MicroRNA-520a discourages lung cancer pathogenesis and progression involving the downregulation of RRM2 and Wnt signaling deficits

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Research

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Abstract

Background

Increasingly evidence has noted the critical functions of microRNAs (miRNAs) in disease control including cancer progression. This paper aimed to explore the functions of miR-520a in lung cancer (LC) and the downstream molecules implicated.

Methods

Aberrantly expressed miRNAs in LC tissues were screened out by miRNA microarrays. miR-520a expression in LC tissues and cell lines was determined, and the correlation between miR-520a level and survival rate of patients was analyzed. Altered expression of miR-520a was introduced to evaluate its function in LC cell malignant behaviors. The target mRNA and the potential signaling pathway mediated by miR-520a were figured out. Xenograft tumors were induced in mice to test the role of miR-520a in tumorigenesis *in vivo*.

Results

Poor expression of miR-520a was found in LC tissues and cell lines. A higher miR-520a level indicated a better survival rate in LC patients. Overexpression of miR-520a led to declines in cell viability, proliferation, migration, invasion and resistance to apoptosis. The target mRNAs of miR-520a were enriched on the Wnt signaling. miR-520a inactivated the Wnt pathway. miR-520a could bind to RRM2 and downregulate RRM2 expression in LC cells. Overexpression of RRM2 promoted the malignant behaviors of cancer cells, but this promotion was inhibited by miR-520a. Overexpression of miR-520a also inhibited the tumor growth and metastasis in nude mice.

Conclusion

The present study provided evidence that miR-520a could inhibit LC progression through RRM2 down-regulation and Wnt signaling deficit. This paper may offer novel ideas concerning LC treatment.

Introduction

Cancer remains the greatest healthy concern across the globe, and lung cancer (LC) is the most prevailing cancer type which occupies nearly a quarter of cancer-related mortality according to the Cancer Statistics in 2020 [1]. According to the pathological type, LC is categorized into two main subtypes including non-small cell lung cancer (NSCLC, taking up to nearly 85% of all cases) and small cell lung cancer (15% of all cases) [2]. In China, LC has the greatest mortality rate among all cancer types [3]. Despite the improvements in traditional therapeutic regimens including surgery, radiotherapy and chemotherapy in

the past decades, the treating outcome of LC patients remains unfavorable [4]. A major reason is that a considerable number of LC patients are initially found at advanced stages with metastatic properties, and the overall 5-year survival rate of these patients are extremely low at about 5% [1]. Identifying new interventions targeting LC growth and metastasis is of great importance to offer better opportunities and hopes to LC patients.

Gene-based therapy has been a promising target for disease treatment and aroused wide concerns. Approximately 97% of all human genomes are transcribed to non-coding (nc) RNAs and can regulate the molecular processes at DNA-RNA-protein levels including in tumorigenesis, though without protein-coding capacities [5]. microRNAs (miRNAs) are a largely studied short ncRNAs which are renowned for their capacity in post-transcriptionally controlling gene expression by primarily interacting with the 3'-UTR of target mRNAs [6]. Owing to the potent gene-modifying functions, miRNAs can regulate diverse fundamental cellular processes including apoptosis, proliferation, maintenance of cell differentiation, and tumorigenesis in several cancers including LC [7]. The microarray analysis in this study identified miR-520a as an aberrantly lowly expressed miRNA in LC tissues. miR-520a has been found as a tumor inhibitor in many human malignancies. It showed inhibitory functions on proliferation, invasion and migration in gastric cancer cells by targeting spindle and kinetochore associated 2 [8]. The miR-520a-3p has been documented to be implicated in several competing endogenous RNA (ceRNA) networks (miRNAs working as sponges for other ncRNAs), whose downregulation by other RNAs may facilitate NSCLC malignancy [9, 10]. But the independent roles of miR-520a in LC and the downstream molecules involved remains largely unknown. Here, the integrated bio-information analyses suggested that ribonucleotide reductase subunit 2 (RRM2) is a potential mRNA target of miR-520a. Overexpression of RRM2 has been confirmed with oncogenic roles in many malignancies including gastric cancer [11] and pancreatic cancer [12]. Herein, we hypothesized that miRNA-520a could inhibit LC progression in tumor growth and metastasis regards, with both cell and animal experiments performed to validate this hypothesis.

Materials And Methods

Clinical sample collection

During the period from January 2015 to January 2016, 24 patients pathologically diagnosed as LC and admitted into Shandong Provincial Chest Hospital were recruited into the research. Patients with other chronic diseases, or with chemo/radio therapy history, or with a family history of malignancy were excluded. The tumor tissues and the adjacent normal tissues (over 5 cm away from tumor) were resected during surgery and instantly frozen in liquid nitrogen and then in a -80°C ice box. A three-year follow-up study was performed on each patient. This study was ratified and supervised by the Ethics Committee of Shandong Provincial Chest Hospital. Signed informed consent was collected from each eligible patient. The characteristic information of all participants is presented in Table 1.

Hematoxylin and eosin (HE) staining

The collected lung tissues from patients and liver and lung tissues from mice (see below) were embedded in paraffin and cut into slices, which were successively baked at 60°C for one hour, soaked in xylene I, II and III (10 minutes for each), soaked in different concentrations of alcohol for 5 min, washed in water for 5 minutes. Afterwards, the slices were stained in hematoxylin solution for 8 minutes, differentiated in 0.5% hydrochloric acid-ethanol mixture for 10 seconds, soaked in ammonia for 40 seconds, and then stained with 0.5% eosin for 5 minutes. The slices were washed in distilled water for 1 minute after each staining. Next, the slices were soaked in xylene and alcohol again, sealed with neutral balsam (Solarbio, Science & Technology Co., Ltd., Beijing, China), and then observed under a microscope (BX53, Olympus Optical Co., Ltd, Tokyo, Japan) with 5 random fields selected. The nuclei were stained in red, while the cytoplasm in pink. Three independent experiments were performed.

Microarray analysis

Total RNA from 15 mg either LC tissues or normal lung tissues was extracted using a miRNeasy Kit (Qiagen GmbH, Hilden, Germany) and a mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Carlsbad, CA, USA), respectively, and the RNA purity was examined by a Nano Drop (Thermo Fisher Scientific). Subsequently, 100 ng RNA was collected and labeled using a miRNA Complete Labeling and Hyb Kit (Agilent Technologies, Inc. Wakefield, MA, USA). Then, the samples were hybridized with the Human V2 mi RNA Microarrays (Agilent) at 200 rpm at 60°C for a total of 20 hours. The images were scanned and analyzed using a DNA Microarray Scanner (Agilent), while the data were obtained and analyzed by GeneSpring GX (Agilent).

Bio-information analysis

Data were analyzed using R-Language Package (version 3.6.3). The microarray results were analyzed using a limma Package (<http://www.bioconductor.org/>). Differentially expressed miRNAs were screened out with $|\log_2FC| > 2.0$ and $p < 0.01$ as the criteria, and the heatmap was produced using a pheatmap Package (<https://cran.r-project.org/web/packages/pheatmap/index.html>). Target mRNAs of miR-520 were predicted on several online systems including StarBase (<http://starbase.sysu.edu.cn/>), TargetScan (www.targetscan.org), miRDB (www.mirdb.org) and miRbase (www.mirbase.org). The predicted results were compared using a gplot package (<https://cran.r-project.org/web/packages/gplots/>), and the GO enrichment analysis was performed using a clusterProfiler package (<http://www.bioconductor.org/>).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from tissues and cells was separated by the TRIzol Reagent (Thermo Fisher). Then, the RNA was transcribed to cDNA by a high-capacity cDNA Reverse Transcription Kit (Thermo Fisher). Subsequently, real-time qPCR was performed using TaqMan microRNA assay kits (Thermo Fisher) on a 7900HT fast Real-time PCR system (Applied Biosystems, Foster City, CA). The fold change of the acquired Data was measured using the $2^{-\Delta\Delta Ct}$ method with U6 as the internal control for miRNA while GAPDH for RRM2. The primer sequences are exhibited in Table 2.

Cell culture and transfection

LC cell lines H273, H23, A549 and H460, pulmonary epithelial cell line 16HBE, and HEK293T cells were purchased from ATCC (Manassas, VA, USA). The cells were cultivated in RPMI-1640 (Gibco, Thermo Fisher) containing 10% fetal bovine serum (FBS, North Andover, MA, USA) at 37°C with 5% CO₂. The miR-520 mimic and RRM2 overexpressing (OE) vector were synthesized by RiboBio Co., Ltd. (Guangzhou, Guangdong, China). Cell transfection was performed on a Lipofectamine 2000 DNA Transfection Reagent (Thermo Fisher). In brief, when the cell confluence reached 80%, the Lipofectamine Reagent was diluted in Opti-MEM, while another Opti-MEM was used to dilute DNA. The diluted DNA was added in the diluted Lipofectamine 2000 at a ratio of 1:1 and incubated for 5 minutes. After that, the cells were added with DNA-lipid compound and cultured at 37°C for 2 days.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Well growing cells were resuspended to 5×10^4 /mL and sorted into 96-well plates at 100 µL per well till the cells reaching a density of 5×10^3 cells/well (the marginal pores were filled with sterile phosphate buffer saline (PBS) to eliminate the potential edge effect). Three duplicated groups were set for each group. The plates were incubated in a 37°C incubator with 5% CO₂. One plate was taken out for measurement every 24 hours. In brief, each well was loaded with 20 µL MTT solution (Solarbio) and then incubated for four more hours. Then, the culture medium in wells was discarded, followed by loading of 150 µL dimethyl sulphoxide to fully dissolve the crystal violet. Then the optical density at 570 nm of each well was determined using a microplate reader (Varioskan LUX, Thermo Fisher). The obtained data were analyzed to produce an MTT-proliferation curve.

5-ethynyl-2'-deoxyuridine (EdU) labeling assay

Exponentially growing cells were sorted into 6-well plates. When the confluence got to 80%, 10 µM EdU solution was loaded into each well (Genecopoeia, USA). Then the cells were incubated for 2 hours, washed in PBS, fixed in 4% paraformaldehyde for 30 minutes, incubated in glycine solution, and rinsed with PBS containing 0.5% TritonX-100. Subsequently, the cells were added with Andy Fluor™ 594 azide, then incubated in the dark for 30 minutes, washed in methanol and PBS for two times, and then loaded with 4', 6-diamidino-2-phenylindole (DAPI) and incubated for 20 minutes. The labeling was observed under a fluorescent microscope (XSP-BM13C, Shanghai CSOIF. Co., Ltd., Shanghai, China) at a 400 × magnification with 3 random fields included. The EdU-positive cells were stained in red and all cells were stained in blue by DAPI. The cell proliferation rate = number of proliferation cells/total cells × 100%. Three independent experiments were performed.

Transwell assay

The 24-well plates were used for Transwell assays. The Matrigel gel (Corning, Corning, NY, USA) was diluted in serum-free RPMI-1640 (Gibco, Thermo Fisher) at a ratio of 5:1 and then loaded onto the apical chamber (80 µL in each apical chamber) and maintained for 1-2 hours, while each basolateral chamber

was filled with 500 μ L 10% FBS-RPMI-1640. The cells were seeded into 24-well plates and incubated for 8 hours. Subsequently, the gel and cells in the apical chambers were removed, while the invaded cells were stained by crystal violet staining solution (Beyotime Biotechnology Co., Ltd., Shanghai, China) for 10 minutes. Then the staining was observed under a microscope (BX53, Olympus Optical Co., Ltd., Tokyo, Japan) at a 400 $\times$ magnification with 5 random fields selected. The number of invaded cells were photographed and analyzed, and the average value of 3 duplicated cells were evaluated. The cell migration was determined in a similar manner without Matrigel gel pre-coating in the apical chambers. The number of migrated cells in the basolateral chambers were calculated as well.

Flow cytometry

Apoptosis of cells was determined by an Annexin V-FITC Apoptosis Detection Kit (Nacalai, Tesque, Japan). The treated cells were cultivated at 37°C with 5% CO₂ for 48 hours, centrifuged at 1000 rpm for 3 minutes, washed in PBS for twice, and then centrifuged again. Then, 20 μ L Annexin V Binding Buffer (10 $\times$) was added into 200 μ L PBS. Cells were resuspended in PBS, and then filled with 10 μ L Annexin V-FITC conjugate and 5 μ L PI solution, and then cultivated at room temperature in the dark for 15 minutes. Then the cells were added with 300 μ L binding buffer, and the excitation wavelength at 488 nm was determined using a flow cytometer (Beckman Coulter, USA).

Dual luciferase reporter gene assay

The 3'UTR sequences of RRM2, SDC1, and YWHAZ containing miR-520a binding sequences were inserted to the pGL3 vectors (Ke Lei Biological Technology Co., Ltd., Shanghai, China) to construct pGL3-based wide type (WT) vectors, and the corresponding mutant type (MT) vectors were constructed as well with the mutated binding sequences. Well-constructed vectors and the reference vectors were co-transfected with miR-520a or mimic control in HEK293T cells using a Lipofectamine 2000 Kit (Thermo Fisher). Forty-eight hours later, the cells were collected and lysed, and the relative luciferase activity was evaluated using a Luciferase Reporter Assay Kit (K801-200, Biovision) and a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Three independent experiments were performed to collect the average value.

Western blot analysis

Cells were centrifuged and washed in PBS, resuspended in Tris containing protease inhibitor (50 mM Tris-HCL, 150 mM NaCl). The protein concentration was determined using a PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Then, an appropriate volume of protein was separated on 8%-12% SDS-PAGE and transferred onto PVDF membranes (LMAI Bio, Shanghai, China). The membranes were blocked and washed in 5% skimmed milk for an hour, followed by an incubation with the primary antibodies to B-cell lymphoma-2 (Bcl-2, 1:1000, sc-7382, SANTA CRUZ, CA, USA), Bcl-2-associated x, (Bax, 1:5000, sc-7480, SANTA CRUZ), caspase-3 (1:1000, ab208161, Abcam, Inc., Cambridge, MA, USA), cleaved-caspase-3 (1:500, ab2302, Abcam), β -catenin (1:1000, ab208161, Abcam), Cyclin D1 (1:500, sc-8396, SANTA CRUZ) and β -actin (1:1000, #3700, Cell Signaling Technology) at 4°C for 16 hours. After that, the cells

membranes were washed and further incubated with secondary antibodies to IgG (1:5000, ab205719, Abcam) and cleaved-caspase (1:10000, ab205718, Abcam) at 20°C for 2 hours. The protein bands were developed using enhanced chemiluminescence Western Blotting Substrate (Solarbio) and the images were taken and analyzed using a Western Blot Imaging System (FluorChem M, ProteinSimple). Three independent experiments were performed.

Xenograft tumors in nude mice

A total of 12 female nude mice (strain: BALB/c; 3-4 weeks old, 14 ± 2 g) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were fed at constant 25°C and 45% humidity with free access to food and water. The mice were randomly allocated into 4 groups (miR-520a control, miR-520a mimic × A549/H460 cells), 3 in each. As for cell implantation, the exponentially growing A549/H460 cells with stable miR-520a mimic and mimic control transfection were adjusted to 1×10^7 cells/mL. Then, each mouse was transplanted with 20 μ L cell suspension through axillary injection, and then the growth of tumor *in vivo* was photographed and recorded. The tumor volume was recorded every 7 days, which was calculated as $a \times b^2/2$ (where 'a' refers to the long diameter while 'b' refers to the short one), and a growth curve was produced. Twenty-eight days later, the mice were euthanized by overdose of 1% pentobarbital sodium (150 mg/kg), and the tumor was collected and weighed. All procedures were performed in line with the guidelines of the Animal Ethics Committee of Shandong Provincial Chest Hospital. Great attempts were made to reduce the usage and pain of animals.

Tumor metastasis *in vivo*

Another 12 nude mice were numbered by weight and allocated into 4 groups for tumor metastasis measurement. The cells with stable transfection were injected into mice through the caudal veins, and the mice were euthanized by overdose of 1% pentobarbital sodium on the 45th day post injection. Thereafter, the lung and liver tissues of mice were collected, embedded in paraffin, cut into slices, and subjected to HE staining to observe the formation of metastases. The staining was observed under the microscope with 5 non-overlapping fields included.

Statistical analysis

SPSS 22.0 (IBM Corp. Armonk, NY, USA) was used for data analysis. Measurement data were exhibited as mean $\pm$ standard derivation (SD). Data were analyzed using the *t* test (two groups) and one-way or two-way analysis of variance (ANOVA) followed with Tukey's multiple comparison test (multiple groups). The *p* value was obtained from two-tailed tests, and *p* < 0.05 was regarded to have statistical significance.

Results

miR-520a is poorly existed in LC tissues and cells

Tissue samples from 3 LC patients were collected and observed. It was found that the cells in the LC tissues presented destructed cell structure and shrinking nuclei, indicating severe lung injury (Fig. 1A). Then, the RNA in tissues was extracted for microRNA analysis, which suggested that miR-520a is notably down-regulated in tumor tissues (Fig. 1B). A similar result was found by RT-qPCR, which identified lower expression of miR-520a in LC tissues than that in the paired adjacent tissues (Fig. 1C). The following study suggested that patients with higher levels of miR-520a have better prognosis and longer lifetime (Fig. 1D). In addition, miR-520a expression in LC cell lines H273, H23, A549 and H460 and in normal pulmonary epithelial cell line 16HBE was determined as well. The RT-qPCR results suggested that all LC cell lines showed decreased miR-520a expression as relative to the 16 HBE cells (Fig. 1E). Among them, the A549 and H460 cells, with lowest expression of miR-520a, were transfected with miR-520 mimic or the mimic control for the subsequent experiments (Fig. 1F).

Artificial up-regulation of miR-520a inhibits LC progression

Following the above findings and administration, it was found that over-expression of miR-520a by miRNA mimic led to a notable decline in cell number of EdU-positive cells in both A549 and H460 cells with reduced cell viability (Fig. 2A). The MTT assay results suggested that both the number of proliferated cells and the proliferation rate of cells were reduced following miR-520a overexpression (Fig. 2B). In terms of cell migration and invasion, the Transwell assay results suggested notable declines in cell migration rate and number of migrated cells, as well as declines in number of invaded cells in both A549 and H460 cell lines following miR-520a mimic administration (Fig. 2C-D). In addition, according to the flow cytometry, it was found that miR-520 overexpression led to increased number PI-Annexin V-double-positive cells, indicating an increase in cell apoptosis rate (Fig. 2E). From the molecular perspective, it was found that miR-520a mimic led to a declined level of Bcl-2 but increased levels of Bax and cleaved caspase-3 (Fig. 2F). Collectively, these results suggested that miR-520a could inhibit the progression of LC.

miR-520a directly binds to RRM2 to mediate the Wnt signaling pathway

To further identify the potential downstream molecules implicated in the effect of miR-520a, we first predict the target mRNAs of miR-520a on StarBase, TargetScan, miRDB and miRBase, with 219 mRNAs found intersected (Fig. 3A). Then, a GO enrichment analysis was performed, which identified that the Wnt signaling was highly enriched by these genes (Fig. 3B) and a total of 18 genes were enriched in this signaling pathway. Then, the levels of Wnt signaling pathway-related factors Cyclin D1 and β -catenin in A549 and H460 cell lines were measured. The western blot analysis results identified that the Cyclin D1 and β -catenin levels in both cell lines were declined following miR-520a overexpression (Fig. 3C), indicating that miR-520a inactivated the Wnt signaling. Subsequently, we tested the expression of mRNAs enriching in the Wnt signaling in tissues using RT-qPCR. The results suggested that the expression of SDC1, YWHAZ and RRM2 was notably increased in tumor tissues as compared to the paired normal tissues (Fig. 3D). Then the luciferase assays validated that only RRM2 presented a targeting relationship with miR-520a (Fig. 3E). The correlation analysis showed an inverse relationship between miR-520a and

RRM2 in LC tumor tissues (Fig. 3F). Accordingly, the RRM2 expression was found higher in LC cell lines than that in 16HBE cells (Fig. 3G). Also, miR-520a mimic was found to decrease the RRM2 expression in A549 and H460 cells (Fig. 3H). In addition, overexpression of RRM2 in cells resulted in increased levels of Cyclin D1 and β -catenin in A549 and H460 cells (Fig. 3I), indicating that miR-520a might inactivate the Wnt signaling pathway through down-regulating RRM2 expression.

miR-520a inhibits the oncogenic effects of RRM2 on LC progression

To confirm the involvement of RRM2 in the miR-520a-mediated events in LC, A549 and H460 cells introduced with RRM2 OE vector were further transfected with miR-520a mimic (Fig. 4A). It was found that the increased cell viability by RRM2 OE vector transfection alone was reversed by further miR-520a transfection (Fig. 4B). In addition, co-transfection of miR-520a and RRM2 OE vector resulted in a decreased number in migrated cells and invaded cells, as well as increased number of apoptotic cells compared to the RRM2 OE vector transfection alone (Fig. 4C-E). These results, collectively, suggested that miR-520a directly binds to RRM2 to inhibit the malignant behaviors of LC cells.

miR-520a inhibits tumor growth and metastasis *in vivo*

A549 and H460 cell lines with stable miR-520a mimic/control transfection were implanted into nude mice. Then the tumor volume in mice was measured every 7 days, and it was found that over-expression of miR-520a led to a significant decline in tumor growth rate in mice (Fig. 5A). Twenty-eight days later when the mice were euthanized, it was found that miR-520a mimic also led to reduced tumor weight in nude mice (Fig. 5B). In addition, the HE staining results suggested that the number of metastatic nodules in lung (Fig. 5C) and liver (Fig. 5D) tissues was decreased by miR-520a mimic.

Discussion

LC has been remaining as the most prevailing cancer and consequently the biggest cause of cancer-morbidity worldwide with the survival rate of patients at late stages particularly low, leaving understanding the biology of LC and identifying novel molecules involved in pathogenesis of great urgency [13]. miRNA-targeted therapies, including miRNA replacement and miRNA reduction where oligonucleotides, virus-based constructs or small molecule compounds are administrated to restore expression of suppressive miRNAs or inhibit expression of oncogenic miRNAs [14]. The present study evidenced that miR-520a played potent tumor suppressing roles in LC development in both cell models and animal models, during which the target down-regulation of RRM2 and Wnt signaling defect were involved.

A dysregulated miRNA profiling is often linked to the onset and development of diseases including cancers. Here, a miRNA microarray analysis suggested that miR-520a was down-regulated in LC tumor samples. This was validated by RT-qPCR, which presented that miR-520a was expressed at poor levels in LC tissues and cell lines as relative to the normal ones. A higher level of miR-520a was associated with a better prognosis in LC patients, and artificial upregulation of miR-520a inhibited proliferation, migration,

invasion, and resistance to death of LC cell lines. Emerging study have emphasized the core functions of miRNAs in LC pathogenesis. For instance, miR-218 was found to act as a strong tumor suppressor in LC, inhibiting the malignant behaviors of LC cells and tumor growth by targeting interleukin-6 receptor and JAK3 [15]. On the other hand, miR-421 was found as an oncogene, which promoted proliferation and invasion potentials in NSCLC cells by targeting PCDC4 [16]. In terms of miR-520a, it was reported with tumor-inhibiting roles in many neoplastic diseases. Upregulation of miR-520a-3p by lidocaine, for example, was found to block proliferation and trigger apoptosis of colorectal cancer cells [17]. The similar trends were found in osteosarcoma cells [18] and breast cancer [19]. As for in LC, recent studies have noted the involvement of miR-520a in several ceRNA networks with tumor-suppressing functions as well [9, 10]. Here, our study validated the independent anti-cancer roles of miR-520a. In addition, the increased cleaved caspase-3 level and Bax/Bcl-2 ratio identified the pro-apoptotic role of miR-520a in a cytokine perspective. Furthermore, the growth and metastases of xenograft tumors in nude mice were also inhibited by overexpression of miR-520a.

The direct targets of miR-520a were little concerned in the above-mentioned researches concerning its role in LC. In the present study, an integrated bioinformation analysis based on the data from 4 well-known RNA prediction systems was performed, with 219 mRNAs were found intersected, namely, the common outcomes. Then a GO enrichment analysis found that the Wnt signaling pathway was enriched by these mRNAs. The Wnt signaling is important for the development and homeostasis by regulating their endogenous stem cells, whose aberrant activation has been recognized as a critical factor in the onset, maintenance and development of many cancers [20]. There is no exception for LC, in which Wnt inactivation has been reviewed as a promising therapeutic option for LC treatment [21, 22]. In addition, it was found that miR-520a led to declines in Cyclin D1 and β -catenin levels in cells, indicating the implication of Wnt defect in the miR-520a-mediated events. Furthermore, our study found that SDC1, YWHAZ and RRM2 were enriched in this signaling pathway, while only RRM2 was confirmed to have a binding relationship with miR-520a according to the luciferase assays. Then we found RRM2 was highly expressed in LC tissues and cell lines. RRM2 is a cell cycle dependent factor that was suggested to play oncogenic roles in many cancers such as adrenocortical cancer [23], glioma [24] and neuroblastoma [25]. Interestingly, RRM2 has also been involved in several ceRNA networks and its upregulation has been found to trigger cell proliferation, drug-resistance and tumor growth in LC [26, 27]. Importantly, artificial up-regulation of RRM2 in cells promoted cell viability, migration, invasion and resistance to apoptosis. Accordingly, further experiments found that overexpression of miR-520a blocked the above events, indicating that RRM2 is a downstream target of miR-520a during its regulation in LC progression.

Conclusion

To sum up, the current study evidenced that miR-520a could inhibit malignant behaviors of cells and tumor growth in LC by directly binding to RRM2 and the subsequent Wnt signaling defect. These findings can provide novel insights into the gene-based therapy for LC treatment. We also hope more researches in this field will be conducted to develop more understandings to improve the therapeutic efficacy in LC treatment.

Abbreviations

ANOVA, analysis of variance; Bax, Bcl-2-associated X; BCA, bicinchoninic acid; Bcl-2, B-cell lymphoma-2; ceRNA, competing endogenous RNA; DAPI, 4', 6-diamidino-2-phenylindole; EdU, 5-ethynyl-2'-deoxyuridine; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE staining, Hematoxylin and eosin (HE) staining; LC, lung cancer; mean $\pm$ SD, mean $\pm$ standard derivation; miRNA, microRNA; MT, mutant type; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; ncRNAs, non-coding RNAs; NSCLC, non-small cell lung cancer; RRM2, ribonucleotide reductase subunit 2; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PVDF, polyvinylidene fluoride; WT, wild type.

Declarations

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Authors' contributions

Yi Xie conceived the study and participated in its design and coordination; Congyu Xue and Yi Xie performed all experiments; Shuai Guo and Lei Yang analyzed and interpreted the data; The draft was improved through discussion and editing by all the authors who read and approved the final manuscript.

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Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

This study was ratified and supervised by the Ethics Committee of Shandong Provincial Chest Hospital. Signed informed consent was collected from each eligible patient. All procedures were performed in line with the guidelines of the Animal Ethics Committee of Shandong Provincial Chest Hospital. Great attempts were made to reduce the usage and pain of animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* 2020;70(1):7-30.
2. Qiang H, Chang Q, Xu J, Qian J, Zhang Y, Lei Y, et al. New advances in antiangiogenic combination therapeutic strategies for advanced non-small cell lung cancer. *J Cancer Res Clin Oncol.* 2020;146(3):631-645.
3. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, et al. Cancer statistics in China, 2015. *CA Cancer J Clin.* 2016;66(2):115-132.
4. Liu M, Zhang H, Li Y, Wang R, Li Y, Zhang H, et al. HOTAIR, a long noncoding RNA, is a marker of abnormal cell cycle regulation in lung cancer. *Cancer Sci.* 2018;109(9):2717-2733.
5. Braicu C, Zimta AA, Harangus A, Iurca I, Irimie A, Coza O, et al. The Function of Non-Coding RNAs in Lung Cancer Tumorigenesis. *Cancers (Basel).* 2019;11(5).
6. Harrandah AM, Mora RA, Chan EKL. Emerging microRNAs in cancer diagnosis, progression, and immune surveillance. *Cancer Lett.* 2018;438:126-132.
7. Wu KL, Tsai YM, Lien CT, Kuo PL, Hung AJ. The Roles of MicroRNA in Lung Cancer. *Int J Mol Sci.* 2019;20(7).
8. Su H, Ren F, Jiang H, Chen Y, Fan X. Upregulation of microRNA-520a-3p inhibits the proliferation, migration and invasion via spindle and kinetochore associated 2 in gastric cancer. *Oncol Lett.* 2019;18(3):3323-3330.
9. Liu Y, Lin X, Zhou S, Zhang P, Shao G, Yang Z. Long noncoding RNA HOXA-AS2 promotes non-small cell lung cancer progression by regulating miR-520a-3p. *Biosci Rep.* 2019;39(5).
10. Wan Y, Yao Z, Chen W, Li D. The lncRNA NORAD/miR-520a-3p Facilitates Malignancy in Non-Small Cell Lung Cancer via PI3k/Akt/mTOR Signaling Pathway. *Onco Targets Ther.* 2020;13:1533-1544.
11. Zhong Z, Cao Y, Yang S, Zhang S. Overexpression of RRM2 in gastric cancer cell promotes their invasiveness via AKT/NF-kappaB signaling pathway. *Pharmazie.* 2016;71(5):280-284.
12. Xia G, Wang H, Song Z, Meng Q, Huang X, Huang X. Gambogic acid sensitizes gemcitabine efficacy in pancreatic cancer by reducing the expression of ribonucleotide reductase subunit-M2 (RRM2). *J Exp Clin Cancer Res.* 2017;36(1):107.
13. Arbour KC, Riely GJ. Systemic Therapy for Locally Advanced and Metastatic Non-Small Cell Lung Cancer: A Review. *JAMA.* 2019;322(8):764-774.
14. Xue J, Yang J, Luo M, Cho WC, Liu X. MicroRNA-targeted therapeutics for lung cancer treatment. *Expert Opin Drug Discov.* 2017;12(2):141-157.
15. Yang Y, Ding L, Hu Q, Xia J, Sun J, Wang X, et al. MicroRNA-218 functions as a tumor suppressor in lung cancer by targeting IL-6/STAT3 and negatively correlates with poor prognosis. *Mol Cancer.*

- 2017;16(1):141.
16. Yang YN, Bian LQ, Ling XD, Fang CY, Jiang SL. MicroRNA-421 promotes proliferation and invasion of non-small cell lung cancer cells through targeting PDCD4. *Pathol Res Pract*. 2019;215(10):152555.
 17. Qu X, Yang L, Shi Q, Wang X, Wang D, Wu G. Lidocaine inhibits proliferation and induces apoptosis in colorectal cancer cells by upregulating mir-520a-3p and targeting EGFR. *Pathol Res Pract*. 2018;214(12):1974-1979.
 18. Zhang B, Yu L, Han N, Hu Z, Wang S, Ding L, et al. LINC01116 targets miR-520a-3p and affects IL6R to promote the proliferation and migration of osteosarcoma cells through the Jak-stat signaling pathway. *Biomed Pharmacother*. 2018;107270-282.
 19. Li J, Wei J, Mei Z, Yin Y, Li Y, Lu M, et al. Suppressing role of miR-520a-3p in breast cancer through CCND1 and CD44. *Am J Transl Res*. 2017;9(1):146-154.
 20. Duchartre Y, Kim YM, Kahn M. The Wnt signaling pathway in cancer. *Crit Rev Oncol Hematol*. 2016;99141-149.
 21. Stewart DJ. Wnt signaling pathway in non-small cell lung cancer. *J Natl Cancer Inst*. 2014;106(1):djt356.
 22. Xi Y, Chen Y. Wnt signaling pathway: implications for therapy in lung cancer and bone metastasis. *Cancer Lett*. 2014;353(1):8-16.
 23. Grolmusz VK, Karaszi K, Micsik T, Toth EA, Meszaros K, Karvaly G, et al. Cell cycle dependent RRM2 may serve as proliferation marker and pharmaceutical target in adrenocortical cancer. *Am J Cancer Res*. 2016;6(9):2041-2053.
 24. Sun H, Yang B, Zhang H, Song J, Zhang Y, Xing J, et al. RRM2 is a potential prognostic biomarker with functional significance in glioma. *Int J Biol Sci*. 2019;15(3):533-543.
 25. Li J, Pang J, Liu Y, Zhang J, Zhang C, Shen G, et al. Suppression of RRM2 inhibits cell proliferation, causes cell cycle arrest and promotes the apoptosis of human neuroblastoma cells and in human neuroblastoma RRM2 is suppressed following chemotherapy. *Oncol Rep*. 2018;40(1):355-360.
 26. Huang N, Guo W, Ren K, Li W, Jiang Y, Sun J, et al. LncRNA AFAP1-AS1 Supresses miR-139-5p and Promotes Cell Proliferation and Chemotherapy Resistance of Non-small Cell Lung Cancer by Competitively Upregulating RRM2. *Front Oncol*. 2019;91103.
 27. Yang Y, Li S, Cao J, Li Y, Hu H, Wu Z. RRM2 Regulated By LINC00667/miR-143-3p Signal Is Responsible For Non-Small Cell Lung Cancer Cell Progression. *Onco Targets Ther*. 2019;129927-9939.

Tables

Table 1 The Detailed Characteristics of 24 Patients

Item	Group	n
Gender	Male	14
	Female	10
Age	≤ 60	11
	> 60	13
TNM	I and II	5
	III and IV	19
Lymph node metastasis	Negative	6
	Positive	18
Tumor size	≤ 3cm	15
	> 3 cm	9
History of smoking	ever	16
	never	8

Note: TNM, Tumor node metastasis

Table 2 Primer sequences in RT-qPCR

Gene	Primer sequence (5'-3')
miR-520a	F: GCCACCACCATCAGCCATAC
	R: GCACATTACTCTACTCAGAAGGG
RRM2	F: CCTGACTGTAAATCGTCCAGTGGTA
	R: AGTTTGGAAGCGACTGTAGGT
U6	F: CTCGCAGCGCTTCGACA
	R: ACTTGCGGCTTCACGAATT
GAPDH	F: GATATATGATCAGTCATCTGC
	R: TTGGATGATCTGGTTTAGCG

Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; RRM2, ribonucleotide reductase subunit 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

Figures

Figure 1

miR-520a is poorly expressed in LC tissues and cells. A, LC tissues and the adjacent normal tissues observed by HE staining; B, differentially expressed miRNAs between cancer and the adjacent normal tissues screened out by miRNA microarray analysis; C, miR-520a expression in LC and the adjacent normal tissues determined by RT-qPCR (* $p < 0.05$, two-way ANOVA); D, overall survival rate of patients with different levels of miR-520a according to a 3-year followed-up study; E, miR-520a expression in LC cell lines and in normal pulmonary epithelial cell line 16HBE determined by RT-qPCR (* $p < 0.05$, one-way ANOVA); F, miR-520a expression in A549 and H460 cells after miR-520a mimic/control transfection determined by RT-qPCR (* $p < 0.05$, two-way ANOVA).

Figure 2

Artificial up-regulation of miR-520a inhibits LC progression. A, viability of A549 and H460 cells determined by EdU labeling assay (* $p < 0.05$, two-way ANOVA); B, proliferation of A549 and H460 cells after miR-520a transfection determined by MTT assay (* $p < 0.05$, two-way ANOVA); C-D, changes in number of migrated (C) and invaded cells (D) detected using the Transwell assays (* $p < 0.05$, two-way ANOVA); E, apoptosis rate of A549 and H460 cells determined by flow cytometry (* $p < 0.05$, two-way ANOVA); F, protein levels of apoptosis-related factors Bcl-2, Bax, caspase-3 and cleaved caspase-3 determined by western blot analysis (* $p < 0.05$, two-way ANOVA).

Figure 3

miR-520a directly binds to RRM2 to mediate the Wnt signaling pathway. A, a Venn diagram for the intersected target mRNAs of miR-520a predicted on StarBase, TargetScan, miRDB and miRbase; B, the enriched signaling pathway by the putative mRNAs identified by GO enrichment analysis; C, protein levels of Cyclin D1 and β -catenin in A549 and H460 cells after miR-520a mimic transfection determined by western blot analysis (* $p < 0.05$, two-way ANOVA); D, expression of Wnt-enriched mRNAs in LC tumor tissues determined by RT-qPCR (* $p < 0.05$, two-way ANOVA); E, binding relationships between miR-520a and SDC1, YWHAZ or RRM2 validated through dual-luciferase reporter gene assays (* $p < 0.05$, two-way ANOVA); F, correlation analysis of the RRM2 and miR-520a expression in tumor tissues; G, RRM2 expression in LC cell lines H273, H23, A549 and H460 and in normal pulmonary epithelial cell line 16HBE determined by RT-qPCR (* $p < 0.05$, one-way ANOVA); H, RRM2 expression in A549 and H460 cells after miR-520a mimic transfection detected by RT-qPCR (* $p < 0.05$, two-way ANOVA); I, protein levels of Cyclin

D1 and β -catenin in A549 and H460 cells after RRM2 overexpression determined by western blot analysis (* $p < 0.05$, two-way ANOVA).

Figure 4

miR-520a inhibits the oncogenic effects of RRM2 on LC progression. A, miR-520a expression in A549 and H460 cells co-transfected with RRM2-OE and miR-520a mimic/control determined by RT-qPCR; B, viability of cells determined by EdU labeling assay; C-D, number of migrated (C) and invaded (D) cells determined by Transwell assays; E, apoptosis rate of cells determined by flow cytometry; in all panels, * $p < 0.05$, two-way ANOVA.

Figure 5

miR-520a inhibits tumor formation and metastasis in nude mice. A, role of miR-520a mimic in tumor volume changes (* $p < 0.05$, two-way ANOVA); B, role of miR-520a mimic in tumor weight change (* $p < 0.05$, two-way ANOVA); C-D, formation of metastatic nodules in lung (C) and liver (D) nodules in rats (* $p < 0.05$, two-way ANOVA).