Identification of key pathways and genes in miR-30e regulating osteogenesis in aortic smooth muscle cells using bioinformatics analysis

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Abstract
Background: Cardiovascular disease (CVD) is an important problem that threatens the health of all mankind and remains the world’s first cause of morbidity and mortality. In this study, we identified the gene characteristic during vascular calcification and explored their potential mechanisms.

Results: Gene expression profiles of GSE65435 were downloaded from GEO database. The GSE65435 dataset contained 18 samples, the 3 SMCs + miR-30e samples and 3 SMCs + ct-miR samples were selected. The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) enrichment analyses were implemented, and gene signal network and pathway relation network of the differentially expressed genes (DEGs) were analyzed by GCBI. In total, 620 differentially expressed genes were identified in SMCs + miR-30e samples, including 249 up-regulated genes and 371 down-regulated genes. GO analysis summed up that DEGs were significantly concentrated in negative regulation of apoptotic process. KEGG pathway analysis showed that DEGs were enriched in PI3K-Akt signaling pathway.

Conclusions: This study showed that the identified DEGs increased the molecular mechanisms underlying the development of vascular calcification and might be used as new diagnostic and therapeutic strategies for the treatment of vascular calcification.

Introduction
According to the World Health Organization (WHO) 2011 report, cardiovascular disease (CVD) is the primary problem that threatens the health of all mankind and remains the world’s first cause of morbidity and mortality [1]. The number of cardiovascular diseases exceeds cancer, diabetes, chronic respiratory disease, etc. [2]. The data for 2008 showed that 30% of the total number of deaths worldwide are due to cardiovascular diseases, the vast majority of which were coronary heart diseases and stroke, and this trend is expected to continue [3]. In 2030, the number of death due to heart disease and stroke may increase from 18 million to 23.3 million and cardiovascular disease will continue to be the major cause of death [4]. In China, according to the China’s health statistics released by the ministry of health, a total of 27,160,00 people died of cardiovascular diseases in 2007, becoming the first cause of death [5]. CVD pathogenesis has been a hot topic in the field of cardiovascular research, it has been commonly recognized that smooth muscle cell proliferation, inflammatory mediators, vascular endothelial dysfunction and lipid deposition are involved, among which vascular calcification plays an important role in the occurrence and development of a variety of cardiovascular diseases such as coronary heart disease, hypertension, degenerative heart valvular disease and cardiomyopathy [6]. Cardiovascular calcification is similar to the bone formation during the embryonic period, a variety of genes and proteins in minerals and bone metabolism have a certain regulatory role in vascular calcification [7].

MicroRNA (miRNA) is very important for gene regulation. Recent studies have shown that some miRNA can affect the process of bone formation by regulating the target genes, and could also have a regulatory role in the process of vascular calcification [8]. MiR-30e was reported to have the ability to induce adipogenic differentiation and reduce bone formation differentiation in stromal cells by targeting Lrp6 [9]. The article “miR-30e targets IGF2-regulated osteogenesis in bone marrow-derived mesenchymal stem cells, aortic smooth muscle cells, and ApoE2/2 mice” aimed to examine the role of miR-30e in vascular calcification. This article concluded that miR-30e inhibited the osteogenesis in MSCs and SMCs by targeting IGF2 and suppressed their differentiation into adipogenic or smooth muscle lineage [10]. In this study, there is an experiment that SMCs were treated with ct-miR or miR-30e and draw a conclusion that miR-30e inhibited the osteogenesis in MSCs and SMCs and suppressed their differentiation into adipogenic or smooth muscle lineage. The results of gene sequencing of two groups mice were upload to GEO DataSets (GSE65435) [11]. To further show the function of miR-30e of SMCs at the molecular level and explore the possible candidate biomarkers for diagnosis, prognosis, and drug targets, we are analyzing their biological functions and pathways.

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Key words: Vascular calcification; miR-30e; differentially expressed genes (DEGs); gene ontology (GO); Kyoto Encyclopedia of Genes and Genomes pathway (KEGG)

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Materials and methods

Microarray data
Gene expression profiles of GSE65435 were downloaded from the GEO database. GSE65435, which was based on Affymetrix GPL6246 platform (Affymetrix Mouse Gene 1.0 ST Array), was submitted by Wen, et al. The GSE65435 dataset comprised 18 samples. In this study, we selected the 6 samples, describing the effect of miR-30e in aortic smooth muscle cells differentiation.

Identification of DEGs
The raw data files were uploaded to the website of Gene-Cloud of Biotechnology Information (GCBI) [12]. SMCs + miR-30e samples were used as the test group and the SMCs + ct-miR used as the control group in this data. In the analysis process, we defined P = 0.05, Q = 0.05 and fold change = 2.0.

Gene ontology and pathway enrichment analysis of DEGs and network analysis
All the analysis was completed by the GCBI. In the gene ontology analysis, FDR = 0.05 and P = 0.05. In the pathway enrichment analysis, P = 0.05. Then we analyzed gene signal network to gene ontology analysis and analyzed pathway relation network to pathway enrichment. Then, networks were built by the cytoscape (3.4.0) software.

Results

Identification of DEGs
The samples were composed by 3 test samples and 3 control samples. Based on the P < 0.05 and fold control (FC) > 2.0 standards, a total of 620 genes were identified after the analyses of GSE65435, of which 249 were up-regulated and 371 were down-regulated (Figure 1). The gene Saa3 had a largest difference between SMCs + miR-30e group and SMCs + ct-miR group. The expression of gene Saa3 in SMCs + miR-30e group is 199.97 times in SMCs + ct-miR group. As showed in Table 1, the top ten DEGs were Saa3, Serpine2, Cd34, Grem1, Chi3l1, Hp, Selp, Ch25h, Clmp and Sfrp1.

GO term enrichment and KEGG pathway analysis
We analyzed DEGs by GCBI online tools which are mainly based on the algorithms of miR and target scan, to identify over represented GO categories and KEGG pathways. GO analysis indicated that up-regulated DEGs were significantly concentrated in biological processes (BP), including the cell cycle, cell division, and cell proliferation.

To identify the biological functions of these genes, GO and pathway enrichment analysis were implemented, respectively. As illustrated in Figure 2, the top ten regulated GOs sensitive to high concentration of miR-30e were positive regulation of angiogenesis, inflammatory response, positive regulation of apoptotic process, negative regulation...
of cell proliferation, negative regulation of apoptotic process, cell adhesion, positive regulation of transcription from RNA polymerase II promoter, apoptotic process, protein phosphorylation and multicellular organismal development. And the genes of each function were listed in Table 2. GO analysis obviously suggested that high concentration of miR-30e could affect expression of many miRNAs, through many crucial functions such as regulation of angiogenesis, inflammatory response and positive regulation of apoptotic process of the mice with high expression of miR-30e. Combining with the KEGG database, we analyzed the pathways in which the putative target genes were involved. As illustrated in Figure 3, the top ten deregulated pathways sensitive to high concentration of miR-30e were PI3K-Akt signaling pathway, apoptosis, and promote the activation of adipocyte cells, leading to the differentiation of adipocytes in SMCs. Furthermore, enriched KEGG pathways might play an important role in apoptosis induced by miR-30e. So, we speculated that the down-regulated genes were related with apoptosis, and promote the activation of adipocyte cells, leading to the differentiation of adipocytes in SMCs. Therefore, the process of osteoblast apoptosis, and promote the activation of adipocyte cells, leading to the differentiation of adipocytes in SMCs. Furthermore, enriched KEGG pathways might play an important role in apoptosis induced by miR-30e.

Discussion

Vascular calcification is a complex biological process associated with aging and degenerative changes [13]. Studies have shown that vascular calcification is an active process that similar to bone formation and it is regulated by multiple factors involving vascular smooth muscle cells, macrophages, endothelial cells, fibroblasts, multiple signal molecules (AKT, KLFS, Smads etc.). Atherosclerosis risk factors include dyslipidemia, hypertension, diabetes, renal failure and this can promote the occurrence and development of arterial calcification [14,15]. The pathogenesis of vascular calcification is currently considered to be related with intravascular bone formation [16]. From the article “miR-30e targets IGF2-regulated osteogenesis in bone marrow-derived mesenchymal stem cells, aortic smooth muscle cells, and ApoE2/2 mice”, we known that miR-30e inhibited the osteogenesis in SMCs by targeting IGF2 and suppressed their differentiation into adipogenic or smooth muscle lineage [10].

In this study, gene expression data of 3 SMCs + miR-30e samples and 3 SMCs + ct-miR samples were recovered from the GEO dataset under the accession number GSE65435. The study analyzed 620 DEGs between SMCs + miR-30e samples and SMCs + ct-miR samples, among which 249 genes were up-regulated and 371 were down-regulated. Interestingly, the top 10 DEGs were all down-regulated. So, we speculated that the down-regulated genes were related with osteogenesis. For purpose of better concluded the reciprocity of DEGs, we further analyzed GO analysis and KEGG pathway analysis.

The GO term analysis indicated that DEGs were mainly concluded negative regulation of apoptotic process, cell adhesion, and positive regulation of transcription from RNA polymerase II promoter. It is suggested that miR-30e may promote the process of osteoblast apoptosis, and promote the activation of adipocyte cells, leading to the differentiation of adipocytes in SMCs. Furthermore, enriched KEGG pathways might play an important role in apoptosis induced by miR-30e. Therefore, the process of osteoblast apoptosis, and promote the activation of adipocyte cells, leading to the differentiation of adipocytes in SMCs. Furthermore, enriched KEGG pathways might play an important role in apoptosis induced by miR-30e.

Table 1. The top 10 mRNAs with high degrees of DEGs.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Gene Description</th>
<th>d Score</th>
<th>Fold Change</th>
<th>Gene Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saa3</td>
<td>NM_011315</td>
<td>Mus musculus serum amyloid A 3 (Saa3), mRNA</td>
<td>-31.68102</td>
<td>-199.976269</td>
<td>down</td>
</tr>
<tr>
<td>Serpin2</td>
<td>NM_009255</td>
<td>Mus musculus serine (or cysteine) peptidase inhibitor, clade E, member 2 (Serpin2), mRNA</td>
<td>-28.431854</td>
<td>-55.253872</td>
<td>down</td>
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<tr>
<td>Cd34</td>
<td>NM_001111059</td>
<td>Mus musculus CD34 antigen (Cd34), transcript variant 1, mRNA</td>
<td>-19.894471</td>
<td>-12.007931</td>
<td>down</td>
</tr>
<tr>
<td>Grem1</td>
<td>NM_011824</td>
<td>Mus musculus gremlin 1 (Grem1), mRNA</td>
<td>-19.045846</td>
<td>-20.450459</td>
<td>down</td>
</tr>
<tr>
<td>Chh1lI</td>
<td>NM_007695</td>
<td>Mus musculus chitin 3-like 1 (Chh1lI), mRNA</td>
<td>-18.246297</td>
<td>-13.716394</td>
<td>down</td>
</tr>
<tr>
<td>Hl</td>
<td>NM_017370</td>
<td>Mus musculus haptoglobin (Hl), mRNA</td>
<td>-17.482949</td>
<td>-30.85244</td>
<td>down</td>
</tr>
<tr>
<td>Selp</td>
<td>NM_011347</td>
<td>Mus musculus selectin, platelet (Selp), mRNA</td>
<td>-15.500173</td>
<td>-8.72038</td>
<td>down</td>
</tr>
<tr>
<td>Ch2sh</td>
<td>NM_009890</td>
<td>Mus musculus cholesterol 25-hydroxylase (Ch2sh), mRNA</td>
<td>-15.265039</td>
<td>-22.893805</td>
<td>down</td>
</tr>
<tr>
<td>Clmp</td>
<td>NM_133733</td>
<td>Mus musculus CXADR-like membrane protein (Clmp), mRNA</td>
<td>-15.021017</td>
<td>-11.558135</td>
<td>down</td>
</tr>
<tr>
<td>Sfrp1</td>
<td>NM_013834</td>
<td>Mus musculus secreted frizzled-related protein 1 (Sfrp1), mRNA</td>
<td>-14.351045</td>
<td>-46.482443</td>
<td>down</td>
</tr>
</tbody>
</table>

Figure 2. Significantly changed GOs of predicted target genes.

Gene Signal Network and pathway Relation Network

We performed pathway relation network analysis to draw a reciprocity network covering 35 significantly changed pathways (Figure 4). Among them, MAPK signaling pathway (degree = 16), apoptosis (degree = 15), pathways in cancer (degree = 15) and cell cycle (degree = 12) showed highest degree, suggesting that these four pathways might play an important role in apoptosis induced by miR-30e treatment. The top 10 significantly changed pathways were listed in Table 4. Based on the obvious regulated GOs and pathways, we selected intersected genes and further constructed mRNAs-GO-networks to screen the key regulatory functions of the identified mRNAs and their target genes, respectively. As shown in Figure 5 and Table 5, the top rated 10 mRNAs including IL-6, Myd88, Fos, Ppap2b, Tlr2, Tlr4, Hk2, Wnt4, C3 and Gstk1. Apart from the gene Tlr4, the other mRNAs were down-regulated by miR-30e treatment in mice.

pathways of DEGs including PI3K-Akt signaling pathway, pertussis, cytokine-cytokine receptor interaction. Previous studies showed that PI3K-Akt signaling pathway is a key pathway for osteoclast activation [17,18]. MiR-30e promotes osteoclast activation by active this pathway, and then leads to osteoblasts reduction. The pertussis toxin-insensitive CCR5 signaling in macrophage pathway was inhibited in the SMCs + miR-30e group and inhibited calcium transport into cells [19]. This pathway also contributed to the reduction of osteogenesis in the SMCs cells. The cytokine-cytokine receptor interaction targets on cells and promotes adipogenic differentiation [20,21].

We also analyzed the mRNAs pathway network with DEGs and list the top degree genes: IL-6, Myd88, Fox, Ppap2b, Trl2, Trl4, Hk2, Wnt4, C3 and Gata1. Among them, IL-6 expression in the highest degree. Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine [22,23]. T cells and macrophages secreted the interleukin 6 and stimulate the immune response. In addition, IL-6 also secreted by the osteoblasts to stimulate osteoclast formation [24]. Smooth muscle cells of many blood vessels also secreted IL-6 as a pro-inflammatory cytokine that miR-30e acts in the regulation of IL-6 [25]. The second pathway...
gene myeloid differentiation primary response gene 88 (MYD88) is a key linker in the Toll-like receptor (TLR) signaling pathway and plays an important role in the transmission of upstream information and disease progression [26]. Toll-like receptors (TLRs) are a class of proteins that play an important role in the innate immune system [27]. From this, we speculate that SMC differentiation is related to the immune system. Ppap2b lets it to regulate vascular and embryonic development by inhibited LPA signaling, which is associated with many human diseases, including cardiovascular disease and cancer, as well as developmental defects [28]. Tlr2 also belong to Toll-like receptor (TLR) signaling pathway. The above results indicated that multiple factors in the body affected the osteogenic differentiation of SMC.

Module analysis of the mRNAs GO network showed that the SMC osteogenesis differentiation was associated with MAPK signaling pathway, apoptosis, and pathways in cancer. The MAPK pathway communicated a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. MAPK signaling pathway also promoted the differentiation of osteoclasts, and related with calcification in SMCs [29]. Studies have also showed that MAPK pathway is associated

<table>
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<th>Pathway ID</th>
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<th>Degree</th>
<th>Pathway Feature</th>
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<tr>
<td>4010</td>
<td>MAPK signaling pathway</td>
<td>16</td>
<td>down/up</td>
</tr>
<tr>
<td>4210</td>
<td>Apoptosis</td>
<td>15</td>
<td>down</td>
</tr>
<tr>
<td>6200</td>
<td>Pathways in cancer</td>
<td>15</td>
<td>up/down</td>
</tr>
<tr>
<td>4510</td>
<td>Focal adhesion</td>
<td>12</td>
<td>up/down</td>
</tr>
<tr>
<td>4115</td>
<td>p53 signaling pathway</td>
<td>9</td>
<td>down/up</td>
</tr>
<tr>
<td>4060</td>
<td>Cytokine-cytokine receptor interaction</td>
<td>9</td>
<td>down/up</td>
</tr>
<tr>
<td>4310</td>
<td>Wnt signaling pathway</td>
<td>8</td>
<td>down</td>
</tr>
<tr>
<td>10</td>
<td>Glycolysis / Gluconeogenesis</td>
<td>7</td>
<td>Up/down</td>
</tr>
<tr>
<td>4620</td>
<td>Toll-like receptor signaling pathway</td>
<td>6</td>
<td>down/up</td>
</tr>
<tr>
<td>4630</td>
<td>Jak-STAT signaling pathway</td>
<td>6</td>
<td>up/down</td>
</tr>
</tbody>
</table>
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Table 5. The top 10 mRNAs with high degrees of mRNAs-pathway-network.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Feature</th>
<th>Biotype</th>
<th>Gene Description</th>
<th>Degree</th>
</tr>
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<tr>
<td>Il6</td>
<td>down coding</td>
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<td>&quot;Mus musculus interleukin 6 (Il6), mRNA.&quot;</td>
<td>20</td>
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<tr>
<td>Myd88</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus myeloid differentiation primary response gene 88 (Myd88), mRNA.&quot;</td>
<td>10.5</td>
</tr>
<tr>
<td>Fes</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus FBJ osteosarcoma oncogene (Fos), mRNA.&quot;</td>
<td>7.5</td>
</tr>
<tr>
<td>Ppap2b</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus phosphorous acid phosphatase type 2B (Ppap2b), mRNA.&quot;</td>
<td>6</td>
</tr>
<tr>
<td>Trx2</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus toll-like receptor 2 (Trx2), mRNA.&quot;</td>
<td>4.5</td>
</tr>
<tr>
<td>Tlr4</td>
<td>up coding</td>
<td></td>
<td>&quot;Mus musculus toll-like receptor 4 (Tlr4), mRNA.&quot;</td>
<td>4.5</td>
</tr>
<tr>
<td>Hk2</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus hexokinase 2 (Hk2), mRNA.&quot;</td>
<td>2</td>
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<tr>
<td>Wnt4</td>
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<td>&quot;Mus musculus wingless-related MMTV integration site 4 (Wnt4), mRNA.&quot;</td>
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<tr>
<td>C3</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus complement component 3 (C3), mRNA.&quot;</td>
<td>1</td>
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<tr>
<td>Gstk1</td>
<td>down coding</td>
<td></td>
<td>&quot;Mus musculus glutathione S-transferase kappa 1 (Gstk1), nuclear gene encoding mitochondrial protein, mRNA.&quot;</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 5. mRNAs gene network. According to the interactions between miRNAs and the intersected target genes, miRNAs-gene-network was constructed to illustrate the key regulatory functions of the identified miRNAs and their target genes.

with cancer [30]. The results showed that calcification in SMCs also related with pathways in cancer. We hypothesized that when a cancer-related pathway is activated, it causes a series of cellular responses, including vascular calcification.

Conclusion

In this study, we provide an integrated bioinformatics analysis of DEGs, which may be included in the progress of differentiation of SMCS. The study showed a series of important targets for future research into the molecular mechanisms and biomarkers. We hope that these genetic analyzes contribute to the study of vascular calcification.

Availability of data and material

miR-30e targets IGF2-regulated osteogenesis in bone marrow-derived mesenchymal stem cells, aortic smooth muscle cells, and ApoE2/2 mice.

Competing interests

The authors have no conflicts of interest to disclose.
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None.

Authors’ contributions
Zhi X., Chen X. and Su J.C. designed this study. Zhi X. analyzed the data. All authors read and approved the final manuscript.

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References
10. Ding W (2016) miR-30e targets IGFB2-regulated osteogenesis in bone marrow-derived mesenchymal stem cells, aortic smooth muscle cells, and ApoE (-/-) mice. Cardiovasc Res 109: 373-373. [Crossref]