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Original Article

Up-regulated and hypomethylated genes are causative factors and diagnostic markers of osteoporosis

Jiang Minbo[1], Chen Feng[2], Hongli Wen[3], Muhammad Jamil[4], Heng Zhang[1], Mostafa A Abdel-Maksoud[5], Adel M Zakri[6], Taghreed N Almanaa[5], Akram A Alfuraydi[5], Bandar M Almunqedhi[5]

[1]Department of Orthopedic, Shanghai Songjiang District Central Hospital, Shanghai 201699, China; [2]Department of Orthopedics, Hongqi Hospital, MuDanjiang 157011, Heilongjiang, China; [3]Department of Foreign Language, MuDanjiang Medical University, MuDanjiang 157011, Heilongjiang, China; [4]PARC Arid Zone Research Center, Dera Ismail Khan 29050, Pakistan; [5]Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia; [6]Department of Plant Production, College of Food and Agricultural Sciences, King Saud University, Riyadh 11451, Saudi Arabia

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Abstract: Background: Due to the lack of sensitive diagnostic biomarkers for osteoporosis (OP), there is an urgent need to identify and uncover biomarkers associated with the disease in order to facilitate early clinical diagnosis and effective intervention strategies. Methods: GEO2R was employed to conduct a screening of differentially expressed genes (DEGs) within the transcriptome sequencing data obtained from blood samples of OP patients within the GSE163849 dataset. Subsequently, we conducted expression confirmation of the identified DEGs using an additional dataset, GSE35959. To further explore Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, MicroRNA (miRNA) interactions, and drug predictions, we employed the DAVID, miRTarBase, and DrugBank databases. For validation purposes, clinical OP samples paired with normal controls were collected from the Pakistani population. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to assess the expression levels of DEGs and miRNA, while targeted bisulfite sequencing (bisulfite-seq) analysis was used to investigate methylation patterns. DNA and RNA from clinical OP and normal control samples were extracted using appropriate methods. Results: Out of total identified 269 DEGs, EGFR (epidermal growth factor receptor), HMOX1 (heme oxygenase-1), PGR (progesterone receptor), CXCL10 (C-X-C motif chemokine ligand 10), CCL5 (C-C motif chemokine ligand 5), and IL12B (interleukin 12B) were prioritized as top DEGs in OP patients. Expression validation of these genes on additional Gene Expression Omnibus (GEO) dataset and Pakistani OP patients revealed consistent significant up-regulation of these genes in OP patients. Receiver operating characteristic (ROC) analysis demonstrated that these DEGs displayed considerable diagnostic accuracy for detecting OP. Targeted bisulfite-seq analysis further revealed that EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B were hypomethylated in OP patients. Moreover, has-miR-27a-5p, a common expression regulator of the EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B was also significantly down-regulated in OP patients. Conclusion: The DEGs that have been identified hold significant potential for the future development of diagnostic and treatment approaches for OP in preclinical and clinical applications.

Keywords: Osteoporosis, biomarker, expression analysis

Introduction

Osteoporosis (OP) has become a prevalent clinical concern, particularly in aging populations, and is characterized by the deterioration of bone microarchitecture, leading to a reduction in bone mass and strength [1, 2]. OP commonly results in fractures, with the spine being the most frequently affected site, although fractures may also occur in the hip, forearm, and proximal humerus [3].

Based on medical literature, the development and progression of this disease involve various factors. These include genetic abnormalities, gender, age, steroid production, lifestyle choices, and a range of environmental factors [4-6]. Apart from these factors, inadequate calcium

Table 1. Clinical characteristics of the OP patients

Sr. No	Gender	Age (Years)	Case	BMI
1	Female	46	Advance	22.1
2	Female	65	Advance	24.2
3	Female	41	Advance	20.7
4	Female	65	Advance	24.5
5	Female	43	Advance	24.2
6	Female	53	Advance	20.2
7	Female	65	Advance	28.1
8	Female	59	Advance	18.9
9	Female	45	Advance	18.2
10	Female	67	Advance	24.1
11	Female	41	Advance	21.3
12	Female	39	Advance	25.2
13	Female	53	Advance	20.7
14	Female	57	Advance	21.1
15	Female	43	Advance	19.1

BMI = body mass index.

intake, smoking cigarettes, and excessive alcohol consumption are additional secondary factors contributing to OP [7, 8]. Commonly referred to as a "silent disease", OP is characterized by its asymptomatic nature until the occurrence of a fracture.

At present, treatment approaches for OP involve pharmaceutical interventions. However, this treatment method is deemed unsatisfactory due to its time-consuming nature, high cost, and the potential for adverse side effects associated with the medications. To date, researchers worldwide have endeavored to investigate the underlying mechanisms of OP. For instance, a study revealed the association between the components of the Wnt signaling pathway, such as Wnt3a, secreted frizzled-related protein 1, sclerostin, and low-density lipoprotein receptor-related protein 5, and alterations in bone mineral density (BMD) within the skeletal structure [9]. Clinically, the assessment of bone mineral density (BMD) plays a significant role in defining the presence of OP, with heritability estimates ranging from 0.5 to 0.9. BMD measurement serves as a crucial parameter in evaluating the occurrence of OP [10]. Hence, the measurement of BMD is a crucial clinical biomarker for OP. Nevertheless, the underlying pathways contributing to this condition have not been extensively investigated. Therefore, there is a need to identify hub genes associated with OP, which may serve as potential therapeutic targets.

It is worth emphasizing that microarray data analysis can be employed to identify key genes and gene regulatory networks linked to a particular disease [11]. In this study, we obtained the OP microarray dataset from the Gene Expression Omnibus (GEO) database and conducted data processing to identify differentially expressed genes (DEGs) in blood samples obtained from OP patients and normal individuals. Subsequently, we proceeded to validate the expression and assess the evolution of promoter methylation levels for the identified DEGs using clinical samples. Moreover, we also predicted DEGs regulatory MicroRNA (miRNA) and performed its expression analysis in OP patients. In summary, the primary objective of this study was to identify key genes implicated in the development and progression of OP. These genes hold potential as biomarkers and therapeutic targets for individuals affected by OP.

Methodology

Sample collection for molecular analyses

The study methodologies in accordance with the Helsinki Declaration [12] have been approved by the Ethical and Research Committee (approval letter # AZRC-113) of Pakistan Agriculture Research Center (PARC) Arid Zone Research Center, Dera Ismail Khan-29050, Pakistan. The present study included a total of 15 OP subjects who visited the District Head Quarter (DHQ), Teaching Hospital, Dera Ismail Khan, Khyber Pakhtunkhwa (KPK), Pakistan and voluntarily participated. Informed written consent was obtained from all participants. During the recruitment process, comprehensive information was collected, including details on nutrition, overall health, complete medical history, fracture history, and associated risk factors. To conduct the study, a 5 cc blood sample was obtained from each patient. Additionally, as a control group, 5 cc blood samples were collected from 15 normal individuals. Serum was separated, and the blood cells were stored at -80°C. The clinical characteristics of the enrolled OP patients are presented in **Table 1**.

Data resource for hub gene prediction

To identify DEGs, we initially obtained the GSE163849 dataset from the GEO database [13, 14] available at http://www.ncbi.nlm.nih.gov/geo/. This dataset consisted of expression profiles from 4 normal individuals and 4 OP patients. Additionally, we retrieved an additional dataset, GSE35959, from the GEO database to validate the expression levels of the identified hub genes. The GSE35959 dataset comprised expression profiles from 5 normal individuals and 14 OP patients. Combining these two datasets, we obtained expression profiles from 9 normal individuals and 18 OP patients. Both the GSE163849 and GSE35959 datasets were based on the GPL20115 platform.

Screening of differentially expressed genes

The GSE163849 dataset was analyzed with the help of the "limma" package in R software 3.6.1 [15]. After analysis, to screen for DEGs, we first annotated the probes in the obtained GSE163849 data file. Probes that did not match with any gene symbol were excluded from further analysis. In cases where multiple probes corresponded to a single gene, we calculated the average value of these probes to obtain the final expression value. Additionally, we obtained the FPKM (Fragments Per Kilobase Million) expression values from the GSE163849 dataset. These expression values were then subjected to differential expression analysis using the limma package in R [16, 17]. DEGs were selected based on the criteria of $|\log2FC| > 1$ and a p-value < 0.05, serving as the cutoff for significance.

Nucleic acid isolation

Total cell DNA from blood samples was extracted using the organic method [18], while total RNA was extracted using the Trizol method [19, 20]. We employed the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to assess the concentration and purity of the extracted DNA and RNA, ensuring that the A260/A280 ratio fell within the range of 1.8 to 2.0.

Library preparation for targeted bisulfite sequencing analysis

In brief, total DNA (1 μg) was fragmented into approximately 200-300 bp fragments using a Covarias sonication system (Covarias, Woburn, MA, USA). Following purification, the DNA fragments underwent repair and phosphorylation of blunt ends using a mixture of T4 DNA polymerase, Klenow Fragment, and T4 polynucleotide kinase. The repaired fragments were then 3' adenylated using Klenow Fragment (3'-5' exo-) and ligated with adapters containing 5'-methylcytosine instead of 5'-cytosine and index sequences using T4 DNA Ligase. The constructed libraries were quantified using a Qubit fluorometer with the Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and sent to Beijing Genomic Institute (BGI), China for targeted bisulfite sequencing. Following sequencing, the methylation data were normalized into beta values.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation analysis of DEGs

The specific protocols are as follows: First, the PrimeScript™ RT reagent kit (Takara, Japan) was used according to the manufacturer instructions for reverse transcription of the extracted RNA from OP patients and control samples into complementary DNA (cDNA). This kit includes reverse transcriptase enzymes and required buffers for reverse transcription, ensuring precise conversion of RNA into cDNA. This critical step involved the addition of specific primers (Oligo dT) and followed temperature-controlled incubation, meticulously executed per the manufacturer's guidelines. Subsequent to cDNA synthesis, an ABI ViiA 7 Real-Time PCR System (Thermo Fisher, USA) was employed for RT-qPCR due to its precision and reliability. SuperReal SYBR Green Premix Plus, sourced from Tiangen Biotech in China, served as the fluorescent dye to enable accurate measurement of target gene expression levels in strict accordance with the manufacturer's recommended protocol. The GAPDH gene was selected as the internal reference gene to normalize target gene expression. RT-qPCR reactions were meticulously conducted in 96-well plates to accommodate multiple samples efficiently. To ensure robust and reliable results, all experimental runs were performed in triplicate independently, minimizing potential variability and enhancing the accuracy of gene expression analysis.

Table 2. List of the primers

Sr. no	Gene ID	Primer ID	Primer sequence (5'-3')	Product size (bp)
1	2597	GAPDH-F	ACCCACTCCTCCACCTTTGAC	110
		GAPDH-R	CTGTTGCTGTAGCCAAATTCG	
2	1956	EGFR-F	GAGCTCTTCGGGGAGCAG	131
		EGFRF-R	GTGATCTGTCACCACATAATTACCTTTCT	
3	3162	HOMOX1-F	CCAGGCAGAGAATGCTGAGTTC	144
		HOMOX1-R	AAGACTGGGCTCTCCTTGTTGC	
4	5241	PGR-F	AATGGAAGGGCAGCACAACT	192
		PGR-R	TGTGGGAGAGCAACAGCATC	
5	3627	CXCL10-F	GGTGAGAAGAGATGTCTGAATCC	102
		CXCL10-R	GTCCATCCTTGGAAGCACTGCA	
6	6352	CCL5-F	CCTGCTGCTTTGCCTACATTGC	125
		CCL5-R	ACACACTTGGCGGTTCTTTCGG	
7	3593	IL12B-F	GACATTCTGCGTTCAGGTCCAG	143
		IL12B-R	CATTTTTGCGGCAGATGACCGTG	

The emitted fluorescence signals during RT-qPCR were captured using the Applied Biosystems Sequence Detection Software (SDS v1.3.1). Subsequently, calculations were performed to determine Cq/CT (cycle quantification/cycle threshold), ΔCT, and ΔΔCT values, following the established 2-ΔΔCT method [21]. The stepwise procedure for implementing the 2-ΔΔCT method is elaborated below: ΔCT = CT (a target gene) - CT (a reference gene).

ΔCT for the target sample is CTD - CTB, and the ΔCT for the reference sample is CTC - CTA. The ΔΔCT is the difference in ΔCT as described in above formula between the target and reference samples, which is ΔΔCT = ΔCT (a target sample)-ΔCT (a reference sample) = (CTD - CTB)-(CTC - CTA).

After calculating gene expression, a student t-test was applied to find differences in the expression levels of DEGs between OP and normal control samples. A p-value < 0.05 was considered a significant difference. Following primers of each DEG and control gene (GAPH) were synthesized by the ORIGENE Company, USA, for RT-qPCR analysis of the hub genes (**Table 2**).

Receiver operating curve (ROC) generation

Based on the RT-qPCR expression data, ROC curves of identified DEGs expression were generated using the SRPLOT web source (https://bioinformatics.com.cn/srplot).

Functional enrichment analysis

Functional enrichment profiling of the DEGs was carried out using the DAVID tool [22]. Functional enrichment includes Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. GO is further divided into biological processes (BP), and cellular components (CC), molecular functions (MF) analyses [23]. A $P < 0.05$ was used as the cut-off criterion for the functional enrichment analysis.

miRNA prediction analysis

To predict potential regulatory miRNAs of the DEGs, we used miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) [24] database. After searching for DEGs-associated miRNAs, the miRNA-mRNA regulatory network was incorporated using Cytoscape software.

RT-qPCR analysis of has-miR-27a-5p expression

To analyze has-miR-27a-5p expression, we conducted real-time PCR using the PrimeScript® miRNA RT-PCR kit (Takara) following the guidelines provided by the manufacturer. For normalization of miRNA expression, U6 snRNA was utilized in this analysis. The relative expression levels were determined using the 2-ΔΔCt method [25]. Student t-test [21] was used to evaluate differences in the expression levels between OP and the normal control group. The following primers were used for the expression analysis of has-miR-27a-5p and U6-snRNA.

U6-snRNA-F 5'-UUCUCCGAACGUGUCACGUTT-3'; U6-snRNA-R 5'-ACGUGACACGUUCGGAGAA-TT-3'; miR-27a-5p 5'-AGGGCUUAGCUGCUUG-UGAGCA-3'.

Primers were synthesized by the ORIGENE Company, USA.

Drug prediction analysis

DrugBank (http://www.drugbank.ca) database [26] is a comprehensive and widely used online

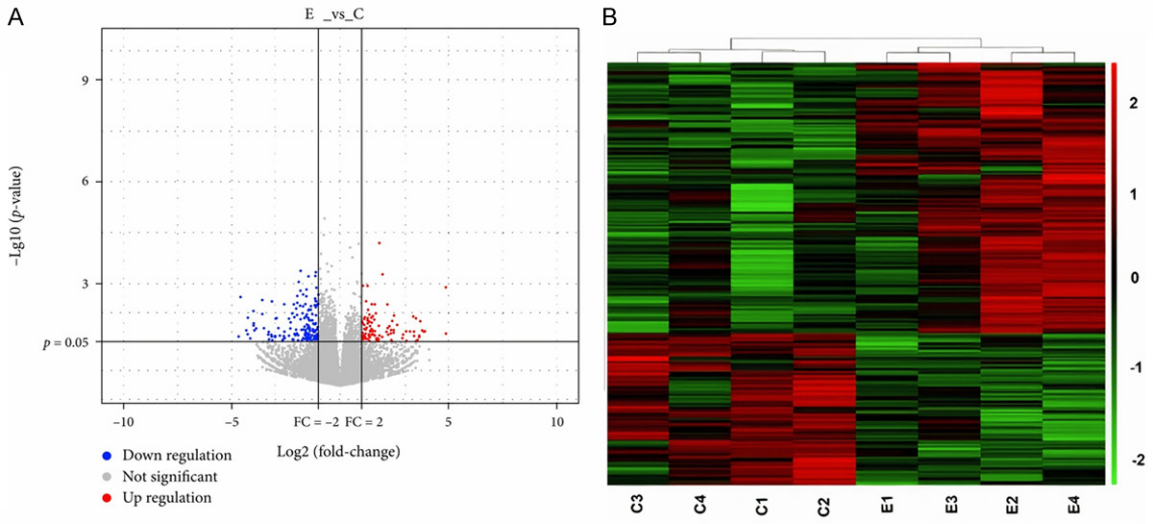

Figure 1. Comprehensive visualizations of differentially expressed genes (DEGs) in the GSE163849 dataset. A. Volcano Graph: This plot displays the DEGs detected in the GSE163849 dataset, showcasing their significance and fold changes. The x-axis represents the log2 fold changes, while the y-axis denotes the -log10 adjusted *p*-values. Up-regulated genes are highlighted in red, and down-regulated genes are indicated in blue. Non-significant genes are depicted in gray. B. Heatmap: The color-coded heatmap graphically represents the expression levels of DEGs identified in the GSE163849 dataset. Rows correspond to individual genes, and columns signify different samples. The color gradient varies from green (low expression) to red (high expression), facilitating immediate visual comparison.

database that provides valuable information on drugs, their targets, mechanisms of action, and drug-drug interactions. It offers a vast collection of data, including pharmacological and pharmaceutical details, as well as links to external resources. In this study, the DrugBank database was used to evaluate DEGs-associated potential targeted drugs.

Results

The GSE163849 dataset was chosen, and DEG analysis was performed using the "limma" package in software version 3.6.1. A total of 269 DEGs were identified, comprising 97 up-regulated genes and 172 down-regulated genes ($P < 0.05$). **Figure 1A** illustrates the plotted distribution of all 269 DEGs, with blue representing down-regulated genes, red representing up-regulated genes, and gray indicating other DEGs. Furthermore, the heat map in **Figure 1B** visually depicts the expression levels of all DEGs, revealing distinct clustering patterns between the OP and control groups.

Functional enrichment analysis of DEGs

Following that, we conducted an analysis to determine the top 10 enriched Gene Ontology (GO) terms across three aspects: CC, BP, and MF (**Figure 2A**, **2B**). The CC analysis revealed significant enrichment of DEGs in terms such as "myofibril", "sarcomere", "band", and "disc". In the MF analysis, the top three enriched terms were "actin-binding", "serine-type endopeptidase activity", and "growth factor binding". In the BP analysis, DEGs were found to be significantly enriched in processes related to the regulation of "smooth muscle cell promotion", "response to a toxic substance", and "negative regulation of cytokine production" (**Figure 2A**). The chord diagram reveals the involvement of few DEGs, such as ligp1, Il12b, Xcl1, Cxcl10, Ccl7, Ccl5, Mmp12, and F3 in cytokine-mediated signaling pathways. Regarding lymphocyte migration, the implicated genes are Tbx21, Xcl1, Cxcl10, Ccl7, and Ccl5 (**Figure 2B**). Additionally, the enriched KEGG pathways include "cytokine-cytokine receptor interaction", "Toll-like receptor signaling pathway", "chemokine signaling pathway", and "HIF-1 signaling pathway" (**Figure 2C**).

Prioritizing DEGs for characterization as molecular biomarkers of OP and their expression confirmation on the additional Gene Expression Omnibus dataset

Based on the log2FC and *p*-value, the top six DEGs in GSE163849 dataset, including EGFR

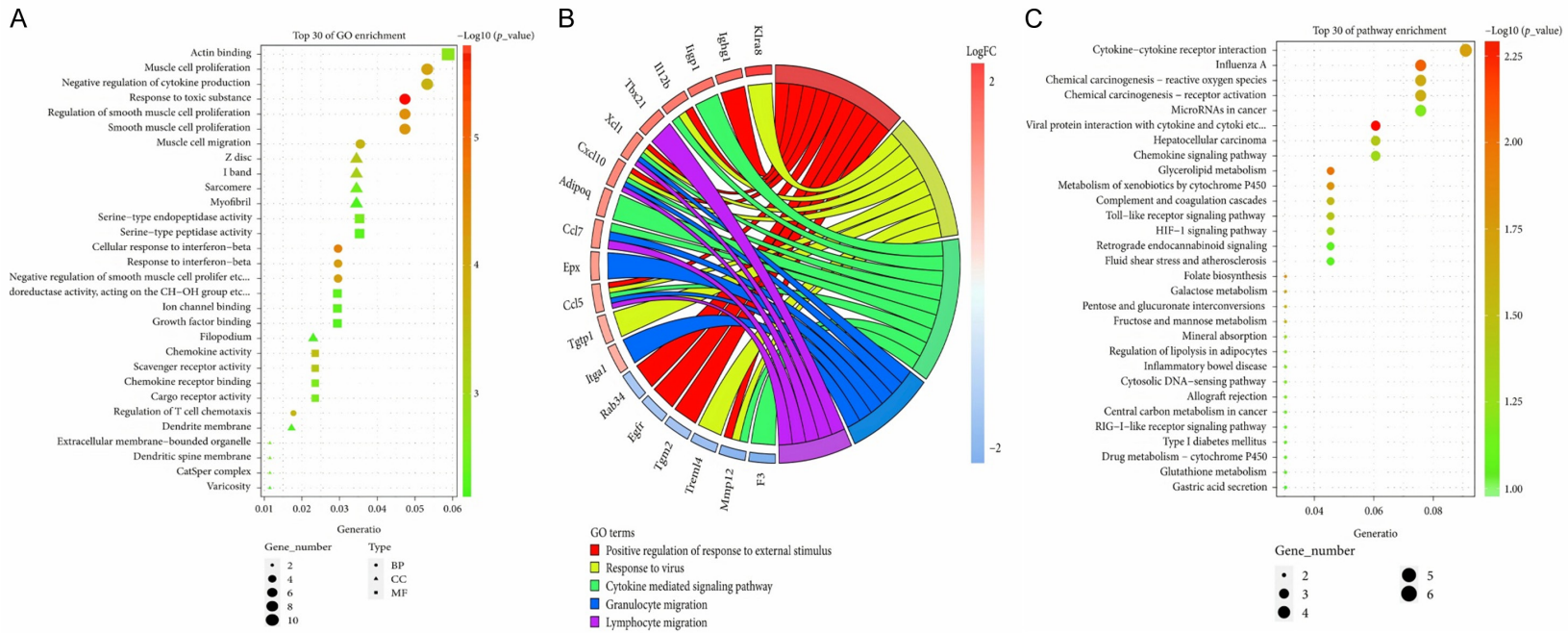

Figure 2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of differentially expressed genes (DEGs) in GSE163849. (A) GO enrichment showcases significant biological processes (BP), molecular functions (MF), and cellular components (CC). (B, C) KEGG analysis in parts B and C detail metabolic and signaling pathways influenced by DEGs, respectively.

(epidermal growth factor receptor), HMOX1 (Heme Oxygenase-1), PGR (Progesterone receptor), CXCL10 (C-X-C Motif Chemokine Ligand 10), CCL5 (C-C Motif Chemokine Ligand 5), and IL12B (interleukin 12B) were prioritized for their characterization as a molecular biomarkers of OP due to following reasons: By prioritizing these top DEGs, the risk of false positive findings will be reduced and the accuracy of downstream analyses will be enhanced. Moreover, the top DEGs often possess high biological relevance, being associated with critical pathways and functions. By studying these DEGs, valuable insight into the molecular mechanisms underlying OP will be gained, potentially leading to the discovery of novel therapeutic targets or diagnostic markers.

To confirm the expression level of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B, we further analyzed an additional GEO OP dataset (GSE35959) and observed significant up-regulation of these genes in OP patients within this independent dataset (**Figure 3**).

Validation of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B gene expression in clinical OP samples

To validate the results obtained from the GEO expression dataset, cDNA from both OP and control blood samples was utilized for RT-qPCR analysis of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B. The results, as depicted in **Figure 4A**, demonstrated a significant increase in the expression levels of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B in the OP sample group (n=15) compared to the control group (n=15, p-value < 0.05). Additionally, the ROC curves for EGFR (AUC: 0.714, p-value < 0.05), HMOX1 (AUC: 1.0, p-value < 0.05), PGR (AUC: 0.671, p-value < 0.05), CXCL10 (AUC: 1.0, p-value < 0.05), CCL5 (AUC: 0.91, p-value < 0.05), and IL12B (AUC: 0.671, p-value < 0.05) exhibited significant diagnostic potential, sensitivity, and specificity (**Figure 4B**).

Targeted bisulfite sequencing analysis to analyze promoter methylation levels of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B in clinical OP samples

In order to assess the extent of promoter methylation in the biomarker genes EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B within clinical OP samples, we enrolled a total of 15 individuals diagnosed with OP, along with 15 healthy individuals from the Pakistani population (detailed clinical information can be found in **Table 1**). In both the OP and control groups, a high rate of bisulfite conversion (C to T) exceeding 99% was observed, and there were no notable differences in the read mapping rate between the two groups. Following stringent quality control measures, all 15 samples from the OP group and 15 samples from the control group were deemed suitable for subsequent analysis.

Our analysis revealed a significant pattern of hypomethylation across all candidate genes (EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B) in OP samples compared to the control group (**Figure 5**). This finding suggests that hypomethylation may have a substantial impact on the dysregulation of these differentially expressed genes (DEGs).

miRNA prediction and expression analysis of miR-27a-5p

Following our miRNA prediction analysis, we identified a total of 140 miRNAs that target all six differentially expressed genes (EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B) (**Figure 6A**). By examining the degree of centrality, we further narrowed down the candidates and found that hsa-miR-27a-5p potentially regulates all of the DEGs collectively (**Figure 6B**).

To validate hsa-miR-27a-5p as a common regulator of gene expression, we conducted RT-qPCR to analyze its expression in the OP sample group compared to the control group. Our analysis demonstrated a significant decrease (p-value < 0.05) in the mRNA levels of hsa-miR-27a-5p in the OP group when compared to the control group (**Figure 6C**).

Drug prediction analysis

For OP patients, medical treatment is typically the primary approach to managing the disease. Consequently, it becomes crucial to carefully select appropriate candidate drugs. In our present study, we utilized the DrugBank database to explore potential therapeutic drugs for OP based on the identified DEGs. Notably, our investigation yielded several drugs deemed suitable for the treatment of OP with respect to

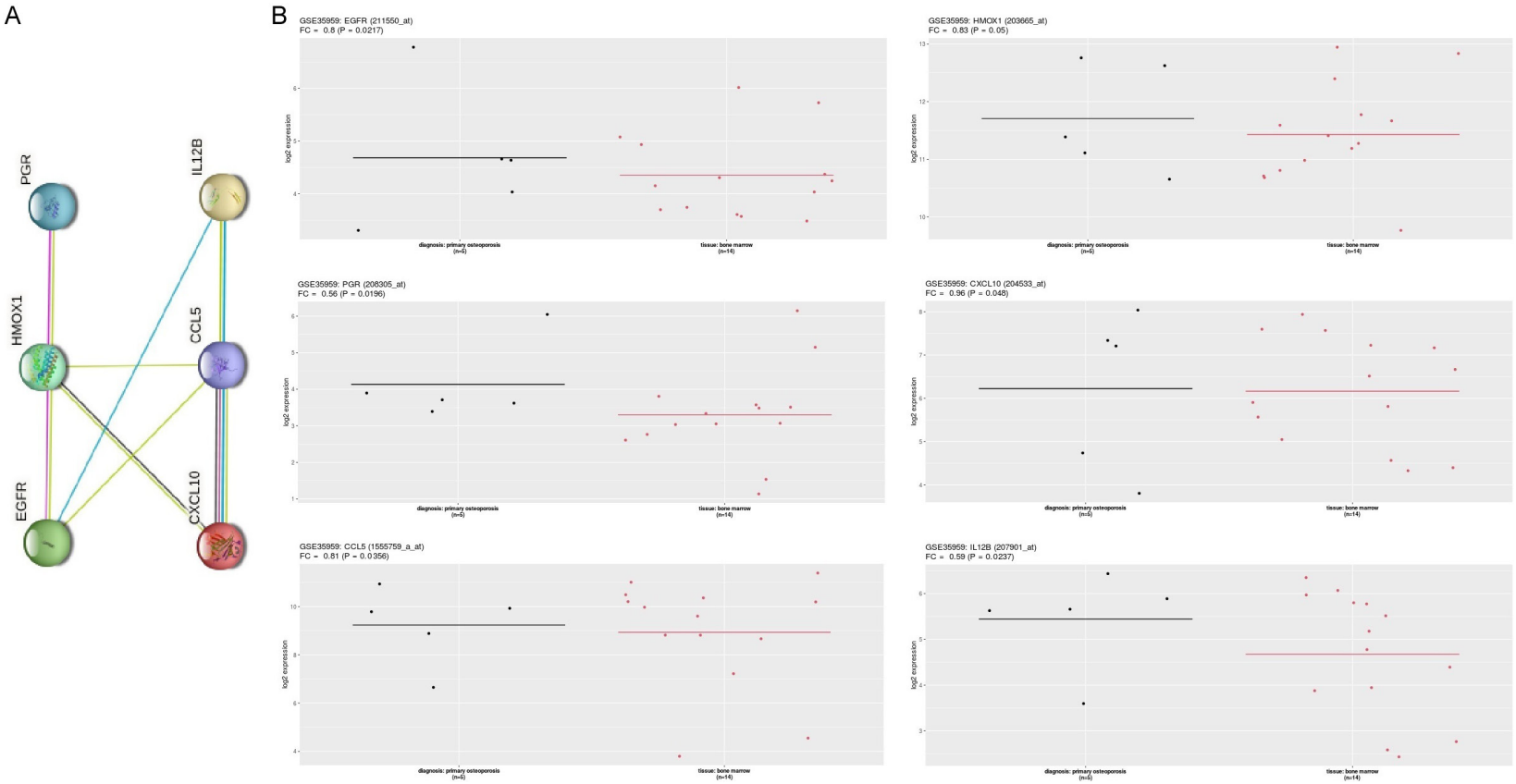

Figure 3. Comprehensive protein-protein interaction (PPI) network and validation of top six DEGs. A. The PPI network of the top six differentially expressed genes (DEGs), highlighting their relational intricacies and potential functional clusters. B. The expression validation analysis of these top six DEGs using the GSE35959 dataset. A *P*-value less than 0.05 served as the selection criteria for statistical validity.

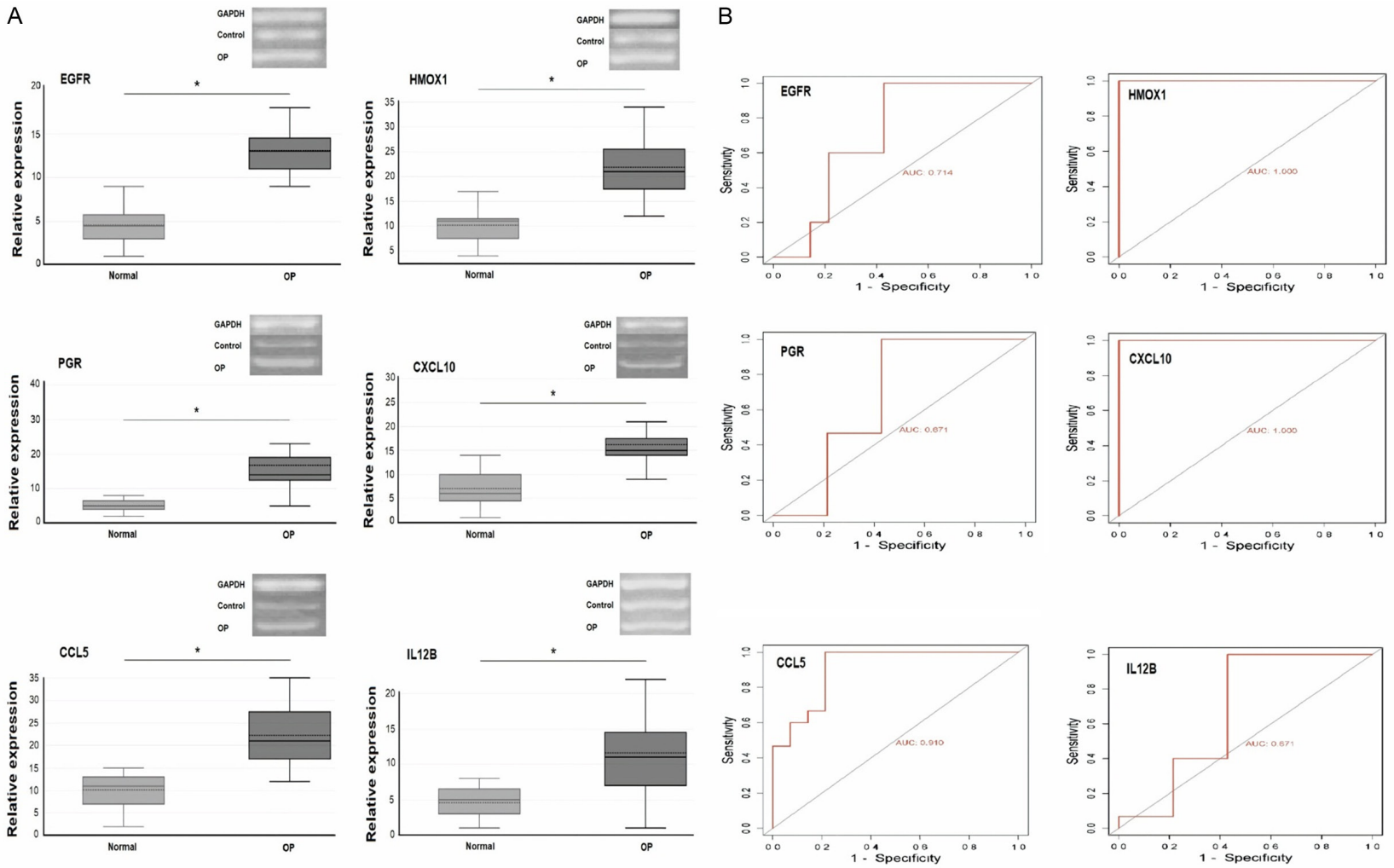

Figure 4. Detailed expression and diagnostic potential of top six DEGs in Pakistani osteoporosis (OP) patients. A. The relative expression levels of the top six differentially expressed genes (DEGs) in both Pakistani OP patients and normal control samples, quantified using real-time quantitative PCR (RT-qPCR). B. The receiver operating curves (ROC) generated based on RT-qPCR expression data of the top six DEGs, offering insights into their diagnostic accuracy and performance. A *P*-value of less than 0.05 serves as the selection criteria for confirming statistical significance.

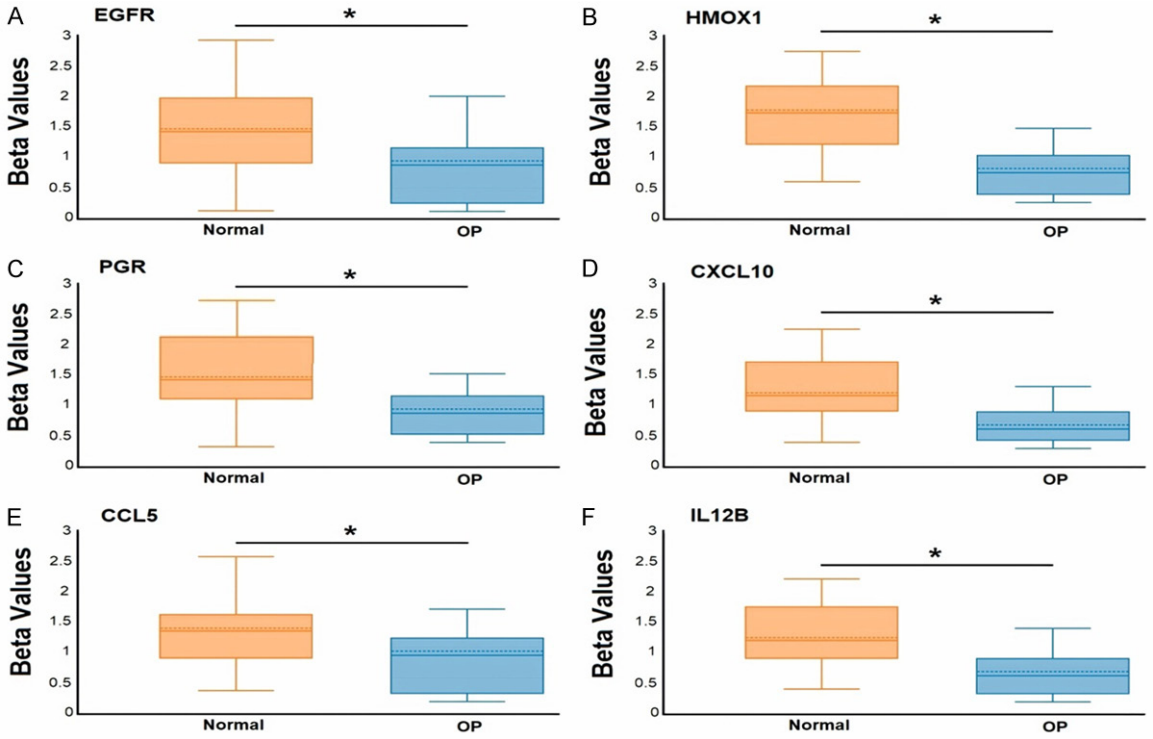

Figure 5. Comprehensive methylation profiling of top six DEGs in Pakistani osteoporosis (OP) patients versus controls. A. Methylation profiling of EGFR. B. Methylation profiling of HMOX1. C. Methylation profiling of PGR. D. Methylation profiling of CXCL10. E. Methylation profiling of CCL5. F. Methylation profiling of IL12B. A stringent statistical threshold of $P < 0.01$ was applied for selection criteria. EGFR = Epidermal growth factor receptor, HMOX1 = Heme Oxygenase-1, PGR = Progesterone receptor, CXCL10 = C-X-C Motif Chemokine Ligand 10, CCL5 = C-C Motif Chemokine Ligand 5, and IL12B = interleukin 12B.

identified DEGs, namely Acetaminophen, Cyclosporine, Deferoxamine, and Afimoxifene (**Table 3**).

Discussion

Every year, OP continues to impact millions of individuals worldwide [27]. This condition primarily manifests as compromised bone microarchitecture and the progressive loss of bone mass and strength, particularly among the elderly population [27]. Timely treatment of OP can help prevent osteoporotic fractures. However, the current approach to treating OP is associated with limited effectiveness due to its time-consuming nature, high cost, and potential for adverse side effects from medications [28]. Hence, further investigations delving into bone biology, underlying molecular pathways, and signaling networks associated with OP will contribute to a better understanding of this disease. Such studies hold the potential to uncover novel treatment approaches and methods. Furthermore, since OP often remains clinically silent until a fracture occurs, the timely diagnosis of this condition is of utmost importance. Early detection is crucial for initiating appropriate treatment and alleviating the patient's pain [29]. The present manuscript utilizes a diverse range of bioinformatics and molecular experiments with the aim of identifying novel biomarkers and potential treatment targets for OP.

The first step involved analyzing the expression profiles of OP patients and normal individuals in the GSE163849 dataset to identify DEGs. Through this analysis, a total of 269 DEGs were identified between the OP and normal groups. Among these, 97 genes were found to be up-regulated, while 172 genes were down-regulated ($P < 0.05$). Notably, the top six DEGs in this dataset, which exhibited the most significant differential expression between the OP and normal control groups, were EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B, all of which were up-regulated.

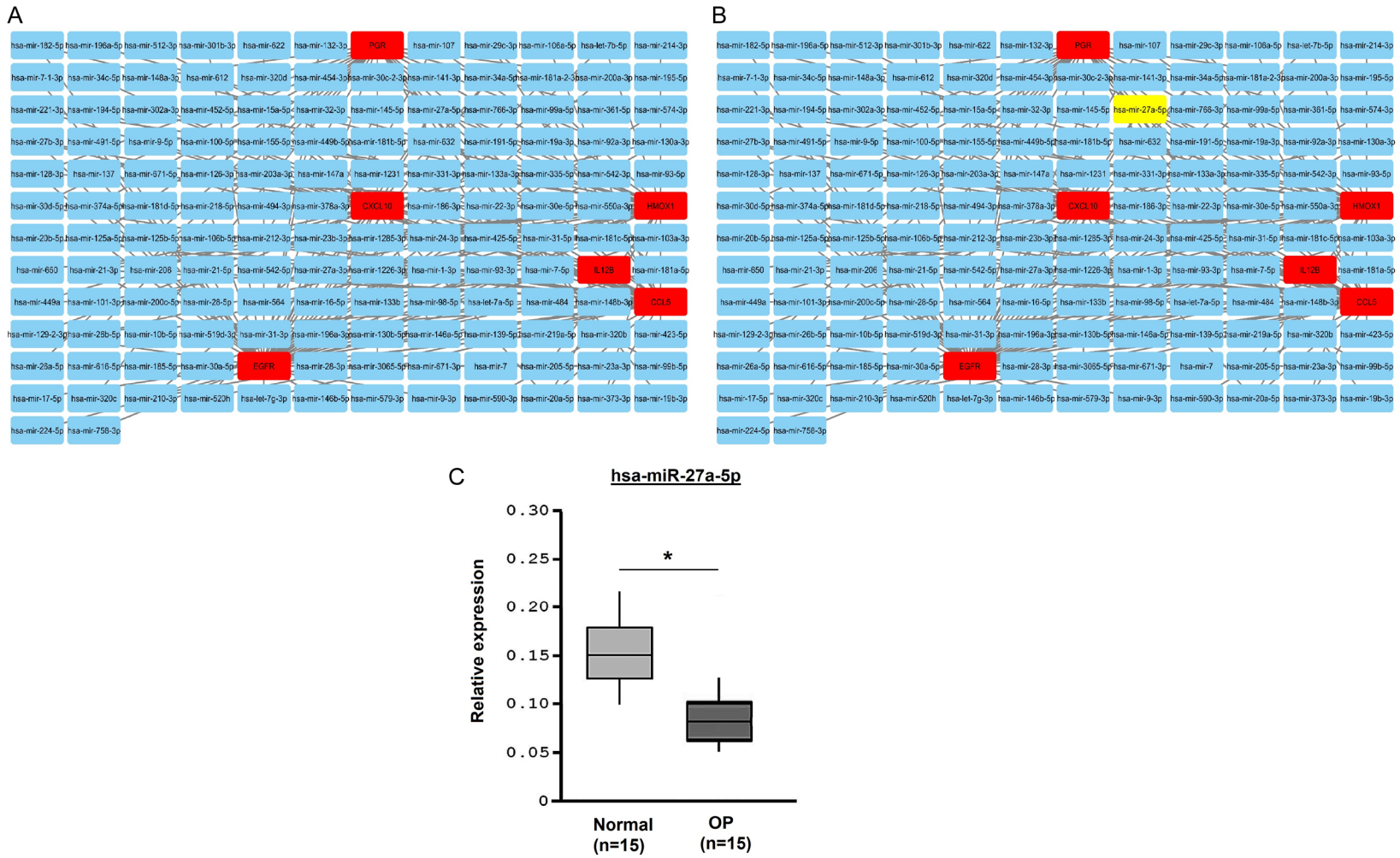

Figure 6. Elucidating miRNA-DEG associations with multi-tiered analysis. (A) Global network of computationally predicted miRNAs targeting the DEGs, offering an overarching view of potential regulatory relationships, (B) Specific interaction between the meaningful miRNA, has-miR-27a-5p, and the DEGs, aiming to detail the regulatory landscape. (C) RT-qPCR expression profiling of has-miR-27a-5p, providing experimental confirmation. Node colors are indicative: blue nodes represent miRNAs, red nodes are the DEGs, and the yellow node specifically designates has-miR-27a-5p. miRNA = microRNA, DEG = differentially expressed gene, and RT-qPCR = reverse transcription-quantitative polymerase chain reaction.

Table 3. DrugBank-based DEGs-associated drugs

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	EGFR	Acetaminophen	Decrease expression of EGFR mRNA	A20418	Approved
		Alitretinoin		A20508	
2	HMOX1	Cyclosporine	Decrease expression of HMOX1 mRNA	A21868	Approved
		Deferoxamine		A22066	
3	PGR	Afimoxifene	Decrease expression of PGR mRNA	A20491	Approved
4	CXCL10	Acetaminophen	Decrease expression of CXCL10 mRNA	A20420	Approved
		Cyclosporine		A21868	
5	CCL5	Cyclosporine	Decrease expression of CCL5 mRNA	A23761	Approved
6	IL12B	Cyclosporine	Decrease expression of IL12B mRNA	A23761	Approved

The subsequent step involved validating the expression of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B using an additional GEO (GSE35959) dataset and clinical OP samples obtained from the Pakistani population. By analyzing this additional GEO dataset and performing RT-qPCR analysis on OP patients from Pakistan, we consistently observed up-regulation of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B DEGs in comparison to control samples. Moreover, bisulfite-seq analysis indicated that these DEGs (EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B) exhibited hypomethylation patterns in Pakistani OP patients.

EGFR is a transmembrane receptor implicated in OP [30]. EGFR plays a role in bone remodeling by promoting osteoblast proliferation and differentiation, leading to increased bone formation [30]. It also influences osteoclast activity, affecting bone resorption [30]. Dysregulation of EGFR signaling is observed in osteoporotic bone tissues, contributing to disrupted bone homeostasis. A study by Zhang et al. conducted on postmenopausal women with OP showed up-regulated EGFR expression in blood samples compared to healthy controls [31]. Similarly, Wang et al. reported increased EGFR levels in osteoporotic bone samples from elderly women [32]. Furthermore, dysregulated EGFR signaling has been associated with impaired osteoblast differentiation and decreased bone formation [33].

HMOX1 plays a crucial role in the development and progression of OP, a condition characterized by reduced bone density and increased fracture risk [34]. HMOX1 is an enzyme responsible for the breakdown of heme into biliverdin, carbon monoxide (CO), and iron. It exerts protective effects by acting as an antioxidant and anti-inflammatory agent [35]. In OP, dysregulation of HMOX1 expression and activity has been observed, leading to increased oxidative stress, inflammation, and impaired bone remodeling [35]. A previous study showed decreased HMOX1 expression in OP patients compared to healthy controls, accompanied by elevated oxidative stress markers [36]. Another study demonstrated that HMOX1 deficiency led to accelerated bone loss and impaired bone formation in mice [37]. Additionally, it was also found that up-regulation of HMOX1 alleviated OP-related bone loss and inflammation [38].

The PGR, a nuclear hormone receptor, is involved in regulating the effects of progesterone, a hormone important in female reproductive processes [39]. Studies have shown that PGR expression in bone cells, such as osteoblasts and osteoclasts, influences bone remodeling and turnover. A previous study demonstrated reduced PGR expression in osteoblasts and osteocytes of postmenopausal women with OP compared to healthy controls [40]. Another study found that polymorphisms in the PGR gene were associated with decreased bone mineral density and increased fracture risk in postmenopausal women [41]. Additionally, it was also found that PGR deficiency in mice led to impaired bone formation and increased bone resorption [42].

CXCL10 and CCL5, two chemokines of the CXC and CC families respectively, have been implicated in the pathogenesis of OP [43]. Multiple studies have highlighted their role in bone metabolism and inflammation. CXCL10 and CCL5 promote the recruitment and activation

of immune cells, including osteoclasts, leading to increased bone resorption and decreased bone density [43]. Several studies have demonstrated the dysregulation of CXCL10 and CCL5, in OP, suggesting their potential involvement in the disease. For instance, an earlier study showed increased CXCL10 levels in the bone marrow of postmenopausal women with OP compared to healthy controls [44]. Moreover, another study found elevated CCL5 expression in osteoporotic patients, and its levels correlated with decreased bone mineral density [45]. Another study by Paula-Silva et al. demonstrated that CXCL10 and CCL5 enhanced osteoclast differentiation and bone resorption in vitro [46].

IL12B, a subunit of the interleukin-12 (IL-12) cytokine, is already known to play an important role in the pathogenesis of OP [47]. Studies have suggested a role for IL12B in regulating bone metabolism and inflammation [47]. Previously published literature has provided evidence of dysregulation of IL12B in OP, suggesting its potential involvement in the disease. For instance, a previous study found elevated IL12B expression in OP patients compared to healthy controls, correlating with disease severity [48]. Another study demonstrated that IL12B level was positively associated with markers of bone loss and negatively correlated with bone mineral density [49].

Recent literature has shed light on the involvement of epigenetic modifications, such as DNA methylation, in the development and progression of OP [50]. Therefore, we further analyzed the promoter methylation levels of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B through bisulfite-seq analysis in OP patients belonging to the Pakistani population. Results of the analysis indicated hypomethylation of these genes as a major reason behind their over-expression in OP patients. To date, there has been a lack of investigation into the methylation levels of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B in patients with OP within the medical literature. As a result, this particular study represents the initial exploration of the methylation aspect concerning EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B in the context of OP.

Moreover, in this study, we explored has-miR-27a-5p miRNA as the common expression regulator of the EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B genes. Several studies have suggested that has-miR-27a-5p may play a role in the development and progression of OP. Research has shown that has-miR-27a-5p is up-regulated in osteoporotic bone tissue and osteoporotic animal models [51]. High levels of has-miR-27a-5p have been associated with the inhibition of osteoblast differentiation, which is the process by which bone-forming cells mature and produce new bone tissue [52]. The inhibition of osteoblast differentiation can lead to reduced bone formation and contribute to the development of OP [52]. However, in this study, we observed significant down-regulation of has-miR-27a-5p in OP samples as compared to the control group. This scenario indicates that the down-regulation of has-miR-27a-5p is a key factor contributing to the dysregulation of EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B in patients with OP.

Conclusion

Our study has identified six crucial genes (EGFR, HMOX1, PGR, CXCL10, CCL5, and IL12B), which hold promise in establishing a diagnostic model for OP. The identification of these genes presents potential molecular targets that can greatly contribute to the clinical diagnosis and treatment of OP.

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Disclosure of conflict of interest

None.

Address correspondence to: Muhammad Jamil, PARC Arid Zone Research Center, Dera Ismail Khan 29050, Pakistan. E-mail: jamilmatrah@parc.gov.pk; Heng Zhang, Department of Orthopedic, Shanghai Songjiang District Central Hospital, Shanghai 201699, China. E-mail: erop227455@163.com

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