

# Identification of potent novel biomarkers related to Osteoporosis through bioinformatics approach

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## Research Article

**Keywords:** Osteoporosis, DEGs, KEGG, Gene Ontology, Protein-protein interaction

**Posted Date:** January 31st, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1311533/v1>

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# Abstract

## Objective

Calcium is fundamental component of bone tissue. It is present in extracellular fluid in ionized form, some are bounded with albumin while a few are of the complex anionic form. Calcium regulates many biochemical processes. Loss of calcium causes hypocalcemia further lead to osteopenia and osteoporosis. Proton pump inhibitor causes mal-absorption of calcium that leads to poor bone metabolism might causes hip fracture. Some studies via microRNA gene regulatory networks have been analyzed in the present study.

## Methods

Microarray gene expression dataset has been retrieved by using NCBI gene expression Omnibus (GEO) database. Benjamini and Hochberg algorithm was used for identifying DEG's and pre-processing of datasets. Heatmap plot and principal component analysis plot were generated by using online tool ClustVis for DEG's. PPI network and sub network were analyzed by finding functional interactions among protein via online tool STRING v 10.5. DEG's functional pathways analysis has been done by using DAVID (Database for Annotation, Visualization and Integration Discovery) software.

## Results

A number of 3390 differentially expressed genes (DEG's) were identified. ....were up-regulated and were down-regulated genes. DEGs related to Hypocalcemia and Bone signaling were 53, osteogenesis and bone signaling both were 86, whereas, 8 DEG's related to hypocalcemia, Osteogenesis, and Bone signaling. The network of protein-protein interaction and sub network was having ITCH, CKAP4(Up) and FBXW11, RAB37(down) DEGs were concerned in hypocalcemia and were forming hub nodes while CHML, ATP11A, TMEM30A (Up) and YWHAE, AP1M1, FYN(down) DEG's forming were all related to bone signaling. Functional enrichment analysis was performed notably enriched major molecular functions, biological processes and cellular components of novel DEG's (FDR<0.05) related to hypocalcemia and osteoporosis were found.

## Conclusion

Thus by concluding the genes ie; ITCH, CKAP4, FBXW11, RAB37, CHML, ATP11A, TMEM30A, YWHAE, AP1M1, FYN, CTNNB1, UBE2D1, RAP1A, EGFR, MAPK1 and AKT1, whether are unregulated or downregulatd in the diseased tissue samples, and plays a essential role in disease progression. These hub genes are occupied in different biological, molecular and cellular functions which might be related to

bone signaling or osteoporosis. For the further cross validation wet lab experiments are required to validate gene roles.

## Introduction

Calcium is a basic elemental constituent of human body. It is a fundamental constituent of bone structural design and is necessary for deposition of bone mineral throughout the life [1]. About 99% of the body calcium constitutes bone tissue, and remaining are found in the extracellular fluid (ECF)[2]. About 50% of calcium that's present in ECF is active in ionized form, 40% is bounded in albumin, while 10% is in complex anionic form i.e., phosphate, citrate, sulphate and lactate[3]. Calcium regulates many biochemical processes like blood coagulation, intracellular signal transduction, neural transmission, muscle function, cellular membrane integrity and cellular enzymatic activities, cell differentiation, and bone mineralization[4].

Osteoporotic fractures now causing an enormous public health issue globally and it has been estimated that, around 10 million Americans, over the age of 50 years, have been suffering with it[5]. Accordingly a clinical data analysis estimated that about 1.5 million people per year undergoes an osteoporotic fracture and this can lead to poor health quality and increased risk of death rates[6]. Serum calcium levels <8mg/dL or ionized calcium <4.4mg/dL causes hypocalcemia, while in infants of weight~1500g at birth time, <7mg/dL of the total serum calcium level is leads to hypocalcemia[7]. It's well known that hypocalcemia leads to osteopenia and further osteoporosis (loss of bone density)[8]. In most cases at its early stages neonatal hypocalcemia are asymptomatic, but in late/advanced stages aponea, cyanosis, poor feeding, vomiting, tachycardia, heart failure, prolonged QT interval, tremor, laryngospasm, jerking and twitching episodes, tetany and seizures are the main generalized clinical features[9]. Primarily three major hormones regulate the calcium homeostasis in our body which are; parathyroid hormone (PTH), 1, 25-dihydroxyvitamin D-3 (Vitamin D3), and calcitonin, and these three-control calcium transport in our gut, bone and kidney[10].

Gastric acid suppression via Proton pump inhibitors (PPIs) causes malabsorption of calcium and hypergastrinemia, and may results in poor bone metabolism through path of induction of hyperparathyroidism[11]. What's more, the diagnosis of OP is intricate until the episode of bone fractures. So that further study is necessary in order to trace out biomarkers and therapeutic targets that will say something about pathogenesis and molecular mechanisms of osteoporosis. Mutations have been detected in various genes associated with osteoporosis, like CSTA gene interacts with various genes, involved in epidermal development and maintenance, and is associated with the immune regulation of osteoclast[12]. Additionally, FGF21 is inversely coupled with regional bone mass density, affecting the bone development, while its main function is to regulate glucose and lipid metabolism[13]. Even though previous researches have detected regulatory genes and protein linked with osteoporosis[14], still there is need to decipher the candidate biomarkers related to osteoporosis. To generate narrative promises for osteoporosis research, some studies via microRNA gene regulatory networks have been explored here[15].

## Material And Methods

### Retrieval of microarray gene expression dataset

Using NCBI Gene expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) [16] one raw microarray gene expression was retrieved having ID: GSE35958, which includes osteoporosis samples allied to MED12 mutation and MED12 wild type mutation. The [HG-U133\_Plus\_2] Affymetrix Human Genome U133 Plus 2.0 Array [CDF:Brainarray ENSG Version 14.1] platform was used. In wild type and mutational MED12 osteoporosis samples the study of differential gene expression was done using various bioinformatics tools.

### Identifying DEG's and pre-processing of datasets

Benjamini and Hochberg algorithm was used for processing the raw data and was performed next to the retrieval of raw gene expression. Furthermore, log transformation was implemented to the data set by avoiding limma precision weights and force normalization was done. Probe dataset values related to the particular gene were averaged and then with the help of BiGGESTs software up-regulated and down regulated genes were selected.

Applying GEO2R(<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) tool, probe level symbols were exchanged to the gene level symbols. For the assortment of DEG's, parameters were adjusted as,  $p \text{ value} < 0.05$  and threshold  $\log_{2}FC$  values for up regulated genes  $> 0.1$  and  $< -0.1$  for down regulated genes. Through volcano plot screened DEG's were shown that emphasizes the upregulated and down regulated genes. A mean difference graph were plotted with  $\log_{2}$  fold variant versus average  $\log_{2}$  expressed gene, with the help of LIMMA package of GEO2R [17].

### Generation of heatmap plots and principal component analysis plot

Heatmap plot and principal component analysis (PCA) plot were constructed by applying an online tool ClustVis[18] for DEGs. It was unable to generate whole dataset profile of PCA, since this tool can support file size only up to 2MB hence only DEGs were analysed for its principal component.

### Construction of PPI network and sub network

For the findings of functional interactions amongst protein, STRING v 10.5 [19](<https://www.string-db.org/>), an online tool was used here. For the current study, preferred DEG's with included parameter of combined score  $> 0.9$  were put forwarded for analysis. For the various networks and co-networking creation bioinformatics software Cytoscape V 3.2.1(<http://www.cytoscape.org>)[20] was applied, and while constructing networks degree and edge betweenness criteria were employed.

### DEG's functional and pathways analysis

A large set of functionally annotated genes records were integrated by using an online tool DAVID (Database for Annotation, Visualisation and Integrated Discovery) software [21] (<https://david.abcc.ncifcrf.gov>). DAVID v 6.8 tools were applied for performing gene Ontology (GO) enrichment analysis that includes analysis of molecular function (MF), cellular component (CC) and biological process (BP). Based upon closely associated function DAVID uses an entire set of genes that is based on hypergeometric distribution.

## Results

### Figure 1

Data normalization and DEG's visualization via Box plot (A), UMAP plot (B), Volcano plot (C) and MD plot (D).

### Figure 2

Heat map and Venn diagram of the differentially expressed genes (DEGs). Blue to orange gradation is for small to large changes in gene expression values.

### Figure 3

Venn diagram showing DEGs related to Hypocalcemia, Osteogenesis and Bone Signaling.

**Figure 4:** Protein-protein interaction (PPI) of DEG's. Red-circle: up-regulated genes; blue-circle: down-regulated genes. Blue diamond for similar related genes and lines shows the correlation between genes, where thickness of lines (edges), is proportional to the combined scores.

### Figure 5

Gene Ontology (GO) enrichment analysis for up-regulated and down-regulated DEG's, and KEGG pathway enrichment for DEG's (5C).

### Table 1

GENE ONTOLOGY (GO) analysis for novel DEGs related to hypocalcemia and osteoporosis.

## Differentially expressed genes (DEGs)

A total of 3390 DEG's were identified initially out of which ... were found to be up-regulated while ... were found to be down-regulated and these were selected on the basis of their average gene expression values. Out of 3390 DEGs, only 2473 DEGs were found to be significant with p-values (<0.05). In total 2473 DEGs, here 1929 DEG's were found to be novel in which DEGs related to only Hypocalcemia, Bone Signaling and Osteogenesis were 4, 1147 and 1 respectively in their number. While DEGs related to Hypocalcemia and

Bone Signaling were 53; Osteogenesis and Bone Signaling Both were 86; and DEGs related to Hypocalcemia, Osteogenesis and Bone Signaling were 8 in number.

## **Principal component and hierarchical clustering analysis of DEGs**

Uniform manifold approximation and projection plot (umap) shows the clustering of dataset with neighborhood scoring 4. Heat-map was constructed for DEGs, which shows a data matrix where coloring gives an overview of the numeric differences for gene expressions with upregulation in orange and downregulation in blue color code. While gradation from blue to orange shows changing of gene expressions from small to large.

## **The protein-protein interaction and Sub-network construction**

Based on the combined score calculated by STRING, a total of gene pairs (combined score>0.9) was found to interact together, forming a unique network having nodes and edges respectively. From main network, sub-networks were extracted separately (Figure 4B), the clustering coefficient and edge betweenness were taken as a basic criterion for the selection of hub nodes.

This main network was having ITCH, CKAP4 (Up) and FBXW11, RAB37 (down) DEGs who were involved in hypocalcemia and were forming hub nodes while CHML, ATP11A, TMEM30A (Up) and YWHAE, AP1M1, FYN (down) DEGs forming hub nodes were all related to bone signaling. The up DEGs like CTNNA1, UBE2D1, RAP1A, EP300, CDC42 and down DEGs like EGFR, MAPK1, AKT1, VEGFA, TP53 were all involved in other pathways.

## **Gene Ontology and pathway enrichment analysis**

Functional enrichment analysis was performed and significantly enriched major molecular functions, biological processes and cellular components of novel DEGs (FDR<0.05) related to hypocalcemia and osteoporosis were listed in Tables 1.

GO enrichment analysis identifies novel DEGs to be involved majorly in biological processes like Osteoblast, myoblast, endodermal cell differentiation; regulation of potassium, chloride transmembrane transport; positive regulation of hormone biosynthesis, mesenchymal and endothelial; organization of extracellular matrix and actin cytoskeleton. While novel DEGs are a part of cellular components like lysosomal, endosome and plasma membrane; synapse and postsynaptic density; cell-cell junction and focal adhesion; cytosol, actin cytoskeleton, cytoplasm and nucleoplasm. Novel DEGs are involved in molecular functions like binding of metal ion, histone acetyltransferase, ephrin receptor, enzyme, laminin, chromatin, sequence-specific and transcription regulatory DNA, identical protein, ion-channel, transcription factor, proteins; ATPase coupled transmembrane transporter and transcription factor activity. Further KEGG pathway reveals that novel DEGs were involved in major significant pathways like NF-kappa B, PI3K-Akt, Wnt and Epithelial cell signaling; regulation of actin cytoskeleton;

## Discussion

Osteoporosis is a systemic bone disease, which leads to the deterioration of microstructure of bone tissues causing lowering of the bone mass and so consequent fracture. The imbalance of bone remodeling process leading to bone resorption and its main pathophysiological process of osteoporosis[22]. Various types of non-skeletal factors add to fracture risk and thus, diagnostic tool for osteoporosis is the estimation of risk factors and Bone Mass Density measurement[22]. [22]. Bone is an active tissue that is continuously remodeled with the help of specific bone forming cells, osteoblast and bone resorbing cells, osteoclasts. The imbalance of bone metabolism like decreasing bone resorption causes lower blood calcium level, called as hypocalcemia. Hypocalcemia is defined as blood calcium level below 8.5mg/dL or ionized blood calcium level lower than 4.6mg/dL in blood plasma[23]. It is most common in advance stage prostate cancer patients and about approximately in 30% of cases[24]. Osteogenesis and bone formation involve many genes, some have positive whilst some have negative impacts[25]. Like WNTB increases osteoblast activity[26] and RUNX2, a transcription factor involved in osteoblast differentiation and shortage of sclerostin (SOST) enhances bone formation [27]. Signaling pathways play a decisive role in the regulation of osteoblasts and osteoclasts that regulate the bone turn over. Osteoclast activation escort to the loss of bone, and at present therapeutic agents for osteoporosis mainly works on inhibition of bone resorption [28]. PTHrP1-36 stimulates bone formation by reducing bone resorption. Member of TGF that's Activin A stimulates osteoclastogenesis and also involved in the process of inhibition of bone mineralization [29].

Here, using different bioinformatic tools a high throughput gene expression datasets of osteoporosis vs control were studied and as the output it has revealed about 1929 were novel DEGs out of 3390. From total of 1929 novel DEGs, about 1147 DEGs were related to bone signaling only; 86 were related to osteogenesis and bone signaling both; 53 in hypocalcemia and bone signaling both; 8 DEGs were together involved in hypocalcemia, osteogenesis and bone signaling while only 4 were related to hypocalcemia and 1 in osteogenesis.

The hypocalcemia related novel DEGs forming hub nodes are mainly ITCH, CKAP4, FBXW11 and RAB37 from these the first two are upregulated and rest two are downregulated respectively.

ITCH (Itchy E3ubiquitin protein ligase) gene, a regulator of ubiquitination of T-cell receptors, and any mutation in ITCH gene can cause syndromic Multisystem Autoimmune Disease with acute liver failure[30].

CKAP4, Cytoskeletal-associated protein 4; also known as p63, CLIMP-63 or ERGIC-63, is a 63kDa type II transmembrane (TM) protein, residing in endoplasmic reticulum or in intermediate compartment of Golgi. CKAP4 protein is upregulated in hepatocellular carcinoma tissues and has been identified as specifically in it [31].

FBXW11, a F-box family members contributing to tumorigenesis and tumour development [32]; [33]. A recent work revealed the role of Fbxw11 in the proliferation of lymphocytic leukemia cells and implies

that it can be served as a potential molecular target for the disease treatment [34].

RAB37 is a small GTPase, playing important roles in several cellular processes through intracellular membrane traffic. It has been identified as a tumour suppressor and regulates exocytosis of several proteins including TIMP metalloproteinase inhibitor 1 (TIMP1) [35].

Similarly, the bone signaling novel DEGs forming hub nodes mainly include CHML, ATP11A, TMEM30A, YWHAE, AP1M1 and FYN from these the first three are upregulated and rest three are downregulated respectively.

CHML (Choroideremia-like) protein is essential for the prenylation modification of various Rab proteins and it exerts biological effects on vesicle trafficking and signal transduction. CHML gene is now considered as an independent factor to evaluate the prognosis of Multiple Myeloma, and is associated with poor survival of myeloma cells [36].

However, ATP11A, a ubiquitously expressed gene in various tissues and deleterious effect are lethal for an organism. It plays an important role in myotube formation, while detailed cellular function of ATP11A is still remain exclusive. Mutation in this gene affects localization of Golgi and plasma membrane and Phosphatidylserine flippase activity [37].

TMEM30A (Transmembrane protein 30A), is a ubiquitously expressed terminally-glycosylated membrane protein [38]. The TMEM30A phospholipid flippase complex plays role in cell migration via the formation of membrane ruffles as a result of phospholipid translocation [39].

YWHAE (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Epsilon), is a protein coding gene and its association has been found with Endometrial Stromal Sarcoma and Kidney Clear Cell Sarcoma. Mutation in this gene has now been considered a risk for Major Depressive Disorder in the Han Chinese Population [40].

AP1M1 (Adaptor protein-1  $\mu$  subunit-1), mediates late secretory and vascular traffic and is required for growth [41]. Adaptor proteins (AP) are complexes predominantly as coat proteins of membrane vesicles in post-Golgi trafficking of mammalian cells. AP-1 is crucial for cell division and plant growth.

FYN, is a Src family non-receptor tyrosine kinase, which interacts with tau via SH3 domain [42], where tau is enriched in axons and regulates the microtubule assembly. Fly is found to be critical for neurofibrillary tangle formation and tau hyperphosphorylation and if depletion in Fly occurs it causes tau induced neuropathy [43].

GO cluster analysis identified several other major up regulated novel DEGs like; CTNNB1, UBE2D1, RAP1A and few major down regulated DEGs like EGFR, MAPK1 and AKT1 which were forming hub nodes.

Among these CTNNB1 ( $\beta$ -catenine-1), a fundamental component of the canonical Wnt signaling pathway that controls cell growth and cell adhesion [44]. Nonsense and missense mutations in CTNNB1 were

identified in patients with ASD [45] and intellectual disability (ID) [46].

UBE2D1 has a crucial role in hepatocellular carcinoma progression, it is one of the family members of E2 ubiquitin conjugating enzyme, mediating the ubiquitination and degradation of tumor suppressor protein p53[47].

RAP1A, a small G protein similar to Ras oncogene and has role in different cellular processes [48]. Other studies show that RAP1A mediates Glioblastoma cell proliferation [49] and oral cavity squamous cell carcinoma [50].

EGFR (Epidermal Growth Factor Receptor (EGFR), a member of the ErbB family of receptor tyrosine kinase (RTK) proteins, is aberrantly expressed in tumors [51]. The ZNF216 (zinc finger 216) in human carcinoma cells has been proved to be a potential regulator of EGFR activity [52].

MAPK1 (Mitogen-activated protein kinase 1) is a serine/threonine kinase that plays critical roles in several cellular processes like cell proliferation, survival, adhesion, migration via phosphorylation of hundreds of nuclear and cytosolic substrates in the cell. It is a master regulator of stem cell differentiation and is responsible for stem cell fate [53].

A recent study tells MAPK-RAP1A signalling plays an important function as clinical diagnosis and prognostic value in Hepatocellular carcinoma (HCC), and is related with immune infiltration and clinical prognosis [54].

AKT1 (Protein kinase B) is a member of AGC family of serine-threonine kinases and transduces signals through the phosphoinositide 3-kinase (PI3K)/AKT cell-signalling cascade. It is involved widely in signal transduction, metabolism, cell-cycle regulation, transcription, cell-proliferation and angiogenesis processes[55].

## Conclusion

Further we can now conclude, that these genes ie; ITCH, CKAP4, FBXW11, RAB37, CHML, ATP11A, TMEM30A, YWHAE, AP1M1, FYN, CTNNB1, UBE2D1, RAP1A, EGFR, MAPK1 and AKT1, whether are upregulated or downregulated in the diseased tissue samples, but must be playing a crucial in the disease progression. These hub genes are involved in different biological, molecular and cellular functions which can directly or indirectly may be related to osteoporosis or hypocalcaemia or bone signalling. Most of these hub genes have their roles in cancer progression while their role in occurrence of osteoporosis has to be depicted. But a clear picture of these genes' role needs wet lab experiments, observations, analysis and further cross validations.

## Declarations

### Ethics approval and consent to participate

Not applicable

### **Consent for publication**

Not applicable

### **Availability of data and materials**

Request for additional materials can be addressed to the Ravi Bhushan. All the relevant data are enclosed in the manuscript and provided as supplementary file. The RNA-seq. raw data were retrieved from NCBI's Gene Expression Omnibus and are accessible through GEO accession number i.e. GSE35958 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35958>).

### **Competing interests**

The authors declare that they have no any conflict of interest.

### **Funding**

Not applicable

### **Credit authorship contribution statement**

R Bhushan, K Kusum, Khusboo .:Methodology, Investigation, Writing - review & editing.

RB:Visualization, Investigation, Validation.

Kusum kusum: Manuscript writing, review and editing.

Ravi Bhushan: Data curation, Software, Conceptualization, review and editing .

R S More: Conceptualization,Writing – review and editing.

### **Declaration of competing interest**

The authors have declared no conflict of interest.

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# Table 1

Table 1 is available in the Supplementary Files section.

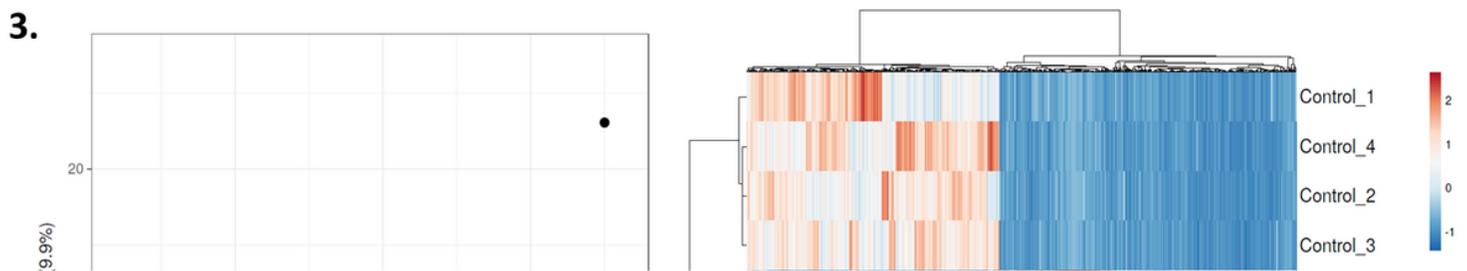
## Figures

### Figure 1

Data normalization and distribution via Box plot, and UMAP plot.

### Figure 2

DEG visualization through volcano plot, MD plot, and venn diagram.



### Figure 3

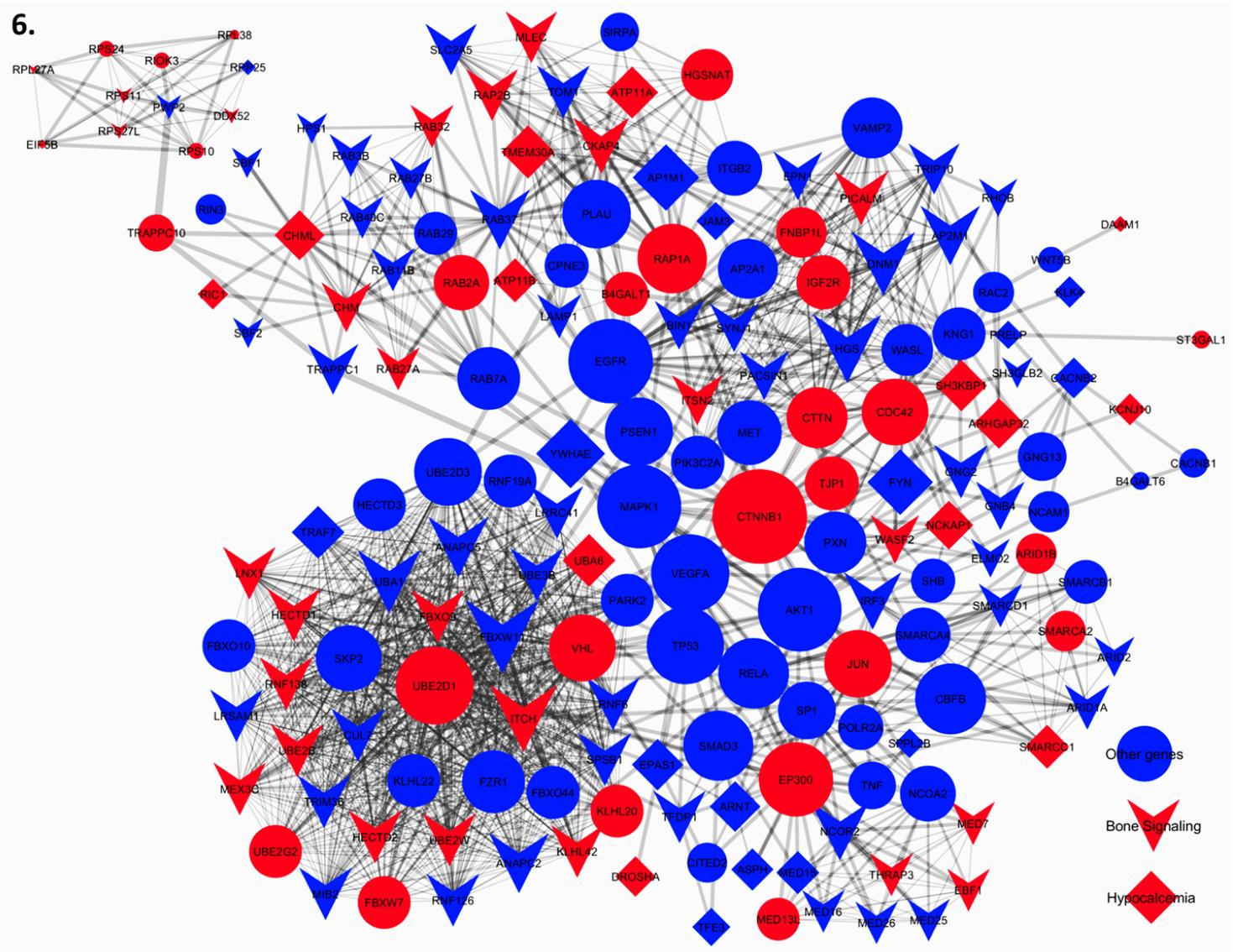
Heat map and principal component analysis of the differentially expressed genes (DEGs). Blue to orange gradation is for small to large changes in gene expression values.

### Figure 4

Novel DEGs identification related to Hypocalcemia, Osteogenesis and Bone Signaling.

**Figure 5**

Protein-protein interaction (PPI) network of DEG's. Red-circle: up-regulated genes; blue-circle: down-regulated genes. Blue diamond for similar related genes and lines shows the correlation between genes, where thickness of lines (edges), is proportional to the combined scores.



**Figure 6**

Protein-protein interaction (PPI) network of novel and reported DEG's related to bone signaling and hypocalcemia. Red-circle: up-regulated genes; blue-circle: down-regulated genes. Blue diamond for similar related genes and lines shows the correlation between genes, where thickness of lines (edges), is proportional to the combined scores.

**Figure 7**

Gene Ontology (GO) enrichment analysis for up-regulated and down-regulated DEG's, and KEGG pathway enrichment for DEG's.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [DEGs.xlsx](#)