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ORIGINAL ARTICLE

Tissue expression levels of miR-29b and miR-422a in children, adolescents, and young adults' age groups and their association with prediction of poor prognosis in human osteosarcoma

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Abstract Osteosarcoma is the most common type of bone cancer in children and adolescents. MicroRNAs (miRNAs) play important roles in the development, differentiation, and function of different cell types and in the pathogenesis of various human diseases. miRNAs are differentially expressed in normal and cancer cells. The investigation of miRNA expression between healthy subjects and patients with osteosarcoma is crucial for future clinical trials. In this study, the expression levels of miRNAs were detected by qRT-PCR. Correlation between expression levels of tow miRNAs and different clinicopathological characteristics were analyzed using the χ^2 test. Survival rate was detected using the log-rank test and Kaplan–Meier method. qRT-PCR was shown that expression levels of miR-29b and miR-422a were strongly decreased in osteosarcoma bone tissue compared with non-cancerous bone tissues. Our result indicated that the low expression levels of miR-29b and miR-422a showed strong correlation with tumor size (P=0.20; 0.029), advanced TNM stage (P=0.0[illegible]0.012), distant metastasis (P=0.008; 0.019), and grade of tumor (P=0.009; 0.016). Kaplan–Meier survival analysis showed that the low expressions of miR-29b/miR-422a were correlated with shorter time overall survival (log-rank test, P=0.009; P=0.013). Moreover, multivariate Cox proportional hazards model indicated that miR-29b and miR-422a (P=0.024; P=0.016) were independent prognostic markers of overall survival of patients. Our result indicated that downregulation of miR-29b and miR-422a may be linked to the prediction of poor prognosis, indicating that miR-29b and miR-422a may be a valuable prognostic marker for osteosarcoma patients.

Keywords miR-29b and miR-422a · Survival · PCR · Osteosarcoma · Pathology

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Introduction

Osteosarcoma (OS) is a destructive primary bone tumor in children and adolescents that is a tumor with high metastatic potential [1, 2]. Despite advances of therapeutic strategies such as surgery and chemotherapy, long time survival rate of patients suffering from advanced osteosarcoma remains very low to date [3, 4]. Therefore, it is required to identify biomarkers, molecular mechanisms, and therapeutic targets for osteosarcoma. MicroRNAs (miRNAs) are small, non-coding RNA molecules that are significant markers for prognosis and diagnosis of cancer and promising targets for treatment. miRNAs have important roles in a number of biological functions such as embryogenesis, cell fate specification, cellular proliferation, differentiation, and apoptosis through alteration

of the targets expression by both downregulation and upregulation [5, 6]. Dysregulation of different microRNAs has been reported in various human cancers [6, 7]. It is clear that they can function either as tumor-suppressor or oncogene in different human malignancies depending on their target mRNAs [8, 9]. Nevertheless, the roles of miRNAs in biology of cancer are still required to be studied. Downregulation of miR-29b has been suggested in many kinds of cancer, and most of the mentioned studies focused on the mechanism by which miR-29b acts as inhibitor of tumorigenesis [10, 11, 12]. A recent study indicated that miR-422a was downregulated in OS tissues, and it could be a valuable diagnostic and predictive marker [13]. However, the roles of miR-29b and miR-422a in osteosarcoma and their relationship with clinicopathological factors need further investigation. We aimed the expression pattern of miR-29b and miR-422a in osteosarcoma tissues and their relationship with clinicopathological factors.

Materials and methods

Patients and clinical specimens

We recruited 51 patients with osteosarcoma from different hospitals in Tehran, Iran, between April 2009 and December 2014. In addition, adjacent normal bone tissues were also collected.

Patients underwent surgery without chemotherapy or radiotherapy before surgery. Clinical data was obtained from the database of the patients. The tissues were obtained from surgical specimens reviewed by pathologist. All the specimens were stored at −80 °C until use. The study was confirmed by the research ethics committee, and the clinicopathological features were categorized in Table 1.

Quantitative reverse transcriptase PCR

We used quantitative real-time PCR to assess the expression level of miR-29b and miR-422a in osteosarcoma patients and

Table 1 The relationship of miR-29b and miR-422a expression with clinicopathological factors of osteosarcoma

Clinicopathological features	No. of cases (51)	Expression of miR-29b		expression of miR-422a		*P* value of miR-29b	*P* value of miR-422a
		Low=31	High=20	Low=29	High=22		
Gender							
Male	30	18	12	19	11	0.672	0.618
Female	21	13	8	10	11		
Age							
Children, adolescents	20	13	7	15	5	0.528	0.412
Young adults	31	18	13	14	17		
Tumor diameter (cm)							
≤5	33	14	19	18	15	0.020	0.029
>5	18	17	1	11	7		
Location							
Distal	24	13	11	10	14	0.412	0.406
Proximal	27	18	9	17	10		
Tumor grade							
Low	22	11	11	10	12	0.009	0.016
High	29	20	9	19	10		
Histological type							
Osteoblastic	23	14	9	13	10	0.454	0.483
Chondroblastic	14	8	6	8	6		
Mixed	10	6	4	5	5		
Fibroblastic	4	3	1	3	1		
TNM stage							
I+II	34	15	19	14	20	0.001	0.012
III+IV	17	16	1	15	2		
Distant metastasis							
Yes	18	13	5	13	5	0.008	0.019
No	33	18	15	16	17		

normal tissues. In brief, total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). The expression levels of miRNAs were quantitated using the TaqMan miRNA assay kit (Applied Biosystems). Real-time PCR was performed using Rotor Gene 6000 Real-Time PCR (Qiagen, Germany) with an Invitrogen kit and a TaqMan universal PCR master mix was also applied. U6 snRNA was utilized as an endogenous control. Fold change expression of miRNAs were calculated using ΔΔ-CT method ($\Delta\Delta Ct = \Delta Ct_{tumor\ samples} - \Delta Ct_{control\ sample}$).

Statistical analysis

All data presented as mean±SD. SPSS 18.0 software (SPSS Inc., USA) were used to analyze all variables. Differences between groups were evaluated using Student's *t* test. Moreover, the relationship between miRNA expression and various clinicopathological factors was analyzed using the χ^2 test. Survival time was followed up to the date of death, and survival rate was determined using the log-rank test and Kaplan–Meier method. Prognostic values of clinicopathological factors were evaluated using Cox proportional hazards model. $P<0.05$ was statistically significant.

Results

Downregulation of miRNAs

The expression levels of miR-29b and miR-422a in patients were compared with those of adjacent normal bone tissues. qRT-PCR has shown that expression levels of miR-29b and miR-422a were strongly decreased in osteosarcoma bone tissue (mean±SD, 3.17±1.39; 3.74±0.57) compared with noncancerous bone tissues (mean±SD, 9.67±2.55; 6.16±1.03; $P=0.001$; $P=0.006$; Fig. 1).

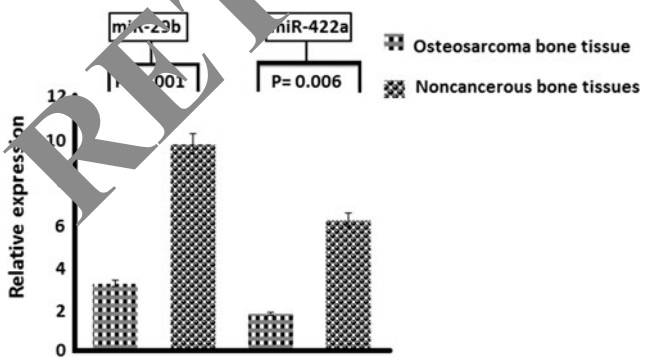

Fig. 1 The relative expression of miRNAs in osteosarcoma tissues and their corresponding normal samples

Association with clinicopathologic features

The patients were divided into low- and high-expression groups according to the median expression level. Donregulation of miR-29b and miR-422a expression levels showed strong correlation with large tumor size ($P=0.20$; 0.029), advanced TNM stage ($P=0.001$; 0.012), distant metastasis ($P=0.008$; 0.019), and grade of tumor ($P=0.009$; 0.016).

No significant correlation was found between miR-29b/miR-422a expression levels and age groups ($P=0.528$; 0.412), gender ($P=0.672$; 0.618), location ($P=0.412$; 0.406), and histological type ($P=0.454$; 0.482; Table 1).

Association of miRNA expression with prognosis in patients

The low expressions of miR-29b/miR-422a were correlated with shorter time overall survival (log-rank test, $P=0.009$; $P=0.013$; Figs. 2 and 3) based on Kaplan–Meier survival analysis. According to the multivariate Cox proportional hazards model, miR-29b and miR-422a ($P=0.024$; $P=0.016$) were independent prognostic markers for overall survival of patients (Tables 2 and 3).

Discussion

To date, there are no available biomarkers routinely utilized that have diagnostic and predictive values. Therefore, more specific prognostic biomarkers are required for advancing the therapeutic strategies for osteosarcoma patients. qRT-PCR has shown that expression levels of miR-29b and miR-422a were strongly decreased in osteosarcoma bone tissue compared with noncancerous bone tissues. Moreover, decreased expression of miR-29b and miR-422a was strongly linked to large tumor size, advanced

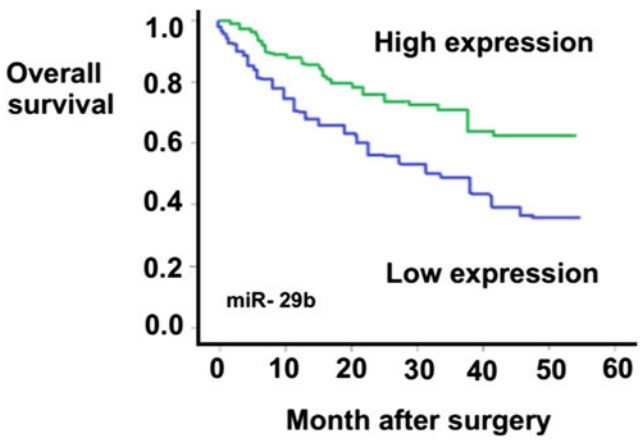

Fig. 2 Correlation between miR-29b expression and survival time in patients

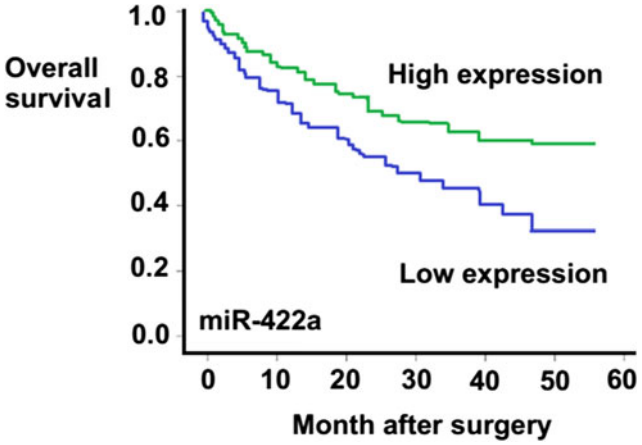

Fig. 3 Correlation between miR-422a expression and survival time in patients

TNM stage distant metastasis, and grade of tumor. These result indicated that low expression of miRNAs may be correlated with progression of osteosarcoma. Dysregulation of different microRNAs has been suggested in osteosarcoma tissues [7, 14, 15, 16]. Previous studies have reported that miR-29b was downregulated in many kinds of cancer [11, 12, 17, 18]. MiR-29b directly regulates VEGF as tumor-suppressive gene, a chaperone involved in the tumorigenesis in osteosarcoma tissues. It has reported that restoration of miR-29b inhibited cell proliferation, migration, and invasion in cancer. Cheng et al. suggested that miR-29b has oncogenic potential and targets the TET2 gene, which acts as tumor-suppressor frequently mutated in hematopoietic malignancies [18]. It has been reported that miR-29 functions as a regulator in many kinds of cancers, through its targeting. It acts as a tumor-suppressor and inhibits cell proliferation, differentiation, metastasis, and chemosensitivity [19]. On the other hand, Gougelet et al. suggested that miR-422a was downregulated in OS tissues, and it could be a valuable diagnostic and predictive marker [13]. There have been some studies which indicated that miR-422a play an important role in human diseases. Previous studies indicated that decreased expression of

Table 2 Multivariate analysis of miR-29b expression for prognostic factors

Clinicopathological characteristics	HR	95 % CI	*P* value
Gender	1.217	0.683–1.839	0.741
Age	1.374	0.593–3.373	0.431
Tumor grade	3.194	2. 235–11.216	0.009
Location	0.792	0.734–3.021	0.583
Distant metastasis	3.945	2.383–13.326	0.003
TNM stage	3.642	2.684–12.127	0.004
Histological type	2.439	1.321–3.484	0.453
miR-29b expression	2.778	1.453–8.793	0.024

Table 3 Multivariate analysis of miR-422a expression for prognostic factors

Clinicopathological characteristics	HR	95 % CI	*P* value
Gender	0.925	1.275–2.128	0.673
Age	1.143	1.056–2.478	0.567
Tumor grade	3.015	2. 352–9.215	0.005
Location	0.874	0.738–2.843	0.512
Distant metastasis	3.601	2.632–14.12	0.002
TNM stage	3.972	2.991–14.10	0.001
Histological type	2.127	1.425–3.120	401
miR-422a	2.918	1.7 –9.698	0.016

miR-422a may play a protective role in colorectal tumor and can act as inhibitor of pathways that stimulate tumor cell proliferation in osteosarcoma [19, 20]. In the current study, the low expressions of miR-29b and miR-422a were correlated with shorter time overall survival based on Kaplan–Meier survival analysis. Multivariate Cox proportional hazards model showed that miR-29b and miR-422a were independently correlated with overall survival of patients, suggesting that these miRNAs could be as independent prognostic factor of overall survival in patient suffering from osteosarcoma.

In conclusion, our result indicated that downregulation of miR-29b and miR-422a may be linked to the prediction of poor prognosis, indicating that miR-29b and miR-422a may be a valuable prognostic marker for osteosarcoma patients.

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Conflicts of interest None

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