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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#These authors contributed equally to this work as co-first author

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 THCA occurrence 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: CO2 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 Bio-technology Co., Ltd. Inverted fluorescence microscope, was provided by Shanghai Yuyan Scientific Instrument 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 Xinhua Biotechnology Co., Ltd. Micro-pipette, 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. Ultra-low temperature refrigerator, provided by Shanghai Fuze Trading Co., Ltd. Poly gel, provided by Beijing YITA Biotechnology 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 transfecct 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 μL of empty medium and 5 μL transfection reagent and 5 μL sirna 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. The optical density (OD) was measured at 450 nm. The statistical results were statistically significant.

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 μL of empty medium and 5 μL transfection reagent and 5 μL sirna 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.

Table 1. Dual luciferase reporter gene analysis.

<table>
<thead>
<tr>
<th></th>
<th>miR-NC</th>
<th>miR-145overexpression</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAB5C protein</td>
<td>1.03±0.08</td>
<td>0.43±0.06</td>
<td>13.416</td>
<td>0.001</td>
</tr>
<tr>
<td>RAB5C mRNA</td>
<td>1.06±0.09</td>
<td>0.53±0.09</td>
<td>9.311</td>
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</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
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Table 3. The impact of miR-145 and RAB5C on PTC cell proliferation.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>n</th>
<th>24h</th>
<th>96h</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-NC</td>
<td>5</td>
<td>0.18±0.02</td>
<td>1.32±0.23</td>
<td>11.041</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-145 overexpression</td>
<td>5</td>
<td>0.19±0.02</td>
<td>0.53±0.18</td>
<td>4.197</td>
<td>0.003</td>
</tr>
<tr>
<td>Control-shRNA</td>
<td>5</td>
<td>0.19±0.03</td>
<td>1.28±0.22</td>
<td>10.977</td>
<td>0.001</td>
</tr>
<tr>
<td>RAB5C -shRNA</td>
<td>5</td>
<td>0.18±0.03</td>
<td>0.49±0.16</td>
<td>4.258</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Note: Compared with the respective control group, *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.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>n</th>
<th>Number of migrating cells</th>
<th>Number of invading cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-NC</td>
<td>5</td>
<td>540±38</td>
<td>1168±86</td>
</tr>
<tr>
<td>miR-145 overexpression</td>
<td>5</td>
<td>257±34*</td>
<td>854±42*</td>
</tr>
<tr>
<td>Control-shRNA</td>
<td>5</td>
<td>554±36</td>
<td>1147±73</td>
</tr>
<tr>
<td>RAB5C -shRNA</td>
<td>5</td>
<td>238±30*</td>
<td>786±52*</td>
</tr>
</tbody>
</table>

Note: Compared with the respective control group, *P<0.05.

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

<table>
<thead>
<tr>
<th>Grouping</th>
<th>n</th>
<th>p-ERK</th>
<th>t-ERK</th>
<th>p-ERK/t-ERK</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-NC</td>
<td>5</td>
<td>0.48±0.13</td>
<td>1.06±0.17</td>
<td>0.45±0.15</td>
</tr>
<tr>
<td>miR-145 overexpression</td>
<td>5</td>
<td>0.85±0.11a</td>
<td>0.95±0.14</td>
<td>0.89±0.16a</td>
</tr>
<tr>
<td>Control-shRNA</td>
<td>5</td>
<td>0.53±0.10</td>
<td>1.04±0.16</td>
<td>0.47±0.11</td>
</tr>
<tr>
<td>RAB5C -shRNA</td>
<td>5</td>
<td>0.98±0.13a</td>
<td>0.88±0.14</td>
<td>1.11±0.17a</td>
</tr>
</tbody>
</table>

Note: Compared with the respective control group, aP<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), MUC-1 (11), and p70S6 kinase (12). Overexpression of miR-145 enables WNT by directly targeting WNT2B in vitro/β-catenin signaling pathway inhibited 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, p33 can inhibit c-myc by introducing miR-145, so miR-145 directly targets c-Myc (17). It also has been proved that 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 PTC pathogenesis 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.

Acknowledgements
None.

References