European Review for Medical and Pharmacological Sciences 2019; 23: 1203-1213

Upregulation of miR-504-3p is associated with favorable prognosis of acute myeloid leukemia and may serve as a tumor suppressor by targeting MTHFD2

S.-M. LI, Y.-Q. ZHAO, Y.-L. HAO, Y.-Y. LIANG

Department of Hematology, Jining No. 1 People's Hospital, Jining City, Shandong Province, China

Abstract. – OBJECTIVE: Deregulated expression of miRNAs contributes to the development of acute myeloid leukemia (AML). miR-504-3p, one of these miRNAs, has been found have upregulated expression in various human malignancies. Our present study aimed to detect the expression of miR-504-3p and its biological effect in AML.

PATIENTS AND METHODS: Real-time quantitative PCR was applied to evaluate the expression level of miR-504-3p in AML cell lines and the serum from AML cases. The correlations between miR-504-3p and AML patients' clinicopathological characteristics, as well as AML patients' overall survival, were statistically assessed. Moreover, we investigated the effect of miR-504-3p knockdown on AML cells by CCK-8, Transwell assays and flow cytometry, *in vitro*. The Western blot, RT-PCR and luciferase reporter assay were performed to evaluate the relationship between miR-504-3p and its downstream target genes. Finally, the biological function of MTHFD2 was also analyzed.

RESULTS: The expression levels of miR-504-3p were significantly down-regulated in the serum of AML patients and cell lines, and its low expression was positively associated with advanced clinical stages and poor prognosis of AML patients. Functional assays indicated that overexpression of miR-504-3p leads to AML cell growth arrest, invasion and migration inhibition, and elevated rates of apoptosis. We also found that miR-504-3p regulated the expression of MTHFD2 by binding to its 3'-UTR, and knockdown of MTHFD2 significantly suppressed AML cells proliferation, migration and invasion, and promoted apoptosis.

CONCLUSIONS: Our findings provide important evidence that supports the role of miR-504-3p as a tumor suppressor in AML via the inhibition of MTHFD2 expression.

Key Words
miR-504-3p, Tumor progression, AML, Prognosis, MTHFD2.

Introduction

Acute myeloid leukemia (AML) is a group of hematopoietic malignancies, which is characterized by arrested differentiation and abnormal proliferation[1,2]. AML incidence increases with age and the formation of AML has been associated with multiple factors, including radiation, chemical degradation, genovariation and viral infection[3,4]. Although AML treatment has significantly improved in recent years, with improvements in risk assessment, post-remission chemotherapy and hematopoietic stem-cell transplantation, the relapse remains a major cause of failure and the clinical outcome of pediatric AML is still poor[5,6]. Thus, the research on the molecular mechanisms underlying the progression of AML is of clinical significance for developing new therapeutic targets and approaches for AML.

MicroRNAs (miRNAs), a class of endogenous, noncoding, short (18-25 nucleotides in length), small RNA molecules, are regarded as negative regulators of gene expression at the posttranscriptional level[7]. They have been identified to repress the translations and/or promote the degradations of their target mRNAs by specifically binding to the 3' untranslated regions of the mRNAs[8,9]. Although miRNAs have been considered to be "noise", more and more evidences indicate that miRNAs involve in various cellular processes, including cell proliferation, apoptosis, cell cycle, migration and invasion, and stem cell renewal[10,11]. Interestingly, growing studies show that Growing evidences show that miRNAs modulate cell proliferation, cell cycle, apoptosis, migration, invasion, and metastasis in human cancers, such as lung cancer, cervical cancer, glioma[12-14]. On the other hand, several studies also reported that miRNAs

Corresponding Author: Yan-yan Liang, MD; e-mail: kkkshdbb7741@126.com

display functional roles in regulating tumorigenesis and progression of AML by acting as tumor suppressor or oncogenes[15,16]. Recently, although a large number of miRNAs have been reported to be dysregulated in AML, the biological function of most of them remains unknown[17,18]. MiR-504-3p, located at Xq26.3, was a novel tumor-related miRNA. Recently, its dysregulation and biological function have been reported in several tumors[19-21]. However, the role of miR-504-3p in progression of tumors was opposite in different tumors. In AML, the expression profile and biological function of miR-504-3p have not been investigated. In this study, we analyzed the expression of miR-504-3p and its clinical significance in AML patients. Furthermore, functional assays were performed to explore the function of miR-504-3p in AML cells proliferation, apoptosis, migration and invasion. To our best knowledge, this is the first study to report the role of miR-504-3p and its potential mechanism in progression of AML.

Patients and Methods

Clinical Specimens

The present study included 134 patients with AML and 41 healthy subjects. The peripheral blood of the AML patients was collected at Jining NO.1 People's Hospital from April 2010 to May 2013. The present study was approved by the Research Ethics Committee of the Jining NO.1 People's Hospital. Informed consent was obtained from all subjects involved in this study. All the patients were radiotherapy and chemotherapy naïve prior to this study. All the specimens were processed for extraction of RNA, or were stored at -80°C for later use. All the patients' clinical features were listed in Table I. The control group consisted of 41 healthy volunteers with no clinical symptoms of cancer or other diseases.

Cell Culture

The human leukemia cell lines (HL-60 and THP-1) and the human normal stromal cells HS-5 were obtained from the Chinese Academy of Sciences Cell Bank (Xuhui, Shanghai, China). The cells were all maintained in 10 cm dishes (Corning, Costar, NY, USA) in complete medium supplemented with 10% fetal bovine serum (FBS; EverGreen, Hangzhou, Zhejiang, China) containing Penicillin-Streptomycin Solution for cell culture (Procell, WuHan, Hubei, China) in a humidified air at 37°C with 5% CO_2.

Cell Transfection

The miRNA mimics including miR-504-3p mimic, negative control mimic (NC-mimic) were purchased from Genscript Co., Ltd. (Nanjing, Jiangsu, China). The siRNA specific against MTHFD2 (si-MTHFD2) and negative control siRNA (si-Control) were purchased from GeneChemCo., Ltd. (Pudong, Shanghai, China). After seeded the corresponding HL-60 or THP-1 cells in 6-well plates (Excell Bio, Taicang, Jiangsu, China), the miRNA mimics or siRNAs were transfected using Lipofectamine 3000 reagents (Invitrogen, Carlsbad, CA, USA).

Reverse Transcription-Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cultured cells or AML samples using total RNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The miRNA was isolated using miRcute miRNA isolation kit (Tiangen, Haidian, Beijing, China) in accordance with the manufacturer's instructions. Then, 2 μg total RNA were reverse transcribed to cDNA using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, Liaoning, China) and qRT-PCR assay was performed using TransStart Green qPCR SuperMix (Transgen Biotech, Haidian, Beijing, China) on a Stratagene MX3005P Real-time PCR instrument (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. For the mature miR-504-3p detection, TransScript Green miRNA Two-Step qRT-PCR SuperMix (TransGen Biotech, Haidian, Beijing, China) were applied. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. The relative expression levels of mRNA and miR-504-3p were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method. All the primers were summarized in Table II.

Western Blot Analysis

Total proteins of the HL-60 and THP-1 cells were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Excell Bio, Taicang, Jiangsu, China) containing protease inhibitor cocktail (ApexBio Technology, Yangpu, Shanghai, China). The BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was then carried to detect the concentration of proteins. After the protein lysates was electrophoresed on SDS-PAGE, they were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and then the membrane was incubated with the pri-

Table I. Association between serum miR-504-3p expression and different clinical features in pediatric AML patients.

Clinical variables	No.	Serum miR-504-3p expression		p
		High	Low	
Gender				NS
Male	71	31	40	
Female	63	37	26	
Age (year)				NS
< 20	62	28	34	
≥ 20	72	40	32	
BM blasts, %				NS
< 50	67	32	35	
≥ 50	67	36	31	
WBC counts, ×10⁹/L				NS
< 10	66	35	31	
≥ 10	68	33	35	
PLT counts, × 10⁹/L				NS
< 50	59	29	30	
≥ 50	75	39	36	
Extramedullary disease				NS
Absent	77	40	37	
Present	57	28	29	0.008
FAB subtype				
M0	10	6	4	
M1	26	17	9	
M2	51	25	26	
M4	31	11	20	
M5	16	9	7	
Cytogenetics				0.003
Favorable	47	34	13	
Intermediate	42	22	20	
Unfavorable	45	12	33	
Complete remission				0.008
Yes	88	52	36	
No	46	16	30	

mary antibodies for MTHFD2 (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (ProteinTech, Wuhan, Hubei, China). The protein bands were determined using corresponding secondary antibodies, enhanced chemiluminescence (ECL) reagents (Meilun, Dalian, Liaoning, China) and a GE Typhoon FLA9500 Gel Imaging System (GE Healthcare, Pudong, Shanghai, China). The optical density of the protein bands was analyzed by ImageJ software (NIH, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8) Assay

A CCK-8 assay kit (Yeasen, Pudong, Shanghai, China) was utilized to analyze the cell proliferation following the manufacturer's protocol. Briefly, cells were plated in 96-well plates (Corning, Costar, NY, USA) at a density of 1×10^3 cells per well. At the indicated time points, 10 µl CCK-8 solution was added into each well and the cells were continued to be cultured at 37°C for an additional 2-4 h. The absorbance of each well at the wavelength of 450 nm was then evaluated with an enzyme-linked immunosorbent assay microplate reader.

Table II. The primers for PCR.

Primer Name	Sequences
miR-504-3p: Forward	AGTGCAGGGCAGGGTTT
miR-504-3p: Reverse	TGGTGTCGTGGAGTCG
MTHFD2: Forward	GATCCTGGTTGGCGAGAATCC
MTHFD2: Reverse	TCTGGAAGAGGCAACTGAACA
GAPDH: Forward	TGGCCTTCCGTGTTCCTAC
GAPDH: Reverse	GAGTTGCTGTTGAAGTCGCA
U6: Forward	GCGCGTCGTGAAGCGTTC
U6: Reverse	GTGCAGGGTCCGAGGT

Cell Cycle and Apoptosis Detection

Cell cycle and apoptosis analyses were conducted on a BD FACSCalibur system (FACScan; BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle was examined using a cell cycle detection kit purchased from Key-Gen Biotech. Co., Ltd. (Haidian, Beijing, China). In short, the HL-60 or THP-1 cells were collected and re-suspended in solution containing 500 µl propidium iodide (PI) (50 µg/ml), 100 µg/mL RNase A and 0.2% Triton X-100 for 30 min at 4°C in the dark. Subsequently, the cells were washed with PBS and the cell cycle was measured by flow cytometry. For cell apoptosis detection, an Annexin V/PI apoptosis assay Kit (Beyotime, Pudong, Shanghai, China) was employed. Briefly, after the HL-60 or THP-1 cells were collected and re-suspended in the binding buffer, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and PI for 15-20 min in the dark at room temperature. Then, the flow cytometry was utilized to detect the apoptotic rates of HL-60 and THP-1 cells.

Transwell Assays

The migratory and invasive abilities of HL-60 and THP-1 cells were evaluated by transwell assays. The upper chambers of transwell inserts (8 µM pore sized, BD Biosciences, Franklin Lakes, NJ, USA) were coated with matrigel (1:8, BD Biosciences, Franklin Lakes, NJ, USA) for invasion assay, while migration assay without matrigel. In short, cells were collected and suspended in serum-free medium. Next, the cells were added into the upper side of the transwell chambers and the lower chambers were filled with 350 µl volume of medium supplemented with 20% fetal bovine serum (FBS) (EverGreen, Hangzhou, Zhejiang, China) as chemoattractant. After incubating for 24 h, the cells that did not invade through the membrane were removed by a cotton swab, and the cells on the bottom of the membrane were fixed with methanol (Beyotime, Pudong, Shanghai, China) and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). After washing with PBS for three times, the images were taken with a microscopy microscope (Carl Zeiss, Jena, Germany).

Luciferase Reporter Assays

The luciferase reporter assay was performed to determine whether MTHFD2 was a direct target of miR-504-3p in HL-60 and THP-1 cells. Briefly, the 3'-UTR of MTHFD2 mRNA containing wild-type (MTHFD2 WT) or mutant (MTHFD2 MUT) binding sequence of miR-504-3p were constructed into pGL3 Basic vector (Promega, Madison, WI, USA) through standard molecular cloning approaches by Generay Biotechnology Co., Ltd. (Songjiang, Shanghai, China). Subsequently, HL-60 and THP-1 cells were seeded in 24 well plates (Excell Bio, Taicang, Jiangsu, China) at the density of 1×10^5 cells per well. Then, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was utilized to transfect pGL3-MTHFD2 WT or pGL3-MTHFD2 MUT plasmids as well as miR-504-3p mimic or NC mimic. Finally, the luciferase activity was assessed by Dual Luciferase reporter assay kit (Promega Corporation, Madison, WI, USA).

Statistical Analysis

All statistical analyses were conducted using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used to compare the mean values between two groups. Comparisons between multiple groups were performed by one-way ANOVA, and comparisons between two groups were performed by the Student-Newman-Keuls (SNK) method. A chi-square test was used to analyze the relationship between miR-504-3p and various clinicopathologic parameters. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. A Cox's regression model was used for univariate and multivariate analysis. Statistically significance was considered as p-values < 0.05.

Results

Downregulation of miR-504-3p in AML Patient Samples and Cell Lines was Correlated with Pathogenic Condition and Prognosis

Firstly, we performed RT-PCT to investigate whether serum miR-504-3p expression was dysregulated in AML patients. As shown in Figure 1A, we found that the patients with AML had a lower serum miR-504-3p expression level than the healthy controls ($p<0.01$). Then, we also observed that the AML patients with the M5 subtype had lower serum miR-504-3p levels than those with other subtypes. Furthermore, our results showed that AML patients in favorable risk cytogenetic subgroup had dramatically higher serum miR-504-3p expression than those in intermediate or unfavorable risk cytogenetic subgroup (both $p<0.05$). Then, the expression levels of miR-

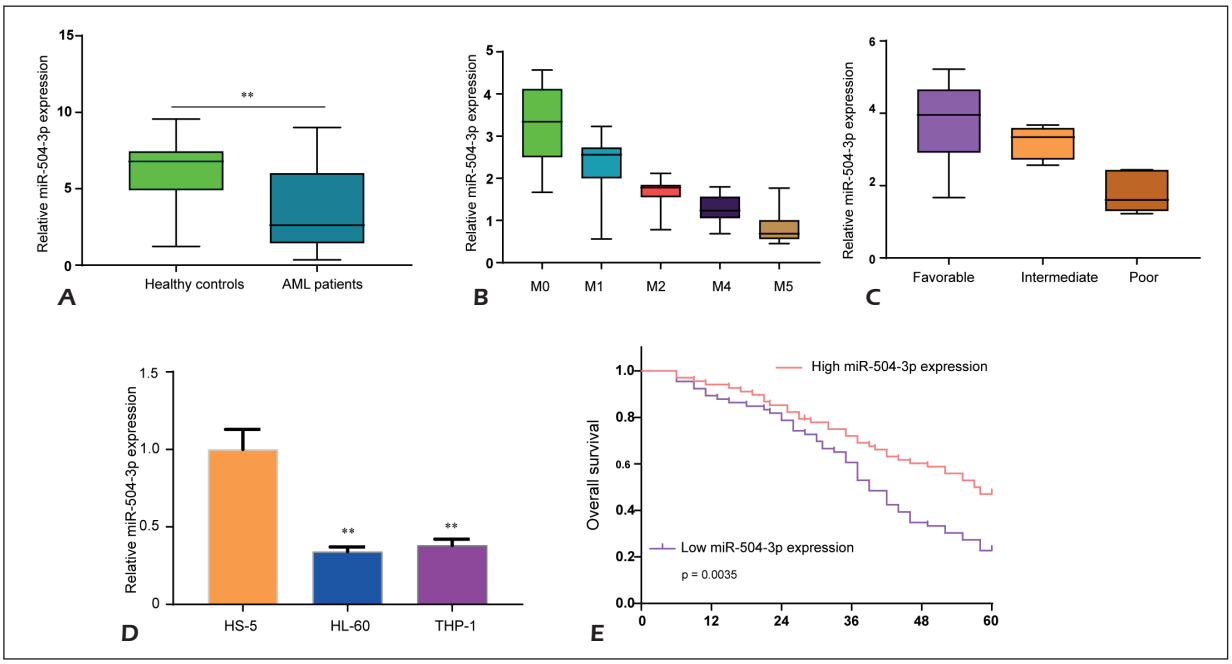

Figure 1. Down-regulated serum miR-504-3p expression was negatively correlated with prognosis of AML. **A**, Serum miR-504-3p levels in AML patients were significantly lower when compared to healthy controls. **B**, Serum miR-504-3p was reduced in AML patients with advanced stages. **C**, Serum miR-504-3p levels in pediatric AML patients with favorable risk cytogenetics were greatly higher than those with intermediate/unfavorable risk cytogenetics. **D**, Serum miR-504-3p expression was downregulated in the AML cell lines HL-60 and THP-1 compared with HS-5. **E**, Overall survival rate in patients with low serum miR-504-3p expression level was significantly worse than that in patients with high serum miR-504-3p expression level (log-rank test, p=0.0035). *p<0.05, **p<0.01.

504-3p and in two AML cell lines and HS-5 cell line were detected. As shown in Figure 1D, we found that miR-504-3p expression was significantly down-regulated in both HL-60 and THP-1. Thus, our findings suggested that dysregulation of miR-504-3p may be involved in the progression of AML. In order to study the association of miR-504-3p expression with clinicopathologic data, the expression levels of serum miR-504-3p in AML patients were categorized as low or high in relation to the mean value. As shown in Table I, we found serum miR-504-3p expression was strongly associated with FAB subtype (p=0.008), cytogenetics (p=0.003), and complete remission (CR) achieving (p=0.008). However, there was no association between serum miR-504-3p expression and other clinical features. In addition, Kaplan-Meier survival analysis and log-rank tests were carried out on the basis of post-operative survival time and patients' characteristics. As shown in Figure 1E, the results indicated that patients in the low serum miR-504-3p expression group had significantly poorer prognosis than those in high serum miR-504-3p expression group (p=0.0035). Of note, the univariate proportional hazard model also revealed a statistically significant correlation between overall survival and cytogenetics (p=0.013), complete remission (p=0.004), and miR-504-3p expression (p=0.001, Table III). More importantly, multivariate analysis confirmed that miR-504-3p expression (RR=2.945, 95% CI: 1.215-4.568, p=0.005), in addition to cytogenetics and complete remission, was an independent prognostic marker for AML patients (Table III).

The Effects of miR-504-3p on the Proliferation, Cell Cycle, Apoptosis, Migration and Invasion of HL-60 and THP-1 Cells in Vitro

Given that miR-504-3p was downregulated in AML tissues and cell lines, it is necessary to evaluate whether enhancing miR-504-3p expression could affect biological activity in AML cells. To achieve this, HL-60 and THP-1 cells were transfected with negative control miRNA mimic (NC mimic) or miR-504-3p mimic, respectively. Firstly, we performed qRT-PCR to determine the expression of miR-504-3p in HL-60 and THP-1 cells. The data demonstrated that the relative expression levels of miR-504-3p was successfully increased in HL-60 and THP-1

Table III. Univariate and multivariate analysis of overall survival in AML patients.

Variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	p	RR	95% CI	p
Gender	1.721	0.784-2.325	0.166	–	–	–
Age	1.643	0.855-2.217	0.189	–	–	–
BM blasts, %	1.544	0.578-2.132	0.245	–	–	–
WBC counts, ×109/L	2.216	1.122-2.657	0.096	–	–	–
PLT counts, × 109/L	1.423	0.739-2.234	0.162	–	–	–
Extramedullary disease	1.674	0.844-2.457	0.149	–	–	–
Cytogenetics	2.896	1.327-4.428	0.013	2.556	1.159-3.943	0.025
Complete remission	3.217	1.558-4.532	0.004	2.784	1.237-4.165	0.009
miR-504-3p expression	3.856	1.479-5.438	0.001	2.945	1.215-4.568	0.005

cells transfected with miR-504-3p mimic compared with the cells transfected with NC mimic (Figure 2A). Subsequently, CCK-8 assays were carried out to assess the effects of miR-504-3p on the proliferation of HL-60 and THP-1 cells. The results indicated that transfection of miR-504-3p mimic remarkably suppressed the proliferative rates of both HL-60 and THP-1 cells (Figure 2B and C). In addition, HL-60 and THP-1 cells transfected with miR-504-3p mimic were further determined by flow cytometry analysis to assess their cell-cycle stages and cell apoptosis. As the data shown in Figure 2D, overexpression of miR-504-3p in HL-60 and THP-1 cells was found to induce G0/G1 cell cycle arrest. The data of apoptotic analysis also revealed that transfection of miR-504-3p mimic resulted in a remarkable decline of apoptotic rates in HL-60 and THP-1 cells (Figure 2E). Moreover, the transwell migration assays suggested that the number of migrated HL-60 or THP-1 cells was significantly reduced after transfection with miR-504-3p mimic (Figure 2F). Analogously, the transwell invasion assays demonstrated that the invasive abilities of HL-60 and THP-1 cells were dramatically decreased when they were transfected with miR-504-3p mimic (Figure 2G). To sum up, these data demonstrated that overexpression miR-504-3p not only inhibited the proliferation, migration and invasion of HL-60 and THP-1 cells, but also induced the cell cycle arrest and cell apoptosis, which indicated that miR-504-3p played crucial roles in modulating the biological functions of AML cells.

MTHFD2 was the Direct Target of miR-504-3p in HL-60 and THP-1 Cells

To identify the target genes of miR-504-3p regulating, we next searched the publicly available database, miRDB (http://www.mirdb.org/), and selected MTHFD2 as the potential downstream target gene (Figure 3A). As predicted, the data of luciferase assays indicated that ectopic expression of miR-504-3p was able to abrogate the luciferase activity of HL-60 and THP-1 cells transfected with MTHFD2 wild-type 3'-UTR (MTHFD2 WT) plasmids, while there were no remarkable inhibition effects on HL-60 and THP-1 cells transfected with MTHFD2 mutant 3'-UTR (MTHFD2 MUT) plasmids (Figure 3B). Besides, qRT-PCR assays revealed that overexpression of miR-504-3p led to a significant decline of MTHFD2 mRNA expression (Figure 3C). Similarly, the results of Western blot assays also demonstrated that enhancing the expression of miR-504-3p dramatically reduced the protein expression in HL-60 and THP-1 cells (Figure 3D). Furthermore, the correlation between the expression of miR-504-3p and MTHFD2 in AML patient samples was evaluated by Pearson's correlation analysis. The results confirmed that there was a negative correlation between MTHFD2 expression and miR-504-3p expression in AML samples (Figure 3E). Collectively, our results demonstrated that miR-504-3p repressed MTHFD2 by directly binding to its 3'UTR.

Depression of MTHFD2 modulated the proliferation, cell cycle, apoptosis, migration and invasion of HL-60 and THP-1 cells

To further elucidate the effects of miR-504-3p on proliferation, cell cycle, apoptosis, migration and invasion of AML cells through MTHFD2 repression, siRNAs specific against MTHFD2 (si-MTHFD2) were transfected into HL-60 and THP-1 cells. As expected, qRT-PCR assays suggested that the si-MTHFD2 siRNAs were able to reduce

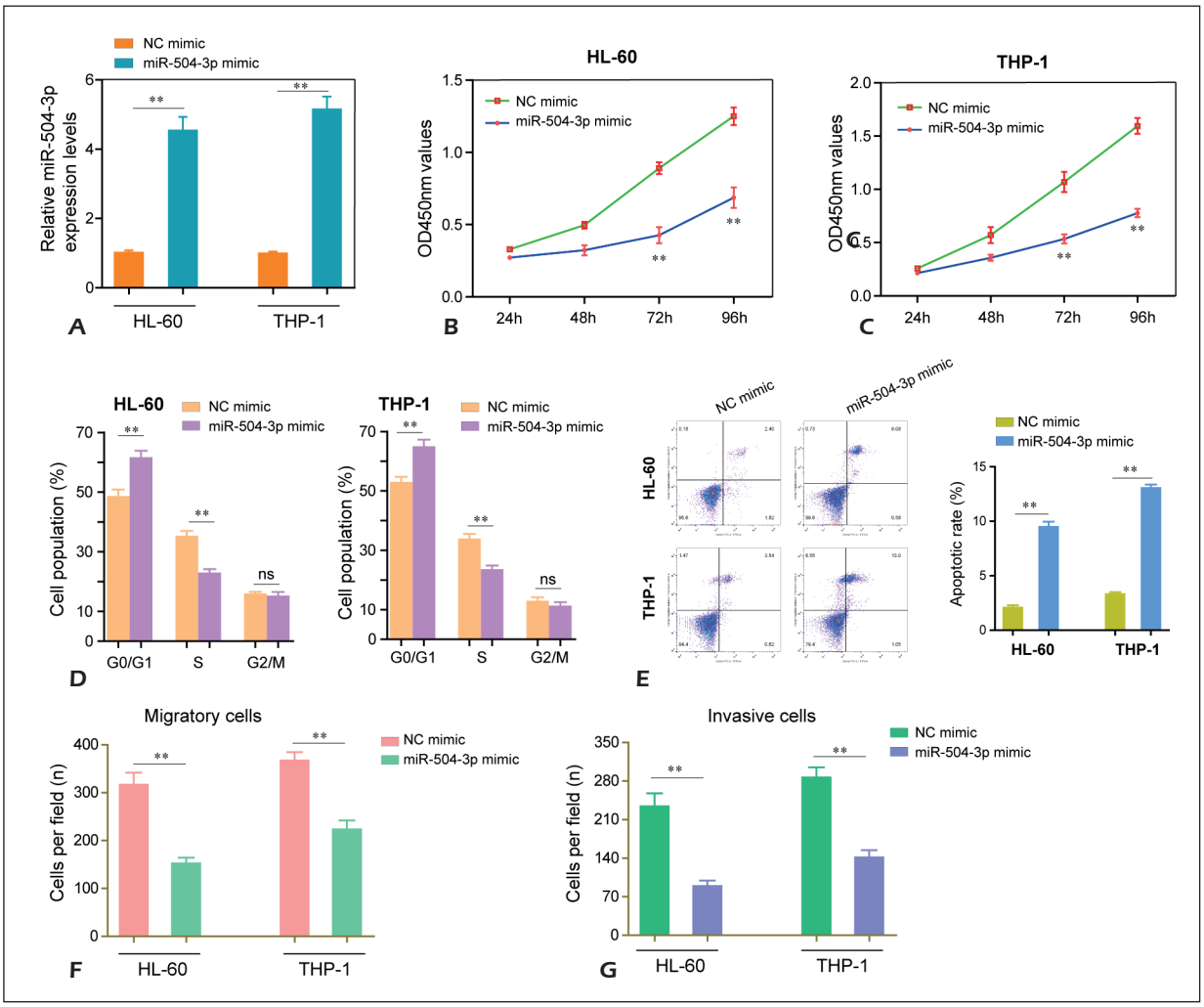

Figure 2. Overexpression of miR-504-3p affected the proliferation, cell cycle, apoptosis, migration and invasion of HL-60 and THP-1 cells. **A**, Expression of miR-504-3p in HL-60 and THP-1 cells was examined by qRT-PCR assays. (**B** and **C**) The proliferation rates of HL-60 and THP-1 cells were determined by CCK-8 assays. **D**, Cell cycle analysis of HL-60 and THP-1 cells after treatment with miR-504-3p mimic or NC mimic. **E**, The apoptotic rates of HL-60 and THP-1 cells transfected with miR-504-3p mimic or NC mimic were determined by flow cytometry analysis. **F**, Transwell analysis of HL-60 and THP-1 cells migration after transfecting with miR-504-3p mimic or NC mimic. **G**, The transwell invasion assays assessed the invasive cell number of HL-60 and THP-1 cells. *p<0.05, **p<0.01.

the relative mRNA levels effectively in AML cells (Figure 4A). Thereafter, we employed CCK-8 assays to assess the alteration of proliferative rates in HL-60 and THP-1 cells when MTHFD2 was silenced by siRNAs. As the data presented in Figure 4B and C, depletion of MTHFD2 resulted in a notable reduction of proliferation in HL-60 and THP-1 cells compared with the control cells. Additionally, the flow cytometry analysis was further conducted to evaluate the cell cycle and apoptosis of HL-60 and THP-1 cells. According to the data, knockdown of MTHFD2 markedly induced cell cycle arrest at G0/G1 stage (Figure 4D). The results of apoptosis assays by flow cytometry clearly proved that the apoptotic rates of HL-60 and THP-1 cells transfected with si-MTHFD2 were obviously increased when compared with the controls (Figure 4E). Moreover, the cell migratory and invasive capabilities were evaluated using transwell assays. The results suggested that suppressing the expression of MTHFD2 significantly reduced the migratory abilities of HL-60 and THP-1 cells (Figure 4F). Similarly, by conducting transwell invasion assays, we found that silence of MTHFD2 was able to inhibit the invasive capabilities of HL-60 and THP-1 cells (Figure 4G). Taken together, these findings suggested that repression of MTHFD2 impaired cellular proliferation and invasion in HL-

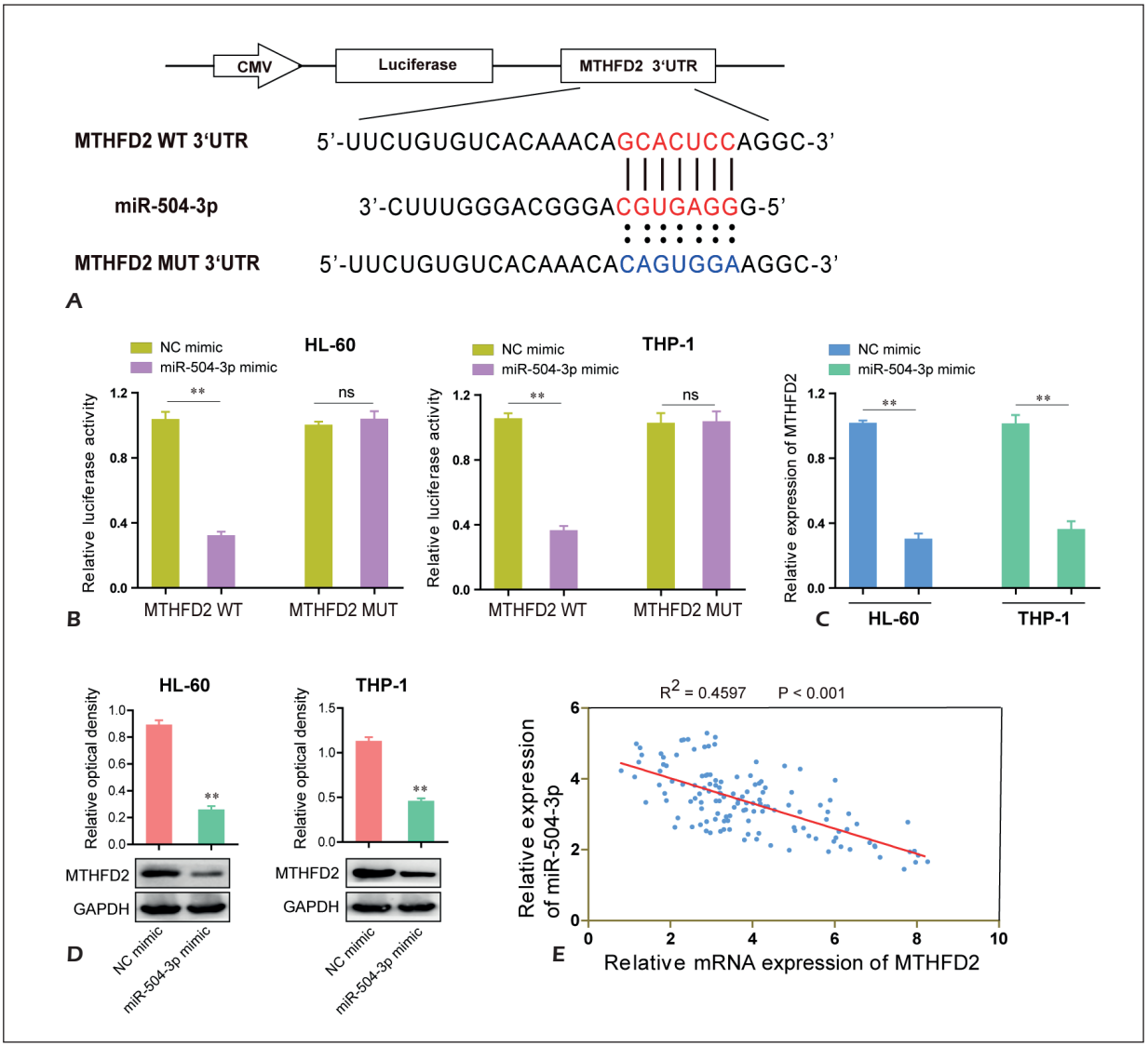

Figure 3. miR-504-3p directly targeted MTHFD2 in AML cells. **A**, The putative and mutated miR-504-3p binding sequences in the 3'-untranslated region (3'-UTR) of MTHFD2 were predicted by bioinformatics tool "miRBD". **B**, Luciferase reporter assays evaluated the luciferase activity of HL-60 and THP-1 cells under various experimental conditions. **C**, The relative mRNA expression of MTHFD2 detected by qRT-PCR assays. **D**, The protein expression of MTHFD2 detected by Western blot. **E**, The relationship between miR-504-3p and MTHFD2 expression evaluated by Pearson's correlation analysis. *p<0.05, **p<0.01.

60 and THP-1 cells, which implied that MTHFD2 was involved in modulating the development and progression of AML.

Discussion

AML is a heterogeneous disease that is associated with a very poor prognosis. Identification of novel diagnostic and prognostic biomarkers for AML patients is very important for clinical management[22,23]. Recently, the researches on the role of miRNAs highlighted its potential to be novel biomarkers for various tumors, including AML[24,25]. In this study, our attention focused on a tumor-related miRNA whose expression pattern and function in AML have not been reported. We found that miR-504-3p expression was significantly down-regulated in both AML patients and cell lines. Low serum miR-504-3p expression was positively associated with FAB subtype, cytogenetics, and complete remission (CR) achieving, suggesting that miR-504-3p may act as a negative regulator in clinical progression of AML. Clinical survival assay showed this trend that low

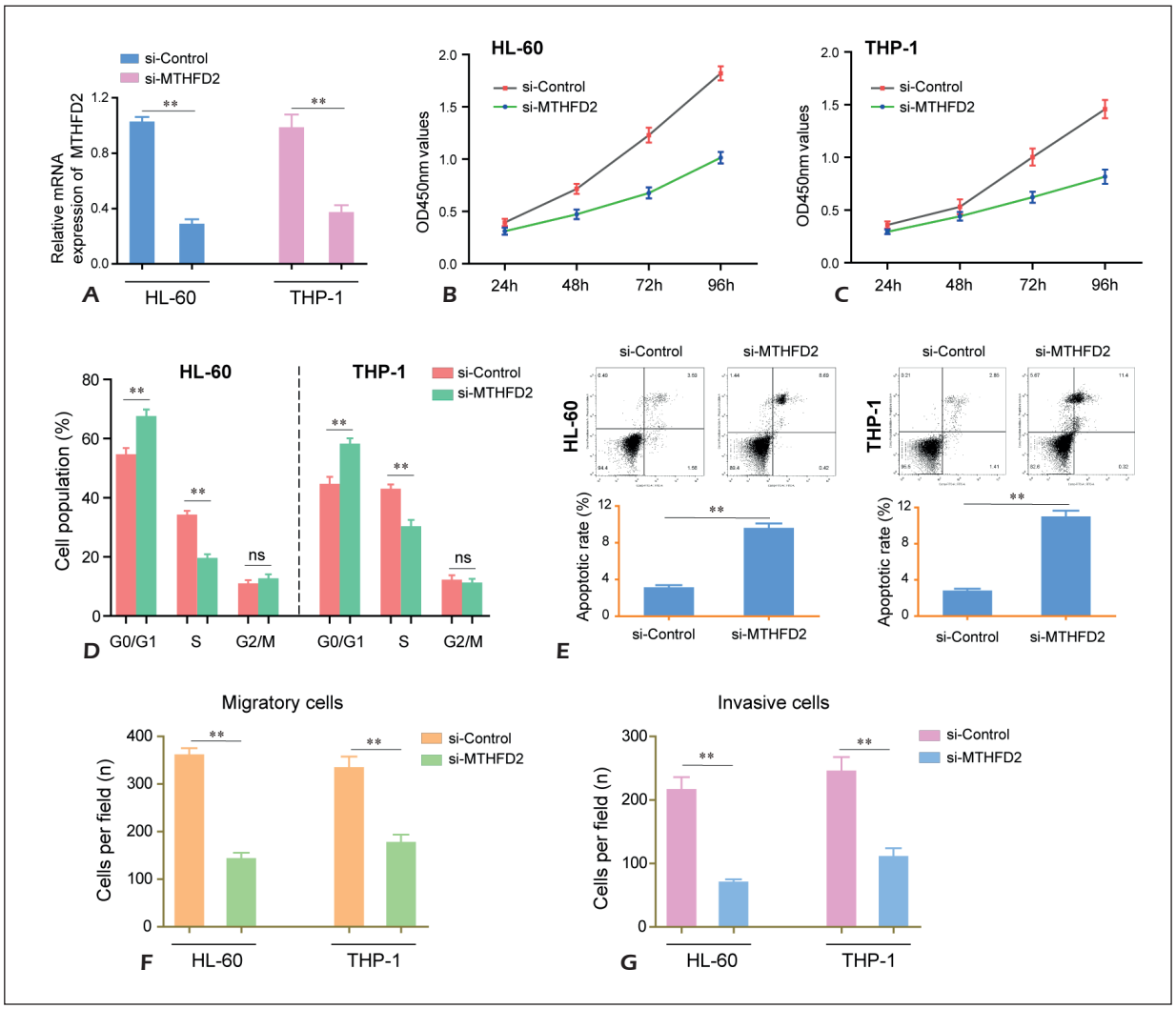

Figure 4. Knockdown of MTHFD2 inhibited cell proliferation, induced cell cycle arrest and apoptosis, and suppressed cell migration as well as invasion *in vitro*. **A**, qRT-PCR assay confirmed that MTHFD2 was downregulated in si-MTHFD2 transfected HL-60 and THP-1 cells, compared with those transfected with si-Control. (**B** and **C**) CCK-8 assays evaluated the proliferative rates of HL-60 and THP-1 cells transfected with si-MTHFD2 or si-Control. **D**, Decreased expression of MTHFD2 induced HL-60 and THP-1 cells cycle arrest at G0/G1 stage, as detected by flow cytometry. **E**, The apoptotic rates of HL-60 and THP-1 cells were evaluated by flow cytometry analysis. **F**, Downregulated MTHFD2 expression inhibited migratory capabilities of HL-60 and THP-1 cells, as determined by transwell migration assays. **G**, The invasive abilities of HL-60 and THP-1 cells assessed using transwell invasion assays. **p*<0.05, ***p*<0.01.

serum miR-504-3p expression was associated shorter overall survival. Most importantly, the results of multivariate analysis revealed that miR-504-3p expression could be an independent prognostic marker for AML patients. To our best knowledge, this study is the first to report that miR-504-3p was frequently dysregulated in AML and may be used as a potential prognostic biomarker.

miR-504-3p, located at Xq26.3, has been shown to be involved in various biological processes, including adipogenesis, angiogenesis and proliferation and cell cycle[26,27]. Growing evidence indicated that miR-504-3p acted as a tumor suppressor or oncogene in several tumors. For instance, Quan et al[28] reported that miR-504 levels were significantly down-regulated in hepatocellular carcinoma and served as a tumor suppressor by inhibiting the proliferation and invasion in hepatocellular carcinoma cell lines via modulating Frizzled-7-mediated-Wnt/β-catenin signaling. Ye et al[29] showed that miR-504 was notably downregulated in non-small cell lung cancer and associated with advanced clinical stages. *In vitro* assay revealed that overexpression of miR-504 could inhibits cell prolif-

eration and invasion by targeting LOXL2. On the other hand, Cai et al[30] indicated that miR-504 was highly expressed in osteosarcoma and its forced expression promotes tumour growth and metastasis, and induces apoptosis in human osteosarcoma by targeting TP53INP1 both *in vitro* and in *vivo*. Those results indicated that the role of miR-504 in different tumor was varied. In this study, based on clinical data, we hypothesized miR-504-3p as a tumor suppressor in AML. Then, we performed gain-of-function experiments. Our results indicated that ectopic expression of miR-504-3p suppressed the proliferation, migration and invasion of AML cells. Our results together with previous studies indicated that miR-504-3p functioned as a tumor suppressor miRNA in AML.

Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), located on chromosome 2, is a mitochondrial enzyme with methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase activities[31,32]. MTHFD2 is the most distinctively expressed metabolic enzyme between cancer cells and normal cells, including normal proliferating cells[33]. Scholars[34,35] showed that MTHFD2 was frequently highly expressed in several tumors and may serve as a tumor promoter by promoting cells proliferation. Of note, knockdown of MTHFD2 in AML cells decreased growth, induced differentiation, and impaired colony formation in primary AML blasts, suggesting that it have potential to be a drug targeting[36,37]. In this work, bioinformatics analysis from TargetScan database indicated that MTHFD2 was one of miR-504-3p's targets and this was confirmed by luciferase reporter assay and Western blot assay. For the first time, we found that MTHFD2 was inversely correlated with miR-504-3p levels. The rescue assay confirmed that up-regulation of MTHFD2 could to some extent offset miR-504-3p's tumor suppressive effects. Taken together, miR-504-3p acted as a tumor suppressor in gastric cancer by downregulating MTHFD2.

Conclusions

To the best of our knowledge, the present study was the first to indicate that miR-504-3p was significantly in AML patients and was also significantly associated with aggressive tumor phenotype and poor prognosis in patients with AML. Overexpression of miR-504-3p significantly suppressed AML cells proliferation and metastasis by targeting MTHFD2. Thus, our findings provided basic information to better understand AML biology and could help to facilitate the development of miRNA-based anti-tumor strategies.

Conflict of Interests

The Authors declare that there are no conflicts of interest.

References

1) Haouas H. Angiogenesis and acute myeloid leukemia. Hematology 2014; 19: 311-323.
2) Rashidi A, Fisher SI. Acute myeloid leukemia following solid organ transplantation: entity or novelty? Eur J Haematol 2014; 92: 459-466.
3) Rowe JM. The increasing genomic complexity of acute myeloid leukemia. Best Pract Res Clin Haematol 2014; 27: 209-213.
4) Al-Hussaini M, DiPersio JF. Small molecule inhibitors in acute myeloid leukemia: from the bench to the clinic. Expert Rev Hematol 2014; 7: 439-464.
5) Estey EH. Acute myeloid leukemia: 2014 update on risk-stratification and management. Am J Hematol 2014; 89: 1063-1081.
6) Roboz GJ. Current treatment of acute myeloid leukemia. Curr Opin Oncol 2012; 24: 711-719.
7) Bak RO, Mikkelsen JG. miRNA sponges: soaking up miRNAs for regulation of gene expression. Wiley Interdiscip Rev RNA 2014; 5: 317-333.
8) Bekris LM, Leverenz JB. The biomarker and therapeutic potential of miRNA in Alzheimer's disease. Neurodegener Dis Manag 2015; 5: 61-74.
9) Sand M. The pathway of miRNA maturation. Methods Mol Biol 2014; 1095: 3-10.
10) Tutar Y. miRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol 2014; 15: 429.
11) Hammond SM. An overview of microRNAs. Adv Drug Deliv Rev 2015; 87: 3-14.
12) Sun Q, Jiang CW, Tan ZH, Hou LY, Dong H, Liu K, Sun G, Liu YJ, Wang YQ, Lu XC, Liu H. MiR-222 promotes proliferation, migration and invasion of lung adenocarcinoma cells by targeting ETS1. Eur Rev Med Pharmacol Sci 2017; 21: 2385-2391.
13) Wei WF, Zhou CF, Wu XG, He LN, Wu LF, Chen XJ, Yan RM, Zhong M, Yu YH, Liang L, Wang W. MicroRNA-221-3p, a TWIST2 target, promotes cervical cancer metastasis by directly targeting THBS2. Cell Death Dis 2017; 8: 3220.
14) Bai QL, Hu CW, Wang XR, Shang JX, Yin GF. MiR-616 promotes proliferation and inhibits apoptosis in glioma cells by suppressing expression of SOX7 via the Wnt signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 5630-5637.
15) Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol 2014; 9: 287-314.
16) Yan TH, Qiu C, Sun J, Li WH. MiR-877-5p suppresses cell growth, migration and invasion by

targeting cyclin dependent kinase 14 and predicts prognosis in hepatocellular carcinoma. Eur Rev Med Pharmacol Sci 2018; 22: 3038-3046.

17) Hartmann JU, Brauer-Hartmann D, Kardosova M, Wurm AA, Wilke F, Schodel C, Gerloff D, Katzerke C, Krakowsky R, Namasu CY, Bill M, Schwind S, Muller-Tidow C, Niederwieser D, Alberich-Jorda M, Behre G. MicroRNA-143 targets ERK5 in granulopoiesis and predicts outcome of patients with acute myeloid leukemia. Cell Death Dis 2018; 9: 814.

18) Han GT, Sun ZL. Up-regulation of serum miR-4262 predicts clinical outcome of patients with acute myeloid leukemia. Eur Rev Med Pharmacol Sci 2017; 21: 2172-2176.

19) Cui R, Guan Y, Sun C, Chen L, Bao Y, Li G, Qiu B, Meng X, Pang C, Wang Y. A tumor-suppressive microRNA, miR-504, inhibits cell proliferation and promotes apoptosis by targeting FOXP1 in human glioma. Cancer Lett 2016; 374: 1-11.

20) Zhao L, Tang M, Hu Z, Yan B, Pi W, Li Z, Zhang J, Zhang L, Jiang W, Li G, Qiu Y, Hu F, Liu F, Lu J, Chen X, Xiao L, Xu Z, Tao Y, Yang L, Bode AM, Dong Z, Zhou J, Fan J, Sun L, Cao Y. miR-504 mediated down-regulation of nuclear respiratory factor 1 leads to radio-resistance in nasopharyngeal carcinoma. Oncotarget 2015; 6: 15995-16018.

21) Soutto M, Chen Z, Saleh MA, Katsha A, Zhu S, Zaika A, Belkhiri A, El-Rifai W. TFF1 activates p53 through down-regulation of miR-504 in gastric cancer. Oncotarget 2014; 5: 5663-5673.

22) Weisberg E, Sattler M, Manley PW, Griffin JD. Spotlight on midostaurin in the treatment of FLT3-mutated acute myeloid leukemia and systemic mastocytosis: design, development, and potential place in therapy. Onco Targets Ther 2018; 11: 175-182.

23) Zhang Q, Dai K, Bi L, Jiang S, Han Y, Yu K, Zhang S. Pretreatment platelet count predicts survival outcome of patients with de novo non-M3 acute myeloid leukemia. PeerJ 2017; 5: e4139.

24) Weng H, Lal K, Yang FF, Chen J. The pathological role and prognostic impact of miR-181 in acute myeloid leukemia. Cancer Genet 2015; 208: 225-229.

25) Zhang X, Wang Y, Guo Q, Diao Y, Liu H, Song G, Wang W, Zhang Z, Yin H, Li L. Prognostic role of microRNA-155 in patients with leukemia: a meta-analysis. Clin Chim Acta 2018; 483: 6-13.

26) Yang MH, Lin BR, Chang CH, Chen ST, Lin SK, Kuo MY, Jeng YM, Kuo ML, Chang CC. Connective tissue growth factor modulates oral squamous cell carcinoma invasion by activating a miR-504/FOXP1 signalling. Oncogene 2012; 31: 2401-2411.

27) Hu W, Chan CS, Wu R, Zhang C, Sun Y, Song JS, Tang LH, Levine AJ, Feng Z. Negative regulation of tumor suppressor p53 by microRNA miR-504. Mol Cell 2010; 38: 689-699.

28) Quan H, Li B, Yang J. MicroRNA-504 functions as a tumor suppressor in hepatocellular carcinoma through inhibiting Frizzled-7-mediated-Wnt/beta-catenin signaling. Biomed Pharmacother 2018; 107: 754-762.

29) Ye MF, Zhang JG, Guo TX, Pan XJ. MiR-504 inhibits cell proliferation and invasion by targeting LOXL2 in non small cell lung cancer. Biomed Pharmacother 2018; 97: 1289-1295.

30) Cai Q, Zeng S, Dai X, Wu J, Ma W. miR-504 promotes tumour growth and metastasis in human osteosarcoma by targeting TP53INP1. Oncol Rep 2017; 38: 2993-3000.

31) Fu C, Sikandar A, Donner J, Zaburannyi N, Herrmann J, Reck M, Wagner-Dobler I, Koehnke J, Muller R. The natural product carolacton inhibits folate-dependent C1 metabolism by targeting FolD/MTHFD. Nat Commun 2017; 8: 1529.

32) Pike ST, Rajendra R, Artzt K, Appling DR. Mitochondrial C1-tetrahydrofolate synthase (MTHFD1L) supports the flow of mitochondrial one-carbon units into the methyl cycle in embryos. J Biol Chem 2010; 285: 4612-4620.

33) Koufaris C, Gallage S, Yang T, Lau CH, Valbuena GN, Keun HC. Suppression of MTHFD2 in MCF-7 breast cancer cells increases glycolysis, dependency on exogenous glycine, and sensitivity to folate depletion. J Proteome Res 2016; 15: 2618-2625.

34) Gustafsson Sheppard N, Jarl L, Mahadessian D, Strittmatter L, Schmidt A, Madhusudan N, Tegner J, Lundberg EK, Asplund A, Jain M, Nilsson R. The folate-coupled enzyme MTHFD2 is a nuclear protein and promotes cell proliferation. Sci Rep 2015; 5: 15029.

35) Liu F, Liu Y, He C, Tao L, He X, Song H, Zhang G. Increased MTHFD2 expression is associated with poor prognosis in breast cancer. Tumour Biol 2014; 35: 8685-8690.

36) Pikman Y, Puissant A, Alexe G, Furman A, Chen LM, Frumm SM, Ross L, Fenouille N, Bassil CF, Lewis CA, Ramos A, Gould J, Stone RM, DeAngelo DJ, Galinsky I, Clish CB, Kung AL, Hemann MT, Vander Heiden MG, Banerji V, Stegmaier K. Targeting MTHFD2 in acute myeloid leukemia. J Exp Med 2016; 213: 1285-1306.

37) Gu Y, Si J, Xiao X, Tian Y, Yang S. miR-92a inhibits proliferation and induces apoptosis by regulating methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) expression in acute myeloid leukemia. Oncol Res 2017; 25: 1069-1079.