Low-expression of miR-7 promotes cell proliferation and exhibits prognostic value in osteosarcoma patients

Shouying Liu1*, Changxi Zhou2*, Changbao Zhu3*, Qiuhe Song4*, Ming Wen5, Ye Liu6, Huaijie An7

1Department of Orthopaedics, The 253th Hospital of The Chinese People’s Liberation Army, Hohhot 010051, China; 2Department of Nanlou Respiratory Diseases, PLA General Hospital, Beijing 100853, China; 3Department of Orthopaedics, The 474th Hospital of The Chinese People’s Liberation Army, Urumchi 830013, China; 4Department of Dermatology, Attached Hospital of Jiujiang University, Jiujiang 332000, China; 5Department of Human Resources, Human Resources China Communications Construction Company Ltd, Beijing 100088, China; 6Department of Anesthesiology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing 100026, China; 7Center of Basic Medical Sciences, Navy General Hospital of PLA, Beijing 100037, China. *Co-first authors.

Received December 14, 2015; Accepted February 25, 2016; Epub August 1, 2017; Published August 15, 2017

Abstract: Aim: MicroRNAs (miRNAs) play important roles in occurrence and development of osteosarcoma. Previous studies had verified the role of microRNA-7 (miR-7) in various diseases, especially in cancers. Our purpose in this study was to investigate the values of miR-7 in development and prognosis of osteosarcoma. Methods: QRT-PCR was used to measure the expression of miR-7 in osteosarcoma tissues, adjacent tissues and healthy tissues as well as in osteosarcoma cell lines MG63, U2OS and normal osteoblastic cell line hFOB1.19. CCK-8 and siRNA assays were performed to estimate the effect of miR-7 in the process of cell proliferation. The Kaplan-Meier and Cox regression analysis were performed to detect the prognostic values of the miR-7 in osteosarcoma patients. Results: The results demonstrated that miR-7 expression decreased in osteosarcoma tissues and cell lines compared with the controls. Proliferation assay declared that the cell proliferation was accelerated by down-regulation of miR-7. Kaplan-Meier exhibited that the overall survival time of low-miR-7 expression was shorter than those with high-miR-7 expression (P=0.001). Cox regression analysis revealed that Enneking, distant metastasis and recurrence were all prognostic factors just like low-miR-7. Conclusion: The expression of miR-7 was lower in osteosarcoma tissues and cell lines and miR-7 acted as a tumor suppressor. The low-expression of miR-7 was associated with clinicopathologic characteristics (age, tumor site, Enneking, therapies). Moreover, miR-7 might be an independent prognostic marker and promote cell proliferation in osteosarcoma.

Keywords: Osteosarcoma, miR-7, cell proliferation, prognosis

Introduction

Osteosarcoma, a most common malignancy of bone mainly arises from the metaphysis of the long bones of adolescents and young adults. It accounts for 5% of all pediatric tumors and 8.9% of cancer-related deaths in children [1, 2]. The characteristics of osteosarcoma include easy relapse and metastasis, high malignancy and invasion. Over 50% osteosarcoma patients were diagnosed as suffering metastasis which led to low cure rate and low 5-years’ survival rate [3]. Recently, as the development of comprehensive therapies, the 5-year’ survival rate has risen to 60-70% [4]. However, the clinical prognosis is still poor when patients develop recurrent or metastatic osteosarcoma. Meanwhile, the fundamental molecular mechanisms of the development and progress of osteosarcoma is still obscure. Therefore, it is important to explore the mechanisms and pathogenesis of osteosarcoma and develop strategies for diagnosis, treatment and prognosis of this disease.

MicroRNAs (miRNAs) which are a class of small endogenous non-coding RNA with a length of 18-25 nucleotides [5], have been confirmed to be related to many progresses of various cancers. The differential expressions of miRNAs between tumor tissues and healthy tissues make them either act as oncogenes or tumor suppressors in different cancers. MiRNAs control the expression of its target genes via spe-
The role of miR-7 in osteosarcoma patients

Specific sites binding with the 3'-untranslated regions (3'-UTR) of the target-mRNAs at post transcriptional level [6]. Besides, in the important disease processes such as cell growth, cell cycle, apoptosis, migration and invasion, miRNAs have been reported to play vital roles. So far, more than 1900 human miRNAs regulating about 60% of the genes in mammals have been identified [7]. MicroRNA-7 (miR-7) is an evolutionary conserved miRNA with three different genomic loci in humans [8]. And it served as a tumor suppressor in several human cancers [9]. However, the function of miR-7 in osteosarcoma is unknown.

In this study, we aimed to detect the expression of miR-7 in osteosarcoma tissues and cell lines, then explore the effects of miR-7 on osteosarcoma cell proliferation, further analyze the prognostic value of miR-7 expression on osteosarcoma patients.

Materials and methods

Clinical samples collection

The current study was conducted in Chinese PLA General Hospital and permitted by the Ethic Committee of the hospital. Patients who were collected in the research were diagnosed as osteosarcoma during 2013-2015. None of them had received any chemical and physical treatments. The clinicopathologic characteristics of all patients including age, gender, tumor site, Enneking, histological type, surgical method, distant metastasis and recurrence were recorded in database. Besides, written informed consent had been signed in advance.

Tumor tissues and adjacent tissues from 87 patients with osteosarcoma were obtained, besides, 30 healthy tissue samples were obtained as control. There were 35 females and 52 males with a median age of 32.72±16.60. The tissue samples were quickly frozen in liquid nitrogen and stored at -80°C for RNA extraction.

A 5-years’ follow-up was performed with all osteosarcoma patients. The overall survival time was defined as the diagnosed time to the last day of follow-up. The information about follow-up was updated each three months through telephone or questionnaire. The data were recorded in a database.

Cell culture and transfection

Human osteosarcoma cell lines MG63, U2OS and human normal osteoblastic cell line hFOB1.19 were purchased from the Type Culture Collection of the Chinese Academy of Science. MG63 and U2OS cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) while hFOB 1.19 cells were cultured in DMEM/F-12 (1:1; HyClone, Logan, UT, USA). 10% fetal bovine serum (FBS; Gibco, NY, USA), 1% penicillin/streptomycin and 2 mM gluta-
The role of miR-7 in osteosarcoma patients

Results

MiR-7 expression significantly decreased in osteosarcoma tissues and cell lines

Table 1. Relationship between clinicopathologic characteristics and miR-7 in patients with osteosarcoma

<table>
<thead>
<tr>
<th>Clinicopathologic characteristics</th>
<th>n</th>
<th>miR-7 expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30</td>
<td>43</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>≥30</td>
<td>44</td>
<td>9</td>
<td>35</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur</td>
<td>35</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>Tibia</td>
<td>24</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Humeral bone</td>
<td>20</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>Others</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Enneking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>46</td>
<td>35</td>
<td>11</td>
</tr>
<tr>
<td>III</td>
<td>41</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoblastic</td>
<td>19</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Chondroblastic</td>
<td>30</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Fibroblastic</td>
<td>26</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Telangiectatic</td>
<td>12</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Therapies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neoadjuvant chemotherapy</td>
<td>20</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Resection</td>
<td>41</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>Postoperative chemotherapy</td>
<td>26</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>44</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Present</td>
<td>43</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>40</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Present</td>
<td>47</td>
<td>25</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 1. Relationship between clinicopathologic characteristics and miR-7 in patients with osteosarcoma

The expression of miR-7 was reported to be down-regulated in osteosarcoma in previous study [10]. To further validate this point, we detected miR-7 expression in both osteosarcoma tissues and cell lines with qRT-PCR. The result demonstrated that the expression of miR-7 was lower in osteosarcoma tissues than adjacent tissues and healthy tissues (Figure 1A, P<0.001). Besides, in the osteosarcoma cell lines (MG63 and U2OS), the expression of miR-7 was significantly decreased compared

RNA extraction and qRT-PCR

Total RNA was isolated from all tissues and cell cultures with Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase was conducted to synthesize the first chain of cDNA with TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then, qRT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). U6 acted as an endogenous control. The relative expression quantification of miR-7 was evaluated by comparative cycle threshold (CT) method.

Cell proliferation assay

The ability of cell proliferation was analyzed via cell Counting (CCK8) Kit-8 (Dojindo, Japan). After transfection, MG63 and U2OS cells viability at different time points (24, 48, and 72 h) was measured at 450 nm with an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA). Each sample was in triple.

Statistical analysis

Statistical analysis was carried out with SPSS version 13.0 software (SPSS Inc, IL, USA). All quantified data was presented as mean ± SD. The expression level of miR-7 was analyzed by T test. The relationship between clinicopathologic characteristics and miR-7 expression was estimated with chi-square test. Kaplan-Meier and cox regression analysis were taken to estimate the association of clinicopathologic characteristics, miR-7 expression and the overall survival. The difference was considered to be significant when P<0.05.

Results

MiR-7 expression significantly decreased in osteosarcoma tissues and cell lines

The expression of miR-7 was reported to be down-regulated in osteosarcoma in previous study [10]. To further validate this point, we detected miR-7 expression in both osteosarcoma tissues and cell lines with qRT-PCR. The result demonstrated that the expression of miR-7 was lower in osteosarcoma tissues than adjacent tissues and healthy tissues (Figure 1A, P<0.001). Besides, in the osteosarcoma cell lines (MG63 and U2OS), the expression of miR-7 was significantly decreased compared
The role of miR-7 in osteosarcoma patients

Figure 2. Cell proliferation assay of MG63 (A), U2OS (B) cell transfected with miR-7 siRNA or with control vector at an indicated time.

Figure 3. Kaplan-Meier survival curve analysis of patients with osteosarcoma. Compared with the high-miR-7 expression group, the patients with low-miR-7 expression level had shorter survival time ($P=0.001$).

The role of miR-7 in osteosarcoma patients

The role of miR-7 in osteosarcoma patients was investigated. Patients were divided into high-miR-7 expression (high-miR-7) group and low miR-7 expression (low-miR-7) group according to the median expression quantity of miR-7. 46 patients were attributed to high-miR-7 group while the others belonged to low-miR-7 group. Kaplan-Meier showed that patients with low-miR-7 expression had shorter survival time than those with high-miR-7 expression. Log rank test verified that the difference between two groups was significantly ($P=0.001$) (Figure 3).

Relationship between clinicopathologic characteristics and miR-7

The miR-7 expression was lower in osteosarcoma tissues and cell lines. To investigate whether clinicopathologic characteristics affected the expression of miR-7, we analyzed the relationship between clinicopathologic characteristics and miR-7. The results manifested that age ($P<0.001$), tumor site ($P<0.001$), Enneking ($P<0.001$) and therapies ($P<0.001$) were closely related to the expression of miR-7 (Table 1).

Down-regulation of miR-7 promoted cell proliferation

We then investigated the impact of miR-7 on osteosarcoma cell lines. The outcome revealed that down-regulation of miR7 significantly promoted cell proliferation in both tumor cell lines (MG63 and U2OS) compared with normal osteoblastic cell line (hFOB-1.19) (Figure 2A, 2B).

Association between miR-7 and overall survival

The osteosarcoma patients were divided into high miR-7 expression (high-miR-7) group and low miR-7 expression (low-miR-7) group according to the median expression quantity of miR-7. 46 patients were attributed to high-miR-7 group while the others belonged to low-miR-7 group. Kaplan-Meier showed that patients with low-miR-7 expression had shorter survival time than those with high-miR-7 expression. Log rank test verified that the difference between two groups was significantly ($P=0.001$) (Figure 3).
The role of miR-7 in osteosarcoma patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enneking</td>
<td>2.182</td>
<td>1.048-4.690</td>
<td>0.012</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>5.623</td>
<td>1.501-21.058</td>
<td>0.010</td>
</tr>
<tr>
<td>Recurrence</td>
<td>3.697</td>
<td>1.149-11.898</td>
<td>0.028</td>
</tr>
<tr>
<td>High-miR-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Low-miR-7</td>
<td>6.504</td>
<td>1.637-25.846</td>
<td>0.008</td>
</tr>
</tbody>
</table>

All clinicopathologic characteristics and miR-7 were put into multivariate analysis to test the prognostic value of them by cox regression analysis. Enneking (HR=2.182, P=0.012), distant metastasis (HR=5.623, P=0.010), recurrence (HR=3.697, P=0.028) and low-miR-7 (HR=6.504, P=0.008) were related to the prognosis of osteosarcoma. And they might be independent prognostic markers in osteosarcoma (Table 2).

Discussion

With the wide study of various detecting techniques and further research of miRNAs, the relationships between miRNAs and the occurrence as well as development of multiple tumors have attracted more and more attentions. The potential oncogene or antitumor effects make miRNAs expect to be molecular markers in the diagnosis and prognosis of cancers that provide new therapy strategies.

The impact of miRNAs on osteosarcoma has been reported in many studies. For instance, down-regulation of miR-21 inhibited cell invasion and migration according to regulating the expression of PECK [11]. The over expression of miR-151-3p, miR-191, miR-542-3p, miR-135b, miR-214, the decrease of miR-143, miR-199a-3p, miR-183, miR-34a, miR-218 mainly participated in tumor cell proliferation, migration, cell cycle, apoptosis and so on [12-19]. Besides, miR-223, miR-26a, miR-183, miR-9 were verified to be related to the prognosis of osteosarcoma [20-23].

MiR-7 was found by Mariana et al in 2001 and then has been identified to be dysregulated in some human cancers [24]. MiR-7 has been characterized as a tumor suppressor and played roles in inhibiting cell growth, proliferation, apoptosis, migration and invasion in several types of cancer. For instance, Kefas et al, and Wang et al both found that miR-7 was down-regulated in glioblastoma, the differences were that miR-7 decreased viability and invasiveness of primary glioblastoma lines in Kefas et al’s study while it inhibits cellular growth and glucose metabolism by regulating the IGF-1R/Akt signaling pathway in the study of Wang et al [25, 26]. Down-regulation of miR-7-mediated IGF1R reduced the IGF1-induced activation of AKT leading to the inhibition of cell proliferation and cell-cycle arrest, and an enhanced apoptotic rate in the study of Jiang et al [27]. In the study of Fang et al, miR-7 as a tumor suppressor could not only abolish the tumorigenesis but reverse the metastasis of hepatocellular carcinoma through regulating the PI3K/Akt/mTOR-signaling pathway [28]. MiR-7 targeted different genes was considered to be an tumor suppressor and inhibited cell proliferation as well as increased apoptosis in colorectal cancer both in the study of Zhang et al and Xu et al [29, 30]. Moreover, Li et al, found that miR-7 reduced or inhibited cell proliferation, induced cell apoptosis by suppressing the expression of both EGFR and RAF-1 oncogenes in Lewis lung cancer [31]. The over-expression of miR-7 could suppress cell viability and promoted cell apoptosis regulated by oncogene XIAP in cervical cancer cells according to Liu et al [32]. The up-regulation of miR-7 arrested cell cycle at G1 to S transition in hepatocellular carcinoma while it inhibited cell growth and invasion in pancreatic cancer cells via Zhang et al and Ma et al [33, 34]. However, the value of miR-7 in osteosarcoma was still unclear.

In the present study, we detected the expression of miR-7 in osteosarcoma tissues and cell lines. Meanwhile, we analyzed the function of miR-7 in the progress of osteosarcoma. The result showed that miR-7 decreased in osteosarcoma and might serve as a tumor suppressor. Cell proliferation analysis revealed that miR-7 was positive to the proliferation of osteosarcoma cancer cell lines MG63 and U2OS compared with normal cell line hFOB1.19. The study also determined the relationship of clinicopathologic characteristics and miR-7 expression as well as the association between miR-7 expression and overall survival. Age, tumor site, Enneking and therapies were considered to be related to the expression of miR-7 which
enhanced the view that miR-7 plays a role in the progress of osteosarcoma. The results of Kaplan-Meier and cox regression analysis manifested that miR-7 impacted the overall survival of osteosarcoma patients and could be as an independent prognostic marker. Besides, Enneking, distant metastasis and recurrence had a predictive meaning in osteosarcoma.

In conclusion, miR-7 is low-expression in osteosarcoma and may function as a tumor suppressor. Besides, miR-7 promoted osteosarcoma cell proliferation and took prognostic value in osteosarcoma patients. The suppressive function of miR-7 may be helpful for the forecast of osteosarcoma and provide new therapy strategies.

Disclosure of conflict of interest
None.

Address correspondence to: Ye Liu, Department of Anesthesiology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing 100026, China. Huaijie An, Center of Basic Medical Sciences, Navy General Hospital of PLA, Beijing 100037, China. E-mail: liue3hfsdf@sina.com

References
The role of miR-7 in osteosarcoma patients


