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Original Article

Bioinformatics analysis of gene expression profiles in B cells of postmenopausal osteoporosis patients

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ABSTRACT

Objective: The aim of this study was to gain a better understanding of the molecular mechanisms and identify more critical genes associated with the pathogenesis of postmenopausal osteoporosis (PMOP).
Materials and Methods: Microarray data of GSE13850 were download from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were identified either in B cells from postmenopausal female nonsmokers with high bone mineral density (BMD) compared with those with low BMD (defined as DEG1 group) or in B cells from postmenopausal female smokers with high BMD compared with postmenopausal female nonsmokers with high BMD (defined as DEG2 group). Gene ontology and immune-related functional enrichment analysis of DEGs were performed. Additionally, the protein–protein interaction network of all DEGs was constructed and subnetworks of the hub genes were extracted.
Results: A total of 51 DEGs were identified in the DEG1 group, including 30 up- and 21 downregulated genes. Besides, 86 DEGs were identified in the DEG2 group, of which 46 were upregulated and 40 were downregulated. Immune enrichment analysis showed DEGs were mainly enriched in functions of CD molecules and chemokines and receptor, and the upregulated gene interleukin 4 receptor (*IL-4R*) was significantly enriched. Moreover, guanine nucleotide-binding protein G (GNAI2), filamin A alpha (FLNA), and transforming growth factor-β1 (TGFB1) were hub proteins in the protein–protein interaction network.
Conclusion: IL-4R, *GNAI2*, *FLNA*, and *TGFB1* may be potential target genes associated with the pathogenesis of PMOP. In particular, *FLNA*, and *TGFB1* may be affected by smoking, a risk factor of PMOP.

Introduction

Postmenopausal osteoporosis (PMOP), is a common skeletal disorder affecting postmenopausal women due to simultaneous existence of independent predisposing factors, such as aging, continuous calcium loss, and estrogen deficiency [1]. PMOP is characterized by excessive bone resorption by osteoclasts (OCs) over bone formation by osteoblasts (OBs), leading to low bone mineral density (BMD; T score for BMD < 2.5) and decreased bone strength, which predisposes to an increased risk of fragility fracture [2,3]. It is estimated that one-third of adult women have osteoporosis-related fractures in their lifetime, which is a major public health concern [4]. A better understanding of the mechanisms will contribute to the development of novel and effective treatment approaches for PMOP.

Ample evidence has demonstrated that the imbalance between bone formation and resorption is the main mechanism of osteoporosis [5]. Recently, studies have shown that the mutation of osteoclast genes such as parathyroid hormone 1 receptor (*PTH1R*), colony stimulating factor 1 (*CSF1*), and osteoporosis related genes such as, collagen, type I, α1 (*COL1A1*), and low-density lipoprotein receptor-related protein 5 (*LRP5*), are significantly associated with PMOP [5–7]. In addition, there has been an increasing body of evidence showing that the immune system has a strong association with bone metabolism [8]. For instance, Breuil and colleagues [9] first demonstrated that the number of B lymphocyte and memory B lymphocytes was reduced in osteoporotic females. Xiao et al [10] demonstrated that downregulation of estrogen receptor 1 and mitogen activated protein kinase 3 in B cells could regulate secretion of factors, causing increased osteoclastogenesis, which was

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involved in the etiology of PMOP. Receptor activator of nuclear factor-κB ligand (RANKL) binds to its receptor RANK on the precursors of OCs and stimulates osteoclastogenesis [11]. Recently, Xu et al [12] indicated that the activation of apamycin complex 1 (mTORC1) in B lymphocytes could stimulate the formation of OCs via regulation of β-catenin and RANKL/osteoprotegerin, causing osteoporosis. However, the roles of B lymphocytes in bone metabolism and PMOP remains to be elucidated, especially at the gene expression level. It has also been reported that tobacco smoking can inhibit the activity of osteoblasts [13,14]. In addition, Yoon et al [15] reported that tobacco smoking was associated with reduced BMD and increased the risk of fracture. Thus, tobacco smoking may be a risk factor for PMOP. Understanding the molecular mechanism of PMOP and the effects of tobacco smoking on PMOP are of critical importance for management policy.

Recently, Xiao et al [10] performed a genome-wide expression study on human B cells between 10 low and 10 high BMD postmenopausal women and identified that a novel estrogen receptor 1 and mitogen activated protein kinase 3-centered gene network may contribute to the etiology of PMOP. In this study, we downloaded the microarray data of GSE13850 from a public database, which was also deposited by Xiao et al [10], and analyzed the differentially expressed genes (DEGs) of B cells between PMOP (with high BMD) and normal postmenopausal women (with low BMD) to gain better insights into the immune-associated mechanisms of PMOP. In particular, B cells of PMOP female smokers and PMOP female nonsmokers were also used to search DEGs to explain the effects of tobacco smoking on PMOP in this study. In addition, gene ontology (GO) and immune-related functional enrichment analysis of DEGs were performed. Additionally, the protein–protein interaction (PPI) network of all DEGs was constructed and subnetworks of the hub genes in the PPI network were extracted and analyzed. The present study aimed to identify more critical genes and functions associated with the mechanisms of PMOP, which can be exploited to develop efficient diagnostic and therapeutic approaches.

Materials and methods

Affymetrix microarray data

The microarray data of GSE13850, deposited by Xiao et al [10] on September 30, 2009, were obtained from Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). The platform of this dataset is GPL96 [HG-U133A] Affymetrix Human Genome U133A Array. B cells were isolated from the whole blood from 40 postmenopausal women, including 20 nonsmokers with low or high BMD (10 low vs. 10 high) and 20 smokers with low or high BMD (10 low vs. 10 high), using the B cell-positive isolation method (Invitrogen Life Technologies Dynal Biotech Inc., Waltham, MA, USA). Then, total RNA was extracted and used for gene expression microarrays.

Data preprocessing and screening of DEGs

The raw data were downloaded and preprocessed using the robust multiarray average algorithm [16] in R/Bioconductor Affy package (http://www.bioconductor.org/packages/release/bioc/html/affy.html) [17]. The procedure of data preprocessing included quantile normalization and $\log_2$ transformation. The probe-level data in .cel files were converted into gene expression measures.

The limma package (http://bioconductor.org/packages/release/bioc/html/limma.html) [18] in R was used to identify DEGs either in B cells from postmenopausal female nonsmokers with high BMD compared with those with low BMD (defined as DEG1 group) or in B cells from postmenopausal female smokers with high BMD compared with postmenopausal female nonsmokers with high BMD (defined as DEG2 group). A corrected p-value < 0.1 and $|\log_2$ fold change (FC)| > 0.5 were chosen as the thresholds for DEG screening.

GO enrichment analysis

GO analysis is commonly used for functional studies of large-scale transcriptomic or genomic data [19]. The Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) provides a set of data-mining tools and assists in the interpretation of genome-scale datasets by expediting the transition from data collection to biological annotation [20]. In this study, to better understand the functional relevance of the identified DEGs and explore PMOP-related genes, DAVID was used to search for over-representation in GO terms in biological process (BP) category of the identified DEGs. GO BP terms with p-value < 0.005 based on a hypergeometric test [21] were defined to be statistically significant.

Immune-related functional enrichment analysis

Immunome database (http://bioinf.uta.fi/Immunome/) is a reference set of human immune system-related genes and proteins and contains different categories of immune genes, including cellular immunity, humoral immunity, inflammation, and chemokines and their receptors [22]. Based on this database, the hypergeometric test was used to evaluate whether the identified DEGs in the DEG1 group or the DEG2 group were significantly enriched in the immune-related function. A p value < 0.05 was used as the cutoff criterion.

Construction of PPI network

The Human Protein Reference Database (http://www.hprd.org/) provides curated proteomic information pertaining to human proteins, including PPIs [23]. In the present study, PPI network of all DEGs identified in the DEG1 and DEG2 groups was constructed with PPIs information derived from the Human Protein Reference Database. Besides, the network was visualized using Cytoscape software (http://www.cytoscape.org/) [24], which can provide several integrative and interactive visualization and analysis tools. In addition, the topological properties of the PPI network, including average shortest path, clustering coefficient, the closeness centrality and degree distribution were analyzed using Cytoscape software (http://www.cytoscape.org/) [24]. Degree distribution was calculated by counting the number of interactions (edges) between various nodes (proteins) of the PPI network. Nodes in the PPI network with higher degrees (highly connected nodes) were deemed to be hub proteins, which play essential roles in the PPI network. Moreover, subnetworks with hub genes as the center were extracted from the PPI network.

Results

Screening of DEGs

A total of 51 DEGs were identified in the B cells from the DEG1 group (postmenopausal female nonsmokers with high BMD compared with those with low BMD), comprising 30 upregulated genes and 21 downregulated genes, such as LIM domain containing 2 (*LIMD2*), lymphocyte specific protein 1 (*LSP1*), and ATPase Ca^{2+} transporting (*ATP2A3*). By contrast, a total of 86 DEGs were screened out in the DEG2 group (postmenopausal female smokers

with high BMD compared with postmenopausal female nonsmokers with high BMD), containing 46 up- and 40 downregulated genes, such as proplatelet basic protein (*PPBP*), hemoglobin beta (*HBB*) and histone cluster 1 H4C (*HIST1H4C*). The detailed information of all the DEGs identified in the DEG1 group and DEG2 group are listed in Tables S1 and S2, respectively.

GO enrichment analysis

The results of GO enrichment analysis showed that the DEGs identified in the DEG1 group were enriched in 61 GO BP terms and the DEGs identified in the DEG2 group were enriched in five GO BP terms. All the 61 GO BP terms enriched by DEGs in the DEG1 group was presented in Table S3. The top five GO BP terms enriched by DEGs in the DEG2 group as well as the GO BP terms enriched DEGs in the DEG2 group that were associated with immunity are listed in Table 1. From the results, we found that DEGs in the DEG1 group were mainly connected with immune response, such as *immune effector process*, *cell activation involved in immune response*, and *lymphocyte activation involved in immune response*. By contrast, among the GO terms enriched by DEGs in the DEG2 group, only one GO term was associated with immune response, namely *regulation of immune system process*. The other GO BP terms enriched by DEGs in the DEG2 group were identified to be connected with cell differentiation (e.g., *regulation of erythrocyte differentiation* and *regulation of megakaryocyte differentiation*), *activation of mitogen-activated protein kinase kinase activity* and *histone mRNA catabolic process*.

Immune-related functional enrichment analysis

The DEGs were identified to be concerned with immune-related functions in the above functional enrichment analysis. Hence, we intended to screen out the detailed immune-associated functions in which the DEGs in the DEG1 group and DEG2 group were involved. DEGs in the DEG1 group and DEG2 group were imported into the Immunome database and were subjected to analysis under the criteria of $p < 0.05$. The results showed that DEGs in the DEG1 and DEG2 groups were both significantly related to *CD molecules* and *chemokines and receptor*. In addition, the overlapping DEG interleukin 4 receptor (*IL-4R*) between DEG1 and DEG2 groups was involved in these two immunity related functions. Moreover, compared with DEGs in the DEG2 group, DEGs in the DEG1 group were also concerned with antigen processing and presenting, suggesting that this function may be a special pathway for DEG1 and is not associated with smoking. The results of enrichment analysis using the Immunome database are shown in Table 2.

Construction of PPI network and sub-network extraction

A PPI network based on all DEGs identified in the DEG1 and DEG2 group was constructed, comprising 737 nodes (proteins) and 774 edges (interactions). From the PPI network, we found that several proteins were with higher node degrees and were considered as hub proteins, such as filamin A-α (*FLNA*, degree = 62), guanine nucleotide-binding protein G (*GNAI2*, degree = 48), ataxia telangiectasia mutated (*ATM*, degree = 39), junction plakoglobin (*JUP*, degree = 38) and transforming growth factor-β1(*TGFB1*, degree = 33). Then, we extracted a subnetwork containing the top five hub proteins (*FLNA*, *GNAI2*, *ATM*, *JUP*, *TGFB1*) as centers and the subnetwork is illustrated in Figure 1. Among them, *FLNA*, *TFGB1*, and *JUP* were the overlapping DEGs both in the DEG1 and the DEG2 group; all of these three genes were upregulated in the DEG1 or in the DEG2 group. *GNAI2* belonged to DEGs identified in the DEG1 group and was identified to be upregulated. *ATM* was one of the upregulated DEGs identified in the DEG2 group. Moreover, except the module containing *ATM*, the other four modules were highly connected through some non-DEGs such as presenilin 1 (*PSEN1*) and decorin (*DCN*).

Discussion

PMOP, which is characterized by decreased BMD and microarchitectural deterioration of bone tissue, represents an incremental medical and economic threat among the aged population

Table 2
Immune-related enrichment analysis of differentially expressed genes (DEGs).

DEG	Immune function	*p*	Gene symbol
DEG1	Antigen processing & presenting	1.28×10^{-2}	CD1C, TAPBP
	CD molecules	2.42×10^{-2}	IL-4R, CD1C, CD74, IL2RG
	Chemokines & receptor	4.74×10^{-2}	IL-4R, IL2RG, IRF2
DEG2	CD molecules	3.86×10^{-2}	IL-4R, GP1BB, IL13RA1, PDCD1, LILRA4
	Chemokines & receptor	1.20×10^{-2}	IL-4R, IL7, IL13RA1, PF4, PPBP

DEG1 = DEGs between PMOP and normal postmenopausal women; DEG2 = DEGs between PMOP female smokers and PMOP female nonsmokers; PMOP = postmenopausal osteoporosis.

Table 1
Gene ontology enrichment analysis of differentially expressed genes (DEGs).

DEG	GO category	GO term	*n*	Genes	*p*
DEG1	GO:0001773	Myeloid dendritic cell activation	2	TGFB1, CD74	2.47×10^{-3}
	GO:0002252	Immune effector process	7	CD1C, CD74, FCER2, IL4R, STXBP3, TGFB1, DDX58	2.55×10^{-3}
	GO:0002263	Cell activation involved in immune response	4	CD1C, IL4R, STXBP3, TGFB1	1.36×10^{-3}
	GO:0002274	Myeloid leukocyte activation	5	CD74, IL4R, SPI1, STXBP3, TGFB1	9.05×10^{-5}
	GO:0002285	Lymphocyte activation involved in immune response	3	CD74, IL4R, TGFB1	3.66×10^{-3}
	GO:0002366	Leukocyte activation involved in immune response	4	CD74, IL4R, STXBP3, TGFB1	1.36×10^{-3}
	GO:0002376	Immune system process	15	CD1C, CD74, FCER2, IGHD, IL2RG, IL4R, SPI1, STXBP3, TAPBP, TGFB1, VASP, DDX58, KLF13, ATPIF1	2.66×10^{-3}
	GO:0002460	Adaptive immune response based on somatic recombination of immune receptors built from	4	CD74, FCER2, IL4R, TGFB1	4.34×10^{-3}
DEG2	GO:0000186	Activation of MAPKK activity	2	MAP3K1, NRAS	3.68×10^{-3}
	GO:0002682	Regulation of immune system process	13	ATM, F7, FOXO3, HLA-DQB1, HMGB2, IL4R, IL7, MAP3K1, PDCD1, PF4, KLF13, PLEKHA1, APOBEC3F	1.70×10^{-3}
	GO:0045646	Regulation of erythrocyte differentiation	3	FOXO3, HMGB2, KLF13	8.00×10^{-4}
	GO:0045652	Regulation of megakaryocyte differentiation	2	HMGB2, PF4	1.74×10^{-3}
	GO:0071044	Histone mRNA catabolic process	2	ATM, TUT1	1.74×10^{-3}

DEG1 = DEGs between PMOP and normal postmenopausal females; DEG2 = DEGs between PMOP female smokers and PMOP female nonsmokers; GO = gene ontology; PMOP = postmenopausal osteoporosis.

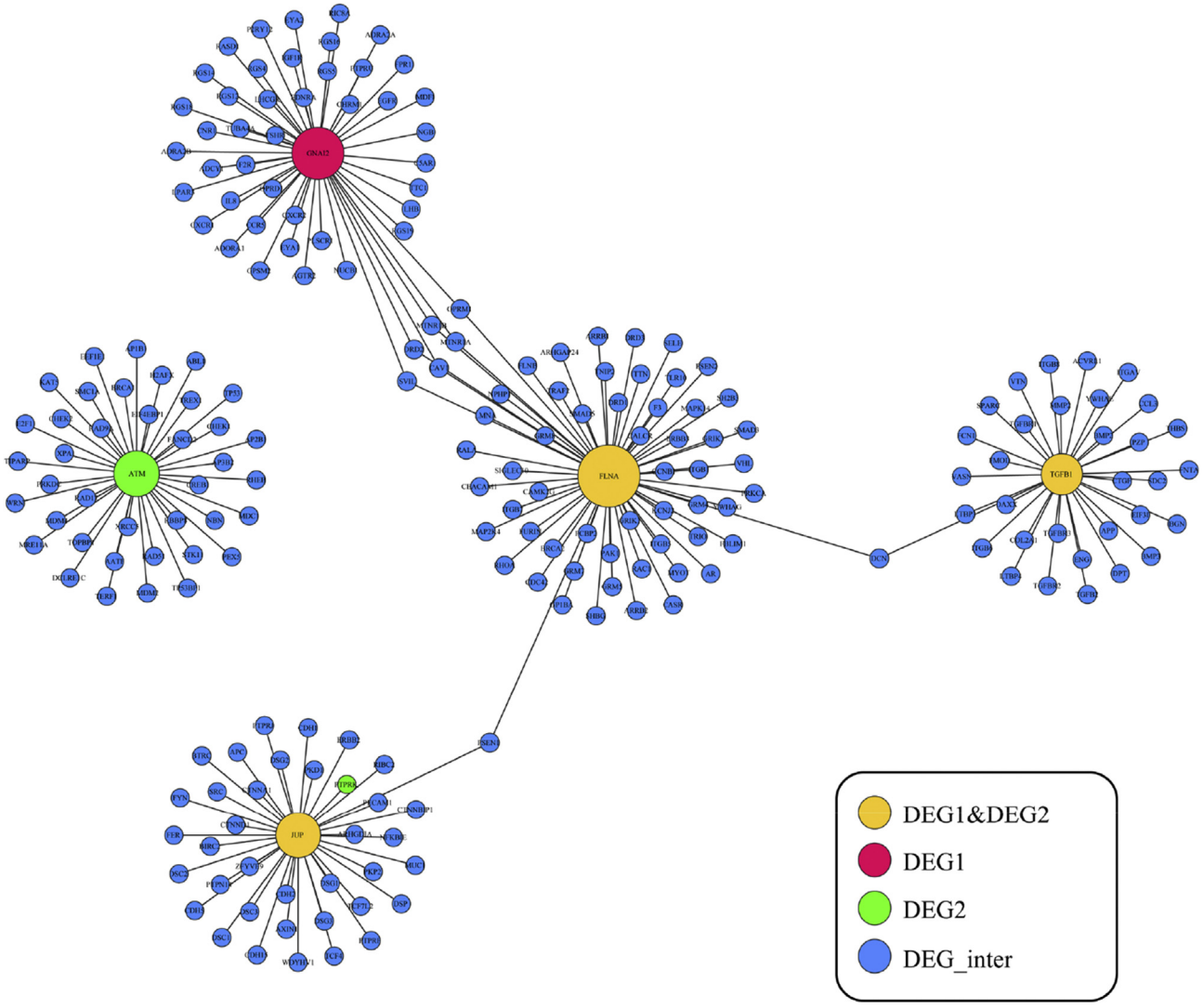

Figure 1. Protein–protein interaction network analysis of differentially expressed genes (DEGs) of DEG1 and DEG2. The red nodes stand for genes in DEG1. The green nodes stand for genes in DEG2. The yellow nodes stand for the overlapping DEGs between DEG1 and DEG2. The blue nodes represent other genes in Human Protein Reference Database. DEG1 = DEGs between postmenopausal osteoporosis (PMOP) and normal postmenopausal females; DEG2 = DEGs between PMOP female smokers and PMOP female nonsmokers.

worldwide [25]. It has already been demonstrated that lymphocytes are important in bone turnover [26]. However, few studies investigated the role of B cells in the mechanisms of PMOP based on gene expression patterns. In the current study, a total of 51 DEGs were identified in B lymphocytes from postmenopausal female nonsmokers with high BMD compared with those with low BMD (defined as DEG1 group), including 30 up- and 21 downregulated genes. Besides, 86 DEGs were identified in B lymphocytes from postmenopausal female smokers with high BMD compared with postmenopausal female nonsmokers with high BMD (defined as DEG2 group), of which 46 were upregulated and 40 were downregulated. Functional enrichment analysis showed that these DEGs were mainly enriched in functions associated with immune system. In addition, the upregulated gene *IL4R* was identified to be enriched in different immune-related functions. Moreover, *FLNA*, *GNAI2*, and *TGFB1* were identified to be hub proteins in the PPI network.

Evidence has shown that tobacco smoking is a risk factor for osteoporosis [13,14]. In this study, we also intended to gain further insights into the mechanisms underlying smoking-related PMOP and to investigate the potential genes and functions associated with the smoking-related PMOP. A total of 86 DEGs were identified in B lymphocytes from the DEG2 group (postmenopausal female smokers with high BMD compared with postmenopausal female nonsmokers with high BMD). However, it is possible that the DEGs identified in the DEG2 group were simply associated with smoking, and had nothing to do with PMOP. Thus, we carried out the comprehensive analysis with the combination of DEGs identified in the DEG1 group and DEGs identified in the DEG2 group. We suggested that the overlapping DEGs both in the DEG1 and DEG2 group or the highly connected hub genes in the PPI network with differential expression may play essential roles in the mechanisms of PMOP or in the mechanisms of smoking-related PMOP.

In the present study, we found that the overlapping upregulated gene *IL4R* (both identified in the DEG1 group and DEG2 group) was enriched in different immune-related functions. Vallé et al [27] demonstrated that B cell proliferation could be stimulated by CD40 in synergy with IL-4. The effect of IL-4 is mainly mediated by the IL-4 receptor α chain. Moreover, the study of Ferrer et al [28] showed that CD40+IL4R stimulation could promote an increased expression of some molecules that are mainly expressed on B cells, such as B-cell maturation antigen and could promote cell viability

and activation in normal B cells. In addition, activated B lymphocytes can be the cellular source of RANKL-mediated osteoclastogenesis for bone resorption [29]. Besides, ample evidence has demonstrated that RANKL plays an essential role in mediating the increase in bone resorption in early postmenopausal women [30]. In the present study, the immune-related functional enrichment analysis using the Immunome database showed that DEGs in the DEG1 group and DEG2 group were mainly concerned with *CD molecules* and *chemokines and receptors*, in which the upregulated gene *IL4R* was significantly enriched. Taken together, we suggested that *IL4R* may play a critical role in the pathogenesis of PMOP through stimulating B cell activation and promoting osteoclastogenesis.

By contrast, *FLNA* (overlapping upregulated DEG), *GNAI2* (upregulated DEG in the DEG1 group), and *TGFB1* (overlapping upregulated DEG) were identified to be with higher node degrees in the PPI network. The protein encoded by *GNAI2* is an α-subunit of guanine nucleotide binding proteins (G proteins). Evidence demonstrates that B cell chemoattractant signaling is highly dependent upon the G-proteins $G_{\alpha i2}$ and $G_{\alpha i3}$ [31]. Additionally, the significance of B cells had already been highlighted in the etiology of osteoporosis [10]. Thus, we inferred that *GNAI2* may be a key gene associated with the etiology of osteoporosis following menopause. By contrast, FLNA was shown to be required for osteoclastogenesis by regulating monocyte migration via Rho GTPases [32]. Thus, increased FLNA may promote OCs formation, which is significantly associated with the incidence of PMOP. Moreover, TGFB1 is a multifunctional set peptides that controls proliferation, differentiation, and survival of lymphocytes in the immune system [33]. Lymphocyte activation could induce RANKL expression, which results in bone loss and increased osteoclastogenesis [34]. Quinn et al [35] reported that TGFB1 increased OC formation via action on OC precursors. In accordance with the previous studies, we found that *TGFB1* and *FLNA* were upregulated and overlapping genes in the DEG1 and DEG2 groups. Thus, we suggested that *GNAI2*, *FLNA*, and *TGFB1* may play essential roles in the pathogenesis of PMOP. In particular, *FLNA* and *TGFB1* may be affected by smoking, a risk factor of PMOP. However, further experiments are needed to determine our findings.

This study has several limitations. Firstly, this is a computational study based on the bioinformatics approaches, which gives simulated results. The lack of cross-check and further experimental verification were the limitation of our investigation. Statistical validation using other datasets or validation using meta-analysis may be used to cross-check our results. Furthermore, we intend to carry out experimental verification in our future studies to verify these results using different methods such as quantitative real-time reverse transcription–polymerase chain reaction, and Western blot analysis. Secondly, the number of DEGs identified in this study were relatively small, possibly due to low sample size in the datasets used in our study. Further investigations based on preliminary *in vitro* studies and clinical information based on larger samples may be required.

In conclusion, our study showed that immune-related functions may play an important role in PMOP. Besides, *IL4R*, *GNAI2*, *FLNA*, and *TGFB1* may be essential in the pathogenesis of PMOP through affecting osteoclastogenesis. In particular, *FLNA* and *TGFB1* may be affected by smoking, a risk factor of PMOP. The results may provide a theoretical guidance for further investigations on the relationship between B cells and osteoporosis and contribute to a better understanding of the pathogenesis of PMOP.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tjog.2016.04.038.

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