ADCY2, ADCY5, and GRIA1 are the key genes of cAMP signaling pathway to participate in osteoporotic spinal fracture after the manipulation of Wnt signaling

Type

Research paper

Keywords

Wnt signaling pathway, Spinal Fractures, GRIA1, cAMP signaling

Abstract

Introduction
Osteoporotic spinal fracture, characterized by high morbidity and mortality, has become a health burden for the aging population. The inactivation of the Wnt signaling has been proved to promote osteoporotic fractures. Our study is to identify the key genes, miRNAs, and pathways that possibly lead to osteoporosis and osteoporotic spinal fracture after the aberrant activation or mutation of Wnt signaling pathway.

Material and methods
Impute R package was used to screen out the differently expressed genes (DEGs) and differently expressed miRNAs in GEO datasets. STRING and Metascape were used to construct protein-protein interactions (PPI) network, gene ontology (GO) enrichment and pathway enrichment. The relative expression of ADCY2, ADCY5, and GRIA1 in bone tissues was measured by RT-qPCR.

Results
562 DEGs were screened out using Impute R package, and a PPI network involving the 562 DEGs was constructed using STRING and Metascape. GO enrichment and pathway enrichment showed that the 562 DEGs were associated with membrane protein-related signaling pathways. Then, 75 genes between the target genes of miR-18a-3p and 562 DEGs were overlapped using Venny 2.1.0. Finally, the cAMP signaling pathway was identified as the key pathway, whilst ADCY2, ADCY5, and GRIA1 were identified the key genes that possibly participate in osteoporotic spinal fracture after the manipulation of Wnt signaling pathway, which was further proved by their excessive downregulation in osteoporotic patients with spinal fracture.

Conclusions
The results demonstrated that ADCY2, ADCY5, and GRIA1 were the key genes to regulate the cAMP signaling pathway in osteoporotic spinal fracture after abnormal Wnt signaling.

ADCY2, ADCY5, and GRIA1 are the key genes of cAMP signaling pathway to participate in osteoporotic spinal fracture after the manipulation of Wnt signaling

Running title: cAMP pathway regulates spinal fracture

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Introduction: Osteoporotic spinal fracture, characterized by high morbidity and mortality, has become a health burden for the aging population. The inactivation of the Wnt signaling has been proved to promote osteoporotic fractures. Our study is to identify the key genes, miRNAs, and pathways that possibly lead to osteoporosis and osteoporotic spinal fracture after the aberrant activation or mutation of Wnt signaling pathway.

Material and methods: Impute R package was used to screen out the differently expressed genes (DEGs) and differently expressed miRNAs in GEO datasets. STRING and Metascape were used to construct protein-protein interactions (PPI) network, gene ontology (GO) enrichment and pathway enrichment. The relative expression of ADCY2, ADCY5, and GRIA1 in bone tissues was measured by RT-qPCR.

Results: 562 DEGs were screened out using Impute R package, and a PPI network involving the 562 DEGs was constructed using STRING and Metascape. GO enrichment and pathway enrichment showed that the 562 DEGs were associated with membrane protein-related signaling pathways. Then, 75 genes between the target genes of miR-18a-3p and 562 DEGs were overlapped using Venny 2.1.0. Finally, the cAMP signaling pathway was identified as the key pathway, whilst ADCY2, ADCY5, and GRIA1 were identified the key genes that possibly participate in osteoporotic spinal fracture after the manipulation of Wnt signaling pathway, which was further proved by their excessive downregulation in osteoporotic patients with spinal fracture.

Conclusions: The results demonstrated that ADCY2, ADCY5, and GRIA1 were the key genes to regulate the cAMP signaling pathway in osteoporotic spinal fracture after abnormal Wnt signaling.

Keywords: Spinal Fracture; Wnt Signaling Pathway; GRIA1; cAMP signaling

Introduction

Osteoporosis is a common disorder caused by the imbalance between osteoblastic bone formation and osteoclastic bone resorption [1]. Age is proportional to the risk of development of osteoporosis [2]. Due to the absence of estrogen, women after menopause were at the highest risk of getting osteoporosis according to a previous study [3]. Certainly, the absence of androgenic hormones could also cause osteoporosis in men [4]. Besides, other factors also led to osteoporosis such as metabolic diseases, anorexia nervosa, thyroid and renal dysfunctions or dietary as well as lifestyle habits like low calcium intake or immobilization [5].

Osteoporosis can lead to a reduction in bone mass, deterioration in bone microarchitecture, susceptibility to skeletal fragility, and increased risk of fracture [6, 7]. The patients suffering osteoporotic fractures particularly spinal fracture are characterized by high morbidity and mortality so that the quality of life is significantly decreased. It has been reported that up to one-third of patients will sustain a new fracture within 5 years after the initial fracture [8]. Although anti-osteoporosis drugs reduce the risk of osteoporotic fractures by 20-70% in clinical trials depending on the drug and fracture type, the persistence with osteoporosis therapy is poor, and the one-year persistence ranges from 18 to 78% between studies in the real-world [9-14]. In fact, the postmenopausal women over age 55 are sensitive to the osteoporosis-related spinal fracture [15]. Therefore, understanding the key mechanism of osteoporotic fractures is crucial for treating spinal fracture.

Wingless-related integration site (Wnt)/β-catenin signaling pathway is the key pathway of bone metabolism to regulate bone mass. The defective Wnt signaling causes several monogenic skeletal disorders such as osteoporosis-pseudoglioma syndrome, van Buchem disease, and sclerosteosis [16-18]. For example, WNT7B enhanced the ability of bone formation by increasing osteoblast activity to increase bone mass [19]. Glucocorticoids depressed bone formation by inhibiting Wnt/β-catenin signaling pathway [20]. Laine CM *et al.* found that the mutation of Wnt1 could decrease the activity of the Wnt/β-catenin signaling pathway in bone leading to the decrease of the number of bone cells, damage of bone formation, low bone mass, and skeletal fragility [21]. If the skeletal fragility happened in vertebrae, the spinal fracture might be caused by simple movements such as coughing or sneezing. Mäkitie RE *et al.* also proved that impaired WNT/β-catenin signaling progressively changed the the spinal structures, which increased the risk of compression fractures especially after the age of 50 [22].

In this study, the expression profiles of mRNA and miRNA after manipulation of Wnt signaling were obtained from GEO DataSets. Then, the bioinformatic analysis including GO enrichment, KEGG enrichment, Reactome pathway, and PPI network was performed to analyze the key pathway, miRNAs, and genes after the mutation of the Wnt signaling pathway in osteoporosis and osteoporotic fractures. In the long term, our study should contribute to the treatment of osteoporotic fractures especially spinal fracture.

Materials & Methods

Clinical samples

A total of 42 patients diagnosed with osteoporosis and spinal fracture between Sep 2018 and March 2020 and 45 age-matched healthy donors with unintentional spinal fractures (served as normal controls) were enrolled in this study. The small needle bone biopsies from spine were obtained from the subjects. All participants signed written informed consents before biopsy collection. This study has been approved by the Ethics Committee of the Affiliated Huai'an Hospital of Xuzhou Medical University and The Second People's Hospital of Huai'an.

Data collection and array data analysis

Two expression profile data sets, GSE34747 and GSE103473, relating to osteoporosis and peripheral and spinal fracture were downloaded from GEO database (https://www.ncbi.nlm.nih.gov/gds/). The differently expressed genes (DEGs) of GSE34747 between Wnt activation samples (n=3) and normal samples (n=3) were identified using impute R package. The differently expressed miRNAs of GSE103473 between Wnt1 mutation samples (n=12) and normal samples (n=12) were identified using impute R package. The DEGs and differently expressed miRNAs were selected with the log |fold change| value ≥1 and P-value ＜ 0.05. The common genes between DEGs of GSE34747 and the target genes of miRNAs of GSE103473 were overlapped by Venny 2.1.0.

The construction and analysis of protein-protein interactions (PPI) network

To construct the PPI network, DEGs were uploaded to STRING (https://string-db.org/) and

Metascape (http://metascape.org/gp/index.html#/main/step1), respectively. STRING was an online tool to predict and visualize the PPI which includes direct and indirect associations. Metascape was an online gene annotation and analysis tool, which could analyze and visualize the PPI network. The analysis of PPI by Metascape algorithm depends on BioGrid, InWeb_IM, OmniPath databases. Molecular Complex Detection (MCODE) was applied to identify connected network components.

Gene ontology (GO) enrichment and pathway enrichment analysis

GO enrichment of DEGs including biological process, molecular function, and cellular component was analyzed using STRING and Metascape. Reactome pathway database was a relational database of signaling and metabolic molecules. STRING PPI network construction was performed to analyze the Reactome pathways of DEGs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways containing the information of the network of genes or molecules were also analyzed by STRING and Metascape.

Quantitative Real-time PCR (RT-qPCR)

Total RNA was extracted from bone tissues from spines using TRIzol reagent (Invitrogen, USA) and quantified using NanoDrop 2000 (Thermo Fisher Scientific, USA). Then 2 ug RNA was subjected to reverse transcription PCR to generate cDNA through the use of PrimeScriptVR RT reagent Kit (Takara, Japan), the qRT-PCR was then conducted to detect the expression of target genes using SYBR Premix Ex Taq (Takara, Japan). GAPDH was applied as the internal control, and the gene expression was calculated using $2^{-\Delta\Delta CT}$ method. The measurement data were shown as mean ± standard deviation (SD), and the difference between two groups was analyzed by student's *t*-test using GraphPad Prism 8.0 (GraphPad Software, USA). Statistical significance was considered when $P<0.05$.

Results

GO enrichment and Reactome pathway enrichment of 562 DEGs using STRING

Wnt signaling was associated with osteoporosis and could be activated by lithium. GSE34747 including the LiCl-stimulated samples (Wnt activation samples) and normal samples was analyzed by R software. Hierarchical clustering analysis showed that the datasets were well clustered: most genes in Wnt activation samples and normal samples tended to be grouped in two clusters, while there were limited overlapped parts (Fig.1A). For the functional enrichment analysis by STRING, 562 DEGs were finally selected with the fold change value ≥1 and P-value < 0.05 and displayed the complicated PPI network (Fig.1B). GO enrichment showed that the biological process of 562 DEGs was associated with the chemical stimulus, the molecular function of 562 DEGs was associated with receptor activity, and cellular component was associated with membrane (Fig.1C). Meanwhile, Reactome pathway analysis revealed that GPCR was the key signaling which had been proved to be related to osteoporosis. These results demonstrated that 562 DEGs might be associated with membrane protein-related signaling pathways.

The analysis of process enrichment, pathway enrichment, and PPI network of DEGs using Metascape

To further identify the function of 562 DEGs, another algorithm, Metascape, was used to analyze and visualize the key processes and pathways. As shown in Fig.2A, the calcium signaling pathway which was consistent with the GO analysis results of STRING, was the key pathway. In addition, the PPI network constructed by Metascape displayed 5 MCODEs (Fig.2B). The top 3 MCODEs were calcium signaling pathway, cAMP signaling pathway, and anterograde trans-synaptic signaling. The results of Metascape identified that the calcium signaling pathway and cAMP signaling pathway were the key pathways.

The identification of overlapping genes between the target genes of miRNAs and DEGs

GSE103473 was the miRNA profile of spinal fracture involving the Wnt1 mutation samples and normal samples. Hierarchical clustering analysis showed that most miRNAs in Wnt mutation samples and normal samples tended to be grouped in two clusters, while there was some degree of overlapping (Fig.3A). miR-34a-5p, miR-22-3p, miR-143-5p, miR-18a-3p, miR-31-5p, and miR-223-3p were the top 6 differently expressed miRNAs of GSE103473. Venny 2.1.0 was then used to select the overlapping genes between the target genes of the top 6 differently expressed miRNAs and the formerly identified 562 DEGs of GSE34747. Due to the most overlapping genes in miR-18a-3p, miR-18a-3p and overlapping 75 genes were screened out, which was associated with osteoporosis and spinal fracture (Fig.3B-G).

The identification of key genes using STRING and Metascape

To explore the biological functions of 75 genes involving in osteoporosis and peripheral and spinal fracture, STRING was first used to construct the PPI network of the 75 genes. The PPI network analysis showed that ADCY5, ADCY2, MLLT4, and GRIA1 were the genes associated with the cAMP signaling pathway (Fig.4A). Similar to the result of STRING, process and pathway analysis by Metascape also revealed that the cAMP signaling pathway was the key pathway (Fig.4B). Compared the results of STRING with Metascape, ADCY2, ADCY5, and GRIA1 were identified as the significant genes that were associated with the cAMP signaling pathway and possibly participate in osteoporosis and spinal fracture when Wnt signaling was manipulated (Fig.4C).

The expression of ADCY2, ADCY5, and GRIA1 in osteoporotic patients with spinal fracture

To further investigate the association of ADCY2, ADCY5, and GRIA1 with spinal fracture, we collected bone tissues from osteoporotic patients with spinal fracture (n=42) and age-matched healthy donors (n=45), and examined the expression of ADCY2, ADCY5, and GRIA1 mRNA by RT-qPCR. The results showed an approximately 50% downregulation of ADCY2 mRNA in the bone tissues of osteoporotic patients compared with the healthy donors (normal group) (Fig. 5A). Meanwhile, the expression of ADCY5 and GRIA1 mRNA exhibited a 60% downregulation in the bone tissues of osteoporotic patients (Fig. 5B-C). These results indicated that ADCY2, ADCY5, and GRIA1 might play a regulatory role in the occurrence of osteoporosis with spinal fracture.

Discussion

Spinal fracture induced by osteoporosis has become a health burden of the aging population, especially in postmenopausal women over 55. The activation of the Wnt/β-catenin signaling pathway has been proved to prevent osteoblast and osteocyte apoptosis so it acts as a negative role in osteoporosis [20]. In this study, the GO analysis and Reactome pathway analysis revealed that 562 DEGs might be associated with membrane protein-related signaling pathways especially calcium signaling pathway and cAMP signaling pathway. By analyzing the miRNA microarray, the 75 target genes of miR-18a-3p were screened out for further PPI network construction and GO term enrichments. Both STRING and Metascape enrichments identified that the cAMP signaling pathway was a crucial pathway. By comparing the results of STRING and Metascape, ADCY2, ADCY5, and GRIA1 were thought to be the key genes participating in osteoporosis and spinal fracture after the manipulation of Wnt signaling. Lastly, the aberrantly downregulated ADCY2, ADCY5, and GRIA1 in osteoporotic patients with spinal fracture further suggested the potential role of the three genes in the pathogenesis of osteoporotic spinal fracture.

Wnt/β-catenin signaling pathway promoted the regeneration of osseous tissue by stimulating proliferation and differentiation of osteoblasts [23, 24]. The Wnt proteins are secreted glycoproteins, which can stimulate the signaling pathway by binding to LRP5/6 and co-receptor Fizzled [25]. Then, the receptors including Dsh, Axin, and APC can inhibit the activity of glycogen synthase kinase 3 (GSK3) to prevent the phosphorylation of β-catenin [26]. The phosphorylation of β-catenin results in the degradation of β-catenin so that the Wnt/β-catenin is inactivated. Kim JH *et al.* found that β-catenin expression in bone tissues from patients suffering from osteoporotic fractures was reduced, indicating that the decrease of β-catenin could cause osteoporotic fractures [24]. Additionally, the Wnt/β-catenin signaling pathway have also been reported to participate in the regulation of chondrocyte proliferation and apoptosis in osteoarthritis [27]. In our study, impaired Wnt signaling led to significant miR-18a-3p upregulation that possibly participated in osteoporosis and spinal fracture. 75 genes that were both target genes of miR-18a-3p and DEGs caused by proactive Wnt activation went through STRING and Metascape interrogation, which demonstrated that the cAMP signaling pathway was the key pathway that might be associated with Wnt activation or mutation. We then concluded that the activation or inactivation of the Wnt signaling pathway could affect the cAMP signaling, which therefore affecting osteoporosis, and spinal fracture processes.

Cyclic 3',5'-adenosine monophosphate (cAMP) is an important second messenger in bone homeostasis, which acts as a prominent role in determining the fate of cells. The intracellular cAMP level could be elevated by activating the G-protein-coupled receptor (GPCR) that was the major mediator of bone remodeling by inhibiting osteoblasts apoptosis and enhancing osteoblasts differentiation [28]. In the present study, the Reactome pathways analysis exhibited that signaling by GPCR was closely associated with Wnt activation, which was consistent with the result of 75 overlapping genes' enrichment analysis. cAMP has been proved to inhibit osteoblast proliferation by suppressing the MAP kinase pathway [29]. In addition, a network pharmacological study demonstrated that cAMP signaling pathway was one of the critical pathways that were closely related to the bone formation and resorption [30]. Weivoda MM *et al.* investigated the relationship between cAMP and Wnt pathway and found that Wnt3a suppressed osteoclast differentiation by activating the cAMP/PKA pathway [31]. Together with the previous studies and our bioinformatic analysis, we believed that the cAMP signaling pathway was closely associated with the Wnt signaling, and thus may participate in osteoporotic fractures.

Adenylate cyclase, a family of membrane bound enzymes that catalyze the formation of cyclic AMP from ATP under the stimulation of G-protein signaling, is the direct regulator of cAMP signaling pathway [32]. Adenylate cyclase family consists of nine members, naming ADCY1~ADCY9 [33]. It has been evidenced that the blockage of cAMP/PKA/CREB signaling through inhibiting the activity of adenylate cyclase could repress Icariin-induced osteogenesis [34]. Another report proved that the stimulation of adenylyl cyclase could activate cAMP-mediated MAPK signaling and induce the expression of Runx2 in osteoblasts to accelerate bone regeneration [35]. Given the potential regulation of cAMP signaling on osteoporosis, the key catalytic enzymes of cAMP signaling, the adenylate cyclase, can be speculated to play a regulatory role in the development and progression of osteoporotic spinal fracture. Particularly, ADCY6 was demonstrated to promote the proliferation and differentiation of osteoblasts in osteoporotic rats through activating Rap1/MAPK signaling pathway [36]. It was also once reported that ADCY3 had a positive effect on bone formation [37]. Interestingly, a research on ADCY5 knock-out mouse models suggested that ADCY5 protected the mice from bone density reduction and susceptibility to fractures of aging [38]. ADCY2 was also identified to be a potential regulator in osteoporotic spinal fracture by our study, however, there was no study supporting this. Based on the close relationship between ADCY2 and cAMP signaling, we believe that it is worth studying the effects of ADCY2 in osteoporotic spinal fracture. On the other hand, Glutamate Ionotropic Receptor AMPA Type Subunit 1 (GRIA1), belonging to a family of AMPA receptors, only have been proved to be a tumor suppressor gene in human osteosarcoma [39]. Therefore, the identified genes ADCY2, ADCY5, and GRIA1 are of high significance in regards to osteoporosis and potentially spinal fracture, thus need to be further studied, which may provide the new therapeutic strategies for osteoporotic fractures.

During our search on gene expression profiling GEO data series in spinal fracture, we found only GSE34747 dataset. The limited number of samples is a drawback indeed; however, there has been no more other profiling data available and we currently don't have enough fund to conduct our own gene profiling experiment. We will certainly consider to conduct the gene expression profiling of our own data in the future. In addition, we have supplied the PCR verification results, which to some extent makes the results more reliable.

Conclusion

In conclusion, we identified that the cAMP signaling pathway was associated with the activation or inactivation of the Wnt signaling pathway in osteoporotic fractures. Meanwhile, ADCY2, ADCY5, and GRIA1 were associated with osteoporotic fractures involving the Wnt pathway and cAMP pathway due to the little studies on these genes in osteoporotic fractures. Our findings might provide novel therapeutic strategies for osteoporotic fractures.

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None.

Conflict of interest

The authors declare no conflict of interest.

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Legend

Figure 1 **Functional enrichment analysis of 562 DEGs in GSE34747.** (A) Heat map of DEGs in GSE34747. Red color revealed the upregulated genes while the green color revealed the downregulated genes. (B) PPI network for DEGs was constructed using STRING. (C) GO enrichment and Reactome pathways enrichment of DEGs were analyzed by STRING. DEGs, differentially expressed genes. PPI, protein-protein interactions. GO, gene ontology. FDR, false discovery rate.

Figure 2 **The analysis of GO enrichment, KEGG enrichment, and PPI network of 562 DEGs using Metascape.** (A) The top 20 pathway and process enrichment was displayed using different colors. (B) The contruction of PPI network using Metascape. The top 3 MCODE were displayed. DEGs, differentially expressed genes. PPI, protein-protein interactions. GO, gene ontology. KEGG, Kyoto Encyclopedia of Genes and Genomes. MCODE, Molecular Complex Detection.

Figure 3 **The target genes of top 6 differently expressed miRNAs were screened out using R software and Venny 2.1.0.** (A) Heat map of differently expressed miRNAs in GSE103473 using R software. (B-G) Venny 2.1.0 was applied to identify the target genes of miRNAs which also belonged to DEGs in GSE34747. DEGs, differentially expressed genes. -, low expression. +, high expression.

Figure 4 **ADCY2, ADCY5, and GRIA1 were identified as key genes by STRING, Metascape and Venny 2.1.0.** (A) The PPI network of 72 target genes of miR-18a-3p was constructed by STRING. The genes involving cAMP signaling pathway were list. (B) cAMP signaling pathway was the key pathway by Metascape analysis. Different colors represented different processes and pathways. (C) The common genes (ADCY2, ADCY5, and GRIA1) from Metascape and STRING involving cAMP signaling pathway by Venny 2.1.0. PPI, protein-protein interactions.

Figure 5 **ADCY2, ADCY5, and GRIA1 mRNA expression in osteoporotic patients with spinal fracture.** (A-C) The relative expression of ADCY2 (A), ADCY5 (B), and GRIA1 (C) mRNA in the bone tissues of osteoporotic patients with spinal fracture and the healthy control was detected by RT-qPCR. The data were shown in mean ± SD. Statistics analysis was conducted using Mann Whitney test. Normal: the healthy control.

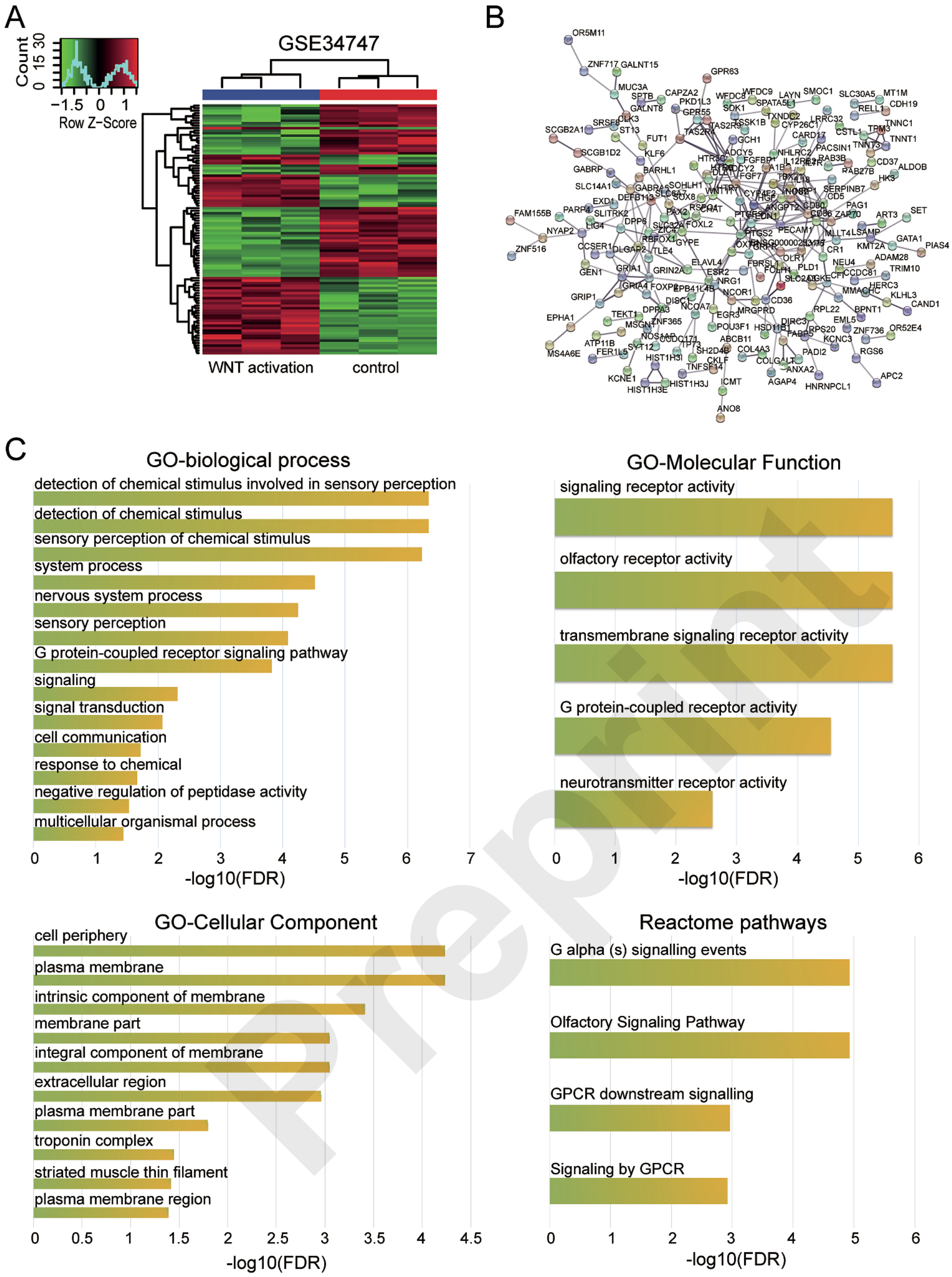
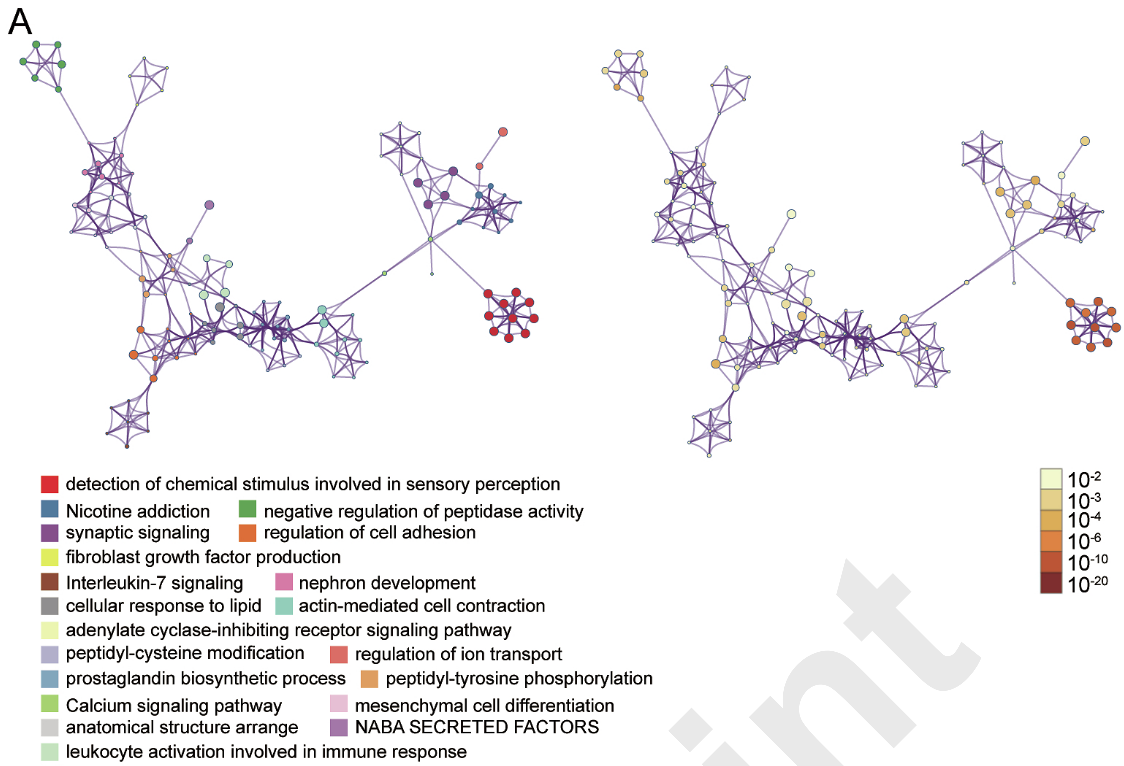
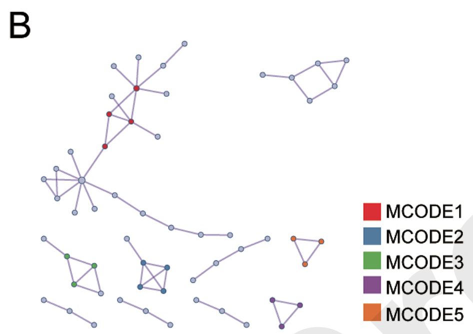


Figure 1 Functional enrichment analysis of 562 DEGs in GSE34747.





GO	Description	Log10(P)
hsa04020	Calcium signaling pathway	-6.5
hsa04024	cAMP signaling pathway	-6.2
GO:0098916	Anterograde trans-synaptic signaling	-5.8

Figure 2 The analysis of GO enrichment, KEGG enrichment, and PPI network of 562 DEGs using Metascape.

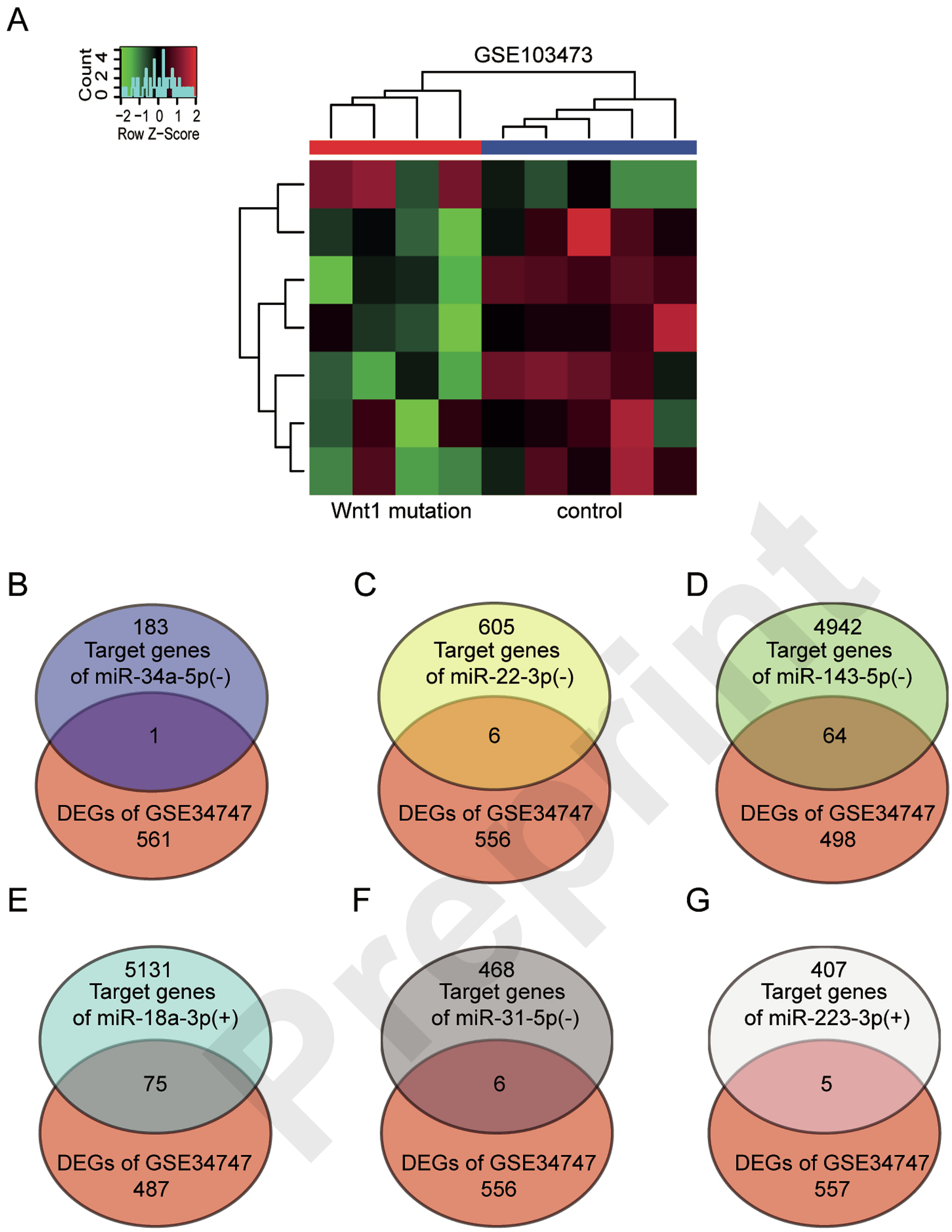


Figure 3 The target genes of top 6 differently expressed miRNAs were screened out using R software and Venny 2.1.0.

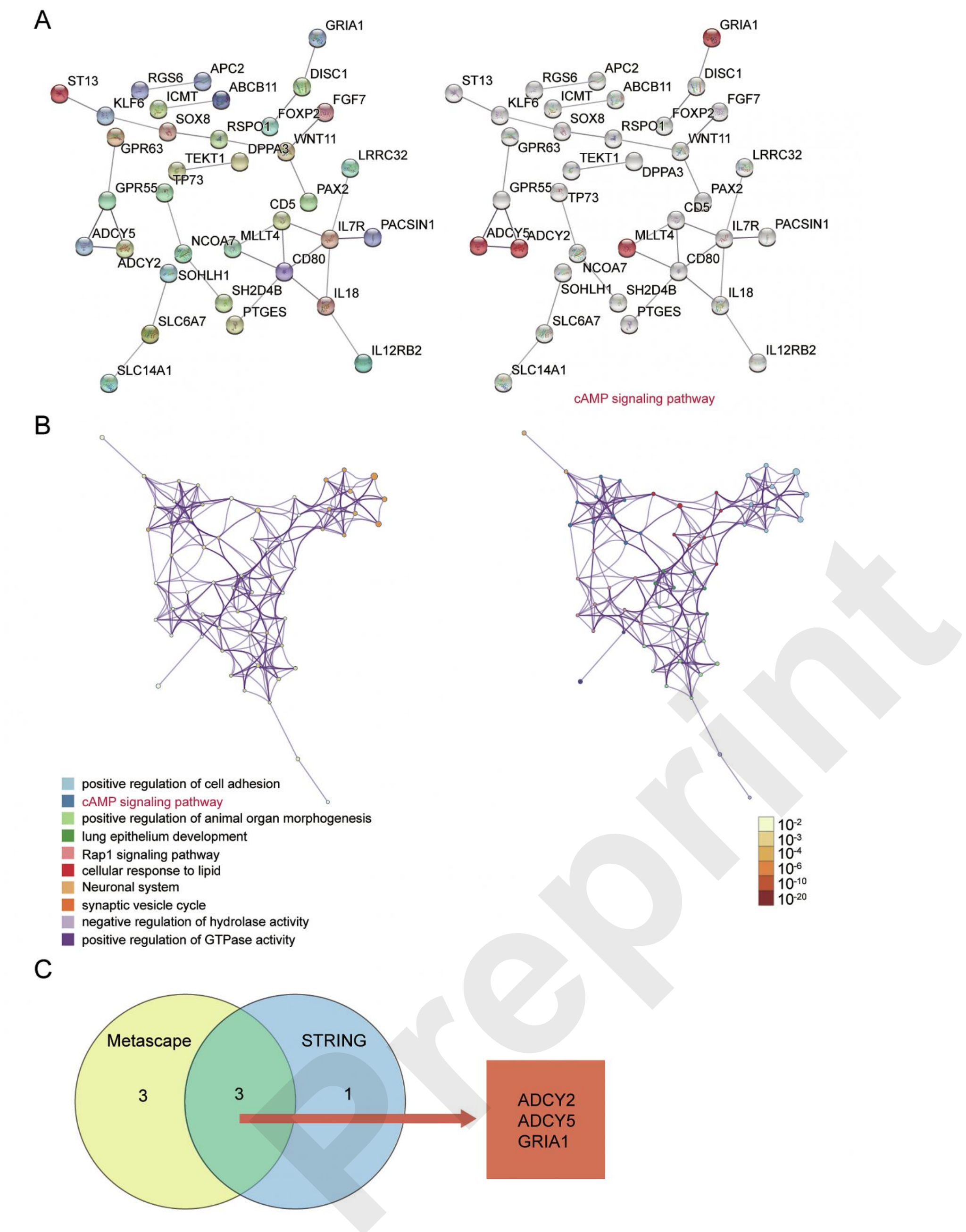
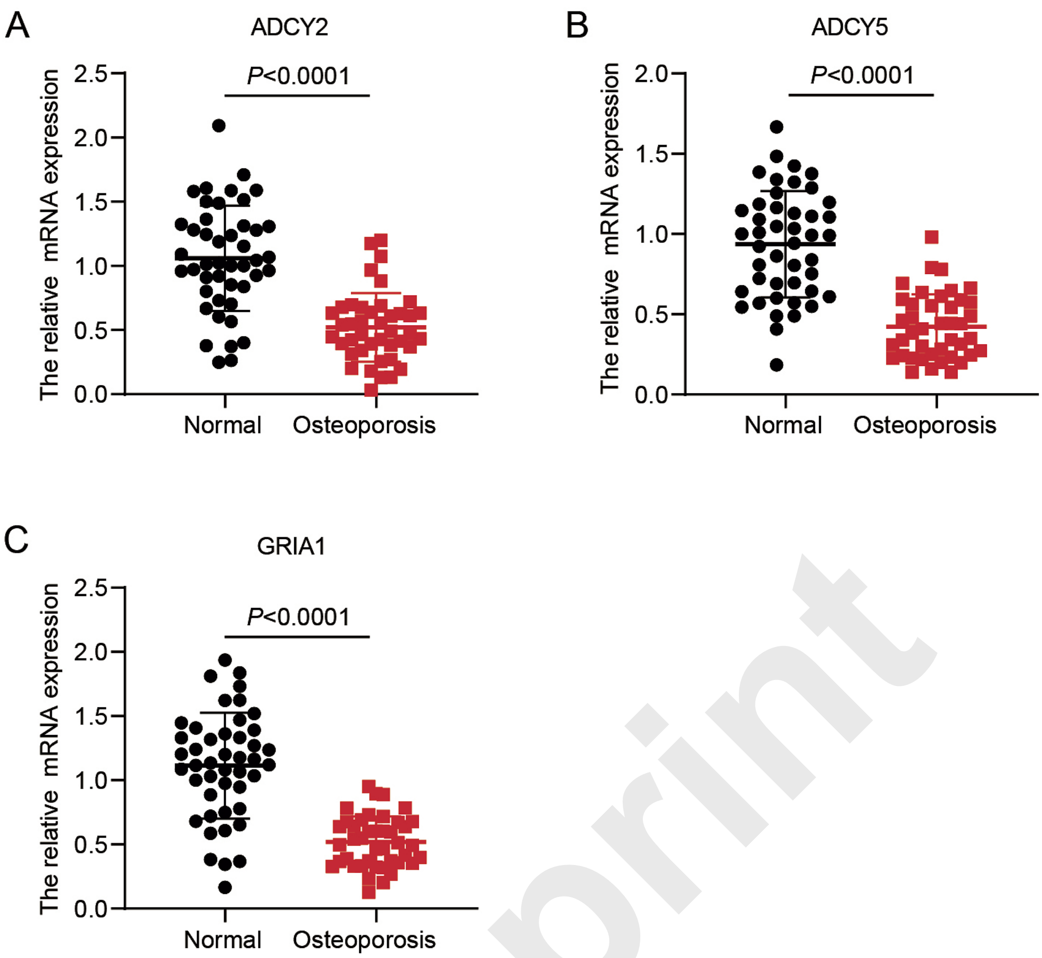

A
GRIA1
DISC1
FGF7
FOXP2
RSPO1
WNT11
DPPA3
LRRC32
PAX2
CD5
IL7R
PACSIN1
MLLT4
CD80
IL18
IL12RB2
PTGES
SH2D4B
SOHLH1
SLC6A7
SLC14A1
NCOA7
ADCY2
ADCY5
GPR55
TP73
TEKT1
GPR63
SOX8
KLF6
ST13
RGS6
APC2
ICMT
ABCB11
cAMP signaling pathway
B
positive regulation of cell adhesion
cAMP signaling pathway
positive regulation of animal organ morphogenesis
lung epithelium development
Rap1 signaling pathway
cellular response to lipid
Neuronal system
synaptic vesicle cycle
negative regulation of hydrolase activity
positive regulation of GTPase activity
10-2
10-3
10-4
10-6
10-10
10-20
C
Metascape
STRING
3
3
1
ADCY2
ADCY5
GRIA1

Figure 4



A
ADCY2
P<0.0001
The relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Normal
Osteoporosis
B
ADCY5
P<0.0001
The relative mRNA expression
2.0
1.5
1.0
0.5
0.0
Normal
Osteoporosis
C
GRIA1
P<0.0001
The relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
Normal
Osteoporosis

Figure 5