Long noncoding RNA BANCR promotes lymphangiogenesis and lymph node metastasis of pancreatic carcinoma by regulating VEGF-C/VEGFR-3 signaling pathway

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Research Article

Keywords: BANCR, Pancreatic carcinoma, VEGF-C/VEGFR-3, MLVD

Posted Date: December 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3774468/v1

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Additional Declarations: No competing interests reported.
Abstract

Background

Long noncoding RNAs (lncRNAs) have proved to play important roles in the tumorigenesis and development of pancreatic carcinoma (PC). The aim of our study is to investigate the expression of long noncoding BRAF-activated noncoding RNA (BANCR) and its role in PC lymph node metastasis.

Methods

BANCR expression was detected in PC tissues by using Real-time quantitative PCR (RT-qPCR). The correlation between BANCR expression and lymph node metastasis was analyzed. Immunofluorescence staining was used to determine microlymphatic vessel density (MLVD) of peritumoral tissues. The correlation between MLVD and lymph node metastasis and the association between BANCR expression and MLVD were also analyzed. VEGF-C and VEGFR-3 expressions in PC cells were detected by western blot in vitro, and the regulatory role of BANCR on VEGF-C/VEGFR-3 was identified by cell transfection techniques. The transfected PC cells were co-cultured with human lymphatic endothelial cells (HLECs) and the MLVD was detected by the magnification inverted fluorescence microscope.

Results

BANCR expression was remarkably increased in PC tissues, especially in the lymph node metastasis group (P < 0.0001). MLVD of the PC tissues in the lymph node metastasis group was significantly higher than the group without lymph node metastasis (P < 0.0001). The BANCR expression level significantly correlated with MLVD (P < 0.05). The result was further verified in PC cell lines. Depletion of BANCR inhibits lymphangiogenesis and down regulates VEGF-C/VEGFR-3 (P < 0.05). However, Overexpression of VEGF-C could counteract the inhibitory effect of siBANCR on lymphangiogenesis.

Conclusions

BANCR promotes lymphangiogenesis and lymph node metastasis of PC by regulating the VEGF-C/VEGFR-3 signaling pathway.

Introduction

Pancreatic carcinoma (PC) is a common and highly malignant tumor of the digestive system. Although progress has been made in the diagnosis and treatment of PC in recent years, PC remains one of the deadliest malignancies, with a five-year survival rate of only about 6%[1]. Patients commonly lack clinical symptoms at the early onset of disease. PC is mainly diagnosed at an advanced, often metastatic stage, which is one of the main reasons for its poor overall prognosis and survival[2]. Lymph node metastasis,
as is well-known, is a poor prognostic factor of PC[3, 4], which influences the clinical stage and treatment of patients with PC. Despite numerous studies demonstrated that lymphatic vessels invasion and subsequent lymph nodes metastasis are early and important events during PC progression[5, 6], the mechanisms that lead PC cells to separate from the primary focus, invade lymphatic vessels and metastasize to lymph nodes remain largely unknown.

Studies of human pancreatic cancer tissues have confirmed a role for tumor-associated lymphangiogenesis (TALA) in lymph node metastasis. Kurahara et al found that high lymphatic vessel density (LVD) in pancreatic head cancer predicted increased lymph node metastasis and decreased survival[7]. Increased peritumoral LVD in human PC tissues correlated with increased lymphatic vessel invasion and more lymph node metastasis, a similar study of patient samples also showed[8]. Numerous studies have shown that the VEGF-C/VEGFR-3 signaling pathway plays an important role in promoting lymphangiogenesis in a variety of malignant tumors, including but not limited to renal cell cancer, colorectal cancer, breast cancer, etc.[9–12], similarly, Ochi et al found that VEGFR-3 combined with VEGFC to stimulate the formation of lymphatic vessels in PC, which induced the formation of new lymphatic capillaries and increased the risk of lymph node metastasis[13]. However, relatively little is known about the regulatory mechanism regulating the VEGFC/VEGFR-3 pathway in PC.

Long noncoding RNA (lncRNA) is a class of RNA molecules over 200 nucleotides long which, due to the lack of open reading framework, does not have the ability to encode protein. LncRNAs were once regarded as “noise” in the transcription process and ignored. Increasing numbers of studies, however, have determined its importance in tumor progression through affecting cell proliferation, apoptosis, epithelial-to-mesenchymal transition, migration, invasion and lymph node metastasis. BRAF-activated non-protein coding RNA (BANCR) is a lncRNA with 693 bp in length and located in a gene desert region on 9q21.11-q21.12. Studies have shown that BANCR is upregulated in gastric, colorectal cancer and hepatocellular carcinoma, and plays an important role in tumorigenesis and development[14–16]. However, studies on BANCR in PC are few, especially in lymphangiogenesis and lymph node metastasis of PC. In our present work, we aim to examine the role of BANCR in PC lymph node metastasis. The results identified that upregulated expression of BANCR was detected in ex-vivo in PC tissues or in-vitro in PC cells and may promote lymphangiogenesis and lymph node metastasis through the VEGF-C/VEGFR-3 pathway.

**Materials and Methods**

**Patient data and tissues Collection**

PC tissues and adjacent healthy tissues were obtained from 36 patients with pancreatic ductal adenocarcinoma who were subjected to surgical resection in The Beijing Luhe Hospital, Capital Medical University from January 2019 to December 2021. All patients involved did not receive preoperative radiotherapy, chemotherapy, traditional Chinese medicine treatment or any other type of treatment, and had no family history of the disease. The diagnosis for all patients with PC was confirmed by
postoperative pathology, and tumor node metastasis (TNM) staging and tumor grading were performed based on the PC staging criteria of American Joint Committee on Cancer[17]. This study was approved by the Ethics Committee of The Beijing Luhe Hospital, Capital Medical University, and all patients had signed written informed consent before performing this study. The clinical and pathologic features of patients with PC are summarized in Table 1.
Table 1
Characteristics of patients with pancreatic ductal adenocarcinoma.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NO. of patients(n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>43</td>
</tr>
<tr>
<td>Max</td>
<td>79</td>
</tr>
<tr>
<td>Median</td>
<td>70</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
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<tr>
<td>2 or less (T1)</td>
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</tr>
<tr>
<td>2.1 to 4 (T2)</td>
<td>14</td>
</tr>
<tr>
<td>Over 4 (T3)</td>
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</tr>
<tr>
<td>Involves celiac axis, superior mesenteric artery, and/or common hepatic artery (T4)</td>
<td>8</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
</tr>
<tr>
<td>No</td>
<td>16</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
</tr>
<tr>
<td>No</td>
<td>30</td>
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<td>Histological grade</td>
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<tr>
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<tr>
<td>Poor (G3)</td>
<td>5</td>
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<td></td>
</tr>
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<td>14</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
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</table>
Total RNA extraction and quantitative real-time polymerase chain reaction analysis

Total RNAs were extracted from tissues samples using TRIzol reagent (Invitrogen). After digestion with DNase I (DNA-free kit, Ambion, Austin, Texas), reverse transcription was conducted to transcribe the isolated RNA into complementary DNA (cDNA) by reverse transcriptase (Takara, Dalian, China) using the stem-loop RT primer. Next, the cDNAs were amplified using SYBR Green real-time polymerase chain reaction (RT-PCR; Takara). The levels of BANCR quantified by quantitative RT-PCR were standardized to that of GAPDH. All primers used for qRT-PCR in this study are presented in Table 2.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>5'-3'Sequence</th>
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<tbody>
<tr>
<td>GAPDH Forward</td>
<td>TCAAGAAGGTGGTGGAAGCAGG</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>TCAAAGGTGGAGGAGTGGGT</td>
</tr>
<tr>
<td>BANCR Forward</td>
<td>GAGCCTTGCCAGTTCCATT</td>
</tr>
<tr>
<td>BANCR Reverse</td>
<td>TGCAGAGGAGATTCAGGT</td>
</tr>
</tbody>
</table>

Immunohistology and determination of microlymphatic vessel density (MLVD)

For immunofluorescence studies, specimens were fixed for 20–25 min in 4% paraformaldehyde (PFA), rinsed in PBS, transferred into 10 and 30% sucrose in PBS, and embedded in tissue freeze medium (Tissue Tek, Sakura Finetek Zoeterwoude, NL). Sections of 12–14 µm were incubated with the following primary antibodies: mouse-anti-human D2-40/podoplanin (1:200, Dako, Hamburg, Germany; #M3619). Secondary antibodies were: goat-anti-mouse Alexa 488/594 (#A11001; #A21135). Sections were counterstained with DAPI and mounted under cover slips with Fluoromount-G (Southern Biotechnology, US). Photos were taken with AxioImagerZ1 (Zeiss, Göttingen, Germany). The positively stained D2-40 protein was mainly located in the cytoplasm and/or cell membrane of the lymphatic endothelium, and was presented as a red color. The determination of microlymphatic vessel density (MLVD) was according to the method previously described by Shinichi Aishima et al[18], which was used to observe and select 5 regions with maximum MLVD (hot spots) under a magnification inverted fluorescence microscope (magnification, x100). Five optic fields were counted under x200 magnification (covering an area of 0.74 mm²) and the average MLVD value was used. All these assessments were made by two independent observers. MLVD was defined as the number of vessels/mm².
Cell lines and Culture Conditions

PC cell line (PANC-1) and human lymphatic endothelial cells (HLECs) were purchased from Guangzhou Genio Biotech Co., Ltd and cultured in incubator with 100% humidity and 5% CO₂ at 37°C. The PANC-1 cells were cultured in RPMI-1640 (HyClone; Cytiva) and the HLECs were cultured in Endothelial Cell Medium (ScienCell Research Laboratories, Inc.), each supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin-streptomycin; Thermo Fisher Scientific, Inc.).

Cells transfection in vitro

Short-interfering RNAs (siRNA) targeting BANCR, and their corresponding negative control (siNC) were all purchased from Ribobio (Guangzhou, China). The siBANCR sequence was 5'GGUGTG GCGUCT UGCUUT T-3'. The siNC sequence was 5'GGCCGGUTTCCUUTTCUGCG-3'. Before transfection, PANC-1 cells were cultured until 60% confluent and then washed with phosphate-buffered saline (PBS). Next, siRNAs were transfected into PANC-1 using Lipofectamine RNAiMAX (Invitrogen) in the light of the manufacturer's recommended protocol, and 40 nM of siRNA was transfected for 48 hours. Because the lentiviral vector contains a fragment of the green fluorescent protein (GFP) gene, successfully transfected cells can observe green fluorescence under a fluorescence microscope.

Western blotting

The transfected cells were examined by western blotting. Total protein was extracted under different treatment conditions using RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) and protein concentration was quantified using the BCA method (Thermo Fisher Scientific, Inc.). A total of 20 µg protein/lane was loaded for electrophoresis. SDS-PAGE on a 10% gel was performed at a constant voltage of 100 V for 40 min. After electrophoresis, the electroporation apparatus was used to transfer the resolved proteins to a PVDF membrane using a constant current of 250 mA for 2 h. The membranes were subsequently blocked at room temperature for 1 h with 5% skimmed milk/TBS-0.1% and Tween-20 (TBST) solution. Primary antibodies against VEGF-C (cat. no. 22601-1-AP; 1:1,000; ProteinTech Group, Inc.) and VEGFR-3 (cat. no. ab27278; 1:1,000; Abcam) were used. A GAPDH antibody (cat. no. ab9485; 1:3,000; Abcam) was also used to detect GAPDH as an internal reference. The membrane was incubated overnight with the primary antibodies at 4°C[19]. After washing the film with TBS-0.1% and Tween-20 (TBST) three times, the film was incubated with the secondary anti-body (Alexa Fluor® 568; cat. no. ab175473; 1:5,000; Abcam) at room temperature for 1 h. TBST was used to wash the films again three times, after which the signals were developed and visualized using an ECL reagen (Thermo Fisher Scientific, Inc.). A CanoScan Lide 120 scanner (Canon, Inc.) was used to scan the film for densitometric analysis. Densitometric analysis was performed using ImageJ 1.48 (National Institutes of Health).

Lymphangiogenesis experiments

PC cells stably transfected with siBANCR or siNC were digested and HLEC cells were added to establish a mixed cell suspension. In order to make subsequent co-culture HLEC cells and PANC-1 cells easily distinguishable under the fluorescence microscope, we fluorescently labeled HLEC cells with Dil.
the day before co-culture. DiI (cell membrane red fluorescent probe) is a lipophilic membrane dye that can diffuse laterally into the cell membrane and gradually stain the entire cell membrane. The mixed cell suspension (PANC-1:HLEC, 1:1; 7.5x10³ cells/well) was seeded on a Matrigel basement membrane (BD Biosciences) coating in 96-well plates at 100 µl/well, and the cells were cultured under the aforementioned conditions. The formation of microlymphatic vessels was observed after culturing for 12 h.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v8.0.1 (GraphPad Software, Inc.). Continuous variables were presented as the mean ± SD of three independent experiments, and the difference between two groups was analyzed using unpaired Student's t-test. The categorical data was analyzed using χ² test. Mann-Whitney U test was used to evaluate significant differences for the RT-qPCR data. Spearman's correlation analysis was used for correlation analysis. P < 0.05 was considered to indicate a statistically significant difference.

Results

BANCR upregulation in PC tissues, which is linked to lymph node metastasis

The expression levels of BANCR in tumor and adjacent normal tissues of 36 patients with PC were measured by qRT-PCR. Detailed information for these patients is listed in Table 1. Data show that BANCR level was higher in PC tissues than corresponding healthy tissues (2.644 ± 0.24 vs. 1.252 ± 0.136, t = 5.045, P < 0.0001) (Fig. 1A). As shown in Fig. 1B, the expression level of BANCR was significantly higher in the lymph node metastasis group compared with in the non-lymph node metastasis group. A statistical difference in BANCR level was present between tissue samples with no known metastasis (n = 16) and tissue samples with positive lymph node metastasis (n = 20). The abovementioned data indicate that BANCR expression was upregulated in PC, especially in cancerous tissue samples with positive lymph node metastasis.

MLVD in PC intra-tumoral tissues and its association with lymph node metastasis and BANCR expression

Given that lymphangiogenesis serves an important role in the process of lymph node metastasis, the lymphatic marker D2-40 was used to stain lymphatic vessels and MLVD was analyzed. Immunofluorescence staining revealed that the staining for D2-40 + cells was mainly located in the cytoplasm of lymphatic endothelial cells and was presented as a red color (Fig. 2A). As shown in Fig. 2B by immunofluorescence, the MLVD of peritumoral tissues in 20 cases with positive lymph node metastasis was 20.02 ± 6.219, which was significantly higher than the 16 cases without lymph node metastasis (13.02 ± 5.251). Spearman's correlation analysis demonstrated a positive correlation between
BANCR expression and MLVD in PC tissues (Fig. 2C). This result indicates that upregulation of BANCR can promote tumoral MLVD and lymphangiogenesis. As mentioned above, there is significant correlation between MLVD and lymph node metastasis. Therefore, it is possible that BANCR promotes PC lymph node metastasis through facilitating lymphangiogenesis.

**Depletion of BANCR suppresses the PC lymphangiogenesis in vitro**

**BANCR promotes PC lymphangiogenesis via VEGF-C/VEGFR-3**

Tumor lymphangiogenesis, largely driven by VEGF-C/VEGFR3 signaling axis, is important for lymphatic metastasis. To investigate whether BANCR regulates VEGF-C/VEGFR-3 to affect PC lymphangiogenesis, we silenced BANCR in PANC-1 cells by transfection with siBANCR. Furthermore, western blot was used to detect the protein expression of VEGF-C and VEGFR-3 (Fig. 4A). The full-length blots are presented in Supplementary Fig. 1. The results showed that down-regulation of BANCR can suppress VEGF-C protein expressions significantly (Fig. 4B: 1.585 ± 0.28 vs. 0.769 ± 0.075, t = 2.819, P = 0.048). As discussed above, siBANCR could inhibit lymphangiogenesis, however, it could be reversed by up-regulating VEGF-C expression. Herein, the magnification inverted fluorescence microscope showed that the MLVD was significantly higher in the si-BANCR/VEGF-C group compared to the si-BANCR/NC group (12.5 ± 1.336 vs. 5.625 ± 0.944, t = 4.202, P = 0.0009) (Fig. 4C-D).

**Discussion**

Increasing evidence suggests that lncRNAs are involved in various pathways. LncRNAs can play an oncogenic or a tumor suppressive role. For example, Kong et al found that lncRNA-CDC6 could function as ceRNA and promote the proliferation and metastasis of breast cancer cells[20]. LncRNA MALAT1 can promote tumorigenesis through Wnt/β-catenin pathway, EMT, PI3K/AKT pathway, ERK/MAPK pathway and angiogenesis[21]. On the other hand, lncRNA MEG3 could inhibit the proliferation and metastasis of gastric cancer[22]. As a result, lncRNAs play a crucial part in tumorigenesis, progression, and metastasis. The long noncoding RNA BRAF-activated noncoding RNA (BANCR), 693-bp in length and located on chromosome 9, was found in 2012 by Flockhart et al[23]. Large amounts of studies had shown that BANCR dysregulated in various types of malignant diseases and involved in occurrence and development of tumors such as thyroid cancer[24], esophageal squamous cell carcinoma[25] and hepatocellular carcinoma[26]. Wu et al discovered that BANCR expression was positively associated with tumor stage and metastasis in PC, and BANCR may promote pancreatic cancer tumorigenesis through miR-195-5p/Wnt/β-catenin axis [27]. It is established that lymph node metastasis is an important factor affecting the prognosis of pancreatic cancer patients, nevertheless, it remains unclear whether BANCR play a role in PC lymph node metastasis. In our study, we found that BANCR is highly expressed in pancreatic cancer and significantly associated with MLVD and lymph node metastasis. Although the detailed molecular
mechanism still remains to be elucidated, evidently IncRNA-mediated cell signaling is an important contributing factor.

Vascular endothelial growth factor C (VEGF-C) is a member of the vascular endothelial growth factor family, and previous studies have demonstrated that VEGF-C participates in the lymphangiogenesis of a variety of malignant tumors, resulting in the lymph node metastasis of tumors and thus affecting prognosis [9–12]. In this study, we found that down-regulation of BANCR can suppress VEGF-C protein expressions significantly in PC cells *in vitro*, which further suggests that BANCR might be involved in the regulation of the VEGF-C/VEGFR-3 signaling pathway. Meanwhile, immunofluorescence staining was used to determine MLVD, and the results show that MLVD in PC tissues with lymph node metastasis was higher than that in PC tissues without lymph node metastasis, suggesting that lymphangiogenesis may play a key role in PC lymph node metastasis.

Furthermore, we found that overexpression of VEGF-C could counteract the inhibitory effect of siBANCR on lymphangiogenesis and MLVD has rebounded through Lymphangiogenesis experiments, suggesting that BANCR may promote lymphangiogenesis in PC by regulating the VEGF-C/VEGFR-3 signaling pathway, thus promoting lymph node metastasis in PC.

**Conclusion**

BANCR may play an important role in lymphangiogenesis and lymph node metastasis of PC by regulating the VEGF-C /VEGFR-3 signaling pathway, and anti-lymphangiogenesis therapy targeting this process is expected to be an effective way to prevent lymph node metastasis of PC, thus significantly improving the prognosis of patient with PC.

**Declarations**

**Institutional Review Board Statement:** This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Institutional Research Ethics Committee on Genetic Analysis at Beijing Luhe Hospital Capital Medical University (approval no. 2022-LHKY-030-02).

**Informed Consent Statement:** The institution's Ethics and Research Committees of Beijing Luhe Hospital Capital Medical University approved the study. All patients signed informed consent for participation in the study, which was conducted according to the Declaration of Helsinki.

**Consent for publication:** All authors have read and agreed to the published version of the manuscript.

**Data Availability Statement:** All data and materials are available in the main text.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Funding:** This study was supported by grants from the Beijing Natural Science Foundation (Shaolong Hao, funding number: 7234377), the Capital Health Development Research Program (Wei Han, funding
number:2022-2-7081), the Science and Technology Program of Tongzhou (Wei Han, funding number: KJ2022CX016).


**References**


Figures
Figure 1

BANCR was overexpressed in PC, especially in the lymph node metastasis group. BANCR expression was analyzed by qRT-PCR in (A) PC tissues (n=36) and normal pancreatic tissues (n=36), and (B) LNM (n=20) and no LNM (n=16) groups. *P 0.005. PC, pancreatic cancer; LNM, lymph node metastasis.

Figure 2

MLVD was elevated in PC tissues with LNM and positively correlated with the expression level of BANCR. (A) D2-40 stains lymphatic endothelial cells red in PC tissues. (B) The MLVD of the peritumoral tissues in the lymph node metastasis group was significantly higher than the group without lymph node metastasis. (C) The peritumoral MLVD was positively correlated with BANCR expression. *P<0.05. MLVD, microlymphatic vessel density.
Depletion of BANCR inhibits PC lymphangiogenesis in vitro. (A) HLEC was stained red and PANC-1 was stained green by immunofluorescence. The number of lymphatic vessels in the siBANCR group in the field of view was significantly lower than that in the siNC group. (B) After calculation, the MLVD reduced after knocking down BANCR compared with the normal control group. *P<0.05. HLEC, human lymphatic endothelial cells.
Figure 4

BANCR promotes PC lymphangiogenesis through VEGF-C/VEGFR-3. (A-B) VEGF-C/VEGFR-3 expressions measured by western blot after transfection with siBANCR compared with vector control. After down-regulation of BANCR, the expressions of VEGF-C/VEGFR-3 were also decreased. (C-D) Immunofluorescence staining was performed to evaluated the MLVD. Overexpression of VEGF-C could counteract the inhibitory effect of siBANCR on lymphangiogenesis, and MLVD has rebounded. *$P<0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.docx