The circRNA-MYLK plays oncogenic roles in the Hep-2 cell line by sponging microRNA-145-5p

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Abstract. For the exploration of circular RNA light chain kinase (circRNA-MYLK), siRNA#1 and siRNA#2 targeting circRNA-MYLK as well as microRNA(miR)-145-5p inhibitor were transfected. Viability was valued with the CCK-8. The protein expression was examined relying on Western blot. The expression of circRNA-MYLK or miR-145-5p was tested depending on qRT-PCR. The apoptotic/migration/invasion rate was separately measured by the Annexin v-FITC/PI with flow cytometer or chambers assays. CircRNA-MYLK was overexpressed in tumor tissue. Silencing circRNA-MYLK induced the inhibitions of viability, invasion and migration, as well as the blocks of MEK/ERK and NF-κB cascades, however, silencing circRNA-MYLK led to provoking of apoptosis. Besides, circRNA-MYLK silencing stimulated the over-production of miR-145-5p, whose silencing abolished the effects of siRNA#1 and siRNA#2 of circRNA-MYLK on those factors above. The circRNA-MYLK had oncogenic roles via targeting miR-145-5p in the Hep-2 cell line via stimulating MEK/ERK and NF-κB cascades.

Key words: Laryngocarcinoma — circRNA-MYLK — miR-145-5p — MEK/ERK — NF-κB

Introduction

Laryngocarcinoma is considered as one of the most widespread tumors, and it is responsible for the high incidences mortality of tumor diseases (Lampri et al. 2015; Li et al. 2016). There are some treatment methods for this disease including the surgery, chemo-radiotherapy as well as utilization of Chinese herbs (Lambert et al. 2010; Liu et al. 2010). Nonetheless, they are restrained by poor efficiency or obvious side effects (Miao et al. 2013). Recently, numerous studies have revealed that non-coding RNAs (ncRNAs) serve essential actions in the expanding of laryngocarcinoma (Chen et al. 2018). However, the pathogenesis of laryngocarcinoma and the potential mechanism of ncRNAs have not been elucidated fully.

Lots of researches demonstrated that circular RNAs (circRNAs) could control cellular reactions and tumor progression (Chen et al. 2015, 2017). Sun and the colleagues stated that circRNAs had vital characters in the tumorigenesis of laryngeal squamous cell carcinomas (LSCC) and had potentials to function in the diagnosis and development of LSCC (Xuan et al. 2016). Moreover, it has been reported that circRNA-myosin light chain kinase (MYLK), which is derived from the MYLK gene, is over-expressed in breast cancer (BC), being connected with the stage and grade of BC (Zhong et al. 2017). What’s more, in hepatocellular carcinoma (HCC) experiment, circRNA-MYLK targeted the microRNA-362-3p (mir-362-3p)/Rab23 axis and thus to be positively related to the occurrence and expansion of this tumor (Li et al. 2019). More important, circRNA-MYLK was beneficial to the survival of prostate cancer (PCa) cells by adjusting the proliferation and migration procedures (Dai et al. 2018), indicating the possibilities that circRNA-MYLK got involved in the progression of tumors.

Over the past decades, miRNAs have been thoroughly identified as key regulators in diverse tumors, playing oncogenic or anti-tumor effects through their downstream genes or signaling cascades. For example, miR-451a was down-regulated in papillary thyroid cancers, in which abundant miR-451a could contribute to cancer cells apoptosis but
inhibit the proliferation and epithelial-mesenchymal transition (Fan et al. 2019). miR-362-3p could be a prognostic biomarker for cervical adenocarcinoma (ADC) and squamous cell carcinoma (SCC) through a regulatory network (Song et al. 2019). Importantly, the miR-145-5p had some connections with the functional mechanism of taurine up-regulated 1 (TUG1) in laryngocarcinoma. Generally TUG1 was conductive to the enhancement of laryngocarcinoma through silencing miR-145-5p (Zhuang et al. 2019). The direct modulation of the circRNA-MYLK and miR-145-5p is still uncertain.

In this current experiment, we wondered how circRNA-MYLK served in laryngocarcinoma in vitro leaning on the Hep-2 cell line.

Materials and Methods

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. We obtained informed consents from every patient, and our research was with the permission of the Medical Ethics Committee of the Weifang People's Hospital.

Clinical specimens

These material tissues were obtained from the patients of Weifang Hospital (Weifang, China) who never got treatment before. Besides, our present research was performed with the permission of every patient and the Medical Ethics Committee of the Weifang Hospital.

Cell cultivation

Hep-2 cell line from the ATCC (Manassas, Virginia, USA) was considered as the experimental object. Those cells were cultivated through the Dulbecco's modified Eagle medium (DMEM) medium (BD Biosciences, San Jose, CA, USA), including 10% serum as well as growth factors (all from Gibco, Waltham, Massachusetts, USA), at 37°C, until 80–90% attachment was observed.

Cell transfection

Small-interfering RNAs were introduced to disturb the production of circRNA-MYLK, which was individually called siRNA#1 and siRNA#2. The siRNA#1, siRNA#2 and the miR-145-5p inhibitor (Sangon Biotech, Shanghai, China) were individually synthesized to induce alterations of circRNA-MYLK and miR-145-5p in Hep-2 cell line. Reagent of lipofectamine 3000 (Roche, Basel, Switzerland) helped to perform this transfection experiment. After three days, we harvested the highest efficiency. In addition, the nucleotide sequences of siRNA#1 and siRNA#2 were as following, siRNA#1: 5'-ACT ACAGGTATGGCCTCACAA-3'; siRNA#2: 5'-TACAGGTATG-GCCTCACAAGT-3'.

Cell viability assessing

Cell viability was assessed through the CCK-8. In the beginning, Hep-2 cells were divided into different groups to undergo diverse stimulus at 37°C. After that, the CCK-8 was injected and mixed with those Hep-2 cells for another 1 h. Based on that, the suspension absorbance of Hep-2 cell line was assessed leaning on a microplate reader (Bio-Rad, Hercules, California, USA).

Apoptosis assay

Totally, those treated Hep-2 cells, which had been adjusted and cultivated in a 6-well plate, were washed and collected. Besides, adherent cells were digested by means of trypsin (Polyplus transfection, Strasbourg, Bas-Rhin, France). With the re-suspension of those cells, Annexin V-FITC and propidium iodide (PI) were mixed to incubate with the cells for 10–16 min for flow cytometer detecting avoiding light.

Migration and invasion assay

The migratory rate of Hep-2 cell line was checked by a two-chamber assay. Followed by a classical nurturing, the upper chamber was filled with cells suspension and the lower chamber was filled with the normal medium (BD Biosciences). After washing, cells were subsequently treated by methanol (Beyotime Biotechnology, Shanghai, China) and crystal violet (Sigma-Aldrich, St. Louis, Missouri, USA) (10–20 min). In the end, we counted the migrated cells under a Canon microscope (Tokyo, Japan) depending on five fields that were randomly selected. Additionally, here, the invasive capability of cells was assessed by a similar method. Generally, the participation of the matrigel glue (Millipore) was the only difference between migration detection and the invasion valuation.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

By means of lysing function of trizol reagent (Solarbio, Beijing, China) and the protective effects of DNasel (Promega,
Madison, Wisconsin, USA), total RNA were extracted from Hep-2 cells. Furthermore, the SuperScript™ III kit (Solarbio) and Taqman Master series kits (HyClone, Logan, UT, USA) were introduced to the qRT-PCR assay. We took β-actin or U6 as the internal reference. The corresponding primer sequences were including circRNA-MYLK: Forward 5'-CAGTGCTGTTTGTCA-3', Reverse 5'-TCGGAGCCTT-GACTCCAG-3'; U6: Forward 5'-CTGGTCCTCAGCACA-3', Reverse 5'-AACGCTCAAGATTTGCGT-3'; miR-145-5p: Forward 5'-ACACTC CAGCTGGGGTC-3', Reverse 5'-TGGTGTCG TG-GAGTCG-3'; GAPDH: Forward 5'-TATGATGATATCAATCATTACAAGGGTAG T-3', Reverse 5'-TGATCCTCA AACTCAT-TAC-3.

**Western blot**

We prepared the RIPA buffer (Beyotime Biotechnology) at a 4°C atmosphere in advance. Once the proteins were isolated from cells, the protease inhibitor (Qiagen, Hilden, Germany) was utilized for protein protection. Those target proteins were transferred into a PVDF membrane (HyClone) by the way of electrophoresis. The primary antibodies used here consisted of anti-Bcl-2 (ab32124, Abcam, Cambridge, UK), anti-Bax (ab32503), anti-cleaved-caspase 9 (ab3234), anti-matrix metalloproteinase (MMP) 2 (ab37150), anti-cleaved-caspase 3 (ab2302), anti-MMP 9 (ab73734), anti-inhibitor of metalloproteinase 1 (TIMP)-1 (ab38978), anti-vimentin (ab29547), anti-mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinases (ERK) kinase (MEK) (ab215263), anti-p-ERK (ab194754), anti-p-ERK (ab201015), anti-ERK (ab17942), anti-p-NF-xB-p65 (ab222494), anti-nuclear factor kappa-B (NF-xB)-p65 (ab207297) and anti-β-actin (ab16039). All of them were attached to the target protein bands at 4°C overnight, before the goat anti-rabbit (HRP) (ab7090) was introduced to the membrane at 25°C (1 h). Image Lab™ Software (Bio-Rad) was responsible for protein bands quantification.

**Statistical analysis**

The experimental analysis could be attributed to the SPSS (19.0), showing as the mean ± standard deviation (SD). t-test or analysis of variance (ANOVA) was responsible for p values, which less than 0.05 was adopted with statistically significant.

**Results**

**CircRNA-MYLK is highly expressed in tissues of patients with laryngeal cancer**

The RNA expression of circRNA-MYLK was tested leaning on qRT-PCR. By contrast, the circRNA-MYLK had higher production level in the tumor tissue (p < 0.001, Fig. 1A). Moreover, the siRNA#1 and siRNA#2 of circRNA-MYLK were produced and transfected into the Hep-2 cells. According to the detection outcomes, the production of the circRNA-MYLK was significantly constrained by the siRNA#1 (59%) and siRNA#2 (70%) (both p < 0.001, Fig. 1B). This data told that the over-production vectors were successfully constructed in the Hep-2 cells.

**Knockdown of circRNA-MYLK inhibits contributes to cell survival**

The cell viability was separately and significantly decreased about 28% or 37% due to the transfections of siRNA#1 and siRNA#2 (both p < 0.05, Fig. 2A). Additionally, the apoptotic rates (2.8 or 2.2 times) (both p < 0.001, Fig. 2B) and the generation of Bax (1.9 or 2.5 times), cleaved caspase 3 (3 or 4.5 times) and cleaved caspase 9 (3.5 or 3 times) were significantly stimulated with the inhibition of circRNA-MYLK (all p < 0.001), while the Bcl-2 was significantly and individually reduced about 61 or 57% (both p < 0.001, Fig. 2C, D). These results displayed that circRNA-MYLK was probably conductive to the viability but prevented apoptosis in Hep-2 cells.

**Knockdown of circRNA-MYLK suppresses Hep-2 cells migrations and invasion**

The migration rate (36 or 41%) (both p < 0.05, Fig. 3A) and the invasion rate (57 or 63%) (both p < 0.001, Fig. 3B), as well as the productions of the MMP-2 (39 or 48%) and MMP-9 (47 or 50%) (p < 0.05, p < 0.01 or p < 0.001) were significantly reduced by the knockdown of circRNA-MYLK (both p < 0.001).

**Figure 1.** circRNA-MYLK was over-expressed in laryngocarcinoma tissues. After the preparation, the production level of circRNA-MYLK in laryngocarcinoma or normal tissues (A), and after the transfection of siRNA#1 and siRNA#2 (B), the circRNA-MYLK level were detected by qRT-PCR. *** p < 0.001. CTRL, control; NT, none tumor; NC, negative control; T, tumor; circRNA-MYLK, circular light chain kinase; siRNA, small interfering RNA.
0.001) and the vimentin (54 or 41%) ($p < 0.05, p < 0.01$) were extremely restrained on account of the silencing of circRNA-MYLK, excluding the TIMP-1 ($p < 0.01$ or $p < 0.001$, Fig. 3C, D). All data displayed that circRNA-MYLK was probably conducive to the migrations and invasion of Hep-2 cells.

**Figure 2.** Knockdown of circRNA-MYLK inhibited cell viability but promoted apoptosis. After the transfection of siRNA#1 and siRNA#2, the cell viability (A) and the apoptotic rate (B) was explored by CCK-8 or Annexin V-FITC/PI-flow cytometer; and the apoptosis-related proteins (C, D), were examined by Western blot. $^*$ $p < 0.05$, $^{***}$ $p < 0.001$. FITC, fluorescein isothiocyanate; CCK-8, cell counting kit-8; PI, propidium iodide. For more abbreviations, see Fig. 1.

**Figure 3.** Knockdown of circRNA-MYLK suppresses Hep-2 cells migrations and invasion. After the transfection of siRNA#1 and siRNA#2, the migration rate and invasion rate were detected with migration assays (A, B) and the migration and invasion proteins were examined by Western blot (C, D). $^*$ $p < 0.05$, $^{**}$ $p < 0.01$, $^{***}$ $p < 0.001$. MMP 2/9, matrix metalloprotein-2/9; TIMP, tissue inhibitor of metalloprotease 1. For more abbreviations, see Fig. 1.
Knockdown of circRNA-MYLK induces high expression of miR-145-5p

The RNA expression level of miR-145-5p was individually and remarkably elevated about 0.89 or 1.2 times by siRNA#1 and siRNA#2 (both \( p < 0.01 \), Fig. 4A). Additionally, the miR-145-5p inhibitor led to an especially reduction of 69% of miR-145-5p \(( p < 0.01, \) Fig. 4B). All data revealed that the miR-145-5p was negatively controlled by circRNA-MYLK. The miR-145-5p silent mutant was successfully constructed.

Silencing circRNA-MYLK induces apoptosis and inhibitions of cell viability, migration and invasion through up-regulating miR-145-5p

As was shown in our investigation, miR-145-5p inhibitor apparently elevated the cell viability (both 0.19 times) (both \( p < 0.05 \), Fig. 5A). The apoptotic rate (26 or 29%) (both \( p < 0.05 \), Fig. 5B) and productions of apoptosis-related proteins such as Bax (27 or 40%) (both \( p < 0.05 \)), cleaved-caspase 3 (51 or 31%) (\( p < 0.05 \) or both \( p < 0.01 \)) and cleaved caspase 9 (60 or 50%) (\( p < 0.01 \) or \( p < 0.001 \)) (\( p < 0.05 \), \( p < 0.01 \) or \( p < 0.001 \)), excluding the Bcl-2 (0.61 or 0.73 times) (both \( p < 0.05 \)) were down-regulated by the miR-145-5p inhibitor (Fig. 5C, D). Furthermore, the migration rate (0.27 or 0.34 times) (both \( p < 0.05 \), Fig. 5E) as well as the invasion rate (0.37 or 0.66 times) (both \( p < 0.05 \), Fig. 5F) of the Hep-2 cell, and the productions of these migration or invasion-related proteins such as MMP-2 (both 0.4 times), MMP-9 (0.44 or 0.34 times) and vimentin (0.91 or 0.39 times) (all \( p < 0.05 \)) were significantly stimulated by miR-145-5p inhibitor, which induced an decline of TIMP-1 (39 or 33%) (both \( p < 0.05 \), Fig. 5G, H). Those results suggested that the miR-145-5p inhibitor significantly dropped the influences of the siRNA#1 and siRNA#2 targeting circRNA-MYLK. The miR-145-5p, which was probably a target of the circRNA-MYLK, was disadvantageous to the growth of the Hep-2 cells.

CircRNA-MYLK silencing blocks MEK/ERK and NF-kB cascades by up-regulating miR-145-5p

Obviously, the protein expression levels of the p-MEK and p-ERK (Fig. 6A), as well as the p/t-MEK (44 or 55%) and p/t-ERK (36 or 51%) (both \( p < 0.05 \), Fig. 6B) were significantly decreased by the siRNA#1 or siRNA#2 targeting circRNA-MYLK. Moreover, the influences of the siRNA#1 or siRNA#2 on the p-MEK and p-ERK, as well as the p/t-MEK (0.38 or 0.64 times) and p/t-ERK (0.72 or 0.68 times) (both \( p < 0.01 \), Fig. 6A, B) were disturbed by the miR-145-5p inhibitor with significant increase on these elements. Additionally, the productions of p-NF-kB-p65 (Fig. 6C) and p/t-NF-kB-p65 (49 or 38%) (both \( p < 0.05 \) or both \( p < 0.01 \) or \( p < 0.001 \)) were inhibited by the siRNA#1 or siRNA#2 (both \( p < 0.05 \), Fig. 6D) but were enforced by the miR-145-5p inhibitor (1.17 or 1.23 times) (\( p < 0.01 \) or \( p < 0.001 \)). These data displayed that siRNA#1 and siRNA#2 targeting circRNA-MYLK deactivated the MEK/ERK and NF-kB signaling cascades in the Hep-2 cell line by promoting miR-145-5p. We could guess that circRNA-MYLK had the potentials to trigger the signaling cascades by targeting miR-145-5p.

Discussion

We noticed that circRNA-MYLK was overexpressed in laryngocarcinoma tissues. What’s more, the viability, invasion and migration as well as the MEK/ERK and NF-kB cascades were significantly reduced in the Hep-2 cell line by siRNA#1 and siRNA#2 targeting circRNA-MYLK. Furthermore, miR-145-5p was negatively modulated by circRNA-MYLK. The anti-tumor impacts of siRNA#1 and siRNA#2 were completely eliminated by miR-145-5p inhibitor, indicating a target relationship between the circRNA-MYLK and the miR-145-5p.
Figure 5. Silencing circRNA-MYLK induced apoptosis and inhibitions of cell viability, migration and invasion via up-regulating miR-145-5p. After the transfections of relative elements, cell viability (A) and the apoptotic rate (B) was tested by CCK-8 or Annexin V-FITC/PI-flow cytometer; the migration rate and invasion rate were detected with chamber assays (E, F); the apoptosis-related proteins (C, D), and the migration and invasion proteins (G, H) were examined by Western blot. * p < 0.05, ** p < 0.01, *** p < 0.001. miR-145-5p, microRNA-145-5p; FITC, fluorescein isothiocyanate; MMP 2/9, matrix metalloprotein-2/9; TIMP, tissue inhibitor of metalloprotease 1; PI, propidium iodide; CCK-8, cell counting kit-8. For more abbreviations, see Fig. 1.
CircRNAs are ncRNAs that have a stable and conserved loop structure (Li et al. 2015). Copious evidences have demonstrated that circRNAs are significantly involved in the progressions of tumors. What’s more, circRNA-MYLK is beneficial to the survival of prostate cancer (PCa) cells by adjusting the proliferation and migration procedures, representing a relationship between the circRNA-MYLK and tumors. Our outcomes observed that deviant expression of circRNA-MYLK was related to the tumorigenesis of laryngocarcinoma. CircRNA-MYLK silencing led to evident inhibition of cells survival. Among them, the converting of the apoptosis, migration and invasion is considered as the typical feature of cancer cells, while apoptosis is cell death response with unique morphological features and evasion of apoptosis represents one of the hallmarks of cancer (Pisitkorn et al. 2016). Just like what had been proved by Li et al. (2019), that knockdown of circRNA-MYLK significantly restrained HCC cells invasion and migration in Huh7 and Hep3B cells. In addition, Dai et al. (2018) observed abundant circRNA-MYLK in PCA samples and circRNA-MYLK was overexpressed to promote cells proliferation, invasion, and migration.

What’s more, miR-145-5p is a member of the miRNAs family, and it has pivotal influences in adjusting cellular processes in diverse tumors (Mei et al. 2017; Wang et al. 2019), including LSCC. Zhang et al. found that miR-145-5p was connected with the suppression of LSCC (Gao et al. 2019). Out of question, an ingenious phenomenon was obtained in our investigation. The miR-145-5p, which was a downstream target of the circRNA-MYLK, was overexpressed to disturb the influences of siRNA#1 and siRNA#2 in Hep-2 cell line. Just like that, Zhao’s team turned out that plentiful circCEP128 modulated the miR-145-5p and the myeloid differentiation factor 88 (MyD88) to accelerate the progression of bladder cancer through MAPK cascade (Sun et al. 2019).

Being involved in the pathogenesis and development processes, ERK acts in various cancers such as prostate cancer, kidney cancer, colon cancer and esophageal squamous cell carcinoma (ESCC) (Wang et al. 2017). Moreover, the MEK-
ERK cascade was conductive to the growth of laryngeal cancer cells, which was simulated by the phosphotyrosine phosphatase 2 (SHP2) (Gu et al. 2014). SH3-domain GRB2-like 2 (SH3GL2) was implicated in the modulation of apoptosis through the MEK/ERK signaling cascade via regulating epidermal growth factor receptor (EGFR) in the Hep2 cell line (Shang et al. 2010). Besides, it had been revealed that the carboplatin combined with the 9-hydroxyphophoribde α to prevent the metastasis of laryngeal cancer due to the MEK/ERK pathway (Mao et al. 2016). It has been investigated that the NF-κB is a major factor that was able to control tumor apoptosis, angiogenesis and invasiveness (Fan et al. 2013). Moreover, NF-κB is quite implicated in the development of laryngocarcinoma. For instance, it had been revealed that testes-specific protease 50 (TSP50) silencing repressed the proliferation but induced apoptosis in HEP2 cell line in a NF-κB-mediated signaling transduction way (Zhang et al. 2013). Besides, it has been demonstrated that NF-κB silencing pathway got involved in the apoptosis modulation in laryngocarcinoma cell lines caused by the thrichosanthin (TCS) (Zhang et al. 2013).

These conclusions told information that MEK/ERK and NF-κB signaling cascades might have pivotal potentials to regulate the expansion of laryngocarcinoma. We observed in our study that knockdown of circRNA-MYLK targeted the miR-145-5p to block the MEK/ERK and NF-κB cascades. We could assume that circRNA-MYLK silencing stimulated the miR-145-5p to exert anti-tumor effects, which was relying on the blocks MEK/ERK and NF-κB pathways. This result could be partly confirmed by a few previous investigations. For example, the circRNA-MYLK was highly expressed to promote cell growth and metastasis by binding to miR-29a through the activation of relative signaling pathways in bladder carcinoma (Zhong et al. 2017). This conclusion revealed that circRNA-MYLK probably functioned in cancers or tumors by controlling signaling pathways. Based on that, circRNA-MYLK probably was a crucial factor for signaling cascades controlling in many tumors, albeit enough evidences had not been accessed. Additionally, miR-145-5p participated in the regulation of the MEK/ERK pathway and thus mediated the protective effects of GEN on H9c2 cell line (Su et al. 2018). miR-145-5p displayed suppressive influences in esophageal squamous cell carcinoma (ESCC), and the expression level of miR-145-5p in cells was tightly connected with the sensitivity of NF-κB cascade (Mei et al. 2017).

Above all, we found that circRNA-MYLK silencing might exhibit anti-tumor roles in the Hep-2 cell by enhancing miR-145-5p through the blocks of MEK/ERK and NF-κB pathways.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest. The authors declare that they have no competing interests.

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Authors’ contributions. Conceived and designed the experiments: Xiaoyan Sun. Performed the experiments and analyzed the data: Yao Chen, Yanmei Wang, Congcong Li, Xuechang Li, Tiejun Yuan, Shuqin Yang. Wrote the manuscript: Yao Chen, Xiaoyan Sun.

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