Long non-coding RNA BANCR promotes proliferation, invasion and migration in esophageal squamous cell carcinoma cells via the Raf/MEK/ERK signaling pathway

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Abstract. Esophageal squamous cell carcinoma (ESCC) is a major histological type of esophageal cancer, identified as a leading cause of tumor-associated death worldwide. In addition, long non-coding RNA (lncRNA) BRAF-activated non-coding RNA (BANCR) expression is increased in the plasma of patients with ESCC, which can be reversed by tumor resection. Thus, the aim of the present study was to investigate the underlying mechanism of BANCR in ESCC progression. The relative mRNA expression of BANCR was determined via reverse transcription-quantitative PCR. The cell behaviors of Eca-109 cells were detected using Cell Counting Kit-8, colony formation, wound healing and Transwell chamber assays. Finally, the expression levels of proteins involved in the Raf/MEK/ERK signaling pathway and cell metastasis were analyzed with western blotting. The results revealed that lncRNA BANCR was highly expressed in ESCC cells compared with in normal esophageal cells. BANCR overexpression enhanced proliferation, migration and invasion of ESCC cells, and BANCR silencing exerted opposite effects. Moreover, BANCR overexpression induced activation of the Raf/MEK/ERK signaling pathway in ESCC cells. Notably, U0126, a specific MEK inhibitor, decreased MEK and ERK expression, and blocked the promotive effects of BANCR overexpression on the proliferation, migration and invasion of ESCC cells. Overall, lncRNA BANCR facilitated the proliferation, migration and invasion of ESCC cells via the Raf/MEK/ERK signaling pathway. Thus, lncRNA BANCR may be a promising target for inhibiting ESCC growth and metastasis.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is defined as the major histological type of esophageal cancer (1,2) and as the seventh leading cause of tumor-associated death globally (3). ESCC is one of the most malignant and aggressive tumors worldwide, with ~410,000 deaths occurring annually (4). According to previous findings, the 3-year overall survival rate in certain areas of China was only ~30% after radiotherapy alone, whereas the recurrence rate was as high as 60-80% due to the existence of certain risk factors (5,6). Despite progress in diagnostic technologies and therapeutics, including chemoradiotherapy and surgery, most patients are diagnosed at the advanced stage and the prognosis of patients with ESCC remains unsatisfactory owing to recurrence and metastasis (7-9), with an overall 5-year survival rate <30% (10). Previous studies have reported that several important parameters, such as life style as well as environmental and genetic factors, contribute to ESCC tumorigenesis (11,12); however, the underlying mechanisms triggering ESCC progression have not been fully defined.

Long non-coding RNAs (lncRNAs) are a class of non-protein coding transcripts >200 nucleotides in length (13). lncRNAs serve a crucial role in a variety of processes in diseases, and their alterations are recognized as drivers or suppressors for cancer progression, including ESCC (14,15). Rapidly accumulating evidence has suggested that lncRNA BRAF-activated non-coding RNA (BANCR) has crucial roles in the progression of various malignancies, including colorectal cancer (16), papillary thyroid carcinoma (17) and endometrial cancer (18), via promoting proliferation, migration and invasion of cancer cells. Moreover, a previous study has suggested that BANCR is highly expressed in tumor tissues compared with in non-cancerous tissues of patients with ESCC and its expression level was positively associated with tumor differentiation, metastasis and tumor stage (1). Moreover, increased

BANCR expression was observed in the plasma of patients with ESCC, which was then decreased following tumor resection (5). Additionally, BANCR has been revealed to exert regulative effects on the migration and invasion of ESCC cells via the Wnt/β-catenin signaling pathway (19). BANCR expression was also negatively associated with the survival rate of patients with ESCC, suggesting BANCR as a novel tumor biomarker for the early detection of ESCC progression (5). It has been reported that BANCR mediates ESCC progression by regulating insulin-like growth factor 1 receptor expression via microRNA-338-3p (20). However, the underlying mechanism of BANCR in ESCC pathogenesis has not been fully understood. Thus, the aim of the present study was to further investigate the molecular mechanism of BANCR in ESCC progression.

The aim of the present study was to investigate the role of BANCR in the pathogenesis of ESCC, and study its potential molecular mechanism. Thus, ESCC cells were transfected with overexpression plasmid and short hairpin RNAs (shRNAs) to regulate BANCR expression in order to determine whether BANCR could regulate the proliferative, migratory and invasive capabilities of ESCC cells.

Materials and methods

Cell culture and transfection. ESCC cell lines, including Eca-109, KYSE30, KYSE150 and TE-1, together with the normal esophageal Het-1A cell line, were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cell lines were cultured in RPMI-1640 medium with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., USA) at 37˚C with 5% CO_2. The MEK inhibitor, U0126 (Selleck Chemicals), was diluted using DMSO to a working concentration of 20 μM. Eca-109 cells were treated with U0126 for 24 h at 37˚C.

Eca-109 cells at the logarithmic growth phase were transfected with 100 nM BANCR short hairpin (sh)RNAs or plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 6 h and incubated for another 24 h before subsequent experimentation, according to the manufacturer's instructions. The shRNAs were obtained from Shanghai GenePharma Co., Ltd. The shRNA sequences were as follows: sh-BANCR-1 forward, 5'-GGACUCCAUGGCAAACGUUTT-3' and reverse, 5'-AACGUUUGCCAUGGAGUCCTT-3'; sh-BANCR-2 forward,5'-GGAGUGGCGACUAUAGCAATT-3'andreverse, 5'-UUGCUAUAGUCGCCACUCCTT-3'; and shRNA-NC (scrambled)forward,5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. The full-length sequences of BANCR (NR_047671) were inserted into pcDNA3.1 vectors (Invitrogen; Thermo Fisher Scientific, Inc.) to construct BANCR overexpression plasmid. The Eca-109 cells transfected with BANCR overexpression plasmid were classified into the BANCR group, and empty pcDNA3.1 vectors were used as the control group.

Reverse transcription-quantitative PCR (RT-qPCR). After transfection, total RNA was isolated from Eca-109 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed using the PrimeScript RT kit (Takara Bio, Inc.) to synthesize the cDNA. The prepared reaction mixture was incubated at 37˚C for 60 min after brief centrifugation (14,000 x g, 10 min), followed by incubation at 85˚C for 5 min for RT. Relative expression levels were determined using quantitative primers and SYBR® Premix Ex Taq™ reagent (Takara Bio, Inc.). The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 5 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. The results were calculated using the $2^{-\Delta\Delta Cq}$ method (21). The primers used for qPCR were: BANCR forward, 5'-ACAGGACTCCATGGCAAACG-3' and reverse, 5'-ATGAAGAAAGCCTGGTGCAGT-3'; and GAPDH (internal reference) forward, 5'-GGTCTCCTCTGACTTCAACA-3' and reverse, 5'-AGCCAAATTCGTTGTCATAC-3'.

Cell viability assay. The survival rate of Eca-109 cells in each group was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. Briefly, Eca-109 cells ($2x10^3$ cells/well) were seeded into 96-well plates and transfected with BANCR overexpression plasmids or sh-BANCR, as aforementioned. After incubation for 24, 48 or 72 h, the CCK-8 reagent (10 μl/well) was added to Eca-109 cells for another 2 h. Absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay plate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. After transfection, Eca-109 cells ($1x10^3$/well) were seeded into 6-well plates. The cells were cultured for 14 days at 37˚C without disturbance during the period of incubation to form the cell clusters. Subsequently, cell colonies were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet solution for 30 min at room temperature before being imaged, and colonies with diameters >0.5 mm were imaged and counted using a digital camera (Nikon Corporation).

Wound healing assay. Eca-109 cells ($2x10^5$ cells/well) were seeded in 6-well plates and transfected until cell confluency reached ~80-90% on the following day. A straight linear wound was gently induced using 200-μl sterile pipette tips, and the cells were cultured in RPMI-1640 culture medium with 1% FBS at 37˚C for 24 h. Subsequently, the wound was observed and photographed under a fluorescence microscope (Olympus IX53; Olympus Corporation) at five random fields (magnification, x200). The average distance between cells was calculated relative to the control by using ImageJ software (version 1.48; National Institutes of Health) to determine the wound closure rate.

Transwell chamber assay. After transfection, Eca-109 cells ($1x10^5$ cells/well) were resuspended in FBS-free RPMI-1640 medium and seeded into the upper chambers of Transwell plates precoated with Matrigel at 37˚C for 4 h. The lower chamber was filled with 500 μl culture RPMI-1640 medium containing 10% FBS, and then maintained at 37˚C in 5% CO_2 for 24 h. After incubation, the membrane was fixed with 4% paraformaldehyde for 30 min at 37˚C. The remaining cells on the upper surface of the filter membrane were removed gently, while cells at the lower surface of the membrane were stained

with 0.1% crystal violet for 30 min at room temperature. The cells were captured in five random fields with a fluorescence microscope (Olympus IX53; Olympus Corporation) at x200 magnification.

Western blotting. Proteins were extracted from transfected Eca-109 cells using RIPA lysis buffer (Takara Bio, Inc.) containing protease inhibitors according to the manufacturer's instructions, and then quantified using a BCA assay kit. Total proteins (20 μg/lane) were loaded and separated via SDS-PAGE on 10% gel, and then separated proteins were transferred onto PVDF membranes. After membranes were blocked with 5% skimmed milk for 2 h at room temperature, primary antibodies against the following proteins were used overnight at 4˚C: Matrix metalloproteinase 2 (MMP2; 1:1,000; cat. no. ab181286; Abcam), MMP9 (1:1,000; cat. no. ab76003; Abcam), phosphorylated (p)-c-Raf (1:1,000; cat. no. 9423; Cell Signaling Technology, Inc.), c-Raf (1:1,000; cat. no. 9422; Cell Signaling Technology, Inc.), p-MEK1/2 (1:1,000; cat. no. 9121; Cell Signaling Technology, Inc.), MEK1/2 (1:1,000; cat. no. 9122; Cell Signaling Technology, Inc.), p-ERK1/2 (1:500; cat. no. 9101; Cell Signaling Technology, Inc.), ERK1/2 (1:1,000; cat. no. 9102; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.). After washing with PBS, the membranes were incubated with HRP-conjugated secondary antibodies (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 2 h, and then visualized using ECL chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). Finally, the gray values of bands were detected using ImageJ software (version 1.48; National Institutes of Health) and normalized to GAPDH.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 (IBM Corp.), and data are presented as the mean ± SEM from three independent experiments. Comparisons of multiple groups were analyzed with one-way ANOVA followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LncRNA BANCR is highly expressed in ESCC cells compared with in normal esophageal cells. To assess BANCR expression in ESCC cells, RT-qPCR was applied in the four ESCC cell lines, Eca-109, KYSE30, KYSE150 and TE-1, and one normal esophageal cell line, Het-1A. As shown in Fig. 1, BANCR expression was significantly increased in ESCC cells compared with in Het-1A cells. Moreover, the Eca-109 cell line exhibited the highest level of BANCR expression and was therefore used for subsequent experiments.

lncRNA BANCR overexpression promotes the proliferation, migration and invasion of ESCC cells, and BANCR silencing induces the opposite effects. To evaluate the efficiency of BANCR plasmids in Eca-109 cells, RT-qPCR was performed. The results revealed that cells transfected with BANCR had a significantly higher BANCR expression compared with control or vector cells (Fig. 2A). Subsequently, CCK-8 and colony formation assays were performed to determine the viability and proliferative ability, respectively. The findings indicated that BANCR overexpression significantly enhanced the viability and proliferative ability of Eca-109 cells (Fig. 2B and C), suggesting the promoting effect of BANCR overexpression on the proliferation of ESCC cells. Additionally, wound healing and Transwell chamber assays were performed to detect the migratory and invasive abilities, respectively. The data revealed that the migratory and invasive capacities were significantly elevated in Eca-109 cells from the BANCR group compared with in those of the control and vector groups (Fig. 3A-D). Furthermore, western blot analysis was performed to determine the expression levels of MMP2 and MMP9, two markers associated with tumor invasion and metastasis. As shown in Fig. 3E, BANCR overexpression led to a significant upregulation of MMP2 and MMP9 expression in Eca-109 cells. These data suggested that BANCR overexpression enhanced the proliferation, migration and invasion of ESCC cells.

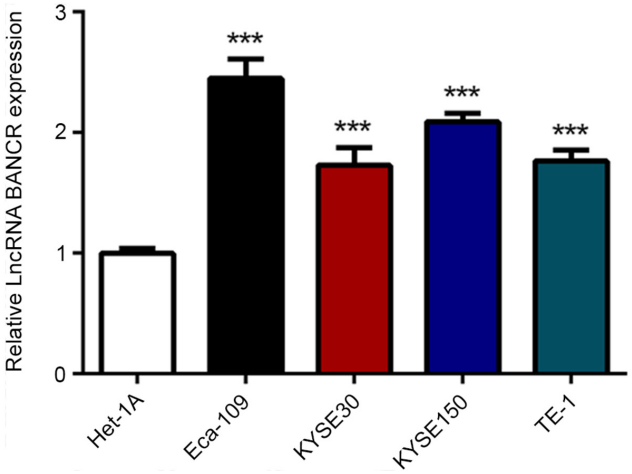

Figure 1. lncRNA BANCR is highly expressed in esophageal squamous cell carcinoma cells. Relative BANCR expression was quantified by reverse transcription-quantitative PCR. Data are presented as the mean ± SEM from three independent experiments. ***$P<0.001$ vs. Het-1A. lncRNA BANCR, long non-coding RNA BRAF-activated non-coding RNA.

Role of BANCR in the proliferation and migration of ESCC cells. To confirm the role of BANCR in the proliferation and migration of ESCC cells, the behaviors of Eca-109 cells were analyzed under the condition of BANCR silencing. sh-BANCR was established to downregulate BANCR expression. RT-qPCR demonstrated that a significantly lower BANCR expression was observed in cells from the sh-BANCR-2 and shRNA-BANCR-1 groups, with a lower BANCR expression in the sh-BANCR-2 group (Fig. 4A). Therefore, shRNA-BANCR-2 was employed for further study. In contrast to BANCR overexpression, BANCR silencing significantly suppressed the viability and proliferative ability of Eca-109 cells (Fig. 4B and C), as well as the migratory and invasive abilities (Fig. 5A-D). Additionally, BANCR-knockdown resulted in a significant decrease in MMP2 and MMP9 expression (Fig. 5E). These data indicated that BANCR silencing inhibited the proliferation, migration and invasion of ESCC cells.

BANCR overexpression induces activation of the Raf/MEK/ERK signaling pathway in ESCC cells. To

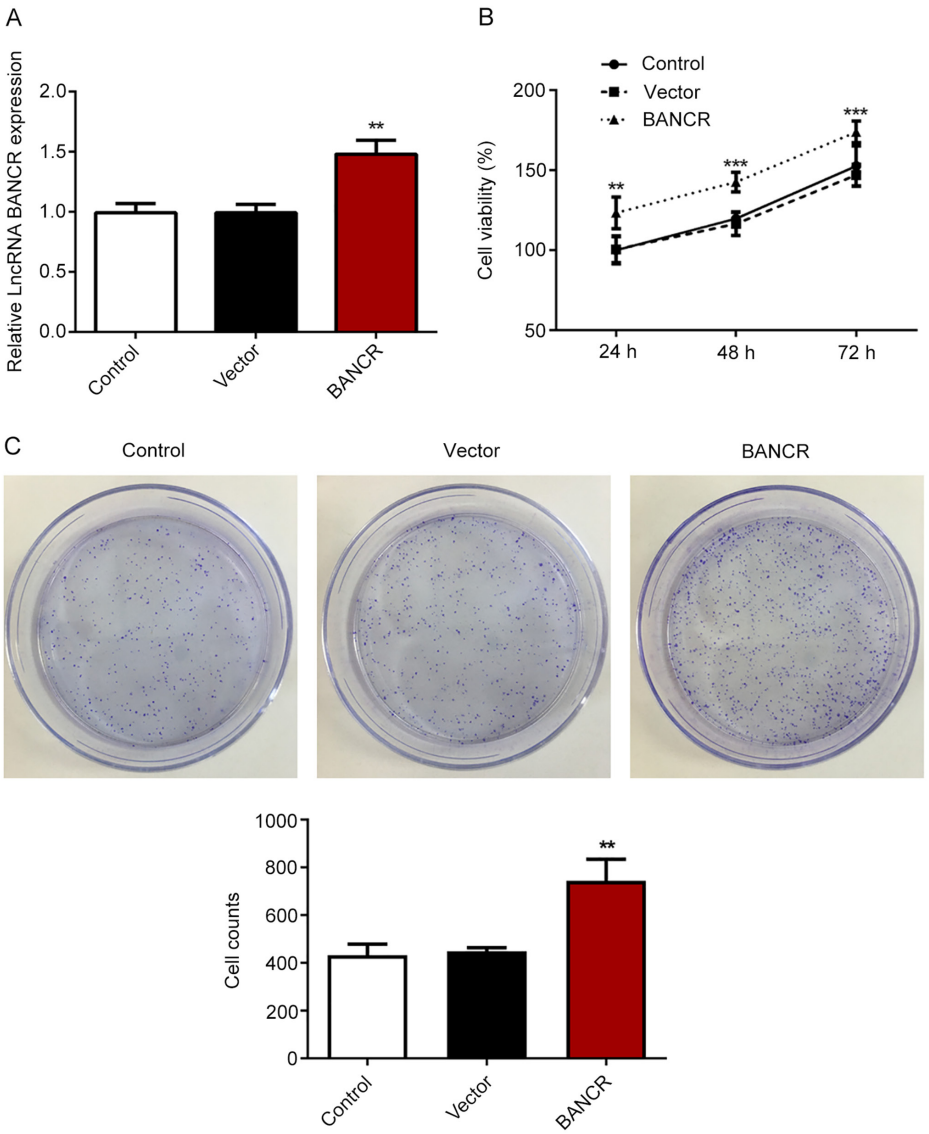

Figure 2. lncRNA BANCR overexpression increases the viability and proliferative capability of esophageal squamous cell carcinoma cells. (A) Relative BANCR expression was quantified by reverse transcription-quantitative PCR in Eca-109 cells transfected with or without BANCR overexpression plasmid. (B) Viability of Eca-109 cells was evaluated using the Cell Counting Kit-8 assay. (C) Cell proliferation was assessed via colony formation assay. Data are presented as the mean ± SEM from three independent experiments. **P<0.01 and ***P<0.001 vs. vector group. lncRNA BANCR, long non-coding RNA BRAF-activated non-coding RNA.

investigate the underlying mechanism of BANCR in ESCC progression, the phosphorylation levels of c-Raf, MEK1/2 and ERK1/2 were detected using western blot analysis. The results revealed that BANCR overexpression significantly increased the levels of p-c-Raf, p-MEK1/2 and p-ERK1/2 (Fig. 6A), suggesting that the Raf/MEK/ERK signaling pathway may be involved in the role of BANCR in the proliferation, migration and invasion of ESCC cells.

BANCR activates the proliferation, migration and invasion of ESCC cells via the Raf/MEK/ERK signaling pathway. To further investigate the mechanism of BANCR/Raf/MEK/ERK signaling in ESCC progression, the specific MEK inhibitor (U0126) was employed. Western blot analysis revealed that U0126 treatment significantly attenuated the increased levels of p-MEK1/2 and p-ERK1/2 induced by BANCR overexpression (Fig. 6B). In addition, U0126 treatment significantly suppressed the viability and proliferative ability of Eca-109 cells transfected with BANCR (Fig. 7A and B). Furthermore, migration and invasion were significantly inhibited in the U0126+BANCR group compared with in the BANCR group (Fig. 7C-F), as well as MMP-2 and MMP-9 expression (Fig. 7G). These data revealed that U0126 treatment reversed the effects of BANCR overexpression on proliferation, migration and invasion of ESCC cells.

Discussion

At present, a variety of lncRNAs have been identified as master regulators of gene expression, and their alterations drive or impede cancer progression (22). Tumorigenesis regulated by lncRNAs is a complicated and multi-stage process, involving complex cell signaling pathways. Rapidly accumulating evidence has suggested that lncRNAs serve an important role in oncogenesis, representing a strategy of lncRNAs as biomarkers for the diagnosis, prognosis and therapy of various

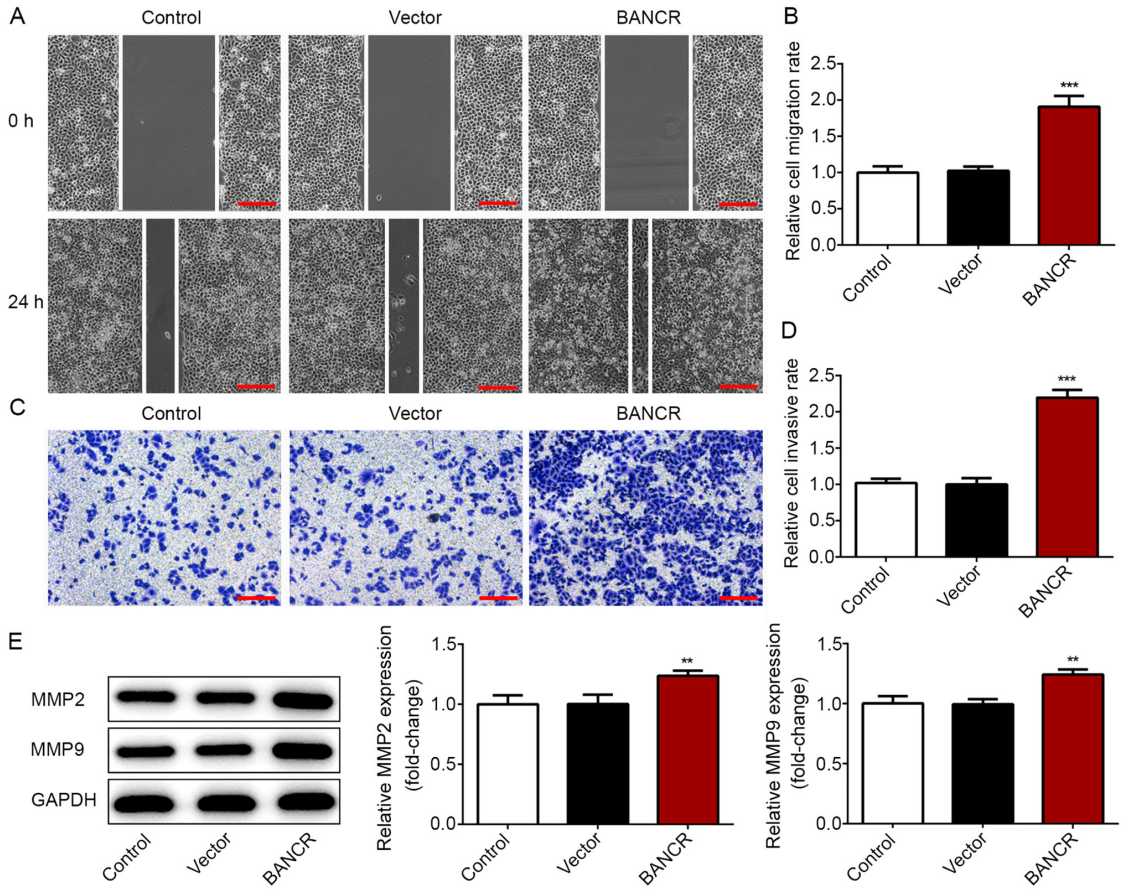

Figure 3. lncRNA BANCR overexpression promotes the migration and invasion of esophageal squamous cell carcinoma cells. (A and B) Migratory ability of Eca-109 cells transfected with or without BANCR overexpression plasmid was determined by wound healing assay (scale bar, 100 μm). (C and D) Invasive ability of Eca-109 cells transfected with or without BANCR overexpression plasmid was analyzed using Transwell chamber assay.(scale bar, 100 μm). (E) Protein expression levels of MMP2 and MMP9 were detected via western blot analysis in Eca-109 cells transfected with or without BANCR overexpression plasmid. Data are presented as the mean ± SEM from three independent experiments. **P<0.01 and ***P<0.001 vs. vector group. lncRNA BANCR, long non-coding RNA BRAF-activated non-coding RNA; MMP, matrix metalloproteinase.

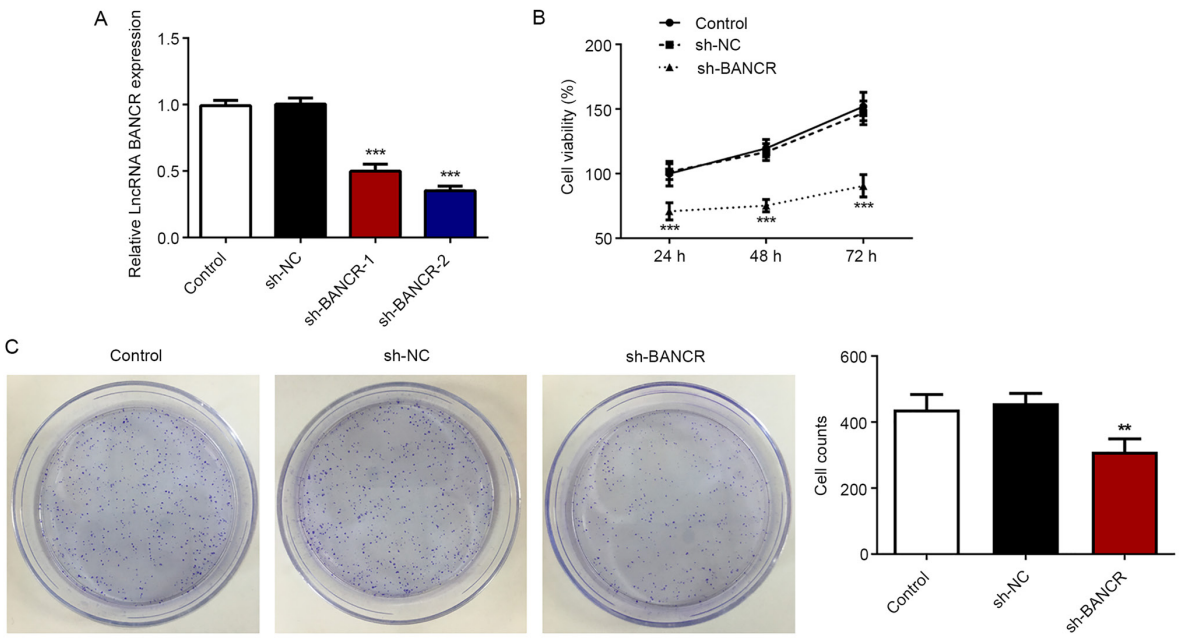

Figure 4. lncRNA BANCR downregulation decreases the viability and proliferative capability of esophageal squamous cell carcinoma cells. (A) Relative BANCR expression was quantified by reverse transcription-quantitative PCR in Eca-109 cells transfected with or without sh-BANCR. (B) Viability of Eca-109 cells was evaluated using the Cell Counting Kit-8 assay. (C) Cell proliferation was assessed by colony formation assay. Data are presented as the mean ± SEM from three independent experiments. **P<0.01 and ***P<0.001 vs. sh-NC group. lncRNA BANCR, long non-coding RNA BRAF-activated non-coding RNA; sh, short hairpin RNA; NC, negative control.

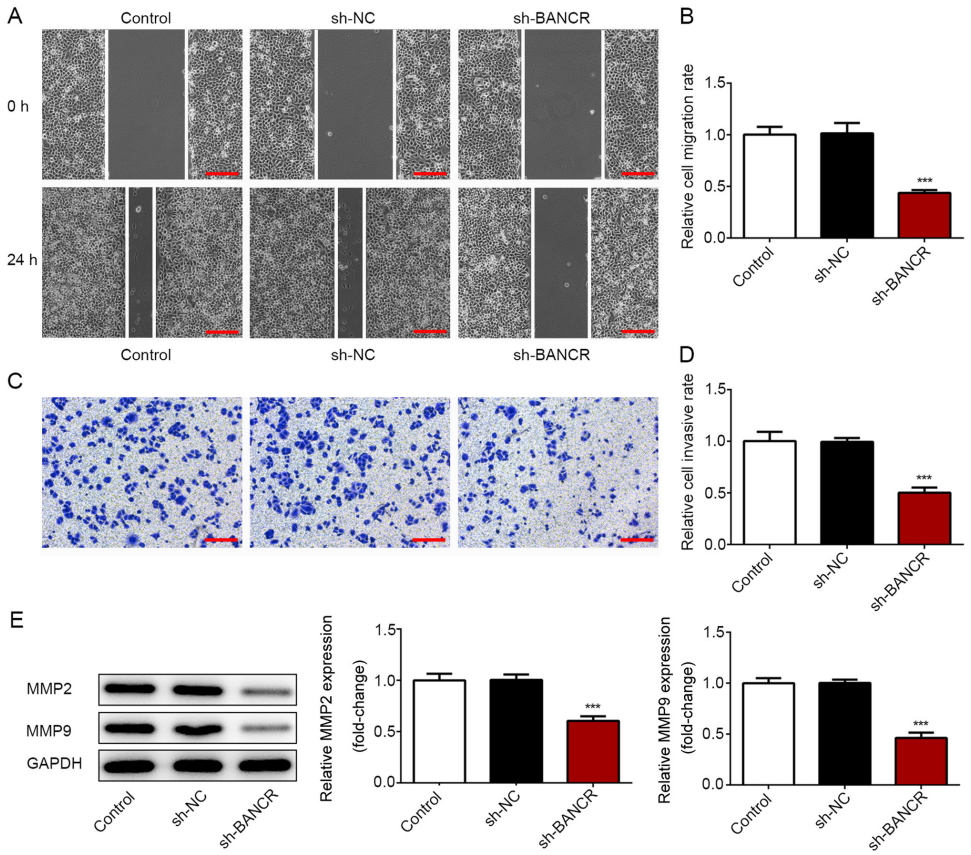

Figure 5. lncRNA BANCR silencing inhibits the migration and invasion of esophageal squamous cell carcinoma cells. (A and B) Migratory ability of Eca-109 cells transfected with or without sh-BANCR was determined by wound healing assay (scale bar, 100 μm). (C and D) Invasive ability of Eca-109 cells transfected with or without sh-BANCR was analyzed using Transwell chamber assay (scale bar, 100 μm). (E) Protein expression levels of MMP2 and MMP9 were detected via western blot analysis in Eca-109 cells transfected with or without sh-BANCR. Data are presented as the mean ± SEM from three independent experiments. ***P<0.001 vs. sh-NC group. lncRNA BANCR, long non-coding RNA BRAF-activated non-coding RNA; MMP, matrix metalloproteinase; sh, short hairpin RNA; NC, negative control.

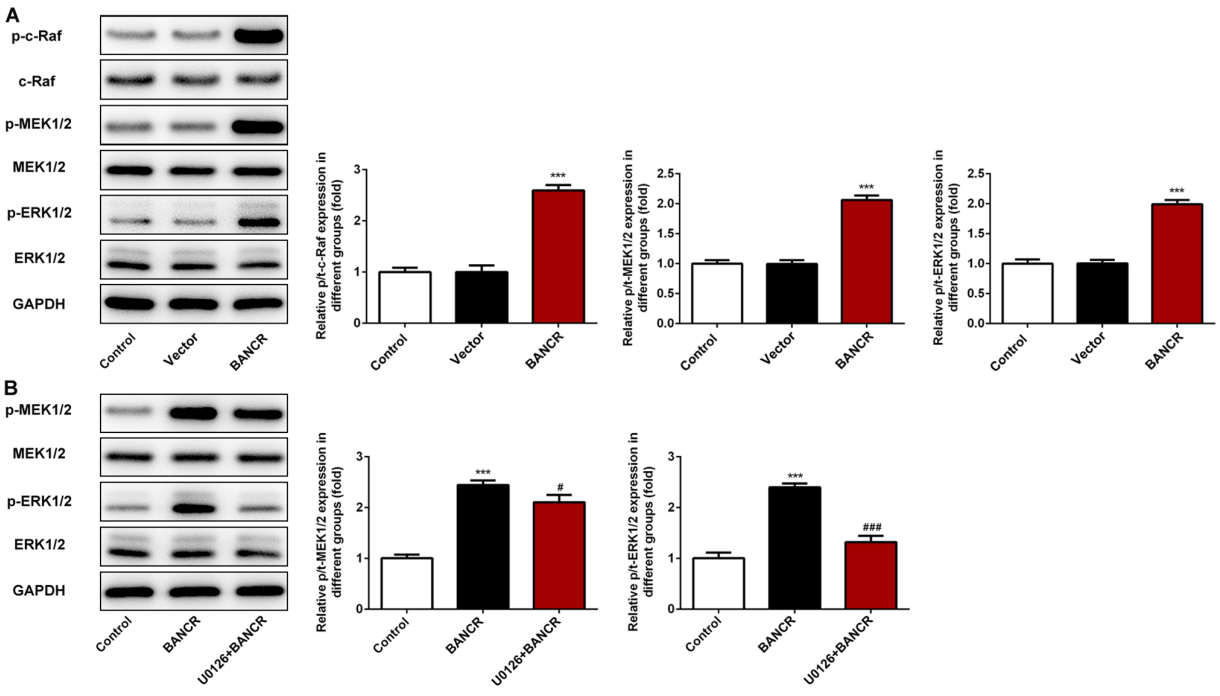

Figure 6. lncRNA BANCR overexpression induces activation of the Raf/MEK/ERK signaling pathway in esophageal squamous cell carcinoma cells. Protein expression levels were evaluated by western blotting in Eca-109 cells (A) transfected with or without BANCR overexpression plasmid, and (B) treated with U0126. Data are presented as the mean ± SEM from three independent experiments. ***P<0.001 vs. control group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. BANCR group. lncRNA BANCR, long non-coding RNA BRAF-activated non-coding RNA; p, phosphorylated; t, total.

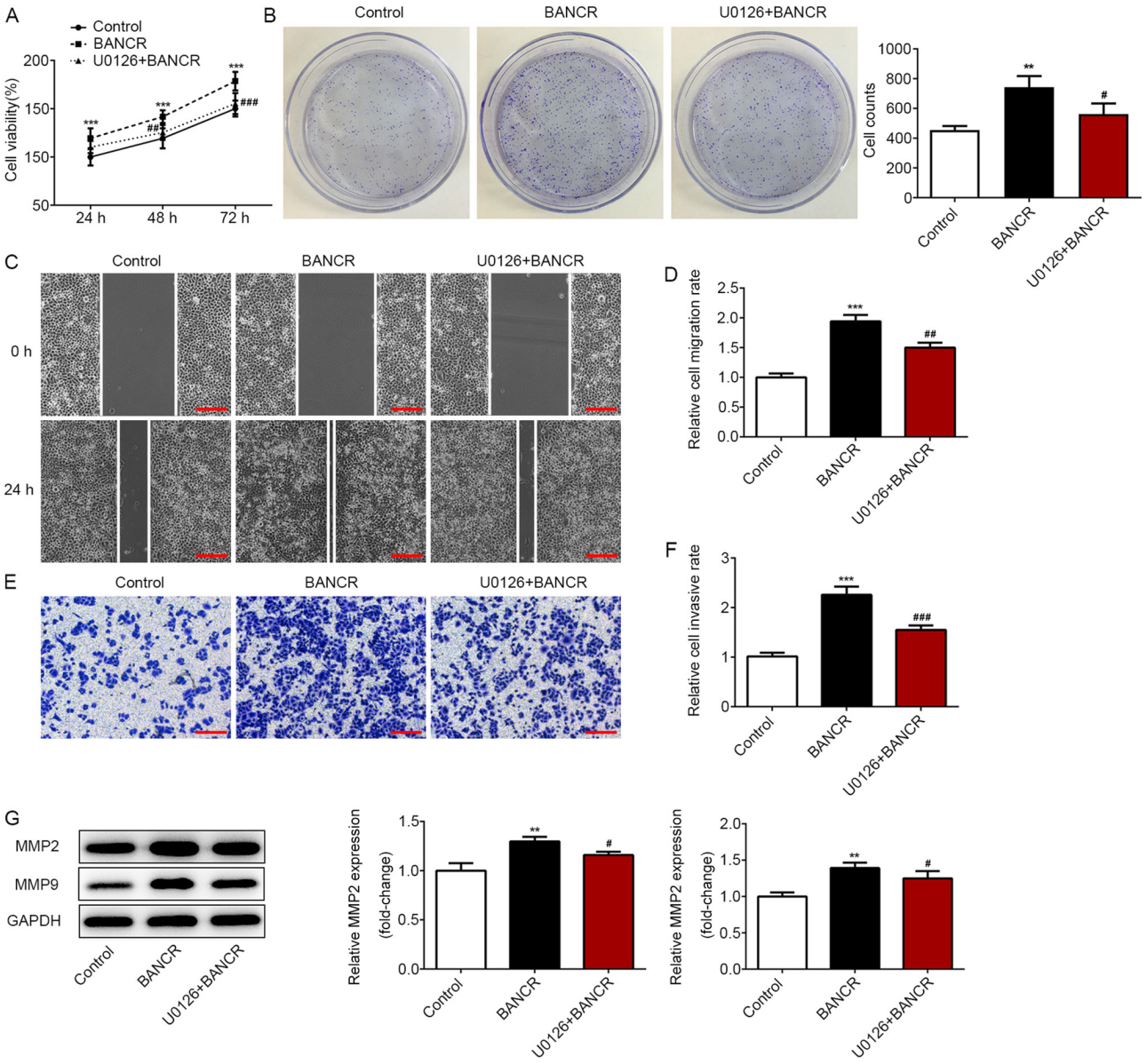

Figure 7. U0126 treatment reverses the impacts of BANCR overexpression on the proliferation, migration and invasion of esophageal squamous cell carcinoma cells. (A) Viability of Eca-109 cells was evaluated using the Cell Counting Kit-8 assay. (B) Cell proliferation was assessed by colony formation assay. (C and D) Migration of Eca-109 cells was determined by wound healing assay (scale bar, 100 μm). (E and F) Invasion of Eca-109 cells was analyzed using Transwell chamber assay (scale bar, 100 μm). (G) Protein expression levels of MMP2 and MMP9 were detected via western blotting in Eca-109 cells. Data are presented as the mean ± SEM from three independent experiments. **P<0.01 and ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. BANCR group. BANCR, BRAF-activated non-coding RNA; MMP, matrix metalloproteinase.

types of cancer, including head and neck cancer and thyroid cancer (23,24). Recently, increasing evidence has shown that lncRNA BANCR may be an oncogene or a tumor suppressor in various human malignancies, including colorectal (16), pancreatic (25), non-small cell lung (26) and ovarian cancer (27). Notably, BANCR expression is significantly increased in tumor tissues of patients with ESCC compared with in non-cancerous tissues, and its level is positively associated with tumor differentiation, metastasis and tumor stage (1). However, the molecular mechanism of BANCR in the pathogenesis of ESCC remains unexploited. Thus, it is of great importance to investigate the underlying mechanism of BANCR in ESCC progression.

Previously, BANCR expression has been observed to be upregulated in colorectal cancer tissues, and BANCR downregulation decreases cell proliferation and invasion, but activates apoptosis (16). Moreover, BANCR expression has been reported to be upregulated in retinoblastoma tissues and cell lines, and positively associated with tumor size and optic nerve invasion (28). Additionally, BANCR-knockdown significantly suppresses the proliferation, migration and invasion of retinoblastoma cells (28). In the present study, it was observed that BANCR expression was higher in ESCC cell lines than in normal esophageal cells. In addition, BANCR overexpression enhanced the proliferation, migration and invasion of ESCC cells, while BANCR silencing exhibited opposite effects, which was consistent with a previous study (28). The current findings indicated that BANC acted as an oncogene in ESCC progression.

Increasing evidence has revealed that the Raf/MEK/ERK signaling pathway is closely associated with tumorigenesis

in multiple types of malignant tumor, including colon and lung cancer (29-31). A previous study has revealed that diallyl disulfide treatment elevates the apoptotic rate by downregulating the RAF/MEK/ERK signaling pathway in esophageal carcinoma cells (32). Additionally, BANCR promotes the migration and invasion of cancer cells and induces epithelial-mesenchymal transition in thyroid cancer through the Raf/MEK/ERK signaling pathway (17). In the present study, BANCR overexpression induced activation of the Raf/MEK/ERK signaling pathway in ESCC cells, suggesting the participation of the Raf/MEK/ERK signaling pathway under BANCR in ESCC progression. In the current study, U0126, a specific MEK inhibitor, decreased MEK and ERK expression, and blocked the promotive effects of BANCR overexpression on the proliferation, migration and invasion of ESCC cells. Overall, the present results strongly suggested that lncRNA BANCR accelerated proliferation, migration and invasion of ESCC cells via the Raf/MEK/ERK signaling pathway.

In conclusion, the current findings provided evidence to demonstrate that BANCR expression was upregulated in ESCC cell lines. In addition, lncRNA BANCR overexpression promoted the proliferation, migration and invasion of ESCC cells, which was significantly reversed following BANCR downregulation. Thus, lncRNA BANCR may be a promising target for inhibiting ESCC cell migration.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XY and GY designed the experiment and drafted the manuscript. XY, MH and GY performed the experiments and analyzed the data. GY reviewed the manuscript. XY and GY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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