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Identification of candidate genes for myeloma-induced osteocyte death based on microarray data

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Abstract

Background: The study was aimed to investigate the molecular mechanisms of osteocyte death in multiple myeloma (MM) patients.

Methods: GSE27372 was downloaded from Gene Expression Omnibus, including three HOB-01 (osteocyte cell line) control samples and three HOB-01 samples co-cultured with JN3 (human MM cell line). After the differentially expressed genes (DEGs) were identified by Student's *t* test method, enrichment analyses were performed for them using DAVID software. Using TRANSFAC, TSGene, and tumor-associated gene (TAG) databases, functional annotation was conducted for the DEGs. Additionally, protein-protein interaction (PPI) network and sub-network analyses were performed using STRING database and Cytoscape software.

Results: Total 393 DEGs were identified, including 22 transcription factors (e.g., *KLF4* and *IRF8*) and 37 TAGs. Enrichment analysis suggested that *EGF*, *S1PR1*, and *NPY1R* were enriched in the function of circulatory system development. *EGF* (degree = 31) and *EGR1* (degree = 19) had high degrees and interactions in the PPI network. In the sub-network, *S1PR1*, *C3AR1*, and *NPY1R* could interact with each other.

Conclusions: These DEGs might participate in the osteocyte apoptosis induced by myeloma cells. These findings might provide a theoretical basis for a better understanding of the osteolysis in MM patients.

Keywords: Multiple myeloma, Osteocyte, Differentially expressed genes, Enrichment analysis, Protein-protein interaction network

Background

Multiple myeloma (MM) originates in neoplastic plasma cell disorder, and it is characterized by the clonal proliferation of malignant plasma cells in the bone marrow. As the second most general hematological cancer, the incidence of MM worldwide is about 1.5/100,000 new case [1]. It is also found that the incidence of MM is higher in men than in women, as well as in black than white in the USA [2]. MM is always associated with organ dysfunction, especially osteolysis [3]. Although the survival rate of MM patients is increased due to the advanced medicines [4], MM continues to be considered as an incurable disease, and further study is required to

fully understand the potential molecular mechanisms of osteolysis in MM patients.

To adopt a volume appropriate for the local environment, the bone continuously remodels to keep the balance between bone formation and resorption mediated by osteocytes, osteoblasts, and osteoclasts [5]. Osteocyte represents the most abundant cell type in the bone and is actively involved in bone turnover. Dickkopf 1 (*DKK1*) levels increase in the peripheral blood and bone marrow plasma of MM patients and relate to the development of osteolytic lesions [6, 7]. Tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) produced by myeloma cells is positively correlated with osteolytic markers (such as urinary deoxypyridinoline and serum calcium), indicating that *TRAIL* may function in osteolysis of MM patients [8]. Via promoting the expression of receptor activator of NF- κ B (*RANK*) in osteoclast precursors,

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c-Akt (*AKT*) plays a role in the osteoclast formation and bone osteolysis induced by MM [9]. Giuliani et al. found that MM cells promoted osteocyte death and altered the transcriptional profile in osteocyte [10]. However, they did not further perform comprehensive bioinformatics analysis to investigate the internal causes. In spite of the above researches, the molecular mechanism of the increased osteocyte death in MM patients is still unclear.

In our study, in order to investigate the molecular mechanism of osteocyte death in MM patients, we re-analyzed the gene expression profile in Giuliani et al. study and identified the differentially expressed genes (DEGs) between normal osteocytes and osteocytes affected by MM cells. The enriched functions and pathways of DEGs were further identified. In addition, we constructed the protein-protein interaction (PPI) network of DEGs. Then, the most significant sub-network was screened out.

Methods

Affymetrix microarray data

Gene transcriptional profile of GSE27372 [10] was downloaded from Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), and GSE27372 was based on the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 array. There

were six specimens in this dataset, including three HOB-01 (osteocyte cell line) control samples and three HOB-01 samples co-cultured with JLN3 (human myeloma cell line).

DEGs screening

AFFY package (version 1.28.0, <http://www.bioconductor.org/packages/release/bioc/html/affy.html>) of Bioconductor [11] was used to pre-process the Affymetrix microarray data. All raw data were scaled according to the robust multi-array average (RMA) method [12] with default settings. After background correction, quantile normalization, and probe summarization, the gene expression matrix was obtained. The Student's *t* test method [13] was adopted to analyze the expression differences between control and co-culture group. For each significant DEG, both *P* value <0.05 and $|\log_2$ fold change (FC)| >0.58 need to be met.

Function and pathway enrichment analyses of DEGs

Functional annotation for DEGs was performed by Gene Ontology [14]. Kyoto Encyclopedia of Genes and Genomes (KEGG <http://www.genome.jp/kegg/pathway.html>) pathway enrichment analysis was used to identify main functional and metabolic pathways involving DEGs. We used *P* value <0.01 as the cut-off criterion for the enrichment analysis which was conducted by the Database for

Table 1 Function enrichment analysis of DEGs

Category	GO ID	GO term	Gene count	<i>P</i> value
Down	GO:0072358	Cardiovascular system development	35	2.22E-15
Down	GO:0072359	Circulatory system development	35	2.22E-15
Down	GO:0048731	System development	73	1.24E-14
Down	GO:0009653	Anatomical structure morphogenesis	55	1.44E-14
Down	GO:0031012	Extracellular matrix	19	5.70E-09
Down	GO:0005583	Fibrillar collagen	5	3.72E-08
Down	GO:0044420	Extracellular matrix part	12	5.72E-08
Down	GO:0005201	Extracellular matrix structural constituent	9	2.38E-08
Down	GO:0005178	Integrin binding	8	5.43E-07
Down	GO:0005198	Structural molecule activity	18	5.64E-06
UP	GO:0048583	Regulation of response to stimulus	67	5.57E-11
UP	GO:0023051	Regulation of signaling	59	2.48E-10
UP	GO:0010646	Regulation of cell communication	59	2.71E-10
UP	GO:0023052	Signaling	97	2.82E-10
UP	GO:0005615	Extracellular space	20	1.72E-03
UP	GO:0044421	Extracellular region part	24	2.83E-03
UP	GO:0031095	Platelet dense tubular network membrane	2	4.40E-03
UP	GO:0043565	Sequence-specific DNA binding	21	5.35E-05
UP	GO:0000982	RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity	7	1.95E-04
UP	GO:0003700	Sequence-specific DNA binding transcription factor activity	25	3.44E-04

GO gene ontology; ID identifier; DEGs differentially expressed genes

Table 2 Pathway enrichment analysis of DEGs

Category	KEGG pathway	Gene count	P value
Down	Protein digestion and absorption	6	1.44E-04
Down	ECM-receptor interaction	6	1.88E-04
Down	Amoebiasis	6	6.24E-04
Down	Focal adhesion	8	7.86E-04
Down	Regulation of actin cytoskeleton	8	1.19E-03
Down	Axon guidance	6	1.74E-03
Down	Melanoma	4	5.42E-03
Down	Hypertrophic cardiomyopathy (HCM)	4	9.38E-03
Up	JAK-STAT signaling pathway	12	3.08E-06
Up	Nicotinate and nicotinamide metabolism	4	4.16E-04
Up	Calcium signaling pathway	8	5.14E-03
Up	VEGF signaling pathway	5	5.68E-03
Up	Phosphatidylinositol signaling system	5	6.34E-03
Up	Cytokine-cytokine receptor interaction	10	6.46E-03

KEGG Kyoto Encyclopedia of Genes and Genomes; DEGs differentially expressed genes

Annotation, Visualization and Integrated Discovery (DAVID; version 2.1b, <http://david.abcc.ncifcrf.gov/>) online software [15].

Transcription factors (TFs) and tumor-associated genes (TAGs) in DEGs

DEGs with the function of transcriptional regulation, namely, differentially expressed transcription factors were selected based on the TRANSFAC database (<http://www.gene-regulation.com/pub/databases.html>) [16]. According to the TSGene database (<http://bioinfo.mc.vanderbilt.edu/TSGene/search.cgi>) [17] and TAG database (<http://www.binfo.ncku.edu.tw/TAG/>) [18], we extracted the TAGs from DEGs, including tumor suppressor genes and oncogenes.

Construction of PPI network and sub-network

The interaction pairs of DEGs were analyzed via online tool Search Tool for the Retrieval of Interacting Genes (STRING; version 9.0, <http://string-db.org>) [19], and

interaction data were downloaded on June 27, 2014. Only the gene pairs which were recorded in database, and experimental validated, text-mined, or co-expressed were used and combined score >0.4 was set as the criterion of PPI. Then, the PPI network was constructed using Cytoscape (version 2.8, <http://cytoscape.org>) [20].

The sub-network analysis of PPI network was performed using the ClusterONE plug of Cytoscape [21]. The pathway enrichment analysis of the genes in the most significant sub-network was performed by using the DAVID online software [15].

Results

MM-induced DEGs in osteocytes

After analyzing the microarray data of control and co-culture groups, a total of 393 DEGs were screened out, including 167 down-regulated genes and 226 up-regulated genes in co-culture group.

Functions and pathways enriched by DEGs

To explore the specific functions and pathways of DEGs, functional and pathway enrichment analyses were performed. The down-regulated genes were most significantly enriched in the functions of cardiovascular system development, circulatory system development (Table 1), and the pathways of protein digestion, absorption, and extracellular matrix (ECM)-receptor interaction pathway (Table 2). Especially, epidermal growth factor (EGF), sphingosine-1-phosphate receptor 1 (S1PR1), and Neuropeptide Y1 receptor (NPY1R) were involved in circulatory system development. Moreover, the up-regulated genes were most significantly enriched in the functions of regulation of response to stimulus and regulation of signaling function (Table 1), as well as the pathways of JAK-STAT signaling, nicotinate, and nicotinamide metabolism (Table 2).

Differentially expressed TFs and TAGs

The TFs and TAGs differentially expressed between control and co-culture groups were further extracted from DEGs. As shown in Table 3, four down-regulated TFs and 18 up-regulated TFs (e.g., Krüppel-

Table 3 Differentially expressed TFs and TAGs

Regulation	TF counts	TFs	TAG counts	Oncogenes	Tumor suppressor genes	Other TAGs
Down	4	TGFB111, NKX2-2, KLF5, ID2	16	RUNX1T1, PDGFRB, CD24	TPM1, SPTBN1, PRICKLE1, KLF5, IGFBP3, FAT4, EFNA1, DNAJB4, DAB2, CADM1	TGFB2, LRRC17, BAMBI
Up	18	SOX9, SOX7, NR3C2, MSX1, MAFF, KLF4, JUNB, IRF8, HOXB6, HMGA2, HES1, OXF1, FOXA2, ETV5, ETV4, ELK3, EGR3, EGR1	21	VEGFA, SPHK1, PDGFRA, JUNB, HMGA2, FOSL1, FGF5, ETV1, CCND1	SPRY2, SOX7, IRF8, IL24, HOPX, FOXA2, EPHB2, ENC1, EGR1, DUSP6	KLF4, EMP1

TF(s) transcription factor(s); TAG(s) tumor-associated gene(s)

like factors 4, *KLF4*; and IFN regulatory factor-8, *IRF8*) were discovered.

Furthermore, 16 down-regulated TAGs and 21 up-regulated TAGs were screened out (Table 3). Among the down-regulated TAGs, there were 10 tumor suppressor genes and 3 oncogenes. Meanwhile, among the up-regulated TAGs, there were 10 tumor suppressor genes (e.g. *IRF8*) and 9 oncogenes.

Construction of PPI network and sub-network

Interactions between DEGs were identified using STRING software. By integrating DEG pairs with combined score >0.4, a PPI network was built (Fig. 1), consisting of 89 down-regulated genes and 113 up-regulated genes. A total of 10 DEGs had connectivity degree larger than 15, e.g., *EGF* (degree = 31) and early growth response 1 (*EGR1*, degree = 19).

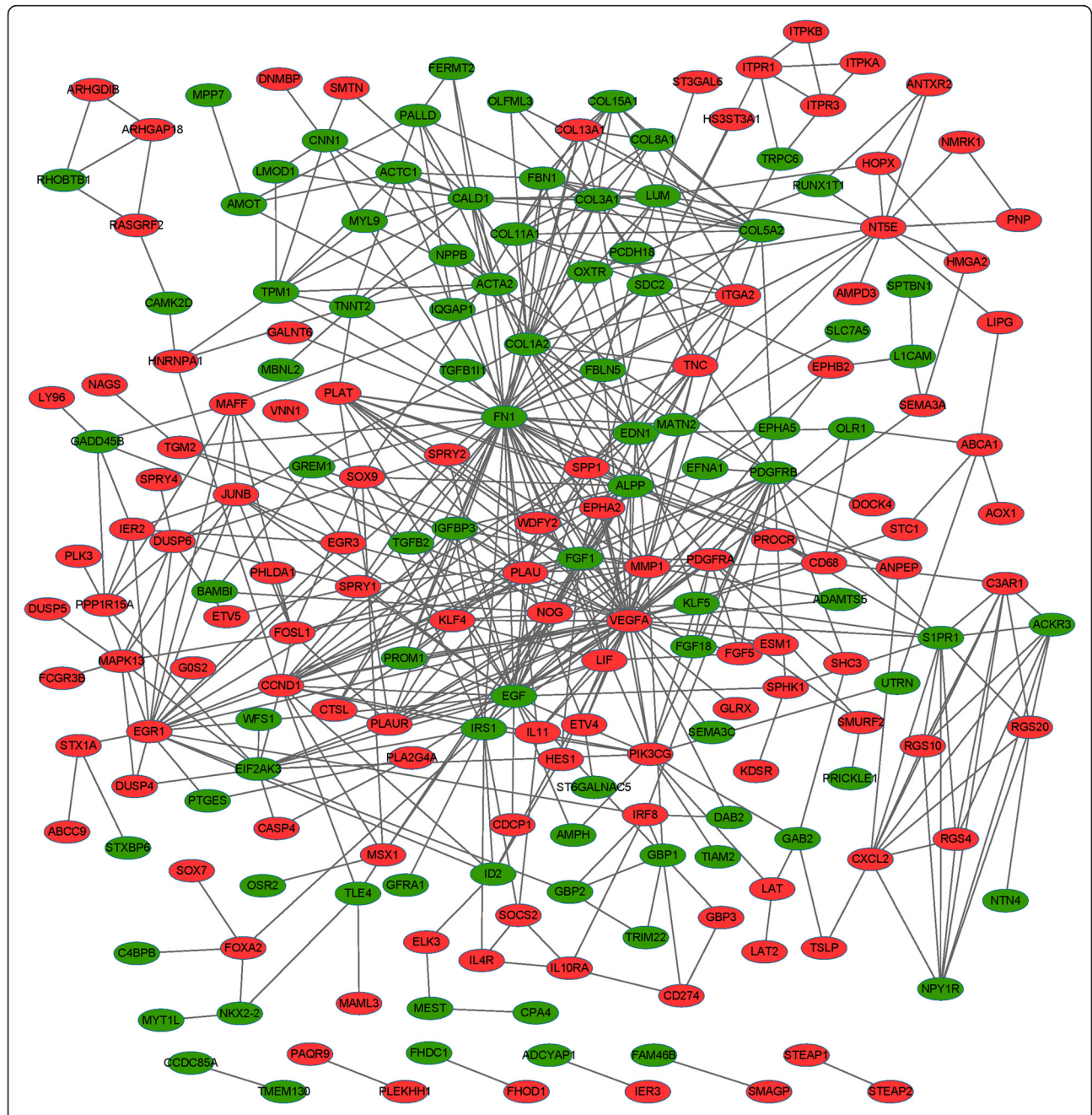


Fig. 1 Protein-protein interaction (PPI) network of differentially expressed genes (DEGs). Red and green nodes represent up-regulated genes and down-regulated genes, respectively

Besides, EGF could interact with EGR1 in the PPI network.

Moreover, a sub-network was obtained from PPI network (P value = 0.0004.3) (Fig. 2), consisting of nine significant DEGs like S1PR1, complement-3a receptor1 (C3AR1), and NPY1R. In the sub-network, S1PR1, C3AR1, and NPY1R had interactions with each other. Furthermore, the up-regulated C3AR1, as well as the down-regulated S1PR1 and NPY1R in the sub-network were enriched in the neuroactive ligand-receptor interaction pathway (Table 4).

Discussion

To gain insight into the molecular mechanisms of the myeloma-induced osteocyte death, gene expression profiles in osteocytes co-cultured with or without myeloma cells were systematically analyzed here. A total of 226 up-regulated genes and 167 down-regulated genes were identified. Then, PPI network was constructed, and sub-network was identified.

TFs like *KLF4* and *IRF8* were up-regulated in osteocytes co-cultured with myeloma cells in comparison with osteocytes cultured alone. Previous study reported that *KLF4* expression is essential for blocking cell cycle and increasing the resistance of MM cells to alkylating agents [22]. As one of the Yamanaka reprogramming factors and TFs, *KLF4* can promote the expression of autophagy-related genes [23]. In addition, silencing of the interferon consensus sequence-binding protein (*ICSBP/IRF8*) gene may be induced by DNA methylation or other mechanisms and correlates with the malignant phenotype of MM [24]. In our study, *KLF4* and *IRF8* were up-regulated TFs, and *IRF8* was a tumor

Table 4 Pathways enriched by the genes in sub-network

KEGG pathway	Gene count	P value	Genes
Neuroactive ligand-receptor interaction	3	1.42E-02	C3AR1, S1PR1, NPY1R

KEGG Kyoto Encyclopedia of Genes and Genomes

suppressor gene. These suggested that *KLF4* and *IRF8* might play a role in osteolysis in MM patients.

In this study, EGF and EGR1 had high connectivity degrees in PPI network. A heparin-binding factor in EGF family, amphiregulin (*AREG*) is overexpressed in primary myeloma cells and can promote growth of bone marrow stromal cell [25]. *EGR-1* induces apoptosis in MM via interacting with *JUN*, and decreased *JUN/EGR-1* can enhance resistance of MM cells to bortezomib [26]. Functional enrichment indicated that *EGF* was involved in circulatory system development. It has been reported that the substances of the circulatory system can induce the apoptosis of tumor cells [27]. EGF could interact with EGR1 in the PPI network, indicating that *EGF* and *EGR1* might be involved in osteocytes apoptosis induced by MM cells through interacting with each other.

Enrichment analysis suggested that *S1PR1*, *C3AR1*, and *NPY1R* in sub-network were enriched in the pathway of neuroactive ligand-receptor interaction, meanwhile *S1PR1* and *NPY1R* were involved in the function of circulatory system development. S1PR1 is a G-protein-coupled receptor which binds to the bioactive signaling molecule sphingosine 1-phosphate (S1P) [28]. It is reported that *S1PR1* and *S1PR2* regulate osteoclast precursor migration between the bone marrow cavities and the circulation [29]. It is shown that component 3 (C3A) and its receptor C3AR play a role in osteoclast formation [30, 31], implying a potential role of *C3AR* in osteocyte death. Thus, *S1PR1* and *C3AR1* may promote the death of osteocytes, and this is consistent with the finding of Giuliani et al. [10]. *NPY1R* is a receptor of neuropeptide Y (*NPY*), which is a neurotransmitter. There is evidence that *NPY* regulates bone homeostasis via actions in peripheral tissues [32]. *NPY1R* is the only Y receptor which is robustly expressed in bone marrow stromal cells [33] and osteoblasts [34], implying a direct role of *NPY1R* in bone remodeling. In addition, the absence of peripheral Y1 receptor will lead to pronounced anabolic effects on the bone [35]. Here, *NPY1R* was significantly down-regulated in osteocytes co-cultured with myeloma cells, inhibiting bone remodeling. In the sub-network, S1PR1, C3AR1, and NPY1R had interactions with each other, suggesting that *S1PR1*, *C3AR1*, and *NPY1R* might function in osteocytes apoptosis induced by MM cells via interactions.

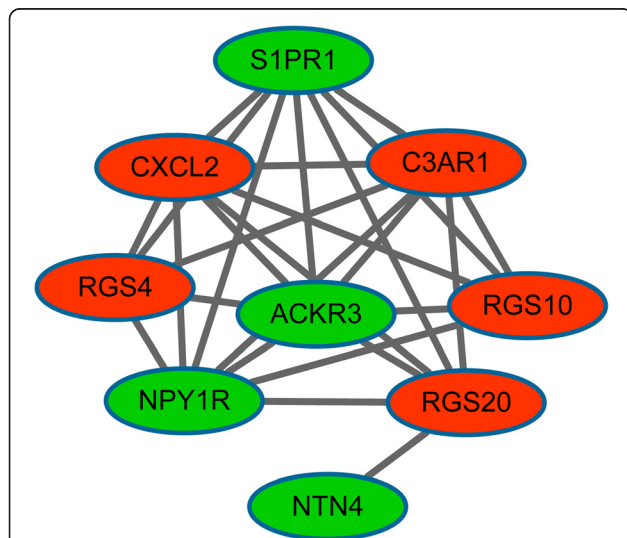


Fig. 2 Sub-network in the protein-protein interaction (PPI) network of differentially expressed genes (DEGs). Red and green nodes stand for up-regulated genes and down-regulated genes, respectively

Conclusions

In conclusion, a total of 393 DEGs were identified between osteocytes co-cultured with and without myeloma cells, and *KLF4*, *IRF8*, *EGF*, *EGRI*, *S1PR1*, *C3AR1*, and *NPY1R* might be involved in osteocyte cell apoptosis induced by MM cells. Here, we studied the potential molecular mechanism of the increased osteocyte death, which may provide a theoretical basis for understanding and treatment of osteolysis in MM patients. However, the present study analyzed gene expression data generated from not primary cells isolated from myeloma patients and healthy controls but cell lines, and the sample size was small. Thus, confirmation of results by quantitative real-time polymerase chain reaction (qRT-PCR) in cell lines followed by the investigation of the roles and functions of candidate genes in cells isolated from MM patients and healthy controls is still needed.

Abbreviations

DEGs, differentially expressed genes; DKK1, Dickkopf 1; ECM, extracellular matrix; EGF, epidermal growth factor; KEGG, Kyoto Encyclopedia of Genes and Genomes; MM, multiple myeloma; NPY1R, neuropeptide Y1 receptor; PPI, protein-protein interaction; RMA, robust multi-array average; S1P, sphingosine 1-phosphate; S1PR1, sphingosine-1-phosphate receptor 1; STRING, Search Tool for the Retrieval of Interacting Genes; TAG, tumor-associated gene; TAGs, tumor-associated genes; TFs, transcription factors; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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Availability of data and materials

The dataset "Gene transcriptional profile of GSE27372" supporting the conclusions of this article can be downloaded from Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). GSE27372 was based on the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array.

Authors' contributions

HT was responsible to the conception and design of the research, acquisition of data, obtaining funding, drafting the manuscript and revision of the manuscript for important intellectual content.

Competing interests

The author declares that they have no competing interests.

Ethics approval and consent to participate

None.

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