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Association of Sequence Variation 713–8delC in Intron 4 of Transforming Growth Factor-β1 Gene and Low Bone Mineral Density in Pakistani Female Osteoporotic Population

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Abstract.- Osteoporosis, a major public health problem, is becoming increasingly prevalent with the aging of the world population. This skeletal disorder is generalized, affecting the elderly, both sexes, and all racial groups' especially postmenopausal women are common victims. It results in fragility of the bone and leads to fractures. Ostopenia is less severe condition with fragile bones. Osteoporosis is polygenic condition in which many genes and environmental factors play key role. Transforming growth factor-beta 1 (TGF-β1) is considered a putative regulator of osteoclastic-osteoblastic interaction (coupling). The present study was done to examine whether a sequence variation of the TGF-β1 gene (713-8delC) is related to bone mineral density (BMD) and osteoporosis in Pakistani local osteoporotic female population. BMD was used as diagnostic tool to identify the subjects suffering from osteoporosis. Subjects were divided into three groups according to BMD *i.e.*, osteoporotic, osteopenic and normal. Fifty samples were collected, 30 from osteoporotic, 11 from osteopenic and 9 from normal females. Sequence was amplified as PCR fragment and RFLP was done using Van91l enzyme to determine the sequence variation. The results showed that 70% of the individuals had a one base deletion in the intron sequence, 8 bases prior to exon 5 (713-8delC), which could influence splicing, while 33% normal women exhibited the 713-8delC. This sequence variation was significantly higher in the osteoporotic group and there was an association between deletion and low BMD ($p < 0.05$). A direct relationship between age and BMD was also established. Average BMD (-3.133 T-score) and age of osteoporotic subjects (52.7 years) was highest among three BMD defined groups, which indicates that with the increase in age, BMD becomes lower and chance of having osteoporosis increases. Most of the post-menopausal women (80%) were found to be osteoporotic in Pakistani local female population.

Key Words: Osteoporosis, bone mineral density (BMD), TGFβ-1

INTRODUCTION

Osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Melton *et al.*, 1992). This disorder is more common in female population all over the world with increasing age. Diagnosis is done by measuring the level of bone mass as bone mineral density (BMD) with or without a fragility fracture (Lane, 2005) which is an important risk factor under strong genetic determination with heritability over 50%. Both environmental and genetic factors affects bone structure and bone density causing osteoporosis (Peacock *et al.*, 2002; Jordan and Cooper, 2002).

Aberrations in bone remodeling lead to bone fragility. In older people, the rate of resorption exceeds the rate of formation (Parfitt, 1987; Erikson 1986), resulting in too little bone, or osteoporosis. Transforming growth factor 1 (TGF-β1) gene has a key role in the regulation of bone metabolism, affecting both bone resorption and formation. It is most abundant growth factor in human bone and is produced by osteoblasts which inhibits osteoclast proliferation activity and stimulates proliferation and differentiation of pre-osteoblasts.

The *TGF 1* gene is located on chromosome 19q13.1-q13.3 (Fujii *et al.*, 1987). It consists of seven exons and very large six introns (Derynck *et al.*, 1987), of which part of exon 5, 6 and 7 encode the active TGF-β1 (Fig. 1). The active form is a 25-kDa disulphide linked dimer that on reduction, yields two identical chains of 112 amino acids (Sporn *et al.*, 1986). It is synthesized and secreted in a latent form as a protein containing 390 amino acids (Kanzaki *et al.*, 1990).

Several studies have investigated the effect of

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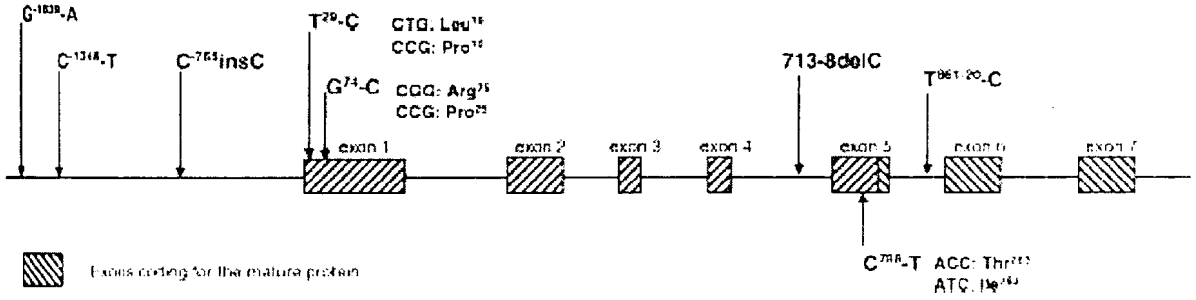

Fig. 1. Gene structure and polymorphism in the TFGß1 gene. Numbers are accordance with Derynck *et al.* (1987) and the important SNPs are G^{-1639}-A, C-1348-T, T^{-29}-C, G^{-74}-C, 713-8delC and C^{788}-T.

Table I.- Bone-related association studies with TGF-pt polymorphisms.

SNP	Position	Population	References
Promoter SNPS			
C-1348T	Promoter	Japan (postmenopausal)	Yamada *et al.* (2001)
		Korea (postmenopausal)	Pak *et al.* (2003)
		Denmark (mixed gender)	Langdahl *et al.* (2003), Grainger *et al.* (1999)
		UK (postmenopausal)	Wells *et al.* (2001)
		Caucasian (nuclear families)	Long *et al.* (2004)
		China (postmenopausal)	Lau *et al.* (2004)
G-1369A	Promoter	UK (postmenopausal)	Wells *et al.* (2001)
		Caucasian, Korea (postmenopausal)	
Coding SNPs			
T29C	Exon 1	Japan (postmenopausal)	Yamada *et al.* (1998, 2000)
Leu10Pro (signal peptide)		Denmark (mixed gender)	Langdahl *et al.* (2003)
		Caucasian (postmenopausal)	Hinke *et al.* (2001), Dick *et al.* (2003)
		UK (postmenopausal)	
		Korea (postmenopausal)	Koh *et al.* (2004)
		China (postmenopausal)	Lau *et al.* (2004)
C788T	Exon 5	UK (postmenopausal)	Wells *et al.* (2001)
Thr263lle (mature peptide)			
Intronic SNPs			
713-8delC	Intron 4	Denmark (pre- and postmenopausal)	Langdahl *et al.* (1997)
		Italy (postmenopausal)	Bertoldo *et al.* (2000)
		UK (postmenopausal)	Wells *et al.* (2001)
		Caucasian (nuclear families)	Long *et al.* (2004)
T861-20C	Intron 5	UK (postmenopausal)	Keen *et al.* (2001)
		Denmark (mixed gender)	Langdahl *et al.* (2003)
		Caucasian (nuclear families)	Long *et al.* (2004)
		China (postmenopausal)	Lau *et al.* (2004)

TGFβ-1 polymorphisms on susceptibility to osteoporosis, BMD and bone turn over. Table I shows the single nucleotide polymorphisms (SNPs) detected in *TGF 1* gene in all bone related association studies performed in different parts of the world. Langdahl *et al.* (1997) for the first time showed that 713-8delC, an intronic polymorphism, is more common among osteoporotic patients. This

association was later confirmed by Bertoldo *et al.* (2000) in an Italian population.

The present study is aimed at establishing a relationship between age groups and BMD of the patients, determining the prevalence of 713-8delC among Pakistani female osteoporotic population, establishing the relationship between specific deletion 713-8delC with low BMD, and comparing the results of osteoporotic patients with osteopenic and normal controls. To accomplish it, Restriction Fragment Length Polymorphism (RFLP) was used to establish the relationship of specific deletion of C with BMD in Pakistani population.

MATERIALS AND METHODS

Sample collection

Blood (3-5 ml) was aseptically drawn from 30 osteoporotic, 11 osteopenic and 09 normal females. BMD was measured by Quantitative Ultrasound Method (QUS) of bones and Hologic Sahara Clinical Bone Sonometer (set on normal Asian BMD values as reference data) which gave both BMD and T-score measurements. Every patient was required to fill in the specially designed questionnaire to determine the risk factors of osteoporosis. Females under medication (steroids, calcium and vitamin D supplements etc.), chronic diseases and hormone replacement therapy (HRT) were excluded.

Genomic DNA isolation

Genomic DNA was isolated from blood samples by Helms (1990) method and visualized on 0.8% agarose gel (Sambrook and Russell, 2001). Isolated DNA was quantified by spectrophotometer (Eppendorf Biophotometer).

Amplification

To amplify 225 bp region in intron 4 of *TGF β -1* gene, PCR was performed (Langdhal *et al.*, 2003) with 100-200ng/50μl genomic DNA, 25μM dNTPs, 0.5μM each primers

Forward 5'-ATTGAGGGCTTTCGCCTTAGCGC-3';
Reverse: 5'- GCGGCCGGTAGTGAACCARGCTT-3')

by e-oligos, 1μ/50μl *Taq* DNA polymerase by Fermentas # EP0402, 1X Taq buffer, 1.5mM $MgCl_2$ and 100-200ng/50μl genomic DNA water in a single reaction tube. The prepared solution was gently vortexed and briefly centrifuged from walls of tubes. The tubes were placed in Applied Biosystem 2720 thermocycler and PCR was run at initial denaturing temperature of 95°C for 5min, followed by 30 cycles each of 95°C for 1min, 61°C for 1min, and 74°C for 5min. The final polymerization temperature was done at 74°C for 5min. The reaction mixture was left at 4°C until it was visualized on 1.5% agarose gel. The specific amplified bands were extracted from the gel by Fermentas DNA extraction kit # K0513 method.

Restriction fragment length polymorphism analysis (RFLP)

The method for RFLP analysis of Langdahl *et al.* (2003) was followed, in which 20μl reaction mixture was prepared by mixing 100-200ng PCR product, 5U Van91l (*Pfl*MI) # ER0711 (10U/μ*l*) by Fermentas and 1X buffer R (10mM Tris HCl (pH 8.5), 10mM MgCl2, 100mM KCl, 0.1mg/ml BSA) and the final volume was made up to 20μl. The prepared solution was spun down for a few seconds and the reaction mixture was incubated at 37°C for 4 hours. After completing incubation, the tubes were kept at 4°C and the DNA bands were visualized by 12% polyacrylamide gel electrophoresis (Sambrook and Russell, 2001). The gel was run at 100V for 2 hours until dye reached two third length of gel. The gel was stained with ethidium bromide solution and was photographed using WEALTE Dolphin-DOC Software.

Statistical analysis

All data were expressed as Mean SEM. Differences in BMD with relation to the sequence variation 713-8delC between the two TGF-β1 genotypes were tested using independent samples T-test. A *P* value of 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 11.5 for Windows.

RESULTS

Attributes of the samples

The data collected is summarized in Table II and the comparison of results indicates the highest

average age in osteoporotic patients than of osteopenic or normal controls. Many of these osteoporotic women were experiencing post-menopausal situation (80%). Average age of puberty was found to be the same in all the three groups. Normal and osteoporotic patients had almost the same mean age and incidence of history of fractures. Incidence of family history of osteoporosis was highest in osteoporotic females.

Table II.- Attributes of osteoporotic, osteopenic patients and the normal controls.

Characteristics	Osteoporotic (n=30)	Osteopenic (n=11)	Normal (n=9)
Age (years)	52.3±15.37	43.73±11.78	48.78±12.6
BMD	3.13±0.44	2.25±0.1	0.81±0.2
Family history of OP (%)	30	18.18	11.11
History of fracture (%)	26.67	9.09	22.22
Postmenopausal (%)	80	54.54	66.67
Age of puberty (years)	13.8±1.92	12.91±1.04	13.89±1.54
Age of menopause (years)	43.29±5.5	39.67±7.1	44.00±3.52

Table III.- Relationship of age groups with BMD

Age group (Years)	Percentage of patients	Average BMD
26-35	20	-2.43 ± 1.06
36-45	14	-2.31 ± 1.01
46-55	28	-2.46 ± 0.80
56 and above	38	-2.7 ± 1.01

Relationship between age and BMD

Data for age of the subjects were classified in four groups and average BMD for each group was calculated. Table III shows the relationship of age with BMD. BMD gradually decreased as the age increased. Women of age 56 years and above had the highest BMD and thus had osteoporosis. Also women of younger age groups had mean BMD in osteopenic range. This indicates that women of younger age group were at higher risk of having osteoporosis in later years.

Genotype analysis

Figure 2 shows the 228bp PCR product of some of the samples, whereas Figure 3 shows the PCR product restricted with *Van911*. After RFLP, the cut and uncut DNA bands were visualized on 12% polyacrylamide gels. The bands obtained were of two types: 228bp (unrestricted DNA) which showed that specific deletion of base Cytosine at 713-8 position (intron 4) of TGFβ-1 gene was absent, while the second type of band obtained was of 203bp (restricted DNA), which was obtained when the specific deletion at specific site was present. The 25bp restricted band could not be seen in polyacrylamide gel because of its very small size.

Table IV.- Relationship of BMD of osteopenic and osteoporotic patients and normal persons with 713-8delC in TGFß1 gene in Pakistani female population.

BMD of	Genotype	
	CC	del c
Normal controls	-0.7 ± 0.07 (n=6)	-1.00 ± 0.00 (n=3)
Osteopenic patients	-2.3 ± 0.00 (n=3)	-2.23 ± 0.04 (n=8)
Osteoporotic patients	-2.95 ± 0.09 (n=9)	-3.21 ± 0.91 (n=21)
Total samples	-2.1 ± 0.25 (n=18)	-2.76 ± 0.14* (n=32)

*P<0.05; CC, no deletion in TFGß1 gene; del C, 713-8delC in TFGß1 gene.

A statistically significant relation between BMD of osteoporotic female population and the sequence variation 713-8delC has been found. A relationship between high prevalence of sequence variation 7138delC in total population and BMD (T-score) has been established. As compared with the population without deletion, there is an inverse relationship between BMD and the sequence variation. High prevalence of sequence variation correlated with decreasing BMD. The opposite relation was found in the case of osteopenic population. There is a significant difference between the patients having deletion with the patients without deletion in total population. Table IV shows relationship of BMD with genotype CC and delC.

DISCUSSION

Osteoporosis is characterized by a combination of low bone mass and deteriorated

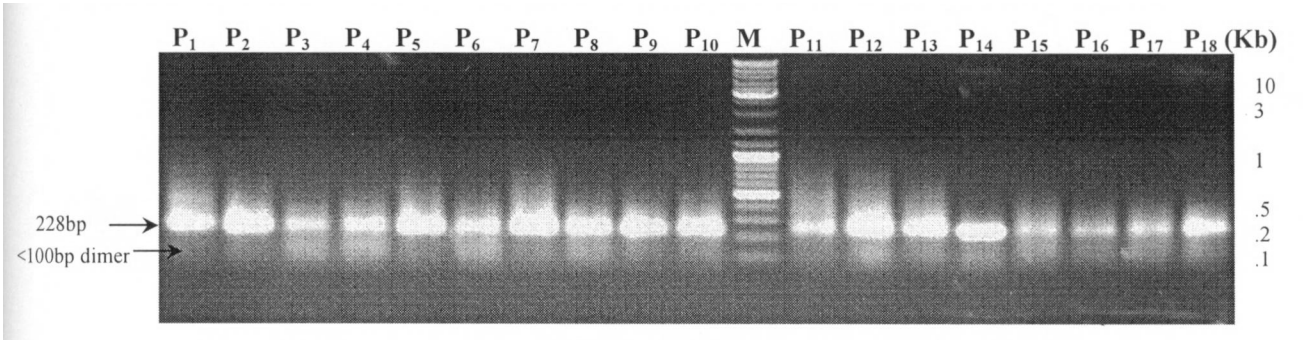

Fig. 2. Agarose gels showing PCR products of sample 1-18 10kb DNA marker was also run along the samples. Size of 10 kb marker is also shown along the gels. P represents PCR product and the number of PCR product is marked according $P_1 – P_{15}$) while M represents the DNA marker. This is a representative gel – all samples have not been shown.

microarchitecture of the bone (Pocock *et al*., 1987). It is very common bone disease as approximately one in three women and one in twelve men will suffer an osteoporotic fracture at some point in their lives, resulting in substantial morbidity, excess mortality and health and social services expenditure (Cooper and Melton, 1992). Low bone mass is the most important risk factor for the development of osteoporotic fractures (Kanis *et al*., 1994). The maximal bone mass of a given individual (peak bone mass) is determined by a combination of genetic and environmental factors, among them diet, physical activity, and hormone status is important to consider (Kelly and Eisman, 1993; Kelly *et al*., 1990, 1991). The influence of genetic factors is highlighted by the fact that daughters of osteoporotic women exhibit lower peak bone mass than daughters of women without osteoporotic fractures (Seeman *et al*., 1989, 1994). Twin and family studies have revealed that genetic factors are responsible for 50%–85% of the inter-individual variation in bone mass (Slemenda *et al*., 1991; Soroko *et al*., 1994). All this clearly shows that BMD, the major factor determining bone strength and consequently osteoporotic fracture risk, can be considered a quantitative polygenic trait.

In current study, numbers of characteristics known to be associated with BMD were evaluated. It has been inferred that most of the osteoporotic women are post-menopausal and have the highest mean for age. Results of this study indicate that as the women ages, the BMD gets lower and chance of experiencing osteoporosis becomes more. Also women of younger age group have mean BMD in osteopenic range. This signifies that younger women are more prone to experiencing osteoporosis later in life. Age of puberty is almost the same in three groups. Normal and osteoporotic females have the highest mean for age of menopause. This contradiction is may be due to small sample size.

It is being considered that family history of osteoporosis is a strong pre-determinant for the person having osteoporosis in future, especially in women (Lane, 2006). One of the major reasons of low trauma fractures has been identified as low BMD (Cooper, 1999; Cummings and Melton, 2002). In this study, considerable number of osteoporotic women reported family history of osteoporosis and previous experience of fracture. Although percentage of the history of fracture in ostoporotic and normal is almost equal however this does not signify that osteoporotic and normal individuals have the same chance of having fracture. This is because normal group has very small sample size.

After the attainment of peak bone mass, bone loss ensues due to bone resorption exceeding bone formation during bone remodeling (Eriksen, 1986). The balance between bone resorption and bone formation seems to be regulated by a variety of growth factors and cytokines, among them transforming growth factor 1 (TGF-β1) is considered to play a very important role (Manolagas *et al*., 1993; Mundy, 1993). In this study, association of sequence variation 713-8delC of TGF-β1 with low BMD was analyzed.

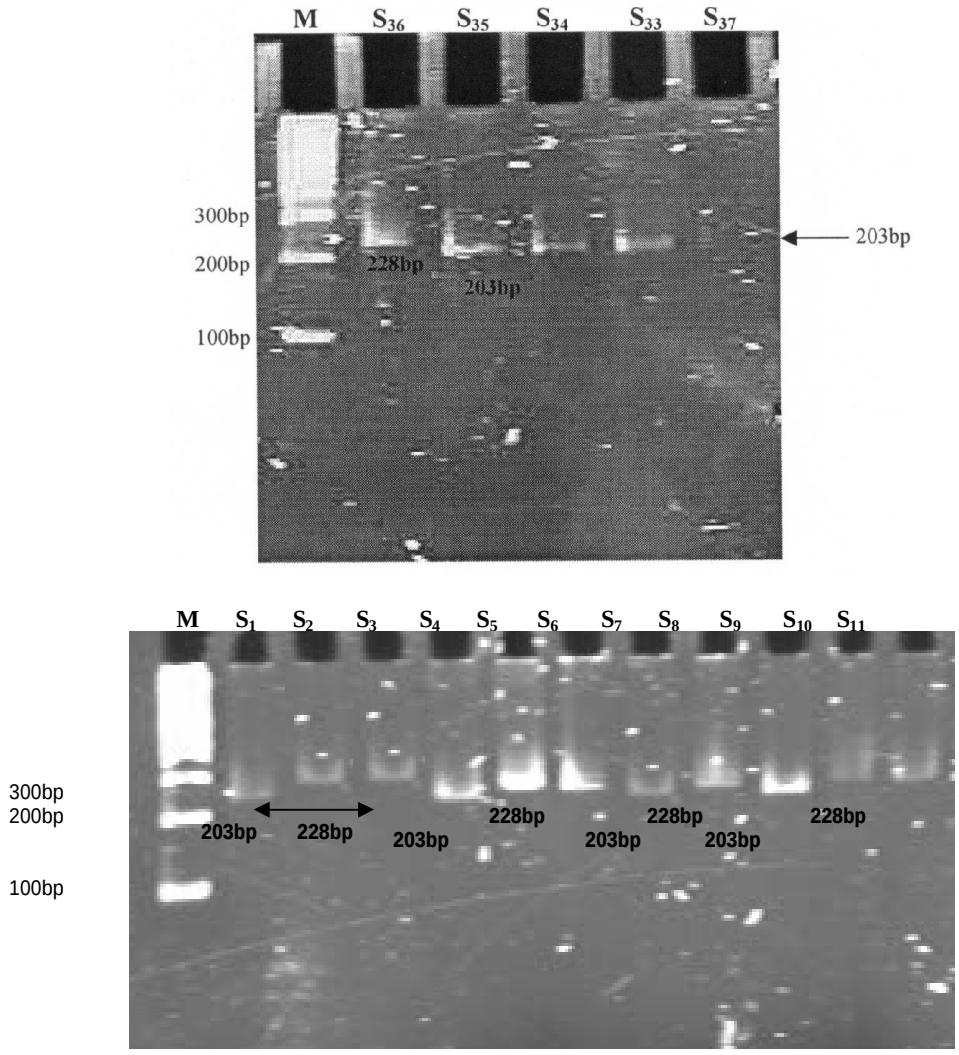

Fig. 3. Polyacrylamide gel of RFLP samples restricted with *Van911*, 10kb DNA marker was also run along the samples. Size of 10 kb marker is also shown along the gels. S represents the loaded sample and the numbers are marked accordingly (S1-S11, S35-37), while M represents the DNA marker, 203bp band shows deletion at 713-8delC, whereas 228bp band show no deletion of base C at 713-8 position in intron of 4 TGF-ß1 gene.

For the detection of sequence variation, RFLP was used as a basic methodology for osteoporotic female samples. Presence of more bands with deletion shows an overrepresentation of a sequence variation (713-8delC) in the TGF-β1 gene in osteoporotic patients. In normal control samples, deletion of base C in TGF-β1 intron 4 was less than osteopenic and osteoporotic patients. In osteoporotic patients, an association was found between the 713-8de1C and BMD. The data was significantly different when compared between deletion of base C (variation) and BMD. In nonosteoporotic normal controls, bone mineral density was unaffected by the presence of this sequence variation.

713-8delC is located in the intron sequence eight base pairs upstream from exon 5. 15% of human genetic diseases are caused by point-sequence variations in splice regions, causing either exon skipping or cryptic splicing (Krawezak *et al*., 1992), Both exon skipping or cryptic splicing would result in a truncated propeptide, and absence of active TGF-β1. Sequence variations from a

theoretical point of view could lead to reduce amounts of TGF-β1 at the tissue level, we have not demonstrated it in this study, and it will require further investigations to draw conclusions on the exact effect of these sequence variations.

Langdahl *et al*. (1997) also demonstrated higher prevalence of the sequence variation 713-8delC in osteoporotic female population than in normal women. This study was also confirmed by Bertoldo *et al*. (2000) who found that Italian women either heterozygote or homozygote for the 713-8delC polymorphism had lower BMD and higher risk of osteoporotic fractures but they could not demonstrate any effect of this polymorphism on bone mass or fracture risk.

In conclusion, our study provides an evidence for association between the 713–8delC of TGF-β1 gene and low BMD in Pakistani local female osteoporotic population. This polymorphism may be one of the most important genetic determinants of bone turnover and bone mass in other populations too. We did not correlate this sequence variation with bone turnover or other biochemical markers. It is still unknown how this polymorphism affects bone mass and further studies on the effect of these polymorphisms on TGF-β1 mRNA production and stability are needed to clarify the mechanisms and pathophysiology underlying the associations with fracture risk and bone mass.

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Conflict of interest

All authors state that there is no conflict of interest.

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