Sox2-dependent inhibition of p21 is associated with poor prognosis of endometrial cancer

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Endometrial cancer is one of the most frequent gynecological malignancies worldwide. Most of the endometrial cancer patients are diagnosed as International Federation of Gynecology and Obstetrics (FIGO) stage I (Table S1). These patients with early stage disease can often be cured by complete surgical removal of the tumor with or without adjuvant chemotherapy. However, about 20% of endometrial cancer cases are diagnosed as the advanced stage, in which tumors have spread to the abdominal or pelvic cavities and/or lymph nodes. The survival rates of patients with advanced disease are poor; in particular, the 5-year overall survival rate for endometrial cancers with FIGO stage III and IV cancers is 20%–26%. An effective chemotherapeutic approach for endometrial cancer has not yet been established and the efficacy of radiotherapy is far from satisfactory. Indeed, radiotherapy does not extend overall survival of patients with this disease.

Several reports have established that multiple transcriptional factors, including sex-determining region Y-box 2 (SOX2), octamer-binding transcription factor (OCT-3/4), and Nanog, comprise a set of essential factors promoting the self-renewal and pluripotency of embryonic stem cells. These factors form a key regulatory network controlling the identity and replication of ES cells with other coregulators. In addition, Kruppel-like factor 4 (KLF4), Myc, and LIN28, have been reported to be critical factors for the establishment of induced pluripotent stem cells from somatic cells. These transcriptional factors regulating the pluripotency of normal stem cells are essential for maintenance of several types of cancer cells. In addition, SOX2 has been reported to be essential for cell survival and cancer progression and initiation in many types of cancers, including glioblastoma, breast cancer, pancreatic cancer, gastric cancer, melanoma, lung cancer, osteosarcoma, and Ewing sarcoma. Recently, we also demonstrated that SOX2 expression was required for the growth and survival of ovarian cancer cells. In most types of cancers, SOX2 acts as an oncogenic transcriptional factor, and thus it may become a novel therapeutic target in these cancers. However, the roles of SOX2 in endometrial cancer are still unclear.
In this study, we examine the role and function of SOX2 in uterine endometrial cancer. We found that SOX2 expression was correlated with poor outcomes in patients with endometrial cancer, and that SOX2 was a critical factor for the proliferation of endometrial cancer cells.

Materials and Methods

Clinical specimens. All 258 uterine endometrial endometrioid adenocarcinoma tumors were resected from patients who had undergone initial surgery at Niigata University Medical and Dental Hospital between 1 January 2006 and 31 December 2013. All patients provided informed consent for participation in the study, and all procedures were performed under a protocol approved by the Ethics Committee of Niigata University. Histopathological diagnoses were based on World Health Organization criteria, tumor grading was based on Gynecologic Oncology Group criteria, and disease stage was assigned according to the FIGO staging system.

Immunohistochemical (IHC) analyses. Primary or mouse xenograft tumors were fixed in neutral formalin, embedded in paraffin, and used for staining with hematoxylin and eosin. For immunostaining, the sections were stained following standard IHC methods, as previously described, with slight modifications. Briefly, after deparaffinization, antigen retrieval was carried out with Target Retrieval Solution (10 mM citrate buffer, pH 6.0; Dako) in a microwave for 30 min at 96°C. Subsequently, the sections were incubated with primary anti-SOX2, anti-p21 (1:200 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-p53 or anti-Ki-67 antibodies (1:200 dilution; Dako) and biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA), followed by incubation with ABC reagent (Dako) and 3,3’-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA). Slides were counterstained with hematoxylin.

IHC scoring. The evaluation of immunostaining was performed independently by two observers (K. Yamawaki and T.I.). Images were taken using a BX51 microscope (Olympus, Tokyo, Japan). Five random bright-field, high-magnification images were evaluated for each sample. Staining of SOX2 and p21 was scored according to nuclear staining of cancer cells. The staining intensity was visually evaluated on a scale of 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining) (Figs 1a and 5a). Samples with moderate or strong nuclear staining (staining intensity scored as 2 or 3) in at least 10% of cancer cells were regarded as “IHC-positive”, and samples with absent or weak staining (staining intensity scored as 0 or 1) and less than 10% staining of cells were regarded as “IHC-negative”. Nuclear staining for p53 and Ki-67 was performed and the stained cancer cells were counted; when more than half of the cancer cells were stained, the sample was regarded as “IHC-positive”.

Cell culture. HEC1, HEC1A, HEC59, HEC151, HEC50B, Ishikawa, and PA-1 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Japan). JHUEM3 and JHUEM7 cells were obtained from RIKEN BioResource Center (Japan). AN3CA cells were obtained from American Type Culture Collection (Manassas, VA, USA). EN cells were a kind gift from Dr. Isaka (Tokyo Medical and Dental University, Japan). EN cells were cultured in Dulbecco’s modified Eagle medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA, USA), and other cancer cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), penicillin, and streptomycin (Gibco). All cells were maintained at 37°C in an atmosphere containing 5% CO2.

Western blot analyses. Cells were lysed in RIPA buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 1 mM EDTA) supplemented with protease inhibitors (Roche). The lysates were separated by SDS-polyacrylamide gel electrophoresis on 5–20% gradient gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Western blot analyses were performed as previously described. Nuclear extracts and extracts of other subcellular fractions were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions.

Immunocytochemical analyses. Adherent cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, washed with 1x PBS, permeabilized in PBS/0.1% Triton X-100 for 5 min at 4°C, and blocked with PBS containing 3% bovine serum albumin (BSA). The fixed cells were then used for immunostaining with anti-SOX2 and anti-phalloidin antibodies (Cell Signaling Technology) and stained with 4,6-diamidino-2-phenylindole (DAPI).

Real-time polymerase chain reaction (PCR) analyses. RNA was isolated from cells with an miRNeasy Mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized from total RNA using a PrimeScript First Strand cDNA Synthesis Kit (Takara, Shiga, Japan). Gene expression levels were measured using SYBR Premix Ex Taq II (Takara) with primers targeting the SOX2 promoter (HA173580; TAKARA) and Taqman gene expression assays (Applied Biosystems, Foster City, CA, USA) with the primer/probe set (Hs01053049_s1) according to the manufacturer’s instructions. Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured to calculate delta Ct values.

siRNA transfection. HEC59, EN, and JHUEM7 cell (2x10^4 cells/well) were transfected with 4 pmol siRNA targeting SOX2 mRNA in the presence of 20 μl HiPerfect (Qiagen) for 2 days. SOX2 siRNAs (si#6: 5’-CTGGCGGAGAATCTCATGTA-TAT-3’; si#7: 5’-CCAUGGGUUCGGUGGUCATT-3’) and control siRNA were purchased from Qiagen.

Cell viability assay. Cell growth was quantified by measuring the amounts of cellular ATP using CellTiter-Glo Luminescence Cell Viability Assays (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The intensity of luminescence was measured using a FLUOROSCAN instrument (Thermo Scientific).

Xenograft establishment. Cells were dissociated into single cells with trypsin/ethylenediaminetetraacetic acid (EDTA; Gibco), suspended in 100 μl medium containing 50% Matrigel (BD Biosciences, Bedford, MA, USA), and used for subcutaneous injection into the flanks of NOD/Shi-scid IL-2rgnull mice (Central Institute for Experimental Animals, Kawasaki, Japan) with a 27-gauge needle. Mice were monitored every 2–3 days until 5 weeks postinjection. All animal experiments and protocols were approved by the Animal Care and Use Committees of Niigata University and performed in accordance with institutional policies.

Cell cycle analysis and cell sorting. Fixed cells in methanol were stained with 25 μg/mL propidium iodide and 50 μg/mL RNase, as previously described. All flow cytometry and cell sorting analyses were carried out using a FACS Aria II (BD Biosciences). Growing cells were incubated with 5 μg/mL Hoechst 33342 (Sigma) for 1 h at 37°C in the dark. After
trypsinization, cells were sorted based on the amount of DNA. \(^{36,37}\)

**Chromatin immunoprecipitation (ChIP) assay.** ChIP was conducted with a SimpleChIP Enzymatic Chromatin IP Kit (#9003; Cell Signaling Technology) according to the manufacturer’s recommendations. Immunoprecipitation was carried out using anti-SOX2 antibodies (#5024; Cell Signaling Technology), normal rabbit IgG (#2729; Cell Signaling Technology) as a negative control, and anti-histone H3 antibodies (#4620; Cell Signaling Technology) as a positive control. Quantification of DNA by real-time PCR was performed as described above with primers targeting the \(CDKN1A\) promoter (#6449; Cell Signaling Technology) and \(RPL30\) promoter (#7014; Cell Signaling Technology).

**Statistical analysis.** Clinicopathological parameters were analyzed using Fisher’s exact test. Univariate survival analysis was performed using the Kaplan-Meier method, and the significance of difference between groups was analyzed using the log-rank test. Multivariate survival analysis was carried out using Cox proportional hazards regression model. For survival analysis, patients who also had other types of cancer, for example, ovarian cancer, or were treated with chemotherapy before surgery were excluded, and a total of 241 patients, including 201 patients with stage I cancer and 31 patients with advanced-stage endometrial cancer, were subjected to survival analysis (Table S2). Differences with \(P\)-values of less than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) or JMP software (SAS Institute Inc., Cary, NC, USA).

**Results**

**SOX2 expression is correlated with histological grade and poor prognosis in endometrial cancer.** To investigate the clinical importance of SOX2 in endometrial cancer, we evaluated SOX2 expression levels in 258 human uterine endometrial endometrioid adenocarcinoma tissues by IHC (Fig. 1a). The patients were stratified into SOX2-positive (red lines, \(n = 8\)) and SOX2-negative (blue lines, \(n = 23\)) groups.
high-grade patients than low-grade ones, and there were significantly correlated with histological grade (Fig. 1b; \( P < 0.0001 \), Fisher’s exact test); 64.0\% (16/25 cases) of high-grade (grade 3) endometrial cancer cases exhibited positive staining for SOX2, whereas only 8.9\% (14/158 cases) of low-grade (grade 1) cases were positive for SOX2 staining. These results were consistent with the findings of previous reports of many types of cancers, including cancers of head and neck, and ovary.\(^{18,38,39}\) The correlation between SOX2 expression and histological grade was also consistent in both early and advanced stage disease (Table S2).

There were no significant differences in the rates of SOX2 positivity between early and advanced stage groups (Fig. S1a). Kaplan–Meier analyses of 31 advanced stage endometrial cancer patients (FIGO stages III and IV) showed that SOX2 expression was associated with poor prognosis (Fig. 1c,d; \( P = 0.0027 \) for progression-free survival [PFS] and \( P = 0.0307 \) for overall survival [OS]). Moreover, multivariate analyses of clinical factors indicated that SOX2 positive staining served as an independent prognostic factor (Table S3). In 201 cases of FIGO stage I endometrial cancer, PFS of SOX2-positive patients was significantly shorter than that of SOX2-negative patients (Fig. S1b; \( P = 0.0049 \)), and univariate analyses demonstrated that multivariate analyses of clinical factors indicated that SOX2 positive staining was significant for PFS (Table S4). However, these two groups do not show any significant difference in OS, potentially because of the good prognosis of patients with early stage endometrial cancer patients,\(^{20}\) or the limited number of cases (Fig. S1c).

It was reported that p53 status is one of the markers useful for this discrimination between low-grade and high-grade endometrioid carcinoma.\(^{40}\) We evaluated the p53 protein expression of the same clinical tissue samples shown in Figure 1(b) (Fig. S1d). Of the total 258 cases, only 13 cases (5.0\%) expressed high levels of p53 expression, which is a hallmark of p53 mutation\(^{41}\) (Fig. S1e). On the other hand, out of 25 cases of high-grade (Grade 3) endometrioid carcinoma, four cases (16.0\%) expressed high levels of p53 expression (Fig. S1f), which is in accord with the relatively high incidence of p53 mutation in high-grade endometrioid carcinoma. Although levels of both p53 and SOX2 increased in high-grade cases, we did not observe statistically significant relationship between p53 status and SOX2 levels (Fig. S1e,f).

**SOX2 is overexpressed in a subset of endometrial cancer cells.** Next, we investigated the expression of SOX2 in 10 endometrial cancer cell lines using western blotting and real-time PCR. A teratocarcinoma cell line PA-1 was used as a positive control for SOX2 expression analysis. We detected SOX2 expression in three endometrial cancer cell lines, HEC59, EN, and JHUEM7 (Fig. 2a). In particular, EN cells expressed high levels of SOX2 comparable to that in PA-1 cells. SOX2 protein and mRNA levels in these cancer cell lines were correlated with each other (Fig. 2a). Based on these results, we used HEC59, EN, and JHUEM7 cells as typical SOX2-positive endometrial cancer cell lines for further experiments.

To investigate the subcellular localization of SOX2 protein in endometrial cancer cells, we performed western blot analysis after subcellular fractionation and immunostaining with anti-SOX2 antibodies. These analyses showed that SOX2 was predominantly localized in the nucleus of HEC59, EN, and JHUEM7 cells; weak SOX2 expression was observed in the cytoplasm (Fig. 2b,c, Fig. S2b). Intriguingly, the expression of SOX2 in respective cells derived from the same cell lines varied (Fig. 2c, Fig. S2b,c). To further characterize this observation, we purified a single cell from EN cells, and the purified cell was cultured for 7 days, following which SOX2 expression in cells derived from a single cell was measured. Nuclear expression of SOX2 in respective EN cells derived from a single cell varied (Fig. S2d). In addition, we divided endometrial cancer cells according to the cell cycle phase (G1 phase and G2/M phase) using Hoechst 33342 staining (Fig. S2e). SOX2 protein and mRNA expression was increased in EN cells during the G1 phase (Fig. S2e). These results suggested that SOX2 expression fluctuated during endometrial cancer propagation, which may contribute to heterogeneous SOX2 expression in clinical endometrial cancer tissues (Fig. 1a).

**SOX2 expression is required for endometrial cancer cell growth.** In order to examine whether SOX2 contributed to endometrial cancer cell proliferation, we evaluated cell proliferation after knockdown of SOX2 using siRNA. Knockdown of SOX2 expression in SOX2-positive cells reduced the proliferation of SOX2-positive cells, including HEC59, EN, and JHUEM7 cells (Fig. 3a,b, Fig. S3a–c).

SOX2-knockdown in endometrial cancer cells increased cell size and altered cell morphology (spreading over the dish), which are reminiscent of senescent cells (data not shown). In fact, SOX2-knockdown cells expressed a senescence marker protein, i.e., β-galactosidase (Fig. 3c, Fig. S3d). Moreover, because cell cycle arrest is a hallmark of cell senescence, cell cycle analysis was performed.\(^{42}\) This analysis showed that cells accumulated in the G1 phase after knockdown of SOX2 expression (Fig. 3d). On the other hand, SOX2-knockdown in endometrial cancer cells did not increase the sub-G1 fraction, representing apoptotic cells (data not shown). Furthermore, knockdown of SOX2 expression in endometrial cancer cells increased the expression of senescence-associated cell cycle...
inhibitor p21 but not p27 (Fig. 3a,e, Fig. S3a,e). Taken together, these results indicated that SOX2 was required for cell cycle progression and for the inhibition of p21 expression in endometrial cancer cells.

To examine whether SOX2 inhibits p21 expression at the transcription level, we examined whether SOX2 binds to the promoter DNA of \textit{CDKN1A} gene encoding p21 protein. ChIP analysis detected specific binding of SOX2 to the \textit{CDKN1A} promoter DNA in both EN and HEC59 cells (Fig. 3f and Fig. S3f). These results indicated that SOX2 represses transcription of p21/\textit{CDKN1A} gene through binding to \textit{CDKN1A} promoter DNA in EN and HEC59 cells.

Because p21 is a potent inhibitor of cell cycle progression, we evaluated the relationship between SOX2 expression and Ki-67 expression in order to examine whether SOX2 expression stimulates cell cycle progression via p21 inhibition. Indeed, in all 258 clinical endometrial cancer samples by IHC (Fig. 4a), expression of Ki-67 was higher in SOX2-positive cases than in SOX2-negative ones (Fig. 4b; \(P<0.0001\), Fisher’s exact test).

**SOX2 expression mediates tumor formation in vivo.** Next, we explored whether SOX2 expression in endometrial cancer cells contributed to tumorigenicity in vivo. EN cells were transfected either with SOX2 siRNA or the control siRNA, and the cells were then inoculated into the flanks of immunocompromised NOG mice. The control EN cells began to generate palpable tumors of up to 5 mm in diameter from 1 week after inoculation; tumors were observed in 8 of 10 inoculated sites at 5 weeks (Fig. 5a). In contrast, SOX2-knockdown EN cells generated smaller tumors that were detectable at 4–5 weeks after injection in only 2 of 12 inoculated sites (Fig. 5b,c). The generated tumors originated from control and SOX2-knockdown EN cells were histologically indistinguishable (Fig. 5a,d). These results indicated that the expression of SOX2 in endometrial cancer cells augments the tumorigenicity in vivo.

**Evaluation of combined SOX2 and p21 expression in advanced endometrial cancer.** The data above indicated that SOX2 in endometrial cancer cells inhibits expression of cell cycle inhibitor p21. Hence, we examined the SOX2 expression was inversely associated with that of p21. IHC analysis of p21 expression (Fig. 6a) of the 31 advanced endometrial cancer cases (Fig. 1d) stratified them into the four groups based on the levels of p21/\textit{CDKN1A} expression (Fig. S4). While the inverse relationship between p21 and SOX2 expression was not clear, further classification based on p53 status suggest that SOX2 expression may be inversely related to that of p21 in cases with high levels of p53 (Fig. S4).

Finally, we determined whether the expressions of SOX2 and p21 in patients with endometrial cancer act as a useful marker for cancer prognosis. Kaplan–Meier analyses showed that the SOX2-positive/p21-negative group showed the most unfavorable prognosis with regard to overall survival (OS;
Fig. 6b. In addition, the SOX2-positive/p21-negative patients showed shorter OS than the other patients (Fig. 6c). These results suggest that the simultaneous evaluation of SOX2 and p21 in advanced endometrial cancer patients provides more accurate information for patient prognosis than either alone, regardless of p53 status.

**Discussion**

In this study we investigated the role of SOX2, an essential multipotent transcription factor, in endometrial cancer. Our findings indicated that expression of SOX2 in endometrial cancer patients were correlated with unfavorable histological...
cancer grade and poor prognosis. We also found that SOX2 augmented cell proliferation of endometrial cancer cells, and p21 was a target of SOX2 in endometrial cancer. Consistent with these observations, SOX2-positive/p21-negative cancers predicted the most unfavorable prognosis in advanced endometrial cancer.

Since the outcomes of advanced endometrial cancer patients are poor, novel prognosis markers are needed to improve therapeutic strategies for advanced cancer. Poorly differentiated tumors have been shown to express high levels of several stemness-related factors that are typically enriched in ES cells. SOX2 is overexpressed in poorly differentiated tumors more often than in well-differentiated tumors in many types of cancers, indicating that SOX2 may act as an effective biomarker predicting the prognosis of cancer patients. Our results were consistent with these findings, and indicated for the first time that SOX2 expression was significantly correlated with histological grade endometrial cancer tissues in both early and advanced stage disease. Thus, SOX2 is a promising candidate biomarker for endometrial cancer.

SOX2 tightly regulates cancer cell proliferation, apoptotic signaling, invasion, and migration in many cancer types. Previous studies have shown that SOX2 is required for positive cell cycle progression and the expression of some cell cycle regulatory genes in various cancer cells. SOX2 augments the proliferation of pancreatic cancer via repressing the expression of cell cycle inhibitors CDKN1A, CDKN1B, and augmenting the expression of cell cycle stimulator CCND3. Knockdown of SOX2 expression in prostate cancer reduces cell proliferation and leads to upregulation of cell cycle inhibitor CDKN1B and downregulation of cell cycle stimulator CCNE1. Our results suggested that SOX2 mediated cancer cell proliferation and that CDKN1A was a direct target of SOX2 in endometrial cancer cells, indicating that SOX2 may be an attractive therapeutic target in endometrial cancer and other types of solid tumors.

Previous studies showed that cancer patients lacking p21 expression tend to have a poorer prognosis than patients with p21 expression in several types of cancer including endometrial cancer. In particular, we revealed that endometrial cancer with high expression level of SOX2 and low expression level of p21 represents an aggressive malignant subgroup with significantly shortened survival. Some other factors including p53, smad, and myc might affect the p21 expression in some cases. Although future prospective studies are needed to validate our results, our findings suggested that simultaneous evaluation of SOX2 and p21 in endometrial cancer may be a useful biomarker for predicting the prognosis of patients. In summary, our findings supported that SOX2 and p21 may be an attractive biomarker for endometrial cancer.
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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Comparison of SOX2 and p53 expression in endometrial cancer.

Fig. S2. SOX2 expression in human endometrial cancer cell lines.

Fig. S3. SOX2 is required to modulate the proliferation of endometrial cancer cells.

Fig. S4. Stratification of SOX2/p21 immunostaining based on p53 expression.

Table S1. Clinicopathological factors of all 258 patients with uterine corpus endometrioid adenocarcinoma presented in Figure 1b.

Table S2. Clinicopathological factors of 241 patients with uterine corpus endometrioid adenocarcinoma presented in Figure 1c, 1d, and Supplementary Figure S1.

Table S3. Univariate (a) and multivariate analyses (b) of prognostic factors for overall survival (OS) and progression-free survival (PFS) in 31 cases of advanced-stage cancer presented in Figure 1c and 1d.

Table S4. Univariate (a) and multivariate analyses (b) of prognostic factors for overall survival (OS) and progression-free survival (PFS) in 201 cases of stage endometrial cancer presented in Supplementary Figure S1b and S1c.

Table S5. List of antibodies used in this study.