LncRNA GAS8-AS1 is a Novel Prognostic and Diagnostic Biomarker for Pancreatic Cancer

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Research

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Abstract

Background: LncRNA GAS8-AS1 inhibits thyroid carcinoma, but its function in other malignancies is unknown. The present study aimed to investigate the involvement of GAS8-AS1 in pancreatic cancer (PC).

Methods: The present study included 68 PC patients (38 males and 30 females, 42- 66 years, 52.1±4.5) and 62 healthy volunteers (28 males and 24 females, 43- 67 years, 52.3 ±4.9). Real-time quantitative PCR, transient cell transfection and in vitro cell migration and invasion assay were applied for the research. In the present study we found that plasma GAS8-AS1 was lower in PC patients than in healthy controls. Downregulation of plasma GAS8-AS1 distinguished early stage PC patients from healthy controls.

Results: Patients with low plasma levels of GAS8-AS1 showed significantly lower 5-year overall survival rate. Plasma levels of miR-1179 were also significantly lower in PC patients than in healthy controls, and were positively correlated with plasma levels of GAS8-AS1 only in PC patients. GAS8-AS1 overexpression resulted in the upregulation of miR-1179. MiR-1179 overexpression also led to the overexpression of GAS8-AS1. Overexpression of both GAS8-AS1 and miR-1179 led to inhibited migration and invasion of PC cells.

Conclusions: Therefore, GAS8-AS1 may promote PC by positively interacting with miR-1179.

Introduction

Pancreatic cancer (PC) is probably the most deadly malignancy and only less than 5% of PC patients can live long than 5 years after initial diagnosis due to the lack of radical treatment and high postoperative recurrence rate (1). Due to the site of origin, PC is usually diagnosed at advanced stages because no serious clinical signs and symptoms can be observed during early stages (2). Chemotherapy is considered as the most appropriate treatment for most PC patients, while the development of chemo-resistance is common and outcomes are poor (3, 4). In addition, incidence of PC also shows an increasing trend, making PC a major burden on public health (5).

Genetic factors are the key players in the development and progression of PC, while the limited number of oncogenes and tumor suppressors identified so far may fail to explain the complex mechanism of the pathogenesis of PC (6, 7). Non-coding RNAs (ncRNAs) lack protein-coding capacity but may participate in cancer biology by regulating expression (8). Long non-coding RNAs (> 200 nt, lncRNAs) and microRNAs (about 22 nt, miRs) have been proved as critical determinants in human cancers (9, 10). LncRNA GAS8-AS1 inhibits thyroid carcinoma (11), but its function in other malignancies is unknown. We performed deep sequencing and found that GAS8-AS1 was downregulated in PC and positively correlated with miR-1179, which inhibits PC (12). The present study was therefore carried out to explore the potential interaction between GAS8-AS1 and miR-1179 in PC.

Materials And Methods

The present study included 68 PC patients (38 males and 30 females, 42–66 years, 52.1 ± 4.5) and 62 healthy volunteers (28 males and 24 females, 43–67 years, 52.3 ± 4.9). Inclusion criteria:1) newly diagnosed PC patients; 2) patients with no history of malignancy. Exclusion criteria:1) patients complicated with other clinical disorders; 2) patients who were treated before admission. All the participants were selected in West China Hospital, Sichuan University. Based on the staging criteria established by AJCC, there were 16, 15, 26, and 11 cases as stage I, II, III and IV, respectively. All healthy volunteers and patients signed informed consent and this study was approved by Ethics Committee of West China Hospital, Sichuan University.

Specimens and cell lines

Blood was extracted from each patient and healthy control before therapy was initiated. Blood was kept in EDTA tubes for 10 min at room temperature, followed by centrifugation at 1200g for 15 min to separate plasma.

Our study included 2 PC cell lines Capan-2 and HPAF-II to perform all in vitro cell experiments. Cells of these two cell lines were purchased from ATCC (USA). The cell culture medium was McCoy's 5a Medium Modified (10% FBS). Cell culture conditions were: 37°C and 5% CO_2.

Follow-up

All patients were followed up for 5 years after admission to record their survival. Follow-up was performed every month through outpatient visit or telephone. Patients who were lost during follow-up or patient died of other diseases or accidence were not included in this study.

Real-time quantitative PCR (qRT-PCR)

RNAzol reagent (Sigma-Aldirch, USA) was used to extract total RNAs from plasma and in vitro cultivated cells. Total RNA samples were subjected to reverse transcription using SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). All PCR reaction systems were prepared using SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific, USA) with 18S rRNA as endogenous control to analyze the expression of GAS8-AS1.

PureLink miRNA Isolation Kit (Thermo Fisher Scientific) was used to extract miRNA samples. miRNA samples were subjected to reverse transcription using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific) was used to prepare PCR reaction systems with U6 endogenous control to analyze the expression of miR-1179.

All PCR reactions were performed in triplicate manner and data were analyzed by $2^{-\Delta\Delta CT}$ method.

Transient cell transfection

GAS8-AS1-expression vectors were constructed by Sangon (Shanghai, China). miR-1179 mimic and miRNA negative control were bought from Sigma-Aldrich (USA). All transient cell transfections were

performed using lipofectamine 2000 (Invitrogen, USA) in strict accordance with manufacturer's instructions. Subsequent experiments were performed at 24h after transfections. Two controls, including control (C, cells without transfection) and negative control (NC, cells transfected with empty vectors or miRNA negative control) were included in this experiment.

In vitro cell migration and invasion assay

Capan-2 and HPAF-II cells of different transfection groups were harvested at 24h after transfection to prepare single cell suspensions at a cell density of 3×10^4/ ml using serum-free McCoy's 5a Medium Modified. Cells suspensions were transferred to the upper chamber with 0.1 ml per well, while the lower chamber was filled with McCoy's 5a Medium Modified containing 20% FBS. To mimic the in vivo invading condition, upper chamber membranes were coated with 50 μg of Matrigel in culture medium at room temperature overnight. After 2 h, membranes of upper chamber were collected, cleaned and stained with 0.5% crystal violet (Sigma-Aldrich, USA) at room temperature for 20 min. Invading and migrating cells were counted under an optical microscope.

Statistical analysis

Three biological replicates were included in each experiment. Differences between PC patients and healthy controls were analyzed by unpaired t test. Differences among different cell transfection groups were analyzed by ANOVA (one-way) and Tukey test. Early diagnostic value of plasma GAS8-AS1 was analyzed using ROC curve analysis with stage I (n = 16) and stage II (n = 15) PC patients as true positive cases and healthy controls (n = 62) and true negative cases. Patients were divided in to high (n = 31) and low (n = 37) GAS8-AS1 level groups based on Youden's index. Survival curves were plotted and compared by K-M method and log-rank t test. Correlations between plasma levels of miR-1179 and GAS8-AS1 were analyzed by linear regression. Differences were statistically significant when $p < 0.05$.

Results

Plasma GAS8-AS1 was downregulated in PC patients and has early diagnostic potentials

Plasma levels of GAS8-AS1 in 68 PC patients and 62 healthy volunteers were measured by performed RT-qPCR. Analysis of RT-qPCR data by unpaired t test showed that plasma levels of GAS8-AS1 were significantly lower in PC patients than in healthy controls (Fig. 1A, $p < 0.05$). Early diagnostic value of plasma GAS8-AS1 was analyzed using ROC curve analysis with stage I (n = 16) and stage II (n = 15) PC patients as true positive cases and healthy controls (n = 62) as true negative cases (Fig. 1B). It was observed that area under the curve was 0.87 (standard error: 0.035, 95% confident interval: 0.80–0.93).

Low plasma levels of GAS8-AS1 were correlated with poor survival

Differences in plasma levels of GAS8-AS1 among patients at different clinical stages were analyzed by one-way ANOVA and Tukey t test. No significant differences in plasma levels of GAS8-AS1 were found among patients at 4 clinical stages (Fig. 2A). Patients were divided in to high (n = 31) and low (n = 37) GAS8-AS1 level groups based on Youden's index. Survival curves were plotted and compared by K-M method and log-rank t test. It was observed that patients with low plasma levels of GAS8-AS1 had significantly lower 5-year overall survival rate (Fig. 2B).

Plasma miR-1179 was downregulated in PC patients and positively correlated with GAS8-AS1

Plasma levels of miR-1179 in 68 PC patients and 62 healthy volunteers were measured by performed RT-qPCR. Analysis of RT-qPCR data by unpaired t test showed that plasma levels of miR-1179 were also significantly lower in PC patients than in healthy controls (Fig. 3A, $p < 0.05$). Correlations between plasma levels of miR-1179 and GAS8-AS1 were analyzed by linear regression. It was observed that plasma levels of miR-1179 were positively correlated with plasma levels of GAS8-AS1 only in PC patients (Fig. 3B), but not in healthy controls (Fig. 3C).

GAS8-AS1 and miR-1179 upregulated each other in PC cells

GAS8-AS1 expression vectors and miR-1179 mimics were transfected into cells of both Capan-2 and HPAF-II cell lines to explore the possible interaction between these 2 factors. Comparing to control (C) and negative control (NC), expression levels of GAS8-AS1 and miR-1179 were significantly increased at 24h after transfection (Fig. 4A, $p < 0.05$). In addition, GAS8-AS1 overexpression resulted in the upregulation of miR-1179 in those cells (Fig. 4B, $p < 0.05$). In addition, miR-1179 overexpression also led to the overexpression of GAS8-AS1 (Fig. 4C, $p < 0.05$).

GAS8-AS1 and miR-1179 resulted in inhibited migration and invasion of PC cells

Transwell migration and invasion assay data were analyzed by one-way ANOVA and Tukey test. Comparing to control (C) and negative control (NC), overexpression of both GAS8-AS1 and miR-1179 led to inhibited migration (Fig. 5A) and invasion (Fig. 5B) of PC cells ($p < 0.05$).

Discussion

It has been reported that GAS8-AS1 inhibited thyroid carcinoma (11), but its function in other malignancies is unknown. The present study first investigated the involvement of GAS8-AS1 in PC and found that GAS8-AS1 may inhibit PC cell migration and invasion by forming a positive feedback regulation loop with miR-1179, which is a tumor suppressive miRNA in PC (12). In addition, plasma GAS8-AS1 may has prognostic and diagnostic values for PC.

PC is a deadly malignancy. Therefore, early diagnosis and accurate prognosis are particularly critical (13, 14). Early detection of PC mainly depends on highly specific and sensitive biomarkers as well as imaging modalities (15). Circulating biomarkers have been widely used in the diagnosis of cancer due to its non-invasive nature and high acceptance rate (16). In the present study we found that plasma GAS8-AS1 was downregulated in PC patients and downregulated plasma GAS8-AS1 distinguished early stage PC patients from healthy controls. In addition, patients with low plasma GAS8-AS1 showed significantly lower overall 5-year survival rate. Therefore, plasma circulating GAS8-AS1 may serve as a potential diagnostic and prognostic biomarker for PC.

The interaction between lncRNAs and miRNAs has been widely investigated in cancer biology. It has been reported that lncRNAs may serve as sponge of miRNAs to inhibit the function of miRNAs (17, 18). Our study found that GAS8-AS1 and miR-1179 may form a positive regulation circle to regulate the migration and invasion of PC cells. However, the mechanism of the interaction between GAS8-AS1 and miR-1179 is unknown. It is likely that certain pathological mediators may mediate the interaction between GAS8-AS1 and miR-1179. This speculation is supported by the observation that plasma levels of GAS8-AS1 and miR-1179 were significantly correlated in PC patients, but not in healthy controls. Studies on the functions of GAS8-AS1 and miR-1179 in cancer biology are rare. Therefore, our future studies will try to identify upper and lower effectors of GAS8-AS1 and miR-1179 in PC to further elucidate the mechanism of the interaction between GAS8-AS1 and miR-1179.

Therefore, GAS8-AS1 was downregulated in PC. GAS8-AS1 and miR-1179 may form a positive feedback regulation circle to inhibit the migration and invasion of PC cells.

Declarations

Authors' contribution

TL: manuscript writing, literature search and data analysis; DH and CT: data analysis and statistical analysis. LGM: research design. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and material

The datasets generated and/or analyzed during the current study are not publicly available due research design, but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University. All procedures performed in studies involving human participants were in accordance with

1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

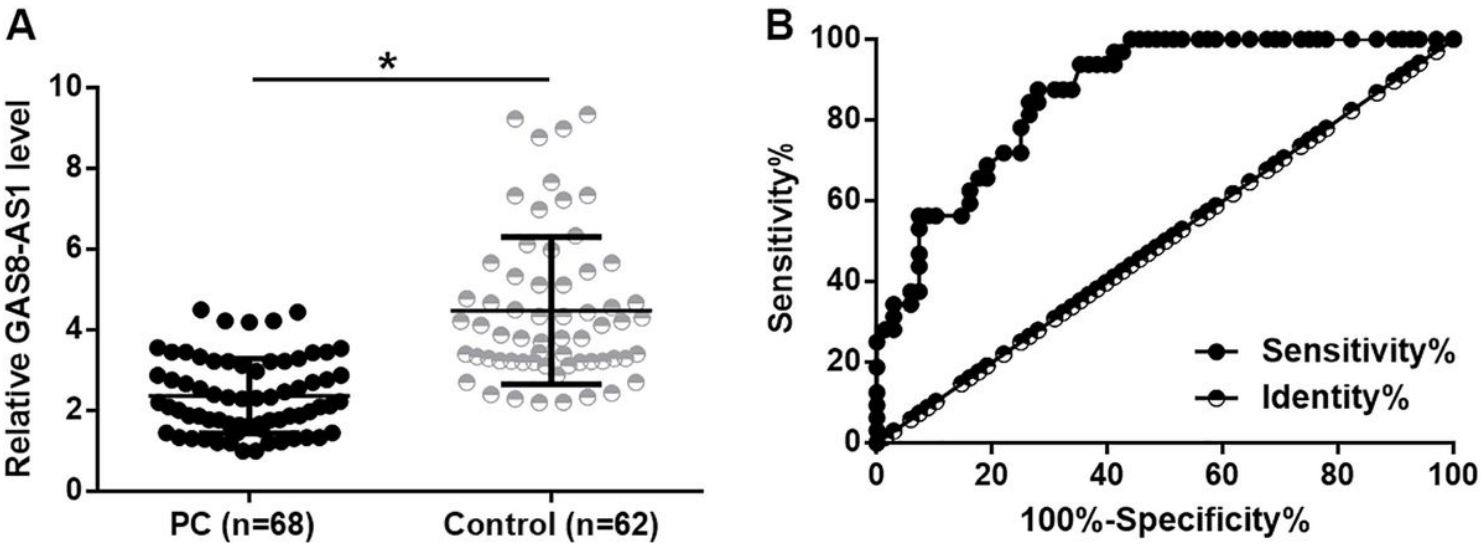

Figure 1

Plasma GAS8-AS1 was downregulated in PC patients and has early diagnostic potentials. Comparisons of RT-qPCR data by unpaired t test showed that plasma levels of GAS8-AS1 were significantly lower in PC patients than in healthy controls (A) (*, $p<0.05$). ROC curve analysis showed that downregulation of plasma GAS8-AS1 distinguished early stage PC patients from healthy controls (B).

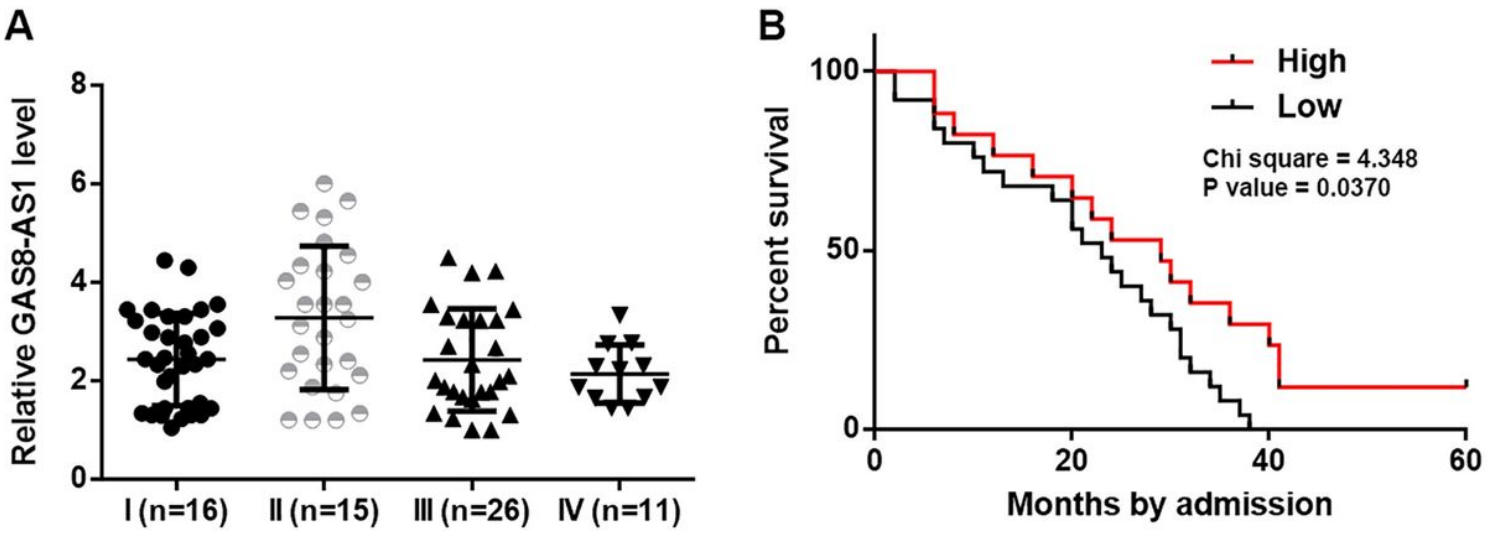

Figure 2

Low plasma levels of GAS8-AS1 were correlated with poor survival. No significant differences in plasma levels of GAS8-AS1 were found among patients at 4 clinical stages (A), (*, $p<0.05$). Survival curve analysis showed that patients with low plasma levels of GAS8-AS1 had significantly lower 5-year overall survival rate (B).

Figure 3

Plasma miR-1179 was downregulated in PC patients and positively correlated with GAS8-AS1. RT-qPCR data analyzed by unpaired t test showed that plasma levels of miR-1179 were also significantly lower in PC patients than in healthy controls (A) (*, $p<0.05$). Linear regression analysis showed that plasma levels of miR-1179 were positively correlated with plasma levels of GAS8-AS1 only in PC patients (B), but not in healthy controls (C).

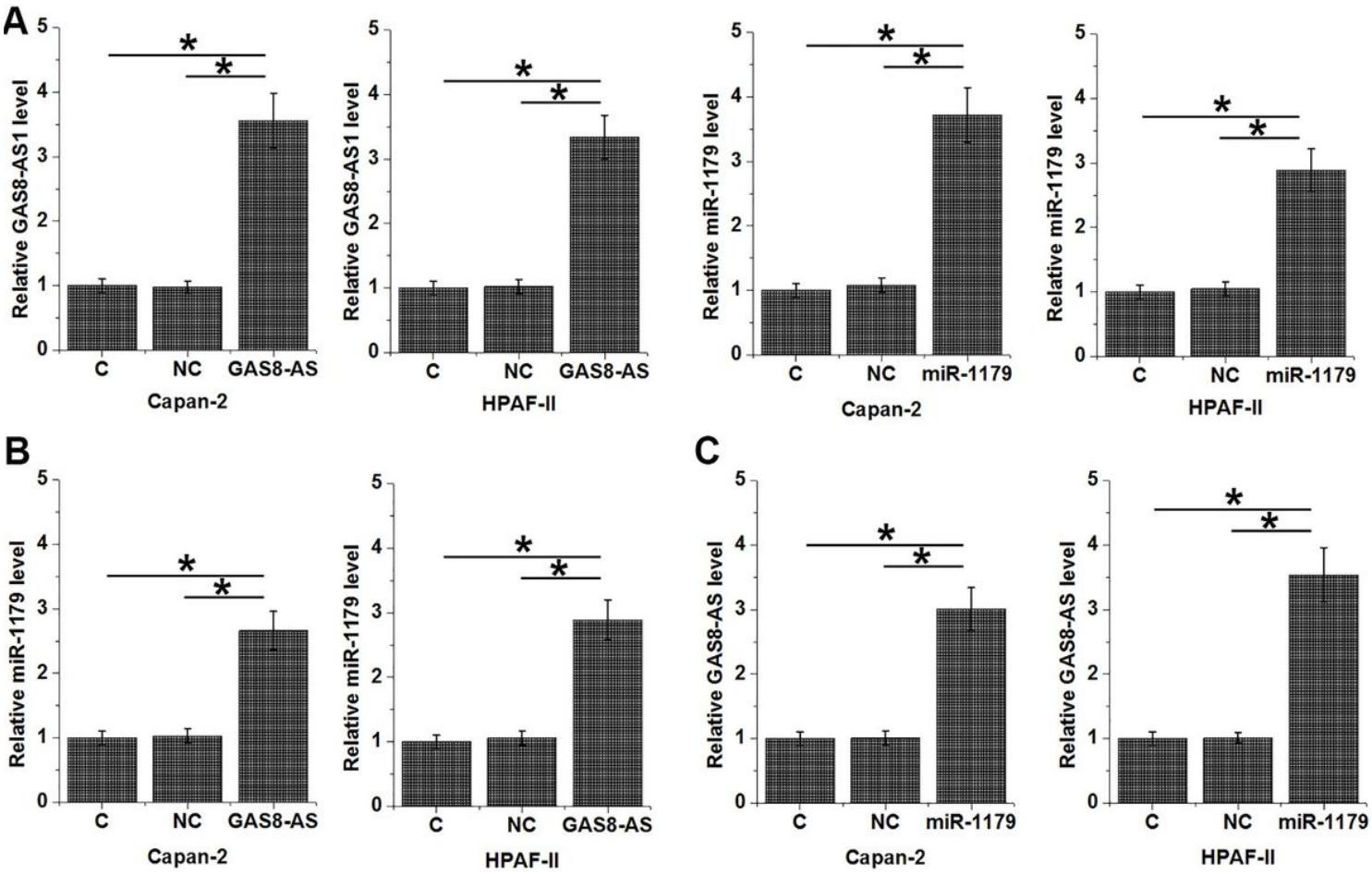

Figure 4

GAS8-AS1 and miR-1179 upregulated each other in PC cells. Comparing to control (C) and negative control (NC), expression levels of GAS8-AS1 and miR-1179 were significantly increased at 24h after transfection (A).GAS8-AS1 overexpression resulted in the upregulation of miR-1179 (B), and miR-1179 overexpression also led to the overexpression of GAS8-AS1 (C), (*, $p<0.05$).

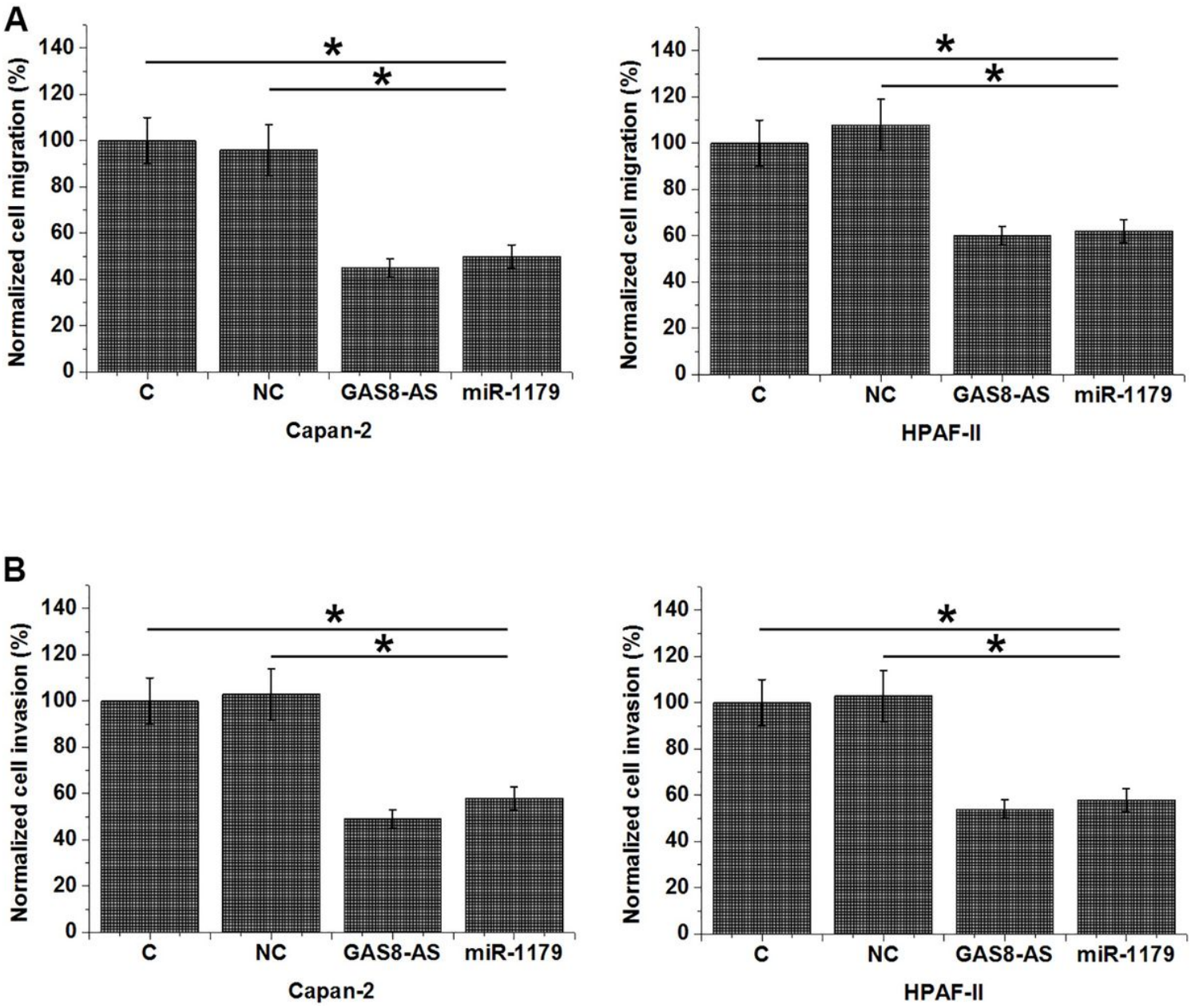

Figure 5

GAS8-AS1 and miR-1179 resulted in inhibited migration and invasion of PC cells. Transwell migration and invasion assay data were analyzed by one-way ANOVA and Tukey test. Overexpression of both GAS8-AS1 and miR-1179 led to inhibited migration (A) and invasion (B) of PC cells (*, $p<0.05$).