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MicroRNA-214 functions as an oncogene in human osteosarcoma by targeting TRAF3

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Abstract. – OBJECTIVE: Osteosarcoma is a malignant bone tumor with high incidence. The prognosis of osteosarcoma is very poor when it is diagnosed with metastasis. Numerous studies have demonstrated that aberrant expressions of microRNAs are involved in cancer initiation and development. However, the potential role of miR-214 in osteosarcoma remains largely unrevealed. The current study investigated the relationship between the miR-214 and TNF receptor-associated factor 3 (TRAF3) in osteosarcoma tissues and cell lines. We also aimed to evaluate the potential roles of miR-214 on the occurrence and metastasis in osteosarcoma and verify its effect on the regulation of TRAF3.

PATIENTS AND METHODS: The miR-214 expression and TRAF3 expression in osteosarcoma tissue samples and cell line were measured using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Followed by transfection assays, transwell assay was conducted to detect the migration and invasion abilities of osteosarcoma cells. Subsequently, Western blotting and luciferase reporter assay were performed in osteosarcoma cells to confirm the target of miR-214.

RESULTS: The results showed that miR-214 expression levels were significantly increased not only in osteosarcoma tissues but also in osteosarcoma cell lines as compared with adjacent normal tissues and matched cell lines, respectively. On the contrary, the TRAF3 expression levels in osteosarcoma tissues and cell lines were frequently decreased compared to the control group. Moreover, TRAF3 was identified as a direct target of miR-214 and the inverse relationship between them was also observed in osteosarcoma tissues. Additionally, we found that miR-214 restoration could significantly promote osteosarcoma cell invasion and migration via targeting TRAF3.

CONCLUSIONS: MicroRNA-214 functioned as an oncogene in osteosarcoma via targeting TRAF3, which may provide new insights into osteosarcoma prevention and treatment.

Key Words
miRNA-214, Oncogene, Human Osteosarcoma, TRAF3.

Introduction

Osteosarcoma is one of the most common malignant bone tumors, primarily affecting adolescents and young adults[1]. In osteosarcoma, tumor cells can form osteoid tissue or immature bone directly; however, osteosarcoma rarely occurs in soft tissues[2]. Currently, existing main standard therapeutic methods for osteosarcoma are chemotherapy and radiation treatments. Although great efforts have been made to promote osteosarcoma treatment, the poor survival rate still exists in patients with metastatic or relapsed osteosarcoma[3]. Previous studies have provided clues regarding the molecular mechanisms underlying the pathogenesis of osteosarcoma. However, the details are still not fully elucidated[4]. Therefore, the knowledge of molecular mechanism underlying osteosarcoma progression is critical to develop advanced therapies and diagnostics for future osteosarcoma studies.

miRNAs are a kind of regulatory RNA molecules which are endogenous and conserved non-coding[5]. Studies have reported that various miRNAs play important roles in regulating gene

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expressions through binding to the 3'-UTR of the target mRNA[6]. Aberrantly expressed miRNAs have been identified in different tumors, implying that miRNAs may be used as diagnostic markers or therapeutic targets[7]. For example, miR-643 was verified to regulate the expression of ZEB1 and inhibit tumorigenesis in osteosarcoma[8]. Overexpression of miR-187 was reported to suppress cervical cancer development *via* targeting HPV16E6[9]. In addition, miR-362-5p acted as an oncogene in hepatocellular carcinoma growth and metastasis *via* targeting CYLD[10]. Moreover, miR-214 has been reported to regulate multiple malignant tumors, including gingival cancer[11], non-small cell lung carcinoma[12] and glioma[13]. However, the functions of miR-214 in osteosarcoma still remain unrevealed. TNF receptor-associated factor 3 (TRAF3), a member of the TRAF family, is ubiquitously expressed in most cells and tissues[14]. TRAF3 interacts with all sorts of biological molecules, exerting multiple biological functions[15]. For example, NDR1 protein kinase accelerates IL-17 and TNF-α-mediated inflammation by competitively binding TRAF3[16]. TRAF3 delays cyst formation induced by NF-κB signaling[17]. TRAF3 enhances TCR signaling by regulating Csk and PTPN22[18]. Previous researchers have shown that TRAF3 acts as an important mediator in myeloid cells, inhibiting NF-kB-inducing kinase (NIK) and the NIK-activated non-canonical NF-κB pathway by promoting NIK ubiquitination and degradation[19]. However, the functions of TRAF3 in osteosarcoma still remain unclear.

Patients and Methods

Patients

Osteosarcoma tissues and corresponding normal tissues were collected from 60 osteosarcoma patients who underwent surgery at our hospital from 2015 to 2017. All of the collected tissue samples were snap-frozen by liquid nitrogen and then stored at -80°C for further use. Written informed consent was obtained from all osteosarcoma patients involved in this study. The current study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China).

Cell Lines

Human osteoblast cell line hFOB1.19 and osteosarcoma cell lines HOS, U2OS were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C in 5% CO_2.

Table I. Correlation of miR-214 expression with the clinicopathological characteristics of the osteosarcoma patients.

Clinicopathological features	Cases (n=60)	miR-214 expression		*p*-value
		High (n=42)	Low (n=18)	
Age (years)				0.5568
> 60	29	20	9	
≤ 60	31	22	9	
Gender				0.5157
Male	31	21	10	
Female	29	21	8	
Tumor size (cm)				0.3264
≥ 5.0	33	26	7	
< 5.0	27	16	11	
TNM stage				0.0056
I-II	25	12	13	
III	35	30	5	
Lymph-node metastasis				0.0062
Yes	36	32	4	
No	24	10	14	
Distant metastasis				0.4904
Yes	31	19	12	
No	29	23	6	

TNM: tumor-node-metastasis.

Table II. Primer sequences for *qRT-PCR*.

Primer	Sequence
miR-214 forward	5'-ATAGAATTCTTTCTCCCTTTCCCCTTACTCTCC -3'
miR-214 reverse	5'- CCAGGATCCTTTCATAGGCACCACTCACTTTAC -3'
U6 forward	5'-CTCGCTTCGGCAGCACA -3'
U6 reverse	5'-ACGCTTCACGAATTTGCGT -3'
TRAF3 forward	5'- TGAGCTGGAGAGCGTAGACA -3'
TRAF3 reverse	5'-AGATCAGCACCCCGTTGTAG -3'
GAPDH forward	5'-ATCACTGCCACCCAGAAGAC -3'
GAPDH reverse	5'-TTTCTAGACGGCAGGTCAGG -3'

U6: small nuclear RNA, snRNA.
TRAF3: TNF receptor-associated factor 3.
GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Cell Transfection

miR-214 mimics, miR-214 inhibitor or TRAF3 siRNA and the corresponding negative controls were purchased from GenePharma (Shanghai, China) and transfected into osteosarcoma cell lines HOS, U2OS by Lipofectamine® 2000 (Invitrogen Carlsbad, CA, USA; Thermo Fisher Scientific, Inc. Waltham, MA, USA) in strict accordance with the manufacturer's instruction.

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

Total RNA from osteosarcoma cells and all the tissue samples were isolated by TRIzol® Reagent (Ambion™) (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted to synthesize cDNA using the Reverse Transcription kit (TaKaRa, Otsu, Shiga, Japan). qRT-PCR was performed using SYBR Green Real-Time PCR Master Mix (TaKaRa, Otsu, Shiga, Japan) on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The primers were as follows: Forward primer for miR-214, 5'-ATAGAATTCTTTCTCCCTTTCCCCTTACTCTCC-3', Reverse primer for miR-214, 5'-CCAGGATCCTTTCATAGGCACCACTCACTTTAC-3'; Forward primer for TRAF3, 5'-TGAGCTGGAGAGCGTAGACA-3', Reverse primer for TRAF3, 5'-AGATCAGCACCCCGTTGTAG-3'; Forward primer for GAPDH, 5'-ATCACTGCCACCCAGAAGAC-3', Reverse primer for GAPDH, 5'-TTTCTAGACGGCAGGTCA GG-3'; Forward primer for U6,5'-CTCGCTTCGGCAGCACA-3', Reverse primer for U6, 5'-ACGCTTCACGAATTT GCG T-3'. The relative expression was analyzed using $2^{-\Delta\Delta CT}$ method.

Transwell Assays

The invasive and migratory abilities of osteosarcoma cell lines HOS, U2OS transfected with miR-214 mimics or inhibitor were tested by transwell assay. For the cell invasion assay, the upper transwell chamber was coated with matrigel and the lower chamber was filled with complete culture medium containing 10% fetal bovine serum (FBS). On the other hand, the transfected cells were seeded and incubated in the upper transwell chamber. After being incubated for 24 hours, the non-invaded cells were removed by cotton swabs. Migratory assay was performed similarly to that of invasion assay, except for matrigel pre-coating. Besides, cell incubation time for migration assay was only 12 hours. Subsequently, cells were fixed and stained with 90% alcohol and 0.1% crystal violet respectively. Then, the cells in a total of more than 5 randomly chosen fields, were counted to determine the migratory and invasive abilities of osteosarcoma cell lines under an inverted microscope (Olympus, Tokyo, Japan).

Western Blotting Analysis

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was used to extract the total proteins from the cultured cell lines. Protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc. Waltham, MA, USA). After being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were then transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). PVDF membrane was incubated with 5% skimmed milk for 1 hour at room temperature and incubated

with the appropriate antibodies at 4°C overnight. The primary antibodies were as follows: a rabbit antibody against TRAF3 (1:1000, ab36988, Abcam, Cambridge, MA, USA) and a rabbit antibody against GAPDH (1:2000, ab128915, Abcam, Cambridge, MA, USA). Next, it was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, ab7090, Abcam, Cambridge, MA, USA) for 1 hour. The enhanced chemiluminescence regents (Thermo Scientific, Waltham, MA, USA) was used to detect the results of antigen-antibody complex on PVDF.

Luciferase Reporter Assay

Osteosarcoma cell lines HOS, U2OS were co-transfected with miR-214 mimics and luciferase reporter vectors containing the wide-type or mutant-type 3'-UTR of TRAF3 using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) was used to detect the luciferase activities 48 hours after transfection.

Statistical Analysis

All above experiments involved in current study were conducted at least three times. All the data were shown as the mean ± SD. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 18.0 (SPSS Inc. Chicago, IL, USA) were applied to perform the statistical analysis. Student's *t*-test was used to determine the statistical significant differences. It was considered to be statistically significant difference when $p<0.05$.

Results

miR-214 was Over-Expressed and TRAF3 Was Down-Regulated in Osteosarcoma

To investigate whether the expression levels of miR-214 and TRAF3 were altered in osteosarcoma, qRT-PCR was performed in osteosarcoma tissues and matched normal tissues. Results demonstrated that miR-214 expression in osteo-

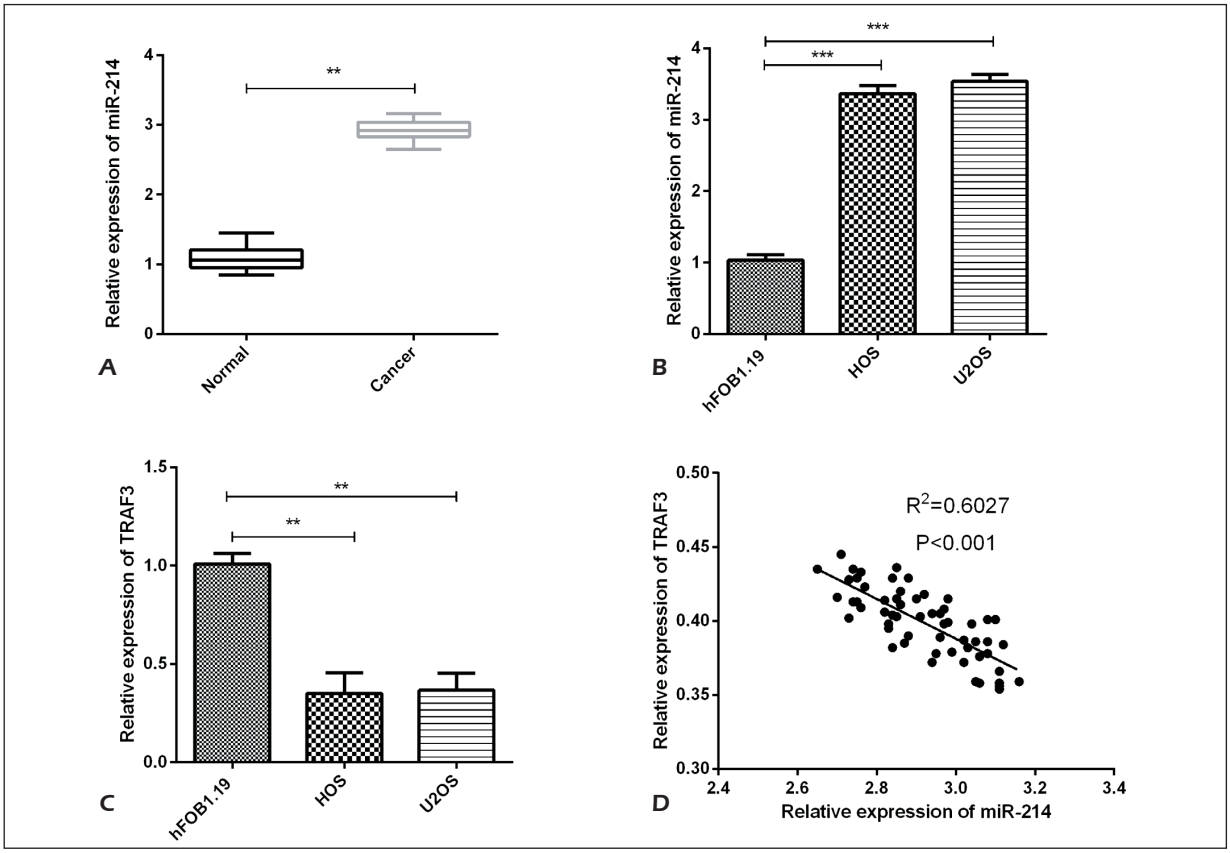

Figure 1. miR-214 expression was elevated and TRAF3 expression was reduced in osteosarcoma tissues and cells. **A**, Expressions of miR-214 in osteosarcoma tissues were detected by qRT-PCR (**$p<0.01$). **B**, qRT-PCR analysis of miR-214 expressions in osteosarcoma cells (***$p<0.001$). **C**, The mRNA expression of TRAF3 in HOS and U2OS cells (**$p<0.01$). **D**, Correlation between miR-214 and TRAF3 expression.

sarcoma tissues was increased significantly in contrast with that in the corresponding normal tissues (Figure 1A, $p<0.01$). Similarly, compared to the human osteoblast cell line hFOB1.19, miR-214 expression in osteosarcoma cell lines was significantly aggrandized (Figure 1B, $p<0.001$). We also measured the mRNA expression of TRAF3 in osteosarcoma cells. However, the results inversely demonstrated that the mRNA expression of TRAF3 in HOS and U2OS cells was decreased significantly in contrast with that in the human osteoblast cell line hFOB1.19 (Figure 1C, $p<0.01$). In addition, we analyzed the correlation of miR-214 and TRAF3 in osteosarcoma. From the results, we could easily know that miR-214 expression was negatively correlated with the expression of TRAF3 (Figure 1D, $p<0.001$).

miR-214 Promoted Cell Invasion and Migration in Osteosarcoma Cell Lines

The HOS and U2OS cell lines transfected with miR-214 mimics or inhibitor were applied to detect the effects of miR-214 on osteosarcoma cell invasion and migration abilities. Firstly, miR-214 mimics or inhibitor was transfected into osteosarcoma cells to over-express or inhibit the miR-214 expression. Then, the efficiencies were confirmed by qRT-PCR. The successful over-expression or inhibition of miR-214 expression in HOS and U2OS cells was respectively confirmed by qRT-PCR analysis (Figure 2A $p<0.01$). Subsequently, the invasion and migration abilities of osteosarcoma cells, which were transfected with miR-214 mimics or inhibitor were observed using transwell assay. The results demonstrated that the migration and invasion abilities of HOS and U2OS cells were remarkably promoted by miR-214 overexpression whereas the inhibition of miR-214 dramatically suppressed the cell migration and invasion capacities (Figure 2B, 2C, 2D and 2E). Taken the above results together, we demonstrated that miR-214 could promote the invasion and migration abilities of osteosarcoma cells.

miR-214 Regulated TRAF3 Expression Through Targeting its 3′-UTR

We explored the correlation between miR-214 and TRAF3 to understand the mechanism of miR-214 in osteosarcoma more clearly. By

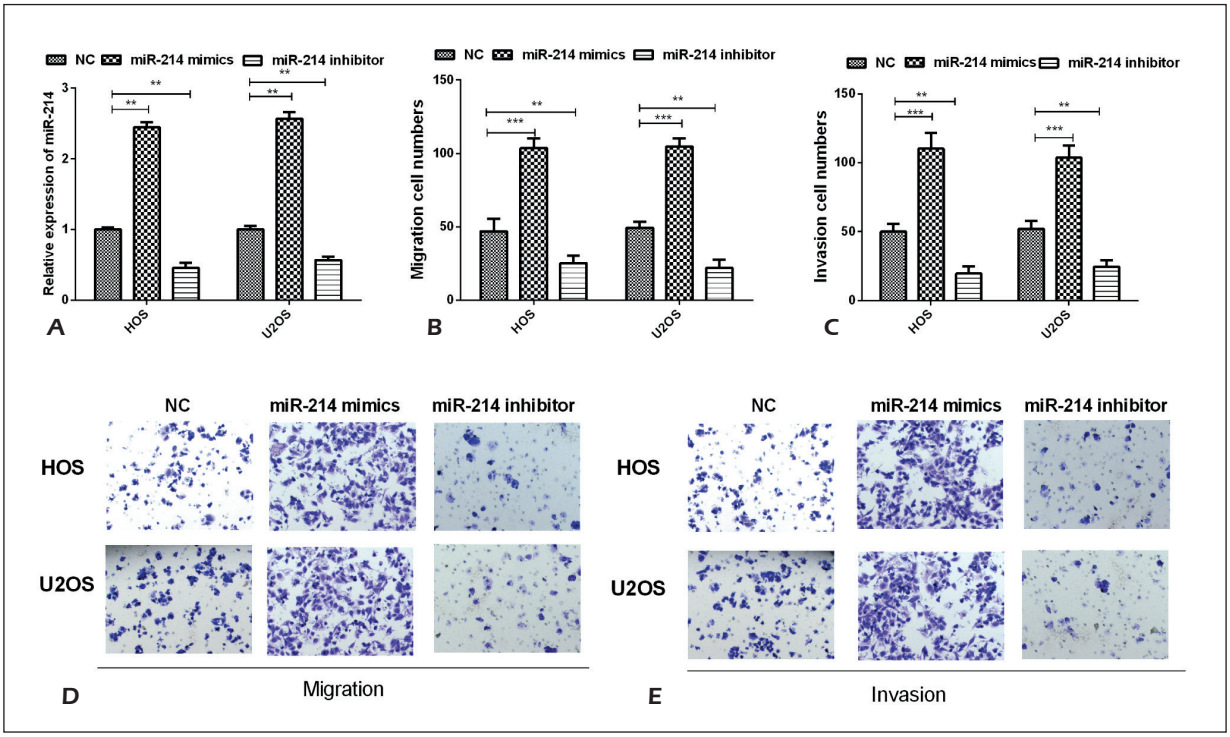

Figure 2. miR-214 suppressed the cell migration and invasion abilities of osteosarcoma cells. **A**, The miR-214 expression was measured by qRT-PCR in HOS and U2OS cells with different transfections (**$p<0.01$). **B-C**, The numbers of migratory (**B**) and invasive (**C**) cells in HOS and U2OS cell lines with different transfections (**$p<0.01$, ***$p<0.001$). **D**, Cell migration was observed by the transwell assay in HOS and U2OS cells with different transfections. **E**, Cell invasion was detected by the transwell assay in HOS and U2OS cells with different transfections.

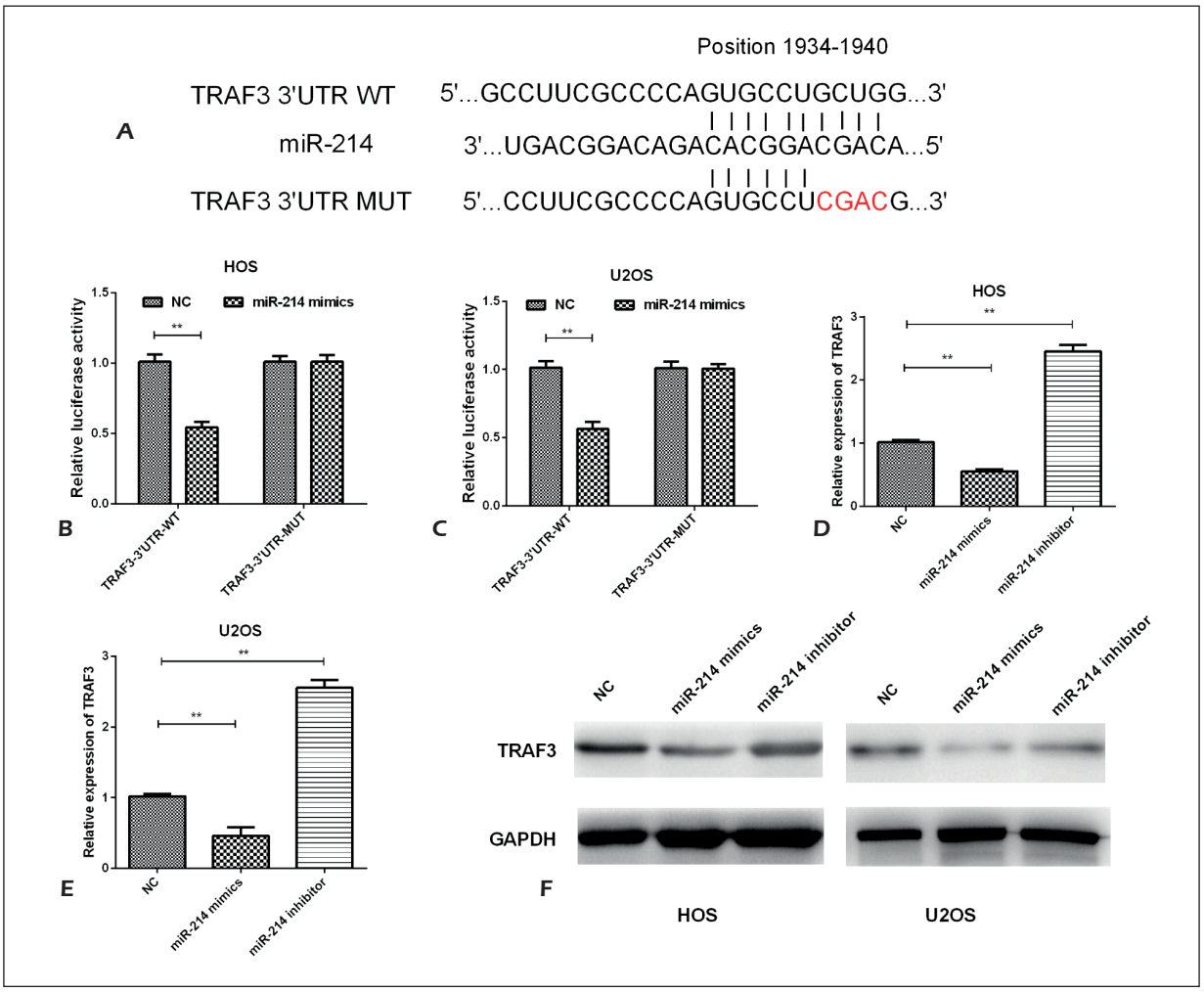

Figure 3. miR-214 regulated TRAF3 expressions via targeting its 3'-UTR. **A**, The target site of miR-214 in TRAF3 sequence was predicted according to the Target Scan. **B**-**C**, The fluorescence activities of the TRAF3 3'UTR was measured by luciferase reporter assay in HOS cells (**B**) and U2OS cells (**C**) that were co-transfected with miR-214 mimics and TRAF3 3'UTR-WT or TRAF3 3'UTR-MUT, respectively (**p<0.01). D, E, qRT-PCR analysis was applied to measure the mRNA expressions of TRAF3 in HOS cells (**D**) and U2OS cells (**E**). **F**, Western blot analysis was applied to measure the protein expression levels of TRAF3 in HOS and U2OS.

means of TargetScan, the target sites of miR-214 in the sequence of TRAF3 were predicted (Figure 3A). To validate the target specificity, luciferase reporter assay was performed by cotransfecting the miR-214 mimics and the luciferase reporter plasmids containing TRAF3 3'-UTR-WT or TRAF3 3'-UTR-MUT into osteosarcoma cells, respectively. We measured the luciferase activities of the TRAF3 3'-UTR to investigate whether TRAF3 was the target of miR-214. The results showed that the relative luciferase activities in both HOS and U2OS cells co-transfected with the TRAF3 3'-UTR-WT vector and miR-214 were significantly decreased compared to the control. However, there was no significant variation in the cells co-transfected with the TRAF3 3'-UTR-MUT vector and miR-214 (Figure 3B and 3C, p<0.01). Additionally, the regulatory effects of miR-214 on TRAF3 were analyzed by qRT-PCR and Western blot analysis. The results indicated that miR-214 could suppress the TRAF3 expression in both HOS and U2OS cells (Figure 3D, 3E and 3F, p<0.01). These results indicated that miR-214 could bind directly to TRAF3 3'-UTR and inhibit TRAF3 expression in osteosarcoma cells.

TRAF3 May Regulate the Effects of Mir-214 on Osteosarcoma Cell Invasion and Migration

To investigate whether the carcinogenic effects of miR-214 on osteosarcoma cell migration and invasion were mediated by TRAF3, we used TRAF3 specific siRNA to knockdown endogenous TRAF3 in HOS and U2OS cells. Then, qRT-PCR assays and Western blot assays were performed to determine the silencing efficiency of TRAF3. The consequences demonstrated that not only the mRNA expression, but also the protein expression were reduced in cells transfected with TRAF3 siRNA in contrast with the control (Figure 4A and 4B, $p<0.001$). Moreover, we investigated whether TRAF3 was instrumental to the invasion and migration abilities of osteosarcoma cells. From the transwell assay results, we found that the deletion of TRAF3 markedly reversed miR-214-medicated promotion of cell invasion and migration in osteosarcoma cells (Figure 4C and 4D, $p<0.01$). These results implied that miR-214 inhibited osteosarcoma cell migration and invasion by targeting TRAF3.

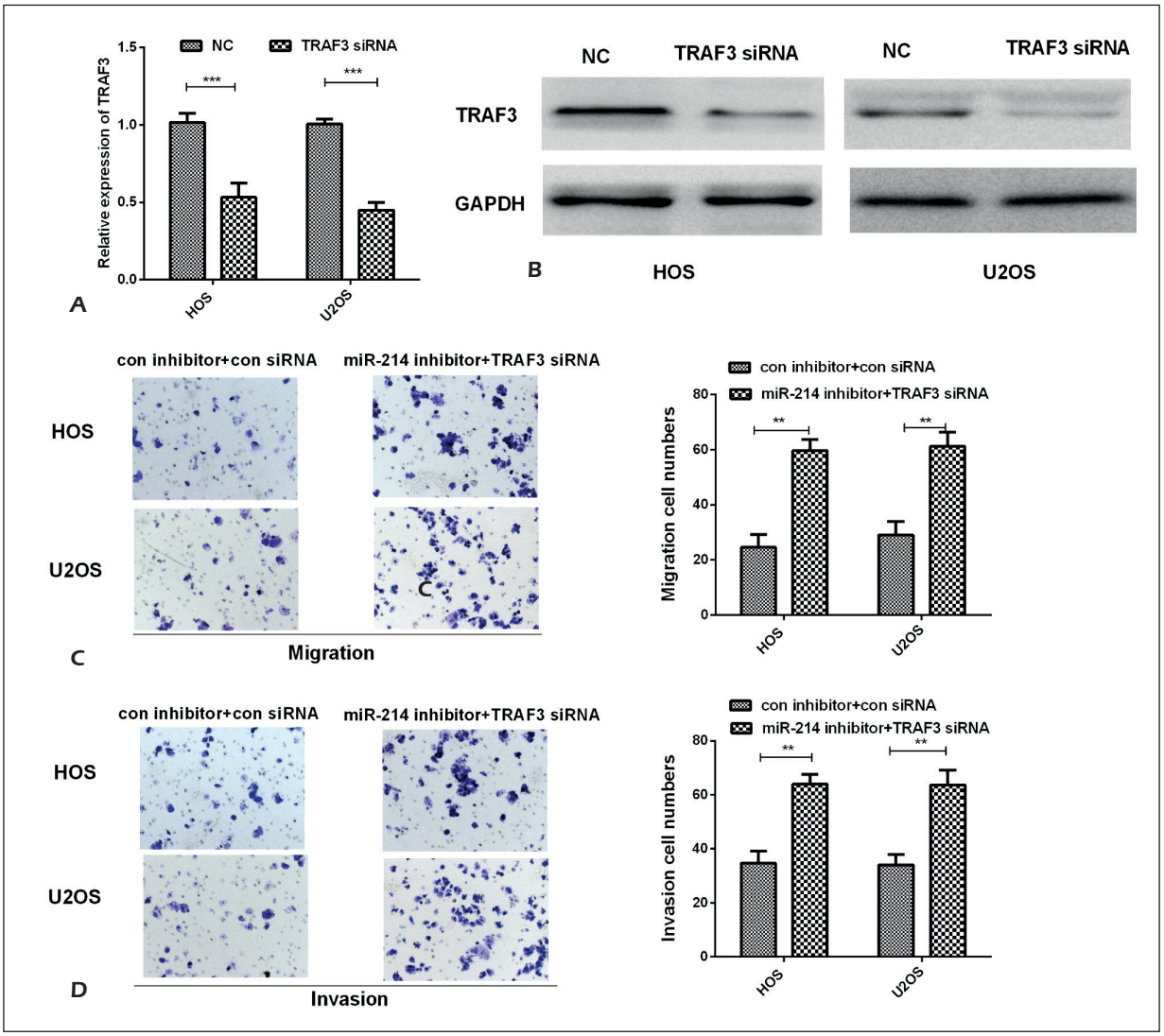

Figure 4. TRAF3 may regulate the functions of miR-214 in osteosarcoma cell invasion and migration. **A**, The mRNA expression levels of TRAF3 in osteosarcoma cells transfected with TRAF3 siRNA was measured using qRT-PCR (***$p<0.001$). **B**, The protein expression levels in osteosarcoma cells transfected with TRAF3 siRNA were measured by Western blotting. **C**, The transwell assay was performed to determine the cell migration ability in osteosarcoma cells co-transfected with TRAF3 siRNA and miR-214 inhibitor. **D**, The cell invasion ability in osteosarcoma cells co-transfected with TRAF3 siRNA and miR-214 inhibitor was measured by the transwell assay.

Discussion

Osteosarcoma is a common malignant bone cancer among children. Although advances have been made regarding the therapeutic strategies for osteosarcoma patients, including surgery and chemotherapy, the treatment of metastatic osteosarcoma remains a challenge and increasing risks of relapse are induced in many patients[20]. Therefore, it is important to elucidate the mechanisms underlying pathogenesis of osteosarcoma and explore novel molecular effective therapeutic approaches to prevent metastasis and improve the survival rate of osteosarcoma patients. For the past few years, emerging evidence has highlighted the role of miRNA in human tumorigenesis[21]. Researches have demonstrated that miRNAs regulate a variety of pathological and physiological processes. In addition, it has been reported that miRNAs participated in the initiation and progression of various tumors[22]. Therefore, identification of osteosarcoma-associated miRNAs as biomarkers for diagnosis, prognosis and therapeutic targets for osteosarcoma treatment is of great importance.

Evidence has demonstrated aberrant expressions of miR-214 are involved in the tumorigenesis of numerous types of tumors. It is indicated that abnormal miR-214 expressions might induce human carcinogenesis through negatively regulating its target genes[23,24]. For example, miR-214 was reported to promote peritoneal metastasis of gastric carcinoma *via* targeting PTEN[25]. In addition, miR-214 could accelerate apoptosis of breast cancer cells by regulating the RFWD2-p53 cascade[26]. Moreover, miR-214 was found to promote metastasis and epithelial-mesenchymal transition in lung adenocarcinoma *via* regulating the suppressor-of-fused protein[27]. miR-214 has been indicated to play important roles in many tumors, but its role in osteosarcoma remains unclear. The current study aimed to investigate the effects of miR-214 on osteosarcoma. Briefly, it revealed that miR-214 was frequently upregulated in osteosarcoma. Moreover, we demonstrated that overexpression of miR-214 could inhibit the migration and invasion of osteosarcoma cells while miR-214 knockdown promoted these biological functions of osteosarcoma cells. Therefore, these data indicated that miR-214 exerted crucial roles in promoting osteosarcoma.

TRAF3, a vital member of the TRAF adaptor family, has distinct cell and context-specific roles[28]. The different members of the TRAF family have divergent and non-redundant roles, but they also have overlapping roles in the control of cellular processes[29]. Investigations[30] indicated that TRAF3 can activate NF-κB through canonical or noncanonical signaling pathways. However, little is known about the possible functions of TRAF3 in osteosarcoma. In current study, we investigate the correlation between miR-124 and TRAF3 in osteosarcoma. Results demonstrated that miR-214 expression was negatively correlated with TRAF3 expression in osteosarcoma tissues. Moreover, we found that TRAF3 was a direct functional target of miR-214 in osteosarcoma cells and regulated the oncogenic functions of miR-214 in osteosarcoma invasion and migration.

Conclusions

We showed that miR-214 was significantly overexpressed in osteosarcoma, and its expression level was negatively correlated with TRAF3 expression. We also observed that miR-214 overexpression prominently inhibited cell invasion and migration by targeting TRAF3 in osteosarcoma. The above findings provided new insights into the mechanisms underlying the oncogenic role of miR-214, providing new direction in developing miRNA-associated therapy for osteosarcoma prevention and treatment.

Conflict of Interests:

The authors declared no conflict of interest.

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