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Reduced expression of miR-564 is associated with worse prognosis in patients with osteosarcoma

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Abstract. – OBJECTIVE: MiR-564 has been reported to be involved in the development of several types of cancers. However, its clinical significance in osteosarcoma (OS) has not been investigated. We aimed to explore the prognostic value of miR-564 in OS patients.

PATIENTS AND METHODS: Quantitative RT-PCR assay was used to detect the expression levels of miR-564 in OS tissues and matched normal bone tissues. The correlation between miR-564 expression and clinicopathological features was analyzed by Pearson's χ^2-test. The disease-free survival and overall survival rates of OS patients were calculated by the Kaplan-Meier method. Cox regression analysis was used to assess factors related to survival.

RESULTS: We observed that the level of miR-564 was significantly reduced in OS tissues compared with that in the paired noncancerous bone tissues ($p < 0.01$). In addition, low miR-564 expression was found to be closely correlated with advanced clinical stage ($p = 0.000$) and distant metastasis ($p = 0.003$). Furthermore, survival analyses showed that patients with low expression of miR-564 had a shorter overall survival ($p = 0.001$) and disease-free survival ($p = 0.001$) than those with high expression of miR-564. Finally, we showed that miR-564 was an independent poor prognostic factor for both 5-year overall survival ($p = 0.001$) and 5-year disease-free survival ($p = 0.001$) through multivariate analysis.

CONCLUSIONS: Overall, our data for the first time suggest that downregulated miR-564 may be used as a novel prognosis predictor of OS.

Key Words:
miR-564, Osteosarcoma, Prognosis.

Introduction

Osteosarcoma (OS) is one of the most common primary malignant tumors, which is prevalent in teenagers and young adults[1]. OS was involved in the regulation of the distal long bones through the formation of neoplastic bone tissue[2]. Recenly, approximately 7000 new cases are diagnosed annually in China[3]. Despite the rapid development in therapeutic strategies, the overall survival rate of osteosarcoma has not been substantially improved[4,5]. For both low- and high-grade OS, metastasis is one of the primary unfavorable factors for prognosis[6]. On the other hand, diagnosis at advanced stage should take the blame for the high mortality rate of OS[7]. Therefore, it is of great significance to investigate biomarkers, which could predict metastasis and identify OS at an early clinical stage to improve the individual treatment.

MicroRNAs (miRNAs) are a group of non-coding RNAs with a size of 21-24 nucleotides and act as post-transcriptional regulators of gene expression[8]. MiRNA plays an important role in normal tissue development and cell differentiation, proliferation, and apoptosis[9,10]. Importantly, several studies[11-15] have confirmed that dysregulation of miRNAs expression occurs in various types of human cancers in breast, lung, liver, pancreas and colon. In malignancy, miRNAs can function as oncogenes or tumor suppressor genes, depending on their targets, which may provide insight into the diagnosis and prognosis of human malignancies[16,17]. MiR-564, a tumor-related miRNA, has been reported to be abnormally expressed in several tumors, including OS[18,19]. Ru et al[19] firstly showed that miRNA-564 expression was down-regulated in OS tissues and cell lines. In subsequent *in vitro* experiment, they found that overexpression of miR-564 suppressed the growth of OS cells by targeting Akt, suggesting that miR-564 served as a tumor suppressor in OS. However, to date, the potential clinical significance of miR-564 in OS patients has not been reported. In this study, we further determined the expression levels of miR-564 in OS samples from our hospital. Then, we firstly explore the association between

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Table I. Correlation of miR-564 expression in osteosarcoma with clinicopathologic characteristics.

Parameters	Group	Total	miR-564 expression		*p*-value
			High	Low	
Age (years)	< 18	116	55	61	NS
	≥ 18	101	55	46	
Gender	Male	111	60	51	NS
	Female	106	50	56	
Tumor site	Femur/Tibia	91	4	50	NS
	Others	126	69	57	
Tumor size (cm)	< 8	130	70	60	NS
	≥ 8	87	40	47	
Serum level of lactate dehydrogenase	Elevated	136	68	68	NS
	Normal	81	42	39	
Serum level of alkaline phosphatase	Elevated	140	73	67	NS
	Normal	77	37	40	
Differentiation	Well and moderate	129	72	57	NS
	Poor	77	37	40	
Clinical stage	IIA	124	76	48	0.000
	IIB/III	93	34	59	
Distant metastasis	Absent	135	79	56	0.003
	Present	82	31	51	

miR-564 expression and clinicopathological parameters in OS. In addition, the potential prognostic values of miR-564 were also firstly analyzed. Our study for the first time reported that miR-564 acted as a favorable prognostic biomarker in OS patients.

Patients and Methods

Patients and Tissue Samples

Between 2009 and 2013, a total of 217 human OS samples and the adjacent non-tumor bone tissue were collected during surgery at The First Bethune Hospital of Jilin University. The samples were biopsy materials and all patients did not previously receive radiotherapy, chemotherapy or immunotherapy. All the tissues were histologically and pathologically diagnosed by two experienced pathologists. All the resected specimens were placed immediately into liquid nitrogen and stored at -80°C. Follow-up was performed every 2-3 months during the first year after surgery until January 2018. Details of clinical and pathological characteristics of the patients are summarized in Table I. All patients showed their full intentions to participate in the present study and a written consent form was obtained from each patient. This study was approved by the Human Ethics Committee of The First Bethune Hospital of Jilin University.

RNA Extraction, Reverse Transcription, and qRT-PCR

In accordance with the manufacturer's instruction, total RNA was extracted from OS tissues by using TRIzol reagent (Life Technologies, Xian, Shaanxi, China). RNA was reverse transcribed into complementary DNA (cDNA) using PrimeScript™ RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China). The expression levels of miR-564 were analyzed using SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). PCR cycles were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. The expression levels of miR-564 were normalized with the GAPDH. The primes of miR-564 and GAPDH were obtained from Applied Biosystems (Foster City, CA, USA). The primers were as follows: miR-564, forward primer: 5'- GGCATAGGCACGGTGUC -3' and reverse primer: 5'-TGGTGTCGTGGAGTCG -3'; GAPDH, forward primer: 5'-AATGACCCCTTCATTGAC-3' and reverse primer: 5'-TCCACGACGTACTCAGCGC-3'. Relative quantification was calculated by using the $2^{-\Delta\Delta Ct}$ method. All qRT-PCRs were performed in triplicate.

Statistical Analysis

SPSS version 19 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The measurement data were expressed by mean ± SD.

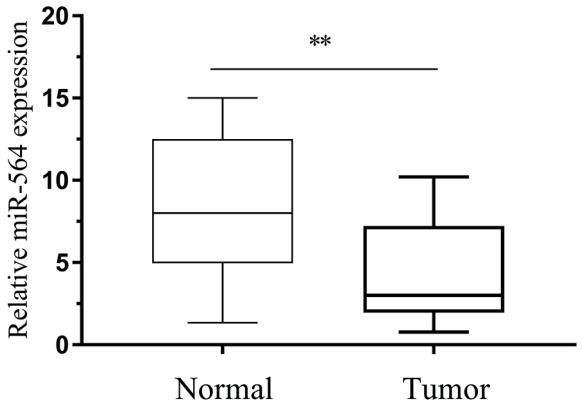

Figure 1. The relative expression levels of miR-564 in OS specimens and matched adjacent noncancerous bone tissue. MiR-564 was significantly downregulated in OS tissues compared with that in paired adjacent non-tumor bone tissues (p <0.01).

Differences between samples were determined by Student's t-test. The relationships between the miR-564 expression level and clinicopathological parameters were analyzed using the Pearson χ^2-test. Survival curves were obtained by using the Kaplan-Meier method and compared by using the log-rank test. Multivariate Cox proportional hazards analyses were used to analyze the independent prognostic factors for survival in OS patients. A $p < 0.05$ was considered to indicate a statistically significant difference.

Results

MiR-564 is Down-Regulated in Human OS Tissues

In order to know the expression manner of miR-564, we measured the levels of miR-564 in 217 pairs of OS and corresponding normal bone tissues by qRT-PCR. As shown in Figure 1, we found that the expression of miR-564 was decreased in OS tissues compared with the paired adjacent normal tissues ($p < 0.01$). Our results indicated that miR-564 downregulation may be involved in the malignant progression of OS.

Expression Levels of miR-564 and Clinicopathological Characteristics in OS

To analyze whether miR-564 was associated with the clinical progression of OS, we tested the association between miR-564 expression and clinicopathological characteristics in the 217 OS patients. According to the median ratio of relative miR-564 expression (5.32) in tumor tissues, the 217 HCC patients were divided into two groups: relative high miR-564 group and relative low miR-564 group. The results of x^2-test showed that low expression of miR-564 was correlated with higher clinical stage ($p = 0.000$) and more distant metastasis ($p = 0.003$). However, miR-564 expression was not correlated with age, gender, tumor size, tumor site and differentiation ($p > 0.05$).

Low miR-564 Expression Predicts Poor Prognosis in OS

To further understand the clinical significance of low miR-564 expression, the prognostic power of miR-564 was determined for overall survival and disease-free survival in 217 OS patients, using the Kaplan-Meier method and log-rank test. Interestingly, the 5-year overall survival ($p = 0.001$, Figure 2) and disease-free survival ($p > 0.000$, Figure 3) of OS patients with low miR-564 expression were shorter compared to those patients with high miR-564 expression. Moreover, the Cox proportional hazards model was employed to determine whether miR-564 could be used to predict the prognosis of OS patients independently. As shown in Table II, we found that clinical stage, distant metastasis and miR-564 expression were independent predictors for overall survival and disease-free survival of patients with OS.

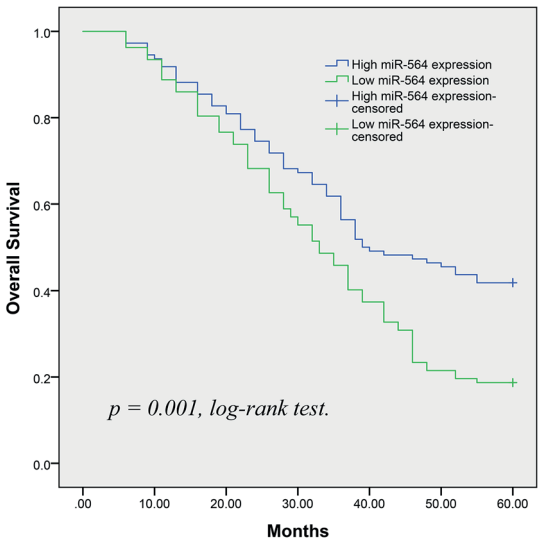

Figure 2. Kaplan-Meier analysis for the overall survival of OS patients with different expression of miR-564. Patients with low miR-564 expression had a shorter overall survival than those low expression ($p = 0.001$).

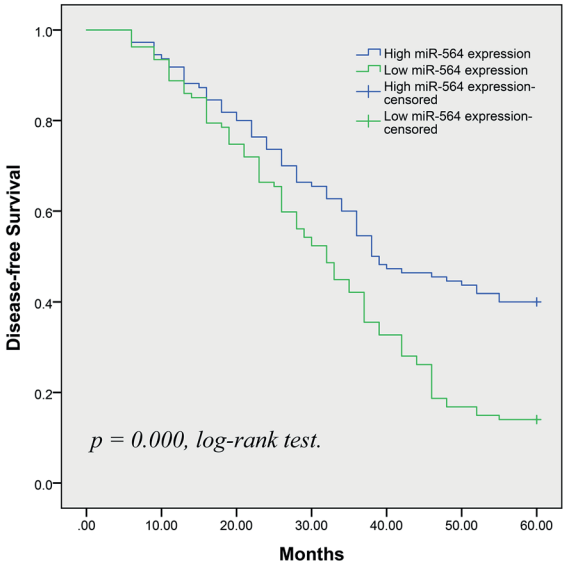

Figure 3. Kaplan-Meier analysis for the overall survival of OS patients with different expression of miR-564. Patients with low miR-564 expression had a shorter disease-free survival than those low expression ($p = 0.000$).

Discussion

OS is characterized by high local aggressiveness, rapid growth rate and early metastasis to lungs[20]. Currently, the 5-year overall and disease-free survival rates for OS patients are around 40-55%[21]. In order to improve the prognosis of OS patients, several clinicopathological features, such as TNM stage and differentiation were used to predict the long-term prognosis of OS patients. However, these clinicopathological features have not been proven to be sufficiently effective[22,23]. Recently, more and more studies focused on the potential possibility of tumor-related miRNAs as clinical biomarkers for predicting the prognosis of OS patients[24]. Importantly, a number of cancer-specific miRNAs have been identified, which may be used as novel biomarkers for diagnosis and prognosis[25-27]. The enormous potential of miRNA used as biomarkers encouraged us to identify more cancer-associated miRNAs and to investigate their clinical significance.

MiR-564, located at 3p21.31, has been reported to be lowly expressed in several cancers and plays a critical role in tumor progression. For instance, Jiang et al[28] reported that miR-564 was significantly lowly expressed in glioblastoma and its overexpression suppressed glioblastoma cell proliferation and invasion by targeting TGF-β1, suggesting miR-564 as a tumor suppressor in this disease. Merve et al[29] found that miR-564 expression was significantly down-regulated in breast cancer and associated with five-year overall survival. In their *in vitro* experiment, it was observed that up-regulation of miR-564 suppressed breast cancer cells migration and invasion by modulating EMT. Meng et al[30] revealed that the expression levels of miR-564 was significantly lower in prostate cancer tissues and cell lines compared with those in normal prostate tissues and cell lines, respectively. Gain-function assay indicated that forced miR-564 expression suppressed metastasis and proliferation of prostate cancer by targeting MLLT3. Yang et al[31] observed that the deregulated expression of miR-564 was associated with poor prognosis and aggressive phenotype of human lung cancer, and promotion of miR-564 inhibits the proliferation and motility of lung cancer cells *in vitro* by targeting ZIC3. Those findings suggested miR-564 as a tumor suppressor in above tumors. Interestingly, Ru et al[19] firstly reported miR-564 as a down-regulated miRNA in

Table II. Multivariate survival analysis of overall and disease-free survivals in 217 osteosarcoma patients.

Variables	Overall survival			Disease-free survival		
	RR	95% CI	p	RR	95% CI	p
Age	1.667	0.682-2.324	0.423	1.442	0.792-2.193	0.238
Gender	2.138	0.793-2.663	0.136	1.783	0.583-2.348	0.113
Tumor site	1.993	0.682-2.332	0.177	2.425	0.893-2.883	0.093
Tumor size	1.532	0.557-2.327	0.113	1.786	0.679-2.669	0.082
Serum level of lactate dehydrogenase	1.889	0.498-3.554	0.153	1.457	0.783-2.784	0.118
Serum level of alkaline phosphatase	1.253	0.475-2.554	0.574	1.032	0.556-2.019	0.234
Differentiation	2.667	0.687-3.453	0.132	2.884	0.783-3.183	0.117
Clinical stage	4.632	1.453-7.673	0.001	5.327	1.667-8.873	0.001
Distant metastasis	3.284	1.236-5.132	0.006	3.742	1.448-4.673	0.001
miR-564 expression	4.253	1.663-8.632	0.001	4.632	1.884-9.376	0.001

OS. They performed cells experiments and found that overexpression of miR-564 inhibited the growth of OS cells via targeting Akt. However, the clinical significance of miR-564 in OS has not been investigated. In this study, we found that the expression levels of miR-564 in OS tissues were significantly lower than those in matched adjacent normal tissues. This results together with previous findings confirmed miR-564 expression was down-regulated in OS. In addition, we found that miR-564 expression was associated with advanced clinical stage and positively distant metastasis. More importantly, we firstly reported that patients with low miR-564 expression showed shorter overall survival and disease-free survival than those with high miR-564 expression. According to multivariate analyses, we confirmed that miR-564 overexpression was an independent favorable prognostic biomarker for OS patients. Of course, due to the limited sample size in our study, further prospective analysis with a large number of OS samples is worth doing to further confirm our findings.

Conclusions

We demonstrated that miR-564 plays an important role in tumor development and metastasis of OS. To our best knowledge, our results for the first time indicate that miR-564 is an important indicator of poor survival rate and an independent prognostic factor for OS patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the surveillance, epidemiology, and end results program. Cancer 2009; 115: 1531-1543.
2) Heymann D, Rédini F. Targeted therapies for bone sarcomas. Bonekey Rep 2013; 2: 378.
3) Zhou W, Hao M, Du X, Chen K, Wang G, Yang J. Advances in targeted therapy for osteosarcoma. Discov Med 2014; 17: 301-307.
4) Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 2006; 32: 423-436.
5) Isakoff MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: current treatment and a collaborative pathway to success. J Clin Oncol 2015; 33: 3029-3035.
6) Tan ML, Choong PF, Dass CR. Osteosarcoma: conventional treatment vs gene therapy. Cancer Biol Ther 2009; 8: 106-117.
7) Federman N, Bernthal N, Eilber FC, Tap WD. The multidisciplinary management of osteosarcoma. Curr Treat Options Oncol 2000; 10: 82-93.
8) Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
9) Ke XS, Liu CM, Liu DP, Liang CC. MicroRNAs: key participants in gene regulatory networks. Curr Opin Chem Biol 2003; 7: 516-523.
10) Grosswendt S, Filipchyk A, Manzano M, Klironomos F, Schilling M, Herzog M, Gottwein E, Rajewsky N. Unambiguous identification of miRNA: target site interactions by different types of ligation reactions. Mol Cell 2014; 54: 1042-1054.
11) Nassar FJ, El Sabban M, Zgheib NK, Tfayli A, Boulos F, Jabbour M, El Saghir NS, Talhouk R, Bazarbachi A, Calin GA, Nasr R. miRNA as potential biomarkers of breast cancer in the Lebanese population and in young women: a pilot study. PLoS One 2014; 9: e107566.
12) Mascaux C, Feser WJ, Lewis MT, Barón AE, Coldren CD, Merrick DT, Kennedy TC, Eckelberger JI, Rozeboom LM, Franklin WA, Minna JD, Bunn PA, Miller YE, Keith RL, Hirsch FR. Endobronchial miRNAs as biomarkers in lung cancer chemoprevention. Cancer Prev Res (Phila) 2013; 6: 100-108.
13) Endo S, Yano A, Fukami T, Nakajima M, Yokoi T. Involvement of miRNAs in the early phase of halothane-induced liver injury. Toxicology 2014; 319: 75-84.
14) Kredo-Russo S, Mandelbaum AD, Ness A, Alon I, Lennox KA, Behlke MA, Hornstein E. Pancreas-enriched miRNA refines endocrine cell differentiation. Development 2012; 139: 3021-3031.
15) Thomas J, Ohtsuka M, Pichler M, Ling H. MicroRNAs: clinical relevance in colorectal cancer. Int J Mol Sci 2015; 16: 28063-28076.
16) Fan H, Liu X, Zheng WW, Zhuang ZH, Wang CD. MiR-150 alleviates EMT and cell invasion of colorectal cancer through targeting Gli1. Eur Rev Med Pharmacol Sci 2017; 21: 4853-4859.
17) Zhou Y, An Q, Guo RX, Qiao YH, Li LX, Zhang XY, Zhao XL. miR424-5p functions as an anti-oncogene in cervical cancer cell growth by targeting KDM5B via the Notch signaling pathway. Life Sci 2017; 171: 9-15.
18) Liang C, Xu Y, Ge H, Xing B, Li G, Li G, Wu J. miR-564 inhibits hepatocellular carcinoma cell proliferation and invasion by targeting the GRB2-ERK1/2-AKT axis. Oncotarget 2017; 8: 107543-107557.
19) Ru N, Zhang F, Liang J, Du Y, Wu W, Wang F, Liu X. MiR-564 is down-regulated in osteosarcoma and inhibits the proliferation of osteosarcoma cells via targeting Akt. Gene 2018; 645: 163-169.
20) Geller D S, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol 2010; 8: 705-718.

21) Gorlick R. Current concepts on the molecular biology of osteosarcoma. Cancer Treat Res 2009; 152: 467-478.

22) Botter SM, Neri D, Fuchs B. Recent advances in osteosarcoma. Curr Opin Pharmacol 2014; 16: 15-23.

23) Sun L, Li Y, Zhang J, Li H, Li B, Ye Z. Prognostic value of pathologic fracture in patients with high grade localized osteosarcoma: a systemic review and meta-analysis of cohort studies. J Orthop Res 2015; 33: 131-139.

24) Zhang J, Yan YG, Wang C, Zhang SJ, Yu XH, Wang WJ. MicroRNAs in osteosarcoma. Clin Chim Acta 2015; 444: 9-17.

25) Tang B, Liu C, Zhang QM, Ni M. Decreased expression of miR-490-3p in osteosarcoma and its clinical significance. Eur Rev Med Pharmacol Sci 2017; 21: 246-251.

26) Han G, Wang Y, Bi W, Jia J, Wang W. MicroRNA-124 functions as a tumor suppressor and indicates prognosis in human osteosarcoma. Exp Ther Med 2015; 9: 679-684.

27) Dong J, Liu Y, Liao W, Liu R, Shi P, Wang L. miRNA-223 is a potential diagnostic and prognostic marker for osteosarcoma. J Bone Oncol 2016; 5: 74-79

28) Jiang C, Shen F, Du J, Hu Z, Li X, Su J, Wang X, Huang X. MicroRNA-564 is downregulated in glioblastoma and inhibited proliferation and invasion of glioblastoma cells by targeting TGF-β1. Oncotarget 2016; 7: 56200-56208.

29) Mutlu M, Saatci O, Ansari SA, Yurdusev E, Shehwana H, Konu O, Raza U, Đahin O. miR-564 acts as a dual inhibitor of PI3K and MAPK signaling networks and inhibits proliferation and invasion in breast cancer. Sci Rep 2016; 6: 32541.

30) Meng FJ, Meng FM, Wu HX, Cao XF. miR-564 inhibited metastasis and proliferation of prostate cancer by targeting MLLT3. Eur Rev Med Pharmacol Sci 2017; 21: 4828-4834.

31) Yang B, Jia L, Guo Q, Ren H, Hu D, Zhou X, Ren Q, Hu Y, Xie T. MiR-564 functions as a tumor suppressor in human lung cancer by targeting ZIC3. Biochem Biophys Res Commun 2015; 467: 690-696.