ROR1 is highly expressed in circulating tumor cells and promotes invasion of pancreatic cancer

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Abstract. Pancreatic cancer (PaC) is an aggressive malignancy, which is associated with high levels of metastasis. Circulating tumor cells (CTCs), which may be considered a functional biomarker and promising treatment strategy for metastasis, are associated with the prognosis and progression of various metastatic cancers, including PaC. Receptor tyrosine kinase-like orphan receptor 1 (*ROR1*) expression contributes to cell metastasis and poor clinical outcomes in malignant tumors. The present study aimed to explore the function of ROR1 in PaC CTCs. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were used to examine the expression of *ROR1*, E-cadherin and N-cadherin. Cell proliferative and invasive ability was assessed by MTT and Transwell assays, respectively. The results revealed that the mRNA and protein expression levels of *ROR1* were augmented in PaC tissues. Furthermore, the mRNA expression levels of *ROR1* were higher in CTCs compared with in peripheral blood cells, and *ROR1* was more highly expressed in CTCs than in cells. Notably, CTCs exhibited a markedly greater proliferative and invasive capacity than PANC-1 and SW-1990 cells, whereas knockdown of endogenous *ROR1* by small interfering RNA led to suppression of the invasion of CTCs. In addition, it was revealed that the mechanism underlying the effects of *ROR1* on PaC CTC metastasis may involve the epithelial-mesenchymal transition process. In conclusion, *ROR1* may be considered a potential biomarker and therapeutic target for the treatment of PaC.

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Introduction

Pancreatic cancer (PaC) is one of the most aggressive and lethal types of tumor, and is the fourth leading cause of cancer-associated mortality worldwide (1). Despite improvement in cancer treatment, the prognosis for patients with PaC remains poor, with a 5-year survival rate of ~5% (2). It has been predicted that, with its current rise in incidence, PaC will become the second leading cause of cancer-associated mortality by 2030 (3). The majority of patients with PaC are not suitable for surgical resection due to local advancement and metastasis, and the current chemotherapeutic regimens for advanced PaC are limited (4). The high proclivity for partial invasion and early progression to distant metastases leads to the poor outcome in PaC (5), thus resulting in the high mortality rate of this malignancy. Furthermore, the current biomarkers poorly predict prognosis of patients with metastatic PaC (6). Therefore, for the clinical treatment of this disease, the selection and identification of valid and reliable biomarkers as prognostic indicators for patients with PaC are important.

Circulating tumor cells (CTCs) are tumor cells that induce metastasis, which is responsible for the majority of cases of cancer-associated mortality (7). CTCs have been widely recognized as tumor- or metastasis-derived cells, which move into the circulatory system from the primary tumor site during surgical resection and lead to disease recurrence in patients with cancer (8-11). CTCs have already been widely used as a biomarker for the assessment of cancer prognosis and response to therapy (12). Previous studies have reported that CTC counts have an association with the prognosis and development of numerous metastatic diseases, including breast, colon, prostate and lung cancers (13-17). Therefore, studying CTCs may improve understanding of the potential mechanisms underlying tumorigenesis and metastasis, offer promising clinical trials for patients with metastatic cancer, and supply a target for designing effective and individualized cancer therapies.

Receptor tyrosine kinase-like orphan receptor 1 (*ROR1*) is an embryonic protein, and a member of the ROR receptor tyrosine kinase family, which serves key roles in cell differentiation and proliferation, angiogenesis, tumor migration and metastasis (18-23). Numerous studies have indicated that *ROR1* is expressed at high levels in various blood and solid malignancies; however, it is lowly expressed in normal adult tissues (24-27). In this regard, ROR1 protein

may be an ideal drug target for cancer therapy; however, the underlying functions of *ROR1* in PaC have yet to be elucidated.

On the basis of these findings, the present study explored the association between *ROR1* and PaC, and revealed that *ROR1* was increased in PaC tissues compared with in noncancerous tissues. In addition, the mRNA expression levels of *ROR1* were increased in CTCs compared with in peripheral blood cells from patients with PaC. Furthermore, the proliferative and invasive capacity was increased in CTCs with high levels of ROR1 compared with in PANC-1 and SW-1990 cells. Furthermore, the present study revealed that downregulating *ROR1* expression suppressed the invasive ability of CTCs. In addition, the results demonstrated that the epithelial-mesenchymal transition (EMT) process may participate in the metastasis of CTCs from primary PaC tissue. Taken together, these results suggested that *ROR1* may be a novel genetic modifier for the progression of PaC.

Materials and methods

PaC tissues and blood samples. A total of 25 (male; mean age, 52.6; age range 35-70 years) human PaC tissue and paired adjacent noncancerous pancreatic tissue samples were acquired after written informed consent was obtained from the participating patients. The patients did not receive partial or systemic treatment prior to tissue sampling, and agreed to receive these treatments at the First Affiliated Hospital of Soochow University (Suzhou, China) between June 2006 and January 2017. Paired adjacent noncancerous pancreatic tissues (≤5 cm from the tumor site) were obtained from patients during surgery. All tissue specimens were immediately snap-frozen in liquid nitrogen following surgery. In addition, peripheral blood specimens were obtained from patients with PaC using vacutainer tubes containing the anticoagulant EDTA. All peripheral blood samples were stored at 4˚C and were processed within 3 days. The present study was approved by the Ethics Review Committee of the First Affiliated Hospital of Soochow University.

PaC cell culture. Human PaC cell lines PANC-1 and SW-1990, and the normal human pancreatic cell line HPDE6-C7 were used in the present study from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were seeded in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), and were cultured at 37˚C in a humidified incubator containing 5% CO_2.

Isolation and culture of CTCs. Peripheral blood cells (PBCs) and CTCs were isolated and cultured with Isolation of Peripheral Blood Mononuclear Cells kit (Qiagen, Inc., Valencia, CA, USA) and the CellSearch Circulating Tumor Cell kit (Menarini Silicon Biosystems, Inc., Bologna, Italy) according to the manufacturer's protocol, respectively. The peripheral blood samples from patients with PaC were pooled into 10 ml vacutainer tubes containing the anticoagulant EDTA on ice. The 2-ml peripheral blood cells were lysed in a conical tube containing 13 ml 1X red blood cell (RBC) lysis buffer (Beijing Leagene Biotech Co., Ltd., Beijing, China) for 10 min at room temperature, and were then centrifuged at 800 x g for 8 min at room temperature. Subsequently, the cells were washed twice by resuspending the pellets in 6 ml 1X PBS and were centrifuged at 800 x g for 3 min at room temperature. Finally, the remaining cells were cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS and incubated in a cell incubator containing 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from PaC tissues or cells and peripheral blood cells using a HP Total RNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and Blood RNA kit (Omega Bio-Tek, Inc.), respectively, according to the manufacturer's protocols. The RNA levels were analyzed using a NanoDrop spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA was reverse transcribed into cDNA with an M-MLV First Strand kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The mRNA expression levels of *ROR1* were quantified using a Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen; Thermo Fisher Scientific, Inc.) on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Sequences of the primers were as follows: *ROR1* (forward), 5'-AGATCACAGCTGCCTTCACTAT-3'; *ROR1* (reverse), 5'-GACATTCTCCAGGATTTCACAT-3'; β-actin (forward), 5'-GGCGGCACCACCATGTACCCT-3'; β-actin (reverse), 5'-AGGGGCCGGACTCGTCATACT-3'. The thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 50˚C for 20 sec and 60˚C for 1 min. Quantification cycle (Cq) values of *ROR1* mRNA were equilibrated to the internal control β-actin mRNA. Relative expression was calculated using the ΔΔCq method (28). All experiments were performed in triplicate.

Western blot analysis. Protein lysates from cells and tissues were obtained using radioimmunoprecipitation lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), which contained protease and phosphatase inhibitors (Sangon Biotech Co., Ltd., Shanghai, China). Protein concentrations were measured using NanoDrop technology (NanoDrop; Thermo Fisher Scientific, Inc.). Protein products (25 μg) were separated by 10% SDS-PAGE and were electrophoretically transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membrane was then incubated with primary antibodies at 4˚C overnight after blocking with 1.5% bovine serum albumin (Beyotime Institute of Biotechnology, Shanghai, China), followed by incubation with secondary antibodies at room temperature for 2 h. An Pierce™ ECL Western Blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) was used to detect protein bands, which were analyzed using Quantity One 4.6 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used to normalize target proteins. Antibodies employed in western blotting were: Rabbit anti-ROR1 (1:200, cat. no. ab15148; Abcam, Cambridge, UK), rabbit anti-E-cadherin (1:800, cat. no. ab15148; Abcam), rabbit anti-N-cadherin (1:1,000, cat. no. ab18203; Abcam), rabbit

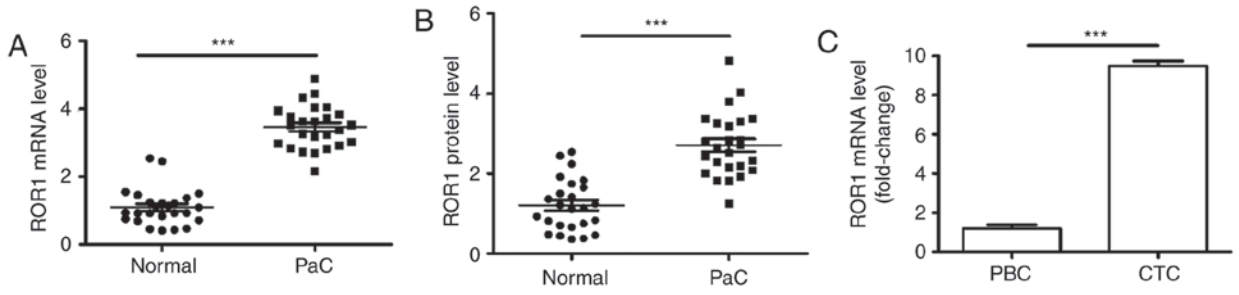

Figure 1. ROR1 expression was upregulated in PaC tissues and CTCs. (A) *ROR1* mRNA expression was detected in 25 paired PaC tissues and adjacent noncancerous tissues by reverse transcription-quantitative polymerase chain reaction. (B) ROR1 protein levels were measured in 25 paired PaC tissues and adjacent noncancerous tissues by western blotting. (C) Abundance of *ROR1* mRNA in CTCs compared with in PBCs. Representative data from three independent experiments were presented as the means ± standard deviation. ***P<0.001. CTC, circulating tumor cells; PaC, pancreatic cancer; PBC, peripheral blood cells; ROR1, receptor tyrosine kinase-like orphan receptor 1.

anti-β-actin (1:2,000, cat. no. ab8227; Abcam) and mouse anti-rabbit secondary antibodies (1:2,000, cat. no. sc-2357; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

RNA interference. The small interfering RNA (siRNA) sequence that directly targets human *ROR1* and the scrambled sequence used as a corresponding negative control (NC) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The target siRNA sequence for *ROR1* was (5'-3'): Sense, UGAACCAAUGAAUAACAUCdTdT and antisense, GAUGUUAUUCAUUGGUUCAdTdT. The NC sequence was (5'-3'): Sense, UUCUCCGAACGUGUCACGUTT and antisense, ACGUGACACGUUCGGAGAATT. CTC cells were seeded into 24-well plates at a density of $5x10^4$ cells/well. On the following day, CTCs were transfected with a mixture containing 100 pmol siRNA and Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37˚C. The cells were harvested after 72 h and Transwell assays were performed.

MTT assay. The proliferation of PANC-1 and SW-1990 cells, as well as CTCs obtained from patient blood samples, was assessed using MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), according to the manufacturer's protocol. A total of 3,000 cells/well were seeded in a 96-well plate and were incubated for 4 days in a humidified atmosphere containing 95% air and 5% CO_2 at 37˚C. Subsequently, 10 μl MTT reagent was added to each well and cultured at 37˚C for 2 h in darkness. Dimethyl sulfoxide was added to dissolve the crystals, and the absorbance was measured at 570 nm every 24 h. Three independent experiments were performed.

Transwell assay. Cell invasion assays were performed using Transwell plates (BD Biosciences, Franklin Lakes, NJ, USA) Cell invasion assays were performed in 24-well Transwell chambers containing polycarbonate filters with 8 mm pores coated with Matrigel. According to the manufacturer's protocol, $5x10^4$ cells were suspended in DMEM containing 1% FBS and were placed into the top chamber, which contained a Matrigel-coated filter. DMEM supplemented with 10% FBS was added to the bottom chamber to be used as a chemoattractant. After 48 h at 37˚C, the DMEM was discarded and cells adhering to the upper side of the membrane were removed with a cotton swab. Cells that had invaded onto the lower side of the membrane were stained with 1% crystal violet for 30 min at room temperature and observed by a light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan). Invasive cells were stained and counted in at least three microscopic fields (magnification, x100). The experiments were independently repeated three times.

Statistical analysis. Differences between two groups were analyzed using Student's t-test (two-tailed). Statistical analyses were conducted using GraphPad Prism 5.02 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Representative data from three independent experiments were presented as the means ± standard deviation or means ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

ROR1 expression is upregulated in PaC tissues and CTCs. Numerous studies have indicated that *ROR1* is increased in several types of blood and solid malignancies (18,27). To investigate whether the expression levels of *ROR1* were elevated in PaC, ROR1 expression was detected in 25 paired PaC tissues and adjacent noncancerous tissues using RT-qPCR and western blotting. The mRNA expression levels of *ROR1* in PaC tissues were significantly increased compared with in paired noncancerous pancreatic tissues (Fig. 1A). Furthermore, the protein expression levels of ROR1 were detected, and the results demonstrated that PaC tissues exhibited significantly increased ROR1 protein expression compared with in the paired noncancerous pancreatic tissues (Fig. 1B). CTCs, also known as metastasis-derived cells, move into the circulatory system from the primary tumor site. Therefore, the difference in *ROR1* mRNA expression between CTCs and PaC blood cells was investigated. The results indicated that the abundance of *ROR1* mRNA in CTCs was ~10-fold higher compared with in peripheral blood cells obtained from patients with PaC (Fig. 1C). These findings indicated that *ROR1* expression was increased in PaC, particularly in CTCs.

Proliferative and invasive ability of CTCs is stronger than PANC-1 and SW-1990 cells. Increased ROR1 expression serves key roles in cell proliferation and invasion (18,27). To

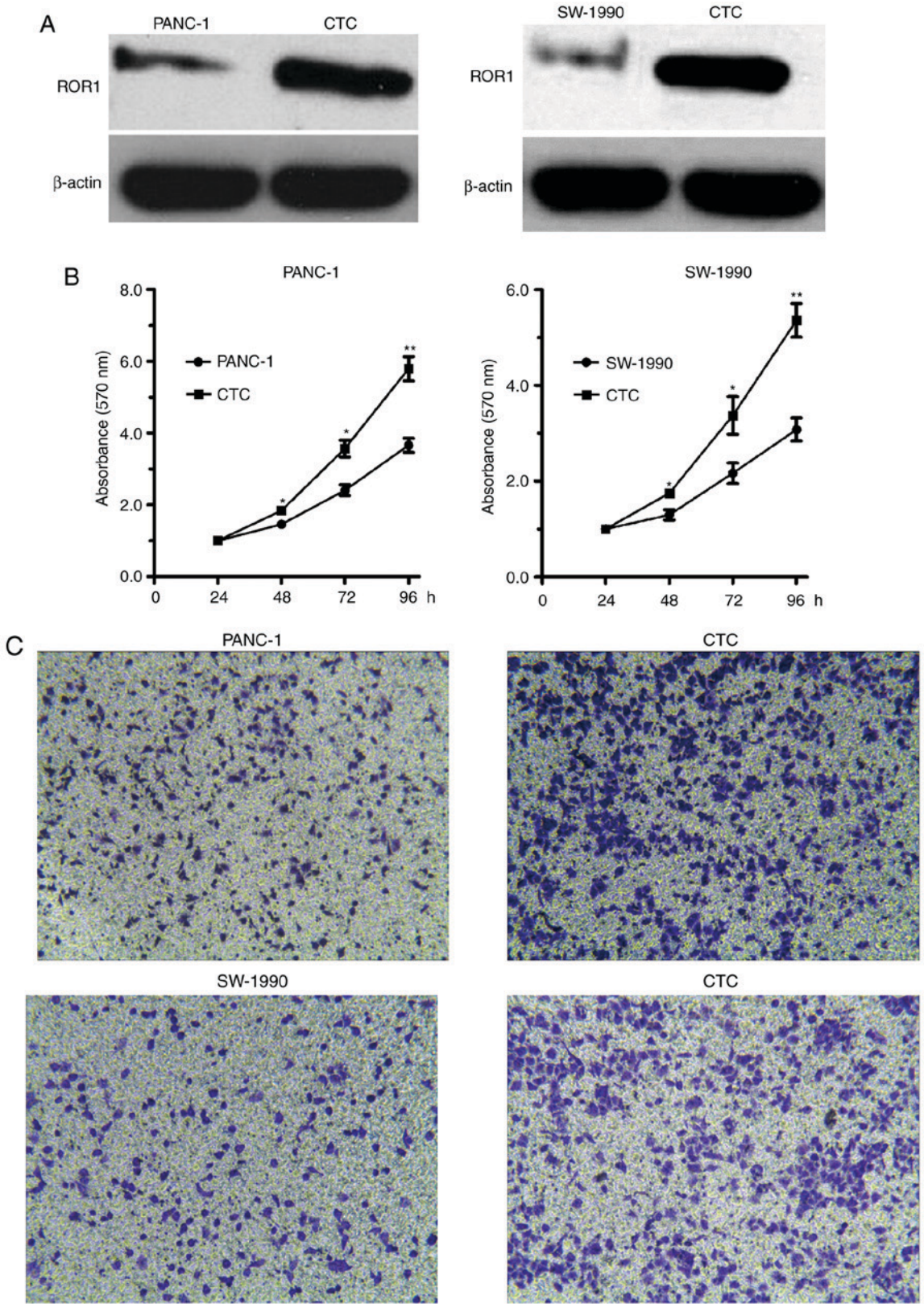

Figure 2. Proliferative and invasive ability of CTCs is stronger than PANC-1 and SW-1990 cells. (A) Western blot analysis of ROR1 in CTCs, PANC-1 and SW-1990 cells. β-actin was used as a loading control. (B) Cell proliferation of CTCs and PANC-1 and SW-1990 cells, as examined by MTT assay at the indicated time points. (C) Invasion of CTCs and PANC-1 and SW-1990 cells, as examined by Transwell assay. Invasive cells were stained and counted in at least three microscopic fields (magnification, x100). Representative data from three independent experiments were presented as the means ± standard error. *P<0.05. CTC, circulating tumor cells; ROR1, receptor tyrosine kinase-like orphan receptor 1.

investigate the role of *ROR1* in PaC cells, and in CTC growth and invasion, MTT and Transwell assays were performed. Initially, the expression levels of ROR1 were detected in CTCs and PANC-1 and SW-1990 cells by western blotting.

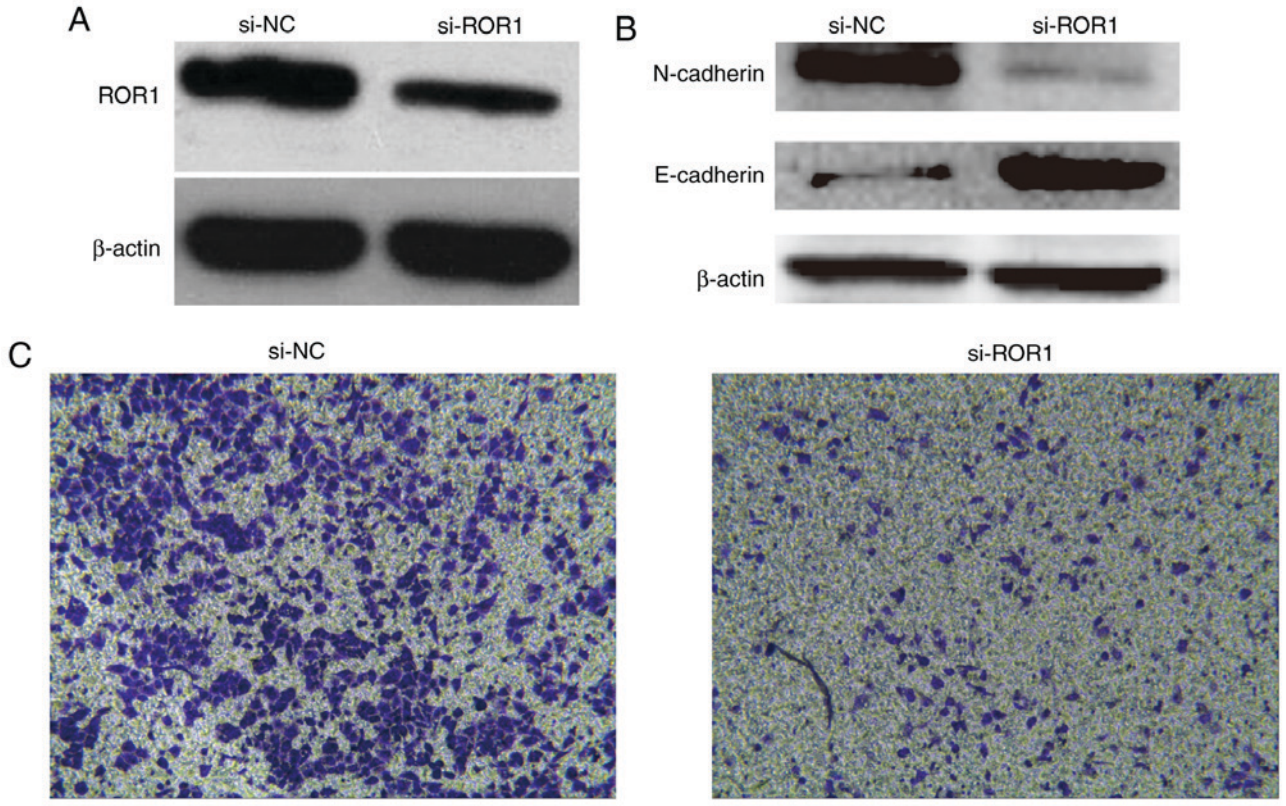

Figure 3. Knockdown of *ROR1* suppresses CTC invasion. (A and B) Western blot analysis of ROR1, E-cadherin and N-cadherin in NC siRNA-transfected CTCs and ROR1 siRNA-transfected CTCs. β-actin was used as a loading control. (C) Invasion of NC siRNA-transfected CTCs and ROR1 siRNA-transfected CTCs was analyzed by Transwell assay. CTC, circulating tumor cells; NC, negative control; ROR1, receptor tyrosine kinase-like orphan receptor 1; si, small interfering RNA.

As illustrated in Fig. 2A, ROR1 expression was markedly increased in CTCs compared with in PANC-1 and SW-1990 cells. Furthermore, compared with in the PANC-1 and SW-1990 cells, CTCs with high ROR1 expression exhibited significantly higher cell viability (Fig. 2B). Consistent with the MTT assay results, CTCs with high ROR1 expression displayed enhanced cell invasive ability compared with PANC-1 and SW-1990 cells (Fig. 2C). Taken together, these results suggested that *ROR1* may serve key roles in the ability of CTCs to mediate PaC growth and invasion.

Knockdown of ROR1 suppresses CTC invasion via regulation of the EMT process. In order to further demonstrate the role of *ROR1* in PaC metastasis, *ROR1* was knocked down in CTCs by siRNA (Fig. 3). Western blot analysis demonstrated that ROR1 expression in ROR1 siRNA-transfected CTCs was markedly reduced compared with in NC siRNA-transfected CTCs (Fig. 3A). Subsequently, a Transwell assay was performed to determine the invasive ability of ROR1 siRNA-transfected CTCs and NC siRNA-transfected CTCs cells. Knockdown of *ROR1* expression markedly prevented CTC invasion compared with in the control cells (Fig. 3C).

The present study explored the mechanism by which *ROR1* regulates cell invasion. EMT serves a key role in tumor cell invasion and metastasis (29). To investigate whether the typical molecular markers of EMT were altered, the protein expression levels of E-cadherin and N-cadherin were examined by western blotting. *ROR1* knockdown by siRNA increased E-cadherin protein expression, but decreased N-cadherin protein expression in CTCs (Fig. 3B). Taken together, these results indicated that knockdown of *ROR1* in CTCs markedly decreased the invasive ability by regulating the EMT process.

Discussion

In the present study, *ROR1* expression was examined in PaC tissues and CTCs, and the roles of *ROR1* were detected in proliferation and invasion of PaC cells and CTCs. In PaC samples, ROR1 was upregulated compared with in the paired noncancerous pancreatic tissues. Notably, the mRNA expression levels of *ROR1* were increased in CTCs compared with in peripheral blood cells from patients with PaC. It was also demonstrated that ROR1 levels were markedly increased in CTCs compared with in PANC-1 and SW-1990 cells. The CTCs, which were obtained from PaC blood samples and contained higher ROR1 levels, possessed stronger proliferative and invasive capabilities compared with the PaC cells, and knockdown of *ROR1* by siRNA reduced the invasive ability of CTCs. In addition, E-cadherin expression was increased and N-cadherin expression was decreased when ROR1 was knocked down in CTCs. Therefore, the EMT process may participate in the metastasis of CTCs from primary PaC tissue. These findings, combined with those of previous studies (24-27), indicated that ROR1 may be a novel tool for the treatment of patients with PaC.

CTCs serve a crucial role in cancer development and metastasis. For example, Chang *et al* (30), reported that tumor marker detection could complement CTC enumeration in predicting progression in patients with metastatic castration-resistant prostate cancer. The basic biological mechanisms of CTCs in facilitating cancer development and metastasis have attracted much attention (31,32). It is of great importance that highly specific molecular markers for CTC-targeted cancer therapy are identified. An increasing number of studies have reported on the expression and function of ROR1 (24-27); however, its regulatory role in CTCs from PaC remains largely unknown.

ROR1 is critically involved in the development and progression of various human cancers. In addition, *ROR1* has been reported to act as a promoter of stem cell tumorigenicity in ovarian cancer, which could be used as an indirect antagonist when its expression is decreased (33-35). In the present study, CTCs exhibited a higher proliferative and invasive potential compared with PANC-1 and SW-1990 cells, thus suggesting that *ROR1* may promote the tumorigenic process in PaC cells. In support of this, Cui *et al* (24), reported that *ROR1* stimulates leukemia-cell activation and enhances disease progression in patients. In addition, Gentile *et al* (23), reported that *ROR1* serves a key role in the malignant phenotypes maintained by the MET proto-oncogene, receptor tyrosine kinase.

In recent years, numerous methods have been applied to detect and quantify CTCs. Furthermore, the number and phenotype of CTCs, which have many similar biological characteristics, can provide information on patient prognosis and treatment efficacy (36-39). The present study demonstrated that the expression of *ROR1* was upregulated in PaC tissues. Notably, the abundance of *ROR1* mRNA in CTCs from patients with PaC was ~10-fold higher compared with its abundance in peripheral blood cells. These findings are in accordance with previous studies, which demonstrated that *ROR1* levels are increased in PaC tissues compared with in corresponding normal tissues (20,22-24). However, there are some contradictory findings with regards to *ROR1* expression in other types of cancer. For example, Balakrishnan *et al* (40), reported that *ROR1* is overexpressed in normal tissues compared with cancer tissues, including parathyroid tissues, pancreatic islets, and regions of the esophagus, stomach and duodenum, with resultant toxicity. These results could be explained by the finding that alterations in metabolic gene expression induced by cancer are heterogeneous in different tumor types (41). PaC is usually treated with interventional therapy, chemotherapy and radiotherapy; however, the efficacy is poor. More researchers have begun to regard molecular targeted therapy as a study focus for the acquisition of an in-depth understanding of the molecular mechanism underlying PaC. The present results indicated that *ROR1* could be knocked down by siRNA, which resulted in prevention of the invasion of CTCs in PaC. A previous study suggested that the number of CTCs in the peripheral blood of patients with several types of metastatic carcinoma is positively correlated with poor clinical prognosis (42). The present results suggested that *ROR1* may exhibit potential for use as a screening tool to optimize the outcome of patients with PaC, and to aid the decision of what population should be screened given the relatively low incidence, but poor survival associated with PaC. Suppressing *ROR1* may be a potential therapeutic approach in PaC.

In recent years, EMT, which serves a key role not only in embryonic development but also in tumor cell invasion and metastasis, has become a focus of attention (28). In the process of EMT, cell-cell junctions, polarity and epithelial cell markers are lost, which is accompanied by the gain of mesenchymal markers, and a motile and invasive phenotype, which may initiate tumor metastasis (43). Wang *et al* demonstrated that microRNA (miR)-30a inhibits EMT and metastasis by targeting *ROR1*, and that the downregulation of *ROR1* by the overexpression of miR-30a could elevate E-cadherin levels and reduce N-cadherin expression in breast cancer (44). The present study revealed that when ROR1 was lowly expressed, the mesenchymal marker N-cadherin was also expressed at low levels, whereas the epithelial marker E-cadherin was highly expressed. These findings are consistent with those of previous studies and further indicated that *ROR1* may facilitate PaC metastasis by modulating the EMT process.

In conclusion, the present findings demonstrated that *ROR1* may be associated with the metastasis of PaC. This study furthers the understanding of the biological regulation of CTCs and suggests a novel rationale and therapeutic strategy for the diagnosis and treatment of *ROR1*-positive CTCs. However, the sample size was relatively small; therefore, there is a need to further validate these results in a larger sample size. In addition, the molecular mechanisms underlying the effects of ROR1 on PaC metastasis requires further research.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

GLX and JS wrote the main manuscript. GLX, JS, WSW and YHX performed the experiments. GLX, WSW and CFN designed the study. WSW and CFN analyzed the data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Committee of the First Affiliated Hospital of Soochow University. Written informed consent was obtained from the participating patients.

Patient consent for publication

Informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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