopic tumors

A primary tumor was minced, disaggregated by Accumax, passed through a 45-μm filter, and then cultured in McCoy's 5A medium supplemented with 10% FBS and puromycin. Primary MES-SA/GFP cells were examined for fluorescent signals under an Axio Observer.A1 inverted microscope (ZEISS, Oberkochen, Germany).

Reagents, antibodies, cell proliferation assay, in vitro growth curve analysis, sphere formation, migration and invasion assays, karyotype analysis, immunohistochemical staining, small animal PET imaging, DNA microarray, quantitative real-time PCR and statistical analysis are described in the supplementary file.

Results

Establishment of the spontaneous pulmonary metastasis model of human uterine sarcoma

To develop an evaluation system for metastasis, two MES-SA/GFP cell lines were generated from independent clones. KSN female nude mice were inoculated subcutaneously with MES-SA/GFP cells. At 5 weeks after transplantation, the extirpated subcutaneous tumor was cut into 2-mm pieces and then directly transplanted into the uterocervical junction of other mice (Fig. 1A). Orthotopic transplanted uterine sarcomas were observed at 3 weeks post-transplantation, and had reached 2341 ± 400 mm^3 at 6 weeks post-transplantation (Fig. 1A and B). In vivo passaging of small tumor pieces from orthotopic tumor-bearing KSN mice to other mice was performed for three passages. In this model, the orthotopic-transplanted tumor exhibited tumorigenicity [65.9% (54/82)]. In mice bearing orthotopic tumors, metastases were observed mainly in the lung and rarely in lymph nodes or peritoneal dissemination. About 72% (39/54) and 15% (8/54) of mice developed pulmonary micro- and macro-(nodules) metastases, respectively (Fig. 1C). Pulmonary metastases were also confirmed by immunohistochemistry (Fig. 1D). Histopathological analysis showed that all of the tumors were undifferentiated uterine sarcomas that were characterized by round-shaped cells with marked cellular pleomorphism, cytologic atypia, hyperchromatic nuclei, high mitotic activity (almost always exceeding 10MF/10HPF), and frequent extensive necrosis (Fig. 2A). Immunohistochemical analysis revealed additional characteristics that are consistent with undifferentiated uterine sarcoma. Ki-67 staining indicated a high proliferation index. We detected overexpression of p53, while the expression of cyclin-dependent kinase inhibitor p16 was partially present in tumors. Immunoreactivity showing diffuse vimentin and sarcomeric actin, CD10, and focally positive alpha-smooth muscle actin indicated a uterine mesenchymal tumor (Fig. 2A). Interestingly, overexpression of GLUT1, GLUT3, and HIF-1 was detected in tumors (Fig. 2B). The tumors also showed high expression of p-AKT and p-mTOR, but not p-MAPK (Fig. 2C). It has been reported that abnormalities in PTEN-AKT-mTOR signaling were detected in sarcomas [12]. These results indicated the tumors were a typical uterine sarcoma with a metastatic potential to the lung. Therefore, this model may be useful for preclinical research.

Correlation between ^{18}F-FDG uptake in primary tumors and pulmonary metastasis

^{18}F-FDG PET shows a correlation between tracer uptake and expression of GLUT1 and Ki-67 in uterine sarcoma [13]. PET imaging showed that ^{18}F-FDG accumulation in orthotopic uterine tumor-bearing mice was higher than that in the control (Fig. 3A and B). Consistent with the report, we observed a marked ^{18}F-FDG standardized uptake values (2.6 ± 0.54, n = 6) in all primary tumors. Interestingly, ^{18}F-FDG uptake in primary tumors had a significant positive correlation with the metastasis score (Fig. 3C, r = 0.893, p = 0.0129). These results indicated that ^{18}F-FDG PET imaging was useful for detection of the primary tumor along with its metastatic cells as well as evaluation of the grade (e.g., metastatic potential) of the orthotopic animal model.