High Expression of CD109 Antigen Regulates the Phenotype of Cancer Stem-Like Cells/Cancer-Initiating Cells in the Novel Epithelioid Sarcoma Cell Line ESX and Is Related to Poor Prognosis of Soft Tissue Sarcoma

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Abstract

Epithelioid sarcoma (ES) is a relatively rare, highly malignant soft tissue sarcoma. The mainstay of treatment is resection or amputation. Currently other therapeutic options available for this disease are limited. Therefore, a novel therapeutic option needs to be developed. In the present study, we established a new human ES cell line (ESX) and analyzed the characteristics of its cancer stem-like cells/cancer-initiating cells (CSCs/CICs) based on ALDH1 activity. We demonstrated that a subpopulation of ESX cells with high ALDH1 activity (ALDHhigh cells) correlated with enhanced clonogenic ability, sphere-formation ability, and invasiveness in vitro and showed higher tumorigenicity in vivo. Next, using gene expression profiling, we identified CD109, a GPI-anchored protein upregulated in the ALDHhigh cells. CD109 mRNA was highly expressed in various sarcoma cell lines, but weakly expressed in normal adult tissues. CD109-positive cells in ESX predominantly formed spheres in culture, whereas siCD109 reduced ALDH1 expression and inhibited the cell proliferation in vitro. Subsequently, we evaluated the expression of CD109 protein in 80 clinical specimens of soft tissue sarcoma. We found a strong correlation between CD109 protein expression and the prognosis (P = 0.009). In conclusion, CD109 might be a CSC/CIC marker in epithelioid sarcoma. Moreover, CD109 is a promising prognostic biomarker and a molecular target of cancer therapy for sarcomas including ES.

Introduction

Epithelioid sarcoma (ES) is a relatively rare and highly malignant soft tissue sarcoma (STS) accounting for <1% of all STSs [1]. The mainstay of treatment is aggressive, radical local resection or amputation. Currently other therapeutic options available for ES are limited. Therefore, a novel therapeutic option needs to be developed.

Recent studies have revealed that several human cancers contain a small subpopulation of cells called cancer stem-like cells (CSCs)/cancer initiating cells (CICs), which are defined by the ability of self-renewal, multi-differentiation potential, and tumorigenesis. Therefore, CSCs/CICs are believed to be responsible for the progression and relapse of cancer [2]. In the current study, we isolated CSCs/CICs based on aldehyde dehydrogenase 1 (ALDH1) activity. Human ALDHs are a family of NAD (P)+-dependent enzymes involved in detoxifying a wide...
variety of aldehydes to their corresponding weak carboxylic acids [3]. They serve to detoxify both xenobiotic aldehydes (e.g., cyclophosphamide) and many other intracellular aldehydes, including ethanol and vitamin A [4]. Therefore, ALDH activity is important for drug resistance and the response to oxidative stress [5]. Recently ALDH1 activity was used, either alone or in combination with cell surface markers, to identify CSCs/CICs in hematologic malignancies and carcinomas derived from the lung and prostate [6-8].

We established a new ES cell line (designated ESX) from a 73-year-old woman. Next, we investigated CICs/CSCs in ES cell lines and isolated CSCs/CICs based on ALDH activity. Finally, we demonstrate that CD109 is a potential CSC/CIC marker that may be useful as a prognostic biomarker and a molecular target of cancer therapy for sarcomas, including ES.

Materials and Methods

Ethics Statement

Mice were maintained and experimented on in accordance with the guidelines of and after approval by the Ethics Committee of Sapporo Medical University School of Medicine, Animal Experimentation Center under permit number 08-006. Any animal found unhealthy or sick was promptly euthanized. All studies were approved by the Institutional Review Board of Sapporo Medical University Hospital. Written informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki.

Primary tumor

A 73-year-old Japanese woman was admitted to our hospital with a 9-month history of swelling of the left thigh. The swelling had gradually enlarged and become painful. A well-demarcated elastic soft mass was palpable in the medial aspect of the left thigh. Magnetic resonance imaging revealed a subcutaneous tumor and lymph node metastases in the inguinal region (Figure S1A). The tumor (3×3 cm) was homogeneously hypointense relative to skeletal muscle in T1-weighted images, whereas it was heterogeneously iso- and hyperintense relative to skeletal muscle in T2-weighted images. Computed tomography revealed no pulmonary metastasis. The serum CA125 level was 6.6 U/ml (normal: <40 U/ml). Open biopsy showed that the tumor was composed of sheets of large cells with vesicular chromatin, prominent nucleoli, and amphophilic cytoplasm, with peripheral palisading of epithelioid cells around necrotic areas (Figure S1B). Immunohistochemical analysis revealed that the tumor was positive for AE1/AE3 and vimentin, but negative for CD34, CA125, and S-100. The tumor was weakly positive for INI1 analyzed by immunohistochemistry, fluorescence in situ hybridization (FISH) analysis revealed the heterozygous deletion of INI1 in 17 of 50 tumor cells (34%) (Figure S1D). Upon these findings, the tumor was diagnosed as proximal-type epithelioid sarcoma. Wide resection of the tumor and lymph node dissection were performed, but systemic chemotherapy was not. Unfortunately, pulmonary metastases developed 12 weeks after surgery and the patient died 16 weeks after the definitive surgery.

Establishment of a new ES cell line, ESX

The resected specimen of the primary tumor was rinsed with phosphate-buffered saline, cut into small pieces with a scalpel and cultured in Iscove’s modified Dulbecco’s Eagle’s medium (IMDM; GIBCO BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Inc., South Logan, UT). The tumors were incubated at 37°C in 5% CO₂. The cell line (ESX) was maintained for more than 24 months.

Cell lines

Human osteosarcoma cell lines (NY, U2OS and HOS), human Ewing sarcoma cell lines (SKES, WES, and RDES), the human synovial sarcoma cell line FUJI, and the human ES cell line VA-ES-BJ were purchased from the Japanese Collection of Research Resources Cell Bank (Tokyo, Japan) and American Type Culture Collection (Manassas, VA, USA). The human synovial sarcoma cell line YaFuSS and FU-EPSS-1 were gifts from Dr J. Toguchida (Kyoto University) [9] and Dr H. Iwasaki (Fukuoka University) [10]. The human osteosarcoma cell line OS2000 and KIKU, and the human malignant fibrous histiocytoma cell lines MCH2003 and MCH2004 were established in our laboratory [11-14].

ALDEFLUOR assay

The ALDEFLUOR kit (StemCell Technologies, Vancouver, Canada) was used to separate the population with high ALDH1 activity. Cells (1×10⁶) were suspended in ALDEFLUOR assay buffer containing an ALDH1 substrate, body-pseudoaldehyde, at the concentration of 1μmol/L and incubated for 50 min at 37°C according to the manufacturer’s protocol. A specific inhibitor of ALDH1, diethylaminobenzaldehyde (DEAB), was used at 50mmol/L as a negative control.

CD109-positive cell sorting

The cells were washed once with PBS and then centrifuged at 440g at 4°C for 5 min using an LX120 (Tomy, Tokyo, Japan). The cell pellets were resuspended and incubated for 60 min at 4°C with a 100-fold dilution of a mouse anti-CD109 antibody (R&D Systems). Then samples were washed with PBS 3 times and stained and incubated for 60 min at 4°C with a 500-fold dilution of an FITC-labeled anti-mouse secondary antibody (KPL, Gaithersburg, MD). Cell sorting was performed using a FACSAria II (BD Bioscience, San Jose, CA). Collected data were analyzed using BD FACSDiva V6.1.3 (BD Bioscience). Propidium isodide (PI; Life Technologies Corp.) was used to stain live cells.

RNA preparation and PCR analysis

Total RNAs were extracted from cells using the RNeasyMini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using iScript III and an oligo(dT) primer (Life Technologies Corp.). Human Multiple Tissue cDNA Panels I and II, and the Human Malignant Mixture Tissue cDNA Panel (Clontech; Mountainview, CA) were used as normal tissue cDNAs. PCR was performed using KOD Dash (TOYOBO, Osaka, Japan) to
detect CD109. The primer sequences used were 5'-TTGAATTCGAGGATCC-3' and 5'-AAAGGUUGGUCAGUAGCACCCA-3'. The PCR mixture was denatured at 98°C for 2 min, followed by 30 cycles at 98°C for 15s, at 55°C for 2s, and at 74°C for 30s. GAPDH and beta-actin were used as internal controls. Real-time PCR was performed using the StepOne system (Life Technologies Corp.). Primers and probes were designed using the TapMan gene expression assay (Life Technologies Corp.). Thermal cycling was performed with 40 cycles of 95°C for 1s, followed by 60°C for 20 min. Each experiment was done in triplicate and normalized to the GAPDH gene as an internal control.

siRNA

CD109 siRNA (siCD109) (5'-AAAGGUUGGUCAGUAGCACCCA-3') was designed using BLOCK-it RNAi (Life Technologies Corp.). Control siRNA was obtained from Life Technologies Corp. The siRNAs were transfected using Lipofectamine RNAiMAX transfection reagent (Life Technologies Corp.)

Spherical colony formation assay

Cells were plated at 1000 cells per well in six-well ultra-low attachment plates (Corning Inc., Corning, NY) and cultured in DMEM/F12 medium with 10ng/ml hEGF, 10ng/ml hbFGF, and 2% B-27 (Life Technologies Corp.) at 37°C in 5% CO₂. On day 7, the number of colonies was counted under an inverted contrast microscope.

Cell proliferation assay

Cells were seeded in duplicate at a density of 2.5×10⁴ cells/well in 24-well plates. On the following day, siRNAs were transfected. At 48 hr, 72 hr and 120 hr cells were trypsinized and counted with a Coulter Counter (Beckman Coulter, Inc. Brea, CA).

Basement membrane matrix invasion assay

Invasiveness was analyzed using a Matrigel™ Invasion Chamber (BD Biosciences). Briefly, 2.5×10⁴ cells were placed on inserts in the wells in IMDM with 10% FBS. After 52 hours, the cells were stained using a Hemacolor staining kit (Merck Millipore, Billerica, MA) and the degree of migration was determined.

Gene expression profiling

RNA from ALDH⁺high cells was labeled with Cy5 dye and RNA from ALDH⁺low cells were labeled with Cy3 dye. The probe mixture was hybridized for 40 hours at 65°C using a Human Whole Genome Microarray (G4112F) (Agilent Technologies, Santa Clara, CA). The array was scanned after washing with a G2565BA Microarray Scanner and fluorescent signals were acquired using Feature Extraction software (Agilent Technologies). The average expression ratio of Cy5 to Cy3 was determined per gene. A dye swap experiment was also done to label ALDH⁺high and ALDH⁺low cells with Cy3 and Cy5, respectively. An average ratio of more than 2.0, reproducible in 2 experiments, was determined to indicate differential up-regulation in ALDH⁺high cells. The accession number of ArrayExpress is E-MEXP-3826. We focused on membrane protein-related genes for cell sorting and as therapeutic targets using antibodies. Therefore, we selected membrane protein-related genes based on information obtained from GeneCards (http://www.genecards.org) (Table S2), followed by screening of mRNA expression in ALDH⁺high and ALDH⁺low cells by RT-PCR (data not shown).

Xenografting

ALDH⁺high and ALDH⁺low cells freshly sorted from cell lines were washed and suspended in PBS. Then the cells (1×10⁶, 1×10⁷ and 1×10⁸ cells in 50μl of PBS) were mixed with an equal volume of Matrigel (BD Science) and subcutaneously injected into the bilateral sides of the lower back in female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prdkcscid/J, Charles River Laboratory, Yokohama, Japan). Tumor growth was monitored weekly for 10 weeks. Then the xenografted tumors were resected and analyzed.

Immunohistochemical staining

Formalin-fixed paraffin-embedded sections from 80 STS patients who underwent resection for stage I-III tumors and chemotherapy or radiotherapy for stage IV tumors between 2004 and 2009 in the Sapporo Medical University Hospital were used for CD109 staining as previously described [15]. The reactivity of the anti-CD109 antibody was determined by the staining pattern of the tumor cell membrane and graded as follows: 0 (no staining), 1 (partial staining of the membrane), 2 (mild to moderate circumferential staining of the membrane) and 3 (strong circumferential staining of the membrane). If the score was 2 or 3 in more than 10% of the tumor cells, it was considered to be positive. The clones used, antigen retrieval methods, and commercial sources of the antibodies used in the study are listed in Table S1.

Statistical methods

The Mann-Whitney test was used to compare in vitro data and the differences in tumor volume using IBM SPSS Statistics (IBM Corp., Armonk, NY). The Fisher exact test was used to compare the associations between the CD109 expression level and clinicopathological factors using SAS software 9.3 (SAS Institute, Cary, NC). Postoperative disease-free survival (DFS) and overall survival (OS) were estimated using Kaplan-Meier plots. Prognostic significance was evaluated by the log-rank test. Univariate and multivariate analyses for hazards ratios (HR) in OS were performed by Cox’s proportional-hazards regression with backward selection using IBM SPSS Statistics. A probability of less than 0.05 was considered statistically significant.

Results

Establishment of the ES cell line ESX

A tumor cell culture obtained from a patient with ES of the left thigh (Figure S1) was maintained for over 1 year and designated ESX (Figure 1A). The ESX cells were spindle-
Identification of an ALDH<sup>high</sup> population in ES cell lines

We performed ALDEFLUOR assay to detect ALDH<sup>high</sup> populations containing CSCs/CICs in the epithelioid sarcoma cell lines. As shown in Figure 2A, all 3 ES cell lines (ESX, VA-ES-BJ, and FU-EPS-1) contained ALDH<sup>high</sup> populations, although the proportion of ALDH<sup>high</sup> cells varied. The mean proportions of ALDH<sup>high</sup> cells were 36.6%, 14.2%, and 13.8% in ESX, VA-ES-BJ, and FU-EPS-1, respectively. The proportions of ALDH<sup>high</sup> cells in ES cell lines were lower than in the other sarcoma cell lines (Figure S2). The proportion of ALDH<sup>high</sup> cells in ESX was significantly higher than in the others (p<0.001) (Figure 2B). We then analyzed the differentiation abilities of ALDH<sup>high</sup> and ALDH<sup>low</sup> ESX cells in vitro 9 days and 12 days after sorting (Figure 2C). ALDH<sup>high</sup> and ALDH<sup>low</sup> cells showed a tendency to differentiate into ALDH<sup>low</sup> and ALDH<sup>high</sup>, respectively. These results could indicate the flexible plasticity of CSCs/CICs of ES cells. The frequency of ALDH<sup>high</sup> cells converted from ALDH<sup>low</sup> cells after in vitro culture was 16.3% on Day 12. On the other hand, when sorted ALDH<sup>high</sup> cells were cultured in vitro the frequency of remaining ALDH<sup>high</sup> cells was 36.2% on Day 12, indicating that ALDH<sup>high</sup> cells could maintain higher enzyme activity than ALDH<sup>low</sup> cells.

Cancer-initiating ability of ALDH<sup>high</sup> cells of ES cell lines

In a previous study, we showed that CSCs/CICs could generate floating spheroid-like bodies in a serum-free medium [16]. We therefore determined whether ALDH<sup>high</sup> and ALDH<sup>low</sup> cells of ESX could generate spherical colonies. As shown in Figure 3A, most ALDH<sup>low</sup> cells died and the others formed a few small colonies. In contrast, the number of colonies derived from ALDH<sup>high</sup> cells was significantly higher than that from ALDH<sup>low</sup> cells (p<0.001) (Figure 3B). Next, we examined the expression of stem/progenitor cell-related genes Sox2, Oct3/4, and Nanog [17,18]. Interestingly, in the ALDH<sup>high</sup> population the mRNA expression of Sox2, Oct3/4, and Nanog was lower than in ALDH<sup>low</sup> (Figure 3C). These findings were in marked contrast to the CSCs/CICs of carcinomas, which suggested that gene expression of stem cell-related genes was not involved in the cancer-initiating ability, at least in epithelioid sarcoma [19]. In the ALDH<sup>high</sup> cells of VA-ES-BJ and FU-EPS-1, the mRNA expression of stem/progenitor cell-related genes was not lower than in ALDH<sup>low</sup> cells (Figure S3).

To determine whether CSCs/CICs were abundant in the ALDH<sup>high</sup> population, we performed xenograft transplantation of ALDH<sup>high</sup> and ALDH<sup>low</sup> cells of ESX, VA-ES-BJ, and FU-EPS-1 into NOD/SCID mice. The sorted ALDH<sup>high</sup> and ALDH<sup>low</sup> cells were injected subcutaneously into mice, and tumor growth was monitored weekly for 10 weeks. In ESX, 1×10<sup>5</sup> ALDH<sup>high</sup> cells showed tumorigenicity in one of four mice. In contrast, 1×10<sup>4</sup> ALDH<sup>low</sup> cells failed to form tumors. On the other hand, 1×10<sup>4</sup> ALDH<sup>high</sup> and ALDH<sup>low</sup> cells both showed tumorigenicity (Figure 3, D and E). However, the frequency of tumor formation was lower for ALDH<sup>low</sup> cells than for ALDH<sup>high</sup> cells (Figure 3F). The histology of the tumors derived from ALDH<sup>high</sup> and ALDH<sup>low</sup> cells of ESX showed no major differences (Figure 3G). In the case of FU-EPS-1, neither ALDH<sup>high</sup> nor ALDH<sup>low</sup> cells could form tumors. In VA-ES-BJ, ALDH<sup>low</sup> cells formed a tumor but ALDH<sup>high</sup> cells did not (Figure 3F). These findings suggested that ALDH<sup>high</sup> cells of ESX contained a higher number of tumorigenic cells compatible with CSCs/CICs than ALDH<sup>low</sup> cells. In contrast, we considered that VA-ES-BJ and FU-EPS-1 did not contain a CSCs/CICs population in ALDH<sup>high</sup> cells that was similar to that of ESX. Therefore, ESX was used for the further characterization of the ALDH<sup>high</sup> population containing CSCs/CICs.

Identification of the novel marker CD109 for the CSCs/CICs of ES

We screened the upregulated genes in ALDH<sup>high</sup> cells using a cDNA microarray to identify membrane protein-related genes for cell sorting and therapeutic targets using antibodies. The upregulated membrane protein-related genes in ALDH<sup>high</sup> cells of ESX are summarized in Table S2. Among them, we selected CD109, which was upregulated in ALDH<sup>high</sup> cells, as a representative marker (Figure 5A). The other molecules listed in Table S2 were not upregulated in ALDH<sup>high</sup> cells assessed by RT-PCR (data not shown). CD109, a GPl-anchored glycoprotein, was originally identified as a leukemia antigen. It has been reported that CD109 is expressed on activated T lymphocytes and platelets, endothelial cells and a subpopulation of CD34+ hematopoietic stem and progenitor cells [20,21]. CD109 has also been reported as the cell surface antigen on acute myeloid leukemia [20]. On the other hand, in the other ES cell lines there was no difference of CD109 mRNA expression between ALDH<sup>high</sup> and ALDH<sup>low</sup> cells.
supported the hypothesis that CD109 could be essential to maintain the cancer-initiating ability of CSCs/CICs in ALDH\textsuperscript{high} cells of ESX.

We assessed the CD109 mRNA expression profiles in sarcomas and human fetal and normal adult tissues by RT-PCR. In addition to ES, CD109 mRNA expression was observed in 9 of 14 (64\%) sarcoma cell lines (Figure 5B). As
Figure 2. Identification of an ALDH\textsuperscript{high} population in ES cell lines. A. All 3 ES cell lines, ESX, VA-ES-BJ and FU-EPS-1, demonstrated ALDH activity. FACS analysis of ALDH1 expression in cells and the DEAB control.
B. The proportions of ALDH\textsuperscript{high} cells in ESX, VA-ES-BJ and FU-EPS-1. Bars represent mean±SEM (n=4) of multiple experiments. ***p<0.001, determined by the Mann-Whitney test.
C. Differentiation of ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells in vitro. Sorted ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells were analyzed on days 0 (immediately after sorting), 9 and 12. Representative fluorescence-activated cell sorting analysis is shown.

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Figure 3. Cancer-initiating ability of ALDH\textsuperscript{high} cells \textit{in vitro} and \textit{in vivo}.  

A. The features of spherical colonies derived from resultant ALDH\textsuperscript{high} cells and ALDH\textsuperscript{low} cells of ESX. 

B. The number of spherical colonies from ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells of ESX. Bars represent mean±SEM (n=6). ***p<0.001, determined by the Mann-Whitney test. 

C. The mRNA expression of stem/progenitor cell-related genes. RNA was isolated from freshly sorted spheroid cells (1×10\textsuperscript{5}) on day 7. Bars represent mean±SEM. *p<0.05, determined by the Mann-Whitney test. 

D. The features of xenotransplanted cells \textit{in vivo}. Macroscopic features of 1×10\textsuperscript{4} ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells of ESX in an NOD/SCID mouse at 10 weeks after xenotransplantation. 

E. Tumor growth curve of ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells of ES cell lines in NOD/SCID mice. 

F. Tumorigenesis of ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells of ES cell lines in NOD/SCID mice. 

G. H&E of the xenotransplanted tumors derived from ALDH\textsuperscript{high} and ALDH\textsuperscript{low} cells of ESX (1×10\textsuperscript{4}) (above; scale bar 50μm, below; 20μm). 

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Figure 4. Invasive ability of ALDH$^{\text{high}}$ cells of ESX. A. The features of invasion derived from resultant ALDH$^{\text{high}}$ and ALDH$^{\text{low}}$ cells. Invading cells are indicated by circular dots and arrows.
B. The number of invasive cells. Bars represent mean±SEM (n=4). ***p<0.001, determined by the Mann-Whitney test.
C. The mRNA expression of EMT-related genes. Bars represent mean±SEM. *p<0.05, determined by the Mann-Whitney test.
Figure 5. CD109 expression in sarcoma cells and normal adult and fetal tissues. A. Expression of CD109 mRNA in ES cell lines. Bars represent mean±SEM. ***p<0.001, determined by the Mann-Whitney test. N.S.: not significant.

B. Expression of CD109 mRNA in human sarcoma cell lines. Cell lines of epithelioid sarcoma (FU-EPS-1 and VA-ES-BJ), osteosarcoma (OS2000, KIKU, NY, U2OS, Saos-2, HuO9 and HOS), Ewing sarcoma (SKES, WES and RDES), synovial sarcoma (Fuji and YaFuSS) and malignant fibrous histiocytoma (MFH2003 and MFH2004) were used.

C. Expression of CD109 mRNA in human fetal tissues (upper panel) and human adult tissues (lower panel). ESX was used as a positive control.

D. Immunohistochemistry of CD109 in normal adult tissues.

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shown in Figure 5C, CD109 mRNA was weakly expressed in fetal skin, spleen, liver, lung, kidney, heart, pancreas and colon tissues. In adult tissues, CD109 mRNA was also weakly expressed in the lung, heart, small intestine, and testis. However, CD109 protein was expressed in alveoli of the lung, but not in heart and colon tissues (Figure 5D). These results suggested that CD109 could be an antigen highly expressed in various human sarcomas.

**CD109 is associated with cancer-initiating ability and TGFβ/Smad signaling**

The proportion of CD109-positive cells was 0.2% in ESX (Figure 6A), which was lower than that of ALDH<sup>high</sup> cells (Figure 1A). However, higher expression of ALDH1 mRNA was detected in CD109-positive cells than in CD109-negative cells (Figure 6B). Moreover, the number of spherical colonies derived from CD109-positive cells was higher than that from CD109-negative cells (Figure 6C and D). In addition, the expression of stemness-related genes was lower in CD109-positive cells than in CD109-negative cells (Figure 6E). These features of CD109-positive cells were similar to those of ALDH<sup>high</sup> cells and suggested that CD109 might regulate cancer-initiating ability in ESX.

Next, we examined the effect of CD109 knockdown on cell proliferation. ESX cells were treated with siCD109, trypsinized and counted after 48 hr, 72 hr and 120 hr. Expression of both mRNA and protein of CD109 was downregulated (Figure S4, A, B and C). As shown in Figure 7A, siCD109 significantly inhibited cell proliferation ($p<0.05$). These results also supported the idea that CD109 plays an important role in cancer-initiating ability.

CD109 is a component of the TGF-β1 receptor 1 complex and negatively regulates TGF-β/Smad signaling [22]. Therefore, we hypothesized that CD109 could positively regulate ALDH activity and negatively regulate TGFβ1R1 expression in the TGF-β/Smad signaling pathway. We examined whether downregulation of ALDH1A1 and upregulation of TGFβ1R1 were induced by silencing of CD109. As shown in Figure 7B, ALDH1A1 mRNA was downregulated in siCD109-transfected ESX cells. On the other hand, TGFβ1R1 mRNA was upregulated in the siCD109-transfected cells (Figure 7C). Moreover, silencing of CD109 reduced the ALDH<sup>high</sup> population in ESX cells (Figure 7, D and E). These findings suggested that CD109 positively regulated ALDH1A1 activity and negatively regulated the TGF-β/Smad signaling pathway.

**CD109 protein is associated with poor prognosis in soft tissue sarcoma patients**

To determine the clinical relevance of CD109 expression in soft tissue sarcomas (STSs), we evaluated CD109 expression by immunohistochemistry in the primary extremity lesions of 80 STSs. The clinical characteristics of these patients are summarized in Table S3. Representative staining patterns with the anti-CD109 antibody are shown in Figure 8A. Positive expression of CD109 protein was identified in 18% (15/80) of the STSs. Higher expression of CD109 was significantly associated with histologic grade, tumor stage, and distant metastases ($p=0.021$, 0.0012, and 0.0003 respectively). However, no other significant correlation was found between CD109 expression and other clinical parameters of the STSs. As shown in Figure 8B and 8C, positive CD109 expression, including in well-differentiated liposarcomas, was significantly associated with decreased probabilities of overall survival (OS) and disease-free survival (DFS) ($P=8.3\times10^{-4}$ and 4.5×10<sup>-4</sup>, respectively). Moreover, excluding well-differentiated liposarcomas, positive CD109 expression was also significantly associated with decreased probabilities of OS and DFS ($P=0.006$ and 0.049, respectively). The OS rates at 5 years were 46.7% for CD109-positive patients and 85.3% in those who were CD109 negative. Several variables were tested to assess whether they had an impact on survival. Univariate and multivariate analyses revealed that the histologic grade and expression of CD109 were independent risk factors for poor outcome (Table 1, Table 2). The hazard ratio (HR) of OS for the CD109-positive group was 3.85 (95% confidence interval CI, 1.40-10.55). Thus, CD109 might be a predictive biomarker for both distant metastasis and the prognosis of STSs in the clinical setting.

**Discussion**

In the present study, we (i) established and characterized the new ES cell line ESX; (ii) demonstrated that the ALDH<sup>high</sup> population of ESX contained CSCs/CICs showing *in vitro* and *in vivo* tumorigenesis; (iii) found that high expression of CD109 in ALDH<sup>high</sup> cells was important to maintain tumorigenesis as the important feature of CSCs/CICs; and (iv) showed the prognostic impact of CD109 expression on patients with STSs. ES is a rare, slow-growing malignant tumor. With an infiltrative growth pattern and a propensity for extension along fascial planes, and nerves, it is characteristically associated with multiple local recurrences and late metastasis [23]. The effects of multiagent chemotherapy and radiotherapy remain unclear; therefore, novel therapeutic options need to be developed. The establishment of an experimental model is imperative to investigate the biological characteristics of ES and develop novel therapeutic options. To the best of our knowledge, only 11 human ES cell lines have been reported to date [10,24-33]. ESX might therefore facilitate further studies on the biological characteristics of this rare tumor entity.

Using ESX, we tried to identify CSCs/CICs of ES. Previously, we performed side population (SP) analysis to identify CSCs/CICs of sarcomas [16]. However, SP cells were hardly detected in ESX (data not shown). Therefore, we used the ALDEFLUOR assay. In this study, we demonstrated that an ALDH<sup>high</sup> population existed in ESX, and that the ALDH<sup>high</sup> cells possessed repopulating capacity and high tumor-forming ability *in vitro* and *in vivo*. ALDH is a cytosolic isoenzyme involved in the detoxification of intracellular aldehydes by oxidation and conversion of retinol to retinoic acid, and it confers resistance to chemotherapeutic agents such as cyclophosphamide [34]. Therefore, it makes sense that ALDH<sup>high</sup> cells contain CSCs/CICs. Visus et al. were the first to describe the use of ALDH1A1 as a putative CSC/CIC marker in head and neck squamous cell carcinomas [35]. In the field of sarcoma, Awad
Figure 6

A. FACS analysis of CD109 positive cells in ESX was shown.
B. The mRNA expression of CD109 and ALDH1A1. Bars represent mean±SEM. ***p<0.001, determined by the Mann-Whitney test.
C. The features of spherical colonies (indicated by arrows) derived from resultant CD109-positive cells and CD109-negative cells of ESX (original magnification ×40).
D. The number of spherical colonies from CD109-positive cells and CD109-negative cells of ESX. Bars represent mean±SEM (n=3). **p<0.01, determined by the Mann-Whitney test.
E. The mRNA expression of stem/progenitor cell-related genes. Bars represent mean±SEM. *p<0.05, determined by the Mann-Whitney test.

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Figure 7. **CD109 positively regulates ALDH activity and negatively regulates the TGFβ/Smad signaling pathway.**

A. The cell proliferation curve of ESX treated with SiCD109 or control siRNA. Bars represent mean±SEM (n=4). *p<0.05, determined by the Mann-Whitney test.

B, C. mRNA expression of ALDH1A1 (D) and TGFβ1R1 (E). The expression of ALDH1A1 and TGFβ1R1 was evaluated by real-time PCR two days after transfection.

D. FACS analysis of ALDH activity.

E. The proportion of ALDH<sup>high</sup> cells. Bars represent mean±SEM (n=4). *p<0.05, determined by the Mann-Whitney test.

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Figure 8

A. Representative immunostaining of CD109 (original magnification x400).

B, C. Prognosis estimated by Kaplan-Meier plots for patients with soft tissue sarcoma including (B) and excluding (C) the patients with well-differentiated liposarcoma.

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et al. tested ALDH activity in Ewing sarcoma, defining CSCs/CICs as those cells that showed the highest ALDH activity [36]. However, there are no other reports regarding CSCs/CICs of sarcoma. This is the first report identifying and characterizing of CSCs/CICs as those cells that showed the highest ALDH activity [36]. Twist and Snail, which could promote EMT, are also considered to be key factors in the maintenance of CSCs/CICs. Twist and Snail induce not only increased potential for invasiveness and metastases, but also increased ability to form spheres and generate tumors in xenografts [39]. In this study, the ALDH<sup>high</sup> population expressed Twist 1 and Snail 1 at higher levels than ALDH<sup>low</sup> cells. ALDH<sup>high</sup> cells of ESX showed higher tumorigenicity than CD109-negative cells. Therefore we considered that CD109 could be a representative molecule of ALDH<sup>high</sup> cells of ESX promoted cancer-initiating ability as the result of CD109-mediated inhibition of TGF-β.

CD109-positive cells of ESX highly expressed ALDH1 and showed higher tumorigenicity than CD109-negative cells. Furthermore, the expression status of stemness-related genes of CD109-positive cells was similar to that of ALDH<sup>high</sup> cells of ESX. Therefore, we considered that CD109 could be a promising therapeutic target not only for sarcoma but also for epithelial cancer.

CD109 is a TGF-β co-receptor, a component of the TGF-β1 receptor 1 (TGFβ1R1) complex. It accelerates TGF-β receptor degradation and negatively regulates TGF-β/Smad signaling [44,45]. In some human cancers, CD109 actually impairs TGF-β/Smad signaling [46]. TGF-β can play both tumor-suppressive and tumor-promoting roles. Especially in the early phase of cancer initiation, TGF-β acts as an anti-oncogenic factor [47]. In this study, ALDH<sup>high</sup> cells of ESX showed higher tumorigenicity and higher expression of CD109. In addition, silencing of CD109 upregulated TGFβ1R1 mRNA in ESX cells. These findings suggested that CD109 expressed in ALDH<sup>high</sup> cells of ESX promoted cancer-initiating ability as the result of the CD109-mediated inhibition of TGF-β.

CD109-positive cells of ESX highly expressed ALDH1 and showed higher tumorigenicity than CD109-negative cells. Furthermore, the expression status of stemness-related genes of CD109-positive cells was similar to that of ALDH<sup>high</sup> cells of ESX. Therefore, we considered that CD109 could be a representative molecule of ALDH<sup>high</sup> cells. In addition, knockdown of CD109 decreased ALDH1 activity in ESX cells. These results suggested that CD109 might regulate ALDH1 activity and confer the characteristics of CSCs/CICs in ESX cells. However, the proportion of CD109-positive cells was lower than that of ALDH<sup>high</sup> cells of ESX. Therefore we hypothesized that other factors might also regulate ALDH1 activity.

The evaluation of the clinical specimens of STSs revealed a strong correlation between CD109 expression and DFS and OS, suggesting that CD109 could be a promising prognostic biomarker in STSs. Although it is reported that CD109 is preferentially expressed in the early stage of tumorigenesis in

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**Table 1. Univariate analysis of the variables in overall survival.**

| Variable        | Hazard ratio | 95% CI     | P value |
|-----------------|--------------|------------|---------|
| Tumor size      |              |            |         |
| <5cm            | 1            |            |         |
| 5-10cm          | 3.38         | 0.70-16.2  | 0.129   |
| >10cm           | 2.21         | 0.46-10.6  | 0.321   |
| Tumor depth     |              |            |         |
| Deep            | 24.0         | 0.024-24154| 0.367   |
| Histologic grade|              |            |         |
| I               | 1            |            |         |
| II              | 3.18         | 0.28-35.1  | 0.344   |
| III             | 20.4         | 2.66-157   | 0.004   |
| CD109 expression|              |            |         |
| Negative        | 1            |            |         |
| Positive        | 5.72         | 2.13-15.3  | 0.001   |

CI: confidence interval

**Table 2. Multivariate analysis of the variables in overall survival.**

| Variable        | Hazard ratio | 95% CI     | P value |
|-----------------|--------------|------------|---------|
| Histologic grade|              |            |         |
| I               | 1            |            |         |
| II              | 2.03         | 0.177-23.39| 0.570   |
| III             | 13.9         | 1.75-111.7 | 0.013   |
| CD109 expression|              |            |         |
| Negative        | 1            |            |         |
| Positive        | 3.85         | 1.40-10.55 | 0.009   |

CI: confidence interval

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oral tumor and urothelial carcinomas [43,46], we demonstrated that high expression of CD109 was significantly associated with advanced stage in STSs. These results suggest that the pathophysiological function of CD109 protein in sarcomas is different from that in carcinomas. Therefore, we speculate that CD109 expression in STSs is deeply involved in invasion and metastasis.

In conclusion, we established the novel ES cell line ESX. Next, we investigated CSCs/CICs in ES cell lines and isolated CSCs/CICs based on ALDH activity. Finally, we demonstrated that CD109 is a potential CSC/CIC marker, prognostic factor and molecular target for STSs, including ES.

Supporting Information

Table S1. List of commercial sources of the antibodies used in the study.

Table S2. List of the 37 membrane protein-related related upregulated (rate ≥2.0) genes in ALDH<sup>hi</sup> cells of ESX.

Table S3. Association between CD109 expression and clinical variables.

Figure S1. Clinical characteristics of the origin of the new epithelioid sarcoma cell line. A. Magnetic resonance imaging reveals a subcutaneous tumor (3×3 cm) located in the left thigh (left panel, arrow) and lymph node metastases in the inguinal region (right panel, arrow). B. H&E staining of the primary tumor from a resected specimen reveals typical features of epithelioid sarcoma, showing central necrosis with peripheral palisading of epithelioid cells around necrotic areas (scale bars, left 500μm, right 50μm). C. Immunohistochemical analysis of the primary tumor for AE1/ AE3, vimentin, CD34, CA125, S-100 and INI1 expression (scale bar, 50μm). D. Fluorescence in situ hybridization (FISH) analysis using the INI1/CEP22 deletion probe performed according the protocol we previously described [48]. Heterozygous deletion demonstrated by the lack of one red signal (indicating the INI1 region) was detected (indicated by red arrow). Green signals indicate the centromeric region of chromosome 22.

Figure S2. The proportions of ALDH<sup>hi</sup> cells in the sarcoma cell lines. FACS analysis of ALDH1 activities of the cell lines of osteosarcoma (U2OS and OS2000), synovial sarcoma (Fuji and HS-SYII), Ewing sarcoma (WES and RD- ES) and malignant fibrous histiocytoma (MFH2003 and MFH2004) with and without DEAB control.

Figure S3. The mRNA expression of stem/progenitor cell-related genes in epithelioid sarcoma cell lines, VA-ES-BNJ and FU-EPS-1. RNA was isolated from freshly sorted spheroid cells (1x10<sup>5</sup>) on day 7. Bars represent mean±SEM. *p<0.05, determined by the Mann-Whitney test.

Figure S4. CD109 knockdown using siRNA. A. Real-time PCR analysis of CD109 mRNA expression of ESX after transfection of siCD109 or mock siRNA on day 2. Bars represent mean±SEM. ***p<0.001 was determined by Student’s t-test. B. Western blot analysis of siCD109 cells. Cell lysate with Nonident P-40 detergent solution was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Separated proteins were transferred unto polyvinylidene fluoride membranes and probed with a mouse anti-CD109 antibody (H-7; Santa Cruz Biotechnology, USA). β-Actin was used as a loading control. The anti-CD109 antibody was used at 100-fold dilution. The membrane was visualized with Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol and analyzed using Odyssey Fc Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Anti-β-actin was used as an internal control. C. Real-time PCR analysis of CD109 mRNA expression of ESX after transfection of siCD109 on days 3, 5, 7 and 10.

Author Contributions

Conceived and designed the experiments: ME T. Tsukahara VK T. Torioge TW TY NS. Performed the experiments: ME T. Tsukahara MM M. Kaya SN JN HI. Wrote the manuscript: ME T. Tsukahara NS.

References

1. Weiss SW, Goldblum JR (2008) Enzinger and Weiss’s soft tissue tumors. Mosby, Inc.
2. Jordan CT, Guzman ML, Noble M (2006) Cancer stem cells. N Engl J Med 355: 1253-1261. doi:10.1056/NEJMra061808. PubMed: 16990388.
3. Sládek NE (2003) Human aldehyde dehydrogenases: potential pathological, pharmacological, and toxicological impact. J Biochem Mol Toxicol 17: 7-23. doi:10.1002/jbt.10057. PubMed: 12616643.
4. Vasilious V, Pappa A, Petersen DR (2000) Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. Chem Biol Interact 129: 1-19. doi:10.1016/S0009-2797(00)00211-8. PubMed: 11154732.
5. van den Hoogen C, van der Horst G, Cheung H, Buijs JT, Lippitt JM et al. (2010) High aldehyde dehydrogenase activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. Cancer Res 70: 5163-5173. doi:10.1158/0008-5472.CAN-09-3806. PubMed: 20516116.
6. Cheung AM, Wan TS, Leung JC, Chan LY, Huang H et al. (2007) Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engraftment potential. Leukemia 21: 1423-1430. doi:10.1038/sj.leu.2404721. PubMed: 17476279.
7. Jiang F, Qi Q, Khanna A, Todd NW, Deepak J et al. (2009) Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung.
High Expression of CD109 on Epithelioid Sarcoma

CD109 is a marker for malignant prostate stem cells and predictor of prostate cancer patients‘ outcomes. Lab Invest 90: 399-410. PubMed: 21962895.

Gerharz CD, Moll R, Ramp U, Melvin W, Gabbert HE (1990) Multidirectional differentiation in a newly established human epithelial sarcoma cell line (GRU-1) with co-expression of vimentin, cytokeratins and neurofilament proteins. Int J Cancer 45: 143-152. doi:10.1002/jic.2910450126. PubMed: 2168830.

Roché H, Julia AM, Jozan S, Muller C, Dastugue N et al. (1993) Characterization and chemosensitivity of a human epithelial sarcoma cell line (SARCRC 2). Int J Cancer 54: 663-668. doi:10.1002/ijc.2910540423. PubMed: 8289571.

Sonobe H, Furuhata M, Iwata J, Ota K, Ohtsuki Y et al. (1993) Morphological characterization of a new human epithelial sarcoma cell line, ES20488, in vitro and in vivo. Virchows Arch B Cell Pathol Ind Mol Pathol 63: 219-225. doi:10.1007/BF02895265. PubMed: 7981133.

Cordoba JC, Parham DM, Meyer WH, Douglass EC (1994) A new cytogenetic finding in an epithelial sarcoma, t(8;22)(q22;q11). Cancer Genet Cytogenet 72: 151-154.

Russo JE, Hilton J (1988) Characterization of cytosolic aldehyde dehydrogenase from cyclophosphamide resistant L1210 cells. Cancer Res 48: 2963-2968. PubMed: 3385687.

Viale G, Ito D, Amoscato A, Maciejewski-Franczak M, Abdelasalem A et al. (2007) Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. Cancer 107: 1058-1054. doi:10.1001/2009.506. PubMed: 17074988.

Awad O, Yuste A, Shah P, Gul N, Katri V et al. (2010) High ALDH activity identifies chemotherapy-resistant Ewing’s sarcoma stem cells that retain sensitivity to EWS-FLI1 inhibition. PLO S ONE 5: e13943. doi:10.1371/journal.pone.0013943. PubMed: 2086536.

Yamada C, Shono K, Ito M, Ishikawa Y, Iwasaki H, Ohjimi Y, Ishiguro M, Isayama T, Kaneko Y et al. (2006) Multidirectional differentiation in a newly established human epithelial sarcoma cell line, FU-EPS-1: an epithelioid sarcoma cell-line. Int J Oncol 7: 51-56. PubMed: 21525805.

Ikushima H, Miyazono K (2012) TGF-beta signal transduction in epithelial sarcoma. Cancer 118: 330-338. doi:10.1007/s00776-0007-0663-5. PubMed: 12898310.

Cordoba JC, Parham DM, Meyer WH, Douglass EC (1994) A new cytogenetic finding in an epithelial sarcoma, t(8;22)(q22;q11). Cancer Genet Cytogenet 72: 151-154.
dependent effects of TGF-beta. Cell Tissue Res 347: 37-49. doi: 10.1007/s00441-011-1179-5. PubMed: 21618142.

48. Miura Y, Keira Y, Ogino J, Nakanishi K, Noguchi H et al. (2012) Detection of specific genetic abnormalities by fluorescence in situ hybridization in soft tissue tumors. Pathol Int 62: 16-27. doi: 10.1111/j.1440-1827.2011.02739.x. PubMed: 22192800.