ORIGINAL ARTICLE _

MANCR drives esophageal carcinoma progression by targeting PDE4D

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Summary

Purpose: To explore the role of lncRNA MANCR in regulating in vitro proliferation and apoptosis in esophageal carcinoma cells and in vivo growth of esophageal carcinoma in nude mice.

Methods: MANCR levels in 15 pairs of esophageal carcinomas and non-tumoral tissues were detected by qRT-PCR. In vitro regulations of MANCR on proliferative and apoptotic potentials in TE-1 and EC-109 cells were explored by CCK-8, colony formation assay and flow cytometry. In addition, dual-luciferase reporter assay and rescue experiments were conducted to clarify the potential mechanisms of MANCR on regulating PDE4D. Finally, in vivo role of MANCR in mediating esophageal carcinoma growth was determined in nude mice implanted with EC-109 cells.

Results: MANCR was highly expressed in esophageal carcinomas tissues than non-tumoral ones. MANCR promoted proliferative ability and inhibited apoptosis in TE-1 and EC-109 cells. In nude mice with xenografted esophageal carcinoma, knockdown of MANCR markedly slowed down tumor growth. PDE4D was the target gene binding MANCR, which was downregulated in esophageal carcinoma tissues. Its level was negatively regulated by MANCR. Importantly, PDE4D could abolish the role of MANCR in stimulating the malignant progression of esophageal carcinoma.

Conclusions: LncRNA MANCR is upregulated in esophageal carcinoma cases. Through negatively regulating PDE4D level, MANCR stimulates proliferative ability and inhibits apoptosis in esophageal carcinoma, thus driving the malignant progression.

Key words: LncRNA MANCR; PDE4D; Esophageal carcinoma; Proliferation; Apoptosis

Introduction

Esophageal carcinoma is a common upper gastrointestinal cancer. Globally, there are 482,000 new cases of esophageal carcinoma each year, ranking 8th most-common cancer. It induces 407,000 deaths annually, which is the 6th cancer killer [1,2]. The incidence of esophageal carcinoma is relatively high, causing 286,700 newly onsets and 210,900 deaths each year [3,4]. Esophageal squamous cell carcinoma and esophageal adenocarcinoma are the two major subtypes, and the former one is more common in our country [5]. Clinical symptoms of esophageal carcinoma are atypical in the early phase. With the deterioration of tumor growth, symptoms

often include dysphagia, chest pain, and weight loss [6,7]. Strong invasiveness of esophageal carcinoma results in the high mortality. Only a small number of esophageal carcinoma patients can benefit from progressed surgery, chemotherapy or radiotherapy. Postoperative metastasis and relapse of esophageal carcinoma are common, and its 5-year survival ranges 10-16% [8,9]. The extremely poor prognosis in esophageal carcinoma can be attributed to local infiltration and distant metastasis of cancer cells [9-11]. Therefore, it is of significance to clarify the mechanisms underlying malignant proliferation and metastasis in esophageal carcinoma.

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Epigenetics-associated genes are involved in the occurrence and progression of esophageal carcinoma [12]. Non-coding RNAs are largely expressed in eukaryotes. They are not translated into proteins, but induce the translation of target mR-NAs [13-15]. According to the transcript length, non-coding RNAs are classified into small RNAs and long non-coding RNAs [15]. Tumor-associated miRNAs have been well studied [16]. Recently, lncRNAs have been highlighted because their functions in regulating various biological processes, such as stress response, cell differentiation, apoptosis and pluripotency [17,18]. Dysregulated lncR-NAs will lead to pathological changes, including tumorigenesis [19,20]. Through literature review, IncRNA MANCR is involved in the progression of gastric cancer and breast cancer [21,22]. Its potential involvement in esophageal carcinoma is rarely reported.

We collected clinical samples of esophageal carcinoma and conducted tumorigenicity assay in nude mice in this trial. Biological functions of MANCR in influencing the malignant behaviors of esophageal carcinoma, and the underlying mechanisms are mainly explored. Our findings provide a potential biomarker for screening and diagnosis of esophageal carcinoma.

Methods

Clinical samples of esophageal carcinoma

Esophageal carcinomas tissues and non-tumoral ones were surgically resected from 15 patients and confirmed by H&E staining. They were stored at -80°C and used for RNA extraction. None of recruited patients had preoperative chemotherapy and they were informed that the collected tissue samples were used for experimental studies. This study complied with the Helsinki Declaration Clinical Practice Guidelines and got approval by Ethics Committee of our hospital. Tumor staging and histological subtypes of esophageal carcinoma were assessed according to the criteria proposed by Union for International Cancer Control (UICC).

Cell lines and reagents

Esophageal carcinoma cell lines (OE19, OE33, TE-1, KYSE30 and EC-109) and the normal esophageal epithelial cell line (HEEC) were obtained from ATCC. They were cultured in DMEM (Life Technologies, MD, USA) in a humidified incubator with 5% CO_2 at 37°C. 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin were supplemented.

Transfection

Cells were cultured to at least 30-60% confluence, and transfected with constructed plasmids (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, CA, USA). Transfected cells were collected at 48 h.

Cell proliferation assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

Colony formation assay

Cells were inoculated in a 6-well plate with 200 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Visible colonies were washed in PBS, fixed in methanol for 20 min and dyed in 0.1% crystal violet for 20 min, which were finally captured and calculated.

Flow cytometry

Transfected cells were incubated with Annexin V-FITC and PI in the dark following the recommendation of the commercial kit. Distributions of living cells, necrosis cells and apoptosis cells were determined by flow cytometry. Apoptosis rate was calculated as the ratio of apoptosis cell number to the total cell number.

Quantitative real-time PCR (qRT-PCR)

Cells were lysed in 1ml of TRIzol for collecting total RNAs. To clear genomic DNAs, RNAs were purified by DNase I. Reverse transcription of RNAs was conducted using Primescript RT Reagent (TaKaRa, Otsu, Japan). Subsequently, qRT-PCR was carried out using SYBR[®]Premix Ex Taq[™] (TaKaRa, Otsu, Japan) on the StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Data were processed by iQ5 2.0. Relative level was calculated by $2^{-\Delta\Delta Ct}$ method and normalized to that of GAPDH. The following plasmids were used. MANCR: Forward: 5'-ATGCCACGAGATGGAG-CAAA-3', Reverse: 5'-GTACAGCCTGCTCGGAGAAC-3'; PDE4D: Forward: 5'-AGGGACTCAGGCGTTTTGAT-3', Reverse: 5'-CGCTGTCGGATCGATACAGG-3'; GAPDH: Forward: 5'-CAGAGCTCCTCGTCTTGCC-3', Reverse: 5'-GTCGCCACCATGAGAGAC-3'.

Western blot

Cells were lysed for extracting protein samples, which were quantified by BCA method. Protein samples were electrophoresed in 10% SDS-PAGE, and loaded on PVDF membranes. Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary [anti-PDE4D (1:500) and anti-GAPDH (1:2000)] for 24 h and secondary antibody (1:1000) for 1 h. Band exposure and analyses of grey values were finally conducted.

Dual-luciferase reporter assay

Seed sequences of MANCR binding PDE4D and mutant sequences were inserted into pMIRs for constructing luciferase vectors. Cells were inoculated in 24-well plates, and co-transfected with luciferase vectors and plasmids for 48 h using Lipofectamine 2000, followed by measurement of luciferase activity.

Tumorigenicity assay

Tumorigenicity assay was approved by the Animal Ethical Committee. Nine male nude mice with 8 weeks old were randomly classified into three groups (n=3). Nude mice were implanted with EC-109 cells cotransfected with anti-NC+si-NC, anti-MANCR+si-NC or anti-MANCR+si-PDE4D through subcutaneous administration in the armpit. The width and length of the xenografted tumor were weekly recorded. Six weeks later, mice were sacrificed for harvesting the tumor tissues. Tumor volume = (width²×length)/2.

Statistical analysis

Data were expressed as mean±standard deviation and analyzed by SPSS 22.0. Pairwise differences were compared by the Student's *t*-test. The relationship between MANCR level and clinical features in esophageal carcinoma patients was analyzed by Chi-square test. Kaplan-Meier curves were depicted for survival analysis. p<0.05 was considered as statistically significant.

Results

MANCR was highly expressed in esophageal carcinoma samples

MANCR levels were detected in esophageal carcinomas and non-tumoral tissues. It was highly expressed in esophageal carcinomas tissues (Figure 1A and 1B). Besides, MANCR was upregulated in esophageal carcinoma cell lines as well (Figure 1C).

MANCR promoted proliferative ability and inhibited apoptosis in esophageal carcinoma

MANCR level was intervened in TE-1 and EC-



Figure 1. MANCR was highly expressed in esophageal carcinoma samples. **(A and B)** Differential levels of MANCR in esophageal carcinomas tissues and non-tumoral ones; **(C)** MANCR levels in esophageal carcinoma cell lines. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.



Figure 2. MANCR promoted proliferative ability and inhibited apoptosis in esophageal carcinoma. **(A and B)** Transfection efficacy of pcDNA3.1-MANCR and anti-MANCR in TE-1 and EC-109 cells, respectively; **(C and D)** Viability in TE-1 and EC-109 cells with overexpression and knockdown of MANCR, respectively; **(E and F)** Colony number in TE-1 and EC-109 cells with overexpression and knockdown of MANCR, respectively; **(G and H)** Apoptosis rate in TE-1 and EC-109 cells with overexpression and knockdown of MANCR, respectively; **(G and H)** Apoptosis rate in TE-1 and EC-109 cells with overexpression and knockdown of MANCR, respectively. Data were expressed as mean±SD. **p<0.01.

TE-1 cells overexpressing MANCR, cell viability and colony formation ability were remarkably enhanced (Figure 2C and 2E). On the contrary, knockdown of MANCR decreased viability and colony

109 cells by transfection of pcDNA3.1-MANCR or number in EC-109 cells (Figure 2D and 2F). Flow anti-MANCR, respectively (Figure 2A and 2B). In cytometry was carried out to illustrate the apoptosis in esophageal carcinoma. Apoptosis rate was reduced after overexpression of MANCR, while knockdown of MANCR obtained the opposite trend (Figure 2G and 2H).



Figure 3. MANCR bound to PDE4D. (A) Seed sequences of MANCR binding PDE4D were predicted by bioinformatics tool. (B and C) Luciferase activity in TE-1 and EC-109 cells co-transfected with pcDNA3.1-NC or pcDNA3.1-PDE4D and MANCR-WT or MANCR-MUT; (D) PDE4D level in TE-1 and EC-109 cells with overexpression and knockdown of MANCR, respectively; (E) Differential levels of PDE4D in esophageal carcinomas tissues and non-tumoral ones. Data were expressed as mean±SD. **p<0.01, ***p<0.001.



Figure 4. PDE4D was involved in MANCR-regulated esophageal carcinoma cell phenotypes. (A) Protein level of PDE4D in TE-1 and EC-109 cells regulated by both PDE4D and MANCR; (B and C) Viability in TE-1 and EC-109 cells regulated by both PDE4D and MANCR; (D and E) Apoptosis rate in TE-1 and EC-109 cells regulated by both PDE4D and MANCR. Data were expressed as mean±SD. *p<0.05, **p<0.01.



Figure 5. *In vivo* functions of MANCR and PDE4D on esophageal carcinoma growth. Nude mice were implanted with EC-109 cells co-transfected with anti-NC+si-NC, anti-MANCR+si-NC or anti-MANCR+si-PDE4D. Average tumor volume **(A)** and tumor weight **(B)** in mice of each group; PDE4D **(C)** and MANCR **(D)** levels in xenografted esophageal carcinoma tissues. Data were expressed as mean±SD. *p<0.05, **p<0.01.

MANCR bound to PDE4D

Seed sequences of MANCR binding PDE4D were predicted by bioinformatics tool (Figure 3A). Luciferase activity decreased in TE-1 and EC-109 cells co-transfected with pcDNA3.1-PDE4D and MANCR-WT. However, overexpression of PDE4D did not change luciferase activity in MANCR-MUT, proving the binding between MANCR and PDE4D (Figure 3B and 3C). Overexpression of MANCR downregulated protein and mRNA levels of PDE4D in TE-1 cells. As expected, knockdown of MANCR in EC-109 cells upregulated PDE4D (Figure 3D). In addition, PDE4D was downregulated in esophageal carcinomas tissues (Figure 3E).

PDE4D was involved in MANCR-regulated esophageal carcinoma cell phenotypes

The above data have proven the negative interaction between MANCR and PDE4D. To elucidate the role of PDE4D in esophageal carcinoma progression, co-transfection was performed. Transfection efficacy of pcDNA3.1-PDE4D and si-PDE4D was examined in TE-1 and EC-109 cells, respectively (Figure 4A). Compared with TE-1 cells overexpressing MANCR, those co-overexpressing MANCR and PDE4D presented lower viability (Figure 4B). Co-silence of MANCR and PDE4D in EC-109 cells resulted in higher proliferative ability than those with solely knockdown of MANCR (Figure 4C). In addition, PDE4D was also confirmed to abolish the regulatory effect of MANCR on cell apoptosis of esophageal carcinoma (Figure 4D and 4E).

In vivo functions of MANCR and PDE4D on esophageal carcinoma growth

In vitro influences of MANCR and PDE4D on esophageal carcinoma cell phenotypes were demonstrated. Their *in vivo* functions were thereafter determined by performing tumorigenicity assay in nude mice. Transfected EC-109 cells were implanted

in the armpit of nude mice. Compared with control mice, those administrated with MANCR-silenced EC-109 cells had lower tumor volume and tumor weight of esophageal carcinoma. Meanwhile, their tumor volume and tumor weight were also lower than those with co-silenced MANCR and PDE4D *in vivo* (Figure 5A, B). Esophageal carcinoma tissues were harvested from nude mice after sacrifice at 6 weeks. Higher level of PDE4D was detected in tumor tissues collected from mice with MANCR knockdown than those with co-silence of MANCR and PDE4D, while MANCR level was lower in the former group (Figure 5C, D).

Discussion

Although great efforts have been made on improving therapeutic strategies for esophageal carcinoma, its 5-year survival is far away from satisfy [1-3,5,6]. Local infiltration to surrounding tissues and distant metastasis are the fundamental causes for the poor prognosis in esophageal carcinoma [4-6]. Chemotherapy efficacy is limited by the adverse events, dose-dependent toxicity and extensive harm on normal cells [6-8]. Currently, molecule-targeted therapy has emerged, which has multiple advantages on cancer treatment, such as low dosage, accurate positioning on cancerous foci and less toxic [9-11].

LncRNAs contain more than 200 nucleotides that cannot be translated into proteins [12-14]. They are multi-functional in regulating mRNA degradation, chromosome remodeling and protein activities [14-16]. Previous studies have proposed the potentials of lncRNAs in early-stage diagnosis, development of target drugs and improvement of chemotherapy resistance in esophageal carcinoma [23,24]. MANCR is a newly discovered oncogenic lncRNA [21,22]. It is upregulated in many types of malignant tumors, which provides a favorable environment for malignant proliferation and differentiation [21,22]. Our results consistently detected highly expressed MANCR in esophageal carcinomas tissues. Furthermore, we explored the role of MANCR in influencing cellular functions of esophageal carcinoma. Overexpression of MANCR promoted proliferative ability and inhibited apoptosis in TE-1 cells, whereas knockdown of MANCR yielded the opposite trends in EC-109 cells.

Through bioinformatics analysis, PDE4D was the target binding MANCR. Negatively correlated to MANCR level, PDE4D was downregulated in esophageal carcinomas tissues. Notably, *in vitro* and *in vivo* regulatory effects of MANCR on the malignant progression of esophageal carcinoma could be abolished by PDE4D. We believed that

MANCR may be a promising biomarker for esophageal carcinoma.

Conclusion

LncRNA MANCR is upregulated in esophageal carcinoma cases. Through negatively regulating PDE4D level, MANCR stimulates proliferative ability and inhibits apoptosis in esophageal carcinoma, thus driving the malignant progression.

Conflict of interests

The authors declare no conflict of interests.

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