

MiR-223 down-regulates RHOB to inhibit progression of papillary thyroid cancer

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Research article

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Abstract

Backgrounds The incidence of thyroid carcinoma continues to increase every year. The incidence of papillary thyroid carcinoma (PTC) is the largest of all thyroid cancers, and is the most widely seen. Thus, Do some identification and testing of biomarkers related to tumorigenesis and development, which will help understand and treat PTC.

Methods The expression of miR-223 and RHOB needs to be detected by polymerase chain reaction and western blot, respectively. Thyroid cell-line BCPAP and K1 cells were cultured, transfected for overexpression of miR-223 or RHOB, and evaluated using MTT, Transwell, and apoptosis assays. In order to better understand the relationship between miR-223 and RHOB, dual luciferase reporter gene detection and RHOB rescue experiments were performed. In addition, clinical pathological parameters and correlation analysis of patients with miR-223 were also performed.

Results Aberrant expression of miR-223 was detected in PTC samples. We also determined that miR-223 expression was associated with TNM staging and cervical metastasis. Moreover, miR-223 inhibits the increase in cell number and position, as well as defense against cancer cells and accelerates cell death. Furthermore, miR-223 excessive expression was associated with a significant inhibition of RHOB levels. According to the luciferase test report, it can be known miR-223 was able to bind RHOB 3'-UTR, it is possible to change its stability through these, and finally achieve the purpose of reducing RHOB. Finally, excessive expression of RHOB has undergone earth-shaking changes in the effect of miR-223 overexpression on cell number growth, invasion and disappearance.

Conclusion Aberrant miR-223/RHOB expression took part in PTC make headway and miR-223 inhibition caused RHOB overexpression and cancer progression. Therefore, miR-223 may be a potential therapeutic target for PTC.

Background

The incidence of thyroid cancer has also become higher and higher, but the incidence of thyroid papillary cancer (PTC) has been ranked first [1]. In addition, the fifth most common tumor in women is PTC [2, 3]. The biological behavior of different PTCs varies widely. Some exhibit few clinical symptoms while others are aggressive and may be fatal. This phenomenon is associated with different genetic abnormalities [4]. Therefore, Do some identification and testing of biomarkers related to tumorigenesis and development, which will help understand and treat PTC.

Microrna (miRNAs) are a class of non-coding rna that can be directly contacted with the 3'-untranslation region (3'-UTRs) of a gene to affect the expression of a protein-coding gene[5, 6]. Coding for this gene the miRNA miR-223 in humans is on the X chromosome to q12 this site[7]. Recently, miR-223 phenotypes have been reported in both hematological malignancies [8–12] and in solid tumors, such as breast cancer [13, 14], gastric cancer [15, 16], hepatic cancer [17, 18], and colon cancer [19–21]. However, what kind of function does miR-223 have in PTC? We still have no way to understand. In present study, PicTar,

MiRanda and targetscan were used together to predict the targets of miR-223. RHOB was found to be as a potential target. All eukaryotes contain a small binding protein called Rho GTPases. These are the Ras superfamilies in GTP-binding proteins. In 1985, the first Rho GTPases were found in human body, which were RhoA, B and C, respectively [22]. RHOB promotes proliferation and intrusion of melanoma [23], breast cancer [23–26] and colon cancer [27–29]. Whereas, it is still not very clear about the impact of RHOB on PTC in humans. In the current study, we attempted to identify any inhibiting act of miR-223 in human PTCs and to clear the potential mechanisms involved. The results may help define the process of tumorigenesis, identify a potential biomarker for PTC progression, and determine if miR-223 may be Potential therapeutic targets for PTC a treatment site that is not obvious to PTC.

Methods

Study subjects and patient organized system specimens

A general of 47 couples of PTC and close tissue specimens the source of collection is the patient undergoing surgery in Shengjing Hospital, it is an affiliated hospital of China Medical University, located in Shenyang. Tissue specimens must be stored in liquid nitrogen after separation and the temperature is limited to -80°C . Participants already need to sign a consent form before the study. This program has been adopted by the Ethics Committee of Shengjing Hospital of the Affiliated Hospital of China Medical University. The work must also be carried out under the World Medical Association Ethics Code (Helsinki Declaration). All the samples (including cancer and normal thyroid tissue) are diagnosed by three pathologists to confirm the histologic diagnosis.

Cell culture and transient transfection

Purchased human PTC cells and cell line BCPAP from Shanghai Chinese Academy of Sciences (Shanghai, China). The culture of BCPAP cells requires that the culture environment be RPMI 1640 medium with 10% fetal calf serum (FBS; Gibco, USA). K1 cells were cultured in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA). miR-223 mimics and negative-control (NC negative) were bought from lsbio (Shanghai, China). The overexpression vector and small interfering RNA (siRNA) for RHOB and the respective controls were purchased from lsbio (Shanghai, China). From the manufacturer's instructions we can know to make lipofectamine 2000 (Invitrogen, USA) to transfect cells.

Real-time measurement of the amount of reverse transcriptase polymerase (qRT-PCR)

If we want to extract cell line cells and total RNA in tissues, we need to rely on TRIzol reagents. We can use the integrated miRNA qPCR kit (GeneCopoeia, Rockville, MD, USA) or the Thermo One-Step RT-PCR kit (Thermo Fisher Scientific) for real-time PCR for miRNA expression according to the manufacturer's instructions. The control gene U6 was applied as a command and for normalization. $2^{-\Delta\Delta Ct}$ method was used to analyze relative expression.

Western blotting

Tissue samples or RIPA causes disruption of cultured cells (Sigma-Aldrich) inclusion complete Mini Protease and Phosphatase Inhibit. A Bradford protein using the detection method of Bio-Rad Company of the United States to quantify number of proteins in the lysed samples. The protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane by wet transfer (EMD microporous, USA). Primary antibodies used for blotting included an anti-RhoB antibody (1:100, mouse, BD, USA) and an anti- β -actin antibody (1:2000, Abcam, USA). We can use the ECL Prime Western blotting reagent (Amersham, USA) to detect the signal and make the signal visible to us.

MTT cell viability assays

In vitro growth of BCPAP and K1 cells was assessed using an MTT Cell Proliferation Assay Kit (Invitrogen, USA) according to the manufacturer's agreement. In short, the BCPAP and K1 cells obtained are placed in a 96-well plate for culture. (5000 cells/well). Every 24 h, 10 μ L of MTT stock solution incubate in the medium of each well for 4 h followed by reaction with sodium dodecyl sulfate (SDS)-HCl solution for 30 min. The cells in the 96-well plate were then examined using a BioTek Synergy II microplate reader (BioTek, USA). The cell proliferation rates were determined according to the luminescence readings for each well at an optical density (OD) of 490 nm.

Cell migration and cell invasion assays

Migration experiment inserts with a pore size of 8 μ m (Corning, USA), the ability of cell invasion can be judged by this method. BCPAP and K1 cells were sown place in 6-well plates overnight. Following incubation at 37 °C for 48 h, the cells were trypsinized and counted. Then, 4×10^4 of the resuspended cells were seeded into the top chambers of the Transwell inserts. The medium to be cultured in the lower chamber is RPMI 1640 medium or DMEM medium supplemented with 20% fetal bovine serum. When 48 h have passed, the cells remaining on the upper membrane were removed. The cells that migrated to the lower surface of the membrane were fixed, then stained with 0.5% crystal violet reagent, and finally recorded by a microscope placed under the microscope. Repeat the above experiment three times

Dual-luciferase reporter assay

According to the manufacturer's instructions, we can use the dual luciferase reporter assay to speculate on luciferase activity. Implanted miR-223 mimics or BCPAP and K1 cells used for reverse control were placed in 96-well plates. When 12 h have passed, the cells were further infection 50 ng pGL3-WT-3'-UTR-RhoB or pGL3-Mut-3'-UTR-RhoB using Lipofectamine 2000 reagent. When it has been cultivated for 24 hours, the luminescence was discovered using a Dual-Luciferase Reporter assay system (Promega, USA).

Apoptosis assays

The apoptosis of BCPAP or K1 cells were detected by flow cytometry. Briefly, for Annexin V dyeing, 5 μ L phycoerythrin-Annexin V, 5 μ L propidium iodide (BD Pharmingen), and 300 μ L 1 \times binding buffer were incubated with the cells and allowed to stand for 15 minutes at room temperature. The samples were then analysed within 1 h of staining. Flow cytometry (FACS Canto II, BD Biosciences) was used. Data processing is performed by FlowJo software. The test was repeated three times.

Colorimetric caspase-3 assays

BCPAP and K1 cells were lysed with RIPA buffer and then their protein samples were resolved by Bradford assay. Every 100 μ g of protein was reacted with 10 μ L Ac-DEVD-pNA (Abcam, USA) and incubated for 2 hours at 37 degrees Celsius. The absorbance at 405 nm was measured by a microplate reader (Bio-Tek instrument).

Statistical Analysis

The above experiments were performed three times. SPSS 17.0 software (SPSS, Inc., Chicago, USA) was employed to perform the statistical analyses. The representation of the data is: mean \pm standard deviation. Statistical differences can be analyzed using Student's t test or one-way variance. $P < 0.05$ is a statistically significant difference.

Results

Aberrant expression of miR-223 in human PTC organizations

To evaluate the expression of miR-223 in papillary thyroid carcinoma (PTCs), 47 paired PTC and adjacent non-tumor tissue samples were examined using qRT-PCR. Thirty-eight of the PTC specimens were found to have lower miR-223 expression than the adjacent non-tumor tissue specimens (Figure 1A). We also observed that PTC specimens exhibited lower levels of miR-223 expression than the paired adjacent non-tumor tissues (Figure 1B).

MiR-223 may have inhibited PTC progression and metastasis in patients

After determining the expression of miR-223 in PTC organizations, we then analyzed the correlation between miR-223 and the clinicopathological parameters that can be obtained from clinical experience. We can get the results from Table 1. The expression of miR-223 is not affected by age, sex, and tumor size, multicentricity, or extrathyroidal invasion. However, lower miR-223 levels were detected in patients with advanced TNM stages and with cervical lymph node metastasis. All the above results reflect that the evolution and transfer of PTC may be affected by miR-223.

Overexpression of miR-223 in PTC cells restrained proliferation and migration and promoted apoptosis PTC cells in vitro

MTT assays were used to evaluate the effect of overexpression of miR-223 on the proliferation of BCPAP and K1 cells. We transiently transfer infection BCPAP and K1 cells with miR-223 mimic or negative control (Figure 2A). The forced overexpression of miR-223 inhibited the development of BCPAP and K1 cells emulated to that observed in negative control cells in a time-dependent manner (Figure 2B). The migration potential of BCPAP and K1 cells overexpressing miR-223 can be identified by transwell migration assay. Forced overexpression of miR-223 lead to decreased cell invasion (BCPAP cells, P = 0.035; K1 cells, P = 0.022; Figure 2C). To fix the effect of miR-223 overexpression on cell apoptosis, Annexin-V staining was performed. Overexpression of miR-223 in PTC cells significantly promoted cell apoptosis (Fig. 2D). Similar results were observed for the colorimetric caspase 3 assays (Fig. 2E). Collectively, these data indicated that the overexpression of miR-223 inhibits proliferation and invasion of PTC, accelerates cell death *in vitro*.

MiR-223 directly inhibited RHOB expression in vitro

Based on the bioinformatics analyses (miRWalk, TargetScan, and PicTar), RHOB was predicted to be a potential mark of miR-223. MiR-223 overexpression in PTC cells was related to a significant reduce in RHOB expression beside that in the negative control cells (Figure 3A). Moreover, The relationship between miR and RHOB can be judged by measuring the luciferase reporter gene. Construct a cell that can transfer the infection, which contains the RHOB wild type or the 3'-UTR luciferase reporter plasmid (as shown in Figure 3B). When the wild-type RHOB 3'-UTR plasmid was transfected with the miR-223 mimetic, the activity was significantly inhibited. However, the amount of miR-223 contained did not affect the luciferase activity of the reporter gene containing the mutant 3'-UTRs (as shown in Figure 3C). From the above results can be derived that RHOB was a first-hand mark of miR-223 in PTC.

RHOB overexpression reversed the result of miR-223 overexpression

To promote determine the RHOB-mediated result of miR-223 in PTC, a RHO-specific overexpression vector was transfected into BCPAP and K1 cells overexpressing miR-223 (Fig. 4A), which restored RHOB expression (59.22 ± 4.58 vs. 30.12 ± 3.25 in BCPAP transfected only by mocks, P=0.028; 47.71 ± 2.91 vs. 28.58 ± 2.21 in K1 cells transfected with mimic alone, P=0.048) We discovered that RHOB overexpression restored the inhibitory result of miR-223 on proliferation and invasion in miR-223-overexpression cells (Fig. 4B and 4C). A time-dependent increase in cell proliferation was observed in the BCPAP and K1 cells also overexpressing RHOB compared to that in BCPAP and K1 cells overexpressing miR-223 (10.8 ± 0.7 , 14.2 ± 2.1 , 24.5 ± 3.7 , and $39.4 \pm 5.1\%$ at 24, 48, 72, and 96 h in BCPAP cells, respectively, P = 0.031; 4.5 ± 0.3 , 17.4 ± 2.5 , 22.2 ± 3.1 , and $30.7 \pm 4.3\%$ at 24, 48, 72, and 96 h in BCPAP cells, respectively, P = 0.044).

Furthermore, RHOB overexpression obviously alleviated apoptosis (Fig. 4D and 4E). These data suggested that miR-223 inhibited PTC progression by inhibiting RHOB.

Discussion

PTC is common in all diagnosed thyroid cancers, accounting for 80% of the rate [22]. Although PTC patients always have a good prognosis for overall survival, some patients with advanced stage disease have a poor quality of life. It is therefore important to determine the mechanism of progression for PTC [23].

MiR-223 has been reported to have an inhibitory effect in many solid malignancies. Fabris et al. highlighted miR-223 as a tumor inhibitor in breast cancer [14], Eto et al. have reported miR-223 inhibits gastric cancer progression [15], and Dong et al. have shown that miR-223 is used to control the increase of cell death rate to control the increase of liver cancer cells [18]. Furthermore, several researches have found that miR-223 represses colon cancer progression [19–21]. In the latest research, we observed decreased activity of miR-223 in 38 paired PTC tissue specimens. We also studied the relationship between miR-223 and clinical pathological parameters of PTC, and found that there is a certain relationship between lower miR-223 level and late TNM stage and cervical metastasis. Moreover, BCPAP and K1 cells were used to investigate the effect of aberrant activity of miR-223 in PTC cells. We demonstrated that miR-223 suppression in PTC cells increases the amount, migration, and invasion and promotes apoptosis. So we can get miR-223 to inhibit the progression of PTC.

To reveal the molecular mechanisms of miR-223 in PTC progression, we can speculate on the target of miR-223 with the help of TargetScan, PicTar and miRanda online tools, and further find RHOB as a potential target. All eukaryotes have a small GTP/GDP binding protein called Rho GTPases, which is a GTP-binding protein in the Ras superfamily. RhoA, RhoB and RhoC were the first Rho GTPases discovered in humans in 1985 [22]. Huang et al. showed that gluco-corticoids can increase the amount of Rho-related kinase secretion by 50%, and can also have a positive effect on the migration, invasion and metastasis of melanoma [23]. Moreover, it has been revealed that RHOB promotes growth and invasion of breast cancer cells [24–26] and RHOB promotes colon cancer progression [29–31]. In the latest research, we also used the BCPAP and K1 cells to investigate the relationship between miR-223 and RHOB. MiR-223 overexpression is associated with significant inhibition of RHOB content. Furthermore, we can get from the luciferase test report that miR-223 was able to bind RHOB 3'-UTR. It has a certain influence on its stability, which eventually leads to a decrease in RHOB content. Furthermore, we proved that the overexpression of RHOB deteriorates the effects of miR-223 overexpression on cell proliferation, invasion, and apoptosis.

Overall, our results showed that the aberrant miR-223/RHOB expression was related to PTC progression. The miR-223 inhibition caused RHOB overexpression and cancer progression. Therefore, miR-223 is perhaps a potential therapeutic mark for PTC.

Declarations

Ethics approval and consent to participate

The research had been supported by the Ethics Committee of Shengjing Hospital. Relevant clinical information was gathered from patients' records and informed written consent had been gained. The papillary thyroid carcinoma cell lines BCPAP and K1 were obtained from Cell bank of typical culture preservation Committee of Chinese Academy of Sciences, and did not require ethics approval for their use in this study.

Consent to publication

Not applicable

Availability of data and materials

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

Authors' Contributions

XZ designed the study and drafted the manuscript. WJ reviewed the article. WZ conducted the experiments. All authors have read and approved the final manuscript.

Competing interests

The authors declare there isn't any conflict of interest.

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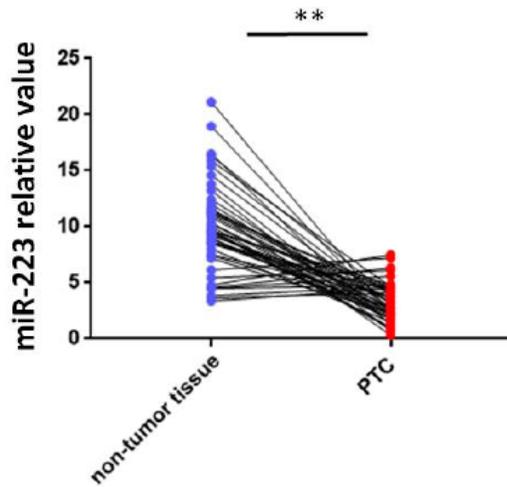
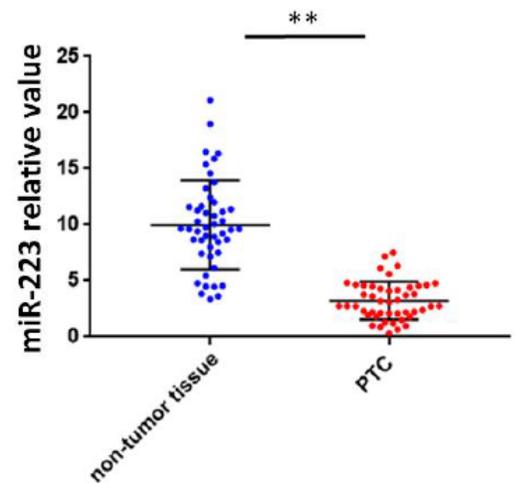
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Table

Table 1. Correlation of miR-223 expression with clinicopathological factors of PTC patients

Parameters	Patients	miR-223	P
Total	47		
Gender			0.489
Male	10 (21.3)	3.09±1.52	
Female	37 (78.7)	3.35±1.88	
Age (Y)			0.624
≥45	29 (61.7)	3.12±1.79	
< 45	18 (38.3)	3.31±1.58	
Tumor size,cm			0.115
≥2	19 (40.4)	3.03±1.82	
< 2	28 (59.6)	3.52±1.63	
Extrathyroidal invasion			0.087
Yes	12 (25.5)	3.05±2.16	
No	35 (74.5)	3.88±1.54	
Multicentricity			0.074
Yes	13 (27.6)	3.01±2.04	
No	34 (72.4)	3.92±1.80	
Cervical metastasis status			0.018
N+	21 (44.7)	2.09±1.45	
N-	26 (55.3)	4.35±1.99	
TNM stages			0.032
I/II	29 (61.7)	4.16±1.86	
III/IV	18 (38.3)	2.17±1.57	
Note: Values in parentheses represent percentages			

Figures

A**B****Figure 1**

Abnormal expression of miR-223 in PTC specimens. (A) Quantification of the process of reverse transcription polymerase reaction(qRT-PCR) analysis of miR-223 expression in 47 paired PTC and adjacent non-tumor tissues. Of the 47 PTC specimens, 38 had lower miR-223 expression than the control. Statistical analysis is carried out by t-test method, and the analysis is performed after logarithmic transformation.($P = 0.002$).Each set of experiments was performed three times. The repetition of a creature is represented by a dot. (B) qRT-PCR split understanding of miR-223 deliverance in PTC and control samples (3.23 ± 1.71 vs. 10.00 ± 3.98 . Perform log transformation on data for split understanding ($P = 0.003$). MiR-223 deliverance was 3.1-twice as much adjacent non-tumor tissue samples than in the PTC specimens. The U6 gene was used as a positive control and for normalization.

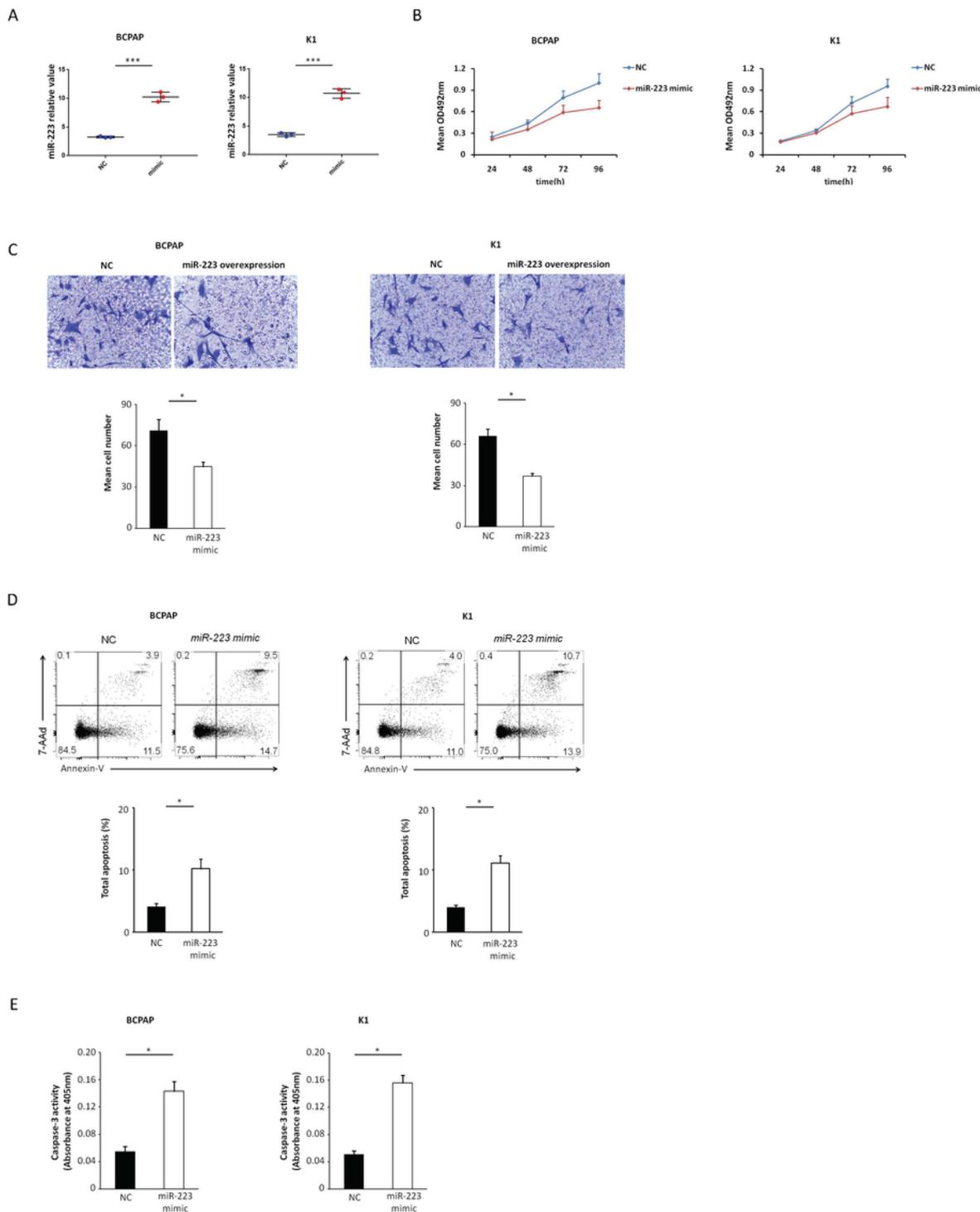


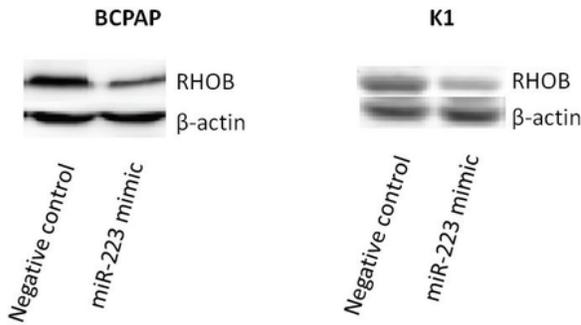
Figure 2

Figure 2

Influent of miR-223 on PTC cell proliferation, invasion, and apoptosis. (A) BCPAP and K1 cells temporarily transfected with miR-223 mimics or negative control (BCPAP: 10.24 ± 0.49 vs. 3.25 ± 0.08 [bridle], $P = 0.033$; K1: 10.70 ± 0.43 vs. 3.51 ± 0.18 [bridle], $P = 0.045$). (B) Cell survival rates determined by MTT assays in miR-223-overexpressed and control BCPAP and K1 cells. Decline with time was found in the cell rate of increase in cell number of the miR-223-overexpression cells (BCPAP cells: $13.06\% \pm 1.1$,

19.1% ± 1.9, 25.8% ± 2.5, and 34.6% ± 4.1 at 24, 48, 72, and 96 h, separately, P = 0.026; K1 cells: 6.4% ± 0.5, 10.1% ± 1.1, 20.7% ± 1.5, and 29.6% ± 3.2 at 24, 48, 72, and 96 h, separately, P = 0.046). (C) Deputation photographs of Transwell aggressive attacks on miR-223 overexpression and negative effects on BCPAP and K1 cells (upper; magnifying power 200) and their quantitative analysis (bottom). (D) Deputypicture and proportion of cell-autonomous ordered death of BCPAP and K1 cells with miR-223-overexpression or negative bridle (*P = 0.039 and 0.021 for BCPAP and K1 cells respectively). (E) Caspase-3 activity in BCPAP and K1 cells with miR-223-overexpression or negative bridle cells (*P = 0.036 and 0.018 for BCPAP and K1 cells respectively). Each set of experiments was done three times. Dots delegatetese ditto.

A



B



C

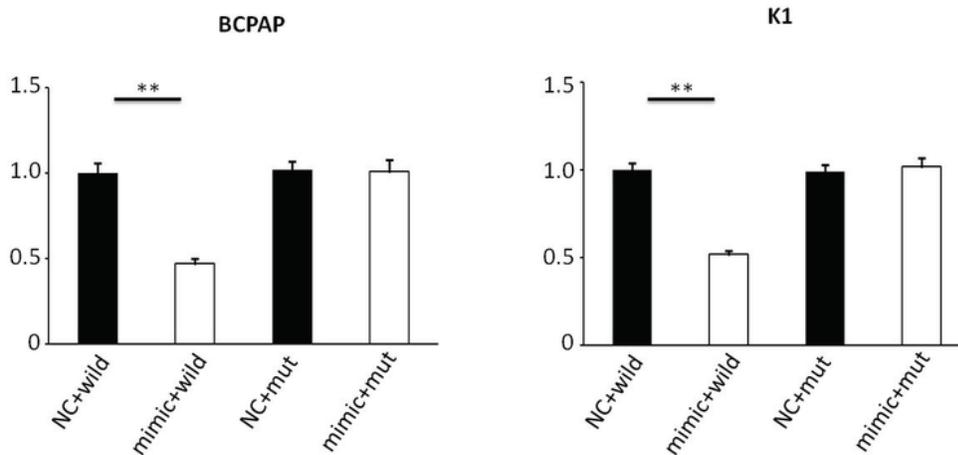


Figure 3

Figure 3

RHOB was a direct target of miR-223 in PTC. (A) Analysis of representative western blot and RHOB proteins by miR-223 simulation or negative comparison analysis of BCPAP and K1 cells (BCPAP: 78.33 ± 6.45 vs. 33.87 ± 3.52 , $P = 0.022$; K1: 61.98 ± 3.22 vs. 29.65 ± 2.38 , $P = 0.040$). β -actin was used as a bridge. (B) miR-223 straight from the shoulder interacted with the 3'-UTRs of RHOB. (C) After co-transfection with miR-223 mimic, the relative luciferase activity of the reporter plasmid containing the

uncultivated-type RHOB 3'-UTR was significantly reduced. But the miR-223 level change did not produce an effect on the luciferase activity of the reporter plasmid with the RHOB mutant 3'-UTRs. These data are the mean \pm SD of the results of three trials that do not interfere with each other. After the logarithmic transformation of the data by two-way analysis of variance, it is statistically organized and analyzed. **, $P = 0.005$ and 0.009 for BCPAP and K1 cells, respectively. Dots delegatetese ditto.

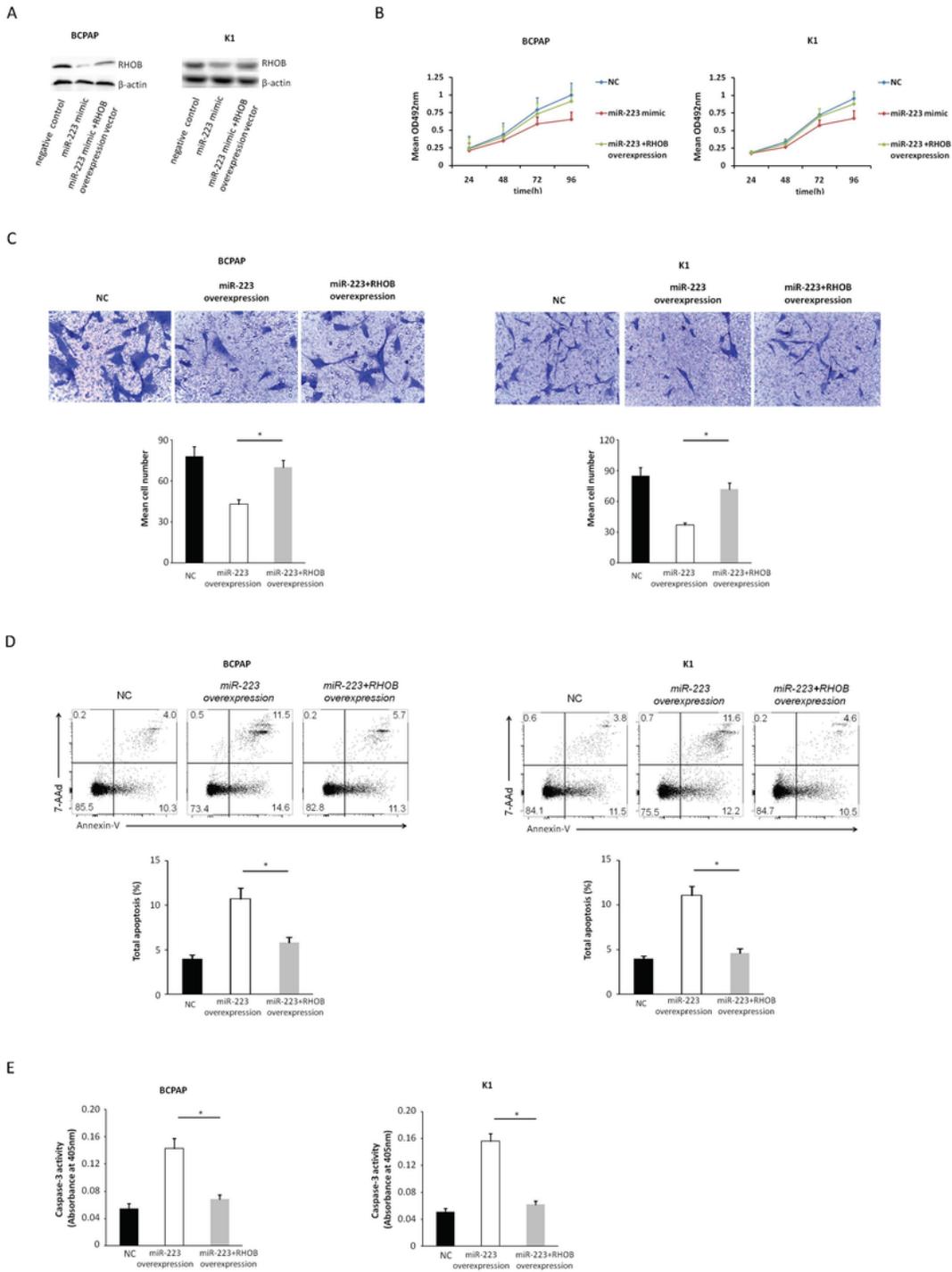


Figure 4

Figure 4

RHOB overexpression restored the effects of miR-223(NC: negative control). (A) Typical western blot findings delivery that transfection of RHOB-overexpression carrier comtrastto restores the depression of RHOB after transfection with miR-223 mimic. (B) Value added rates of miR-223-overexpression BCPAP and K1 cells at different time points after RHOB overexpression. (C) Typical pictures (upper; magnifying power 200) of Transwell aggressive attacks of miR-223-overexpressing BCPAP and K1 cells with or without RHOB overexpression and analysis of specific quantities (bottom) (*P =0.029 and 0.042 for BCPAP and K1 cells, respectively). (D) Typical pictures showing the ratio of BCPAP and K1 cells in apoptosis after transfection with miR-223 mimic with and without RHOB overexpression (*P =0.035 and 0.020 for BCPAP and K1 cells, respectively). (E) Caspase-3 doings of BCPAP and K1cells transfection with miR-223 mimic have and without RHOB over-deliverance (*P =0.018 and 0.013 for BCPAP and K1 cells, respectively).