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Calcitonin receptor (*CTR*) gene polymorphism in a Turkish population using PCR-RFLP assay

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Abstract
C/T polymorphism at the nucleotide position 1340 (codon position 447) in the *CTR* gene has been associated with osteoporosis and kidney stones. This study is set to determine the frequency of such an important polymorphism in the *CTR* gene in a Turkish population to determine their possible susceptibly to the diseases that have been associated in previous studies. CTR gene was amplified by Polymerase Chain Reaction (PCR) from 489 healthy blood donors from Aydın province where is located on the Southern Aegean Region of Turkey. To detect C/T polymorphism in *CTR* gene, a PCR product of 228 bp was digested with restriction enzyme *Alu* I. Data obtained revealed three genotypes so called C/C, C/T and TT of which frequencies were 16.35%, 47.44% and 36.21%, respectively. The frequency of the mutant T allele was 0.5992 in the Turkish population. The Turkish population showed a close similarity with Italian population with regard to genotype and allele frequencies of *CTR* gene. The population studied was in Hardy-Weinberg equilibrium suggesting that there is no strong selection in favor of any genotype. Frequency of heterozygote genotype, which has been suggested being less prone to osteoporosis, was higher than Japanese and Asian populations.

Keywords: Osteoporosis, Calcitonin receptor, polymorphism, Turkish.

Introduction
Calcitonin (CT), discovered by Copp *et al*. in 1962 [1], reduces blood concentration of calcium and secreted by parafollicullar or C cells of thyroid gland [2]. CT is a 3418 Da weight and 32 amino acid length peptide hormone inhibits bone resorption and stimulates renal calcium excretion [3, 4]. Because of the ability of inhibition bone resorption, CT is used in the clinical treatment of osteoporosis [5, 6], paget's disease [7, 8] and hypercalcemia [9]. CT acts its target cells through binding its receptor so that calcitonin receptor (CTR) is activated. CTR contains seven potential transmembrane domains located on the surface of osteoclasts [10] and also occurs on the other cell types such as kidney [11], ovary [12], brain [13] lung [14] and testis [15]. Activation of CTR induces adenylcyclase and causes a decline in the osteoclastic bone resorption [16]. Human *CTR* gene located on 7q 21.3 [17] has several splice variant, two of that known as *CTR*-1 and *CTR*-2 [18]. *CTR*-2 has a single nucleotide polymorphism at 1340 position (C→T) which lead to amino acid change (Pro→Leu) at codon 447 [19]. This base mutation in *CTR*-2 is correspond to at 1377 nucleotide position and in 463 codon position of amino acid sequence in splice variant *CTR*-1 [18]. *CTR* polymorphisms have been used for many association studies due to its occurrence in the coding region [20, 18, 21]. A number of studies have also been carried out on allelic frequencies in different populations [22, 23]. However, to our knowledge, no information exists on polymorphism and distribution of the *CTR* gene in Turkey. The present work aimed to determine the allelic frequencies of the *CTR* gene in the Turkish population that could provide some valuable information for determination of its susceptibility to the diseases associated with them.

Materials and Methods
Study Population
Cross-sectional and Community based study was performed in Aydın province, a major city in the Aegean region of Turkey (western Anatolia). The sample size was calculated as 384 on a prevalence of 50%, d = 0.05, at a confidence level of 95%. A design effect of 1.5 was used to allow for multistage sampling. The calculated study population size was finally 576. Multistage sampling was used in the selection of the study sample. Aydın was separated into five regions according to socio-economic and health data taken from the Directory of Health. When a volunteer applied to the blood donation center, a detailed physical examination was performed by the physician of the center. Healthy volunteers were asked to join the study, and a face-to-face questionnaire was performed. The exclusion criteria were: (1) Not being

accepted as a blood donor as a result of the physical examination; and (2) Only one randomly selected person was included from a family.

The current study was one of two studies to be performed using blood samples from the same population. The study protocol was approved by the Ethical Committee of the Medical School of Adnan Menderes University. Permission for the field study was given by the Turkish Red Crescent. Finally, written informed consent was provided by each participant.

Polymerase Chain Reaction

Blood samples of 2.5 ml were collected in blood collection tubes with EDTA. Genomic DNA was extracted using a Rapid DNA Purification kit, according to the manufacturer's protocol (Epicentre Biotechnologies, USA). To determine polymorphisms, a region of 228 bp in CTR was amplified using the PCR primer sets [21]:

F: 5'-CTCAGTGATCACGATACTGTG-3'

R: 5-ATTCAGTGGAACCAGCGTTGG-3'

PCR was performed in a 25m l mixture of (100–150 ng/ml) DNA, 1X PCR buffer, 1,5 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 uM each primer, and 0.5 U *Taq* DNA polymerase. The amplification conditions were 5 min at 95 ^{0}C, 30 s at 95 ^{0}C, 30 s annealing at 55, 30 s at 72 ^{0}C, and finally 10 min at 72 ^{0}C, for 35 cycles.

PCR-RFLP Assay

For detection of the C/T polymorphism in *CTR*, a PCR product of 228 bp was digested with restriction enzyme *Alu*I (Fermentas, Vilnius, Lithuania), which has a recognition site in this region. Eight microliters of PCR product was digested by *Alu*I (1 unit) in a 25 ul reaction according to manufacturer protocol incubated at 37 ^{0}C overnight. Individual genotypes were determined after electrophoresis of digested PCR products in 3% agarose gel stained with ethidium bromide.

Statistical Analysis

Observed-expected genotype frequencies and deviation from Hardy–Weinberg equilibrium for *CTR* genotypes in the study group were analyzed using Popgene 32. Type 1 error (alpha error) was taken as 0.05.

Results and Discussions

In this study, 54 females and 435 males (total 489 volunteers) were examined. Because of the low number of females participant, the estimated number of individuals that 288 for each gender cannot be reached.

A unique recognition site (5'-AG↓CT-3') takes place at 1340th allelic site on *CTR* gene when C allele mutate to T allele. Therefore after restriction digestion, T allele (Leucine) was cleaved into two fragments of 108 bp and 120 bp while wild type C allele (Proline) remained undigested (Fig. 1).

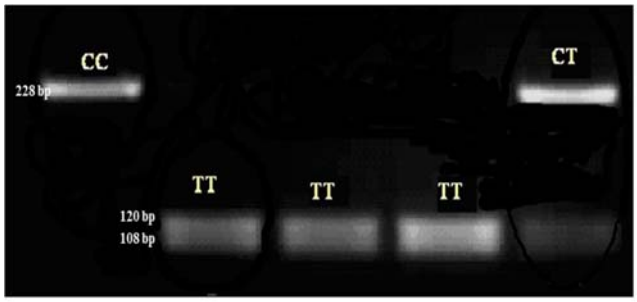

Figure 1. DNA fragment profiles generated by restriction digestion of 228 bp fragment of CTR locus by *Alu*I restriction enzyme.

The allele and genotype frequencies of 489 volunteers including females and males for *CTR* gene at 1340th allelic site are shown in Table 1. The allele frequencies determined for 489 volunteers was 0.4008 for C and 0.5992 for T. The genotype frequency of C/C, C/T, and T/T was found 16.35%, 47.44% and 177 36.21%, respectively. Evaluating males and females allele and genotype frequencies separately, T/T genotype frequency in females was found relatively higher than males genotype frequency. Of 54 females screened for C/T base substitution, genotype frequencies were as follow: C/C, 12.96%, C/T, 46.42% and T/T, 40.62. On the other hand, the genotype frequencies C/C, C/T and T/T in 435 males were 16.78%, 47.35% and 35.87%, respectively. Distribution of genotypes of *CTR* gene in the studied population was in Hardy- Weinberg equilibrium using Chi-square analyses. suggesting that there is no strong selection in favor of any genotype at least until at the age of maturity.

There have been a number of genetic association studies with osteoporosis [21, 24] and calcium oxalate urolithiasis [22, 25] in various populations. The study performed by Masi *et al*. [21] showed that Italian osteoporotic women have less frequently CC genotype with respect to healthy women. Taboulet *et al*. [20] have found association between CTR polymorphism and higher bone density in French women after menopause. Similarly there is another study has been carried out to determine correlation with body mass and bone mineral density in Japanese women [26]. According to their results, C allele was predominant in Japanese population and CTR allele has been associated with body weight in Japanese women. As regards calcium oxalate urolithiasis, Chen *et al*. [22] have found that frequency of TT genotype was higher in patients suffering from calcium oxalate stones than those had no health problems. On the other hand, Bid *et al*. [27] have found C allele more frequent in pediatric nephrolithiasis group. Mittal *et al*. [25] found no association between the C/T polymorphism and urinary stone disease. Even though there are some controversies, *CTR* C/T polymorphism seems have potential effect on the susceptibility of polygenic disease like osteoporosis and kidney stones.

Table 1: Allele and genotype frequencies of C/T polymorphism at 1340th allelic site of CTR gene in the Turkish population

Study Group	Allele	Frequency	Genotype	Frequency	χ^2	p^*
All volunteers n: 489	C T	0.4008 0.5992	C/C C/T T/T	80 (16.35%) 232 (47.44%) 177 (36.21%)	0,086285	0.76895
Females n: 54	C T	0.3704 0.6296	C/C C/T T/T	7 (12.96%) 26 (46.42%) 21 (40.62%)	0,028665	0,865555
Males n: 435	C T	0.4046 0.5954	C/C C/T T/T	73 (16.78%) 206 (47.35%) 156 (35.87%)	0,14475	0,703604

*Hardy Weinberg conformity, *P* value of 0.05 is considered statistically significant

A number of studies have also investigated *CTR* allele variations in different ethnic groups (Table 2). With respect to genotype and allele frequencies, Asian and Japanese populations are the most different ones due to the lower TT and higher CC genotype frequencies. Both populations have higher frequencies of C allele than T relative to other ethnic populations. The American-Caucasian and French-Caucasian populations have lower frequency of CC genotype while in Hispanic and African-American populations both homozygous genotypes are almost equal frequencies and high heterozygous genotypes. When we compared our findings to those reported studies only an Italian populations was close to the Turkish allele frequencies and almost equal genotype distributions. In conclusion, frequency of T allele was found to be close to the C allel in Turkish population giving higher frequency of heterozygote genotype which has been suggested to be less prone to osteoporosis.

Table 2. Genotype and allele frequencies of *CTR* C→T polymorphism in some ethnic populations including the Turkish population

Ethnic group	Genotype Sample size (%)			Allele Sample size (%)	
	TT	TC	CC	T	C
American-Caucasian (24)	104(59.09)	64 (36.36)	8 (4.54)	272(77.3)	80 (22.7)
African-American (24)	23 (25.06)	41 (47.7)	22 (26.7)	87 (50.6)	85 (49.7)
Asian (24)	0 (0)	5 (25)	15 (75)	5 (12.5)	35 (87.5)
Hispanic (24)	10 (20)	26 (52)	14 (28)	46 (46)	54 (54)
Italian (21)	254 (41.4)	136 (44.3)	59 (19.2)	360(58.6)	254 (41.4)
French-Caucasian (20)	105 (48.8)	96 (44.7)	14 (6.5)	306(71.2)	124 (28.8)
Japanese (18)	1 (0.9)	22 (18.8)	94 (80.3)	24(10.25)	210(89.75)
North Indian (23)	6 (5.7)	73 (69.5)	26 (24.8)	85 (40.5)	125 (59.5)
The present study	177(36.2)	232(47.4)	80(16.4)	586 (59.9)	392(40.1)

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References

1. Copp EC, Cameron B, Cheney AGF, Davidson K, Henze K. Evidence for calcitonin—a new hormone from the parathyroid that lowers blood calcium. Endocrinology 1962; 70:638-649.
2. Bussolati G, Pearse AGE. Immunofluorescent localizationbof calcitonin in the 'C' cells of pig and dog thyroid. J Endocrinol 1967; 37:205-209.
3. Warshawsky H, Goltzman D, Rouleau MF, Bergeron JJ. Direct in vivo demonstration by radioautography of specific binding sites for calcitonin in skeletal and renal tissues of the rat. J Cell Biol. 1980; 85:682-694.
4. Friedman J, Raisz LG. Thyrocalcitonin: inhibitor of bone resorption in tissue culture. Science 1965; 150:1465-1467.
5. McDermott MT, Kidd GS. The role of calcitonin in the development and treatment of osteoporosis. Endocr Rev 1987; 8:377-390.
6. Avioli LV. The role of calcitonin in the prevention of osteoporosis, Osteoporosis. Endocrinol. Metab. Clin. North Am 1998; 27(2):411-418.
7. Singer FR, Fredericks RS, Minkin C. Salmon calcitonin therapy for paget's disease of bone the problem of acquired clinical resistance. Arthritis Rheum 1980; 23:1148-1154.
8. Rose De FR, Singer A, Avramides A, Flores R, Dziadiw RK, Baker S *et al*. Response of Paget's disease to porcine and salmon calcitonins. Am J Med. 1974; 56 (6):858-866.
9. Mundy GR, Wilkinson R, Heath DA. Comparative study of available medical therapy for hypercalcemia of malignancy. Am J Med. 1983; 74:421-432.
10. Nicholson GC, Moseley JM, Sexton PM, Mendelsohn FAO, Martin TJ. Abundant calcitonin receptors in isolated rat osteoclasts. J Clin Invest. 1986; 78:355-360.
11. Marx SJ, Woodward CJ, Aurbach GD, Glassman H, Keutmann HJ. Renal receptors for calcitonin receptor: binding and degradation of hormone. J Biol Chem. 1973; 248:4797-4802.
12. Gorn AH, Lin HY, Yamin M, Auron PE, Flannery MR, Tapp DR *et al*. Cloning, characterization, and expression of a human calcitonin receptor from an ovarian carcinoma cell line. J Clin Invest. 1992; 90:1726-1735.
13. Goltzman D. Interaction of calcitonin and calcitonin generelated peptide at receptor sites in target tissues. Science 1985; 227:1343-1345.
14. Fouchereau-Peron M, Moukhtar MS, Benson AA, Milhaud G. Characterization of specific receptors for calcitonin in porcine lung. Proc Natl Acad Sci USA 1981; 78:3973-3975.
15. Chausmer A, Stuart C, Stevens M. Identification of testicular cell plasma membrane receptors for calcitonin. J Lab Clin Med. 1980; 96:933-938.
16. Nicholson GC, Moseley JM, Yates JP, Martin TJ. Control of cyclic adenosine 3', 5' monophosphate production in osteoclasts: calcitonin-induced persistent activation and homologous desensitization of adenylate cyclase. Endocrinology 1987; 120(5):1902-1908.
17. Lin HY, Harris TL, Flannery MS, Aru¡o A, Kaji EH, Gorn A *et al*. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. Science 1991; 254:1022-1024.
18. Nakamura M, Zhang Z, Shan L, Hisa T, Sasaki M, Tsukino R *et al*. Allelic variants of human calcitonin receptor in the Japanese population. Hum Genet 1997; 99:38-41.
19. Taboulet J, Frendo JL, Delage-Murroux R, Pichaud F, de Vernejoul MC, Jullienne A. Evidence for 2 allelic forms of calcitonin receptor gene: distribution in normal and osteoporotic women. (Abstract) J Bone Miner Res. 1996; 11(1):S204.
20. Taboulet J, Frenkian M, Frendo JL, Feingold N, Jullienne A, Vernejoul De MC. Calcitonin reseptor polymorphism is associated with a decrased fracture risk in post-menopausal women. Hum Mol Genet 1998; 7:2129-2133.

21. Masi L, Becherini L, Gennari E, Colli R, Mansani A, Falchetti C *et al.* Allelic variants of human calcitonin receptor: distribution and association with bone mass in postmenopausal Italian women. Biochem Biophys Res Commun 1998; 245:622-626.
22. Chen W, Wu HC, Lu HF. Calcitonin receptor gene polymorphism: A possible genetic marker for patients with calcium oxalate stones. Eur Urol 2001; 39:716-719.
23. Mittal RD, Bid HK, Mittal B. Polymorphism of Codon 447 Calcitonin Receptor Gene in North Indians. Int J Hum Genet. 2003; 3:175-177.
24. Wolfe III LA, Fling ME, Xue Z, Armour S, Kerner SA, Way J *et al*. In vitro characterization of a human calcitonin receptor gene polymorphism. Mut Res 2003; 522:93-105.
25. Mittal RD, Bid HK, Mittal B, Kumar A. Is calcitonin receptor gene (CTR) polymorphism an appropriate marker for calcium oxalate urolithiasis? Int J Hum Genet. 2004; 4:57-60.
26. Nakamura M, Morimoto S, Zhang Z, Utsunomiya H, Inagami H, Ogihara T *et al*. Calcitonin Receptor Gene Polymorphism in Japanese Women: Correlation with Body Mass and Bone Mineral Density. Cal Tissue Int 2001; 68:211-215.
27. Bid HK, Chaudhary H, Mittal RD. Association of vitamin-D and calcitonin receptor gene polymorphismin paediatric nephrolithiasis. Pediatr Nephrol 2005; 20:773-776.