Knockdown of EPHA1 Using CRISPR/CAS9 Suppresses Aggressive Properties of Ovarian Cancer Cells

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Abstract. Background/Aim: Overexpression of erythropoietin-producing hepatocellular A1 (EPHA1), a member of the EPH super family, is frequently observed in various cancer types. The dysregulated interaction of EPHA1 with its ligand Ephrin A1 has been linked to the progression of ovarian cancer (OC). However, the contribution of EPHA1 in the regulation of the aggressive properties of OC cells remains unknown. Materials and Methods: In this study we investigated the differential expression of EPHA1 in human OC cells. The EPHA1 gene was knocked-down using the CRISPR/Cas9 technique to evaluate its effect on the aggressive properties of OC cells. Results: After EPHA1 was knocked-down using a CRISPR/CAS9 genomic editing system in OC cells (SKOV3 and COV504), we observed cell-cycle arrest at the G1/G2 phases in both OC cell lines. Knockdown of EPHA1 in the two OC cells inhibited their aggressive traits, including proliferation, invasion and migration, as well as improving their attachment to extracellular matrix. EPHA1 may play a role in OC through its regulation of multiple signaling pathways, such as matrix metalloproteinase-2 (MMP2), extracellular signal-regulated kinase 2 (ERK2) and proto-oncogene c-MYC. Conclusion: EPHA1 may promote the aggression of some OC cells and, thus, be considered a potential therapeutic target for the treatment of malignant OC.

Ovarian cancer (OC) is the seventh most common type of cancer and cancer-related death in women worldwide and contributes to approximately 4% of all female cancers (1). Although the lifetime risk of OC in women is 1.5%, it is considered as the most deadly of gynecological cancers (2). As OC is often asymptomatic in the early stages, it is often diagnosed when the disease is in the later stages (III and IV). The 5-year survival rate for the women diagnosed with OC at an advanced stage is approximately 19%, despite the 90% rate for patients with early diagnosis (3). However, the diagnosis techniques of OC at its early stages are not well developed and lack accuracy (4). Currently, the etiology of OC is not fully understood despite varied proposed pathogenic hypotheses (5, 6).

With 14 members, the erythropoietin-producing hepatocellular (EPH) receptors represent the largest subfamily of the receptor tyrosine kinases (7, 8). EPH receptors are categorized into two subfamilies, EPHA and EPHB, based on their sequence homology and preferential binding potential to their ephrin-A and ephrin-B ligands, respectively (9, 10). In humans, there is evidence to show expression of nine EPHA (EPHA1-8, 10) and five EPHB (EPHB1-4, 6) receptors, as well as five ephrin-A and three ephrin-B ligands (11). Following activation by the binding of their ephrin ligands, EPH receptors play a variety of roles in physiological and pathological processes, including immune cell development and behaviors, tumorigenesis, tumor-associated angiogenesis and tumor progression (12).

EPH receptor A1 (EPHA1) was originally isolated from erythropoietin-producing hepatocellular carcinoma cell lines (13). The over-expression of EPHA1 has been described in several human cancers, including colorectal (14), breast (15) and prostate (16). However, EPHA1 is found to be down-regulated in non-melanoma skin cancer (17) and glioblastoma. In OC, the expression profile of EPHA1 has not been well-documented. One previous study.

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indicated that the overexpression of EPHA1 and its ligand Ephrin A1 may be correlated with shortened survival in patients with OC (18). Little is known whether EPHA1 plays a role in the aggressive properties of OC cells. In this study, we assessed the expression of EPHA1 in OC cells and attempted to investigate the effect of EPHA1 knockdown (KD), using the CRISPR/Cas9 technique on two OC cell line behaviors.

Materials and Methods

Cell culture. Human OC cell lines, SKOV3 and COV504, were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). COV504 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK) and 1% penicillin/streptomycin solution (Invitrogen). SKOV3 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin solution (Invitrogen). Both cell lines were cultured at 37°C under a humidified atmosphere of 5% CO2.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from cultured cells using TRI reagent (Sigma-Aldrich, Irvine, UK) according to the manufacturer’s instructions and quantified using a Nanodrop spectrophotometer. cDNA samples were generated from the total RNA using GoScript™ Reverse Transcription System kit (Promega, Madison, WI, USA). Subsequently, PCR was conducted using a REDTaq® ReadyMix PCR reaction mix (Sigma-Aldrich) on a 2720 Thermal Cycler (Applied Biosystems, Warrington, UK). The following primers were used: EPHA1: forward, 5'-CCCTGTTCCACTACATCCT-3' and reverse, 5'-CCTTAATGCCACACTACCTT-3' and reverse, 5'-GTCTTTATCATCGTTGCTGAC-3', GAPDH: forward, 5'-GGCTGTTTTAATACCTGTTA-3' and reverse, 5'-ACTGTGATGCTGAGTCTT-3'. The following cycling conditions were used: 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min. PCR products were separated on 1% agarose gel and photographed using a VisiDoc-IT imaging system (Ultra-Violet Products Ltd., San Gabriel, CA, USA).

EPHA1-specific CRISPR/Cas9 vectors and stable cell line establishment. Construction of CRISPR/Cas9 vectors that targeted EPHA1 have been described in one of our previous studies (19). Briefly, human CRISPR/Cas9 guide RNA primers were designed based on the gene EPHA1 (GenBank accession number: NM_005232). The primer sequences for were: EPHA1 casF (guide s): 5'-CACCAGCTGGGGCTGTCGGCAG-3', EPHA1 casR1 (guide as): 5'-AAACACGGCGGGGCGCAGCCACAGAGG-3'. The primer oligos were annealed using the following parameters: 88°C for 2 min, 65°C for 10 min, 37°C for 10 min, 25°C for 5 min. The annealing primer was then purified and cloned into the pSpCas9 (BB)-2A-Puro (PX559) vector (Plasmid #62988; Addgene, Cambridge, MA, USA). The plasmid containing CRISPR/Cas9-EPHA1 was verified by sequencing analysis. Then PX559 vector containing CRISPR/Cas9 backbone and CRISPR/Cas9-EPHA1-gRNA plasmids were transfected into the two OC cells respectively by electroporation using Gene pulser (Bio-Rad, Hercules, CA, USA). After three weeks of selection with 2 µg/ml puromycin, the verified transfecants were cultured in maintenance medium with 0.5 µg/ml puromycin.

Immunofluorescence of EPHA1. Cells were seeded on chamber slides (Nunc™) and incubated overnight. They were then fixed in 100% ethanol for 20 min and then washed with Hank’s Balanced Salt Solution (HBSS). Triton X-100 (0.3%) was used to permeabilize cells before staining. Anti-EPHA1 antibody (#sc-925; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated overnight at 4°C. The slides were then incubated with a specific FITC labelled secondary antibody (Dako, Glostrup, Denmark) for 1 h at room temperature. Fluorescent staining was visualized and captured using an Olympus™ BX51 microscope equipped with a cooled C4742-80 digital camera (Hamamatsu Photonics, Enfield, UK).

Kinexs antibody microarray. Cultured cells were washed twice in ice-cold PBS and then sonicated in standard Kinexus lysis buffer (20 mm MOPS, 1% Triton X-100, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 60 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany), 1 mM DTT). The protein concentration was then quantified with Fluorescence scanning protein dye (Sigma-Aldrich) using bovine serum albumin (BSA) as a standard. Approximately 100 µg of lysate protein samples were shipped in dry ice to Kinexus Bioinformatics Corporation (Vancouver, BC, Canada) for Kinexus™ KAM-880 Antibody Microarray.

Cell cycle analysis. Detached cells were re-suspended in 1 ml of ice cold 70% v/v ethanol and left on ice for 15 min. Fixed cells were then centrifuged at 600 x g for 5 min, washed once with PBS. The pellets were then re-suspended in 500 µl cell cycle staining solution in PBS (50 µg/ml PI from 50x stock solution (2.5 mg/ml), 0.1 mg/ml RNase A and 0.05% Triton X-100), apart from the unstained control. Cells were then incubated at room temperature in the dark for 15 min. Following this, FACS analysis was performed on the BD FACSCanto™ II flow cytometer (BD Biosciences, Oxford, UK) using the FL3 channel (575 nm). The FACS data were analyzed using the FCS Express v6.0 software (De Novo, Los Angeles, CA, USA).

In vitro cell matrix adhesion. Cells (40,000 cells/well) were seeded into a 96-well plate pre-coated with Matrigel (50 µl/well) (BD Biosciences) and incubated at 37°C for 40 min. Following a wash using PBS buffer, the cells were fixed with 4% formalin and stained with crystal violet. After capturing the images, the adherent cells were dissolved with 10% (v/v) acetic acid and absorbance was determined using a plate reader.

In vitro wound healing assay. Approximately 40,000 cells were seeded into a 24-well plate and allowed to reach confluency. The cell monolayer was scraped using a P10 pipette tip. Photographs were taken at 0 and 4 hours after wounding. Migration distances were measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

In vitro transwell invasion and migration assay. A Transwell insert with 8 µm pores was coated with 50 µg of Matrigel for the invasion assay. Non-coated 8 µm pores transwell inserts were used for the migration assay. Forty thousand cells were seeded into each insert for migration and invasion assay, respectively. After 72 h incubation, 1X Enzyme-Free Cell Dissociation Solution (Millipore, Billerica, MA, USA) with 2 µg/ml calecin AM (eBioscience,
Figure 1. Evaluation of EPHA1 knock-down in two ovarian cancer (OC) cells, SKOV3 and COV504. Stable cell lines were established using the EPHA1-gRNA CRISPR/Cas9 vectors (KD) and px459 backbone vector as a control (CTRL). (A) Gene expression of EPHA1 as indicated by reverse transcription-polymerase chain reaction (RT-PCR) using GAPDH as house-keeping gene. (B) Protein expression of EPHA1 from two SKOV3 stable cell lines as indicated by the Kinex antibody array. (C) Immunofluorescence of EPHA1 in two COV504 stable cell lines using an EPHA1 specific antibody (Green) and nuclear counterstain DAPI (Blue).

Hatfield, UK) was added and the transwell inserts were incubated further for 1 h at 37°C. Fluorescence was determined at a wavelength of ex490/em510 nm using the GloMax®-Multi Detection System (Promega).

Electric cell-substrate impedance sensing (ECIS) system-based cell motility assay. The ECIS system (9600 model; Applied Biophysics Inc., Troy, NY, USA) was used to quantify cell migration. 96WIE arrays were used and cells were seeded at 40,000 cells per well in 200 μl DMEM medium. The multi-frequency resistance was recorded using the ECIS system and an electric wound (2,600 Hz, 30 s) was applied after monitoring for 15 h. The data were analyzed using the ECIS-9600 software package.

Western blotting analysis. Western blot was performed as previously reported (20). Briefly, whole cell protein was extracted and quantified by NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) using the BCA protein assay kit. After separation by SDS-PAGE (a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis), the proteins were then transferred to a PVDF
membrane (Millipore). Proteins were probed with primary antibodies anti-ERK or anti-JNK (1:500; Santa Cruz Biotechnology) and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology). The proteins were detected with Z-ECL chemiluminescence kit (Luminata Forte; Millipore, Hertfordshire, UK) and photographed using G-Box gel documentation system (Syngene, Cambridge, UK). GAPDH was used as an internal control. The intensity values were analyzed using the Image J software (NIH).

Statistical analysis. Statistical analysis was performed using SPSS18 (SPSS Inc., Chicago, IL, USA). The Student’s t-test was used for data that were normally distributed. Patients’ survival was analyzed using Kaplan–Meier analysis (21). The differences of the ECIS data were tested using the repeated measures analysis of variance (ANOVA). *p<0.05* was considered statistically significant.

Results

**Knockdown of EPHA1 in OC cell lines.** EPHA1 gene expression was knocked down using the CRISPR/Cas9 method, targeting human EPHA1 genomic DNA. The knockdown of EPHA1 in the SKOV3 and COV504 OC cells was verified using PCR compared to their corresponding vector control cells (Figure 1A). The Kinexus protein array data also confirmed that there was reduction of the EPHA1 protein in SKOV3 cells when EPHA1 was knocked-down (Figure 1B). Immunofluorescence cell staining also indicated a decrease of EPHA1 in COV504 cells (Figure 1C). Therefore, EPHA1 can be knocked down in OC cells using the CRISP/Cas9 technique.
Knockdown of EPHA1 induced G2/G1 cell cycle arrest in OC cells. We evaluated the effect of EPHA1 on the cell cycle of OC cells by FACS. As shown in Figure 2, in the SKOV3 cells with EPHA1 KD, there was increase in cells in the sub-G1 (from 5.03% to 13.13%) and G1 phases (from 46.83% to 61.93%), respectively, and a decrease of cells in the M phase (41.85% to 16.58%) compared to the control with backbone vector (Figure 2A and C). In the COV504 cells, there was also increase in cells in the G1 phase (from 47.71% to 58.33%) and a decrease of cells in the M phase (31.54% to 25.89%) compared to the control with backbone vector (Figure 2B and D). The population of sub-G1 phase COV504 cells was extremely low in the control cells (0.30%) and did not change after EPHA1 KD (0.12%).
Knockdown of EPHA1 reduced cellular adhesion of OC cells to Matrigel. As shown in Figure 3, KD of EPHA1 in COV504 cells decreased the adherent capacity to approximately 24% compared to the control cells (p<0.05). Likewise, KD of EPHA1 in SKOV3 cells also decreased the cellular adhesion capacity to approximately 40%.

Knockdown of EPHA1 inhibited migration capacity of OC cells. We evaluated the migration capacity of the OC cells using the scratch wound-healing assay. EPHA1 KD in COV504 cells decreased the rate of the wound healing from 70.13% to 44.62% 4 hours after scratch wounding. Likewise, EPHA1 KD in SKOV3 cells decreased the rate of wound...
Figure 5. Initial cell spreading/attachment and post-wound migration capacity of the OC cells indicated in the Electric Cell-substrate Impedance Sensing (ECIS) system. (A) The normalized resistance of the cells at 2,000 Hz for the first 15 h indicating the cellular property of spreading and attachment. (B) Following an electric wound (30 s, 2,600 Hz), the normalized resistance of the cells at 64,000 Hz indicating the dynamic change of migration of the OC cells. CTRL, Control; KD, knockdown; OC, ovarian cancer.

Figure 6. Evaluation of proliferation and invasion of OC cells after EPHA1 knockdown. (A) Crystal violet proliferation assay. (B) Transwell invasion property of the OC cells normalized using the control. The transwell inserts with 8 μm pore size were coated with Matrigel gel first to mimic the extracellular matrix in a tumor microenvironment. Data are presented as means with standard deviation (SD) (triple test, n=3). *p<0.05, **p<0.01. CTRL, Control; KD, knockdown; OC, ovarian cancer.
healing from 68.75% to 49.23% 4 hours after scratch (Figure 4). Using the ECIS system, we were able to quantify the attachment and post-wound migration of cells in a high-throughput real-time manner. As shown in Figure 5, The ECIS data at 2,000 Hz indicated that the COV504 cells with EPHA1 KD had a lower level of attachment than the control to the gold electrode (p<0.05). However, the SKOV3 cells with EPHA1 KD did not show significant difference (Figure 5A). Following electric wound in the ECIS system (Figure 5B), both SKOV3 and COV504 cells with EPHA1 KD showed suppressed level of migration (at 64,000 Hz) compared to their control cells after electrical wounding, respectively (p<0.01), supporting the observations from the scratch wounding assays.

Knockdown of EPHA1 inhibited proliferation and transwell invasion of OC cells. The data from the crystal violet proliferation assay indicated that SKOV3 cells with EPHA1 KD decreased the proliferation of cells by approximately 13.79% after culture for 3 days compared to the control (p<0.01; Figure 6A). We also observed an approximately 19.44% decrease of proliferation from the stable COV504 cells with EPHA1 KD compared to the backbone control (p<0.01). The result from the transwell inset invasion assay showed that EPHA1 KD, in SKOV3 cells, led to a decrease in invasion by approximately 22.56% (p<0.05 vs. control). Similarly, EPHA1 KD, in COV504 cells, led to a decrease of invasion by approximately 27.80% compared to the control (p<0.01 vs. control).

Knockdown of EPHA1 inhibited the activity of MMP-2 and c-MYC signaling pathways. We further investigated the possible mechanism that EPHA1 KD had an effect on the behavior of the OC cells. The Western blotting data showed that MMP2 protein expression was down-regulated following EPHA1 KD in both SKOV3 and COV504 cells (Figure 7A). The inhibition of MMP2 activity was also confirmed using gelatin zymography in the two cell types (Figure 7B). It appears that the interaction of P-TY and ERK was inhibited by EPHA1 knockdown. Both ERK1 and ERK2 proteins were up-regulated by EPHA1 KD in SKOV3 cells. However, in COV504 cells with EPHA1 KD, ERK1 was down-regulated,
but ERK2 was up-regulated (Figure 7C). By western blotting, we also found that the c-MYC protein expression was downregulated after EPHA1 KD in both cell types and the reduction was more distinctive in COV504 cells (Figure 7D).

Discussion

Previous studies suggest that EPHA1 may be implicated in the prognosis and progression of various cancers with a diverse manner. For example, EPHA1 is positively associated with tumor proliferative capacity in non-small cell lung carcinoma (22), whilst, in gastric cancer, the EPHA1 transcript expression level appears to be positively associated with tumor size, stage and lymph node metastasis (23). Also, in pancreatic ductal adenocarcinoma, EPHA1 intensity is significantly associated with tumor size and histopathological stage as indicated by immunohistochemical staining (24). However, in clear cell renal cell carcinoma, positive EPHA1 protein staining, as well as a low Ephrin A1 protein levels, are significantly linked to more aggressive tumor features and poor survival (25). In prostate cancer, increased level of EPHA1 protein is frequently correlated with a high Gleason score (16). In contrast, in colorectal cancer (CRC), down-regulation or loss of EPHA1 expression, probably due to DNA hypermethylation, appears to be correlated with invasion, metastasis and poor survival of patients depending on stages (26, 27). However, in OC, the association of EPHA1 with cancer stage is not conclusive. It is reported that increased levels of Ephrin A1, the preferential ligand for EPHA1 and a high-affinity ligand for EphA2, is strongly correlated with poor survival, suggesting that altered Ephrin A1/EPHA1 signaling pathway may be involved in the aggression of this malignancy.

We, herein, took advantage of the potent CRISPR/Cas9 genome editing technique to KD EPHA1 specifically (28, 29). In the stable OC cells with EPHA1 KD, there was accumulative arrest of G1 and/or sub-G1 with corresponding decrease of M phase subsets. There is evidence that the activation of EPHA1 through Ephrin may inhibit ERK, which mediates multiple cancer cell behaviors, including cell cycle phases in a P53-independent manner (30, 31). We further investigated the change of ERK1 and ERK2 protein isoforms. It appears that KD of EPHA1 in SKOV3 cells increases the protein levels of both ERK1 and ERK2. However, in COV504, EPHA1 KD increases the level of ERK2 but decreases ERK1. After phospho-tyrosine proteins are pulled down using a P-TYR monoclonal antibody (P-Tyr) by immunoprecipitation, there is no change of ERK, implying its transcriptional repressor role is independent of its kinase activity (32).

We also observed that EPHA1 KD inhibits the adhesion ability of both OC cell lines tested. One previous study shows that there is interaction between EPHA1 and integrin-linked kinase, which mediates cellular spreading and adhesion to extracellular matrix depending on cell types (33).

We observed that KD of EPHA1 down-regulates c-MYC expression in OC cells. C-MYC is a well-characterized proto-oncogene that has frequently been found to be constitutively activated in various cancer types, including OC (34). As a transcription factor, C-MYC exerts diverse regulatory functions in cancer cells, including cell cycle, cell adhesion, cellular metabolism and apoptotic pathways (35). Therefore, the suppressed aggressive capacity of the OC cells after EPHA1 KD may also attribute to decreased expression of c-MYC.

Our data from the ECIS system further confirm that EPHA1 KD not only inhibited cellular spreading but also post-wound migration of the OC cells. The inhibited invasion by EPHA1 KD may be attributed to the down-regulation of MMP2, as shown by western blotting and zymography. This finding is partially supported by previous study in which EPHA1 silencing causes down-regulation of MMP2 in hepatocellular carcinoma cells (36).

Taken together, our data showed that EPHA1 KD suppresses the malignant properties of the OC cells in different aspects, which include cell cycle arrest, cell adhesion migration, proliferation and invasion. These findings enable us to propose that overexpression or activation of EPHA1 may promote aggression of OC. Therefore EPHA1 may possess potential therapeutic value as an anticancer target in OC patients with EPHA1 or Ephrin A1 overexpression.

Conflicts of Interest

The Authors declare no conflicts of interest.

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