Pakistan J. Zool., vol. 46(1), pp. 261-266, 2014

BMP-2 Polymorphism c.893T>A (rs235768) Does Not Affect Bone Mineral Density in Female Population of Lahore

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Abstract.- Osteoporosis is systemic skeletal disease in which there is a progressive microarchitectural deterioration of bone tissue, generalized reduction in bone mineral density and this increases the person's susceptibility to fractures. It is a polygenic condition in which many genes and environmental factors play key role. Bone morphogenetic protein-2 (*BMP-2*) plays a key role in osteoblast differentiation. In the present study, polymorphism c.893T>A, in *BMP2* gene was assessed for its association with bone mineral density (BMD) variation in osteoporotic female population. Total 50 subjects were initially registered and on basis of BMD, 31 and 19 were diagnosed as osteoporotic and non-osteoporotic individuals, respectively. DNA was isolated from blood samples and 353 bp fragment having site of c.893T>A variation was amplified. RFLP was done using *BsrI* enzyme, to determine the genotype at locus c.893T>A. Osteoporotic group had the higher average age and more number of post-menopausal females. Nevertheless, none of the non-genetic factors that were investigated reached statistically significant association with BMD. Heterozygote genotype, AT, was the most prevalent genotype at this locus and variant homozygote, TT, was the least frequent. Females having variant genotype TT had 14.4% higher BMD than the subjects having wild type genotype, AA. However, genotype at this locus was not found tobe significantly associated with low BMD, thus it does not contribute to osteoporotic phenotype.

Key words: Osteoporosis, bone mineral density, bone morphogenetic protein-2, *BMP-2*-polymorphism.

INTRODUCTION

Osteoporosis is a common systemic skeletal disease characterized by generalized reduction in bone mineral density, microarchitectural deterioration of bone tissue and an increased risk of fracture (Stewart and Ralston, 2000). Osteoporosis and its associated fractures are a major public health concern, because of related morbidity, disability, diminished quality of life, and mortality (Williams and Spector, 2006). Various studies have described BMD as tool for determining bone strength and it falls below -2.5 standard deviations in osteoporotic bone (Stewart and Ralston, 2000; Jordan and Cooper, 2002).

Osteoporosis is a complex polygenic disease that results from an interaction between non-genetic factors and several different genes (Gueguen *et al.*, 1995). Studies of genetic factors can help to find diagnostic markers, therapeutic proteins, and molecular mechanism that govern the mechanism of bone development and metabolism (Brown and Rosen, 2003; Uitterlinden *et al.*, 2006).

BMP-2 is a member of TGF-β superfamily (Kingsley *et al.*, 1994) that plays key role in osteogenic and non-osteogenic process (Christiansen *et al.*, 2000; Sakou, 1998; Wall and Hogan, 1994). It is multi-functional, secretory, signaling, growth factor that acts as cytokine. Jong *et al.* (2004) had shown that *BMP-2* is potent inducer of osteoblast differentiation and it affects bone metabolism. *BMP-2* regulates expression of number of osteoblast related transcription factors and phenotypic genes (Fromigue *et al.*, 1998; Lee *et al.*, 2000; Nakashima *et al.*, 2003; Rawadi *et al.*, 2003) and it is involved in fracture repair process (Bostrom *et al.*, 1995; Tsuji *et al.* 2006).

A reduction in active *BMP-2* levels can result in symptoms that resemble those of osteoporosis, which suggests that osteoporosis may be related to abnormalities in this gene (Urist *et al.*, 1985). The biology of *BMP-2* suggests that polymorphisms that reduce *BMP-2* function or gene expression promote osteoporosis. Several studies have been conducted to determine association between *BMP-2* and osteoporosis with varied results according to population (Choi *et al.*, 2006; Ichikawa *et al.*, 2006; Ozkan *et al.*, 2010). So far, however, nobody had

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0030-9923/2014/0001-0261 $ 8.00/0

looked for polymorphisms in this gene in relation to osteoporosis in Pakistan. This study has investigated the association of variation c.893T>A (rs235768) in *BMP-2* gene with low BMD in female population.

MATERIALS AND METHODS

Bone densitometry and sample collection

Female patients visiting Hijaz Hospital and Naseer Hospital, Lahore were taken as subjects for this study, after informed consent. A standardized questionnaire was prepared and subjects were asked about medical and medication history, family history of osteoporosis, history of fracture by trivial trauma, lifestyle and reproductive factors. Patients taking any medication or suffering from any condition that is known to affect BMD were excluded from the study.

BMD at the heel of foot of each subject was measured by Quantitative Ultrasound using QUS BMD machine (Hologic Sahara®). Blood sample (5 ml) was taken in vacutainers from subject after consent. Subjects were classified on basis of BMD as non-osteoporotic (T-score = ≥ -2.5), and osteoporotic (T-score= < 2.5).

Amplification of BMP-2's *region having SNP rs235768*

DNA was isolated from blood by non-organic method (Grimberg *et al.,* 1989; Miller *et al.,* 1988). Quantity and quality of DNA sample was determined. DNA prep was treated with 1 µl RNase (Fermentas® Cat. #EN0601) in presence of 1X RNase buffer at room temperature for 30 minutes.

Primers were designed to amplify 353 bp fragment having *BMP-2* SNP rs235768 using Primer 3 software. Sequence of forward and reverse primers were

5´ GAAACGAGTG GGAAAACAACC 3´ and
5´ GAGACACCTTGTTTCTCCTCCA 3´,

respectively. *Tm* of forward and reverse primers was 55.9 and 58.1 °C, respectively.

Region of *BMP-2* gene harboring SNP rs235768 was amplified using Fermentas® PCR Kit. Reaction mixture comprised 100-200 ng DNA, 1X PCR buffer, 200 µM dNPTs, 1 µM of each of forward and reverse primers, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase and nuclease free water to make up reaction volume to 50 µl. PCR was carried out on PCR 2720 Thermal cycler (Applied Biosystem) with initial denaturation at 94°C for 5 min, followed by 35 cycles, each of 94°C for 30 sec, 54°C for 45 sec and 72°C for 1 min. Final extension was carried out at 72°C for 5 minutes. Concentrated PCR reaction, 10 µl, was mixed with 2 µl of DNA gel loading dye and run on 1.5% agarose gel with 1X TAE running buffer at 100 V (Sambrook and Russell, 2001).

RFLP analysis of BMP-2 *region harboring rs235768*

RFLP analysis was performed to check the variation rs235768 in *BMP-2* gene. PCR product was eluted from the agarose gel using DNA Extraction Kit (Fermentas® Cat. #K0513) according to manufacturer's protocol. For restriction analysis, purified PCR DNA, 50-100 ng, was mixed with 1 X Buffer B, 15 units of *Bsr*I (*Bse*NI) (Fermentas® Cat. # ER0881) and nuclease free water to makeup 20 µl of reaction volume. Reaction was incubated at 65 °C in water bath for 4 h. Recognition sequence and cutting site of *Bsr*I is as follows:

5´ A C T G G N ↓ 3´
3´ T G A C ↑ C N 5´

Polyacrylamide gel having 12% acrylamide: bisacrylamide, 1X TAE buffer, 300 µl 10% ammonium per sulfate and 90 µl TEMED (CAS #110-18-9 by Bio Basic Inc.) was prepared (Sambrook and Russell, 2001). The restricted samples were mixed with 3 µl DNA loading dye and were loaded in the wells. Electrophoresis was carried out using 1X TAE as running buffer at 100 V until the dye front travelled the two-third of the gel. Gel was stained for 15 min with 1X TAE having 0.01 mg ml^{-1} ethidium bromide. Gel was carefully rinsed with 1X TAE, then observed and photographed using UV transilluminator.

Statistical analysis

All statistical analysis was performed using SPSS version 11.0. Categorical variables and

continuous variables were assessed for their association with BMD in osteoporotic and non-osteoporotic females by chi-square test and Student's t-test, respectively.

Simple linear regression was performed to determine the potential non-genetic covariates of BMD. However, no association was found between any non-genetic factor and BMD. Thus, association between BMD and each genotype was evaluated by ANOVA. Observed and expected frequencies for genotypes were calculated and were assessed for Hardy Weinberg equilibrium using chi-square test. $P<0.05$ was considered significant in all cases.

RESULTS

Attributes of sample

Fifty samples were collected from local females and on basis of BMD, 31 subjects were found to be osteoporotic (T-score= < 2.5) and 19 were non-osteoporotic (T-score = ≥ -2.5). Characteristics of the osteoporotic and non-osteoporotic subjects are given in Table I.

Table I.- Important attributes of samples

	Osteoporotic subjects	Non-osteoporotic subjects	All subjects
No. of Samples	31	19	50
Mean ± SD			
BMD	-3.11±0.44	-2.13±0.28	-2.74±0.61
Age (yrs)	52.61±14.62	45.31±12.94	49.84±14.32
Age at menarche (yrs)	43.36 ± 5.23	42.54±7.27	43.11±5.83
Percentage			
Post-menopausal females	80.64 (25/31)	57.89 (11/19)	72 (36/50)
Family history of osteoporosis	25.80 (8/31)	15.79 (3/19)	22 (11/50)
Experienced fractures	22.58 (7/31)	15.79 (3/19)	20 (10/50)
Smoking	0	0	0
Drinking	0	0	0

For categorical variables the association between the variable and osteoporotic status was calculated by chi-square test and for quantitative variables t-test was used. P value < 0.05 was considered significant. Numbers in brackets indicate the number of sample having the specific attribute compared to total samples.

Average age of osteoporotic females was higher compared to non-osteoporotic females, though not significantly. Average age at menarche did not vary much between the two groups. Although more osteoporotic females reported to be post-menopausal, have experienced fractures and have family history of osteoporosis than non-osteoporotic females but no significant difference was found between the two groups.

Genotypic analysis of variation at nucleotide 893 (rs235768) of BMP-2 *mRNA*

DNA was isolated from the blood samples and *BMP-2* locus harboring SNP rs235768 was amplified in a PCR reaction as 353 bp product. Genotype of nucleotide 893 of *BMP-2* mRNA was determined by subjecting purified PCR product of each sample to RFLP using *Bsr*I enzyme. Nucleotide 893 of *BMP-2* mRNA is a position of SNP rs235768. T (thymine) and A (adenine) at this position are considered as variant and wild-type allele, respectively. After RFLP, restricted sample were run on 12% PAGE, stained and observed (Fig.1).

In both osteoporotic and non-osteoporotic females heterozygote (AT) genotype at the under-study locus was the most prevalent genotype followed by wild type homozygote genotype (AA) and least found variant genotype in both groups was variant homozygote (TT) (Table II). Female population with AA genotype had 14.4% lower BMD compared to females with TT genotype.

None of the attributes of sample in give Table I approached statistical significance (P value < 0.05) as covariates of BMD using stepwise linear regression model fitting for BMD. Therefore, original BMD values were used for ANOVA. This SNP did not show any significant ($P < 0.05$) association with BMD variation when tested using ANOVA. This indicates that *BMP-2* polymorphism at nucleotide 893 of mRNA did not have any influence on BMD in a given female population and thus did not contribute to osteoporotic phenotype.

Chi-square (P value < 0.05) analysis indicated that observed genotypic frequencies were not in agreement with expected ones, so the genotype at this locus did not follow the Hardy-Weinberg equilibrium (Table III).

Table II.- Genotype at nucleotide 893 of *BMP-2* mRNA (SNP rs235768) and BMD of its associated samples.

	Osteoporotic subjects		Non-osteoporotic subjects		All subjects	
	No. of samples	Average BMD	No. of samples	Average BMD	No. of samples	Average BMD
AA	7	-2.82 ± 0.12	2	-2.3 ± 0.1	9	-2.71 ± 0.26
TT	4	-3.3 ± 0.76	1	-2.3	5	-3.1 ± 0.8
AT	20	-3.17 ± 0.4	16	-2.1 ± 0.3	36	-2.69 ± 0.65
	31	-3.11 ± 0.44	19	-2.13 ± 0.28	50	-2.74 ± 0.61

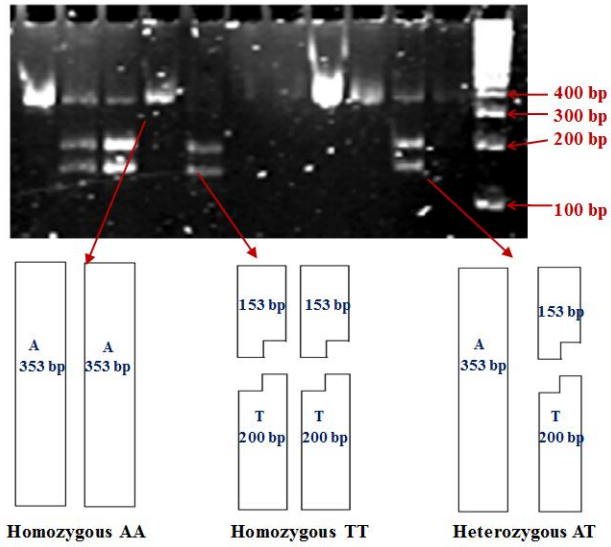

Fig. 1. Results of genotypic analysis of nucleotide 893 of *BMP-2* mRNA (SNP rs235768). Adenine (A) is wild type allele and thymidine (T) is variant allele. PCR product of 353 bp was subjected to restriction reactions using *BsrI* and resulting product(s) were analyzed at 12% PAGE. Enzyme cuts the DNA strand when T (SNP) is present and it does not restrict DNA strand when A (wild type) is present.

Table III.- Genotypic and allelic frequencies at nucleotide 893 of *BMP-2* mRNA (SNP rs235768).

Genotype	Genotypic frequency	Allele	Allelic frequency
AA	0.18	A	0.54
TT	0.1	T	0.46
AT	0.72		

DISCUSSION

Interplay between the genetic and environmental factors control bone turnover and hence influence bone integrity and strength. Any variation in these factors can result in the susceptibility to osteoporosis (Ralston, 2002, 2003). In different populations none or more than one non-genetic parameters like age, BMI, reproductive status, age of menopause, dietary habits have been reported to influence BMD (Choi *et al.*, 2006; Ichikawa *et al.*, 2006; Ozkan *et al.*, 2010). In this study none of the non-genetic factors that were analyzed were found to be associated with low BMD.

BMP-2 is a key morphogen for transcriptional activation of osteoblast lineage commitment and hence influence bone metabolism and strength (Banerjee *et al.*, 2001). A linkage study in Icelanders has first time identified the *BMP-2* gene on chromosome 20p12 as the gene involved in regulation of bone mass (Styrkarsdottir *et al.*, 2003).

c.893T>A (rs235768) SNP in *BMP2* gene was- first time associated with low BMD and thus to osteoporosis in Korean population by Choi *et al.* (2006). This study indicated that genetic effects of *BMD-2* variation on BMD may differ by anatomical site and gender. This transversion of A to T at nucleotide position 224 in exon 3 of *BMP-2* and at nucleotide position 893 in mRNA of *BMP-2* leads to change in codon from AGA to AGT. This nonsynonymous polymorphism results in the change in amino acid at position 190 in *BMP-2* preproprotein from arginine to serine (Styrkarsdottir *et al.*, 2003).

In present study *BMP-2* polymorphism Arg190Ser (c.893T>A) was not found to be associated with low BMD and contribute towards osteoporosis. Similar results had been reported by other studies in Turkish post-menopausal females (Ozkan *et al.*, 2010), Caucasian Americans (Ichikawa *et al.*, 2006), Dutch (Medici *et al.*, 2006)

and Icelandic population (Styrkarsdottir *et al.*, 2003).

Genotypic frequencies at locus c.893T>A were found accordance with Hardy-Weinberg equilibrium in Korean (Choi *et al.*, 2006) and Dutch (Medici *et al.*, 2006) population, which is contrary to our results that indicate that genotypic frequency at aforementioned locus are not in agreement with Hardy-Weinberg equilibrium.

It has been reported that Korean females who were homozygous variant (AA) at c.893T>A locus had 7% lower BMD compared with females who had TT genotype. However, in our population we found much lower BMD (14.4%) in females with AA genotype. In Dutch population (Medici *et al.*, 2006) frequency of variant allele T was 0.4 which is close to the frequency (0.46) that we found in our population. Contrarily, in Turkish population frequency of this genotype was lower (0.33) (Ozkan *et al.*, 2010).

Not much or relatively little information is available on the effect of SNP c.893T>A on *BMP-2* protein structure and function. However, it has been suggested that nonsynonymous polymorphism like c.893T>A may affect transcription, structure, transport, stability or translation of variant mRNA. This may result in inadequate *BMP-2* variant protein both in terms of production and its activity as ligand (Choi *et al.*, 2006).

BMP-2 is evolutionarily a well conserved gene with diverse functions that are regulated by wide array of molecules (Abrams *et al.*, 2004). So it can be assumed that nonsynonymous polymorphisms may have subtle effect on structure and function of these molecules in a temporal, spatial and tissue specific manner.

In conclusion, non-genetic factors and *BMP-2* SNP did not show any association with low BMD in female population under study. Results of this study should be construed prudently and should not be ascertained for overall population because of the limitation of relative small sample size (n=50). It is also possible that another polymorphism of *BMP-2* may be affecting the BMD in a given population. It is likely that for a given polygenic condition there might be other population-specific genetic determinants. Thus, further work will be required to confirm these observations and to study the mechanism by which the coding variants so far identified affect bone cell function and modulate bone mass.

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(Received 3 August 2013, revised 13 December 2013)