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Expression of chromobox homolog 7 (CBX7) is associated with poor prognosis in ovarian clear cell adenocarcinoma *via* TRAIL-induced apoptotic pathway regulation

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Ovarian cancer is the most lethal gynecologic malignancy, and clear cell adenocarcinoma of the ovary (OCCA), in particular, has a relatively poor prognosis among the ovarian cancer subtypes because of its high chemoresistance. Chromobox (CBX) 7 is a polycomb repressive complex 1 component that prolongs the lifespan of normal human cells by downregulating the INK4a/ARF expression which promotes cell-cycle progression. However, recent reports studying the relationship between CBX7 expression and patient survival have differed regarding the tumor cell origins, and the precise role of CBX7 in human carcinomas remains obscure. In this study, we analyzed CBX7 expression by immunohistochemistry in 81 OCCA patients and evaluated its association with their clinical outcomes. Both the overall and progression-free survival rates of the CBX7-positive patients were significantly shorter than those of the CBX7-negative patients ($p < 0.05$). CBX7 knockdown experiments using two OCCA cell lines, TOV21G and KOC-7C, revealed that cell viability was significantly reduced compared to the control cells ($p < 0.001$). Expression microarray analysis revealed that apoptosis-related genes, particularly tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), were significantly upregulated in CBX7 knockdown cells ($p < 0.01$). We further confirmed that CBX7 knockdown resulted in TRAIL-induced apoptosis in the OCCA cells. Thus, in this study, we showed for the first time that CBX7 was associated with a decreased OCCA prognosis. We also successfully demonstrated that the TRAIL pathway is a novel target for CBX7 expression modulation in these cells, and therapeutic agents utilizing the TRAIL pathway may be particularly effective for targeted OCCA therapy.

Ovarian cancer is the most lethal gynecologic malignancy. There is no effective screening method, and most women are diagnosed with advanced-stage disease. In Japan, ovarian clear cell adenocarcinoma (OCCA) accounts for ~25% of all epithelial ovarian cancer (EOC) cases,[1] whereas in North America and Europe, OCCA accounts for only 1–12% of cases.[2] OCCA has been considered to be distinct from high-grade serous adenocarcinoma because of its clinical and biological characteristics. OCCA has been known to be associated with endometriosis,[3,4] and recent studies have suggested that oxidative stress caused by excess iron leads to carcinogenesis in the ovary.[5,6] Advanced stage is associated with poor prognosis in OCCA,[2] and the survival rates in the stages are poorer than those for patients with serous adenocarcinoma,[7] most likely because of OCCA resistance to standard platinum-based chemotherapy.[8] Therefore, it is important to find new therapeutic targets for OCCA.

Chromobox 7 (CBX7) is a member of the polycomb group (PcG) of proteins and is a component of polycomb repressive complex 1 (PRC1). PRC1 can silence the genes that are related to stem cell renewal, differentiation and cancer in conjunction with PRC2.[9] PcG proteins have been reported to be overexpressed and associated with tumorigenesis in a variety of human cancers, mostly by downregulating

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What's new?

Ovarian cancer is the most lethal gynecologic malignancy, with clear celladenocarcinoma of the ovary (OCCA) having a particularly poor prognosis due to high chemoresistance. Chromobox homolog 7 (CBX7) is a polycomb group transcriptional repressor whose role in human cancer remains controversial. Here,the authors showedfor the first time that CBX7 expression is related to worse prognosis in OCCA. Furthermore, knockdown of CBX7 in vitro induced apoptosis in OCCA cell lines, possibly via regulation of the TRAIL-pathway. The findings thus indicate CBX7 as a good prognostic marker, andthe TRAIL-pathway as apotential target for OCCA diagnosis and therapy.

the tumor suppressor genes.[10] CBX7 has been reported to extend the cellular life span by directly repressing the INK4a/ARF locus.[11] Additionally, CBX7 is an oncogene in several human tumors, including follicular lymphoma, prostate and gastric cancers.[12–14] In follicular lymphoma, CBX7 also cooperates with MYC to produce highly aggressive B-cell lymphoma and can initiate T-cell lymphomagenesis by repressing the INK4a/ARF locus.[13] Recently, it has been reported that CDKN2B-AS (ANRIL), a long noncoding RNA also encoded in the INK4a/ARF locus, acts together with CBX7 to repress INK4a/ARF locus genes, such as CDKN2A (p16; p16INK4a) and ARF (p14 ARF) in prostate cancer cells.[15] Furthermore, a genome-wide association study comparing Japanese women with endometriosis to healthy controls revealed that a single nucleotide polymorphism that showed the strongest association with endometriosis was located in the intron of the ANRIL gene.[16] As OCCA is known to have strong association with endometriosis,[4–6] we speculated that CBX7 and ANRIL may play some important roles in the OCCA tumorigenesis. However, in clear contrast, the loss of CBX7 expression has been reported in association with poorer prognoses and more aggressive behaviors of pancreatic, colorectal, and lung cancers.[17–19] It has been shown that CBX7 represses CDH1 (E-cadherin) expression and CCNE1 (cyclin E1) expression in pancreatic and lung cancers, respectively.[20,21] Therefore, the role of CBX7 in cancer still remains controversial. In this study, we attempted to clarify the role of CBX7 in ovarian cancer, specifically OCCA.

Material and Methods

Patients and tissue samples

The ethics committee (Internal Review Board) of the Nagoya University Graduate School of Medicine approved the experiments. The human samples were obtained after each patient provided written informed consent. Formalin-fixed, paraffin-embedded tumor samples from 81 primary OCCA were obtained from the patients who underwent surgical treatment at Nagoya University Hospital from 1986 to 2009 and had clinical follow-up information available. Tumor staging was based on the International Federation of Gynecology and Obstetrics (FIGO) classifications, and reviewed by two expert gynecologists (H.K. and F.K.) for this study. All patients were primarily treated with optimally debulking surgery by skilled surgeons in gynecologic oncology. Thereafter, 75 (93%) of the 81 patients received adjuvant chemotherapy. Beginning in 1997, most of the patients received platinum- and taxane-based agents or CPT-11 chemotherapy. Before 1997, various cisplatin-based chemotherapies were administered. The following chemotherapy regimens were followed: 7% cyclophosphamide 500 mg/m^2, adriamycin 50 mg/m^2, and cisplatin 50 mg/m^2 (CAP), 8% cisplatin and carboplatin (PP), 5% cisplatin, vinblastine, and bleomycin (PVB), 62% paclitaxel 175 mg/m^2, carboplatin AUC5 (TC), 3% docetaxel 70 mg/m^2, carboplatin AUC5 (DC), 4% CPT-11 180 mg/m^2, cisplatin 60 mg/m^2 (CPT-P), 2% other, and 9% unknown. Tumor recurrence or progression was determined by clinical, radiologic, or histologic diagnosis. All histologic diagnoses were specifically reviewed by experts in gynecological pathology (Y.Y. and T.N.) for this study. Endometriosis was defined histologically as the presence of endometrial glands and stromal tissues other than the endometrium or within onethird depth of the uterine myometrium. We also excluded ovarian endometrial cysts lacking epithelium without any atypia for the diagnosis of endometriosis.

Cell culture and cell lines

The human OCCA cell line TOV-21G and a prostate cancer PC3 line were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and the KOC7C line was a generous gift from Dr. Junzo Kigawa (Tottori University, Tottori, Japan). These cells were cultured in an RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% fetal bovine serum at 37°C under a 5% CO_2 atmosphere and were tested and authenticated using the short tandem repeat (STR) method.[22] Human OCCA cell lines JHOC-5, 7, 8, 9 were recently (2009-) obtained from Riken BRC (Tsukuba, Japan) and cultured using Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM:F12) medium (Sigma-Aldrich) with similar conditions. Human endometrial epithelial cells were obtained from normal endometrium of a patient undergoing hysterectomy, and after collagenase treatment, cells were cultured using DMEM:F12 and then infected first with a lentivirus encoding HPV16 E6 and E7 followed by a retrovirus encoding human telomerase similar to previous studies.[23,24] Establishment procedure for the cells (hEEC-N1) was done with written permission, and established cells were confirmed as endometrial epithelial origin by immunohistochemical

analyses for detection of both keratin and vimentin using similar methods as a previous study.[23]

Immunohistochemistry and RNA fluorescence *in situ* hybridization (RNA-FISH)

For immunohistochemistry (IHC), we used anti-CBX7 (ab21873, Abcam, Cambridge, MA) as the primary antibody. DNA probes for RNA-FISH were prepared by PCR amplifying cDNA templates to obtain ~500 bp sized-PCR products using primers 5′-GAATTTGGGAATGAGGAGCA-3′ and 5′-AAGCTGCAAAGGCCTCAATA-3′ for ANRIL and 5′-TCAG AAGGATTCCTATGTGG-3′ and 5′-TCTCCTTAATGTCAC GCACG-3′ for human β-actin, and then labeled with SpectrumOrange using Vysis Nick Translation Kit (Abbott, Abbott Park, IL). For fluorescence IHC and RNA fluorescence *in situ* hybridization (RNA-FISH), cells were grown on a chamber slide, and fixed with 4% paraformaldehyde. For RNA-FISH, after permeabilization using cytoskeletal buffer, slides were hybridized with the labeled probes. For fluorescence IHC, cells were visualized with Alexa Fluor labeled secondary antibody (Life Technologies, Carlsbad, CA). Finally, cells were counterstained with DAPI and then visualized with a fluorescence microscope.

For formalin-fixed, paraffin-embedded ovarian tumor sections, we used polymer-based methods with the EnVision System (DAKO, Glostrup, Denmark). CBX7 staining was interpreted by three independent pathologists (Y.Y., T.N., and S.T.) blinded to clinical data. Because interobserver variability was rather large in samples with 10–50% nuclear staining, we defined the samples that contained nuclear staining >10% of cancer cells as positive, and the others negative.

Small interference RNA (siRNA) transfection

To knockdown CBX7, we used two individual siRNAs: One was an endoribonuclease-prepared siRNA (esiRNA) against CBX7 (EHU035461, Sigma-Aldrich), and the other was designed using the sense sequence 5′-GCATTTGCCCATC TGCCTT-3′; we named these siRNAs siCBX7-1 and siCBX7-2, respectively. We used MISSION siRNA Universal Negative Control (SIC-001, Sigma-Aldrich) as the negative control. KOC7C and TOV21G were transfected with the siRNAs using the Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) at a final concentration of 10 nM, according to the manufacturer's instructions. Analyses were performed 48 hr after transfection.

Quantitative reverse transcriptase-PCR

Total RNA was extracted from the cell lines using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Then, cDNA was synthesized from 500 ng total RNA using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies), and quantitative PCR was performed as previously described.[25] The sequences of primers were as follows: CBX7, forward 5′-GGATGGCCCCCAAAGTACAG-3′ and reverse 5′-TATACCCCGATGCTCGGTCTC-3′; ANRIL, forward 5′-CAACATCCACCACTGGATCTTAACA-3′ and reverse 5′-AG CTTCGTATCCCCAATGAGATACA-3′; CDKN2A, forward 5′-CATAGATGCCGCGGAAGGT-3′ and reverse 5′-CCCGA GGTTTCTCAGAGCCT-3′; TNFSF10A, forward 5′-C CTCA GAGAGTAGCAGCTCACA-3′ and reverse 5′-GCCCA GAG CCTTTTCATTC-3′; for β-actin, forward 5′-CGGGAC CT GACTGACTA-3′ and reverse 5′-GAAGGAAGGCTGGA AG AGT-3′; and TNFSF10, forward 5′-CCTCAGAGAGTAG CA GCTCACA-3′ and reverse 5′-GCCCAGAGCCTTTTCAT TC-3′. The data from the PCR reaction were normalized against the β-actin expression using the comparative Ct method. The transcripts were quantified in duplicates.

Western blot analysis

Whole-cell or tissue lysates were prepared similarly as previously described[26] from cell lines and primary tumor tissues from four OCCA patients. Twenty-microgram proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted on Immobilon P filters (Millipore, Billerica, MA). The following antibodies were used: anti-Cbx7 (1:1,000) (ab21873, Abcam), anti-β-actin (1:2,000) (A5316, Sigma-Aldrich) and anti-PARP (1:1,000) (#9542, Cell Signaling Technology, Beverly, MA). ImmunoStar LD (Wako, Osaka, Japan) was used for chemiluminescence detection.

Cell viability assay

A total of 4,000 cells were transfected with siRNAs in 96-well plates and incubated under 5% CO_2 at 37°C. After 24, 48, 72, and 96 hr, cell viability was assayed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI), according to the manufacturer's instructions.

Migration and invasion assay

The assays were performed using 6.5 mm Transwell plates with 8.0 μm pore polycarbonate membrane inserts (Corning Coaster, Rochester, NY). For the invasion assay, the upper surfaces of the filters were coated with 50 μl of matrigel (Becton and Dickenson, Franklin Lakes, NJ). Next, 1×10^5 cells were seeded in the upper chamber in the culture medium without FBS, and the lower chamber contained 10% FBS. The cells were incubated for 24 hr at 37°C in 5% CO_2. After removing the noninvaded or nonmigrated cells, the remaining cells were stained with Giemsa.

Cell cycle analysis

A total of 5×10^5 cells were washed with PBS, fixed with 70% ethanol at −20°C for at least 30 min, washed again with PBS, and incubated with 0.1 mg/ml RNase A solution (QIAGEN) at 37°C for 20 min. The cells were centrifuged, washed again with PBS, and then incubated with 50 μg/ml of propidium iodide (Sigma-Aldrich) on ice for 20 min. The cell cycle profiles were determined using a FACS Calibur

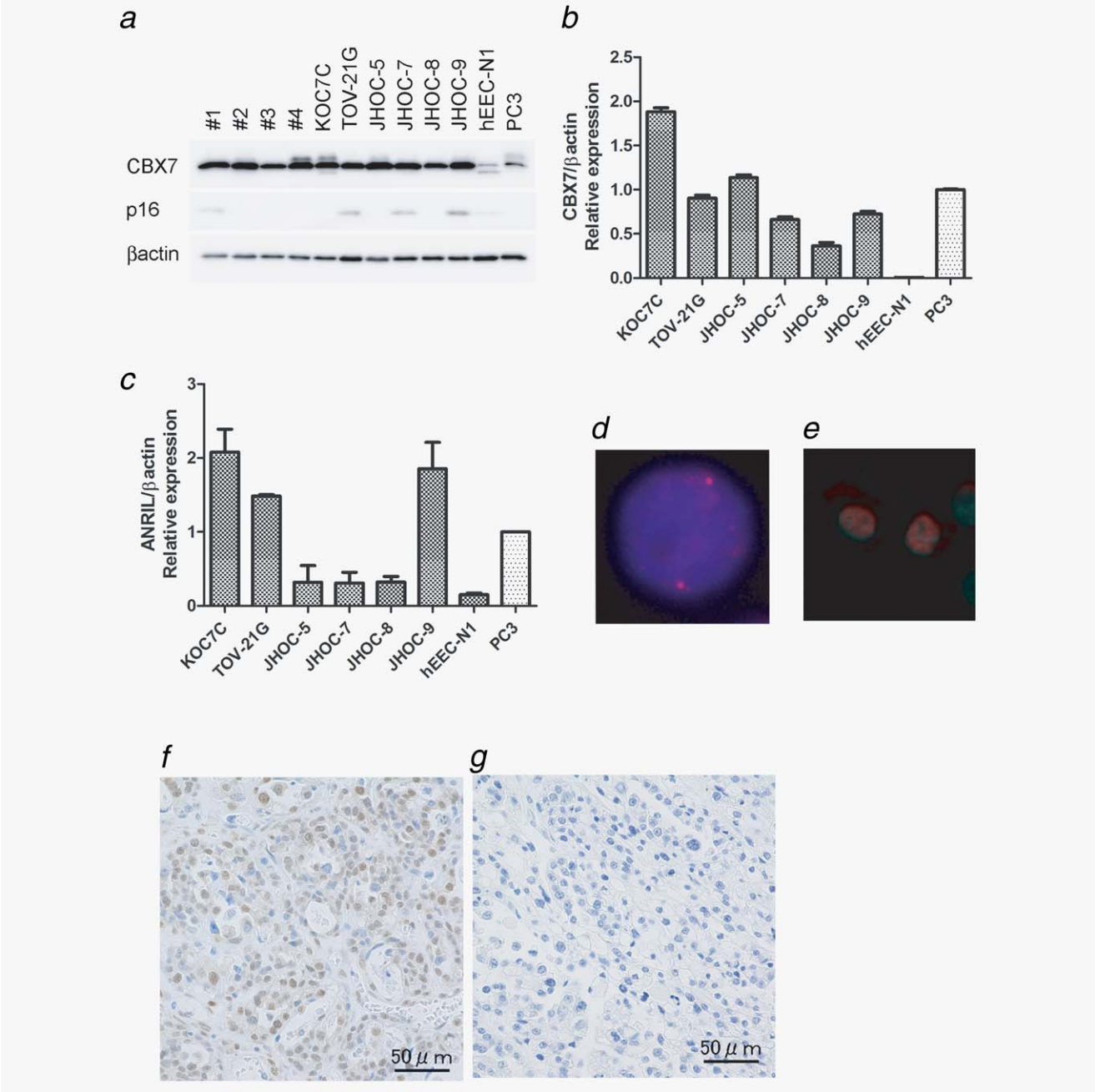

Figure 1. Expression of CBX7 and ANRIL in ovarian clear cell adenocarcinoma (OCCA). (*a–c*) Immunoblotting of four OCCA primary tissue samples (#1,2,3), six OCCA cell lines (KOC-7C, TOV-21G, JHOC-5, -7, -8, -9), a human endometrial epithelial cell line (hEEC-N1) and a positive control prostate cancer cell line, PC3, for detecting CBX7, CDKN2A (p16), and β-actin (*a*). Real-time quantitative reverse transcription PCR analysis using the cells to detect CBX7 (*b*) and ANRIL (*c*). (*d,e*) RNA fluorescence *in situ* hybridization detecting ANRIL (*d*) and fluorescence IHC detecting CBX7 (*e*) in TOV21G cells. Orange signals of both ANRIL and CBX7 are observed in the nuclei of the cells. (*f,g*) Immunohistochemical analysis for CBX7 protein using formalin-fixed paraffin embedded OCCA samples. Intense nuclear staining of the tumor cells is observed (*f*). Negative CBX7 staining (*g*). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

machine (Becton and Dickenson) and analyzed using ModFit LT (Verity Software, Topsham, ME) and CellQuest software (Becton and Dickenson).

Gene expression array analysis

The SurePrint G3 Human Gene Expression 8x60K Microarray (Agilent Technologies, Santa Clara, CA) was used to analyze changes in the mRNA expression levels of the KOC7C and TOV-21G cells after CBX7 knockdown. The microarray targets 27, 958 Entrez Gene RNAs and 7,419 noncoding RNAs. KOC7C and TOV-21G were transfected by siCBX7-1 or negative control siRNA. After 48-hr incubation, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). CBX7 knockdown was confirmed by qPCR. Data

Table 1. Multivariate analysis of clinical factors in relation to survival of patients with OCCA

	OS			PFS		
	HR	95% CI	*p*	HR	95% CI	*p*
Age						
≥50 (*vs*. <50)	0.720	0.380–1.952	0.720	0.459	0.203–1.040	0.459
FIGO stage						
II, III, IV (*vs*. I)	3.811	1.652–8.791	0.002	5.467	2.352–12.706	<0.001
CA125 (U/ml)						
≥35 (*vs*. <35)	2.900	0.181–10.282	0.099	2.562	0.827–7.934	0.103
Year of diagnosis						
1997- (*vs*. -1997)	1.186	0.520–2.702	0.685	1.312	0.594–2.897	0.502
CBX7 expression						
Positive (*vs*. negative)	6.767	1.534–29.856	0.012	8.661	1.946–38.559	0.005

HR, hazard ratio; CI, confidence interval.

analysis was performed using GeneSpring GX11.05.1 software (Agilent Technologies).

TdT-mediated dUTP-biotin nick end labeling assay

We performed the TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay to detect apoptotic cells. The cells were transfected with siRNA on chamber slides and cultured in 5% CO_2 at 37°C for 48 hr. We then used the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore) following the manufacturer's instructions. Briefly, the cultured cells were fixed with 1% paraformaldehyde and permeabilized using a 2:1 ethanol-acetic acid solution, endogenous peroxidase was quenched using 3% hydrogen peroxide in PBS. After detection, hematoxylin was used as a counterstain. We counted positively stained cells in 10 random fields under 400× magnification and calculated the apoptotic cell ratio.

Statistical analysis

Chi-square tests were used to evaluate the associations between the CBX7 status and clinicopathological factors. The overall survival (OS) was calculated from the date of surgery to the date of the last follow-up or the date of death from OCCA. The progression-free survival (PFS) was calculated from the date of surgery to the date of progression/recurrence or to the date of last follow-up. Survival analyses were performed using the Kaplan–Meier method to estimate the OS and PFS, and statistical significance was determined using the log-rank test. A multivariate analysis was performed using a Cox proportional hazards model. Student's *t*-test (for comparison of the two groups), a one-way ANOVA or a two-way ANOVA (for multiple comparisons) was used to evaluate the numerical data. For all of the statistical analyses, $p < 0.05$ was considered statistically significant.

Results

Expression of CBX7 and ANRIL in OCCA

First, we examined the expression of CBX7 protein and mRNA together with ANRIL RNA in six OCCA cell lines and control by immunoblotting and qPCR (Figs. 1*a*–1*c*), and revealed that both genes, CBX7 and ANRIL, were expressed in all OCCA cell lines. Control human endometrial cells also expressed both genes although smaller in amount. CBX7 was detectable by immunoblotting in the four primary OCCA tissue samples (Fig. 1*a*). Furthermore, we analyzed p16 expression in these cell lines. Because JHOC-5 and JHOC-8 had p16 deletion detected by array comparative genomic hybridization analysis (Supporting Information Figure 1), and JHOC-7 and JHOC-9 had relatively high expression of the p16 protein, we selected KOC-7C and TOV21G for further analyses. We further confirmed the expression of ANRIL and CBX7 protein in the nuclei of both cells by RNA-FISH (Fig. 1*d*) and fluorescence IHC (Fig. 1*e*). Then, we analyzed the expression of CBX7 protein in 81 primary human OCCA tissues by IHC staining. Intense nuclear staining was observed in the positive samples (Fig. 1*f*) in clear contrast with no staining in the negative samples (Fig. 1*g*). Of 81 cases, 64 cases (79%) were CBX7-positive and 17 cases (21%) were CBX7-negative. Next, we examined the association between CBX7 expression and clinical factors. Switching to platinum-taxane chemotherapy which improved OS and PFS in women with ovarian cancer[25] was done in 1997 in our institution. Therefore, we speculated that the year of diagnosis may be associated with prognostic outcome. Furthermore, although most patients were not previously clinically diagnosed as endometriosis, pathological examination of the resected tissue material revealed that the more than 80% had endometriosis (52/64 data available patients, see Supporting Information Table 1). Thus, we added the year of diagnoses and also pathologically diagnosed endometriosis in the analysis. As a result, CBX7 expression was not significantly associated with any of the following factors; patient age, FIGO stage, serum CA125 level, the year of diagnosis, or the presence of endometriosis (Supporting Information Table 1).

CBX7 expression is associated with poor prognosis in patients with OCCA

To further evaluate the role of CBX7 in OCCA, we analyzed the relationship between CBX7 expression and OS and PFS

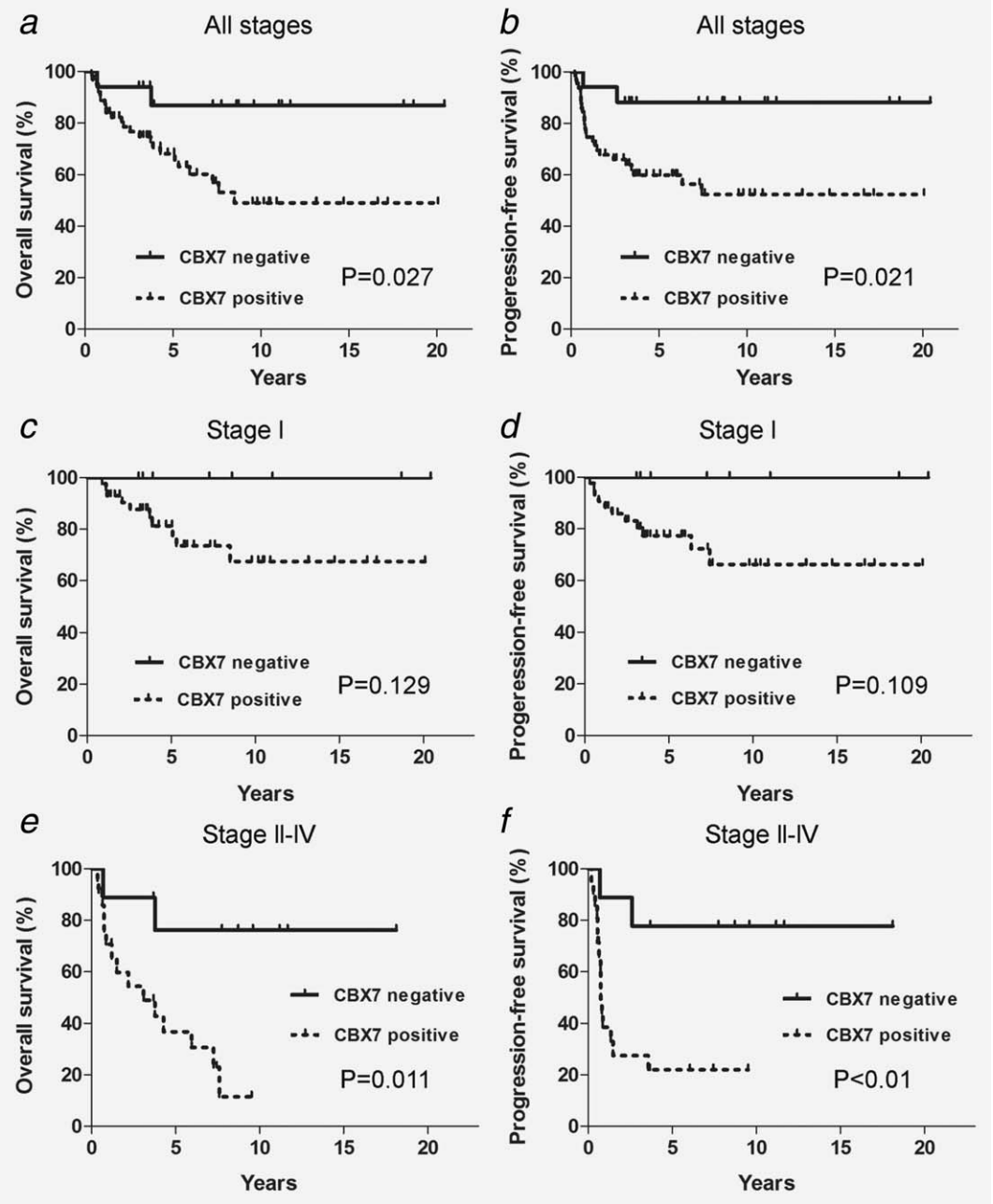

Figure 2. Kaplan–Meier analyses. CBX7 expression is associated with worse OS and PFS in OCCA patients. These Kaplan–Meier survival curves show (*a,b*) OS and PFS in patients with all stages. (*c,d*) OS and PFS in patients with Stage I disease. (*e,f*) OS and PFS in patients with Stages II–IV disease. The solid line indicates the CBX7-negative group, and the dashed line indicates the CBX7-positive group.

using the Kaplan–Meier method together with the log-rank test. The CBX7-positive group had significantly shorter OS ($p = 0.027$, Fig. 2*a*) and PFS ($p = 0.021$, Fig. 2*b*) rates than the CBX7-negative group. However, as most patients with OCCA are diagnosed with Stage I disease (51 cases, 63%), we also analyzed the relationship between CBX7 expression and OS and PFS rates by separating the cases into two groups: the Stage I group and a group consisting of Stages II–IV patients, as categorized at their initial diagnoses. In patients with Stages II–IV disease, the CBX7-positive patients had significantly shorter OS ($p = 0.011$; Fig. 2*e*) and PFS ($p < 0.01$; Fig. 2*f*) rates than the CBX7-negative patients. The CBX7-positive patients tended to have poorer prognoses than the CBX7-negative patients in the Stage I group also, although this difference was not significant (Figs. 2*c* and 2*d*). Similar analysis of endometriosis-positive and negative groups revealed that CBX7 expression was significantly associated with worse OS and PFS rates in the endometriosis-positive group (Supporting Information Figure 2). Endometriosis-negative group also showed a tendency to have worse OS

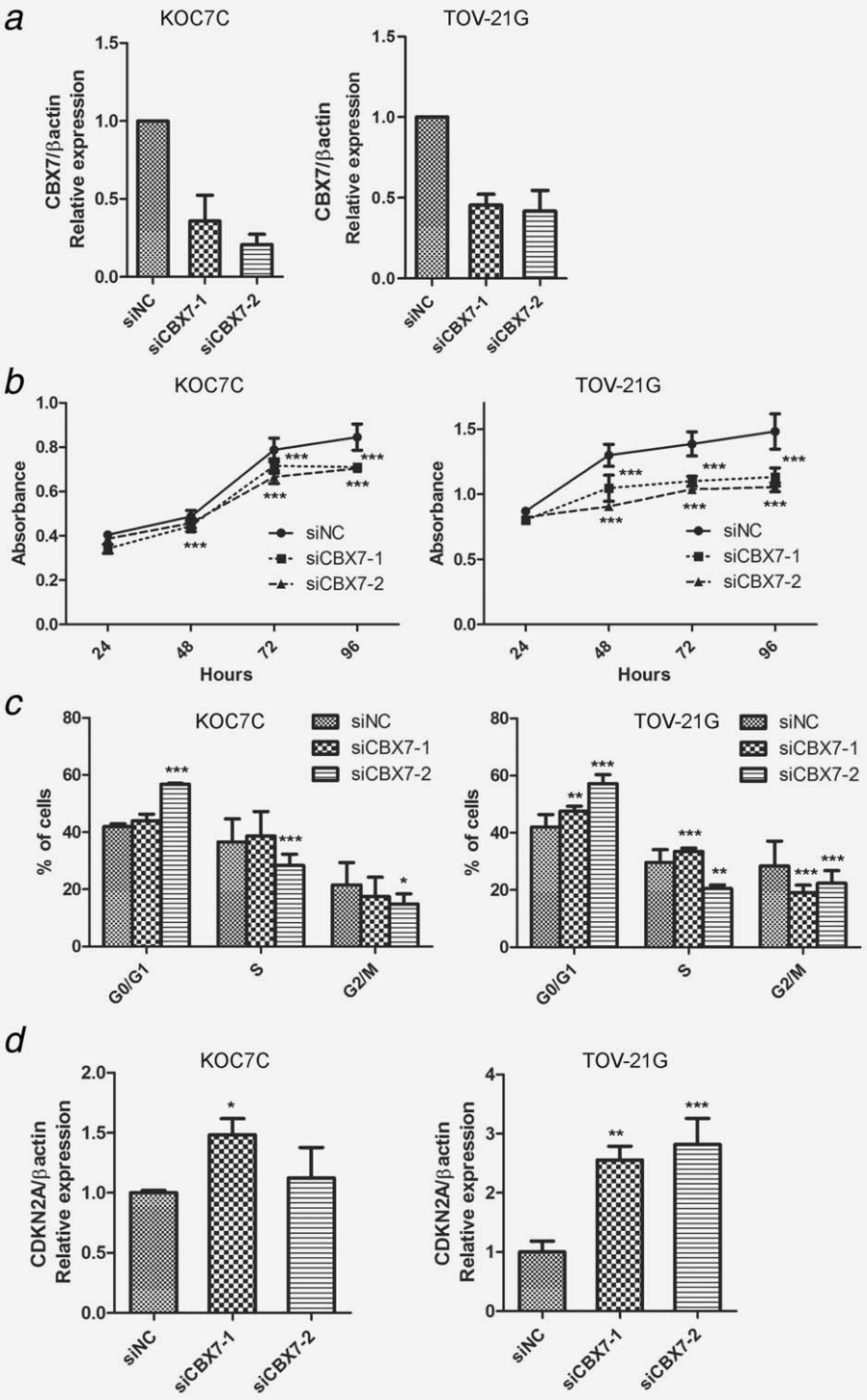

Figure 3. Effects of CBX7 knockdown on OCCA cell lines *in vitro*. After transfection with 2 individual siRNAs (siCBX7-1 and siCBX7-2) or control siRNA in KOC7C and TOV-21G cells, we performed the following experiments. (*a*) The efficacy of two individual siRNAs (siCBX7-1 and siCBX7-2) in KOC7C and TOV-21G measured by quantitative reverse transcriptase-PCR. (*b*) Effects of CBX7 knockdown on cellular viability measured by MTS assay. Hours indicate post-transfection times. (*c*) Cell cycle distribution measured by FACS. (D) Relative expression of CDKN2A (p16) mRNA measured by quantitative reverse transcriptase-PCR compared with control siNC. The means and SD were obtained from three experiments. Statistical analysis was performed to compare CBX7 knockdown and control samples. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

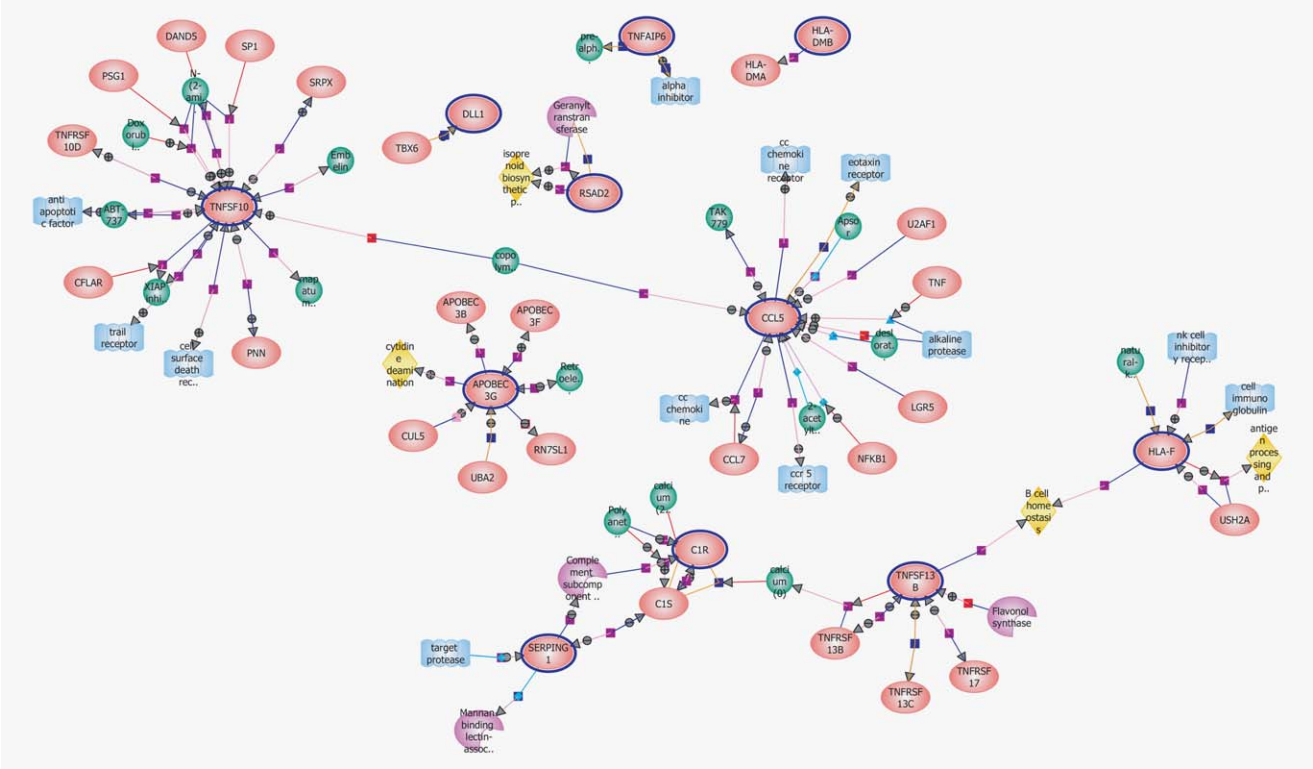

Figure 4. A pathway analysis of 35 genes that had elevated expression levels of more than eightfold compared to CBX7 knockdown groups and the control groups using GeneSpring GX10.02.2 software. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and PFS related to CBX7 expression, but this relationship was not significant probably due to smaller number of the patients. The presence of endometriosis had no correlation with OS or PFS (Supporting Information Figure 3). Thus, CBX7 seemed to be an independent factor associated with poorer prognosis, and we analyzed the factors that contributed to the prognosis using a multivariate Cox proportional hazards model for confirmation. Statistical results showed that CBX7 expression and FIGO Stages II–VI were associated independently with OS ($p = 0.012$ and $p = 0.002$, respectively) and PFS ($p = 0.005$ and $p < 0.001$, respectively) (Table 1). These results suggest that CBX7 may have a valuable role in OCCA disease progression.

CBX7 knockdown decreases cell viability of human OCCA cells

Because immunohistochemical analysis suggested that CBX7 may contribute to the growth of OCCA, we first examined the effect of CBX7 knockdown on the viability of human OCCA cells. We used two different OCCA cell lines, KOC7C and TOV-21G, and two siRNA constructs against CBX7 to perform the knockdown experiments *in vitro*. The knockdown efficacies of the siRNAs were confirmed 48 hr after transfection by quantitative PCR (Fig. 3*a*) and Western blotting. We assessed cell viability using an MTS assay. CBX7 knockdown significantly inhibited the cell viability of both cell lines, and the decrease in cell viability was maximally 30% (Fig. 3*b*). CBX7 knockdown did not affect cell migration or invasion (data not shown). It has been reported that CBX7 represses the INK4a/ARF locus; therefore, we examined the effect of CBX7 knockdown on p16 expression and cell cycle distribution. CBX7 knockdown significantly induced G0/G1 arrest (Fig. 3*c*) presumably *via* increasing the expression of p16 mRNA (Fig. 3*d*).

CBX7 knockdown induced apoptosis in OCCA cells

From our results, CBX7 was shown to play some role in repressing the CDKN2A gene and the progression of the cell-cycle in the OCCA cells. However, the changes were not dramatic, and because CBX7 expression was clearly related to a poorer prognosis in the OCCA patients, we assumed that CBX7 may help regulate other genes, most likely those related to tumor progression in OCCA. Thus, to examine other possible mechanisms, we compared the gene expression profiles of CBX7 knockdown and control samples from the TOV-21G and KOC7C cell lines using an expression microarray (accession number: GSE47146). We identified 35 genes in which the expression levels were upregulated in the CBX7 knockdown groups in common between both cell lines by more than eightfold compared to the control groups and the statistical values were $p > 0.05$ (Supporting Information Table 2). A pathway analysis indicated that CBX7 negatively

regulates apoptosis-related genes, inflammation-associated genes, such as CCL5, and genes associated with immunological phenotypes, such as HLA-F (Fig. 4). Specifically, TNFSF10, a tumor necrosis factor superfamily 10 member, resided in the center of the apoptosis-related genes (Fig. 4). We confirmed that CBX7 knockdown increased TNFSF10 mRNA expression by qPCR (Fig. 5*a*). To confirm that CBX7 knockdown induced apoptosis in OCCA cells, we performed Western blotting to detect the apoptotic markers along with a TUNEL assay. CBX7 knockdown increased the expression of cleaved PARP proteins (Fig. 5*b*), and the number of apoptotic cells detected by the TUNEL method was significantly increased compared with the control group (Figs. 5*c* and 5*d*). Additionally, we performed DNA content analysis using FACS. The percentage of sub-G1 phase cells (Fig. 5*e*) increased more in the CBX7 knockdown cells than in the control cells.

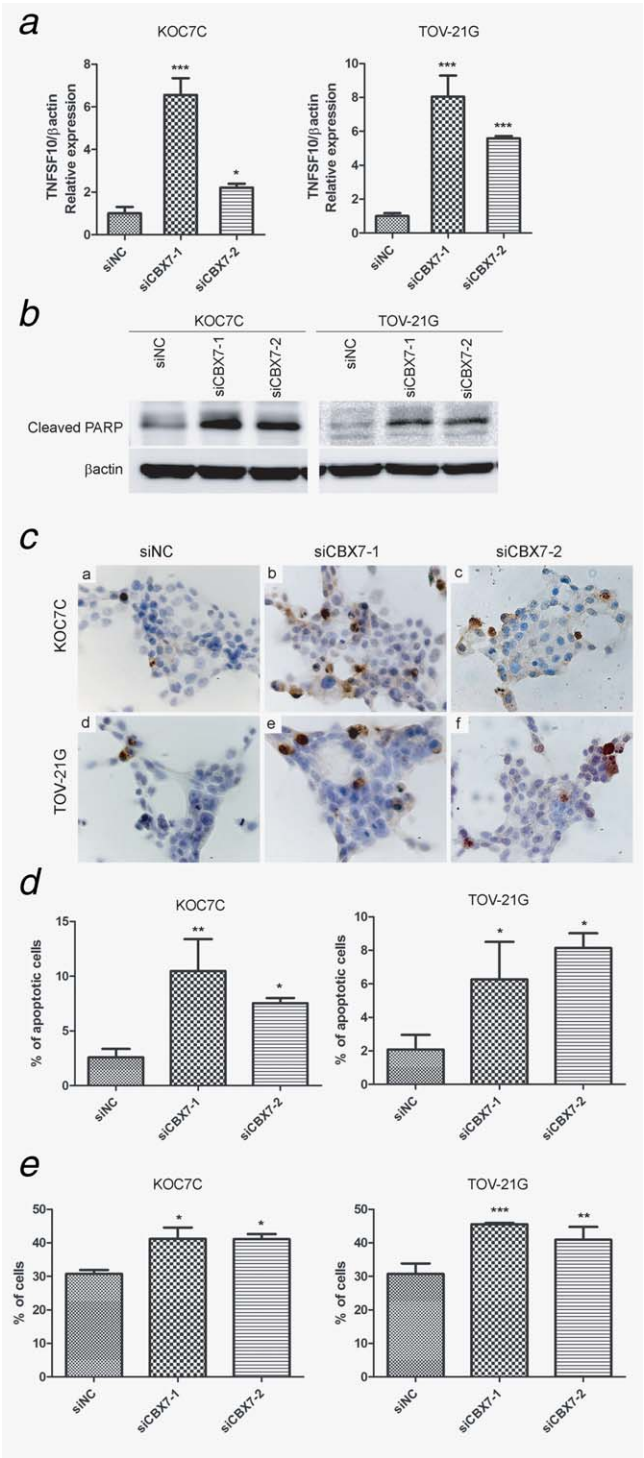

Figure 5. Induction of apoptosis by CBX7 knockdown *in vitro*. After transfection of KOC7C and TOV-21G cells with two individual siRNAs (siCBX7-1 and siCBX7-2) or control siRNA, we performed the following experiments. (*a*) Relative expression of TNFSF10 (TRAIL) mRNA measured by quantitative reverse transcriptase-PCR compared with control. (*b*) Western blotting showed expression of cleaved PARP. (*c*) TUNEL stained cells. (*d*) Percentage of apoptotic cells measured by the TUNEL assay. (*d*) Percentage of sub-G1 cells. siNC indicates the control. Means and SD were obtained from three experiments. Statistical analysis was performed using CBX7 knockdown samples and control samples. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Discussion

OCCA is frequently associated with endometriosis.[4] Histologically defined endometriosis was also found in more than 80% of our cases (Supporting Information Table 1 and Ref. 6) suggesting that endometriosis has an important role in the development of OCCA. Oxidative stress due to excess iron deposition is assumed as the cause of carcinogenesis.[5,6,27] Genome mutations induced by oxidative stress affects specific genes such as the INK4a region,[28] and although less established, frequent amplification of the MET gene is observed in OCCA,[6] which is common with an oxidative-stress induced animal tumor model.[29] ANRIL is a long noncoding RNA that cooperates with CBX7 to suppress the INK4a/ARF region at chromosome 9. Single-nucleotide polymorphism in the 19th intron of ANRIL was shown to have a strong association with endometriosis.[16] Therefore, we speculated that CBX7 may also have a role in OCCA carcinogenesis and progression. As a result, we demonstrated for the first time that CBX7 expression was associated with poorer prognosis in OCCA patients.

CBX7 was initially identified as a factor involved in overcoming cellular senescence and increasing cellular lifespan.[11] CBX7 is one of the PcG proteins, a family that also includes BMI1 and EZH2, and it is a component of PRC1.[9] Most PcG proteins have been known to have strong associations with cancer development, and some of these proteins have also been related to stem cell regulation.[11] EZH2 was recently shown as an tumorigenic independently of PRC,[30] and it is acceptable that other PcG proteins such as CBX7 may also have a distinct oncogenic role. PcG proteins have hundreds of target genes in mammals, and mammalian polycomb

responsive elements are not yet defined.[9] It has been shown that CBX7 plays a critical role in the lymphomagenesis of follicular lymphoma,[13] however, controversial studies have also shown that CBX7 positively regulates E-cadherin expression, thus acting as a tumor suppressor in thyroid cancer.[18,20] CBX7 protein expression loss has also been observed in colon and pancreatic cancer patients with aggressive tumors.[17,31] Most recently, CBX7 knockout mice were shown to have a tumorigenic phenotype that was associated with liver and lung carcinogenesis, and a new role for CBX7, downregulating cyclin E1, was reported.[21] Thus, the precise role of CBX7 in cancer development or progression may be cell-type specific, and in this report, we show for the first time that CBX7 has a progressive role in malignant behavior of OCCA.

It has been reported that PRC1 including CBX7 suppresses the INK4A locus together with ANRIL, which in turn leads to a reduction in the levels of tumor suppressor proteins such as p16 and p14ARF in prostate cancer cells.[12] Because we mainly used formalin-fixed paraffin-embedded tissue for the analyses in this study, we could not determine the relative rate of ANRIL expression in each sample. However, in this study, ANRIL expression was observed in six OCCA cell lines shown by qPCR and also expressed in the nuclei of two cell lines by RNA-FISH. Thus, consistent with previous reports, our data also showed that CBX7, possibly together with ANRIL, could repress the INK4a/ARF locus in the OCCA cells. However, relatively high expression of p16 was detected by immunoblotting in two OCCA cell lines other than KOC7C and TOV21G (Fig. 1*a*). Relative expression increases shown by qPCR in p16 (induced by CBX7 knockdown) were two- to threefold the maximum levels; while this change was not large, it was significant (Fig. 3*d*). On the contrary, G1 arrest induced by CBX7 knockdown was nonsignificant (Fig. 3*c*) in KOC7C cells. We attempted to increase the rate of G1 arrest by ubiquitously demethylating the promotor areas including the p16 promotor by adding 5-azacythidine to the culture medium, and as a result, p16 expression was slightly increased in KOC-7C cells; however, together with CBX7 knockdown, no significant increase in either p16 expression or G1 arrest was observed in these cells (Supporting Information Figure 4). Therefore, although we could not deny the possibility that ANRIL expression level was critical in downregulating the INK4a/ARF locus, but at least, in part, the expression levels of p16 significantly enlarged, and CBX7 knockdown itself was confirmed at both mRNA and the protein level, we considered that there may be other genes that are either directly or indirectly regulated by CBX7.

Interestingly, pathway analysis of the microarray data elucidated that a group of apoptosis-related genes were upregulated by CBX7 knockdown, and TNSF10 was positioned in the center of this group. TNFSF10 is also known as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). TRAIL induces apoptosis by binding to TRAIL-R1 (also known as DR4) and TRAIL-R2 (also known as DR5), and its binding triggers caspase-8 and caspase-3 activations. We further confirmed that CBX7 knockdown significantly induced apoptosis in both cell lines, with two different siRNAs for each one. TRAIL is an important inducer of apoptosis, but the precise mechanism for the regulation of its expression is still unknown.[32] In our study, CBX7 was shown to play a role in inducing the expression of TRAIL and related apoptotic factors. Whether CBX7 together with PRCs directly suppresses any of these molecules remains to be elucidated. TRAIL is an important factor for apoptosis induction in cancer cells, and studies are ongoing to seek novel therapeutic agents, such as TRAIL, and agonistic antibodies against TRAIL receptors are promising new antitumor agents.[33] Recently, however, resistance to TRAIL agonists has been found in some human cancers. This finding suggests that therapy using the TRAIL pathway may be cell-type specific.[34] Our results suggest that TRAIL pathway may be a novel target for CBX7, which may have a biological role in OCCA carcinogenesis and progression. Therapeutic agents utilizing the TRAIL pathway may be particularly effective in OCCA.

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