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Effects of miR-145 targeting rab5c and regulating MAPK / ERK signaling pathway on proliferation and invasion of thyroid papillary carcinoma cells

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ABSTRACT

The objective of this research was to analyze the miR-145 function in thyroid papillary carcinoma cells and explore its possible mechanism. For this purpose, the TPC-1 cell line was selected, miR-145 overexpression and rab5c shRNA lentiviral vector were constructed, and transfected into PTC cells. Luciferase reporter gene was performed to determine the relationship between miR-145 and rab5c, Western blot and qPCR were performed to detach the expression of the related genes, CCK-8 cell proliferation assay and Transwell cell invasion assay were used to determine the proliferation and invasion ability of PTC-1 cells. Results showed that MiR-145 overexpression inhibited the wt-rab5c (wild-type rab5c)luciferase activity, decreased the expression of rab5c mRNA and protein levels in the TPC-1 cell line, inhibited the proliferation and invasion of PTC cell line TPC-1($P < 0.05$). In TPC-1 cells, both miR-145 overexpression and RNA interference with rab5c could increase the expression of the p-ERK protein ($P < 0.05$). In conclusion, MiR-145 inhibits the proliferation and invasion of PTC cells by downregulating rab5c and activating MAPK/ERK pathway in vitro.

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Introduction

According to relevant statistics, there were about 50,000 new patients with thyroid cancer (THCA) in 2020. (1) Papillary Thyroid Carcinoma (PTC), accounts for 85% - 90% of THCA. (2) Although approximately 90% of patients can be clinically cured by standard treatment, (3) nearly 10% of THCA cases have local regional recurrence or distant metastasis, which is a major challenge of THCA treatment. (4) Due to recurrence and metastasis, the prognosis of THCA patients is not optimistic. Therefore, it is necessary to elucidate the mechanism of THCAoccurrence and invasion.

MicroRNA is a small regulatory RNA, which regulates post-transcriptional gene expression. It has been confirmed to be involved in regulating the cell function of tumor cells. MiR-145, as a tumor suppressor gene, has been shown down-regulated in many types of human cancer. It plays a crucial role in tumor occurrence, metastasis, progression, and chemotherapy radiation resistance. (5) It has been found that miR-145 is decreased in PTC tissues and is correlated with clinicopathological features. (6) However, the mechanism is not clear. This study aimed to analyze the function of miR-145 in PTC cells and to clarify its mechanism of action in the development of PTC.

Materials and Methods

General materials

Cell line: TPC-1 cell line was selected and provided by the cell bank of the Chinese Academy of Sciences.

Rab5c shRNA lentiviral vector GV248 was used to target the RAB5C gene.

Experimental instrument: CO_2 cell incubator, provided by Shanghai Fuze Trading Co., Ltd. Ultra-low temperature refrigerator, provided by Shanghai Zoming Machinery Equipment Trading Co., Ltd. The constant temperature water bath is provided by Guangzhou Juneng nano Biotechnology Co., Ltd. Inverted fluorescence microscope, was provided by Shanghai Yuyan Scientific Instrument Co., Ltd. The low-temperature high-speed centrifuge is provided by Promai Precision Medical Technology (Beijing) Co., Ltd. Spectrophotometer, provided by Beijing Zeping Technology Co., Ltd. The microplate reader was provided by Meigu Molecular Instruments (Shanghai) Co., Ltd. PVDF membrane, was provided by Shanghai Jizhi Biochemical Technology Co., Ltd. Transwell cell was provided by Shanghai Yanhui Biotechnology Co., Ltd. Micropipette, provided by Praland (Shanghai) Trading Co., Ltd. Desktop centrifuge, provided by Shanghai Fuze Trading Co., Ltd. The Western blot experimental equipment was provided by Qingdao Feiyoute Testing Co., Ltd.

Experimental reagent: RPMI-1640 cell culture medium, provided by Wuhan purity Biotechnology Co., Ltd. Fetal bovine serum, provided by Lanzhou Rongye Biotechnology Co., Ltd. Qingstreptomycin, provided by Beijing Zeping Technology Co., Ltd. PBS buffer, provided by Beijing YITA Biotechnology Co., Ltd. BCA protein quantification kit was provided by Beijing YITA Biotechnology Co., Ltd. Poly gel, provided by Beijing YITA Biotechno-

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logy Co., Ltd.

Method

Cell resuscitation and culture: take out the cells needed for the experiment from liquid nitrogen, put them in a constant temperature water bath at 37℃, melt them, add the complete medium into a 15ml centrifuge tube in advance, mix them well, and centrifuge at room temperature at 800rpm for 3min. Discard the supernatant, add 1ml of complete medium and mix well. Transfer the liquid into a Petri dish or culture plate containing a complete culture medium, and put it into a constant temperature incubator for further culture.When the observation density is about 85%, the culture medium is sucked out, washed with PBS buffer for one time, and the cell digestion solution is added. The cell morphology is observed under the microscope. When most of the cells in the dish become oval, the cells are digested and neutralized with a complete medium of 3 times the volume of the digestion solution. The cells were collected into a 15ml centrifuge tube and centrifuged at 800rpm for 5min at room temperature. Add 1ml of complete medium, mix well and transfect cells.

Cell transfection: put the required cells into the culture plate or culture dish in advance, and adjust the cell density to 30% - 40%. Lipofectamine RNAi max transfection reagent and required siRNA were prepared in advance and placed on ice for dissolution. Take two sterile DNase / RNase free EP tubes and add 120 μ L of empty medium and 5 μL transfection reagent and 5 μ Lsirna solution, then mix the two tubes of liquid, mix well and incubate at room temperature for 10min. Drop the mixed liquid into the culture plate and put it back into the constant temperature incubator for further cultivation. After 6 h, replaced medium with fresh complete medium.

Observation index

Luciferase reporter assay: a dual luciferase reporter was constructed. And it was transfected into the human papillary thyroid carcinoma cell line.

Cell proliferation assay: CCK-8 purchased from Beyotime, was performed to detach cell proliferation. 2000TPC-1 cells/well were seeded on a 96-well plate. After inoculation (24, 48, 72 and 96 h incubation), 10 μL CCK-8 was added to wells and measured absorbance at 450 nm.

Transwell cell migration experiment: cultured each group cell in a serum-free medium for 1 day. Trypsin digestion, RPMI-1640 cell culture medium was re-suspended according to 5×10^4/well was inoculated into the Transwell upper chamber, and 500 μL of complete cell culture medium was added into the lower chamber. Put it into 37 ℃ and 5% CO_2 incubator for 24h. Take out the upper chamber, fix the cells with 4% methanol for 15min, and rinse them with PBS 3 times. Staining with 2% crystal violet for 15min, and washing with PBS. Observe and photograph under the microscope, and count the cell number.

Transwell cell invasion experiment: add 50 μL of diluted Matrigel gel into each Transwell upper chamber, slowly add it from the edge, and place it in the 37 ℃ incubators for 0.5h before solidification. The cells were cultured for 48h after inoculation. The rest is the same as the Transwell cell migration experiment.

Western blot detection: total protein was extracted with protein lysate, and it was detached by the BCA method, 5 × SDS gel was loaded and boiled. Separated protein by electrophoresis and transferred to PVDF membrane. 10% skim milk was blocked, and incubated 2h (room temperature). The corresponding concentration of primary antibody was added and incubated at 4 ° C overnight. TBST washing.Incubatedsecondary antibody. TBST cleaning, ECL color development and exposure imaging.β-actin, as an internal reference, was used to analyze the relative expression levels of other proteins.

Statistical method

The data were analyzed by SPSS20.0 software and expressed by ($x\pm s$). t-test was used for comparison between groups; the comparison between groups was χ^2 Inspection. $P < 0.05$ means the statistical results were statistically significant.

Results

Verify that RAB5C is a target gene of miR-145

MiR-145 overexpression significantly decreased the luciferase activity of Wt-RAB5C (wild-type RAB5C) ($P < 0.05$). Our results showed that rab5c was a target of miR-145.

MiR-145 overexpression downregulated RAB5C protein and mRNA expression

In Table 2, compared with Mir NC, the miR-145 overexpression group decreased rab5c mRNA and protein levels in the TPC-1 cell line ($P < 0.05$).

Cell proliferation

MiR-145 overexpression inhibited the TPC-1 cell proliferation ($P < 0.05$), and there was no significant difference between the miR-145 overexpression group and the rab5c shRNA group ($P > 0.05$).

Table 1. Dual luciferase reporter gene analysis.

	miR-NC	miR-145overexpression	*t*	*P*
RAB5C protein	1.03±0.08	0.43±0.06	13.416	0.001
RAB5C mRNA	1.06±0.09	0.53±0.09	9.311	0.001

Table 2. The impact of miR-145 overexpression on RAB5C protein and mRNA expression.

	miR-NC	miR-145overexpression	*t*	*P*
RAB5C protein	1.03±0.08	0.43±0.06	13.416	0.001
RAB5C mRNA	1.06±0.09	0.53±0.09	9.311	0.001

Table 3. The impact of miR-145and RAB5C on PTC cell proliferation.

Grouping	n	24h	96h	*t*	*P*
miR-NC	5	0.18±0.02	1.32±0.23	11.041	0.001
miR-145 overexpression	5	0.19±0.02	0.53±0.18[a]	4.197	0.003
Control-shRNA	5	0.19±0.03	1.28±0.22	10.977	0.001
RAB5C -shRNA	5	0.18±0.03	0.49±0.16[a]	4.258	0.003

Note: Compared with the respective control group, [a]$P<0.05$.

Table 4. Transwell cell invasion experiment results. Decreasing the invasion ability of TPC-1 cells by miR-145 overexpression or rab5c inhibition.

Grouping	n	Number of migrating cells	Number of invading cells
miR-NC	5	540±38	1168±86
miR-145 overexpression	5	257±34[a]	854±42[a]
Control-shRNA	5	554±36	1147±73
RAB5C -shRNA	5	238±30[a]	786±52[a]

Note: Compared with the respective control group, [a]$P<0.05$.

Table 5. Expression levels of MAPK / ERK signaling pathway-related proteins.

Grouping	n	p-ERK	t-ERK	p-ERK/t-ERK
miR-NC	5	0.48±0.13	1.06±0.17	0.45±0.15
miR-145overexpression	5	0.85±0.11[a]	0.95±0.14	0.89±0.16[a]
Control-shRNA	5	0.53±0.10	1.04±0.16	0.47±0.11
RAB5C -shRNA	5	0.98±0.13[a]	0.88±0.14	1.11±0.17[a]

Note: Compared with the respective control group,[a]$P<0.05$.

Invasion ability

Transwell cell invasion experiment showed that miR-145 overexpression or rab5c inhibition significantly decreased the invasion ability of TPC-1 cells ($P < 0.05$). See Table 4.

Comparison of protein expression related to Akt/β-catenin signaling pathway in each group of cells

In TPC-1 cells, both miR-145 overexpression and RNA interference with rab5c could increase the expression of the p-ERK protein ($P < 0.05$). See Table 5.

Discussion

MiRNAs play a key role in the gene silencing effect by binding with target mRNA and are involved in regulating cancer cell proliferation, apoptosis, development, stress response and metabolism. (7)MiR-145 is considered a tumor suppressor in various types of human cancer (8,9). It may regulate tumor be had by targeting c-Myc (10), Mucin-1 (11), and p70S6 kinase (12). Overexpression of miR-145 enables WNT by directly targeting WNT2B in vitro/β- inactivation of the catenin pathway inhibits cell proliferation and metastases (13). Similarly, miR-145 is downregulated in esophageal squamous cell carcinoma and it is a candidate target for tumor suppression (14,15). Functional experiments showed that miR-145 was related to the EMT process (14). In addition, miR-145 was lower expressed in MCF-7 cells, and overexpression of miR-145 blocked the growth of MCF-7 cells and induced apoptosis by targeting RTKN. (16) Further, p53 can inhibit c-myc by introducing miR-145, so miR-145 directly targets c-Myc (17). It also has been proved miR-145 is related to PTC tumor diameter, multiple tumors and other clinicopathological features. However, the mechanism of action of miR-145 in PTC is not understood.

RAB5C isoforms have been reported to be involved in cell invasion, they could regulate RAC-mediated cell movement (18) and cohesion (19), promote ovarian carcinogenesis, patriciate in drug resistance of ovarian cancer regulation (20), promote breast cancer invasion through the amap1-prkd2 complex. (21) In this study, miR-145 potential target genes were predicted by bioinformatics software. We confirmed that RAB5C is a potential target of miR-145. In addition, miR-145 negatively regulates the mRNA and protein expression of RAB5C. Interference with RAB5C expression can also inhibit the proliferation and invasion of PTC cells.

In recent years, studies have found that MAPK / ERK signaling cascade activates various receptors to participate in the growth and differentiation of malignant tumors. For example, in U14 cervical cancer mice, miR-92a suppresses immune function by inhibiting PTEN to activate MAPK / ERK signaling pathway. (22) Oxyfadichalcone C also was confirmed to inhibit the proliferation and metastasis of melanoma A375 cells by inhibiting PI3K/Akt and MAPK/ERK pathways. In hepatocellular carcinoma, KCNN4 promotes invasion and metastasis through MAPK/ERK pathway (23). The results showed that both miR-145 overexpression and RNA interference with rab5c could increase the expression of the p-ERK protein, and decrease the proliferation and invasion of PTC by activating MAPK / ERK pathway.

In summary, miR-145 inhibits the proliferation and invasion of PTC cells by targeting RAB5C and activating MAPK / ERK pathway in vitro. This study provides a new molecular regulatory mechanism for PTCpathogenesis and indicates that miR-145 can be used as an inhibitor for

PTC.

Conflicts of interests

The authors state no conflicts of interest in this study.

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