Original Article

Interactions of ABLIM1 and CXCL5 with miRNAs as a prognostic indicator for clinical outcome of osteosarcoma

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Received March 29, 2016; Accepted July 10, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Osteosarcoma (OS) is a kind of high-grade bone-forming malignancy with poor prognosis, causing huge economic losses to the patients and society. Identification of novel biomarkers could be beneficial for the diagnosis and prognosis of OS patients. We aimed to explore the molecular mechanism of osteosarcoma by the differentially expressed miRNAs and mRNAs screened out by microarray data together with the function and metabolic pathway analysis of target genes. Expression data of miRNA and mRNA were downloaded from The Genome Expression Omnibus (GEO) dataset. Differentially expressed miRNAs of osteosarcoma were screened out by two-fold principle. Target genes of differentially expressed miRNAs were extracted out by miRTarBase software. Besides, DAVID data-set was used for the functional annotation. Differentially expressed genes of mRNA microarray were screened out for the study of relation between miRNA and mRNA. A total of 33 differentially expressed miRNAs were screened out, including 27 up-regulated miRNAs and 6 down-regulated miRNAs. Three miRNAs, hsa-miR-182, hsa-miR-486-5p and hsa-miR760, were found to be the most differentially expressed ones, which were all up-regulated and could be used to differentiate osteosarcoma tissues from the normal ones. Besides, two up-regulated genes and fifteen significant down-regulated genes were identified by analysis of mRNA microarray data. Two target genes, the up-regulated gene, ABLIM1 (actin binding LIM protein 1) and the down-regulated gene, CXCL5 (chemokine (C-X-C motif) ligand 5) were recognized as the critical genes. We found that miRNAs interacting with ABLIM1 were hsa-miR-373-3p and hsa-miR-302e while miRNAs interacting with CXCL5 was hsa-miR-3655. The screened miRNAs and critical genes, together with the interactions, could help in better understanding the role of miRNA and mRNA in the occurrence and development of osteoporosis, providing new insights into the treatment of osteosarcoma.

Keywords: Osteosarcoma, miRNA, mRNA, biomarker, pathway analysis

Introduction

Osteosarcoma (OS) is a kind of most common primary malignant bone tumor. Adolescents and young adults, with the age ranging from 15 to 39 years, are more susceptible to osteosarcoma than other people [1-3]. Survival rate of OS seem to reach the plateau despite with the improvement of survival rate thanks to the chemotherapy strategies. Currently, the survival time of 65% patients is five years [4] and it left the problem of significant chemotherapy-related toxicity after treatment [5]. What’s worse, there was still a large number of patients who cannot exempt from amputation and even die due to lung metastasis or relapse [6], causing a huge economic burden to families of patients and the society. Besides, some prior studies demonstrated that a significant risk for OS patients with increasing age was associated with the poorer prognosis of the disease [7, 8]. Therefore, there is still urgent need for the development of new markers that could be helpful for the prediction, therapeutic modalities and the prognostic development of OS disease [4, 9]. Therefore, in this study, we aimed to identify the differentially expressed miRNAs and mRNAs between OS patients and normal ones, with the attempt to find the new markers and therapy targets of OS.
Recent studies showed that DNA damage would lead to genomic instability, malignant transformation, and cell death [6]. MicroRNAs are a series of non-coding RNA molecules that can negatively regulate gene expression and play import roles in various biological processes [10]. Increasing evidence has demonstrated that miRNAs are associated with several types of tumor by regulating cell migration and invasion. DNA microarray technology was used in this study to determine the differentially expressed miRNAs and mRNAs of OS. The new technology allows us to screen tens of thousands of genes simultaneously and be able to look at the molecular mechanism of disease development on a whole genome scale and the method has been used in many studies to identify genes. However, since there has been few public databases built to facilitate data sharing [11-13], it remains a big problem to integrate data from different organism in order to make a reliable hypotheses.

In this study, three significantly up-regulated miRNAs, has-miR-182, has-miR-486-5p and has-miR-760, was identified, which may play important roles in the development and progression of OS. What's more, two genes were found to be regulated by related miRNAs in the interaction analysis, the up-regulated gene ABLIM1 was regulated by has-miR-373-3p and has-miR-302e while the down-regulated gene CXCL5 was regulated by has-miR-3655, suggesting their potential role as a novel diagnostic and prognostic marker of OS.

**Materials and methods**

**Data source of miRNA and mRNA data of osteosarcoma**

The microarray expression data profiling of miRNA and mRNA were downloaded from the Genome expression omnibus (GEO) dataset with the accession number GSE70415. A total of six samples, five osteosarcoma cell lines (MG63, Saos, HOS, NY, Hu09) and one normal mesenchymal stem cells sample (hMSC), were included. The whole datasets was mainly composed of two subsets. The expression dataset of miRNA with osteosarcoma was GSE70367 with the platform of GPL16384 ([miRNA-3] Affymetrix Multiplespecies miRNA-3 Array) while the mRNA expression data of osteosarcoma was GSE70414 with the platform of GPL570 ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array).

**Differentially expressed miRNA screening**

Firstly, information of mature miRNA matching a single probe out of genome from GPL 16384 was screened out. Then, the expression level of normal and osteosarcoma samples of these mature miRNAs was extracted out. Differentially expressed miRNAs were screened out by two-fold principle. To be more specific, if the expression level of miRNA in osteosarcoma was two folds or more than the normal samples, we assumed that these miRNAs were significantly up-regulated in osteosarcoma samples. On the contrary, if the expression level of miRNA in normal samples was two folds or more than the osteosarcoma specimens, we assumed that these miRNAs were significantly down-regulated in osteosarcoma samples. The difference was assumed not significant in other circumstances.

**Cluster analysis and principal component analysis of differentially expressed miRNAs in osteosarcoma**

The heatmap of differentially expressed miRNAs of osteosarcoma were drawn by heatmap package in R language. The most differentially expressed miRNAs between osteosarcoma samples and normal specimens were determined by the heatmap. Then, principal component analysis (PCA) was performed on these miRNAs by the expression value in different
miRNA expression in osteosarcoma

Figure 2. The up-regulated miRNA (A) and down-regulated miRNA (B) between osteosarcoma samples and controls. MG63, Saos, HOS, NY and Hu09 were used as five cell lines representing osteosarcoma. Expression changes with two-folds was used as criterion for differentially expressed miRNAs screening. A total of 27 up-regulated miRNAs and 6 down-regulated miRNAs in the five osteosarcoma cell lines were found out.

Table 1. Information of up-regulated miRNA and down-regulated miRNA of osteosarcoma samples compared to control specimens

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Origin</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-1273d</td>
<td>hsa-mir-1273d</td>
<td>1:10287776-10287861 (+)</td>
<td>GAACCAAUGAGGUGAGGCCUCAGU</td>
</tr>
<tr>
<td>hsa-miR-135b-star</td>
<td>hsa-mir-135b</td>
<td>1:205417430-205417526 (-)</td>
<td>GGAGACCGCCGCCCCGCUGUGAGAU</td>
</tr>
<tr>
<td>hsa-miR-139-3p</td>
<td>hsa-mir-139</td>
<td>11:72326107-72326174 (+)</td>
<td>AGUUGGCCUCACACGCCACACGU</td>
</tr>
<tr>
<td>hsa-miR-153-6p</td>
<td>hsa-mir-153-6p</td>
<td>16:69599711-69599771 (-)</td>
<td>CGGCCCCGCGCCGCUCCGCUCCU</td>
</tr>
<tr>
<td>hsa-miR-182</td>
<td>hsa-mir-182</td>
<td>7:129410223-129410332 (+)</td>
<td>UUUGGCAAGGUAGACUCAACGU</td>
</tr>
<tr>
<td>hsa-miR-200a-star</td>
<td>hsa-mir-200a</td>
<td>1:1103243-1103332 (+)</td>
<td>CAUCCUCCACGAGCCAGUGA</td>
</tr>
<tr>
<td>hsa-miR-208b</td>
<td>hsa-mir-208b</td>
<td>14:23887196-23887272 (+)</td>
<td>GAACCAAAGGUGGAGUCCUUCU</td>
</tr>
<tr>
<td>hsa-miR-302e</td>
<td>hsa-mir-302e</td>
<td>11:7255997-7256068 (+)</td>
<td>ACUGCGCCGCUACACCG</td>
</tr>
<tr>
<td>hsa-miR-3165</td>
<td>hsa-mir-3165</td>
<td>5:140027429-140027511 (+)</td>
<td>GCUUGGCCGCGCCGUGCGUCC</td>
</tr>
<tr>
<td>hsa-miR-3682-5p</td>
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<td>2:54076259-54076342 (-)</td>
<td>CAGUCCGUCCGCUCCGCUCCU</td>
</tr>
<tr>
<td>hsa-miR-3692</td>
<td>hsa-mir-3692</td>
<td>6:157950164-157950232 (+)</td>
<td>UGACAGACAAACAGACACGU</td>
</tr>
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<td>hsa-miR-373-star</td>
<td>hsa-mir-373</td>
<td>19:54291959-54292027 (+)</td>
<td>AAUAGGCAACCAAGGUGUUCU</td>
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<tr>
<td>hsa-miR-4275</td>
<td>hsa-mir-4275</td>
<td>4:28821204-28821290 (+)</td>
<td>CCAUCCUCCACAGU</td>
</tr>
<tr>
<td>hsa-miR-4295</td>
<td>hsa-mir-4295</td>
<td>10:114393929-114394013 (+)</td>
<td>CAGUCCACGGACUCCUCCU</td>
</tr>
<tr>
<td>hsa-miR-4322</td>
<td>hsa-mir-4322</td>
<td>19:10341089-10341161 (+)</td>
<td>CUGGUGCUCGCGCGGCGG</td>
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<tr>
<td>hsa-miR-4323</td>
<td>hsa-mir-4323</td>
<td>19:42637597-42637665 (-)</td>
<td>CAGGCCCAGCCCGACUCCAG</td>
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<tr>
<td>hsa-miR-4504</td>
<td>hsa-mir-4504</td>
<td>14:5076673-50766664 (-)</td>
<td>UUGUGCACAAACAGACAGUCAUG</td>
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<tr>
<td>hsa-miR-451b</td>
<td>hsa-mir-451b</td>
<td>17:27188389-27188456 (+)</td>
<td>UGCAGGCAAGAACUCCUCAU</td>
</tr>
<tr>
<td>hsa-miR-4785</td>
<td>hsa-mir-4785</td>
<td>2:161264321-161264393 (-)</td>
<td>AGAGUGCGCGAGCGCCGCAGC</td>
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<tr>
<td>hsa-miR-4791</td>
<td>hsa-mir-4791</td>
<td>3:19356340-19356423 (-)</td>
<td>UCCUCUAGGACUCGCGCCGCGG</td>
</tr>
<tr>
<td>hsa-miR-486-5p</td>
<td>hsa-mir-486-5p</td>
<td>8:41517959-41518026 (-)</td>
<td>UCCUGUAGGACUCGCGCCGCGG</td>
</tr>
<tr>
<td>hsa-miR-518d-3p</td>
<td>hsa-mir-518d-3p</td>
<td>19:54238131-54238217 (+)</td>
<td>UUGCUCCACGUGUGUGUCC</td>
</tr>
<tr>
<td>hsa-miR-523-star</td>
<td>hsa-mir-523</td>
<td>19:54201639-54201725 (+)</td>
<td>CUCUGAAGGACACGCGCUUCCU</td>
</tr>
</tbody>
</table>
miRNA expression in osteosarcoma

samples, thus we can determine whether these miRNAs can clearly differentiate osteosarcoma samples from normal tissues.

**Prediction of target genes of differentially expressed miRNA and functional enrichment analysis**

Target genes of differentially expressed miRNA verified by experiment were extracted out by miRTarBase database [14]. Then, Go (Gene Ontology) enrichment analysis [15] and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis [16] were conducted by DAVID (Database for Annotation, Visualization, and Integrated Discovery) [17]. In GO enrichment analysis, the p value justified after multi-testing by DAVID software was used as the threshold and p value less than 0.05 was extracted out.

**Screening of differentially expressed mRNA**

Differentially expressed mRNA in osteosarcoma samples were determined by two folds condition and these mRNAs were mapped to the above interaction of miRNA-target genes and mRNA, thus critical genes and interactions associated with osteosarcoma were screened out (Figure 1).

![Cluster analysis of differentially expressed miRNAs of osteosarcoma.](image)

**Figure 3.** Cluster analysis of differentially expressed miRNAs of osteosarcoma. The horizontal axis represents the selected samples and hMSC was the control sample on the separated branch. The vertical axis stands for the result of cluster analysis of differentially expressed miRNA. Three most significant miRNAs, hsa-miR-182, hsa-miR-486-5p and hsa-miR-760, were clustered into one branch, indicating their important role in the occurrence of osteosarcoma disease.
miRNA expression in osteosarcoma

Results

Screening of differentially expressed miRNA in osteosarcoma

Five osteosarcoma cell lines (MG63, Saos, HOS, NY, Hu09) and one control group (hMSC) were included in our downloaded miRNA expression data with the accession number (GSE70415). The up and down-regulated miRNAs were determined between osteosarcoma samples and normal specimens. To be more specific, if the expression value of some miRNA in osteosarcoma samples was at least two folds than normal specimens, we assumed that it was up-regulated in osteosarcoma samples; if the expression value of some miRNA in normal samples was at least two folds than osteosarcoma specimens, we assumed that it was down-regulated in osteosarcoma samples. The usual and specific up and down-regulated miRNAs in osteosarcoma cells were shown in Figure 2.

A total of 1591 miRNAs had the single probe after excluding miRNAs originated from more than one miRNA and these miRNAs were used for the screening of differentially expressed miRNAs. The up and down-regulated miRNAs were display by the form of Wine figure (Figure 2) and list table (Table 1). As can be seen from Figure 2, a total of 393 miRNAs were up-regulated in osteosarcoma tissue and 27 among them were up-regulated in all osteosarcoma samples. As for the down-regulated condition, a total of 417 miRNAs were down-regulated in osteosarcoma samples and six among them were down-regulated in all osteosarcoma samples.

Cluster analysis and principal component analysis of differentially expressed miRNA in osteosarcoma

Heatmap was drawn based on the expression value of the 33 differentially expressed miRNAs and we found that the control group (hMSC) was on one single branch while the osteosarcoma was on the other. Meanwhile, the result of cluster analysis of differentially expressed miRNAs showed that three miRNAs that was most differentially expressed were on the same branch and these miRNAs were hsa-miR-182, hsa-miR-486-5p and hsa-miR760 (Figure 3). Therefore, we assumed that the three differentially expressed miRNAs were most obvious in the occurrence of osteosarcoma disease.

We conducted principal component analysis of miRNA expression value on the top three significantly differentially expressed miRNAs and we found that one dimension principal component had contribution rate of 77.6% while the two dimensions had the contribution rate of 21.55%. The accumulative contributions ratio of the two was 99.15% (Figure 4), which indicated that these three miRNAs can differentiate osteosarcoma samples perfectly from the control group perfectly.

Prediction of target genes of differentially expressed miRNAs in osteosarcoma and functional annotation

Firstly, we extracted the target genes of differentially expressed miRNAs testified by MirTarBase and 2048 target genes corresponding with 28 miRNAs were obtained. The number of target genes corresponding with up-regulated miRNAs was 1866 and the number of target genes matching with down-regulated miRNAs was 67. Functional enrichment annotation was
miRNA expression in osteosarcoma

performed on these target genes and the result of GO enrichment analysis showed that the enriched GO terms of up-regulated miRNAs was much more than that of down-regulated miRNAs. Meanwhile, both target genes of up and down-regulated miRNAs were significantly enriched in the process of transcriptional regulation, confirming the transcriptional regulation role of miRNA in the occurrence of osteosarcoma disease. KEGG enrichment analysis was further conducted on these target genes of up and down-regulated miRNAs. We found that these target genes were significantly enriched in four metabolic pathways respectively and these pathways were all in high correlation with the occurrence of cancer (Figure 5 and Table 2).

**Screening of differentially genes between osteosarcoma samples and normal samples**

Two folds principle was as well used in the determination of differentially expressed mRNA between osteosarcoma samples and normal specimens. By the comparison with the normal samples, two significant up-regulated mRNA in all five osteosarcoma cells were selected while 15 significant down-regulated mRNA were found (Figure 6).

The result of differentially expressed mRNA was mapped to the interaction network of miRNA-target genes mRNA. Two gene, that is, ABLIM1 (actin binding LIM protein 1), up-regulated in osteosarcoma tissue and CXCL5 (chemokine (C-X-C motif) ligand 5), down-regulated in osteosarcoma, were regulated by relative miRNAs. hsa-miR-373-3p and hsa-miR-302e interacted with ABLIM1 gene while hsa-miR-3655 was in the interaction with CXCL5 gene.

**Discussion**

In this study, five osteosarcoma cell lines, MG63, Saos, HOS, NY, Hu09, were used to screen the differentially expressed miRNAs. Genetic, phenotypic and functional characteristics have shown that cell lines can represent osteosarcoma clinical samples robustly [18-20]. As a result, a total of 27 miRNAs were found to be up-regulated in all five cell line while the number of down-regulated miRNAs was six. Three miRNAs, that is, hsa-miR-182, hsa-miR-486-5p and hsa-miR760 were significantly up-regulated in osteosarcoma samples, indicating their potential significant role in the progression of the disease. It’s been reported that dys-regulation of miR-183 significantly impacts tumor metastasis in osteosarcoma via Ezrin [21, 22], however, as for the role of miR-182 in tumoregenesis and metastasis, there hasn’t been much report. The discovery of miR-182 in this study indicated a possible new marker of OS, which needs further research for the specific mechanism. Besides, the result of GO enrichment analysis revealed that target genes...
miRNA expression in osteosarcoma

Table 2. KEGG pathway enrichment analysis of target genes of up-regulated miRNAs

<table>
<thead>
<tr>
<th>Category</th>
<th>Term</th>
<th>Count</th>
<th>%</th>
<th>P-Value</th>
<th>Fold</th>
<th>Enrichment</th>
<th>Benjamini</th>
</tr>
</thead>
<tbody>
<tr>
<td>up-regulated</td>
<td>Systemic lupus erythematosus</td>
<td>28</td>
<td>1.5</td>
<td>2.00E-06</td>
<td>2.7</td>
<td>3.80E-04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Colorectal cancer</td>
<td>21</td>
<td>1.1</td>
<td>3.30E-04</td>
<td>2.4</td>
<td>3.10E-02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endocytosis</td>
<td>35</td>
<td>1.9</td>
<td>6.80E-04</td>
<td>1.8</td>
<td>4.10E-02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adherens junction</td>
<td>19</td>
<td>1</td>
<td>8.30E-04</td>
<td>2.3</td>
<td>3.80E-02</td>
<td></td>
</tr>
<tr>
<td>down-regulated</td>
<td>Endometrial cancer</td>
<td>6</td>
<td>2.4</td>
<td>5.90E-04</td>
<td>8.5</td>
<td>6.20E-02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prostate cancer</td>
<td>7</td>
<td>2.9</td>
<td>1.10E-03</td>
<td>5.8</td>
<td>5.80E-02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pathways in cancer</td>
<td>13</td>
<td>5.3</td>
<td>1.20E-03</td>
<td>2.9</td>
<td>4.10E-02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Focal adhesion</td>
<td>10</td>
<td>4.1</td>
<td>1.30E-03</td>
<td>3.7</td>
<td>3.40E-02</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6. The up-regulated mRNA and down-regulated mRNA in OS samples. MG63, Saos, HOS, NY and Hu09, were used as five cell lines representing osteosarcoma. Expression changes with two-folds was used as criterion for differentially expressed miRNAs screening. The number of up-regulated mRNA was two while the number of down-regulated mRNA was fifteen edmiRNAs.

of differentially expressed miRNAs were mainly enriched in four pathways, that is, regulation of transcription, DNA dependent, regulation of RNA metabolic process, transcription and regulation of transcription, all in high relevance with the occurrence of cancer, confirming their potential role as indicator to clinical outcome of OS patients. Furthermore, target genes of the interested miRNAs were analyzed, which may clearly reflect the roles of miRNAs in tumor progression. The result of miRNA-target genes interactions showed that two genes were regulated by correlated miRNAs. The up-regulated ABLIM1 (actin binding LIM protein 1) was significantly regulated by hsa-miR-373-3p and hsa-miR-302e while the down-regulated gene CXCL5 (chemokine C-X-C motif ligand 5) was regulated by hsa-miR-3655.

It’s been reported that aberrant splicing of ABLIM1 gene was found in the skeletal muscle of patients with DM (dystrophia myotonica) [23], a kind of common form of muscular dystrophy that affects adults. In this study, ABLIM1 was identified as one of the critical genes of OS, and regulated by hsa-miR-373-3p and hsa-miR-302e, providing us with new insights into exploring the molecular mechanism of the role of this gene played in the disease. One of the miRNAs, hsa-miR-373-3p was reported to be able to promote invasion and metastasis of lung adenocarcinoma cells. Si-
miRNA expression in osteosarcoma is frequently accompanied with lung metastasis [24], we assumed that there was somehow correlation between the expression of hsa-miR-373-3p and the occurrence of OS disease, which obviously needed further research.

Another critical gene, CXCL5, has been reported to be an important factor in cancer biology and recent studies demonstrated that CXCL5 directly stimulates cancer cells and endothelial cells proliferation and invasion [25-28]. Prior research revealed that it can promote tumor angiogenesis in many kinds of cancers, such as, non-small cell lung carcinoma and pancreatic cancer to modulate tumor growth metastasis [29, 30]. Besides, 80% of primary lung adenocarcinomas and 65% of lung adenocarcinoma cell lines were observed tumor-specific methylation of CXCL5 [31]. However, the expression of CXCL5 and mechanisms of how CXCL5 functions in OS progress remain elusive.

In summary, we identified three markers hsa-miR-182, hsa-miR-486-5p and hsa-miR760, which may serve as the targets in the developments and treatments of OS. More importantly, two miRNA-target genes interactions were found out, providing new views on diagnosis and prognosis of OS patients.

Disclosure of conflict of interest

None.

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