EphA2-enriched exosomes promote cell migration and are a potential diagnostic serum marker in pancreatic cancer

QIAN WEI[1], LIJUAN WEI[2], JINGYA ZHANG[1], ZE LI[1], HONGLEI FENG[1] and LI REN[1]

Departments of [1]Laboratory and [2]Cancer Prevention Center, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin's Clinical Research Center for Cancer, Tianjin 300060, P.R. China

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Abstract. Pancreatic cancer (PC) is the fourth most common cause of cancer-related mortality worldwide and is characterized by high invasiveness and early metastasis. To identify novel diagnostic markers, the present study aimed to understand the mechanism underlying PC progression. The present study demonstrated that exosomes derived from the highly metastatic Panc-1 PC cell line were internalized by a low metastatic cell line, resulting in increased migration of the latter. Proteomics analysis further revealed that the receptor tyrosine kinase Eph receptor A2 (EphA2) was overexpressed in the Panc-1 exosomes, and these Exo_EphA2 had the ability to transfer metastatic potential to recipient cells. Consistent with this, circulating Exo_EphA2 levels were higher in patients with PC compared with healthy controls. Taken together, these results indicated that Exo_EphA2 acts an oncogene in PC and is a potential tumor maker for PC diagnosis.

Introduction

Pancreatic cancer (PC) is a lethal malignancy, with a 91% mortality rate within five years of diagnosis worldwide (1). Early stage diagnosis of PC is rare, and the majority of patients are diagnosed when the tumor has already metastasized, thereby precluding a curative surgical resection (2,3). The high invasiveness of PC cells and limited treatment options are the major factors underlying its poor prognosis (4,5). Therefore, the present study aimed to understand the mechanism underlying PC metastasis to identify novel biomarkers, and improve the chances of early diagnosis and a favorable clinical outcome.

Exosomes (EXOs) are small vesicles 40-100 nm in diameter that either bleb directly from the plasma membrane or are released when multivesicular bodies fuse with the cell membrane (6,7). Studies have demonstrated that EXOs serve important roles in intercellular signaling, and trafficking of proteins and nucleic acids (8,9). Certain exosomal proteins secreted from cancer cells actively participate in tumor initiation, progression and metastasis (10). In addition, cancer cell EXOs shuttle signaling molecules that reflect their tissue origins (11,12). For example, ovarian cancer cell EXOs promote T cell expansion and protect them against apoptosis (13), whereas mutant KRAS shuttled by colon cancer EXOs induce malignant transformation of wild-type KRAS colon epithelial cells (14). Therefore, the potential clinical applications of EXOs, for example as biomarkers of various types of pathology, have gained attention in recent years (15,16).

The present study demonstrated that receptor tyrosine kinase Eph receptor A2 (EphA2)-expressing EXOs secreted by the highly metastatic PC cell line Panc-1 notably altered the function of low-metastatic BxPC-3 cells. The present results not only provide novel insights into the pathological role of EphA2 in PC but also provide an experimental basis for targeting EXO_EphA2 for early diagnosis of PC.

Materials and methods

Cell culture. The PC cell lines Panc-1 and BxPC-3 were purchased from the American Type Culture Collection. Both cell lines were maintained in RPMI 1640 (HyClone; Cytiva) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C under 5% CO_2.

Isolation of EXO. Cells were cultured in 1640 medium supplemented with 10% FBS until they reached 70% confluence. After removing the medium and washing the cells three times with PBS, fresh serum-free 1640 medium (HyClone; Cytiva) was added, and the cells were cultured for another 48 h at 37˚C. The medium was aspirated and centrifuged for 10 min at 300 x g to remove the dead cells at room temperature, and then at 9,000 x g for 30 min to remove the cell debris at room

Correspondence to: Professor Li Ren, Department of Laboratory, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin's Clinical Research Center for Cancer, 1 West Huan-Hu Road, Ti Yuan Bei, Hexi, Tianjin 300060, P.R. China
E-mail: lirentj@163.com

Abbreviations: PC, pancreatic cancer; EphA2, receptor tyrosine kinase Eph receptor A2; EXO, exosome; TEM, transmission electron microscope

Key words: exosome, Eph receptor A2, migration, pancreatic cancer

temperature. The supernatant was centrifuged at 100,000 x g for 2 h at 4˚C, and the pelleted exosomes were resuspended in PBS, then ultracentrifuged at 100,000 x g for 2 h at 4˚C. The last two steps were repeated, and the purified EXOs were characterized. The protein concentration of EXOs was determined using a BCA kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Electron microscopy. A total of 10 μl purified EXOs (100 μg/ml) was placed on non-glow-discharged carbon-coated grids (300 mesh; Beijing XXBR Technology Co., Ltd.) for 10 min with 4% paraformaldehyde at room temperature and negatively stained with 10 μl 2% uranyl acetate for 1 min at room temperature. The excess solution was removed by wicking onto filter paper, and dried grids were viewed under a FEI/Philips CM12 transmission electron microscope (TEM) operating at 80 KeV (magnification, x100).

Western blotting. Total protein was extracted from cells using RIPA buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). The protein concentration was determined using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of cell lysate (WCLs; 20 μg) or exosome lysate (EXOs; 10 μg) were resolved by Tris-Bis gel electrophoresis using a 4-15% gradient gel (Bio-Rad Laboratories, Inc.). The protein bands were transferred to a nitrocellulose membrane and blocked with 5% skimmed milk in PBS with 0.05% Tween-20 (PBST) at room temperature for 2 h. The blots were probed with anti-tumor susceptibility gene 101 protein (Tsg101; cat. no. ab30871; Abcam, 1:800), anti-CD63 (cat. no. ab68418; Abcam; 1:500), anti-Giantin (GM130; cat. no. ab187514; Abcam; 1:1,000) and anti-EphA2 (cat. no. ab73254; Abcam; 1:1,000) antibodies for overnight at 4˚C. After washing four times with PBST, the membranes were incubated with the secondary antibody (1:5,000) for 1 h at room temperature. Protein bands were visualized using ECL Western Blotting Detection Reagent (cat. no. G075; Applied Biological Materials, Inc.). The band intensities were quantified by ImageJ software (version 1.8.0; National Institutes of Health).

Wound healing assay. Panc-1 ($1x10^5$) and BxPC-3 ($5x10^5$) cells were seeded in 6-well plates and cultured for 24 h to ~100% confluence. The monolayers were gently scratched with 1 ml pipette tips to create a 'wound', and the wells were gently washed with 1X PBS to remove the dislodged cells. The cells were cultured for 48 h with 20 μg/ml Panc-1 or Panc-1^{EphA2-} EXO or 0.5 μg/ml recombinant EphA2 that were added twice (once each at 0 and 24 h) at 37˚C. The scratched areas were photographed at 0, 24 and 48 h post-wounding using a confocal microscope (magnification, x200), and the area was measured using ImageJ (version 1.8.0; National Institutes of Health). Both cell lines were maintained in RPMI 1640 with 1% FBS. The experiment was repeated three times.

Protein identification. The protein extracts were resolved by 15% SDS-PAGE, and the gel was stained with silver nitrate. The appropriate gel pieces were cut and digested, and the resulting peptides were analyzed by positive-ion data-dependent micro-capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an LTQ 2D Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Inc.) as per standard protocol (17). The initial MS scan was followed by eight further MS/MS scans. The proteins were identified by aligning the sequences against the UniProt database (www.uniprot.org) using the Sequest algorithm in Proteomics Browser software (version 2.3.0; Thermo Fisher Scientific, Inc.).

EXO internalization. Panc-1 EXOs were labeled with EXO-Red (cat. no. EXOC300A-1; System Biosciences, LLC) according to the manufacturer's instructions. BxPC-3 cells were plated at $2x10^4$ cells/well on 8-well chamber slides for 24 h and supplemented with 20 μg/ml EXO-Red-labeled Panc-1 EXOs. For microscopy studies, cells were washed three times with PBS, incubated with 4% paraformaldehyde for 15 min at 25˚C, and incubated in DAPI/PBS solution (1:1,000) for 5 min at 25˚C. EXOs were visualized under a laser scanning confocal microscope (magnification, x40; FV-100; Olympus Corporation).

Generation of stable EphA2-knockdown Panc-1 cell line. The plasmid pGLVH1/GFP+Puro, EphA2_shRNA (5'-GAA CTTCAACACAGCCTGG-3') and packaging vector PG-P1-VSVG were prepared by Shanghai GenePharma Co., Ltd., and extracted with high purity and no endotoxins. 293T cells (density, 60-70%) were co-transfected with RNAi-mate (Shanghai GenePharma Co., Ltd.). After 6 h of transfection, they were replaced with a complete culture medium. After 72 h of culture, the supernatant of cells was collected with ultracentrifugation at 40,000 x g for 2 h at 4˚C and concentrated to obtain a high titer lentivirus concentrate for infecting target cells.

Panc-1 cells were transduced with lentivirus ($1x10^9$ TU/ml; dilution, 1:10) and 5 μg/ml polybrene (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C, then the stable transfectants were selected in the presence of 10 μg/ml puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) for 2 days. Decreased EphA2 protein expression levels were confirmed via western blotting as aforementioned. The stable EphA2-Panc-1 cells were maintained in complete RPMI-1640 supplemented with 2 μg/ml puromycin at 37˚C.

Patients and specimens. Serum samples were obtained from 40 patients with PC (median age, 30-70 years; 36 males and 34 females; 20 patients each at stage I+II and stage III+IV) and 30 healthy controls at the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) between March 2019 and September 2019. The disease was staged according to the American Joint Committee on Cancer tumor, node, metastasis classification (18). All experiments involving human specimens were performed in accordance with the 1964 Declaration of Helsinki ethical standards and approved by the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (approval no. bc2019112). Written informed consent was obtained from all patients prior to participation.

Quantification of exosomal EphA2 by ELISA. EXOs were precipitated from 100 μl patient serum, using ExoQuick (System Biosciences, LLC) according to the manufacturer's protocols. EXO pellets were then suspended in PBS and analyzed using an EphA2 ELISA kit (cat. no. DYC3035-2;

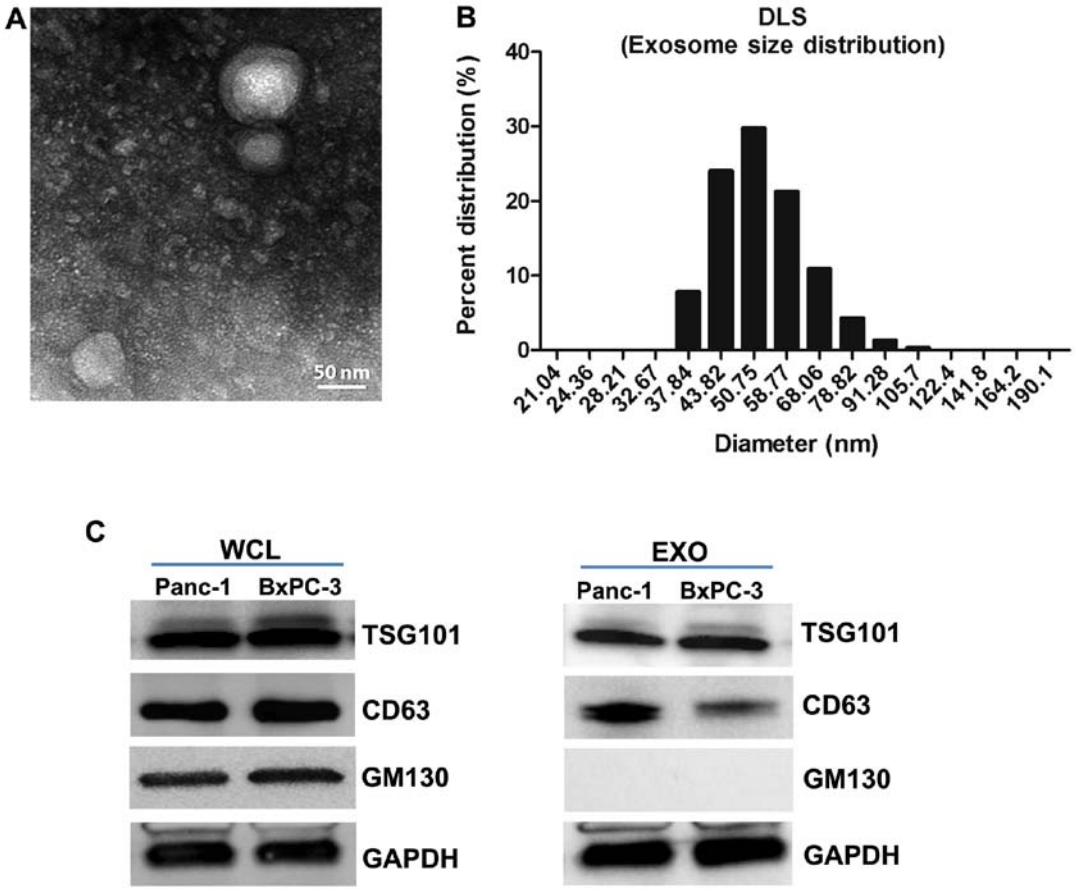

Figure 1. Validation of exosomes. (A) Transmission electron microscopy micrographs showing EXO morphology. (B) Dynamic light scattering showing the size distribution of EXOs (mean diameter, 50.75±20.00 nm). (C) Immunoblots showing the expression levels of indicated proteins in WCL and EXOs. Tsg101 and CD63 were used as positive markers; GM130 was used as the negative marker. EXO, exosome; WCL, whole cell lysate; Tsg101, tumor susceptibility gene 101 protein; GM130, golgin subfamily A member 2.

R&D Systems, Inc.) according to the manufacturer's protocol.

Statistical analysis. Data are presented as the mean ± standard deviation of three biological repeats. Multiple groups were compared using one- or two-way ANOVA with a post hoc Bonferroni test. All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Validation of EXOs derived from PC cells. EXOs released from PC cells were purified and characterized in terms of morphology and exosomal marker expression levels. The distinctive EXO structure was observed via TEM (Fig. 1A), and dynamic light scattering showed a typical size range of 30-100 nm (Fig. 1B). Furthermore, the purified EXOs expressed Tsg101 and CD63, but not the Golgi protein GM130 (Fig. 1C). Thus, these results confirmed the purity of EXO preparation.

Panc-1 EXOs enhance in vitro migration of recipient cells. Panc-1 cells exhibited significantly higher *in vitro* migration ability compared with BxPC-3 cells (Figs. 2A and S1). In order to determine whether Panc-1-derived EXOs enhance the migration of BxPC-3 cells, the latter were incubated with EXOs for 48 h. The percentage of migrating BxPC-3 cells increased significantly at 48 h of exposure to Panc-1 EXOs compared with control cells (Figs. 2B and S1). By contrast, EXOs derived from BxPC-3 cells had no effect on migration (Fig. S2). These results indicated that EXOs secreted by metastatic PC cells enhanced the migration of recipient cells, which is likely by transporting relevant factors.

Panc-1 EXOs are internalized by recipient cells. BxPC-3 cells incubated with Panc-1 EXOs exhibited increased expression of Tsg101 (Fig. 2C and D). The internalization of Panc-1 EXOs by BxPC-3 cells was tracked by labeling EXOs with EXO-Red. Recipient cells internalized these EXOs within 10 h (Fig. 2E).

Analysis of exosomal proteins. EXOs mediate intercellular communication via horizontal transfer of proteins (19). Therefore, it was hypothesized that EXOs derived from Panc-1 and BxPC-3 cells have distinct proteomes and are enriched in metastasis-associated proteins. In order to validate exosomal protein composition, lysates of purified EXOs derived from Panc-1 and BxPC-3 cells were resolved via SDS-PAGE. The silver-stained gels showed distinct protein profiles of EXOs (Fig. 3A), which was confirmed

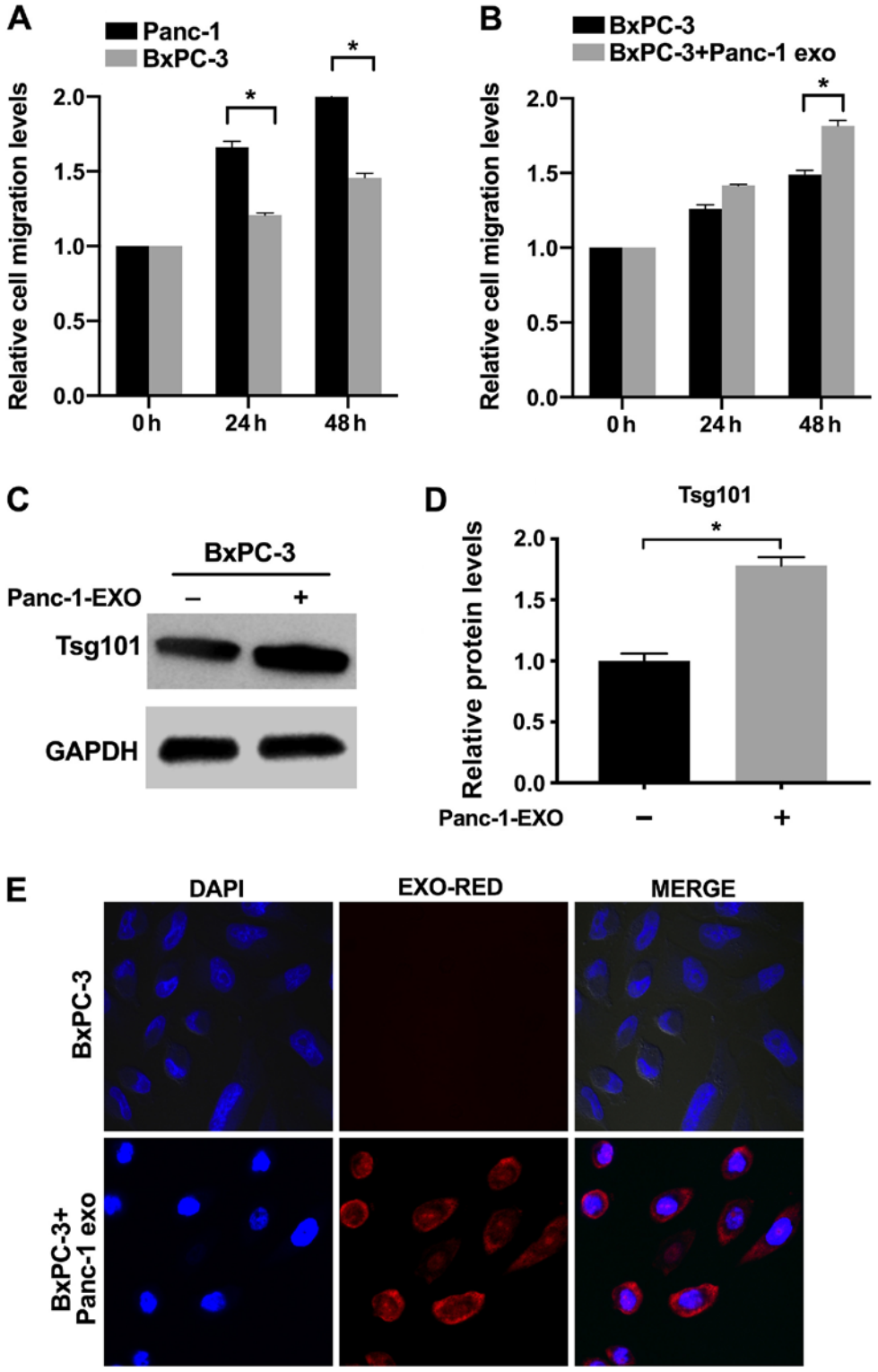

Figure 2. Exosomes enhance cell migration. (A) *In vitro* migration rates of Panc-1 and BxPC-3 cells. (B) Wound-healing assay showing migration of BxPC-3 cells cultured with Panc-1-derived EXOs. (C) Western blotting and (D) quantification of Tsg101 levels in BxPC-3 cells following Panc-1 EXO treatment. (E) Confocal images showing internalization of Panc-1 EXOs by BxPC-3 cells (magnification, x60). *P<0.05 as indicated. EXO, exosome; Tsg101, tumor susceptibility gene 101 protein.

via LC-MS/MS analysis (Table SI). The expression levels of EphA2 were significantly higher in both the lysates and EXOs of Panc-1 compared with BxPC3 cells (Fig. 3B and C) in terms of the LC-MS/MS score. Taken together, these results indicated that the metastatic effects of Panc-1 EXOs are likely mediated by EphA2.

EXO_EphA2 mediates migration of PC cells in vitro. In order to further validate the metastatic function of EXO_EphA2, a stable EphA2^{-} Panc-1 cell line was generated, which exhibited a 70% decrease in EphA2 expression levels compared with control cells (Fig. 4A). Cell migration was significantly decreased in Panc-1^{EphA2-} cells compared with Panc-1 cells (Fig. S3).

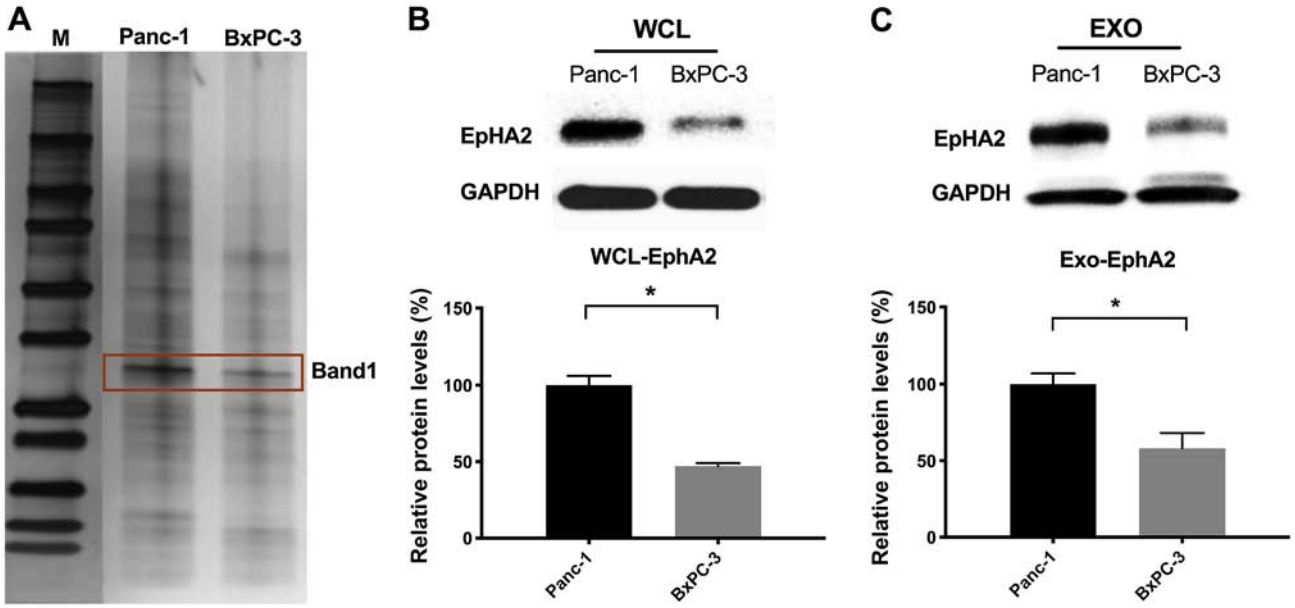

Figure 3. Analysis of exosomal proteins. (A) Silver stained gels showing the protein profiles of Panc-1 and BxPC-3 EXOs. Immunoblots showing EphA2 expression levels in (B) WCL and (C) EXOs of Panc-1 and BxPC-3. *P<0.05 as indicated. EXO, exosome; WCL, whole cell lysate; EphA2, receptor tyrosine kinase Eph receptor A2.

The EXO_EphA2 levels were also significantly decreased in Panc-1^{EphA2-} cells compared with Panc-1 cells (Fig. 4B). Furthermore, unlike EXOs derived from wild-type Panc-1 cells, Panc-1^{EphA2-} EXOs did not significantly increase the migration of BxPC-3 compared with control cells. BxPC-3 cells incubated with recombinant EphA2 did not exhibit increased migration, indicating that exosomal delivery is essential for the oncogenic effects of EphA2 (Figs. 4C and S4). These results indicated that EXO_EphA2 mediates migration of PC cells.

Circulating EXO_EphA2 is a potential diagnostic biomarker for PC. Tumor-derived EXOs are easily detectable in circulation, and therefore are promising diagnostic/prognostic markers for cancer. The levels of circulating EXO_EphA2 were significantly higher in the serum of patients with PC compared with those in healthy controls (Fig. 4D). In addition, levels of circulating EXO_EphA2 were significantly elevated in advanced stage (III+IV) patients compared with those in the early stages (I+II). These results indicated that EXO_EphA2 is a potential diagnostic biomarker for PC.

Discussion

Surgical resection is currently the most effective method for controlling PC. However, only 9-10% of patients are eligible for surgery due to late diagnosis (20). Therefore, the mechanisms underlying PC progression and metastasis need to be elucidated in order to identify novel diagnostic markers (21,22).

There is increasing interest in the molecular biological function of EXOs in cancer. EXOs are secreted vesicles that mediate cellular signaling and trafficking, and serve a key role in cancer progression by transporting oncogenic factors in a paracrine manner (23,24). Yan *et al* (25) demonstrated that EXOs released from stromal cells induce Myc-dependent metabolic reprogramming and promote tumor growth. Similarly, studies have revealed that Wnt5b-harboring EXOs trigger cancer cell migration and proliferation in a paracrine manner (26), and that downregulation of exosomal C-type lectin domain family 3 member B in hepatocellular carcinoma promotes metastasis and angiogenesis via the 5'AMP-activated protein kinase and vascular endothelial growth factor pathways (27). However, EXO secreted by different types of PC cells have not yet been fully characterized. In the present study, EXOs derived from highly metastatic Panc-1 cells enhanced the migratory capacity of low-metastatic BxPC-3 cells, which was associated with high levels of EphA2 expression. Therefore, it was hypothesized that oncogenic EXOs may also endow tumor cells with metastatic abilities in a paracrine manner *in situ*.

EphA2 is overexpressed in melanoma, as well as breast and lung cancer, and is associated with increased tumor progression (28-30). In addition, EXOs released from senescent cells promote cancer cell proliferation by transporting EphA2, indicating its potential as a therapeutic target (31). Zhuang *et al* (32) further demonstrated that EphA2 mediates trastuzumab resistance in breast cancer cells. Consistent with these previous findings, the present results revealed a previously unknown pro-metastatic function of EphA2-enriched EXOs in PC. In the present study, BxPC-3 cells treated with recombinant EphA2 protein did not exhibit increased cell migration. It was hypothesized that the fusion of EXOs with the cell membrane transfers soluble and membrane-associated factors such as EphA2 to recipient cells, which may not be able to internalize the naked recombinant EphA2 protein. These results indicate that exosomal delivery serves a key role in EphA2-mediated cell migration. Since effective prognostic markers for PC remain elusive (33), the present findings are significant in

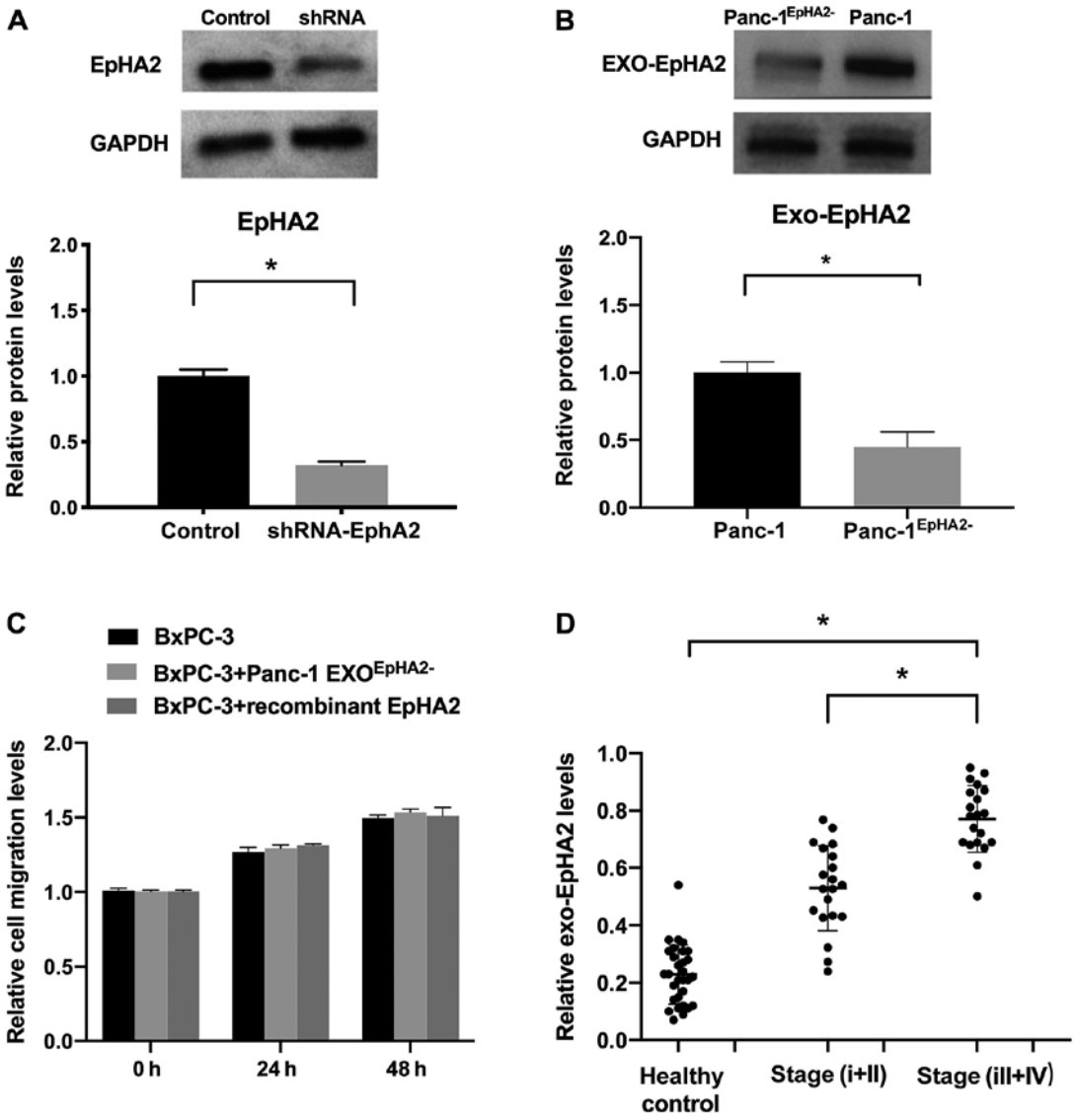

Figure 4. EXO_EphA2 mediates migration. (A) Relative EphA2 levels in Panc-1 cells transduced with EphA2_shRNA and ctrl-shRNA lentiviruses. GAPDH was used as the internal control. (B) Immunoblots showing EphA2 expression levels in EXOs of Panc-1 and Panc1^{EphA2-} cells. (C) Wound-healing assay showing migration of BxPC-3 cells exposed to EXOs derived from Panc-1^{EphA2-} cells and recombinant EphA2. (D) Pre-therapy serum EXO EphA2 levels in controls and patients with pancreatic cancer at various stages. Data are presented as the mean ± SD; *P<0.05. shRNA, small hairpin RNA; ctrl, control; EXO, exosome; EphA2, receptor tyrosine kinase Eph receptor A2.

demonstrating the diagnostic potential of Exo_EphA2 in PC. To the best of our knowledge, the present study is one of few to show that direct exosomal transfer of an oncogenic factor can phenotypically alter recipient cells. Future studies should analyze the circulating levels of Exo_EphA2 in patients with PC to validate its clinical value. Targeting Exo-EphA2 may represent a novel strategy to diagnose metastasis and invasion in PC.

Although EXO_EphA2 likely plays an important role in PC metastasis and appear to be a reliable diagnostic biomarker for patients with PC, there are certain limitations of the present study. In the wound healing assay, cells were maintained with 1% FBS to avoid excessive cell death that may influence the experimental results. Moreover, future studies need to be performed in larger cohorts of patients with PC to validate the clinical utility of Exo_EphA2. In addition, prospective studies are required to further explore the potential prognostic value of EXO_EphA2 for PC.

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Availability of data and materials

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

Authors' contributions

QW and LR conceived and designed the present study. JZ and LW provided the study materials, collected clinical follow-up data and performed the clinical interpretation. ZL and HF performed the experiments. QW performed statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures carried out in studies involving human participants were approved by the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (approval no. bc2019112) and were in accordance with the 1964 Declaration of Helsinki ethical standards. Written informed consent was obtained from all patients prior to participation, and the study was approved by the local Ethical Board.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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