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Research paper

RAB23, regulated by miR-92b, promotes the progression of esophageal squamous cell carcinoma

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ABSTRACT

RAB23, a member of Ras-related small GTPase family, has been reported to be up-regulated in several cancer types. However, its biological functions and the underlying molecular mechanisms for its oncogenic roles in esophageal squamous cell carcinoma (ESCC) remain unknown. In this study, we have shown that the expression of RAB23 was elevated in ESCC tissues and ESCC cells. Overexpression of RAB23 promoted the growth and migration of the ESCC cells, while knocking down the expression RAB23 inhibited the growth, migration and metastasis of the ESCC cells. The molecular mechanism study showed that RAB23 activated beta-catenin/TCF signaling and regulated the expression of several target genes. In the further study, it was found that the expression of RAB23 was regulated by the miR-92b. Forced expression of MiR-92b decreased the mRNA and protein level of RAB23, and RAB23 rescued the biological functions of miR-92b. Taken together, this study revealed the oncogenic roles and the regulation of RAB23 in ESCC, suggesting RAB23 might be a therapeutic target.

1. Introduction

Esophageal cancer is one of the common malignancies worldwide, especially in the East Asia, Africa and North America (Siegel et al., 2016). Esophageal squamous cell carcinoma (ESCC) is the most common clinical type of esophageal cancer in China (Zeng et al., 2016). Although great advances have been made in diagnosis and therapy, ESCC remains to be a devastating malignancy due to late diagnosis and the rapidly aggressive progression. Therefore, exploring the mechanisms for the development and progression of ESCC will benefit the detection, diagnosis and therapy of ESCC.

RAB23, a member of RAB GTPase family, has been considered as the regulator for endocytic recycling and vesicle transportation (Delevoye and Goud, 2015; Klinkert and Echard, 2016; Tang, 2016; Wang et al., 2016a; Wang et al., 2016b). Mutations in RAB23 gene has been reported to cause carpenter syndrome with the abnormal development in neural tube (Ben-Salem et al., 2013; Schmid et al., 2016; Ye et al., 2016). RAB23 has reported to be a negative regulator of Hedgehog signaling by inhibiting the transcriptional activity of Gli1 (Chi et al., 2012; Liu et al., 2015; Sun et al., 2012). RAB23 has been shown to be dysregulated in several cancer types in which RAB23 acted as oncogene or tumor suppressor (Denning et al., 2007; Liu et al., 2007). Some reports have shown that RAB23 was down-regulated in thyroid cancer (including follicular thyroid carcinoma, papillary thyroid carcinoma and follicular variant of papillary thyroid carcinoma) (Liu et al., 2007). However, RAB23 has been reported to be up-regulated in hepatocellular carcinoma and gastric cancer, correlated with clinical features and promoted the growth and invasion of cancer cells (Hou et al., 2008; Liu et al., 2007). Up to date, the expression pattern and the biological functions of RAB23 in ESCC remain unknown.

The expression of RAB23 has been regulated by miR-200b in glioma (Liu et al., 2014; Ye et al., 2014), suggesting the post-transcriptional regulation of RAB23 by microRNA. Previous study has shown that miR-92b was down-regulated in ESCC and miR-92b inhibited the invasion of ESCC cancer cells. The molecular mechanism study revealed that in ESCC cells miR-92b inhibited the expression of integrin αV and the activation of Rac1 (Ma et al., 2016). Whether miR-92b inhibited the invasion of ESCC cells by targeting other genes is still unknown.

In this study, we examined the expression of RAB23 in ESCC tissues using immunohistochemistry and western blot. We also up-regulated and down-regulated Rab23 expression in ESCC cells and examined its effect on cell proliferation and migration. In addition, we investigated the molecular mechanism and explored the regulation of RAB23 by miR-92b.

Abbreviation: ESCC, esophageal squamous cell carcinoma; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; cDNA, complementary DNA; RT-PCR, reverse transcription quantitive PCR; GFP, green fluorescence protein; PBS, phosphate-buffered saline.

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2. Materials and methods

2.1. Cell culture

Het-1A, SHEE, KYSE150, KYSE180, Eca109 and Caes17 cells were obtained from ATCC (American Type Culture Collection). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (FBS, sigma) and antibiotics (sigma). The incubator was filled with a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Patients and specimens

This study was approved by the ethic commitment of Zhengzhou University. 31 ESCC tissues and 31 adjacent non-cancerous tissues were used in this research. Tissues and paired non-cancerous tissues were stored at −80 °C in a freezer.

2.3. Plasmids construction and stable cell lines

MiR-92b was amplified by PCR and inserted into the expression vector pCDH. The coding sequence of RAB23 was amplified by PCR and cloned into the expression vector pcDNA3.1-myc. The expression vectors were transfected into KYSE180 and KYSE150 cells using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. After the selection with G418, the resistant cells were pooled and further confirmed the expression of myc-tagged RAB23 or miR-92b using western blot and qPCR, respectively.

2.4. QPCR analysis

Total RNA and miRNA were isolated from KYSE180 and KYSE150 cells. After the reverse transcription following the manufacturer's instructions, qPCR was performed using a Stratagene M Neasy mini kit or R Neasy kit (Qiagen, Hilden, Germany), respectively. Complementary DNA (cDNA) was randomly primed from 2 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription quantitive PCR (RT-PCR) was subsequently performed in triplicate with QuantiTech SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using the △Ct(U6-miR-92b)for the quantification of the relative mRNA expression level.

For the examination of RAB23 mRNA in ESCC tissues, quantitative real-time PCR was performed using SYBR Green PCR master mix (TAKARA) in a total volume of 8 μl on 7900 Real-Time PCR System (Applied Biosystems) as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s. 18S was used as the reference gene. The relative levels of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method. The sequences of primer are listed as followers: RAB23 forward primer, 5′-GTAGTAGCCGAAGTGGGA-3′; RAB23 reverse primer, 5′-CCTTTGTTTGTTGGGTCTC-3′; 18S forward primer, 5′-GAGAAACGGCTACCACATCC-3′; 18S reverse primer, 5′-CACCAGACTTGCCCTCCA-3′.

2.5. Western blot analysis

Proteins from the cell cultures and ESCC tissues were extracted using RIPA lysis buffer containing protease inhibitors and phosphotase inhibitors. After the centrifugation, the concentration of the proteins was determined using Bradford. Proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with specific antibodies. The immunoreactive protein bands were visualized by ECL kit (Pierce). Antibody to RAB23 (1:1000) was purchased from Abcam, and antibody to GAPDH (1:5000) was purchased from Santa Cruz Biotechnology. Antibodies to Snail, c-Myc (or myc tag), E-cadherin were purchased from Cell Signaling Technology. Secondary antibodies, mouse IgG and Rabbit IgG, were obtained from Cell Signaling Technology.

2.6. Immunohistochemistry

Immunohistochemistry was performed as previously described. Briefly, paraffin-embedded ESCC tissues and paired non-cancerous tissues were subjected to deparaffinization, antigen recovery and incubated with the anti-RAB23 antibody (Abcam; 1:100) for following DAB horseradish peroxidase color development.

2.7. Knocking down the expression of RAB23

The lentivirus particles used to knock down the expression of RAB23 were purchased from the GeneChem (Shanghai, China). Cells were incubated with the virus particles overnight and then selected with puromycine.

2.8. Luciferase assay

Cells were plated in 6-well plate 18 h before transfection. The reporter assays were done including 0.05 μg of Topflash reporter construct, 0.5 μg of expression vector, and 0.02 μg of pRL-TK Renilla luciferase plasmid (internal control for transfection efficiency). Cells were treated with Wnt3a protein 24 h after transfection for another 8 h. Then, cell lysates were prepared and the reporter activity was measured using the dual-luciferase reporter assay system (Promega). Relative luciferase activity was calculated as the ratio of Firefly/Renilla luciferase activity.

2.9. Crystal violet assay

The effects of RAB23 on the growth of ESCC cells were examined using crystal violet assay. Equal number of control cells and experimental cells (1000 cells/well) were seeded in 12-well plates. After 14 days of culture under the standard condition, the medium was removed and the cells were stained with 0.5% crystal violet solution in 20% methanol. After staining for 10 min, the fixed cells were washed with phosphate-buffered saline (PBS) and photographed. The colonies were solved using 1% SDS solution. OD 600 nm was measured.

2.10. Boyden chamber assay

The Boyden chamber assay was performed to examine the effects of RAB23 on the migration of the ESCC cells. Cells (2×10^5) suspended in 0.05 ml medium containing 1% FBS were placed in the upper chamber, and the lower chamber was loaded with 0.152 ml medium containing 10% FBS. 12 h later, cells migrated to the lower surface of filters was detected with traditional H&E staining. The migrated cells were counted under the inverted microscope. Four fields were counted and the average number was obtained. The experiments were repeated for three times.

2.11. MiR-92b target prediction

The computational method TargetScan was selected to predict the potential targets of miR-92b.

2.12. In vivo metastasis assay

KYSE180 cells were forced to express the luciferase gene (KYSE180-Luci). The *in vivo* imaging system (Xenogen) was used to measure the activity of luciferase after administration of luciferin. KYSE180-Luci cells overexpressing si con or si RAB23 (1×10^6 cells in 200 μl PBS) were injected into the nude mice through the tail vein. The metastatic lesions were monitored. Before mice were anesthetized with Forane (Abbott), luciferin (150 mg/kg intraperitoneally) was injected into the