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GENE POLYMORPHISM;

ASSOCIATION OF INTERLEUKIN 6 RECEPTOR GENE POLYMORPHISM WITH REDUCED BONE DENSITY IN POSTMENOPAUSAL WOMEN.

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ABSTRACT… Introduction: Reduced bone density in postmenopausal women is a complex disease with a strong genetic association. Causative factors are both acquired and genetic. Single nucleotide polymorphisms may be associated with genetic predisposition of this condition. **Objectives:** The purpose of this study is to find out an association between single nucleotide polymorphism (rs4845617) of IL-6R (Interleukin 6 receptor) gene and reduced bone density in cases of postmenopausal women and controls. **Study Design:** It is a retrospective case-control study. **Duration:** From March 2017 to Nov 2017. **Setting:** Different hospitals of Sargodha. **Material and Method:** About 30 blood samples were collected from postmenopausal women affected with reduced bone density and 30 healthy age-and gender-matched controls. All the blood samples underwent DNA extraction, Polymerase chain reaction and DNA sequencing techniques. **Result:** In this study, post menopausal women were screened for the rs4845617 in IL-6R gene for their association with reduced bone density. The chi-square test of independence showed that G allele of rs4845617 was significantly associated with reduced bone density in postmenopausal women (OR=2.28, χ^2=8.98, p=0.002). **Conclusion:** Our results signify that this polymorphism may play a role in reduced bone density susceptibility in postmenopausal women.

Key words: Deoxyribonucleic Acid, Genetic Predisposition, Gene, Interleukin 6 Receptor, Reduced Bone Density, Single Nucleotide Polymorphism.

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INTRODUCTION

Reduced bone density explains more than 100 diseases which cover the conditions that affect the joints of body and their surrounding tissues. According to California Arthritis Partnership Program[1], it may lead to Osteoarthritis, Fibromyalgia and Rheumatoid arthritis. Reduced bone density in postmenopausal women is responsible for causing destruction of bone and cartilage. It is one of the leading causes of multiple fractures and may involve dysfunctioning of single or multiple joints.[2]

The peak incidence of the cases is in 4th decade of life. In Pakistan prevalence of reduced bone density in postmenopausal age is 49.3%.[3]

The decreased bone mineral density has an association which systemic complication, and progressive disability. There is a definitive role of inflammatory mediators that include auto antibodies, growth factors, cytokines and chemokines in the progression of the condition.[4]

Cytokines have major role in bone destruction.[5] IL-6 is major cytokine.[6] It is produced by variety of cells and has a key role in inflammation and production of immunological response.[7] IL-6 acts via its receptor IL-6R. It occurs in two forms, one is membrane bound (mIL-6R) and the other is soluble form (sIL-6R). The key actions of IL-6 are mediated by sIL-6R.[8] Although soluble form of IL6R is required for signal transduction and is present in plasma of the healthy persons but its level is found to be increased in certain immunological diseases like rheumatoid arthritis, multiple myeloma and T cell abnormalities such as Aids and adult T cell leukemia.[9] The serum level

of IL6R is also found to be increased in certain acute conditions like myocardial infarction[10] and asthma.[11]

BIOLOGY OF INTERLEUKIN 6 AND ITS RECEPTOR

There are many cells which are involved in the production of IL-6, most important are T cells, B cells, fibroblasts, endothelial cells, monocytes and some tumor cells. The molecular weight of human IL-6 is 21 to 28 kDa.[12] The biochemical composition shows that it contains 212 amino acids. There are two N-glycosylation sites and four cysteine residues. The humans, IL-6 gene contain four exons and four introns. The gene mapping shows that it maps to chromosome 7p21 .The gene for IL-6 contains serum responsive enhancer element and is responsible for induction of cAMP and binding of glucocorticoids. All these have vital role in inflammation, bone density reduction and bone destruction.[13]

Most of the cytokines like IL-1, TNF-α and platelet derived growth factor can induce IL-6 expression in various cell types.[14] In case of reduction of bone density, IL-6 appears to be vital for the stimulation of certain factors.[15] It is also seen that multiple types of stimuli like viral infections, lipopolysaccharides etc are also responsible for induction of IL-6.[16]

The number of amino acids present in IL-6R is 468. Out of which 340 amino acids are present in extracellular portion.[17] IL-6R transmembrane segment contains almost 30 amino acids while intracytoplasmic domain comprises 82 amino acids. There are six N-glycosylation sites and eleven cysteine residues in IL-6R. The intracytoplasmic part of IL-6R does not contain tyrosine kinase domains which is required for signal transduction, this shows it has no enzymatic role in signal transduction of IL-6.[18]

IL-6R is present normally in many cells. One cell may contain hundreds to thousands IL-6 receptors. The number of IL-6R is increased in many diseases. For example in Multiple myeloma, the number of receptors is almost more than double. The regulation of IL-6 is done by negative feedback mechanism via suppression of cytokine signaling.[19]

PROGRESSION OF REDUCED BONE DENSITY BY INTERLUKIN -6 IN CHRONIC INFLAMMATION

Two contrasting features exhibited by IL-6, in many chronic diseases like collagen induced arthritis, autoimmune encephalomyelitis it acts as pro inflammatory. While in case of acute inflammation IL-6 has anti-inflammatory effects.[20]

At the beginning of acute inflammation, there is acute phase response by IL-6. If the activity of IL-6 persists as pro inflammatory cytokine, the acute inflammation becomes chronic and it triggers the production of various cytokines manifestated by immune responses. Several inflammatory diseases like rheumatoid arthritis, systemic juvenile idiopathic arthritis, ankylosing spondylitis, ultimately leads to reduced bone density, have elevated circulatory levels of IL-6.[21]

RISK FACTORS

The risk factors include both the environmental and genetic factors.[22]

Environmental factors

The environmental factors associated with reduced bone density in post menopausal women are

(i) Body mass index
Increased body mass index more risk will be associated.[23]

(ii) Diet
The diet containing insufficient calcium and vitamin D is associated with reduced bone density especially in post menopausal women.

(iii) Smoking
Smokers are more on risk as compared to non smokers.[24]

(iv) Hormonal and reproductive factors
Hormonal and reproductive factors like multiple pregnancies are also considered as risk factors for developing this condition.[25]

Genetic factors

- **Single Nucleotide Polymorphisms (SNPs)**

The inflammatory cytokines are regulated under the strict control of genetic components. These genetic components are responsible for remarkable variations which exist among individuals and are attributed to the polymorphisms in the promoter region of the gene.[26] Studies by several research groups have demonstrated that IL6R SNPs play a major role in reduction of the bone density.[27] Some of them predispose individuals to the development of musculoskeletal diseases. In this study, an association of SNP i.e., rs4845617 of IL6R gene in postmenopausal women with reduced bone density is found out. The aim of this study is to screen the SNP (rs4845617) of IL6R gene in our population and find out whether it is associated with reduced bone density or not. The SNP (rs4845617) is reported to be associated with reduced bone density[28] and is present in 3' UTR (untranslated region).

MATERIALS AND METHODS

Study Design

It is a retrospective case-control study. Thirty cases of post menopausal women with reduced bone density from different hospitals of Sargodha were included in this study. Written Informed consent was obtained from all the patients as well as controls participating in this study. Previously diagnosed cases of Paget's disease, metabolic disorder, endocrinal disease or already undergoing hormonal replacement treatment were excluded from the study.

Cases and Controls

All the cases of postmenopausal women with reduced bone density were examined and diagnosed by qualified gynecologist using standardized measures. Blood samples were taken from subjects and equal number of age- and gender matched controls. Total 30 cases and controls were included in the study.

METHODOLOGY

About 30 blood samples were collected from the cases affected with reduced bone density and 30 healthy controls. 5ml of venous blood samples were collected in 50 ml falcon tubes already containing 400 µl of 0.5 M EDTA. The blood samples were kept frozen at -80°C till the commencement of DNA extraction. Genomic DNA was extracted from whole blood following a Phenol-chloroform standard protocol for genetic analysis.[29]

Agarose Gel Quantification

Quality and quantity of DNA samples was estimated by running samples on 1% agarose gel containing ethidium bromide (electrophoresis). DNA samples of known concentration was also run on the same gel. The concentration of newly prepared samples was measured by comparing them with samples of known concentration. For the record the gel photograph was taken under UV.

Genotyping of SNP (rs4845617)

SNP rs4845617 was analyzed and genotyped in post menopausal women with reduced bone density and controls through direct DNA sequencing of PCR products using primers mentioned in Table-I. The primers flanking the SNP (rs4845617) was designed using Primer3 web browser (http://frodo.wi.mit.edu/).

SNPs	Forward Primer	Reverse Primer
rs4845617 (S1)	GATACGCCC TTTTCTCATC	CTCTACACAC ACTGCGAGTC

Table-I. Sequencing primers for rs4845617

The PCR was done in 25ul reaction volume in 0.2 ml tubes.

The reaction tubes were spin for one or two minutes to collect mixture in the bottom and were subjected to thermo cycling conditions consisting 5 minutes at 95°C for DNA denaturation followed by 30 cycles of amplification each containing 3 steps – denaturation: For one minute at temperature 95°C. Annealing: One minute at 61 °C for primer (rs4845617). Extension: one minute at 72 °C. Final extension at 72 °C for 10 minutes (Figure-1).

PCR products of amplified DNA samples were analyzed on 2% agarose gel. To the amplified samples 5ul loading dye (0.25% bromophenol

blue with 40% sucrose) was added and loaded into wells. Electrophoresis was performed at 120 volts for a period of half an hour in 1 x TAE buffer. Amplified products were visualized and photographed on UV trans illuminator.

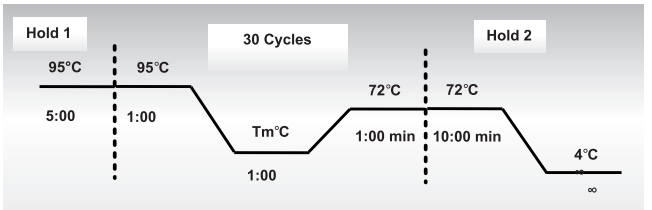

Figure-1. Reaction cycles in PCR

DNA SEQUENCING OF PCR PRODUCTS

PCR products were purified by DNA precipitation for sequencing. A sequencing reaction was performed in 96 well MicroAmp PCR plate, using the BigDye® Terminator v3.1Cycle Sequencing Kit and was analyzed on ABI 3730 Genetic Analyzer.

Sequencing PCR either with forward or reverse primer was setup using the reaction mixture given in Table-II and sequencing conditions in Figure-2.

Reagents	Volume (µl)/ 10µl Reaction
PCR product (10-15 ng/µl)	1.0 µl
5x sequencing PCR buffer	1.0 µl
Primer F/ R (3.2 pmol)	1.0 µl
Big Dye	0.7 µl
ddH_2O	6.3 µl

Table-II. Reaction mixture for sequencing PCR

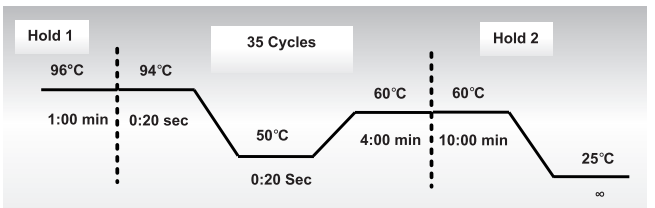

Figure-2. Reaction cycles in sequencing PCR

Analysis of Sequencing Results

The sequencing data was analyzed using Chromas Lite software v2.01. To look out for sequence alterations, the sequence of each exon from all the cases was blast against the normal ITGB3 sequence using BLAST 2 (Basic Local Alignment Search Tool) Sequences Server on NCBI website.[30] It utilizes the BLAST algorithm for pair wise DNA-DNA sequence comparison. The resulting alignments are presented in both graphical and text forms. For every case and control, sequence alteration in rs4537545 was recorded for further statistical analysis.

Statistical Analysis

Data pertaining to participants were implied and analyzed with the Statistical Package for Social Sciences (SPSS) version 20. Demographics of cases and controls were calculated and compared. The association of alleles and genotypes in post menopausal women with reduced bone density and clinical parameters were investigated using the chi-square test of independence with appropriate degree of freedom. The strength of a genetic association was measured by odd ratio (OR). For all statistical analyses, p-values <0.05 were considered significant.

RESULTS

Demographic Results

The quantitative and qualitative parameters are shown in the Table-III.

Quantitative Parameters	Mean ± S.D Total Cases (n) = 60
Age of the patient (years)	44.33 ± 15.68
Body mass index (BMI)	24.03 ± 2.45
Duration of disease (months)	33.93 ± 44.91
Qualitative parameters	n (% frequency)
Multiple fractures	26 (43.3%)
Family history	24 (40%)
Bone & Joint pain	60 (100%)
Multiple Bones & joints involvement	57 (95%)
Decreased Serum Calcium	26(43.3%)

Table-III. Quantitative and qualitative parameters

Genotype and Allele Frequency (Table-IV)

Serial No.	Genotype and Allele Frequencies	Cases		Controls	
		N=30	%	N=30	%
	rs4845617				
I	G	32	53.3	40	33.33
Ii	A	28	46.7	80	66.67
Iii	GG	08	26.7	06	20.0
Iv	AG	16	53.3	09	30.0
V	AA	06	20.0	15	50.0

Table-IV. Shows genotype and allele frequency for rs4845617 in cases of post menopausal women with reduced bone density and controls.

Odds Ratio for rs4845617 (Table-V)

Risk allele (G in rs4845617)	Cases	Controls	Total
Positive	32	20	52
Negative	28	40	68
Total	60	60	120

Table-V

OR = ad/bc = 64 x 80 / 40 x 56 = 5120/2240 = 2.28
Chi-Square (with Yates correction) = 8.98
df = 1 P-value = 0.002
This result is significant at P <0.05.

Association of SNP rs4845617 with reduced bone density in postmenopausal women

The results of genotype frequencies for rs4845617 of IL6R gene are presented in Table-VI. The frequency of homozygous G allele (GG) for rs4845617 of IL6R gene in cases of postmenopausal women with reduced bone density and controls were 26.7% and 20.0% respectively. The frequency of homozygous A allele (AA) for rs4845617 of IL6R gene in cases and controls were 20.0% and 50.0% respectively. Heterozygous AG alleles were 53.3% and 30.0% in patients and controls respectively. The chi-square test of independence showed that allele G was significantly associated with Rheumatoid arthritis in patients (OR=2.28, χ^2=8.98, p=0.002).

rs4845617	Cases (n=30)	Controls (n=30)	χ^2	P value
GG	08 (26.7%)	06 (20.0%)	8.98	0.002[a]
AG	16 (53.3%)	09 (30.0%)		
AA	06 (20.0%)	15 (50.0%)		

Table-VI. Genotype frequencies of the rs4845617 in cases and control subjects

Values are given in numbers and percentage.
[a] P values were calculated by chi-square test (Test for Independence).
χ^2, Chi-square with 1 degrees of freedom.

DISCUSSION

Reduced bone density in post menopausal women may be an immunological disease being explained as chronic inflammation of synovial tissue of joints causing destruction of bone and cartilage. It is a complex disorder represented with various factors including increased production of cytokines, inflammation, immune response, external factors, and genetics.

It has been considered as a chronic inflammatory disease caused by many inflammatory cells and cytokines. Some pro-inflammatory cytokines like IL-1, IL-6, and TNF-α are the main causative elements of immune mediated response. IL-6 is major cytokine. It is produced by variety of cells and has a key role in inflammation and production of immunological response in bone destruction. Detection of cytokine gene polymorphisms distressing the production of inflammatory mediators might lead the way to identify a genetic abnormality of regulation of cytokine cascade that may establish a role in the pathophysiology of the disease.[31]

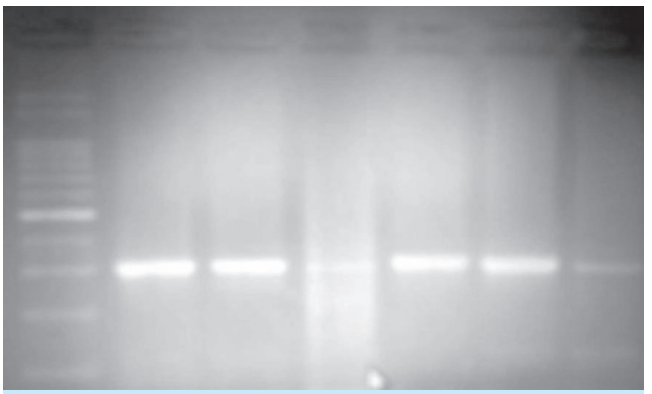

Figure-3. PCR amplification of the target region IL6R gene (300bp) containing rs4845617 polymorphism in cases of postmenopausal women with reduced bone density samples 25-30.

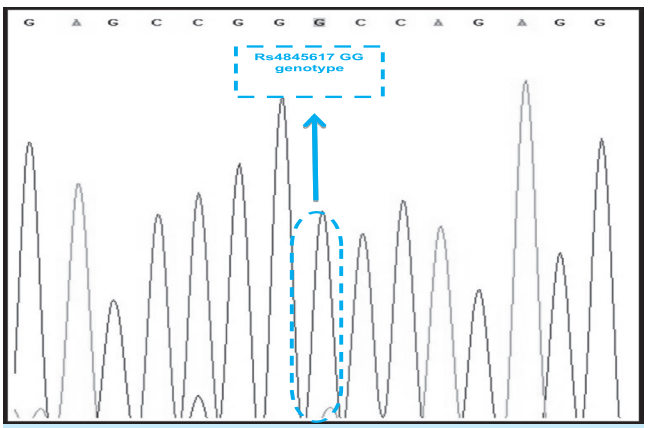

Figure4. DNA sequencing of the target region IL6R gene containing rs4845617 polymorphism in cases of postmenopausal women with reduced bone density sample 25, showing GG genotype frequency.

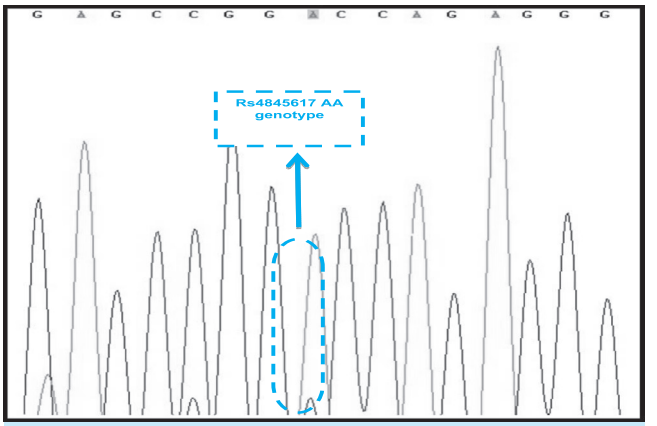

Figure-5. DNA sequencing of the target region IL6R gene containing rs4845617 polymorphism in cases of postmenopausal women with reduced bone density sample 30, showing AA genotype frequency.

In the present study, it is investigated that genetic polymorphism in IL-6R gene is involved in the pathogenesis of reduced bone density in post menopausal women and is confirmed by DNA sequencing technique.

A single-nucleotide polymorphism (SNP, pronounced snip) is a DNA sequence variation occurring when a single nucleotide — A, T, C or G — in the DNA differs between members of a biological species or paired chromosomes in an individual. In different individuals, most of SNPs are represented as only two alleles, for example, two different sequences like CCATGG to CCATAG explaining the alteration of single nucleotide change with two alleles: G and A. SNPs are typically located in non-coding regions of genome. The genomic sharing of SNPs is not homogenous. Also natural selection is provoking the SNP alleles in the adaptable genetic conditions.[32] Apart from natural selection some other features like recombination and mutation frequency can also establish SNP density. SNP density depends upon the existence of nucleotides adjoining each other in particular sequence.[33] SNPs of respective genes of cytokines and/or cytokine receptors are found linked to some human diseases signifying their likely contribution in the pathogenesis. Though their exact involvement in the production of disease cannot be established but they might modify the other key genes, adapt disease manifestations and cause their severity. The IL-6R gene is present on chromosome 1 (1q21), it is shown in a study that IL6R SNPs play a major role in development and progression of reduced bone density in certain immunological diseases.

In the present study, the genotype frequencies of rs4845617 of IL6R gene polymorphisms were described in cases of post menopausal women with reduced bone density and controls.

The present study is the first reporting study about the association of rs4845617 with reduced bone density in post menopausal women in Pakistani population. Previously it was reported to be associated with reduced bone density in Spanish population especially in post menopausal women.[34] It was found to be significantly associated with rheumatoid arthritis in Pakistani population (OR=2.28, χ^2=8.98, p=0.002). It is present in 3' UTR (untranslated region). It may involve in the rate of synthesis of the cytokine. In case of rs4845617, both AG and GG genotype were found to be associated with predisposition of reduced bone density in cases of post menopausal women. While allelic frequencies for A to G alleles in patients and controls significantly supported the association of G allele with the risk of development of this condition (p=0.002).

In conclusion the present study is the first report evaluating rs4845617 of IL6R A/G polymorphism in cases of postmenopausal women with reduced bone density for detection of IL6R genotype frequencies in Pakistani population which was previously considered to be associated with reduced bone marrow density in postmenopausal women of Spanish population.

Although there is no confirmed functional effect of the polymorphism, our results signify that these polymorphisms may play a role in susceptibility of such cases in Pakistani patients. It is further suggested that extensive studies with increased number of samples from different races and ethnic groups of Pakistani population are required to verify results of this study. In future, additional investigations of other polymorphic variants of the IL6R gene and its association with cytokine production in such cases may be supportive

to illuminate the pathogenic mechanism of the disease.

CONCLUSION

Our results signify that this polymorphism may play a role in reduced bone density susceptibility in postmenopausal women.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Shoaib Ahmed	Performed experiment & Methodology.	
2	Bilal Habib	Compile & Analyze resutls.	
3	Amna Mubeen	Literature Review.	
4	Imran Aftab	Abstract & Typhographical error.	
5	Farwa Naqvi	Brief review & helped in finding results.	
6	Amal Shoukat	Spelling & grammer mistakes.	