PHOSPHATE exporter XPR1/SLC53A1 is required for the tumorigenicity of epithelial ovarian cancer

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Abstract
Ovarian cancer is the fifth most common cause of cancer-related death in women. Ovarian clear cell carcinoma (OCCC) is a chemotherapy-resistant epithelial ovarian cancer with poor prognosis. As a basis for the development of therapeutic agents that could improve the prognosis of OCCC, we performed a screen for proteins critical for the tumorigenicity of OCCC using the CRISPR/Cas9 system. Here we show that knockdown of the phosphate exporter XPR1/SLC53A1 induces the growth arrest and apoptosis of OCCC cells in vitro. Moreover, we show that knockdown of XPR1/SLC53A1 inhibits the proliferation of OCCC cells xenografted into immunocompromised mice. These results suggest that XPR1/SLC53A1 plays a critical role in the tumorigenesis of OCCC cells. We speculate that XPR1/SLC53A1 might be a promising molecular target for the therapeutic treatment of OCCC.

KEYWORDS
apoptosis, ovarian cancer, phosphate transporter, proliferation, tumorigenicity, XPR1

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1 | INTRODUCTION

Epithelial ovarian cancer has one of the worst prognoses among gynecologic malignancies. Approximately 60% of epithelial ovarian cancer cases are diagnosed at stages III and IV, and their 5-year survival rate is less than 30%. Epithelial ovarian cancer is classified into four major histological subtypes: serous, clear cell, endometrioid, and mucinous. Ovarian clear cell carcinoma is less sensitive to conventional platinum-based chemotherapy and has worse prognosis than other subtypes. The incidence of OCCC in ovarian cancer is higher in Asia, especially in Japan (25% of ovarian cancers), than in North America and Australia (5%). Mutations in various oncogenes and tumor suppressor genes, including K-ras, p53, ARID1A, and PIK3CA, have been shown to be involved in the development of OCCC. In particular, ARID1A and PIK3CA are mutated in approximately half of the cases of OCCC.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human ovarian cancer OVISE and JHOC5 cells were cultured in DMEM and RPMI-1640 medium (Nissui) supplemented with 10% FBS, respectively. TOV21G cells were cultured in MCDB 105 (50%) Medium 109 (Sigma) supplemented with 10% FBS. ES2, HCT116 (p53^+/−), and HCT116 (p53^−/) cells were grown in McCoy 5A (Sigma) supplemented with 10% FBS.

2.2 | CRISPR-CAS9 screening

CRISPR-CAS9 screening was carried out using TKO CRISPR Library Version 3 (lentiCRISPRv2; Addgene #90294) following the protocols provided in https://www.addgene.org/pooled-library/moffat-crispr-knockout-tkov3/. The OCCC cell lines OVISE, ES2, TOV21G, and JHOC5 were transfected with lentiCRISPRv2 at an MOI of 0.3 followed by selection with puromycin for 2 days. A sample of 2 x 10^7 cells was then pelleted and frozen under liquid nitrogen as a Day 0 sample. Cells were then passaged until they had undergone eight doublings. To maintain sufficient sgRNA coverage, the total number of cells was maintained above 2 x 10^7 for the duration of the culture period. Cells cultured for eight cell doublings from Day 0 were taken as a final time point. Genomic DNA was extracted using the Blood and Cell Culture DNA MidiKit (#13343; Qiagen). One hundred twenty five micrograms of genomic DNA from each sample was split into 2.5 μg fractions and sgRNA sequences were amplified using Herculase II fusion DNA polymerase. Reactions were measured for fragment size using the Agilent 2200 Tapestation and quantified using the KAPA SYBR Fast qPCR Kit (#7959362001; KAPA Biosystems). To generate and analyze sgRNA count data, MAGeCK (version 0.5.9) was used ("mageck count" and "mageck mle --norm-method control --control-sgRNA --cnv-norm" command).

2.3 | Small interfering RNA transfection

Silencer select siRNA targeting XPR1 was purchased from Ambion (siXPR1#1, #17614; siXPR1#2, #17615). Cells were transfected with siRNA using Lipofectamine RNAiMax (Thermo Fisher Scientific). Silencer select siRNA negative control (4390843; Thermo Fisher Scientific) was used as a control.

2.4 | Human ovarian cancer samples

Ovarian cancer tissues were prepared as described previously according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committees of Jikei University School of Medicine and The Institute for Quantitative Bioscience, The University of Tokyo. Informed consent was obtained from all subjects involved in the study.

2.5 | RNA sequencing analysis

Total RNA was extracted using TRIzol reagent (Bioline). For ovarian cancer tissue dataset, cDNA libraries were prepared using the Illumina TruSeq Stranded Total RNA and the Ribo-Zero Gold LT Sample Prep Kit. All libraries were sequenced using an Illumina HiSeq 2500 to create single-end 65 bp reads, which were aligned to the human reference genome build hg38 using STAR.
(paired-end 150 bp reads) were undertaken by AnnoRoad Gene Technology. RSEM was used to calculate transcripts per kilobase million (TPM, Ensembl gene annotation GRCh38). For differential expression analyses, we applied the count data to edgeR. The count data were fitted with a general linear model. Gene Ontology enrichment analysis and GSEA were undertaken using the R package "clusterProfiler". The ovarian normal tissue dataset from GTEx in UCSC Xena (https://xena.ucsc.edu/) was combined with our ovarian cancer tissue dataset (see above), and then normalized by the quantile method using the R package "limma".

2.6 | Cell viability assay

Cells (1 × 10^3 cells) were transfected with siRNA (10 nM) and seeded into 96-well plates. After 24 h, fresh medium was replaced. One, 4, and 6 days after transfection, cell viability was assessed indirectly by measuring the intracellular levels of ATP using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). Luminescence was measured on a Mithras LB 940 (Berthold).

2.7 | Quantitative RT-PCR

Total mRNA was extracted using TRIzol (Bioline) and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara). Real-time PCR was carried out using a LightCycler480 (Roche). The results were normalized against the values detected for GAPDH. Primers used for quantitative RT-PCR are described in Table S1.

2.8 | Immunoblotting

Immunoblotting analysis was carried out as described previously. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and incubated at 37°C for 30 min, then separated by SDS-PAGE and probed with anti-XPR1 (14174–1-AP; Proteintech Group, Inc.), anti-p53 (sc-126; Santa Cruz Biotechnology), or anti-GAPDH (MAB374; EMD Millipore Corp.) Ab.

2.9 | Lentivirus production

Lentiviral vector (CS-Rfa-CG) harboring an shRNA driven by the H1 promoter was transfected with the packaging vectors (pMD2G, psPAX2; Addgene) into 293FT cells using polyethylenimine (Polyscience, Inc.). All plasmids were kindly provided by H. Miyoshi (Riken BioResource Center). Virus supernatants were purified by ultracentrifugation at 106,800 x g for 135 min (SW32Ti rotor; Beckman). Infection efficiency was monitored by GFP expression driven by the CMV promoter. The sequences of shRNAs are shown in Table S2.

2.10 | Mouse xenograft model

OVISE and ES2 cells infected with a lentivirus expressing an shRNA targeting XPR1 were injected s.c. into 7-week-old NOG mice (OVISE) and nude mice (ES2), respectively (NOD/Shi-scid IL-2RγKO for OVISE, BALB/c-nu/nu for ES2; CLEA). Cells (1.0 × 10^6) for each infection were resuspended in a 1:1 mixture of 75 μl PBS and 75 μl Matrigel (Corning Life Sciences). All animal experimental protocols were carried out in accordance with the guidelines of the Animal Ethics Committee of the University of Tokyo.

2.11 | Sub-G1 assays

Cells transfected with siRNA were cultured for 48 or 96 h, and fixed in a 70% ethanol : water solution at ~30°C overnight. Fixed cells were incubated in a 4 mM citric acid (pH 8.0), 200 mM Na_2HPO_4 solution for 20 min, stained with a solution of 10 μg/ml propidium iodide (Sigma) and 10 μg/ml RNase A (Sigma) in 1× PBS for an additional 20 min, and subsequently analyzed on a Sony EC800 Flow Cytometry Analyzer.

2.12 | Statistical analysis

Statistical analyses, including unpaired t-tests and Wilcoxon test, were undertaken using R version 4.0.1 (http://www.r-project.org/).

3 | RESULTS AND DISCUSSION

3.1 | Genome-wide CRISPR/CAS9 screens using OCCC cell lines

To identify genes critical for the proliferation of OCCC, we previously performed CRISPR/CAS9 screens against the OCCC cell lines OVISE, ES2, TOV21G, and JHOC5 (Table 1) using the TKOv3 sgRNA library. From a list of genes selected in this screen (Data S1), we focused on genes that encode membrane proteins overexpressed in cancer cells, which could be suitable targets for Ab drug development. We observed that depletion of 22 genes encoding the SLC family of membrane proteins resulted in a significant decrease in the proliferation of OCCC cell lines (Figure 1A). Furthermore, we analyzed data from the Cancer Dependency Map (https://depmap.org) and found that depletion of SLC family genes such as PHC/
SLC25A3 (mitochondrial phosphate and copper transporter), ZIP10/SLC39A10 (zinc transporter), MDU1/SLC3A2 (glutamate/cystine transporter), GLUT1/SLC2A1 (glucose transporter), and XPR1/SLC53A1 (phosphate exporter) led to a prominent decrease in the growth of ovarian cancer cell lines, including OCCC cell lines (Figure 1B). Although PHC/SLC25A3 had a prominent effect on the

**FIGURE 1** Genome-wide CRISPR/CAS9 screens using ovarian cancer cell lines. (A) Results of CRISPR/Cas9 screens against four types of ovarian cancer cell lines using the TKOv3 single guide RNA library. Green triangles indicate genes essential for growth in the four ovarian cancer cell lines (p < 0.05). Black crosses indicate genes encoding membrane proteins, and red circles indicate genes encoding the SLC family of membrane proteins. (B) Dependency of ovarian cancer cell lines on SLC family genes (data from https://depmap.org). A lower score means that a gene is more likely to be dependent in a given cell line. (C) Quantification of SLC gene family expression from RNA sequencing data. Left panel: normal tissue, n = 200 (from GTEx [genotype tissue expression]); clear cell carcinoma, n = 11; high grade serous adenocarcinoma, n = 8; endometrioid carcinoma, n = 4. Cancer tissues were prepared in our laboratory. Right panel: data of high grade serous adenocarcinoma from UCSC Xena (TCGA TARGET GTEx). PAX8, WT1, and IL6 are controls (PAX8 is known to be upregulated in ovarian cancer, WT1 is downregulated, and IL6 is upregulated in clear cell carcinoma). TPM, transcripts per million. *p < 0.05; **p < 0.01. ns, not significant, Wilcoxon rank sum test

**FIGURE 2** RNA sequencing (RNA-seq) analysis of ovarian clear cell carcinoma (OCCC) cell lines transfected with siRNA targeting XPR1. (A) Volcano plots of RNA-seq data obtained with OCCC cell lines transfected with control siRNA or siRNA targeting XPR1. Genes whose expression was significantly changed by XPR1 knockdown are shown in the volcano plots (logFC > 0.6; p value < 0.05). (B) Gene Set Enrichment Analysis (GSEA HALLMARKS) of RNA-seq data. Five terms common to two or more of the OCCC cell lines are shown. (C) Gene Ontology (GO) overrepresentation analysis of RNA-seq data in (A). The top five terms classified as response to stress in the Biological Process (BP) of GO-slim are shown for each cell line. (D) Venn diagram summarizing the number of genes upregulated by XPR1 knockdown. (E) BP analysis of GO for genes commonly upregulated in more than three cell lines (31 genes in (D))
proliferation of most ovarian cancer cell lines, it is a mitochondrial protein that is not suitable for Ab drug development. Depletion of XPR1 led to a remarkable decrease in the growth of OCCC cell lines compared to other ovarian cancer cell lines. We next examined the expression levels of these SLC family members in ovarian cancers. We observed that ZIP10/SLC39A10, GLUT1/SLC2A1, and XPR1/SLC53A1, but not PHC/SLC25A3 or MDU1/SLC3A2, were upregulated in ovarian cancers compared to normal tissue (Figure 1C). As many studies have already been done on SLC39A10 and SLC2A1, we decided to focus on XPR1.

3.2 Effect of XPR1 knockdown on gene expression profiles of OCCC cell lines

To clarify the effect of XPR1 knockdown on gene expression profiles of OCCC cells, we undertook RNA sequencing and GSEA using OVISE, ES2, TOV21G, and JHOC5 cells transfected with siRNA targeting XPR1 (Figure 2A,B). The GSEA revealed that the p53 pathway was activated in all cell lines. Inconsistent with a previous report, nuclear factor-αB signaling was enhanced in OVISE, ES2, and TOV21G cells. Inflammatory response, epithelial-mesenchymal transition, and RAS signaling were also activated in all of these cell lines. Gene Ontology overrepresentation analysis revealed that expression of genes involved in oxidative stress and other stress responses was enhanced in all cell lines (Figure 2C). The GO biological process analysis indicated that the genes upregulated in common in at least three cell lines (Figure 2D) were enriched for those involved in stress response function (Figure 2E).

3.3 Knockdown of XPR1 suppresses the growth of OCCC cells

We next attempted to examine the effects of RNAi-mediated knockdown of XPR1 on the growth and tumorigenicity of OCCC cells. As OVISE and ES2, but not TOV21G or JHOC5, were tumorigenic in mice, we used OVISE and ES2 in subsequent experiments. We found that siRNA knockdown of XPR1 resulted in a significant decrease in the proliferation of both cell lines in vitro (Figure 3A,B). Consistent with a previous report, the intracellular phosphate level was also decreased (Figure S1). Furthermore, FACS analysis revealed that knockdown of XPR1 induced the accumulation of sub-G₁ cells (Figure 3C). We therefore measured the changes in the expression of marker genes indicating p53-mediated apoptosis: PUMA, NOXA, and GADD45. Quantitative RT-PCR revealed that knockdown of XPR1 in OVISE cells resulted in a marked increase in the expression of these marker genes (Figure 3D). However, knockdown of XPR1 in ES2 cells resulted in a smaller increase in marker expression. This result could be consistent with the fact that ES2 cells harbor a mutation in one allele of p53.

To further clarify the significance of p53 in XPR1 knockdown-induced growth suppression, we compared the effects of siRNA against XPR1 on the proliferation of the colon tumor cell lines HCT116 (p53−/−) and a derivative, HCT116 (p53−/−), in which p53 is disrupted by homologous recombination. We found that knockdown of XPR1 caused both a marked inhibition of the proliferation and an increase in the sub-G₁ population of both cell lines (Figure 4A-D). However, knockdown of XPR1 induced upregulation of PUMA, NOXA, and GADD45 only in HCT116 (p53−/−) cells and not in HCT116 (p53−/−) cells (Figure 4E). These results suggest that knockdown of XPR1 can suppress the proliferation of cancer cells through both p53-dependent and -independent mechanisms.

We next investigated the effects of siRNA-mediated knockdown of XPR1 on the growth of various cancer cell lines (Figures S2A,B). Immunoblotting analysis was also undertaken to examine XPR1 protein expression in these cell lines. We considered XPR1 to be three bands between 63 and 75 kDa (Figure S3A), because exogenously expressed XPR1 migrated to the same position (Figure S3B). Moreover, the intensity of these three bands was decreased in TOV21G cells transfected with siRNAs targeting XPR1 (Figure S3C). Furthermore, we examined the expression levels of XPR1 in various cancer tissues and corresponding normal tissues (Figure S4). For example: in lung cancer, XPR1 expression was higher than normal tissue and XPR1 knockdown was effective against H1299 but not A549 cells; in renal cancer, XPR1 expression was not much different from normal tissue, but XPR1 knockdown was effective against 786O cells; and in colon cancer, XPR1 knockdown was effective against HCT116 (Figure 4C) but not DLD1 cells. These results suggest that the sensitivity of cancer cells to XPR1 knockdown cannot simply be explained by XPR1 expression levels.

3.4 Knockdown of XPR1 suppresses the tumorigenicity of OCCC cells

Finally, we examined the effects of an shRNA targeting XPR1 on the tumorigenicity of OCCC cells. We infected OVISE and ES2 cells with a lentivirus expressing an shRNA targeting XPR1 and transplanted these into immunodeficient mice. The growth of these tumor cells was significantly retarded compared to tumor cells infected with control virus (Figure 5). These results suggest that XPR1 is required for the tumorigenicity of OCCC cells in vivo.

In the present study, we identified the transmembrane protein XPR1 as a critical factor in the proliferation and tumorigenicity of epithelial ovarian cancers, especially OCCC. In addition, we observed that XPR1 is upregulated in epithelial ovarian cancers compared to normal tissue. Thus, XPR1 could be a promising molecular target for the therapy of epithelial ovarian cancer. Knockdown of XPR1 leads to the activation of genes involved in the p53 pathway and responses to inflammation and various stresses, including oxidative stress. Knockdown of XPR1 results in the upregulation of p53 target genes such as PUMA, NOXA, and GADD45 in p53 WT cells, but not in...
FIGURE 3  Effects of XPR1 knockdown on ovarian clear cell carcinoma (OCCC) cell proliferation and expression of p53 target genes.
(A–C) Expression of XPR1 (A), proliferation (B), and sub-G₁ population (C) of OVISE and ES2 cells transfected with siRNAs targeting XPR1 or control siRNA. Results are expressed as the mean ± SD (n = 3). (D) Expression of p53 target genes, PUMA, NOXA, and GADD45, in OVISE and ES2 cells transfected with siRNAs targeting XPR1 or control siRNA. Results are expressed as mean ± SD (n = 3). *p < 0.05; **p < 0.01. ns, not significant, unpaired t-test.
p53-deficient cells. However, XPR1 knockdown induces similar levels of apoptosis in both p53 WT and p53-deficient cells. Thus, XPR1 knockdown-mediated apoptosis can be induced by p53-independent mechanisms in p53-deficient cells. It has been shown that XPR1 acts as a phosphate exporter and regulates phosphate homeostasis in cooperation with the phosphate importer SLC20A2 in an inositol...
polyphosphate-dependent manner. We speculate that XPR1 knockdown induces p53-dependent and -independent apoptosis by dysregulating phosphate homeostasis. In this regard, it is interesting to note that high extracellular phosphate induces apoptosis along with high levels of intracellular phosphate, reactive oxygen species generation, mitochondrial membrane depolarization, and caspase activation. Further investigation is required to clarify the mechanisms underlying apoptosis induced by XPR knockdown.

In conclusion, we have shown that XPR1 is critical for the proliferation and tumorigenicity of epithelial ovarian cancers, especially OCCC. Our results suggest that drugs such as mAbs that inhibit XPR1 function might be useful for the therapeutic treatment of OCCC.

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DISCLOSURE
The authors have no conflict of interest. T.A. is an editorial board member of Cancer Science.

DATA AVAILABILITY STATEMENT
All raw sequence data (FASTQ format) are deposited in NCBI’s Gene Expression Omnibus (GEO) under accession numbers GSE189405(Figure 1A), GSE189553(Figure 1C left), and GSE189552(Figure 2). Code for computational analyses is available from the Lead Contacts, Tetsu Akiyama (akiyama@iqb.u-tokyo.ac.jp) or Tomoatsu Hayashi (hiroton-h@iqb.u-tokyo.ac.jp).

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