Analysis of Osteoporosis Gene Interactome to Identify Heterogenic Genes and Pathways.

A Major Project Dissertation

Submitted in Partial Fulfillment of the Requirement for the Degree of

M.Tech. in Biomedical Engineering

Submitted by

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Certificate

This is to certify that the M. Tech. dissertation entitled **"*Analysis of Osteoporosis gene interactome to identify heterogenic genes and pathways*"**, submitted by *Bharat Singh* (DTU/14/MTECH/103) in partial fulfillment of the requirement for the major project during M.Tech in Biomedical Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work which is to be carry out by him under my guidance.

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Declaration

I declare that my major project entitled "***Analysis of Osteoporosis gene interactome to identify heterogenic genes and pathways***" submitted to Department of Biotechnology, Delhi Technological University is a record of original research work carried out by me at "Genome Informatics Laboratory" under the supervision of **Dr. Yasha Hasija**, Department of Biotechnology.

The material personified in this project report is original and not been submitted for award of any diploma/degree.

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Bharat Singh
2K14/BME/05

Contents

List of figures and schemes

List of tables

Title – *Analysis of Osteoporosis Gene Interactome to Identify Heterogenic Genes and Pathways*

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Abstract

Latest advances in genetics have prompted swift progress towards the efficient identification of genes tangled in complex diseases. Still, the comprehensive understanding about the relation between physiological and molecular mechanism of genes and how they affect disease phenotypes remains a challenge for researchers and clinicians. Here, we wish to identify the osteoporosis disease module, i.e. the indigenous neighborhood of the interactome whose agitation is associated with osteoporosis, and endorse it for functional and pathophysiological application, using both computational and experimental methodologies. Recent studies in osteoporosis suggest that osteoporosis disease module supplemented with uncertain GWAS P-values against certain genetic variations in both diseased and normal conditions; the expression level of genes was different. The osteoporosis module may also contain mechanisms that are collective with other disease modules. We constructed the gene-gene and protein-protein interaction network for 104 genes for 173 reported SNPs accompanied by GO functional enrichment and KEGG pathway enrichment analysis; we recognized the substantial genes of osteoporosis along with their molecular functions. Our analyses exposed polymorphism in SOST and LRP5 as significantly conservative SNPs.

Keywords: GWAS, SNP, Interactome, GO etc.

Introduction

There is collective evidence that disease genes in both complex and monogenic diseases are not scattered arbitrarily on the molecular interface network (interactome), instead of that they tend to work together in analogous biological modules or pathways. Besides this, gene products i.e. proteins associated to the same phenotype have a robust propensity to interact with each other and to make cluster in the similar network vicinity. This recommends the presence of a disease module and an interlinked sub-network that can be systematically allied to a particular disease phenotype. The exact identification of such disease modules could help in revealing the molecular mechanisms causing diseases and detect new genes associated with disease along with the pathways, which ultimately could help in coherent drug target identification. Presently, due to the lack of cellular network maps and listed genes to diseases the exact analysis about disease modules is incomplete. However, that recent advances in interactome mapping and disease gene identification have begun to offer adequate network analysis and precision to enable identification of disease modules for some well-studied complex diseases.

The goal of our work is to clearly implement such approaches to identify drug modules by developing disease module for osteoporosis which counts for approximately 40 million patients in India. Despite the identification of several susceptibility alleles and genes by GWAS and other tools, our awareness about the fundamental etiologic mechanisms accountable for osteoporosis remains limited. Traditional single gene pathway based approaches have been proved to have limited utility due to environmental and genetic factors. In recent years, various approaches have been formulated to find the association between characteristic properties of proteins and protein network to different type of ‘omics’ data to determine novel genes and pathways. These approaches are based on the local impact hypothesis, assuming that if we can identify certain specific disease components, we could be able to identify other disease-related components supposed to exist in their network vicinity. Consequently, each disease can be associated to a definite indigenous neighborhood of the interactome, called the disease module. Our goal, here, is to conclude if a whole network based approach can boost our understanding of the indigenous network neighborhood of a disease using osteoporosis as an example.

Osteoporosis, described by the loss of bone mass and strength, and the advance of microarchitecture damage leading to fragility fractures, has become a substantial clinical problem in health care services associated with aging populations. The vulnerability to osteoporosis is controlled by a variety of factors, such as genetic variants, age, sex steroid

production, lifestyle and environment. A number of studies have explored the pathogenesis of osteoporosis at the molecular levels.

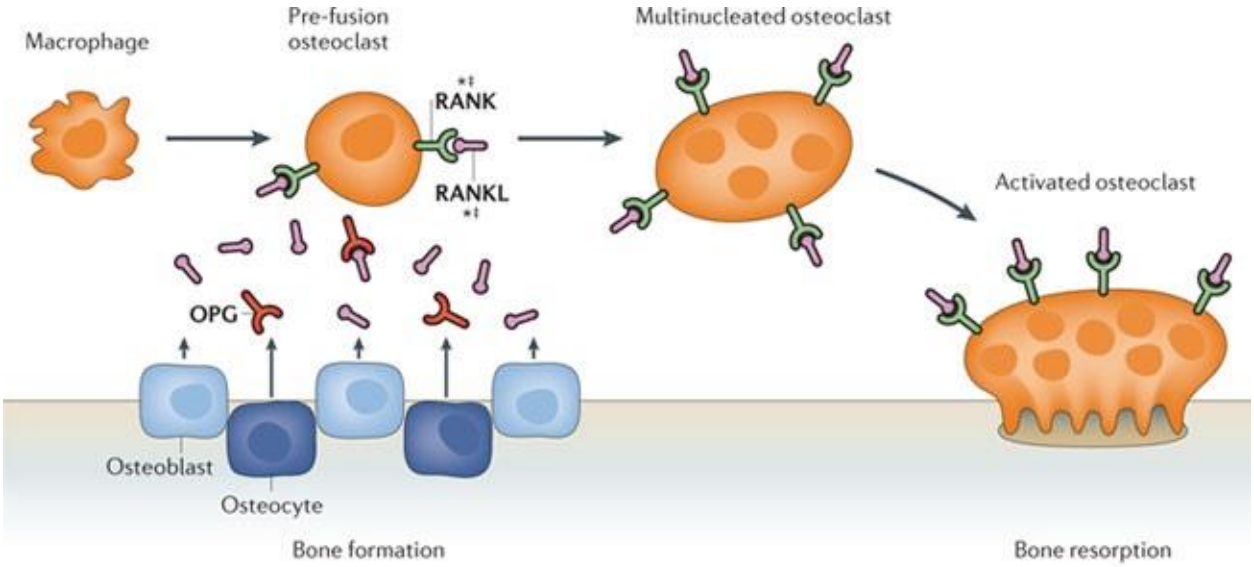

Fig 1: Basic mechanism of bone formation and resorption (Source: Google Image op)

A number of genome-wide association studies (GWASs) have been conducted to identify the genes that regulate Bone mineral density which includes core and accessory genes involved responsible for osteoporosis.

However, the fundamental etiology of osteoporosis is not yet broadly understood and the identification of new therapeutic targets for osteoporosis is required. In order to identify differentially expressed genes we further wish to analyze different osteoporosis module using bioinformatics methods to reveal osteoporosis-specific gene expression patterns and to determine its association indigenous neighborhood our aim is to provide novel targets for the diagnosis and treatment of osteoporosis.

Review of literature

Genetics of osteoporosis

Osteoporosis is somewhat genetically determined. Genetically osteoporosis is polygenic having multiple common polymorphic alleles relating with each other and to determine bone mass environmental factors play a crucial role. A number of studies have endeavored to dissect the genetic factors accountable for the pathogenesis of osteoporosis using candidate gene approach and the genome-wide scanning. However, the results of these studies among unlike populations have been typically inconsistent, signifying genetic heterogeneity of osteoporosis. It is expected that the cohort of genes demonstrating predisposition to the risk of osteoporosis might be different among populations through different ethnic backgrounds. The successful identification of susceptible genes for osteoporosis should demonstrate to be helpful in targeting protective and therapeutic processes to individuals at greater risk and to reduce the effort more cost-effective. Information with concern to genetic variations is also expected to be useful in targeting deterrent or therapeutic measures to individuals genetically determined to have healthier responsiveness. Intestinal calcium absorption is associated to vitamin D receptor gene polymorphisms. Predominantly at lower doses skeletal responsiveness to estrogen, is correlated to polymorphisms in the estrogen receptor-alpha gene. Recently, circulating homocysteine concentrations have been shown to be linked with fracture risk. In postmenopausal women, Folate and vitamin B treatment for reducing serum homocysteine and fracture risk have not been entirely investigated. Nevertheless interaction between methylenetetrahydrofolatereductase(MTHFR) gene polymorphism and folate status on bone phenotypes has been evidenced. Due to latest technological advances, whole-genome association study is becoming more practicable. Genomic information with respect to the predisposition to osteoporosis and the openness to precautionary or therapeutic modalities should increase rather than replace orthodox clinical information.

Prevalence of osteoporosis

Aged people are the firmest growing population in the world and, as people grow older, bone mass deteriorations and the risk of fractures surges. Osteoporosis, demarcated as a skeletal disorder described by compromised bone asset predisposing to an augmented risk of fracture, is a major public health problem thru the world. The social and economic cargo of osteoporosis is increasing gradually because of the aging of the world population. Currently affecting more than

10 million people over the age of 50 in the world, osteoporosis is projected to impact approximately 14 million adults over the age of 50 by the year 2020. Worldwide, approximately 200 million women have osteoporosis. Although the possibility of developing osteoporosis presently is greatest in America and Europe, it will increase in developing countries as population endurance in these countries continues to rise.

Pathogenesis of osteoporosis

Skeletal fragility can result from:

(a) Failure to produce a skeleton of optimal mass and strength during growth

(b) Excessive bone resorption resulting in decreased bone mass and microarchitectural deterioration of the skeleton

(c) An inadequate formation response to increased resorption during bone remodeling.

Role of estrogen in bone resorption

The conception that estrogen deficiency is precarious to the pathogenesis of osteoporosis was based primarily on the fact that postmenopausal women, whose estrogen levels unsurprisingly decline, are at the maximum risk for developing the disease. Morphologic studies and measurements of definite biochemical markers have shown that bone remodeling is enhanced at the menopause, as both markers of bone formation and resoption are increased (Ebeling, P.R., and Parfitt, A.M., et al.). Estrogen deficiency has been considered as the key player in bone loss in women in their 70s and 80s, as evidenced by the fact that estrogen treatment swiftly reduces bone breakdown in older women (Prestwood, K.M., et al.). Fracture risk is inversely associated to estrogen levels in postmenopausal women, and as little as 25% of the dose of estrogen that fuels the breast and uterus is sufficient to reduce bone resorption and increase bone mass in older women (Prestwood, K.M., et. al.). Estrogen is critical for epiphyseal cessation in puberty in both sexes and controls bone turnover in men as well as women. In fact, estrogen has a superior effect than androgen in obstructing bone resorption in men, though androgen may play a silent role (Falahati-Nini, A., et al.). Estrogen actions through 2 receptors: estrogen receptor α (ERα) and ERβ. ERα seems to be the prime mediator of estrogen's actions on the skeleton (Lee et.al). Single nucleotide polymorphisms (SNPs) of ERα may affect bone fragility. In the major study to date, 1 of the SNPs for ERα receptor was associated with a substantial drop in fracture risk, independent of bone mineral density (Ioannidis, J.P., et al. 2004).

The major binding protein Sex hormone–binding globulin (SHBG) for sex steroids in plasma amends the bioavailability of estrogen to hormone-responsive tissues as well as its entry into cells. Epidemiologic studies advocate the role of SHBG in bone loss and fracture risk independently of the effect that it is a binding protein (Goderie-Plomp, H.W., et al.). Whereas estrogen can act on cells of the osteoblastic lineage, its accessory effects on bone might be reliant to actions on cells of the hematopoietic lineage, Indigenous cytokines and growth factors can mediate these effects too. As stated in earlier researches the estrogen affected production of cytokines may be facilitated by T cells (Gao, Y., et al. 2004). An undeviating effect of estrogen in increased osteoclast apoptosis has been accredited to better TGF-β production.

Potential sites of action of estrogen contain effects on T cell cytokine production (i); effects on stromal or osteoblastic cells to amend their production of RANKL or OPG (ii); direct inhibition of differentiated osteoclasts (iii); and effects on bone formation facilitated by osteoblasts or osteocytes to improve the response to mechanical forces introduced by these cells (iv).

Role of calcium, vitamin D, and parathyroid hormone

The concept that osteoporosis is mainly caused due to calcium insufficiency, predominantly in the elderly, was originally counterproposed to Albright's estrogen deficiency theory. Compromised intestinal absorption of calcium due to disease or ageing, Declined calcium intake, as well as lack of vitamin D can result in secondary hyperparathyroidism (Lips, P. 2001). The dynamic hormonal form, 1, 25 dihydroxy vitamin D (calcitriol), is necessary for both optimal intestinal absorption of calcium and phosphorus, it also exerts a negative regulatory effect on parathyroid hormone (PTH) synthesis, so that there are dual pathways that can result in secondary hyperparathyroidism (Lips, P. 2001). Secondary hyperparathyroidism and Vitamin D deficiency can contribute to both accelerated bone loss and increasing fragility, as well as contribute to fall risks by adding to neuromuscular impairment. Clinical trials concerning older individuals at greater risk for calcium and vitamin D paucity indicate that supplementation of above can decrease fracture rates, decrease bone resorption, surge bone mass, and even can decrease the incidence of falling. VDR Polymorphisms have been studied comprehensively, but the results have been capricious, this may be partially due to the fact that the effect of a given polymorphism on this receptor is dependent on an interaction particularly with the calcium and environment (Ferrari, S.L., et al.).

Role of OPG and NF-ĸB

In osteoporosis pathogenicity TNF and TNF receptor family members are also considered as key regulators mainly three of these are involved, osteoblasts produce RANKL (ligand for the receptor activator NF-κB (RANK) on hematopoietic cells), which stimulates the differentiation of osteoclasts and sustains their function. Osteoblasts also conceal and produce a decoy receptor and secrete osteoprotegerin (OPG) that can block RANKL/RANK interactions. Earlier researches suggest that few bone resorption stimulators have been found to escalate RANKL expression in osteoblasts, and certain also decrease OPG expression (Suda, T., et al. 1999). In order to activate RANK, osteoblast cells must physically interact with osteoclasts precursors as these bone cells seem to express the RANKL in membrane bound form. Activated T lymphocytes also produce Soluble RANKL possessing equivalent activity to membrane-bound RANKL in binding to RANK (Kanamaru, F., et al. 2004). Polymorphisms in the OPG gene have been associated with osteoporotic fractures and differences in BMD (Langdahl, B.L., et. al).

Osteoblast differentiation genes

Current discoveries about signal transduction pathways associated to osteoporosis and transcription factors perilous for osteoblast differentiation and function have unlocked new approaches to understand the pathogenesis of osteoporosis. Gene deletion related studies have shown that nonexistence of runt-related transcription factor 2 (Runx2) or osterix (downstream factor), are critical for osteoblast differentiation (Ducy, P et al.). Remarkably, overexpression of Runx2 results in decreased bone mass (Nakashima, K., et al. 2002). Former studies have confirmed the role of these transcription factors in osteoporosis. The recent studies envisage the important role of Wnt signaling pathway in regulation of osteoblast function in determining bone mass and strength (Little, R.D., et al. 2002, Boyden, L.M., et al. 2002). LDL (low density lipoprotein) receptor–related protein 5 (LRP5) interacts with the frizzled receptor to initiate signaling by Wnt ligands. A mutation of LRP5 that leads to constitutive activation can result in an increase in bone density. Deletion of LRP5 results in a severe osteoporotic syndrome associated with atypical eye development (Little, R.D., et al. 2002). Various studies also have suggested that polymorphism of LRP5 is associated with metamorphoses in bone mass and fracture (Bollerslev, J., et al. 2005, Koay, M.A., et al. 2004).

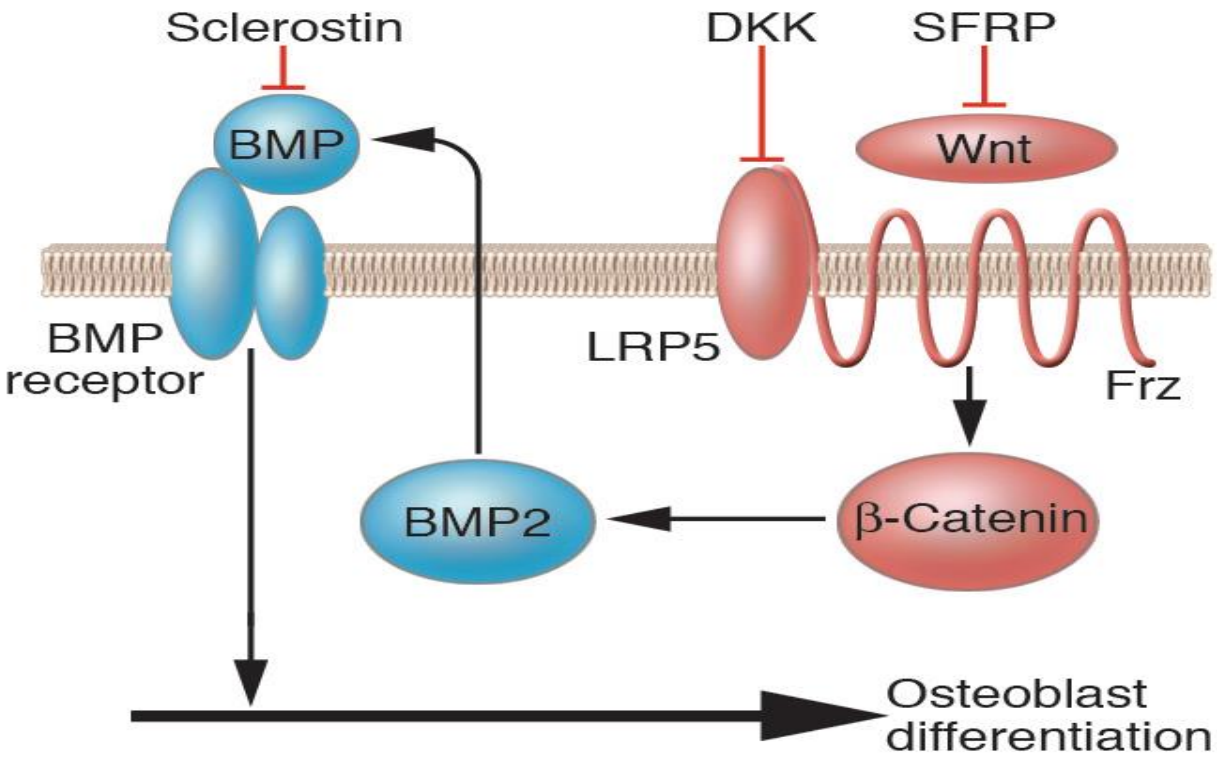

Fig 2: Interaction of the Wnt, BMP, and sclerostin pathways in osteoporosis (Source: Raisz, Lawrence G. "Pathogenesis of osteoporosis: concepts, conflicts, and prospects." *The Journal of clinical investigation* 115.12 (2005): 3318-3325.)

Mutations in LRP5 gene also have been acknowledged in a few patients showing idiopathic juvenile osteoporosis (Hartikka, H., et al. 2005). The detailed mechanisms of osteoblast function alteration by Wnt signaling are not fully understood, but certain clinical evidence suggest that the canonical β-catenin pathway is intricate in the process and that there is communication with bone morphogenetic protein 2 (BMP2) (Mbalaviele, G., et al. 2005). There are various inhibitors reported till date that have been shown to interact with both BMP2 and Wnt signaling pathway. One of these, which inhibits both Wnt signaling and BMP2 is sclerostin, a product of the *SOST* gene (Li, X., et al. 2005, Winkler, D.G., et al. 2004). Van Buchem disease or sclerosteosis (high-bone-mass disorder) is also caused due to the inactivating mutation of this gene (Loots, G.G., et al. 2005, Balemans, W., et al. 2001). Additional potential inhibitory factor to BMP2 and Wnt pathway is secreted frizzled-related protein (SFRP) produced by osteoblasts (Bodine, P.V., et al. 2005).

Disease heterogeneity and need to develop interactome

The Agilent literature search provides a big data to look for the possible explanation of any disease etiology. Due to various recent advances in the field of experimental biological techniques such as tandem affinity purification and other high-throughput methods, there is huge amount of protein-protein interaction (PPI); gene-gene interaction data is publicly available. This amount of data requires new mathematical and computational approaches to be developed to model and evaluate the complex networks that they form.

A first step in the molecular dissection of genetic factors in osteoporosis comprises determining the chromosomal location and the identification and characterization of the set of genes, variants of which are responsible for backing to the genetic susceptibility for the different sub phenotypes of osteoporosis. However the complex character of osteoporosis has makes it quite resistant to the methods of analysis that in the past few decades worked well for the monogenic diseases. Therefore different and often more cumbersome approaches have to be applied.

An accurate model of gene-gene interaction and PPI networks will allow better estimating all types of network statistics along with generating synthetic networks of species for which protein-protein interactions have not been experimentally dogged. This might help understand cellular processes and lead future biological experiments. Therefore, analyzing and modeling these interactome has become a vibrant research area.

Work flow for osteoporosis associated gene polymorphism study

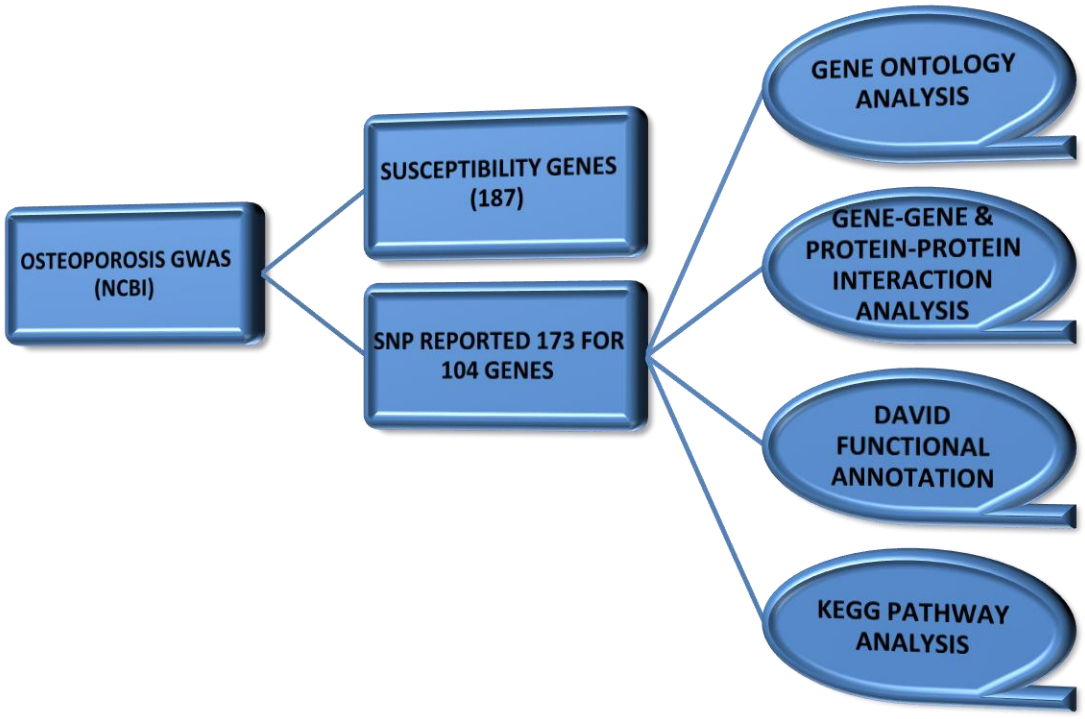

Fig 3: work flow (osteoporosis association with gene polymorphism)

Materials and methods

Data collection

To analyze the association of gene polymorphism and osteoporosis SNP information was obtained from the central PUBMED server. We searched the GWAS article relating to osteoporosis using search term as "OSTEOPOROSIS GENES", "OSTEOPOROTIC GENE POLYMORPHISM", "BONE FRACTURE AND GENE POLYMORPHISM", "BMD AND GENE POLYMORPHISM" and extracted osteoporosis associated SNPs with significant P-value and genes (updated to 1 March 2016).

From the central GENE database provided by NCBI total 187 genes were reported to be associated to osteoporosis, among these 187 genes 173 polymorhisms for 104 genes were found to be positively reported and accessible. Information regarding these polymorphisms were collected and used to develop a data sheet consisting information about following attributes.

1. PMID
2. Gene name
3. RS-ID
4. Type of polymorphism
5. P-value
6. Odds ratio
7. Ethnicity of population
8. Chromosome number
9. Location
10. Variant DNA
11. Variant RNA
12. Variant protein
13. Type of mutation

Gene ontology

The GO delineates concepts/classes used to define gene function, and relationships between these concepts. It categorizes functions along three aspects: **molecular function** (molecular activities of gene associated products) **cellular component** (functional site of the gene products) **biological process** (pathways and greater routes made up of the activities of numerous gene products).

Central gene ontology consortium (http://geneontology.org/) server was used; a widely embraced source of gene functional annotation describing cellular component, molecular function and biological process for the selected gene set.

Gene ontology consortium central server was used for GO analysis with default settings with selecting *Homo sapiens* as the species background. In addition PANTHER (Protein Analysis Through Evolutionary Relationship) classification system which can offer spontaneous visualization of images of GO analysis was used to categorize proteins and their genes in order to simplify high through output analysis.

Developing interactome (Protein-protein and Gene-gene interaction)

A structured network layout explaining network integrity is the core requirement to justify the interaction between genes and proteins. Cytoscape a free software package was used for modeling, visualizing, and analyzing genetic and molecular interaction networks. In order to obtain the gene-gene interaction network a simple excel file containing attribute gene as source and target node along with the p score for each SNP was submitted as input, p-value works as edge attribute and helps in determining the path length between interacting nodes.

Cytoscape's software Core offers basic functionality to layout and probes the network; to visually assimilate the network with phenotypes, expression profiles, and additional molecular states; and to relate the network to databases of functional annotations. Cytoscape Core provides an extensible straightforward plug-in architecture, which allows swift development of supplementary computational analyses and topographies.

After obtaining a gene-gene interaction network the hub gene screening and analysis was performed followed by identification of putative complexes and functional modules.

Maximum of known biological networks contains few hub genes/proteins connected to the maximum nodes expect few hubs having least connections (Lamb et al., 2006). To identify key hub we used the scale free property of network and evaluated the hubs within the interacting network. Further to perform clustering plugin module MCODE was used followed by ontology analysis of hub gene. These investigative results can further confirm the molecular mechanism of osteoporosis and help in finding potentially essential genes.

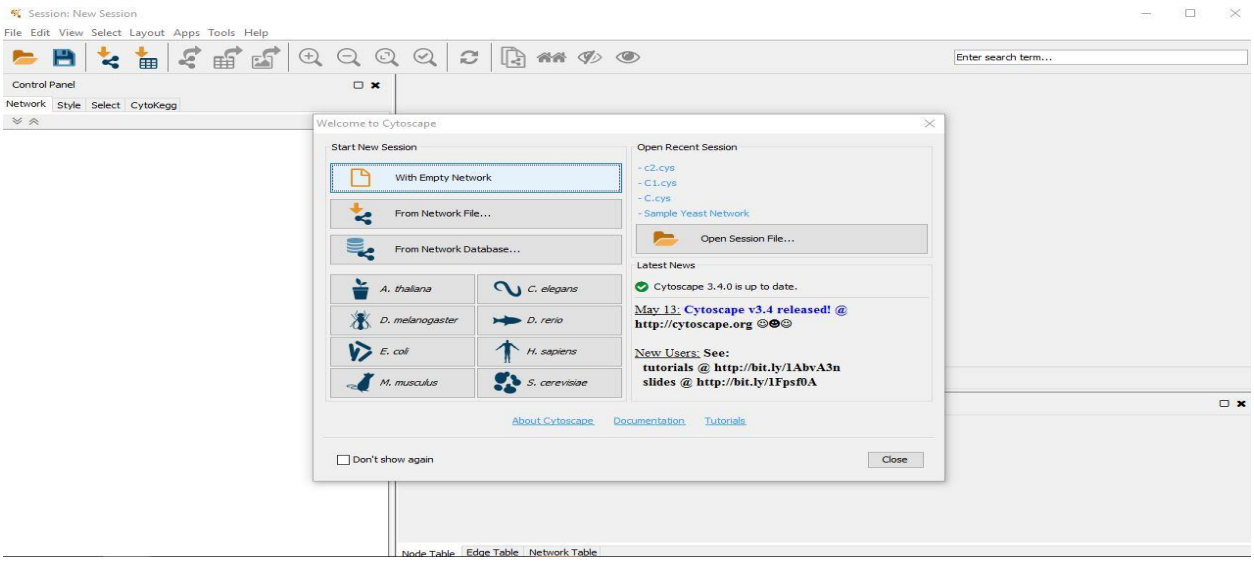

Fig 4: Cytoscape 3.4.0

Respective proteins interaction network was developed using STRING version 10.0 web interface. STRING is a database of well-known and anticipated protein interactions, comprising direct (physical) and indirect (functional) connotations. STRING quantitatively assimilates interaction data produced from four sources: High-Throughput Experiments, Genomic Context, Co-expression profiles (Conserved) and Previous Knowledge. The database currently covers 9.6 million proteins from 2031 organisms. STRING was used to produce osteoporosis gene (protein) network.

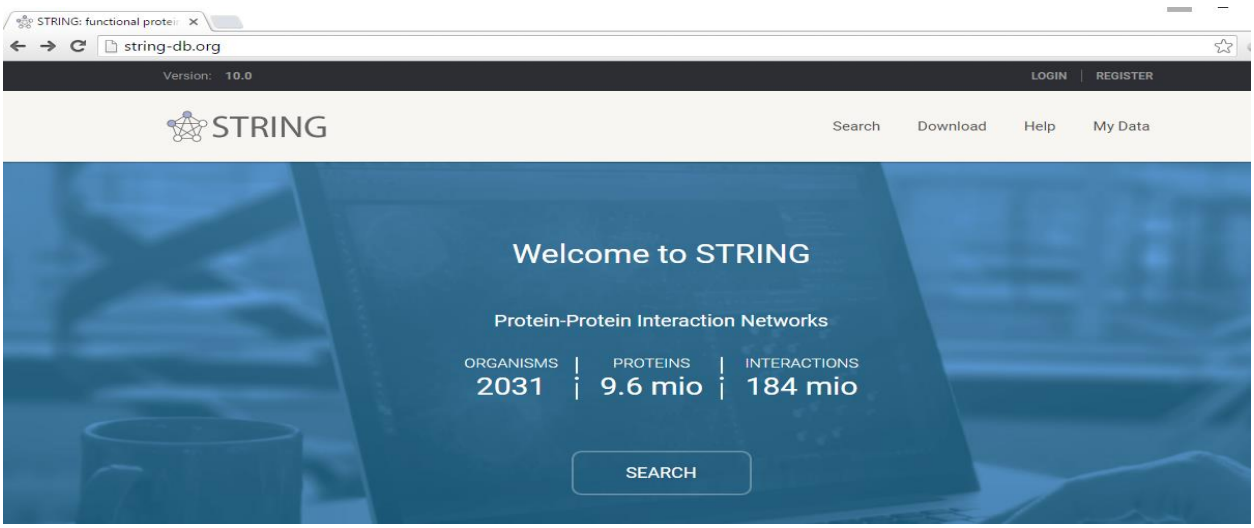

Fig 5: String 10.0

Functional annotation by DAVID (https://david.ncifcrf.gov/home.jsp) and KEGG pathway enrichment analysis

Understanding disease associated non-coding SNPs is a requisite step towards understanding molecular mechanism of multifaceted diseases. DAVID (The Database for Annotation, Visualization and Integrated Discovery) comprises an integrated biological knowledgebase and analytic tools intended at systematically mining biological meaning from hefty gene/protein lists. We used the DAVID to categorize overrepresented KEGG categories in pathways. We engrossed on biological pathway mode to explore KEGG (Kyoto Encyclopedia of Gene and Genome) pathways and performed functional annotation for the complete gene set. We obtained all of the metabolic and non-metabolic pathways and used the DAVID website to perform KEGG pathway cluster analysis for the complete gene set.

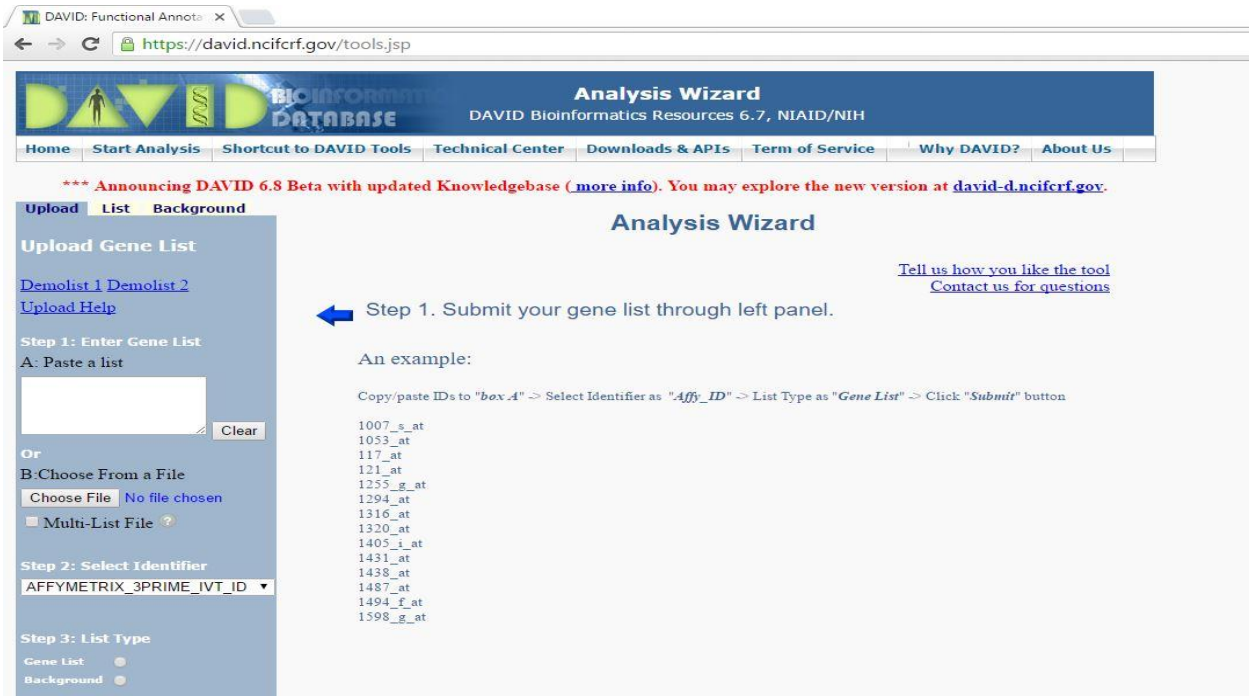

Fig 6: DAVID 6.7

Results

Osteoporosis associated Genes/SNPs

PMID	GENE	RS-ID	POLYMOR	P-VALUE	ODDS RAT	POPULATI	CHROMOS	LOCATION	VARIANT
26078251	VDR	rs2228570	A/C	0.005		Japanese			
26554238	CLDN14	rs219780	A/G			Caucasian	21	36461009	NC_00002
26281333	COL1A1	rs1800012	G/T	0.001		Iranian	17	50200388	NC_00001
25980946	ITLN-1	rs2274907	A/T	<0.001		Caucasian	1	1.61E+08	NC_00000
25931355	TLR4	rs4986791	C/T	>0.05		Polish	9	1.18E+08	NC_00000
25784778	VDR	rs1454385	C/G	<0.05		Chinese	12	47879073	NC_00001
25764158	VDR	rs1544410	A/G	<0.05		Italian	12	47846052	NC_00001
25764158	VDR	rs2228570	C/T	0.047		Italian	12	47879112	NC_00001
25658585	DDR2	rs7521233	C/T	0.000106		Chinese	1	1.63E+08	NC_00000
25658585	DDR2	rs7553831	G/T	0.00013		Chinese	1	1.63E+08	NC_00000
25658585	DDR2	rs6697469	C/G	0.00159	1.42	Chinese	1	1.63E+08	NC_00000
26740865	GSTP1	rs1695	A/G	<0.05		Egyptian	11	67585218	NC_00001
26657339	HSBG	rs1107870	C/T	0.005		Chinese	17	7620390	NC_00001
26657339	CD68	rs9901675	A/G	0.021		Chinese	17	7581494	NC_00001
26657339	HSBG	rs9898876	G/T	0.001	2.16	Chinese	17	7623644	NC_00001
26657339	HSBG	rs2541012	A/G	0.047	1.94	Chinese	17	7625306	NC_00001
26657339	HSBG	rs6259	A/G	0.001	2.15	Chinese	17	7633209	NC_00001
26657339	GBTB4	rs3853894	A/G	0.001	0.42	Chinese	17	7467286	NC_00001
26648999	SAA1	rs12218	A/G	0.001	1.814	Arabian	11	18269774	NC_00001
26628959	COL1A1	rs1107946	A/C	0.74		Caucasian	17	50203629	NC_00001
26628959	COL1A1	rs2412298	C/T	0.55		Caucasian	17	50203294	NC_00001
26600491	IGF1	rs35767	C/T	<0.05		Chinese	12	1.02E+08	NC_00001
26600491	IGF1	rs972936	A/G	<0.05		Chinese	12	1.02E+08	NC_00001
26393357	VDR	rs1544410	A/G	0.429		Spanish	12	47846052	NC_00001
26202809	OPG	rs3102735	C/T	<0.05		Polish	8	1.19E+08	NC_00000
26202809	OPG	rs2073617	C/T	<0.05		Polish	8	1.19E+08	NC_00000
26202809	OPG	rs2073618	C/G	0.42		Polish	8	1.19E+08	NC_00000
26194493	BGLAP	rs1800247	C/T	0.04	0.6	Shanghai	1	1.56E+08	NC_00000
25580429	LRP5	rs3736228	C/T	0.103	1.19	Chinese	11	68433827	NC_00001
25323794	TNFRSF11	rs3134069	A/C	0.032		Slovakian	8	1.19E+08	NC_00000
25138264	TNFSF11	rs2277439	A/G	0.014		Chinese	13	42581307	NC_00001
25138264	TNFSF11	rs2324851	C/T	0.013		Chinese	13	42586794	NC_00001
25138264	TNFRSF11	rs7239261	A/C	0.047		Chinese	18	62337813	NC_00001
25132170	TNFSF11	rs2277438	A/C/G	0.025		Koreans	13	42581032	NC_00001
25132170	FOXL1	rs1004814	A/G	0.012		Koreans	16	86677054	NC_00001
25132170	SOX6	rs7117858	A/G	0.086		Koreans	11	15672916	NC_00001
25132170	ZBTB40	rs6426749	C/G	0.014		Koreans	1	22384980	NC_00000

Table 1: Data sheet

After running a complete search to the NCBI (PUBMED) and dbSNP, a total of 173 SNPs of 104 genes were found to be associated with osteoporosis, BMD or fractures with a significance threshold. Among these SNPs, 104 were mapped to introns, and 9 in 3' and 5' UTR, rest of them were either missense or upstream variant.

Ontology analysis

The results of osteoporosis GWAS-associated genes enrichment by gene ontology consortium and PANTHER is shown in (Fig) Distribution of genes along with all three aspects of ontology is as follows

Genes for **Molecular functions** (Fig) includes, binding (38 genes, 36.50%), receptor activity (25 genes, 24%), catalytic activity (19 genes, 18.30%), structural molecule activity (7 genes, 6.70%), nucleic acid binding transcription factor activity (6 genes, 5.80%), transporter activity (5 genes, 4.80%), enzyme regulator activity (3 genes, 2.90%), translation regulatory activity (1 gene, 1.00%).

Genes for **biological processes** (Fig) includes, cellular process (54 genes, 21.50%), metabolic process (31 genes, 12.40%), developmental process (30 genes, 12.00%), biological regulation (29 genes, 11.60%), response to stimulus (29 genes, 11.60%), immune system process (20 genes, 8%), multicellular organismal process (16 genes, 6.40%), localization (15 genes, 6%), apoptotic process (12 genes, 4.80%), cellular component organization or biogenesis (6 genes, 2.40%), biological adhesion (5 genes, 2.0%) , reproduction (3 genes, 1.20%), and locomotion (1 genes, 0.40%).

Genes for **cellular components** (Fig) were found to be involved in cell part (16 genes, 31.40%), extracellular region (11 genes, 21.60%), membrane (9 genes, 17.60%), organelle (9 genes, 17.60%), extracellular matrix (4 genes, 7.80%), macromolecular complex (2 genes, 3.90%).

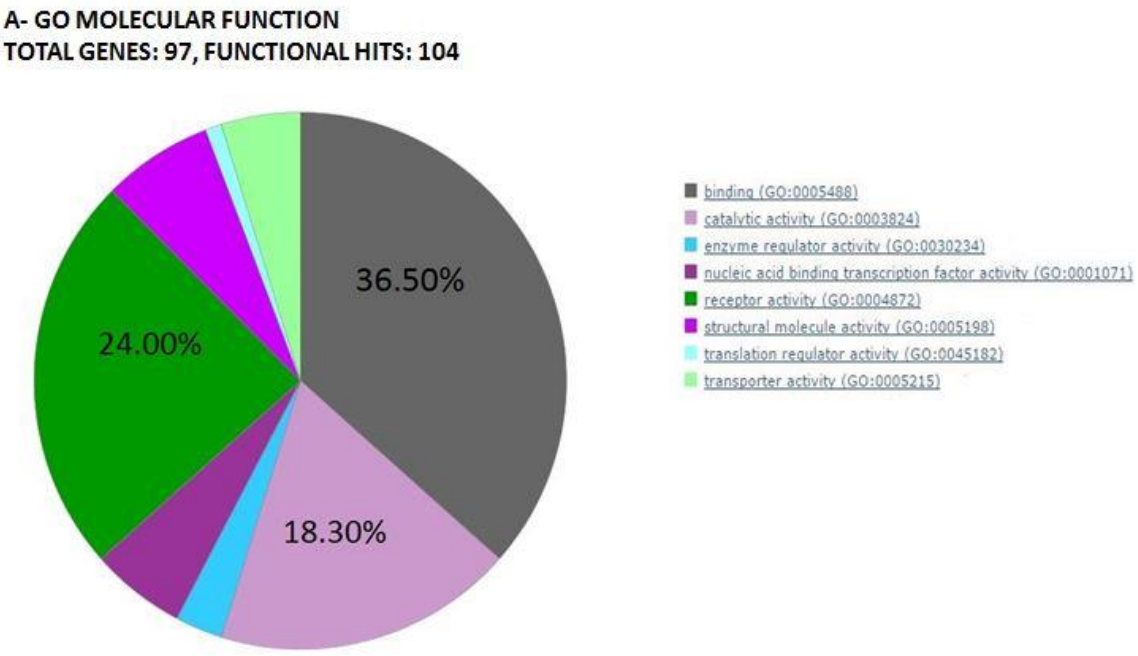

Fig 7: GO molecular function

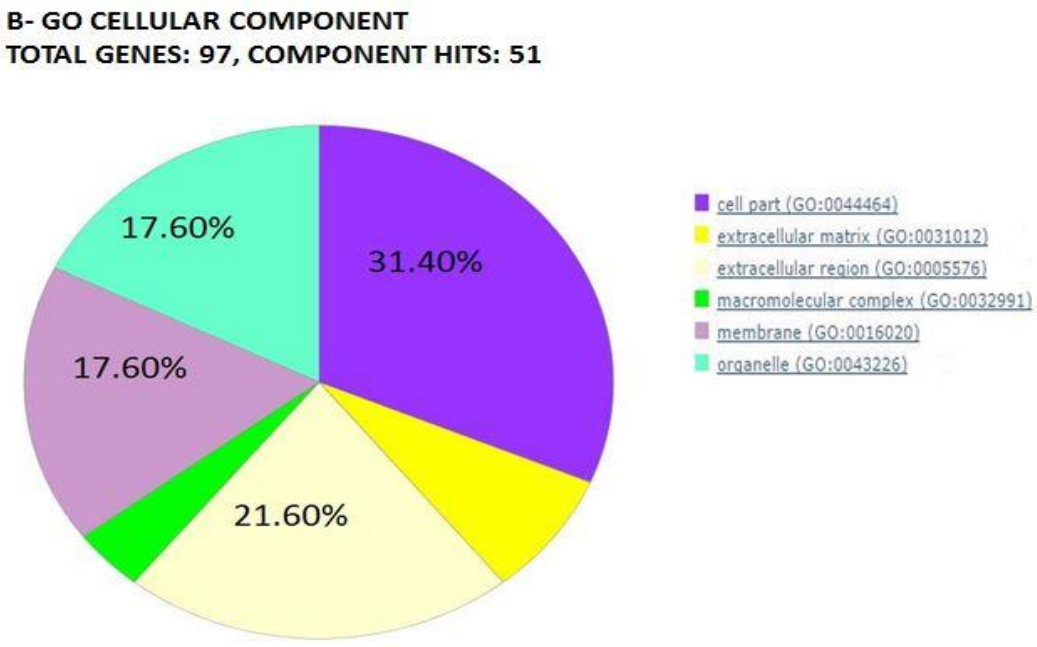

Fig 8: GO cellular component

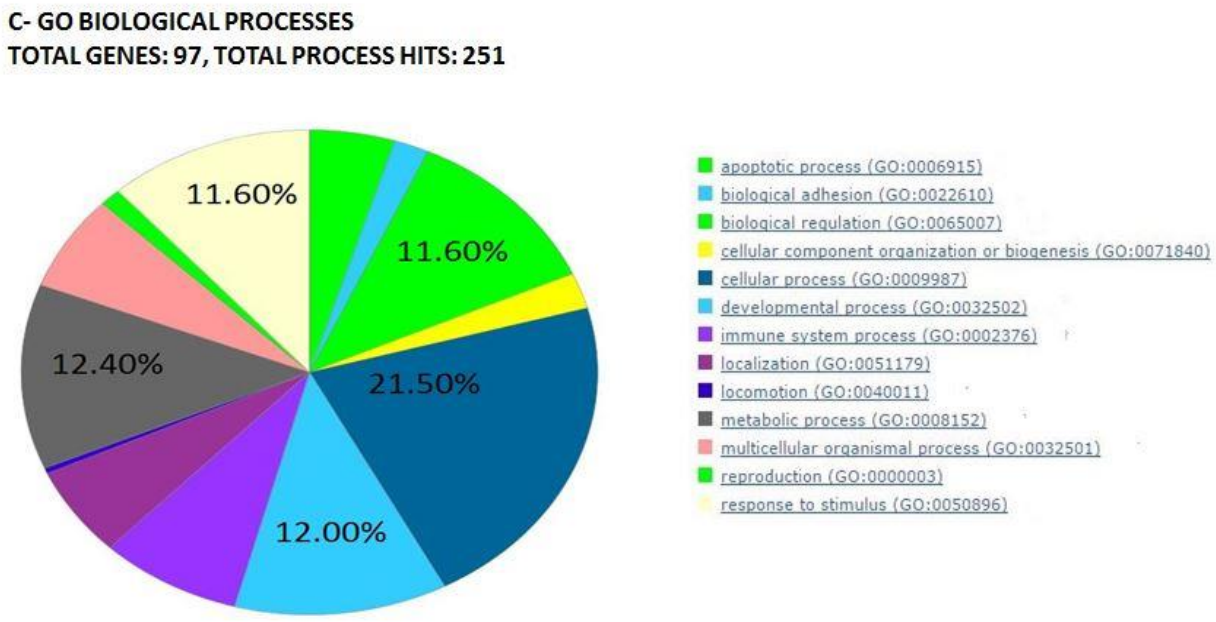

Fig 9: GO biological processes

Gene-gene and Protein-protein interaction analysis

Gene-gene interaction analysis for osteoporosis associated GWAS produced 104 genes displayed a great interaction pattern. The gene-gene interaction was produced using Cytoscape 3.4.0

SOST with highest degree 5 was found to be the hub gene with closeness centrality value of 0.33441558. LRP5 a direct network associate to the SOST gene also showed the second highest value for degree i.e. 10.

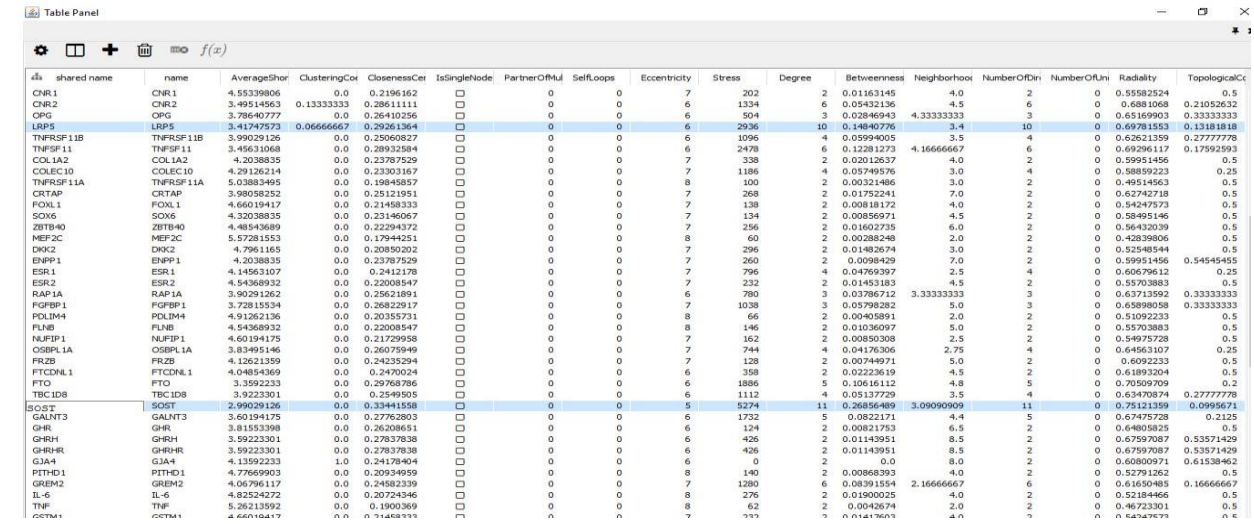
Table Panel

shared name	name	AverageShor	ClusteringCoe	ClosenessCer	IsSingleNode	PartnerOfMul	SelfLoops	Eccentricity	Stress	Degree	Betweenness	Neighborhooc	NumberOfDir	NumberOfUni	Radiality	TopologicalCc
CNR1	CNR1	4.55339806	0.0	0.2196162		0	0	7	202	2	0.01163145	4.0	2	0	0.55582524	0.5
CNR2	CNR2	3.49514563	0.13333333	0.28611111		0	0	6	1334	6	0.05432136	4.5	6	0	0.6881068	0.21052632
OPG	OPG	3.78640777	0.0	0.26410256		0	0	6	504	3	0.02846943	4.33333333	3	0	0.65169903	0.33333333
LRP5	LRP5	3.41747573	0.06666667	0.29261364		0	0	6	2936	10	0.14840776	3.4	10	0	0.69781553	0.13181818
TNFRSF11B	TNFRSF11B	3.99029126	0.0	0.25060827		0	0	6	1096	4	0.05994005	3.5	4	0	0.62621359	0.27777778
TNFSF11	TNFSF11	3.45631068	0.0	0.28932584		0	0	6	2478	6	0.12281273	4.16666667	6	0	0.69296117	0.17592593
COL1A2	COL1A2	4.2038835	0.0	0.23787529		0	0	7	338	2	0.02012637	4.0	2	0	0.59951456	0.5
COLEC10	COLEC10	4.29126214	0.0	0.23303167		0	0	7	1186	4	0.05749576	3.0	4	0	0.58859223	0.25
TNFRSF11A	TNFRSF11A	5.03883495	0.0	0.19845857		0	0	8	100	2	0.00321486	3.0	2	0	0.49514563	0.5
CRTAP	CRTAP	3.98058252	0.0	0.25121951		0	0	7	268	2	0.01752241	7.0	2	0	0.62742718	0.5
FOXL1	FOXL1	4.66019417	0.0	0.21458333		0	0	7	138	2	0.00818172	4.0	2	0	0.54247573	0.5
SOX6	SOX6	4.32038835	0.0	0.23146067		0	0	7	134	2	0.00856971	4.5	2	0	0.58495146	0.5
ZBTB40	ZBTB40	4.48543689	0.0	0.22294372		0	0	7	256	2	0.01602735	6.0	2	0	0.56432039	0.5
MEF2C	MEF2C	5.57281553	0.0	0.17944251		0	0	8	60	2	0.00288248	2.0	2	0	0.42839806	0.5
DKK2	DKK2	4.7961165	0.0	0.20850202		0	0	7	296	2	0.01482674	3.0	2	0	0.52548544	0.5
ENPP1	ENPP1	4.2038835	0.0	0.23787529		0	0	7	260	2	0.0098429	7.0	2	0	0.59951456	0.54545455
ESR1	ESR1	4.14563107	0.0	0.2412178		0	0	7	796	4	0.04769397	2.5	4	0	0.60679612	0.25
ESR2	ESR2	4.54368932	0.0	0.22008547		0	0	7	232	2	0.01453183	4.5	2	0	0.55703883	0.5
RAP1A	RAP1A	3.90291262	0.0	0.25621891		0	0	6	780	3	0.03786712	3.33333333	3	0	0.63713592	0.33333333
FGFBP1	FGFBP1	3.72815534	0.0	0.26822917		0	0	7	1038	3	0.05798282	5.0	3	0	0.65898058	0.33333333
PDLIM4	PDLIM4	4.91262136	0.0	0.20355731		0	0	8	66	2	0.00405891	2.0	2	0	0.51092233	0.5
FLNB	FLNB	4.54368932	0.0	0.22008547		0	0	8	146	2	0.01036097	5.0	2	0	0.55703883	0.5
NUFIP1	NUFIP1	4.60194175	0.0	0.21729958		0	0	7	162	2	0.00850308	2.5	2	0	0.54975728	0.5
OSBPL1A	OSBPL1A	3.83495146	0.0	0.26075949		0	0	7	744	4	0.04176306	2.75	4	0	0.64563107	0.25
FRZB	FRZB	4.12621359	0.0	0.24235294		0	0	7	128	2	0.00744971	5.0	2	0	0.6092233	0.5
FTCDNL1	FTCDNL1	4.04854369	0.0	0.2470024		0	0	6	358	2	0.02223619	4.5	2	0	0.61893204	0.5
FTO	FTO	3.3592233	0.0	0.29768786		0	0	6	1886	5	0.10616112	4.8	5	0	0.70509709	0.2
TBC1D8	TBC1D8	3.9223301	0.0	0.2549505		0	0	6	1112	4	0.05137729	3.5	4	0	0.63470874	0.27777778
SOST	SOST	2.99029126	0.0	0.33441558		0	0	5	5274	11	0.26856489	3.09090909	11	0	0.75121359	0.0995671
GALNT3	GALNT3	3.60194175	0.0	0.27762803		0	0	6	1732	5	0.0822171	4.4	5	0	0.67475728	0.2125
GHR	GHR	3.81553398	0.0	0.26208651		0	0	6	124	2	0.00821753	6.5	2	0	0.64805825	0.5
GHRH	GHRH	3.59223301	0.0	0.27837838		0	0	6	426	2	0.01143951	8.5	2	0	0.67597087	0.53571429
GHRHR	GHRHR	3.59223301	0.0	0.27837838		0	0	6	426	2	0.01143951	8.5	2	0	0.67597087	0.53571429
GJA4	GJA4	4.13592233	1.0	0.24178404		0	0	6	0	2	0.0	8.0	2	0	0.60800971	0.61538462
PITHD1	PITHD1	4.77669903	0.0	0.20934959		0	0	8	140	2	0.00868393	4.0	2	0	0.52791262	0.5
GREM2	GREM2	4.06796117	0.0	0.24582339		0	0	7	1280	6	0.08391554	2.16666667	6	0	0.61650485	0.16666667
IL-6	IL-6	4.82524272	0.0	0.20724346		0	0	8	276	2	0.01900025	4.0	2	0	0.52184466	0.5
TNF	TNF	5.26213592	0.0	0.1900369		0	0	8	62	2	0.0042674	2.0	2	0	0.46723301	0.5
GSTM1	GSTM1	4.66019417	0.0	0.21458333		0	0	7	232	2	0.01417603	4.0	2	0	0.54247573	0.5

Fig 10: Hub protein (Network analyzer view)

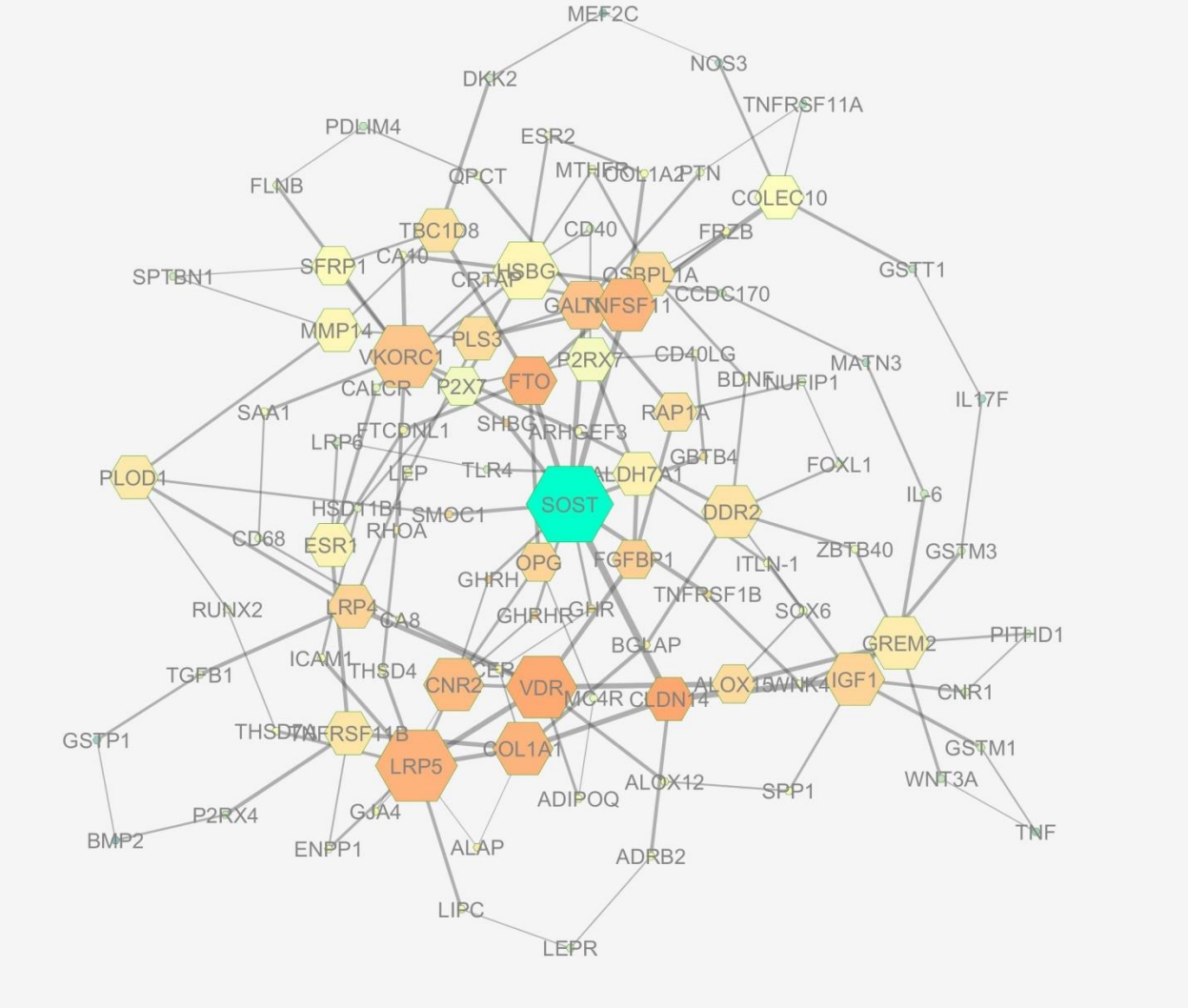

Fig 11: Gene-gene interaction network (produced by Cytoscape 3.4.0)

Network characteristics are as follows

Nodes	**104**
Edges	151
Clustering coefficient	0.22
Network diameter	8
Network density	0.028
Network heterogeneity	0.609
Average no. of neighbors	2.90
Highest degree	11 (SOST)

Table 2: Gene-gene interaction network characteristics

Further to validate the role of SOST respective protein interaction network was developed using STRING Version 10.0 with default settings. The protein-protein interaction network for 104 osteoporosis genes showed substantially reliable interaction.

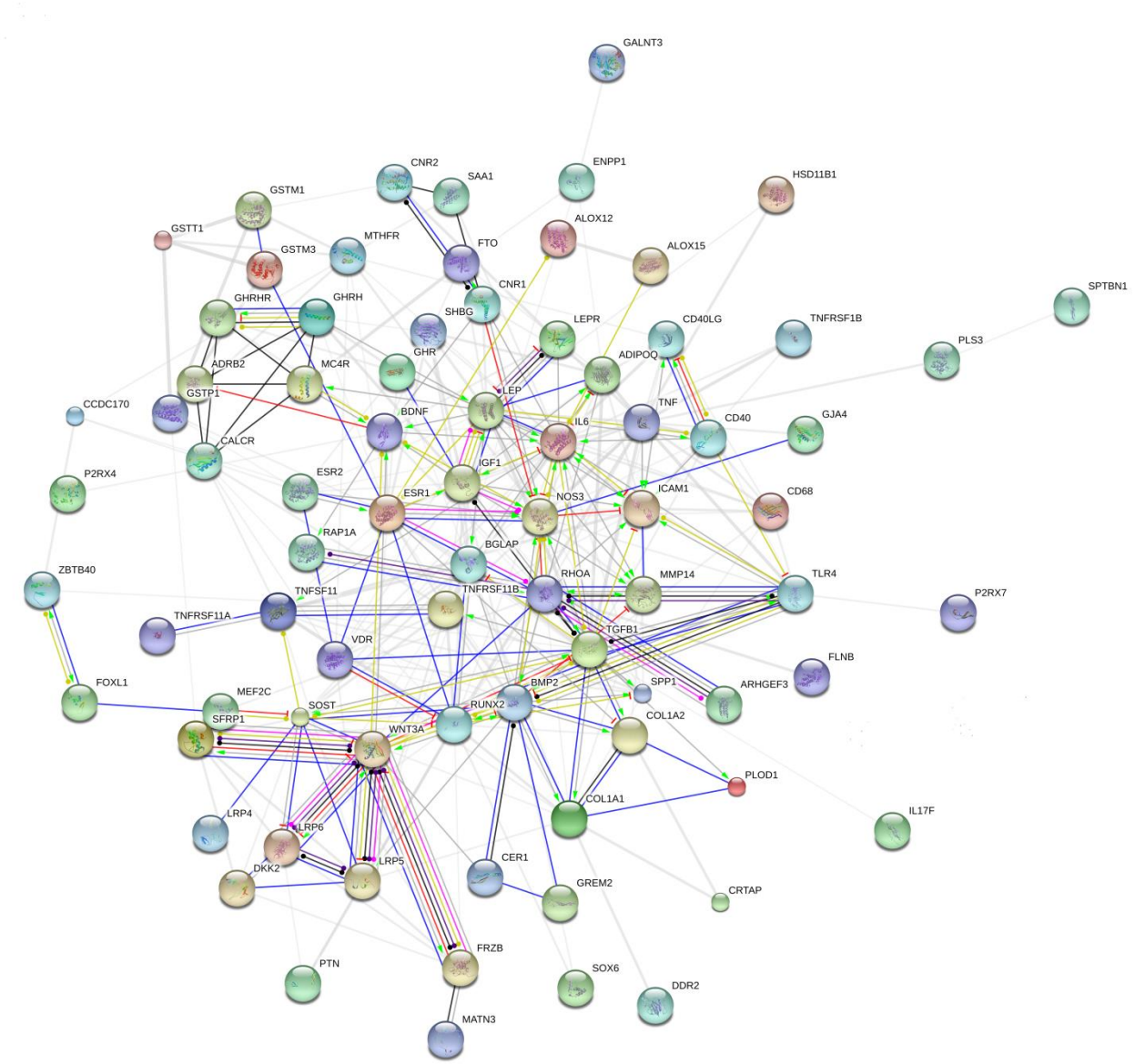

Fig 12: Protein-protein interaction network (evidence view) of osteoporosis associated genes. The nodes and edges represent the proteins (genes) and interactions, respectively. Black means **coexpression**, Red means **gene fusion**, Blue means **cooccurence**, Mustard means **textmining**, Green means **neighborhood**, and Purple means **experiments**.

The network shows hub proteins with resilient connection SOST, WNT3A, FRZB, LRP5, DKK2 and RUNX2. These proteins are involved in Wnt signaling pathway.

Functional annotation

Total 104 unique genes were submitted as gene list among them DAVID database identified a total 95 genes as matching to their database. These 95 DAVID selected genes were then converted to DAVID gene IDs using Gene Accession Conversion Tool.

Based on the enrichment score the submitted gene list was classified into three main groups with enrichment score values of 7.88, 4.26 and 2.38.

Gene group 1 (Enrichment score=7.88)

1	☐	783053	frizzled-related protein
2	☐	803546	gremlin 2, cysteine knot superfamily, homolog (Xenopus laevis)
3	☐	805261	cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis)
4	☐	784005	sclerosteosis
5	☐	822680	dickkopf homolog 2 (Xenopus laevis)
6	☐	786401	secreted frizzled-related protein 1

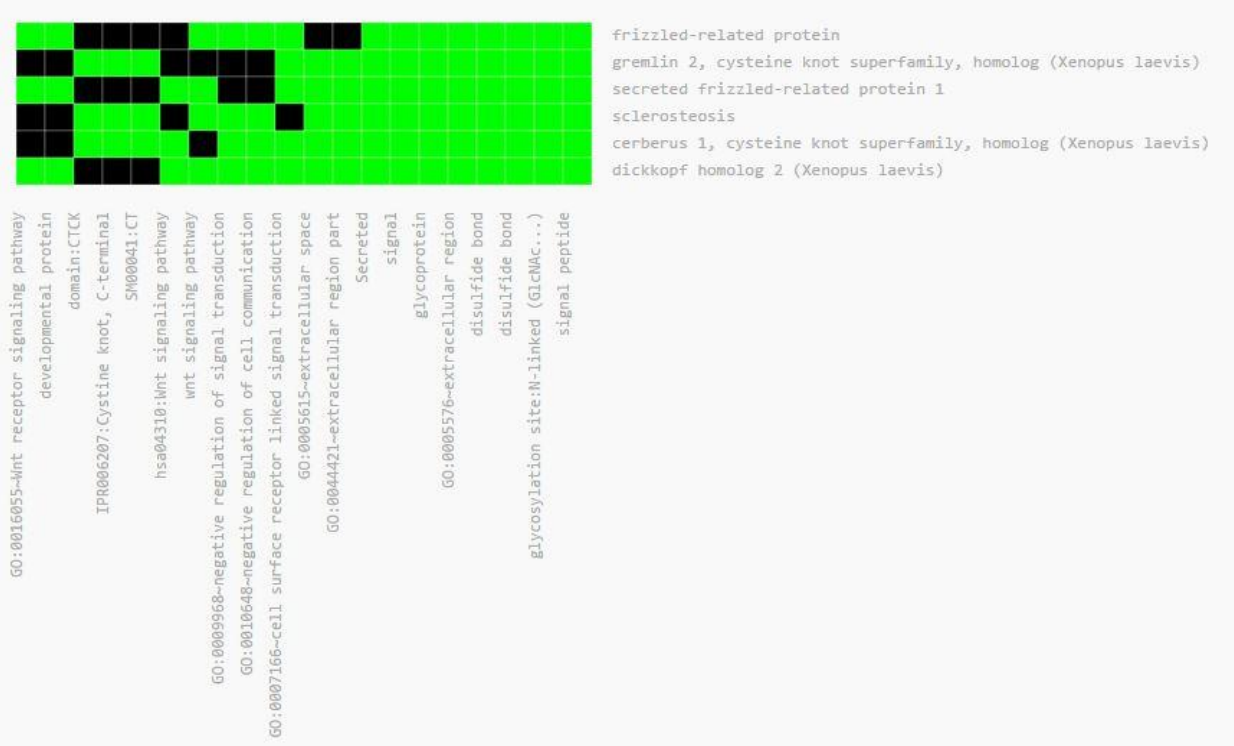

Fig 13: 2D heat map for group 1 genes. Green- corresponding gene term positively associated, Black-corresponding gene term associated not reported yet

To the group 1 functionally related genes showed similarity score as high as 0.77 and as low as 0.36. Sclerosteosis shows high kappa score and also directly associated to the group 1 genes with highest enrichment score of 7.88 so it can be deduced that SOST could play a crucial role in osteoporosis.

Functionally related gene	Kappa score (similarity score)
Dickkopf homolog 2 (xenopus laevis)	0.77
Sclerosteosis	0.68
Germline2, cysteine knot superfamily, homolog (xenopus laevis)	0.64
Frizzled related protein	0.57

Table 3: Kappa score for functionally related genes of group 1

Gene group 2 (Enrichment score=4.26)

1	☐	825920	tumor necrosis factor receptor superfamily, member 1B
2	☐	802013	thrombospondin, type I, domain containing 7A
3	☐	776184	tumor necrosis factor receptor superfamily, member 11a, NFKB activator
4	☐	809821	CD68 molecule

Fig 14: 2D heat map for group 2 genes. Green- corresponding gene term positively associated, Black-corresponding gene term associated not reported yet

In group 2 genes tumor necrosis factor family showed a strong association to osteoporosis with enrichment score of 4.26, further analysis for functionally related genes shows a Kappa value of 0.67 for NFKB activator.

Gene group 3 (Enrichment score=2.38)

1	☐	809178	glutathione S-transferase mu 3 (brain)
2	☐	825037	glutathione S-transferase theta 1
3	☐	780298	glutathione S-transferase mu 1
4	☐	817433	glutathione S-transferase pi 1

Fig 15: 2D heat map for group 3 genes. Green- corresponding gene term positively associated, Black-corresponding gene term associated not reported yet

With enrichment score of 2.38 and Kappa score of 0.97 Glutathione tansferase family (GST) showed a significant association to osteoporosis.

KEGG pathway analysis

We conducted pathway enrichment analysis for the selected gene set using DAVID Version 6.7. KEGG pathways with P<0.01 were considered as significant. A Total of 4 pathways with p value less than 0.01 were found to be significant. The most significant pathway was Cytokine-cytokine receptor interaction pathway with a p value of 8.69E-5 which includes 12 genes from the given gene set, this pathway also showed the minimal FDR (0.0958977) value. The additional

significant pathways included Neuroactive ligand-receptor interaction (P-value=3.44E-04, FDR=0.37874578), Wnt signaling pathway (P-value=0.005418103, FDR=5.81929673), Adipocytokine signaling pathway (P-value=0.005670277, FDR=6.08248497). A total of 35 genes were involved in these pathways.

Category	Term	RT	Genes	Count	%	P-Value	Benjamini
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	RT		12	12.9	8.7E-5	8.4E-3
KEGG_PATHWAY	Neuroactive ligand-receptor interaction	RT		11	11.8	3.4E-4	1.7E-2
KEGG_PATHWAY	Wnt signaling pathway	RT		7	7.5	5.4E-3	1.6E-1
KEGG_PATHWAY	Adipocytokine signaling pathway	RT		5	5.4	5.7E-3	1.3E-1
KEGG_PATHWAY	Glutathione metabolism	RT		4	4.3	1.6E-2	2.7E-1
KEGG_PATHWAY	Focal adhesion	RT		7	7.5	2.0E-2	2.8E-1
KEGG_PATHWAY	Metabolism of xenobiotics by cytochrome P450	RT		4	4.3	2.7E-2	3.1E-1
KEGG_PATHWAY	Drug metabolism	RT		4	4.3	2.9E-2	3.0E-1
KEGG_PATHWAY	Asthma	RT		3	3.2	3.9E-2	3.5E-1
KEGG_PATHWAY	Allograft rejection	RT		3	3.2	5.7E-2	4.4E-1
KEGG_PATHWAY	TGF-beta signaling pathway	RT		4	4.3	6.7E-2	4.6E-1
KEGG_PATHWAY	Toll-like receptor signaling pathway	RT		4	4.3	9.5E-2	5.5E-1
KEGG_PATHWAY	Intestinal immune network for IgA production	RT		3	3.2	9.8E-2	5.4E-1

Fig 16: KEGG pathway enrichment analysis (total hits 71)

Further in four significant pathways related term analysis was conducted which show a similarity score (Kappa score) of 1 to Wnt signaling pathway.

Similarity Score: Very High (0.75-1) High (0.5-0.75) Moderate (0.25-0.5) Low (<0.25)

#	Category	Term	Kappa
1	KEGG_PATHWAY	Wnt signaling pathway	1.00
2	GOTERM_BP_FAT	regionalization	0.82
3	GOTERM_BP_FAT	anterior/posterior pattern formation	0.82
4	GOTERM_BP_FAT	Wnt receptor signaling pathway	0.75
5	GOTERM_BP_FAT	pattern specification process	0.75
6	GOTERM_BP_FAT	gastrulation	0.71
7	SP_PIR_KEYWORDS	wnt signaling pathway	0.69

Fig 16: Kappa score analysis for most significant ($p<0.01$) KEGG pathways

Screening analysis of hub Gene/Protein

After calculating the hub degree of the interacting network we found that SOST has highest hub degree. First neighbor to the hub gene includes following genes GHRH, GHR, SMOC1, HSBG, GALNT3, FTO, TNFSF11, GBT4, CLDN14, and TNFRSF1B.

STRING was then used to find hub protein cluster in which hub protein module existed.

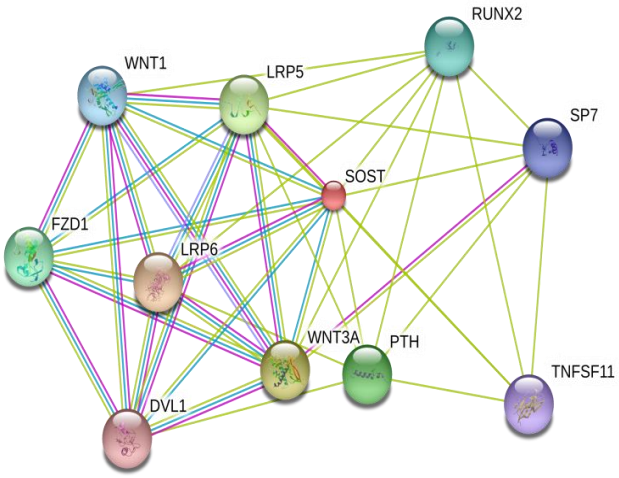

Fig 17: SOST (hub protein) cluster produced by STRING

Successively, GO functional analysis for the cluster gene set was done (GO ID with significant FDR were chosen) which revealed that Wnt signaling pathway was most significant. Wnt signaling pathway has always been reported as a crucial pathway in skeletal homeostasis (Ming-Kang Chang et. al 2013).

GO TERM	GO ID	DESCRIPTION	GENE COUNT	FDR
BP	GO:0045893	Positive regulation of transcription, DNA-templated	11	3.46e-10
BP	GO:0060070	Canonical Wnt signaling pathway	6	2.45e-09
BP	GO:0016055	Wnt signaling pathway	7	6.9e-09
MF	GO:0005109	Frizzled binding	5	1.27e-08
MF	GO:0001664	G-protein coupled receptor binding	6	4.04e-07
MF	GO:0042813	Wnt activated receptor activity	3	4.52e-05
KEGG	04310	Wnt signaling pathway	6	9.53e-09
KEGG	05217	Basal cell carcinoma	4	2.18e-06
KEGG	04916	Melanogenesis	4	1.4e-05
KEGG	04390	Hippo signaling pathway	4	5.67e-05

Table 4: GO functional analysis of hub protein

Discussion and Conclusion

To discover disease heterogeneity, function and mechanisms of osteoporosis GWAS-associated SNPs and genes, were characterized. We conducted GO and pathway analyses, gene-gene interaction analysis, protein-protein interaction analysis and KEGG pathway analysis to look into the insights of osteoporosis GWAS-associated genes. Our analyses exposed polymorphism in SOST and LRP5 as significantly conservative SNPs. The identification and characterization of genes related with osteoporosis has central and pragmatic relevance, as it is presently a common disease.

Our present study acknowledged 187 genes (identified upon manual curation through NCBI-PUBMED) in osteoporosis. We constructed the gene-gene and protein-protein interaction network for 104 genes for 173 reported SNPs accompanied by GO functional enrichment and KEGG pathway enrichment analysis; we recognized the substantial genes of osteoporosis along with their molecular functions. GO enrichment function analysis was expressively characterized by molecular functions which involves binding and receptor activity which supports results of related studies (Filardo, 2002; Bonewald, 2004). The results of KEGG pathway enrichment divulge that cytokine-cytokine receptor interaction pathway (cell signaling), neuroactive ligand-receptor interaction pathway (growth) and Wnt signaling pathway (cancer) pathways were significant metabolic pathways for osteoporosis-related genes. Our interactome module (gene-gene and protein-protein) analysis unveils SOST as hub gene/protein accompanied by LRP5. Direct neighbor to SOST (GHRH, GHR, SMOC1, HSBG, GALNT3, FTO, TNFSF11, GBT4, CLDN14, and TNFRSF1B) can also provide a potential insight about osteoporosis heterogeneity and could prove to be the key regulatory genes of the disease.

SOST expressed in osteocytes codes for a protein sclerostin is a key regulator to Wnt/b-catenin signaling and Wnt/b-catenin signaling/canonical Wnt signaling plays a key role in skeletal homeostasis. In bone, Wnt/b-catenin signaling is required for osteoblastic growth and differentiation (Glass DA, Bialek P, Ahn JD, et al, Kramer I, Halleux C, Keller H, et al.) Polymorphisms of SOST have also been associated with BMD in some population-based studies (Uitterlinden et al. 2004). A sequence of studies investigates the rapport between the *SOST* genotypes and BMD, and the results are erratic. We find the *SOST* polymorphism is significantly associated with BMD (Zhang H, He JW, Wang C et al, He J, Zhang H, Wang C et al.). Second associated hub protein LRP5 have also been proved to be involved in both low and high bone mass in different mutated form (Boyden LM, Mao J, Belsky J, et al and Little RD, Carulli JP,

Del Mastro RG, et al). Sclerostin is an inhibitor of the Wnt/bcatenin signaling pathway as it is a competitive inhibitor to LRP5 (Li X, Zhang Y, Kang H, et al), thus over study identifying SOST and LRP5 as hub gene/protein supports other studies stating the role of Wnt associated biomolecules and verifies the accuracy of the present study. Nevertheless, our pathway enrichment investigations of osteoporosis GWAS-associated SNPs/genes only confirmed well-known cytokine regulated pathways and Wnt signaling pathways, and futile to provide backing for various other mechanisms reported earlier on the basis of physiological confirmation, apparently due to deprived depiction of the processes acute to osteoporosis in existing databases. Nevertheless, this has spawned considerable argument over the potential to target these pathways for the blooming of new agents to tackle diseases of low bone mass, such as osteoporosis.

In conclusion, computational portrayal of osteoporosis GWAS-associated SNPs, emphasized genes (proteins) and pathways those are vital for osteoporosis pathophysiology, thus improving our understanding of the situation and contingent on potential treatment targets. Our results also warrant further experimental test in the future to explore osteoporosis disease heterogeneity.

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