

## ORIGINAL PAPER

# Calcitonin Receptor Alu1 (rs1801197) and Taq1 Calcitonin Genes Polymorphism in 45-and Over 45-year-old Women and their Association with Bone Density

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## ABSTRACT

**Purpose:** Calcitonin receptor gene has also a polymorphism which is associated with bone mass density. This study evaluates the association between calcitonin receptor Alu1 (rs1801197) and Taq1 calcitonin genes polymorphism with bone density rate. **Methods:** In this descriptive-analytical study in 2013 in southwestern Iran, 200 blood samples, per the Cochran sample size formula, were taken from women aged 45 and older. DNA was extracted from the samples using the phenol-chloroform method and the genomic fragments in question were proliferated using the polymerase chain reaction (PCR) method. **Results:** The genotypic distribution of polymorphism Alu1 for TT, TC, and CC genotypes in control group was 31.4%, 38.6%, and 30% and in patients 25.4%, 55.4%, and 19.2%, respectively. There was no significant difference in polymorphism Alu1 between patients and control group and no significant association was found between this gene and bone density rate ( $P > 0.05$ ). All patients and the individuals in the control group exhibited tt genotype for Taq1 calcitonin gene and no significant association was found between these participants and osteoporosis. **Conclusion:** There was no association between two polymorphisms and osteoporosis, and between polymorphism of these two genes and osteoporosis development rate in the participants.

**Key words:** Polymorphism, Taq1 calcitonin gene, Alu1 calcitonin receptor gene, osteoporosis.

## 1. INTRODUCTION

Osteoporosis is a common skeletal disorder, accompanied with decrease in bone mass, deterioration of bone microstructures, and increase in susceptibility to fracture (1). Fracture contributes to increasing morbidity and mortality and imposes high costs on individuals and community. On average, one woman per three and one man per 12 experience fracture during lifetime (2, 3). In the USA, annually 1.5 million fractures occur due to osteoporosis. More than 50% of these patients could not well recover their previous activities and 10% will need long term therapeutic practices. Deep venous thrombosis is high (20-50%) in these patients (1, 4).

Bone mass reaches to its maximum level in early adulthood and then declines gradually as age advances. In women, particularly postmenopausal, as estrogen decreases, intestinal and renal calcium absorption decreases and calcium intake by the bones reaches to its minimum level. Therefore, post-

menopausal estrogen decline is one of the predisposing factors for osteoporosis in postmenopausal women (2, 5). Genetic differences could also contribute to the variety of contents in bone minerals in different ethnics (6).

Calcitonin is a polypeptide hormone, comprising 32 amino acids and contributing mainly to calcium concentration regulation. This hormone is secreted by parafollicular C cells of mammalian thymus and thyroid. A disulfide bond links cysteine 1 to cysteine 7 and a 7-amino acid circular structure is developed at the end of amine. Precursor of pre-procalcitonin has 141 amino acids. Calcitonin gene is located on short arm of chromosome 11 (11p15) (7, 8, 9).

Calcitonin declines the cells' permeability toward calcium, stops osteoclasts' activity, and facilitates and intensifies osteogenesis through stopping calcium's intake from bones and intensifying osteoclastic activities. As a result, the blood's calcium and phosphate decline, which causes decline in hydroxyproline of the blood and urine (7, 8).

Hopner et al. found that calcitonin gene (CT) holds a polymorphic site for TaqI restriction enzyme, this site is located on short arm of chromosome 11, and this polymorphism is associated with several diseases. In this gene, there is another polymorphism called dinucleotide repeat calcitonin, which is associated with bone density rate (9, 19). Miyao et al. investigated the association between the dinucleotide repeat calcitonin in calcitonin polymorphism locus in Japan (9).

Calcitonin receptor is the receptor of calcitonin hormone. Calcitonin receptor gene (*CTR*) is located on long arm of chromosome 7 (7q21). This receptor exists in osteoclasts, renal tract epithelium, testicles, brain, and ovary. Calcitonin activity is mediated through high-affinity receptor, i.e. calcitonin receptor (11, 12).

Calcitonin receptor gene has also polymorphism TaqI which is associated with bone density rate. Masi et al. indicated that this polymorphism was associated with bone density rate and the individuals with genotype *tt* had a less bone density compared with the heterozygous *Tt* individuals (13).

In calcitonin and calcitonin receptor genes, the ability to develop cutting by the enzymes TaqI and AluI causes development of the following genotypes: AluI genotypes *CC*, *TC* and *TT* (14, 15) and TaqI genotypes *TT*, *Tt* and *tt* (16).

The main purpose in treating osteoporosis is to prevent fractures. Recent advances in detecting genetic basis of diseases have opened new horizons. Disease diagnosis through genotype determination is increasing. The pharmacologic and interventional methods available for preventing osteoporosis in the individuals at risk add to the significance of the issue. Also, no study has been yet conducted on the association between molecular basis of bone density decline and the polymorphisms AluI calcitonin receptor in Iran. Therefore, this study was conducted to investigate the association between AluI (rs1801197) calcitonin receptor gene and bone density rate in 45- and above 45-year-old women in southwestern Iran.

## 2. MATERIALS AND METHODS

In this descriptive, analytical study conducted between December, 2012 and September, 2013. This research was approved at research at council and ethics committee of Shahrood University of medical Sciences-Iran. Ethics code: 92-12-17. sample size was calculated by Cochran equation. 45- and over 45-year-old women referring the centers for bone density measurement in southwestern Iran were enrolled based on convenience sampling after the consent to participate in the study was obtained. The questionnaires of clinical and demographic data were filled out. Exclusion criteria were corticosteroids consumption history, ovariectomy or premature ovarian failure, thyroid disease, calcium intake disorders, gastrointestinal and renal diseases, etc. Bone density rate in lumbar vertebrae and femoral neck was measured using dual-energy X-ray absorptiometry (DEXA) and the values were determined by t-score. A 5-ml peripheral blood was taken from each patient in the tubes containing EDTA (0.5 M). DNA was extracted using phenol-chloroform for molecular experimentations (17). Quality and quantity of the extracted DNA were examined by spectrophotometry using Nanodrop.

Sequence of calcitonin and calcitonin receptor genes was

extracted from NCBI database. The primers for the polymorphism AluI and TaqI were designed using online Primer3 software. The used primers are shown in Table 1.

Amplification of fragments containing TaqI calcitonin gene and AluI calcitonin receptor gene polymorphisms was done by PCR. Investigation of PCR products and determination of the genotypes were done using electrophoresis technique on polyacrylamide gel 8% by means of Tris-Borate EDTA (TBE) and loading 6X (Fermentas) buffers (18).

5'-3' Primer sequences	Primer type	Polymorphism type
ATTCAGTGGAAACCAGCGTTGG	F	AluI
CTCAGTGATCACGATGCTGTG	R	AluI
CAGACTGTGCATGCTACA	F	TaqI
CCTGCCTGCAACAGGAC	R	TaqI

Table 1. The sequence of used primers in the study

In each PCR, a vial was considered as negative control to show no contamination with DNA sample. The final volume of the reaction mixture was determined 25 µl per each micro-tube were considered. Thermocycler (PC818, ASTEC, Japan) was used.

The fragments were treated using restriction enzymes of AluI and TaqI (Fermentas). The marker of DNA size used was GeneRuler™ DNA Ladder, ready-to-use. For observing the obtained bands, silver nitrate staining was used.

Restriction fragment length polymorphism (RFLP): RFLP is a method based on longitudinal study of differences in a specific fragment of DNA that is highly capable of detecting some loci. Three cutting enzymes of AluI and TaqI (Fermentase, Germany) were used for RFLP. For this purpose, a mixture containing 10 ml of PCR product, 1 µl of AluI and TaqI, and 2 µl of each enzyme buffer was prepared in a final volume of 30 µl and incubated for 16 h at 37°C. Restriction digestion products obtained in the acrylamide gel were electrophoresed and stained using silver nitrate.

In this study, t-score > - 1 was determined as normal, t-score ≤ - 1 to ≥ - 2 as mild osteopenia, t-score < - 2 to ≥ - 2.5 as severe osteopenia, and t-score < - 2.5 as osteoporosis (19).

The data were analyzed by SPSS software using chi-square and ANOVA. The association between different genotypes of the polymorphisms and bone density rate was investigated by ANOVA. The CI in all experiments was considered 95% and P<0.05 was considered significant.

## 3. RESULTS

Since all women over 45 years old have some bone density decline, we assigned, based on radiographs' t-scores, the individuals with normal bone density and/or mild osteopenia as the control group (70 individuals) and those with severe osteopenia and osteoporosis as the patient group (130 individuals) (Table 2).

The quality of the extracted DNA samples was mostly desirable and A260/A280 between 1.75 and 2. The size of fragments obtained from PCR in the polymorphism TaqI calcitonin gene was 280 bp which produced two 111 and 169-bp fragments after digestion by enzyme TaqI, if the detection site of the enzyme was present. In addition, the length of am-

Variable	Mean ± SD* (Patient group)	Mean ± SD (Control group)	P-Value
Lumbar vertebrae's T-score	- 2.8 ± 0.67	- 0.9 ± 0.8	< 0.0001
Femoral neck's T-score	- 1.3 ± 0.78	- 0.34 ± 0.5	< 0.0001
Age (year)	59.9 ± 8.4	51.3 ± 5.7	< 0.0001
Height (cm)	154.8 ± 3.6	158.5 ± 4.9	< 0.0001
Weight (kg)	66.3 ± 8.09	71.7 ± 9.1	< 0.0001
Lumbar vertebrae's BMD* (g/cm <sup>2</sup> )	0.73 ± 0.1	0.92 ± 0.15	< 0.0001
Femoral neck's BMD (g/cm <sup>2</sup> )	0.76 ± 0.1	0.88 ± 0.11	< 0.0001

Table 2. Characteristics of control and patient groups under study. , \* Bone mineral densityThe number of patient group is 130 and control group is 70.

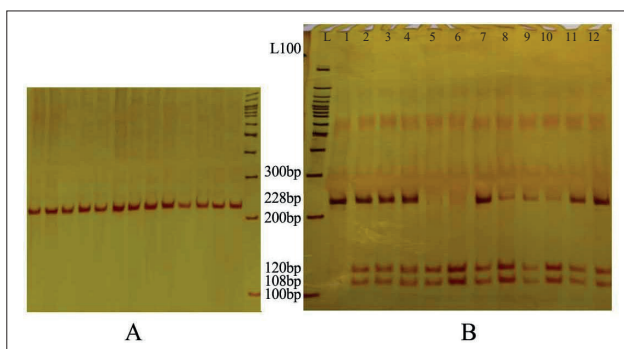


Figure 1. A: PCR product of polymorphism AluI (The size of amplified fragment was 228 bp); B: Enzymatic dissolution of polymorphism AluI (L: DNA ladder. The samples 2, 3, 4, 7-12 had three 228, 108, and 120-bp bands [genotype TC], Sample1 has two 228-bp bands [the individuals with homozygous genotype CC], and samples 5 and 6 had two 108- and 120-bp fragments [genotype TT]).

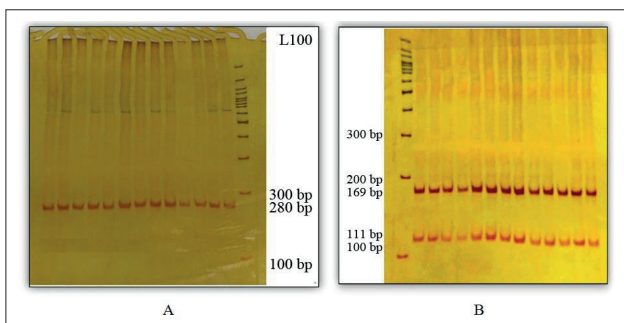


Figure 2. A: The product of polymorphism TaqI PCR (the size of amplified fragments was 280 bp). B: Enzymatic dissolution of polymorphism TaqI (All individuals in case and control groups had genotype tt).

plified fragment in the polymorphism AluI of receptor calcitonin gene was 228 bp which produced two 108 and 120-bp fragments after digestion by enzyme AluI, if the restriction site of the enzyme was present (Figures 1,2).

Genotypic frequency was calculated for the polymorphisms in the two groups (Tables 3-4). For polymorphism AluI in both groups, degree of freedom (df) was considered 2 and p value was obtained > 0.05 (P = 0.16 in the control group and P = 0.443 in the patients); that is, there was no statistically significant deviation for the polymorphism AluI and Hardy-Weinberg equilibrium was noted.

Mean t-score for lumbar vertebrae was -2.933 in the participants with genotype TT, -2.819 in those with genotype CC, and -2.712 in those with genotype TC. df for both lumbar

Polymorphism type	Genotypic frequency		
	genotype	Control group	Patient group
AluI calcitonin receptor gene polymorphism	TT	22 (31.4%)	33 (25.4%)
	TC	27 (38.6%)	72 (55.4%)
	CC	21 (30%)	25 (19.2%)
	total	70 (100)	130 (100%)
TaqI calcitonin gene polymorphism	tt	70 (100%)	130 (100)
	total	70 (100%)	130 (100)

Table 3. The genotypic number and percent of polymorphisms in the two groups

Polymorphism type	genotype	Control group		Patient group	
		The observed individuals	The expected individuals	The observed individuals	The expected individuals
AluI calcitonin receptor gene polymorphism	TT	22	18.2	33	36.5
	Tc	27	35	72	64.8
	cc	21	16.8	25	28.7
	total	70	70	130	130

Table 4. The number of expected and observed individuals for polymorphism AluI in the two groups

vertebrae and femoral neck was 2 and P value for lumbar vertebrae 0.466 and that of femoral neck 0.959, indicating that the polymorphism AluI was not significantly associated with t-score, i.e. bone density.

It is noteworthy that for TaqI all individuals in both groups had the genotype tt, such analyses were not done for this restriction site and its possible association with bone density.

#### 4. DISCUSSION

Osteoporosis is one of the most prevalent metabolic disorders of bone minerals. This disease is multifactorial and is developed by interaction among environmental factors, different genes, and aging. In this study, the association between polymorphism TaqI of calcitonin gene and bone density was investigated in 45- and above 45-year-old women. In other studies, this polymorphism was associated with other diseases, but the association between this polymorphism and osteoporosis has not yet been examined. If this polymorphism is noted in the individuals with osteoporosis, three genotypes are developed. However, the polymorphism of this site, throughout the study, was not noted in the osteoporotic individuals, and all participants had homozygous genotype tt and cutting site was not polymorphic.

Also, in the present study, existence of two allelic forms of calcitonin receptor gene was confirmed due to polymorphic site of AluI. We found that genotypic frequency of heterozygous TC individuals in the patients was higher compared with other genotypes. We also found that heterozygous TC individuals had a higher bone density compared with other genotypes. Therefore, heterozygote genotype of calcitonin receptor gene is more frequent than other homozygote genotypes. Homozygous TT individuals had a lower bone density compared with homozygous CC. However, there was generally no significant association between bone density and no statistically considerable difference in genotypic distribution and allelic frequency between the patients and the control group.

Several studies have been conducted on association between several genes polymorphisms and osteoporosis, including vitamin D receptor gene, collagen Type 1, calcitonin receptor, growth factor, insulin-like, interleukin 6, etc (20).

Nakamura et al. in a study in Japan found an RFLP in calcitonin receptor gene nucleotide 1377 which caused substitution of a leucine amino acid in 463 site with proline. The genotype of heterozygous individuals is manifested as *TC*, leucine homozygous individuals as *TT*, and homozygous proline as *CC* (21). Lack of the remaining proline could change the second structure of calcitonin receptor. This change could decrease calcitonin hormone bond to calcitonin receptor gene and adenylate cyclase response decreases. Therefore, C domain of calcitonin receptor terminal is especially important. Heterozygous genotype or proline/leucine existence changes calcitonin receptor biological activity. Since individuals with heterozygous genotypes produce both alleles, they are somehow superior over other homozygous genotypes.

Some researchers found similar results. For example, Massi et al. in Italy found that frequency of heterozygous *TC* and homozygous *TT* genotypes was high. Also, homozygous *TT* genotype decreased lumbar bone mineral density (BMD) considerably compared with the genotype *CC* (Masi, 1998). In Taboulet et al. study, femoral neck's BMD was higher considerably in heterozygous *TC* individuals compared with homozygous leucine *TT* and homozygous proline *CC*. Fracture risk also was lower in heterozygous individuals (22).

In a study in Turkey, no statistically significant difference was found in genotype and allelic frequency between the patients and the control group (23), which is consistent with the present study. Braga et al. found that polymorphism AluI calcitonin receptor gene in lower ages was associated with vertebral column BMD. That is, this polymorphism was more correlated with bone mass compared with bone loss due to aging (24), which is in contrast to our study. In addition, Tsai, in a study in Taiwan, found that this polymorphism was associated with osteoporosis prevalence and the individuals with the genotype *TT* were at high risk of osteoporosis in lumbar vertebrae and femoral neck (25). In Drews et al. study, BMD was very high in heterozygous individuals and this polymorphism was associated with osteopenia and osteoporosis progress (Drews, 2005). In two studies in Korea and Spain, the polymorphism AluI and BMD were associated (26, 27). In addition, in Charopoulos study, this polymorphism was associated with brachial BMD (28).

However, study of the association between several polymorphic sites of vitamin D receptor and bone density has revealed different findings worldwide. Liu et al. argued that polymorphism TaqI vitamin D receptor was far unlikely to be associated with body mass index variations, but could be only an indicator in a strong, unbalanced link with a close functional mutation (29). Mitra et al. study of the polymorphisms of vitamin D receptor (*Apa1*, *TaqI*, *Fok1*, *Bsm1*) and bone density in Indian postmenopausal women indicated that individuals with the genotypes *aa*, *FF*, and *TT* had a higher bone density compared with the genotypes *AA*, *BB*, *ff*, and *tt* (30). Zhang et al. did not observe any significant association between different polymorphisms of vitamin D genes and bone density in postmenopausal period (31). However, in some studies, polymorphism TaqI was associated with BMD in femoral neck and lumbar neck (7, 27). This inconsistency of the findings could be due to the difference in sample size or inadequate number of the samples, as well as racial differences among different communities. However, the important

finding in the present study and other studies is higher bone density in the individuals with heterozygous *TC* genotype of calcitonin receptor gene compared with homozygous genotypes.

## 5. CONCLUSION

This study indicated no statistically significant deviation for polymorphism TaqI, AluI gene, and calcitonin receptor in the patients and control group. In this study, we used RFLP method to make the findings consistent with those of other studies conducted on this field. The association between other polymorphisms involved in osteogenesis regulation and bone density should be studied. Also, further research is recommended to study calcitonin gene TaqI polymorphism.

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• **Conflict of Interest:** The authors declare no conflict of interests.

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