Original Article
MiR-659-3p regulates the progression of chronic myeloid leukemia by targeting SPHK1

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Abstract: Accumulating evidence shows that microRNAs (miRNAs) are significant regulators of multiple cellular processes including chronic myelocytic leukemia (CML). However, the mechanism of miR-659-3p in CML remains unclear. In this study, we aimed to investigate the potential role of miR-659-3p in CML progression. We found that miR-659-3p was down-regulated in CML. The upregulation of miR-659-3p significantly suppressed the proliferation ability, and enhanced the apoptosis capacity of K562 cells. Simultaneously, we found that sphingosine kinase 1 (SPHK1) was up-regulated in CML. MiR-659-3p performed its function through downregulating SPHK1 by binding to its untranslated region (3'-UTR). These results suggested that miR-659-3p, acted as a tumor suppressor, decreased the proliferation ability of K562 cells, and increased the apoptosis capacity of K562 cells. Therefore, our study provided a new theoretical basis of miR-659-3p which may be a promising approach for CML treatment.

Keywords: miR-659-3p, SPHK1, chronic myeloid leukemia

Introduction

Chronic myeloid leukemia (CML) is a common malignant hematopoietic disorder with the accumulation of a large number of immature white blood cells in the bone marrow and peripheral blood [1-3]. It is characterized cytogenetically by the presence of the Philadelphia (Ph) chromosome and is consistently associated with a reciprocal translocation t [9; 22] [q34; q11] [4]. Approximately 90% of patients with CML have this acquired genetic abnormality. An abnormal Breakpoint cluster region/Abelson oncogene (BCR/ABL) fusion gene which encodes a 210-Kd (P210) fusion protein with prominent tyrosine kinase activity and transforming ability is generated during the translocation [5]. The tyrosine kinase inhibitor (TKI), matinib mesylate is currently used to treat of CML [6, 7]. However, it is effective in controlling but not curing the disease. In addition, some side-effects including diarrhea and allergic reaction remain serious clinical problems [8]. Therefore, a better understanding of the molecular pathogenesis of CML and therapeutic avenues are extremely urgent.

MicroRNAs (miRNAs) are a class of short endogenous non-coding RNAs with 20-25 nucleotides in length. They participate in post-transcriptional regulation by binding to the 3'-untranslated region (3'-UTR) of target mRNAs [9] and play important roles in the biological processes of cell proliferation, differentiation, apoptosis, and invasion [10-13]. It has been confirmed that miRNAs can function as tumor suppressors or oncogenes in various cancers including CML. Much research has shown that abnormal miRNA expression may induce the occurrence of CML [14, 15]. Quan Li et al suggested that miR-130a may function as a novel tumor suppressor in CML by targeting and inhibition of RECK directly [14]. Peng Yang et al demonstrated that miR-362-5p was overexpressed in cells and clinical specimens of CML by down-regulating Growth arrest and DNA damage-inducible (GADD) 45α [16]. However, the functional relevance of miR-659-3p in carcinogenesis and progression of CML remains unclear. We used the prediction algorithms of TargetScan to determine the target genes that mediate the effects of miR-659-3p in CML and selected SPHK1, which has a conservative
miR-659-3p binding site in its 3'UTR with high specificity and is a conservative enzyme that regulates the balance between ceramide/sphingosine and sphingosine-1-phosphate (S1P) levels [14]. It has been demonstrated that SPHK1 mediated cellular behaviors plays a critical role in the regulation of cancer cell survival, proliferation and transformation [15-17]. However, it is not well documented whether SPHK1 is responsible for malignant transformation of CML.

Therefore, in this study, we provided evidence that miR-659-3p expression was significantly decreased in CML. It might function as a tumor suppressor through regulating SPHK1 expression in CML. These results indicated that SPHK1 expression was negatively correlated with the expression level of miR-659-3p in CML, and miR-659-3p down-regulated the expression of SPHK1 by directly targeting the 3'UTR of SPHK1 mRNA. We demonstrated that miR-659-3p inhibited cell proliferation, and promoted cell apoptosis by downregulating the expression of SPHK1. Therefore, miR-659-3p may be a potential biomarker for the detection and treatment of CML.

Materials and methods

Clinical specimens

Sixty-eight patients with CML and sixty-eight healthy donors were included in this study. Patients with CML were recruited from West China Hospital, Sichuan University. Healthy volunteers were recruited from the Sichuan University. The study was approved by Ethics committees of the West China Hospital, Sichuan University. Informed consent was signed by the participants.

Cell lines and transfection

K562 cell lines were purchased from College of Life Science, Hunan Normal University, China. The K562 cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) including 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO₂. For the treatment, 2 x 10⁵ K562 cells were cultured in 6-well plates and then transfected with 200 μl mature miR-659-3p mimic, or inhibitor (Gene-Pharma Co., Ltd., Shanghai, China) for 72 hrs.

All transfections were completed using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols.

Quantitative polymerase chain reaction (qPCR) assay

Total RNA was extracted from the clinical specimens and the treated K562 cells according to the manufacturer's instructions using TRIzol (Invitrogen, Carlsbad, CA, USA) A miScript II RT Kit (Qiagen, Hilden, Germany) was used to reverse transcribe RNA into cDNA and the mRNA expression levels were detected using the SYBR-Green PCR Master Mix kit (Takara). Gene mRNA expression was normalized to β-actin. The target genes and controls were analyzed by qRT-PCR and the reactions were performed on ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA) with primers specific for miR-659-3p (Qiagen, Hilden, Germany). The primer sequences for SPHK1 are: 5’-CAC TGA GCG GCG GAA CCA C-3’ (forward primer) and 5’-GCT GGA CCA CAA CGG GGG A-3’ (reverse primer); The primer sequences for GAPDH are: 5’-TGT TCG TCA TGG GTG TGA AC-3’ (forward primer) and 5’-ATG GCA TGG ACT GTG TAC AT-3’ (reverse primer); The primer sequences for miR-659-3p are: 5’-ACT GCT CGA GCA CTG TCA TTA TTT TCT CAC-3’ (forward primer) and 5’-ATG GCA TGG ACT GTG GTC AT-3’ (reverse primer); The primer sequences for U6 are: 5’-CTC GCT TCG GCA GCA CTG TCA TTA TTT TCT CAC-3’ (forward primer) and 5’-ACT GAG ATC TGC GTT CTT GGG A-3’ (reverse primer); The primer sequences for GAPDH were normalized to GAPDH. All data are performed as the mean ± SD of three independent experiments.

Western blot analysis

Total protein extracted from the treated K562 cells was separated by SDS-PAGE gels based on the molecular weight of the objective proteins. GAPDH was used as a loading control. The protein in gels was transferred to a PVDF membrane (PerkinElmer, Boston, MA). The membranes were blocked in TBST (Tris-buffered saline with 0.5% Triton X-100) containing 5% nonfat milk and probed with primary antibodies against SPHK1 (Abcam, Cambridge, MA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membranes were subsequently washed with 0.1 M
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phosphate buffered saline (PBS) with Tween-20 and then incubated with HRP-conjugated secondary antibodies. Signals were detected using the enhanced chemiluminescence western blotting system (ComWin Biotech, Beijing, China).

Luciferase reporter assay

K562 cells were cultured in a 24-well plate, cotransfected with miR-659-3p mimic, mimic negative control, miR-659-3p inhibitor or inhibitor negative control and WT or Mut 3’-UTR (Whole gene synthesis and then insertion into pGL3, Genema, Shanghai, China) of SPHK1. Cells were collected and washed using PBS and lysed in Passive Lysis Buffer (Promega, WI, USA) at 48 hrs after transfection. The Dual-Luciferase Reporter Assay System (Promega, Wisconsin, WI, USA) was used to analyze the data. Data are shown as the means ± SD of three independent experiments.

MTT assay

Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-triazolium bromide (MTT) assay. The treated cells were seeded in 6-well plates at a density of 1.5 × 10^5 cells per well with complete medium. When the cells adherent, 0.5 mg/mL MTT was added to each well and incubated at 37°C for 4 hrs. After that the supernatant was carefully aspirated and 100 μl of DMSO was added. The absorbance was measured at 490 nm (Thermo, MA, USA).

Flow cytometric analysis of the cell apoptosis

The treated K562 cells suspension was stained with Propidium iodide (PI). The flow cytometry results were analyzed using the FlowJo software. Cells were segmented into four types, such as the viable cells, dead cells, the early stage apoptotic cells, the late stage apoptotic cells, and the dead cells.

Statistical analysis

The data were analyzed by the Student’s t-test and variance (ANOVA) using SPSS 15.0 software (SPSS, Chicago, IL, USA). All results are expressed as means ± SD. P < 0.05 was considered statistically significant.

Results

Expression of miR-659-3p was decreased in CML

The expression level of miR-659-3p was detected in CML patients and healthy donors by qPCR. The results indicated that the expression level of miR-659-3p was significantly lower in CML patients than in healthy donors (***P < 0.001) (Figure 1).

miR-659-3p inhibits the proliferation abilities and promotes apoptosis in K562 cells

Relative miR-659-3p expression level was detected by qPCR. The impact of miR-659-3p expression level on cell proliferation and apoptosis was detected in K562 cells which were transfected with the miR-659-3p mimic, mimic negative control, miR-659-3p inhibitor, or inhibitor negative control. Our results found that the expression level of miR-659-3p was significantly increased in K562 cells transfected with miR-659-3p mimic negative control compared with the control group (***p < 0.001). The expression level of miR-659-3p was significantly decreased in K562 cells transfected with miR-659-3p inhibitor compared with the inhibitor negative control group (***p < 0.001) (Figure 2A). The proliferation ability of K562 cells was detected by MTT assay, and results showed that miR-659-3p inhibited the proliferation capacity of K562 cells (Figure 2B). The apoptosis
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Figure 2. Effect of miR-659-3p on the proliferative ability and apoptosis capacity of K562 cells. K562 cells were transfected with the miR-659-3p mimic, mimic negative control, miR-659-3p inhibitor or inhibitor negative control. A. Relative miR-659-3p mRNA expression level was detected by qPCR. The values of expression level are represented by the mean percent of means ± SD (**P < 0.05). B. MTT assay was performed to determine cell proliferation ability in K562 cells transfected with miR-659-3p mimic, inhibitor, or NC respectively. The values of expression level are given as mean percent of means ± SD (n = 3, **P < 0.05). C. K562 cells were transfected with miR-659-3p mimic, inhibitor or NC and cell apoptosis was detected by Annexin V-PI staining; The apoptosis capacity was significantly decreased in K562 cells transfected with mature miR-659-3p inhibitor compared with the negative control group by Annexin V-PI staining; The apoptosis...
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SPHK1 was a target gene of miR-659-3p in K562 cells

Relative mRNA expression level of SPHK1 was detected by qRT-PCR in K562 cells transfected with miR-659-3p mimic, mimic negative control, miR-659-3p inhibitor or inhibitor negative control. The results showed that the mRNA expression level of SPHK1 was significantly decreased in K562 cells transfected with mature miR-659-3p mimic compared with the control group (***P < 0.01). However, the mRNA expression level of SPHK1 was significantly increased in K562 cells transfected with miR-659-3p inhibitor compared with the control group (**P < 0.01) (Figure 4A). The western blotting experiment also showed a consistent result with qRT-PCR results (Figure 4B). SPHK1 which targeted-regulating miR-659-3p was predicted by the bioinformatic software program TargetScan. According to the results, SPHK1 has a conservative miR-659-3p binding site in its 3'UTR, and the binding to this site has high specificity (Figure 4C). Luciferase assay was performed in K562 cells, which were co-transfected with miR-659-3p and a luciferase reporter containing the full length of SPHK1 3'-UTR (Luc-wt) or a mutant (Luc-mut) in which the four nucleotides of the miR-659-3p-binding site were mutated. Luciferase intensity was measured after transfection for 48 hrs. miR-659-3p inhibitor markedly increased the relative fluorescence value in Luc-wt reporter constructs (***P < 0.001) (Figure 4D). This result confirmed that SPHK1 was a target gene of miR-659-3p in K562 cells.

miR-659-3p inhibits the progression of proliferation and promotes apoptosis of K562 cells

We further detected the effect of miR-659-3p on cell proliferation by MTT assay. Overexpression of miR-659-3p inhibited the proliferation ability of K562 cells in vitro, whereas re-overexpression of SPHK1 (without regulation sites) rescued this inhibition (Figure 5A). However, miR-659-3p inhibitors promoted the proliferation ability of K562 cells in vitro, whereas siSPHK1 further decreased this effect (Figure 5B). Overexpression of miR-659-3p increased the cell apoptosis capacity of K562 cells whereas re-overexpression of SPHK1 decreased this effect; simultaneously, miR-659-3p inhibitors decreased the apoptosis capacity of K562
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Figure 4. SPHK1 is a target gene of miR-659-3p in K562 cells. A. Relative SPHK1 mRNA expression level was detected by qPCR. B. K562 cells were transfected with miR-659-3p mimic, mimic negative control, miR-659-3p inhibitor, or inhibitor negative control, and Western blotting was used to detect the protein levels of SPHK1. GAPDH was used as control. C. WT and Mut 3’-UTR sequences of SPHK1. The wild-type and mutant SPHK1 3’-UTR contained the target sequence of miR-659-3p. D. Relative fluorescence values of SPHK1 3’-UTR (wild type and mutant constructs) were assayed after cotransfection of firefly luciferase constructs containing the SPHK1 wild-type or mutated 3’UTRs and miR-659-3p or inhibitor oligonucleotides for 24 h in K562 cells. All of the error bars indicate means ± SD. Experiments were performed in triplicate. ***P < 0.001 compared with the control group.

cells, whereas siSPHK1 rescued this inhibition (Figure 5C).

Discussion

In recent years, numerous studies have revealed the potential roles of microRNAs as oncogenes or tumor-suppressor genes in cancer by affecting cell proliferation, migration and invasion via the regulation of different target genes [18]. At present, multiple studies have revealed that miRNA expression is a crucial regulator of CML progression, and plays an important role in CML [19-21]. A better understanding of the genes or molecular alterations is likely to be useful in early detection and future targeted treatment strategies of CML. However, the roles of dysregulated miRNAs in CML carcinogenesis remain elusive.

In this study, we provided detailed mechanistic experimental evidence of miR-659-3p in CML by suppressing the expression of SPHK1. First, we found that the expression level of miR-659-3p was down-regulated in CML patients’ samples compared to controls. In addition, our observations indicated that the overexpression of miR-659-3p significantly suppressed the proliferation ability of K562 cells, and enhanced the apoptosis ability of K562 cells, whereas miR-659-3p inhibitor reversed the effects. In conclusion, we suggest that miR-659-3p acts as a novel anti-oncogenic miRNA that exerts an important effect on CML progression.
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Figure 5. Effect of miR-659-3p on proliferation and apoptosis by target SPHK1 in K562 cells. A. MTT assay was used to analyze the proliferation effect of K562 cells transfected with mimics NC, miR-659-3p mimics, miR-659-3p mimics and SPHK1. B. The proliferation ability was detected in K562 cells transfected with inhibitors NC, miR-659-3p inhibitors, miR-659-3p inhibitors and siSPHK1. C. Cell apoptosis was detected by AnnexinV-PI staining.

Then, we used the prediction algorithms of TargetScan to predict the target gene, and SPHK1 was found to be a target gene of miR-659-3p. The miR-659-3p regulated SPHK1 expression through binding to the 3’-untranslational region (3’-UTR). SPHK1 gene is located on human chromosome 17 (17q25.2) and distributed in the cytosol and the plasma membrane. Growth factors, cytokines, hormones and adhesion molecules can up-regulate SPHK1 and make it exhibit intrinsic catalytic activity [22]. A previous study has showed that SPHK1 high expression in tumors is closely associated with poor survival in glioblastoma, gastric cancer, breast cancer and cholangiocarcinoma [23]. In addition, studies have demonstrated that SPHK1 is an important enzyme encoded during neoplastic transformation [24, 25] and it plays...
a critical role in motility and invasion of some cancer cells [15-17] including K562 cells by regulating the sphingolipid rheostat. Therefore, these findings provide a powerful rationale for targeting SPHK1 in CML [26-29].

In the present study, we identified that SPHK1 serves as a direct target gene of miR-659-3p in K562 cells. SPHK1 expression was upregulated in CML, and its expression level was negatively correlated with the expression level of miR-659-3p. To further confirm that miR-659-3p can directly target SPHK1, a knockdown plasmid of SPHK1 (siSPHK1) was used. Our results suggested that miR-659-3p inhibitors promoted the in vitro and decreased the cell apoptosis capacity of K562 cells whereas si-SPHK1 decreased this effect. These results altogether suggest that miR-659-3p was an upstream regulator of SPHK1. MiR-659-3p, by targeting SPHK1, inhibited the proliferation ability of K562 cells and promoted the apoptosis capacity of K562 cells, suggesting that miR-659-3p as a tumor suppressor played a key role in the motility of CML. Therefore, miR-659-3p may provide a potential therapeutic target for CML therapy in the future.

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Disclosure of conflict of interest

None.

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