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SOX17 expression in ovarian clear cell carcinoma

Daichi Kodama[1], Motoki Takenaka[2], Chiemi Saigo[1,3,4], Masako Azuma[1], Yuki Hanamatsu[1,4], Masanori Isobe[2] and Tamotsu Takeuchi[1,4*]

Abstract

Recent studies have revealed that the Sry-related HMG box gene 17 (*SOX17*) plays an important role in ovarian carcinogenesis. Unlike other types of ovarian cancer, ovarian clear cell carcinoma (OCCC) has a distinct pathobiological phenotype, often harboring an *AT-rich interaction domain 1 A (ARID1A)* mutation. In the present study, to determine the SOX17 in OCCC cells, we immunohistochemically examined SOX17 expression in 47 whole-tissue specimens of OCCC. Although not statistically significant, SOX17-high immunoreactivity tended to be related to unfavorable patient outcomes. We also aimed to determine the relationship of SOX17 with ARID1A. Double immunofluorescence staining demonstrated that SOX17 immunoreactivity was not associated with ARID1A immunoreactivity. Immunoblotting revealed that SOX17 was abundantly expressed in cultured OVISE and RMG-V OCCC cells, but not in OVTOKO OCCC cells. Polyubiquitinated bands of SOX17 were observed in MG132 treated OVTOKO, but not in OVISE or RMG-V OCCC cells. Notably, si-RNA-mediated knockdown of a deubiquitinase enzyme, ubiquitin C-terminal hydrolase L1, increased polyubiquitination followed by proteasome degradation of SOX17 in OVISE. These findings indicate that SOX17 is not uniformly and heterogeneously expressed in OCCCs, independent of ARID1A deficiency. Impaired ubiquitin-mediated proteasome degradation may stabilize SOX17 in some OCCC cells.

Keywords ARID1A, Ovarian clear cell carcinoma, Prognosis, SOX17, Ubiquitination

Introduction

Ovarian clear cell carcinoma (OCCC) is defined as ovarian epithelial carcinoma, which demonstrates a unique histopathological phenotype, as "clear cells containing glycogen and/or with the presence of hobnail cells". OCCC accounts for up to 10% of the ovarian cancers in Western countries; however, its prevalence is higher in Asian populations [1]. Moreover, a recent study reported that the incidence of OCCC has increased considerably, accounting for up to 30% of all ovarian cancer cases in Japan [2].

OCCC is a biological and clinical cancer entity distinct from other ovarian carcinomas [3, 4]. OCCC arises from endometriosis or a putative precursor lesion, designated as clear cell adenofibroma, whereas the putative origin of

*Correspondence:
Tamotsu Takeuchi
takeutit08@gmail.com
[1]Department of Pathology and Translational Study, Gifu University School of Medicine, Gifu, Japan
[2]Department of Obstetrics and Gynecology, Gifu University School of Medicine, Gifu, Japan
[3]The United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Japan
[4]Center for One Medicine Innovative Translational Research, Gifu University, COMIT, Gifu, Japan

high-grade serous carcinoma (HGSC), which is a common ovarian cancer, originates from the distal fallopian tube or ovarian surface membrane [5, 6]. *ARID1A* mutations are found in approximately half of the OCCC cases, which are associated with a decrease in ARID1A protein expression as assessed by immunohistochemistry and/or immunoblotting, unlike HGSC [7, 8]. In contrast, *p53* mutations are much less frequent in OCCC than in other histological types of ovarian cancers [9]. Based on poor sensitivity to chemotherapeutic agents, new therapeutic approaches are currently developing, i.e. immune checkpoint blockade, targeting angiogenesis, and exploiting ARID1A synthetic lethal interactions, for patients with OCCC, particularly at advanced stages [10, 11].

Sry-related HMG box gene 17 (*SOX17*) may be an endometrial carcinoma-specific significantly mutated gene [12, 13]. SOX17 protein is also believed to act as a survival master transcription factor in ovarian carcinogenesis [14]; however, these findings were obtained in the study of HGSC. SOX17 immunoreactivity was detected in approximately 60% of OCCC cases using a tissue microarray [15]. Notably, transcriptomic analysis of endometrial epithelium-specific SOX17 knockout mice revealed that SOX17-regulated gene expression patterns markedly overlapped with those of ARID1A-regulated genes [16]. However, the pathobiological properties of SOX17 in OCCC, including its relationship with ARID1A, remain largely unclear. Interestingly, recent advances have highlighted SOX17 in both immune evasion and tumor angiogenesis. Accordingly, it is important to understand SOX17 expression in whole tissue specimens of OCCC.

We have examined carcinogenesis related to the impaired expression and/or function of ARID1A [17–24]. In the present study, we examined SOX17 expression in OCCC using whole tissue specimens from 47 Japanese patients, focusing on its relationship with ARID1A expression. In addition, the SOX17 expression and ubiquitination status of cultured OCCCs were examined. To our knowledge, this is the first manuscript which describe the ubiquitination status of SOX17 in ovarian cancer cells.

Materials and methods

Patient tissue specimens

In this retrospective study, we collected data from all surgically treated patients primarily diagnosed with OCCC. In our institute, the time interval between primary surgery and adjuvant chemotherapy is intended to be less than 40 days after primary surgical resection. Archived pathological tissue specimens from 47 patients with OCCC were used in this study. The study was conducted in accordance with the ethical standards of the Declaration of Helsinki in 1975. The use of tissue samples and the review of clinical records were performed according to protocols approved by the Institutional Review Board of Gifu University Graduate School of Medicine (specific approval number: 2023-080).

Immunohistochemical staining

The detailed procedure for immunohistochemical staining has been previously reported [25, 26]. Briefly, all tissue specimens were surgically obtained, fixed in 10% buffered formalin, and embedded in paraffin. Since Shaker et al. [27] extensively examined SOX17 expression in normal tissue using rabbit monoclonal antibody against human SOX17 (clone EPR20684), we also employed this clone (cat no. AB224637, Abcam, Cambridge, MA, USA) in the present study. The characterization of a murine monoclonal antibody against ARID1A has been previously described [17, 19, 21–23]. Tissues were immunostained with antibodies using the ImmPRESS™ polymerized reporter enzyme staining system (Vector Laboratories, Burlingame, CA, USA).

Double immunofluorescence cytochemical staining was performed as previously reported [28, 29]. After staining with DAPI (4′,6-diamidino-2-phenylindole) to visualize the nuclei, images were acquired using a confocal laser-scanning microscope and the Leica Application Suite X program (Leica TCS SP8, Wetzlar, Germany).

Evaluation of immunohistochemical staining and statistical analysis

We evaluated the immunohistochemical staining results as the percentage of immunoreactivity in OCCC cells that exhibited nuclear SOX17 staining in each representative formalin-fixed and paraffin-embedded whole-tissue specimen. Staining was considered SOX17-low if < 10% of cancer cells exhibited immunoreactivity, and SOX17-high if > 10% based on the criterion described by Asano et al. [30]. All immunostaining results were independently reviewed by two pathologists (CS and TT). In cases of discordant results, the tissue specimens were reviewed by two pathologists to obtain a consensus.

Prognostic information for 41 cases was available. Curves for overall and disease-free survival were drawn using the Kaplan–Meier method, and differences in survival rates were compared using the log-rank test. Statistical significance was set at $P<0.05$. Statistical analyses were performed with EZR version 1.41 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Cell culture

The ovarian cancer cell lines OVISE, OVTOKO, OVSAHO [31], and RMG-V [32] were obtained from the Japan Health Science Research Resources Bank (JCRB;

Osaka, Japan). OVISE, OVTOKO, and RMG-V are clear cell carcinoma cell. In the present study, we employed OVSAHO as a representative HGSC cell line according to the report by Domcke et al. [33]. The cells were cultured in Dulbecco's modified Eagle medium (Gibco Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum. The cells were passaged in our laboratory for no more than 6 months after resuscitation.

Immunoblotting

Immunoblotting was performed according to the method described by Towbin et al. [34] with modifications as previously described [35]. Briefly, cell lysates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with Block Ace (blocking milk; Yukijirushi, Sapporo, Japan) and incubated with the rabbit anti-human SOX17 antibody and murine monoclonal antibodies against HDAC1 (cat no. 5356. Clone 10E2, Cell Signaling Technology, Beverly, MA, USA). For chemiluminescent immunoblot detection, the membranes were incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Cell Signaling Technology), followed by incubation with Western BLoT Ultra Sensitive HRP Substrate (Takara Bio, Ohtsu, Japan). For fluorescent immunoblotting, goat anti-rabbit IgG cross-adsorbed its secondary antibody, Alexa Fluor Plus 800 (cat no. A32735, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and goat anti-mouse IgG (H+L) cross-adsorbed its secondary antibody, Alexa Fluor Plus 647 (Invitrogen; Thermo Fisher Scientific, Inc., cat no. A32728). Images were obtained using the Invitrogen iBright 1500 gel imaging system *a*nd iBright.

Analysis Software (Thermo Fisher Scientific).

Ubiquitination assay

The detailed procedure for the ubiquitin assay, including co-immunoprecipitation, has been previously described [35]. Briefly, the cells were treated with MG132 at concentrations of 10 μM for 16 h. Cell lysates were incubated with the SOX17 antibody, followed by co-immunoprecipitation using Protein A-agarose (Millipore, Billerica, MA, USA). The co-immunoprecipitates were examined using immunoblotting with an anti-ubiquitin antibody (cat no. 3936. Clone P4D1, CST). Protein input for immunoprecipitation was evaluated using immunoblotting with an anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA).

Small interfering RNA (siRNA)-mediated gene silencing

The detailed procedure for siRNA silencing of a target gene has been described previously [28]. In this study, we utilized Ambion siRNAs (Austin, TX, USA), siRNA ID: 105,082 and 105,646, for silencing ubiquitin C-terminal hydrolase L1 (UCHL1). Trilencer-27 Universal scrambled negative control siRNA (OriGene Technologies, Rockville, MD, USA) was used as a non-silencing control. siRNAs were transfected into cells using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cells were used for subsequent studies 48 h after transfection.

Quantitative real-time reverse transcription polymerase chain reaction

cDNA was synthesized from total RNA, and polymerase chain reaction (PCR) was performed using a reverse transcription PCR (RT-PCR) kit (Takara, Seta, Japan) [28]. Normal ovarian cDNA was obtained from Clontech (Palo Alto, CA, USA) The procedure was performed according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green Reaction Kit (Roche Diagnostics, GmbH, Mannheim, Germany) in a LightCycler (Roche Diagnostics) according to the manufacturer's instructions. The following primers were used for the real-time RT-PCR:

SOX17-forward 5′- GATGCGGGATACGCCAGTGA C-3′;

SOX17-reverse 5′- GCTCTGCCTCCTCCACGAA G-3′;

GAPDH-forward 5′-GAAGGTGAAGGTCGGAGT C-3′;

GAPDH-reverse 5′-GAAGATGGTGATGGGATTT C-3′.

The expression of each target gene was analyzed using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen [36] using the LightCycler system. The ΔCT values were normalized to GAPDH in both normal ovary and ovarian cancer cells. The values for the four ovarian cancer cell groups were calculated for each target gene as the fold-change relative to the normal ovary (set to 1.0).

Results

Heterogenous SOX17 immunoreactivity in OCCC

Representative immunohistochemical staining results are shown in Fig. 1. Notably, SOX17 immunoreactivity was heterogeneous in OCCC. In this study, we considered 25 cases with high- and 22 cases with low-SOX17 immunoreactivity (Fig. 1a–c).

Relation between SOX17 immunoreactivity and clinicopathological features

The clinicopathological characteristics of the patients are summarized in Supplementary Tables 1 and Supplementary Fig. 1. A previous study demonstrated that the median age of patients with OCCC was 55 years in over 1,400 patients [37]. Notably, SOX17-high

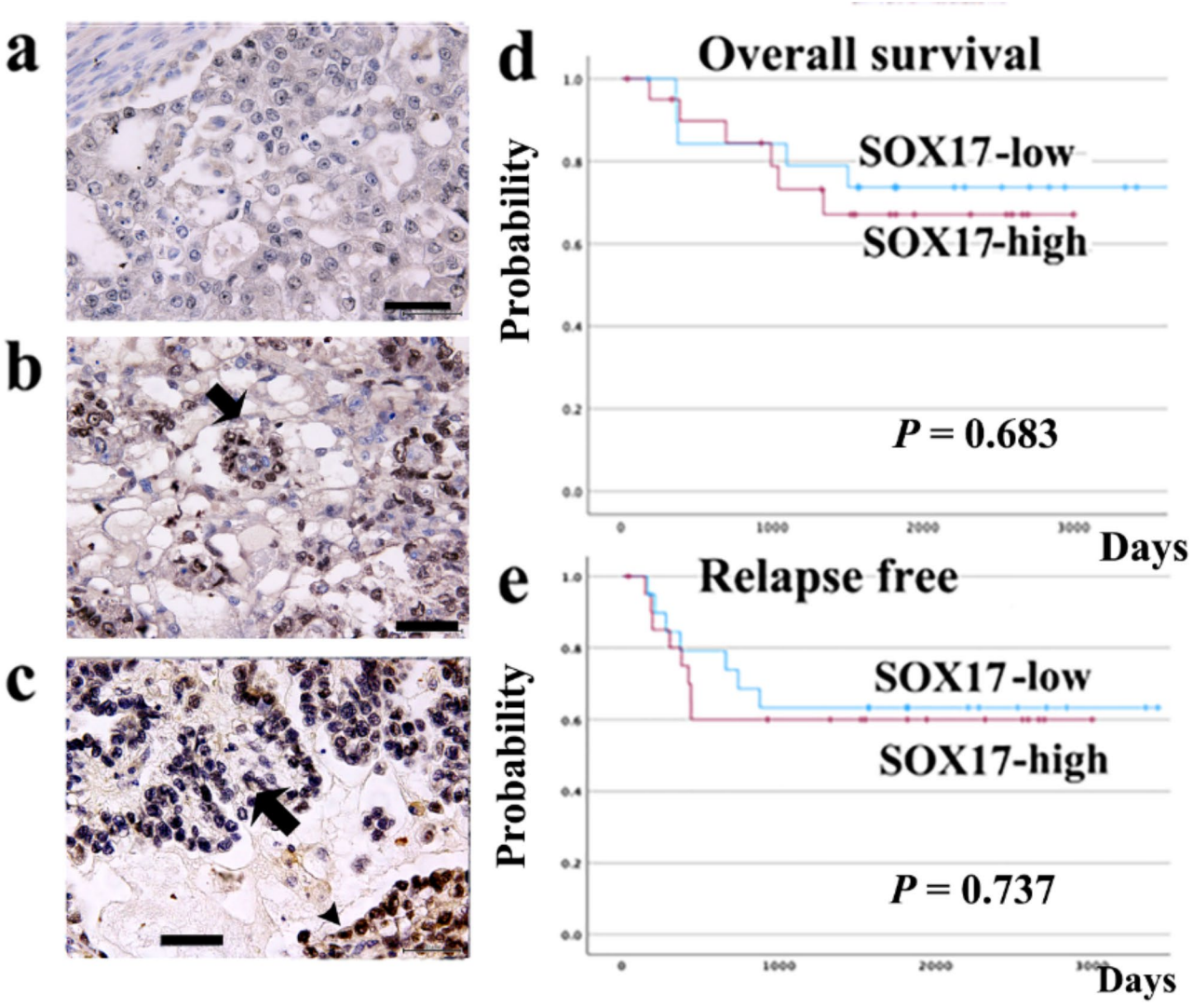

Fig. 1 SOX17 immunoreactivity in OCCC cells. SOX17 immunoreactivity was not diffusely but heterogeneously distributed in OCCC cells. Representative staining of diminished SOX17 expression (**a**). In SOX17-high cases, SOX17 immunoreactivity was focally found in OCCC cells at various intensities (b and **c**). Intermittent SOX17 immunoreactivity is shown (**b**) (arrow indicate the SOX17 immunoreactivity). Intratumoral heterogeneous SOX17 immunoreactivity was found in (**c**) (arrow indicates weak immunoreactivity, while arrowhead indicates strong reactivity). Scale bar, 50 μm

immunoreactivity was found to be significant in patients under 55 years of age.

Survival curves were estimated using the Kaplan–Meier method, and differences in survival were compared using the log-rank test (Fig. 1d and e). SOX17-high immunoreactivity tended to be more common in patients with OCCC who experienced an unfavorable outcome than in patients with SOX17-low OCCC; however, the difference was not statistically significant.

Survival curves were estimated using the Kaplan–Meier method, and differences in survival were compared using the log-rank test (d and e). SOX17-high immunoreactivity tended to be more common in patients with OCCC who experienced an unfavorable outcome than in patients with SOX17-low OCCC; however, the difference was not statistically significant.

Relation between SOX17 and ARID1A immunoreactivities

We examined the relationship between SOX17 and ARID1A expression. Representative results of immunofluorescence staining are shown in Fig. 2. Intratumoral heterogenous SOX17 immunoreactivity was found in both ARID1A-negative tissue specimens (a and b) and ARID1A-positive tissue specimens (c and d). ARID1A-positive cancer cells lacked SOX17 immunoreactivity in several cases (Fig. 2e) but exhibited SOX17 immunoreactivity in other cases (Fig. 2f). We also detected SOX17 immunoreactivity in ARID1A-negative cancer cells (Fig. 2g). Notably, SOX17 immunoreactivity was

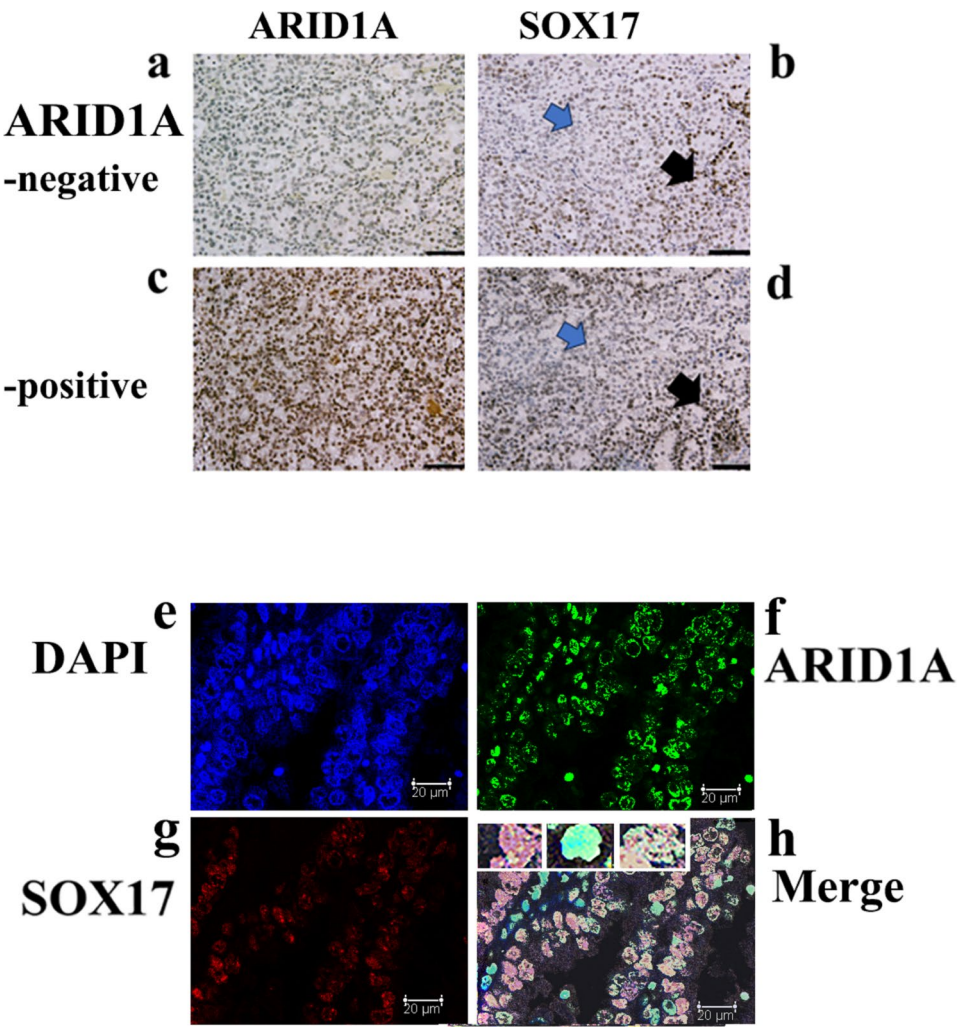

Fig. 2 Representative images of SOX17 immunohistochemical staining in both ARID1A-negative OCCC tissue specimens (**a** and **c**) and ARID1A-positive OCCC tissue specimens (**b** and **d**). Little or no ARID1A immunoreactivity was found in a, while robust ARID1A immunoreactivity was found in c. Immunohistochemical staining demonstrated that SOX17 immunoreactivity was found in both ARID1A-negative tissue specimens (**b**) and ARID1A-positive tissue specimens (**d**). Blue and black arrows indicate SOX17-negative and -positive OCCC cells, respectively. Arrows indicate 100 μm

observed in neighboring ARID1A-positive and -negative cancer cells that exhibited heterogeneous ARID1A expression (Fig. 2f).

Double immunofluorescence staining using antibodies specific to ARID1A (green) and SOX17 (red) with nuclear counterstaining with DAPI (blue) also show the intratumoral heterogeneity of SOX17 expression in OCCC. (e) Cyan nuclear staining was found in the nuclei of cancer cells in a case of ARID1A-positive and SOX17-negative OCCC. (f) Merged immunoreactivity of ARID1A and SOX17 with nuclear counterstaining with DAPI in ARID1A-positive and SOX17-positive OCCC cells is visible. (g): In ARID1A-negative and SOX17-positive OCCC, cancer cells exhibited magenta nuclear staining due to merged red SOX17-immunoreactivity and blue DAPI staining. (h) The neighboring ARID1A-positive and SOX17-positive OCCC cells (indicated with arrowhead) and ARID1A-negative and SOX17-positive OCCC cells (arrow) in a heterogenous ARID1A-loss case. Scale bar, 20 μm.

SOX17 expression in cultured OCCC cell

The representative results are shown in Fig. 3. Immunoblotting revealed a prominent SOX17 protein band in the HGSC cell line OVSAHO. The SOX17 band was also detected in all of the three OCCC cell lines that were examined. However, the SOX17 band signal was considerably weaker in the OVTOKO cells than in the OVISE and RMG-V cells. Notably, polyubiquitinated SOX17 bands were observed in OVTOKO cells, but not in OVISE or RMG-V cells, when these cells were treated with the proteasome inhibitor MG132. In contrast, siRNA-mediated down-regulation of UCHL1 expression

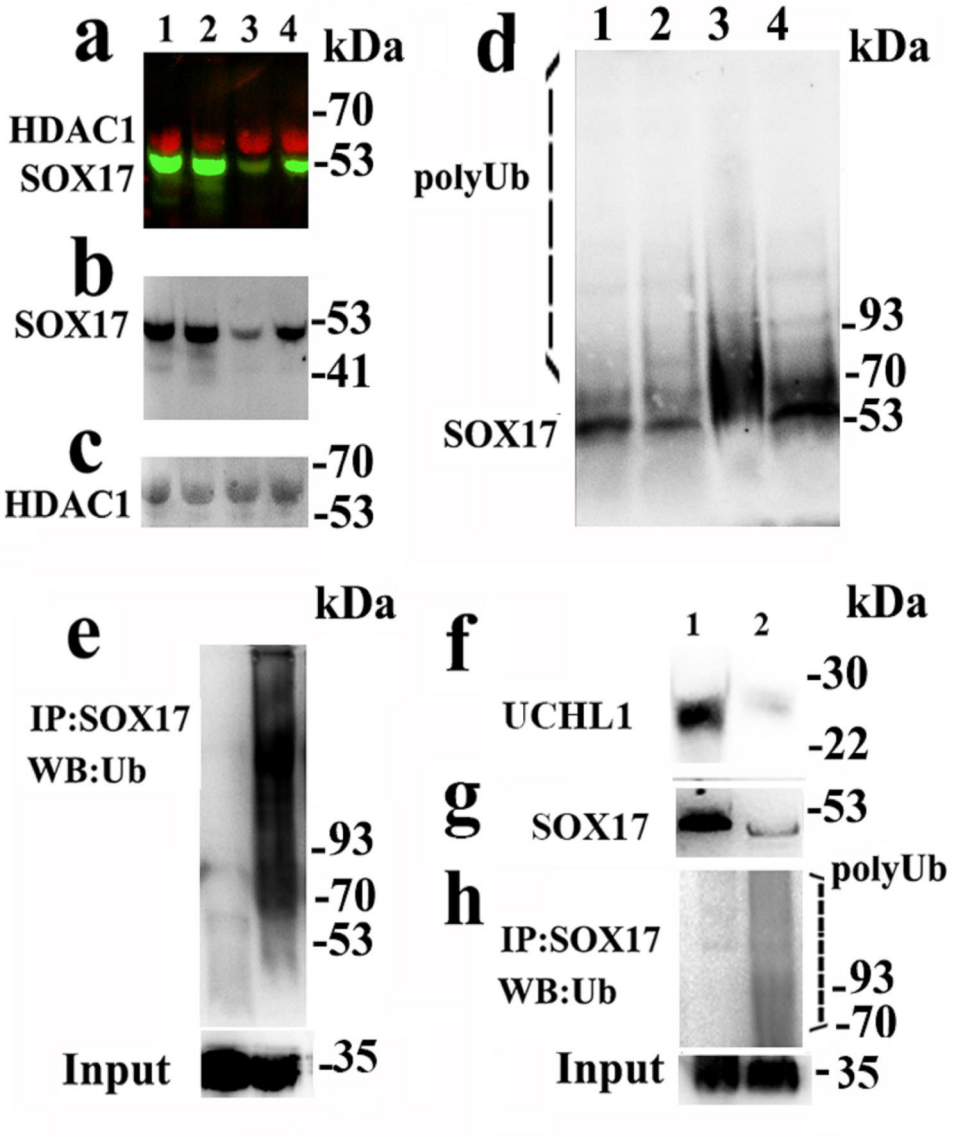

Fig. 3 Immunoblotting results. This procedure revealed a strong SOX17 protein band (green) in a HGSC cell line, OVSAHO (lane 1). The SOX17 band was also found in all of the examined OCCC cell lines (**a**) However, SOX17 band was notably smaller in OVTOKO OCCC cells (lane 3) compared to that of the OVISE (lane2) and RMG-V (lane 4) OCCC cells. The internal control, HDAC1, was stained red. SOX17 and HDAC1 bands were extracted as black and white bands (**b** and **c**, respectively). The polyubiquitinated SOX17 bands were found in OVTOKO cells (lane 3), but were present at diminished levels in the OVSAHO (lane 1), OVISE (lane 2), and RMG-V cells (lane 4) when they were treated with a proteasome inhibitor, MG132 (**d**). Input protein loads of cell lysates that were subjected to co-immunoprecipitation (IP) with anti-SOX17, followed by immunoblotting (WB) with anti-ubiquitin is shown as G3PDH bands (**e**). siRNA-mediated silencing of *UCHL1* decreased the intensity of UCHL1 (**f**) and SOX17 (**g**) protein bands (lane 1, treated with control siRNA; lane 2, treated with Ambion siRNA ID: 105082) and moreover, increased polyubiquitinated SOX17 proteins (**h**). Equal input protein loads are shown as G3PDH bands. Similar results were also obtained using another siRNA ID: 105,646

increased polyubiquitination followed by proteasome degradation of SOX17 in OVISE cells.

We demonstrate the raw western blotting data in the Supplementary Fig. 2.

We also evaluated SOX17 mRNA expression in normal ovaries and in four ovarian carcinoma cell lines.

Interestingly, SOX17 mRNA expression was much higher in OV-SAHO, which closely resembles the.

cognate tumor profiles of HGSC [33], than in the three OCCC cell lines (supplementary Fig. 3).

Quantitative RT-PCR reveals that OV-SAHO, which closely resembles tumor profiles of HGSC [X] (lane 1), expressed abundant *SOX17* mRNA compared to OVISE (lane 2), OVTOKO (lane 3), and RMG-V (lane 4) OCCC cells. As previously reported by Kanai et al. [38] normal ovary showed low expression of *SOX17* mRNA (lane 5). Real-time PCR was performed in triplicate. The values

for the four ovarian cancer cells were calculated in terms of the fold change relative to the mean value for the normal ovary (set to 1.0). Standard deviations were then computed for the triplicate sets.

Discussion

Recently, two research groups reported that SOX17 is a highly sensitive and specific immunomarker for ovarian and endometrial carcinomas [27, 39]. Contrary to this claim, it has been documented that SOX17 is expressed in various tumors, such as gastric cancer [40], lung cancer [41], testicular tumors [42], oligodendroglioma [43], and esophageal cancer [44]. SOX17 also promotes lung cancer metastasis [45, 46].

Regarding SOX17 expression in OCCC, Lin et al. [15], using a tissue microarray, first reported that SOX17 immunostaining was found in approximately 60% of OCCC cases. In the present study, we found that 25 of 47 (53%) OCCC cases exhibited > 10% SOX17 immunoreactivity in whole tissue sections (Fig. 1). SOX17 immunoreactivity was not uniformly or heterogeneously detected in any OCCC tissue specimen (Figs. 1 and 2). The intensity of the SOX17 band varied among the three OCCC cell lines (Fig. 3). Collectively, these results suggest that SOX17 expression is not an inherent phenotype of OCCC.

SOX17 is believed to act as an antagonist of the canonical Wnt/beta-catenin signaling pathway [47]. Since the Wnt/beta-catenin signaling pathway contributes to cancer stem cell renewal, cell proliferation, and differentiation [48], SOX17 could promote OCCC carcinogenesis. However, our preliminary experiments failed to show any significant difference in OCCC cell growth by artificial down-regulation of SOX17 in vitro (data not shown). On the other hand, SOX17 promotes the secretion of angiogenic factors to facilitate tumor neovascularization in ovarian cancer [14]. Moreover, a recent study highlights that SOX17 enables immune evasion during colorectal carcinogenesis [49]. These pleiotropic pathobiological properties of SOX17 might be linked to our present clinicopathological findings that high SOX17 expression tend to be related to the poor outcome of patients with OCCC, despite not being statistically significant. Further extensive studies are desired to explore how and when SOX17 could help or hinder OCCC carcinogenesis.

In the present study, SOX17-high immunoreactivity was found to be significant in younger patients with OCCC, i.e., those under 55 years of age. A previous study demonstrated that patients with OCCC arising in endometriosis were younger than those with OCCC not arising in endometriosis [50]. It may be interesting to explore whether SOX17 plays a role in endometriosis-related carcinogenesis in OCCC.

We also investigated the molecular events involved in SOX17 expression in OCCC. It is well established that OCCC often harbors mutations of *ARID1A*, which lead to insufficient ARID1A expression [7, 51]. ARID1A (also known as SMARCF1 or BAF250) is a DNA-binding element of the chromatin remodeling complex; thus, the loss of ARID1A expression alters the protein profile of various cancers [17, 19, 23]. However, as summarized in Fig. 2, no significant relationship was found between ARID1A and SOX17 expression in OCCC cells. We believe that SOX17 may be related to the carcinogenesis of OCCC in a manner that is independent of the loss of ARID1A expression.

The ubiquitin-proteasome system can degrade SOX17 [52]. Therefore, we investigated the polyubiquitin status of the cultured OCCC cells. As shown in Fig. 3, polyubiquitination bands of SOX17 were found in SOX17-low OVTOKO cells, but not in SOX17-high OVISE or RMG-V OCCC cells when they were treated with MG132. We believe that SOX17 was degraded in OVTOKO cells, but not in OVISE or RMG-V OCCC cells, via the ubiquitin-proteasome system. Moreover, knockdown of UCHL1, which was recently reported to stabilize SOX17 through by deubiquitinating activity in endothelial cells [53], decreased SOX17 expression in OVISE cells. Protein degradation through the ubiquitin-proteasome cascade may contribute to heterogeneous SOX17 expression in OCCC. Walker et al. [49] reported a high mutation frequency in SOX17 in endometrioid endometrial carcinoma; 62 of 539 patients carried mutations. Mutated SOX17 proteins may escape ubiquitin-proteasome degradation, thus accumulating in some OCCCs. In addition, since *UCHL1* could be silenced by hypermethylation of its promoter region in ovarian cancer [54], down-regulation of UCHL1 might lead to instability of SOX17 in OCCCs. However, further studies are needed to verify this hypothesis. Since SOX17 mRNA expression was much lower in the three OCCC cell lines than in the representative HGSC OV-SAHO cells (supplementary Fig. 3), SOX17 expression may be regulated at both the transcriptional and post-transcriptional levels in OCCC.

To our knowledge, this is the first report to describe the prognostic value of SOX17 in OCCC; however, the pathobiological properties of SOX17 expression in OCCC remain unclear. Our preliminary experiments failed to show any significant difference in OCCC cell growth by artificial down-regulation of SOX17 in vitro (data not shown). It was recently shown that SOX17 appears to orchestrate an immune-evasive program during the early steps of colon carcinogenesis [49]. Immune evasion also plays a critical role in the carcinogenesis of OCCC [54]. We are now exploring whether OCCC cells

also employ SOX17 to evade immune response, similar to colon cancer cells.

Recently, a large-scale study using over 500 cases confirmed the lack of prognostic significance for loss of ARID1A immunoreactivity, a reliable surrogate evaluation of ARID1A mutation, in OCCC [55]. The present study indicated that SOX17 was very heterogeneously expressed in the OCCC tissue specimens (Fig. 2). This intratumoral heterogeneity impedes evaluation of the prognostic significance in relation to ARID1A and SOX17 in OCCC. It may be interesting to examine the relation of other relevant genes to ARID1A and SOX17 in clinical samples, and their correlation with prognosis.

In conclusion, the present study demonstrates that SOX17 expression is not an inherent phenotype of OCCC cells. The ubiquitin-proteasome pathway may be responsible for heterogeneous SOX17 expression in OCCC cells without relation to loss of ARID1A expression.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-024-01549-3.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Author contributions

DK performed the experiments and analyzed the data. MT collected and analyzes the data. CS collected tissue specimens, analyzed immunohistochemical staining results, and prepared the manuscript. MA and YH provided technical support and prepared the tissue specimens. MI and TT conceptualized the study design. TT also analyzed data and prepared the manuscript.

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Data availability

Datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval

The study was conducted in accordance with the ethical standards of the 1975 Declaration of Helsinki. This study was approved by the Institutional Review Board of Gifu University Graduate School of Medicine (approval number 2023-080).

Competing interests

The authors have no financial or proprietary interests in any material discussed in this article.

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