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Research article

ASSOCIATION OF CALCITONIN RECEPTOR 1340 C>T POLYMORPHISM WITH PERIODONTITIS SUSCEPTIBILITY

T. Sunitha, M L N Deepika*, G Deepika, B. Srinadh, M. Sujatha and A Jyothy

Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, India
Corresponding Author: Email-mlndeepika@gmail.com

ABSTRACT: Background: Periodontitis is a common multifactorial oral disease and a major cause of tooth loss among adults. The present study was aimed to investigate the role of calcitonin receptor (CTR) gene polymorphism in the causation of periodontitis.

Materials and Methods: A total of 112 subjects comprising of 62 patients and 50 controls were enrolled and recruited from various dental clinics in and around Hyderabad, India. Two milliliter of blood sample was collected from all the subjects. Following extraction of DNA, genotyping for CTR 1340 C>T was performed by PCR-RFLP.

Results: The frequency of CC, CT and TT genotypes in patients was 45%, 42% and 13% while in controls it was 56%, 32% and 12%. The frequency of C and T allele was 0.66 and 0.34 in patients whereas it was 0.72 and 0.28 in controls. The genotype and allele frequencies did not vary between the groups. The genotype frequencies among male and female sub-types revealed a statistically significant difference in female subgroup. The CT genotype and T allele revealed an OR value of 5.62 and 2.40 respectively.

Conclusion: Our study revealed a significant association of this SNP with periodontitis only in females. It also highlights the predisposing role of CT genotype and T allele in the causation of periodontitis. However, replicative studies on the influence of this polymorphism in different ethnic groups may identify the potentiality of this SNP towards periodontitis.

Key words: Periodontitis, Calcitonin receptor, Signal transduction, Bone Mineral Density, Bone resorption

INTRODUCTION

Periodontitis is a common oral disease affecting 20% of adult US population; a major cause of tooth loss among adults and is often linked with severe quality of life impacts (Kimon Divaris, et.al, 2013). The disease is often linked with several systemic conditions such as coronary heart disease [Beck JD, et.al, 1998), pregnancy outcomes (Xiong X, et.al., 1998), diabetes mellitus (Lalla E, Papapanou PN, 2011] and others. The risk factors for periodontitis have been extensively studied and include smoking, diabetes mellitus, in addition to age, race and obesity (Thomas E Van Dyke, et.al, 2005). It is a complex multifactorial disorder and several risk factors such as genetic, metabolic, environmental, microbial factors, aging and poor oral hygiene status are involved in the etiology of the disease (Page RC, Beck JD, 1997). It shows relatively mild phenotype that progresses slowly and is chronic in nature. Certain pathogenic bacteria are also evidenced to be the primary initiator of perionditis (Feng Z, Weinberg A, 2006). Single nucleotide polymorphisms (SNPs) present the most widespread type of sequence variation in genomes, revealing the common genetic mutations that help in determining the risks for various diseases (Fareed M, Afzal M, 2013). Till date, a number of candidate genes such as IL 1, IL 17, TNF-α, TGF, VEGF, MMPs, CSF-1, that play a role in the genetic determination of periodontitis have been identified and investigated [Manuela Ianni, et.al., 2013, Xu J, et.al., 2014, Zuccarello D, et.al., 2014 , Kadkhodazadeh M, et.al., 2013, Pan Y, et.al., 2013, Chen D, et.al., 2014, Schulz S, et.al., 2012, Huang J, et.al., 2014]. One of the important and widely studied candidate genes for periodontitis is calcitonin receptor (CTR) (Marja L Laine, et.al., 2010, Vijayalakshmi R, et.al., 2010 Karthikeyan R, et.al., 2014). It is encoded by CTR gene located on chromosome 7q 21.3, belongs to class II of G-protein coupled receptor and binds specifically to calcitonin; a 32 amino acid peptide hormone produced in the C cells of mammalian thyroid (R D Mittal et.al, 2003) and plays an important role in calcium homeostasis (Xu J, et.al., 2014).

The SNP in the coding region (1340 T>C) (codon 447) of CTR gene causes a proline to leucine substitution in the third intracellular domain of the protein (Laura Masi , Maria Luisa Brandi , 2007, Marc Pondel, 2000). This change alters the secondary structure of calcitonin receptor along with modulating G-protein coupling and signal transduction [Wolfe LA III, 2003]. This SNP was identified to be strongly associated with osteoporosis, a systemic skeletal disease characterized by low bone mass, increased bone fragility and susceptibility to fractures (Geurs NC, et.al., 2003). Periodontitis is an inflammatory disease characterized by loss of connective tissue and alveolar bone. It is a silent disease similar to osteoporosis, causing symptoms until late in the disease process when mobile teeth, abscesses and tooth loss may occur. Since both osteoporosis and periodontitis share common risk factors and as both are bone resorptive diseases, it is hypothesized that osteoporosis could be a risk factor for periodontal disease (Geurs NC, et.al, 2003, Rekha Rani Koduganti, et.al, 2009, Esfahanian V, et.al, 2012). Thus, the present study was aimed to identify the association of CTR 1340 T>C gene polymorphism with periodontitis susceptibility.

MATERIALS AND METHODOLOGY

Study population

A total of 112 subjects were included in the current study. Among them, 62 patients had periodontitis and 50 controls were enrolled and recruited from various dental clinics in and around Hyderabad, India. Patients were selected based on the criteria of 1999 Consensus Classification of Periodontal Diseases (Armitage GC, 1999). Subjects with history or current manifestation of systemic diseases such as diabetes, cardiovascular diseases, hepatitis, HIV and autoimmunity were excluded from the present study. Normal healthy subjects with no history of periodontitis and systemic diseases or other inflammatory diseases were selected as controls. Informed consent from each subject was collected before peripheral blood sampling. Detailed information on clinical and anthropometric measures was recorded through proforma. The Bone Mineral Density (BMD) and CP index were obtained from all the subjects at the time of sample collection from the investigation reports.

Molecular analysis

Five milliliters of blood sample was collected from all the participants and genomic DNA was extracted using standard phenol-chloroform method. For each subject, the CTR 1340 C>T genotyping was performed by polymerase chain reaction using forward and reverse primers. PCR amplification was carried out in a total volume of 10µl containing 50ηg template DNA, 0.15µl of each primer (Bioserve) (Forward: 5′-CTCAGTGATCACGATACTGTG-3′; Reverse: 5′- ATTCAGTGGAACCAGCGTTGG -3′), 1.25 µl PCR buffer with 25mM $Mgcl_2$, 10mM dNTP's (Bangalore Genei) and 1U of Taq DNA polymerase (Bangalore Genei).

PCR was performed in a thermocycler (Biorad) as follows: An initial denaturation step of 5 minutes at 95°C, then 30 cycles consisting of 30 seconds of denaturation at 94°C, 45 seconds of annealing at 53°C and a final extension for 5 minutes at 72°C. The products were analyzed on 2% agarose gel under gel documentation system (Biorad) [Figure 1]. After confirmation, amplified products were then subjected to digestion with 2U of Alu I restriction enzyme (Fermentas, India) for 12 hrs at 37°C and then genotyped using 3.5% agarose gels. An undigested band of 228 bps indicates homozygous CC genotype, two digested products of size 120 and 108 bps represents homozygous TT genotype and the presence of one undigested 228bp band along with 120 and 108 bps corresponds to heterozygous CT [Figure 2].

Statistical analysis

Statistical analysis was performed by SPSS, version 16. Continuous data were expressed as mean ± SD. Allele and genotype frequencies were determined from observed genotype counts. The clinical and anthropometrical variables of patients and controls with continuous variables were compared using Student's *t* test while categorical variables such as allele and genotype frequencies between cases and controls and Hardy-Weinberg equilibrium was calculated by chi-square analysis. The association between genotypes and periodontitis risk was analyzed by calculating the odds ratio (OR) at 95% confidence interval. Statistical tests were two sided and a $p < 0.05$ was considered to be significant.

RESULTS

Characteristics of the study group

Data analysis on a total cohort of 112 individuals (62 patients; 50 controls) revealed that the mean age of the patients and controls at the time of sample collection was 54.0 ± 7.08 years and 34.10 ± 4.77 years respectively and differed significantly between the groups. The frequency of females (55%) predominated in cases and all belonged to postmenopausal period where as in controls there was an increased frequency of males (56%).

The mean Bone Mineral Density (BMD) and CP index were 2.62 ± 0.13 and 3.43 ± 0.36 in patients while they were 1.32 ± 0.26 and 1.23 ± 0.32 in controls respectively. A significant difference was observed between patients and controls with respect to age, BMD and CP index (Table 1).

The genotype and allele frequencies of CTR C>T gene polymorphism in patients and controls are shown in Table 2. The percentage of CC, CT and TT genotypes was 45, 42 and 13 in patients while it was 56, 32 and 12 in controls correspondingly. The genotype and the allele frequencies did not differ significantly between patients and control ($p>0.05$). Patients with CT genotype revealed an OR of 1.53, however did not reach statistical significance. The distribution of CTR genotypes was in agreement with Hardy Weinberg equilibrium in both the groups ($p>0.05$).

For the sake of comparisons, the data was further divided into males and female sub-groups. The distribution of genotypes and alleles when calculated separately for males and females, a significant difference was observed only in female sub-group ($\chi^2= 6.91$; $p=0.03$). Women with CC and CT genotypes revealed an OR value of 0.26 and 5.62 respectively ($p<0.05$). The allelic frequencies varied significantly between the groups and revealed an OR of 0.41 and 2.4 for C and T alleles correspondingly. The genotype and allelic frequencies in males did not differ on comparison between the male patients and controls. The distribution of genotypes was in agreement with Hardy Weinberg equilibrium in male and female subgroups ($p>0.05$) however deviated only in female controls ($p<0.05$).

Table 1: Subject characteristics of the study group

Characteristics	Patients	Controls	p-value
Age	54.0 ± 7.08	34.12 ± 4.77	<0.05
BMD	2.62 ± 0.13	1.07 ± 0.08	<0.05
CP Index	3.43 ± 0.36	1.52 ± 0.04	<0.05

Table 2: Distribution of CTR genotypes and allelic frequencies in patients and controls

Genotype	CC	CT	TT	C	T	Comparisons	OR(CI at 95%)	p-value
Patients 62)	28(45)	26 (42)	8(13)	0.66	0.34	CC vs. CT+TT	0.64(0.30-1.36)	0.34
Controls (50)	28 (56)	16 (32)	6(12)	0.72	0.28	CT vs. CC+TT	1.53(0.70-3.34)	0.33
χ^2 (p-value)	0.96(0.62)			0.58(0.44)		TT vs. CC+CT	1.08(0.35- 3.36)	1.0
HWE	Patients	0.253 (0.615)				C vs. T	0.75(0.41-1.37)	0.44
	Controls	2.12 (0.144)						

*significant at 5% LOS, OR: Odds ratio, CI: Confidence interval, LOS: level at significance

Table 3: Distribution of CTR genotypes and allelic frequencies in males and female sub-groups

Females	**CC**	**CT**	**TT**	**C**	**T**	**Comparisons**	**OR (CI at 95%)**	**p-value**
Patients (34)	14(41%)	16(47%)	4(11%)	0.64	0.36	CC vs. CT+TT	0.26(0.08-0.83)	0.02*
Controls (22)	16(68%)	3(18%)	3(14%)	0.79	0.21	CT vs. CC+TT	5.62(1.40-22.63)	0.01*
χ^2 (p-value)	6.91(0.03)*			4.81(0.02)*		TT vs. CC+CT	0.84(0.16- 4.19)	1.0
HWE	Patients	0.031 (0.859)				C vs. T	0.41(0.22-0.77)	0.008*
	Controls	7.42 (0.006)				T vs. C	2.40(1.28-4.50)	0.008*
Males	**CC**	**CT**	**TT**	**C**	**T**	**Comparisons**	**OR (CI at 95%)**	**p-value**
Patients (28)	14(50%)	10(36%)	4(14%)	0.67	0.33	CC vs. CT+TT	1.15(0.04-3.29)	1.0
Controls (28)	13(46%)	12(43%)	3(11%)	0.67	0.33	CT vs. CC+TT	0.74(0.25-2.17)	0.78
χ^2 (p-value)	0.045(0.97)			0.23(0.87)		TT vs. CC+CT	1.38(0.28-6.86)	1.0
HWE	Patients	0.920 (0.33)				C vs. T	1(0.55-1.80)	1.0
	Controls	0.0009 (0.92)						

*significant at 5% LOS, OR: Odds ratio, CI: Confidence interval, LOS: level at significance

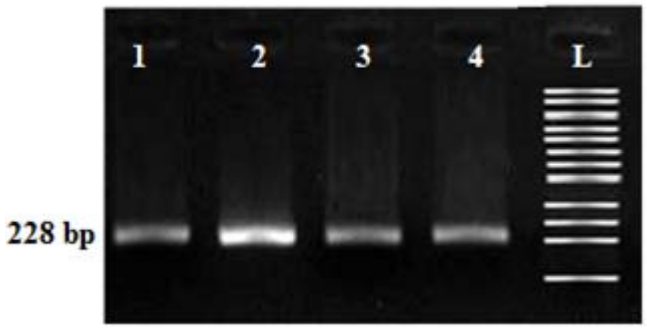

Figure 1: Gel picture showing PCR amplified product of CTR gene

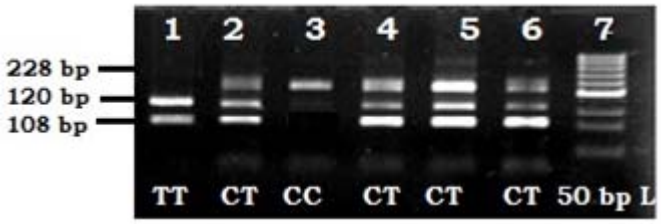

Figure 2: Gel picture showing different genotypes of CTR gene polymorphism

The genotype is CC if the PCR product is not digested by Alu-I enzyme, resulting in 228 bp. TT genotype if the PCR product is digested by Alu-I enzyme in two 120 and 108 bp fragments (TT allelic variant) and CT if half of the PCR product is cut and the other half is uncut resulting in a mixture of 228,120 and 108-bp fragments

DISCUSSION

Periodontitis is a common oral disorder characterized by an inflammatory response towards pathogenic oral bacteria. There is a lack of data regarding prevalence of periodontitis among the Indian population. However, Vandana et al demonstrated that the prevalence increased with age and was significantly more among females [Vandana KL, Reddy MS, 2007]. It is often considered as a multifactorial condition involving both genes and environment. Our study reported a significant involvement of age and BMD similar to the findings of Taboulet et al [Taboulet J, et.al., 1998] where a strong association of CTR polymorphism with osteoporotic fractures and BMD in postmenopausal women was reported. Koduganti et al., in 2009 (Rekha Rani Koduganti, et.al., 2009) reported that the proportion of women with normal BMD, declines with increasing age and this makes them more susceptible to osteroporosis than men. Since, majority of our patients were females belonging to post-menopausal period undergoing hormonal changes (Esfahanian V, et.al., 2012) and showed BMD above 2.5, we infer that these women are osteoporotic and are at higher risk for periodontitis.

A large number of candidate gene polymorphisms have been investigated till date to identify the role of a specific genetic determinant in the causation of periodontitis [18]. Of them, CTR 1340, T>C polymorphism has generated interest precisely since this SNP is associated with reduced BMD and predisposes women to osteoporosis often considered as a risk factor for progression of periodontitis.

Our studies demonstrated predominance of CT (42% vs. 32%) and CC (56% vs. 45%) genotype in patients and controls respectively and these findings were similar to the observation by Masi et al (1998) and Nakamura et al (1997) (Laura Masi , Maria Luisa Brandi , 2007, Nakamura M, et.al.,2001). The distribution of genotypes did not differ significantly between the groups ($p=0.62$), indicating no association of CTR 1340 polymorphism with periodontitis. This discrepancy could be due to ethnic heterogeneity and small sample size. Also, it has been reported by Martin et al that there are wide variations in the efficiency of calcitonin in different receptor/response systems (Martin TJ, et.al.,1995).

Additionally, when the frequency of genotypes was analyzed in males and females separately, a significant involvement of CTR SNP with periodontitis was observed only in females. It also demonstrated that women with T allele (OR: 2.40) or CT genotype (5.62) are at increased risk for periodontitis compared to normal females. Our study was in accordance with the findings of Taboulet et al (Taboulet J, et.al., 1998) in Caucasian population where they observed CT to be associated with higher BMD compared to CC and TT genotypes.

According to Stevenson et al (Stevenson JC, et.al., 1981) postmenopausal women tend to have decreased estrogen and calcitonin levels and upon estrogen supplementation, showed marked increase in the level of calcitonin. Added to that, a proline to leucine change in the CTR alters its secondary structure making it ineffective to bind to calcitonin. This hampered interaction modulates signal transduction pathways in osteoclasts that induces osteoclast motility thereby causing bone resorption. Furthermore, Nosaka reported a correlation between CTR polymorphism and buccal margin bone loss in subjects undergoing implants on maxillae and mandibles, indicating role of CTR polymorphism with periodontitis (Nosaka Y, et.al., 2002, Suzuki A, et.al., 2004).

CONCLUSION

We conclude that the CTR gene polymorphism is not associated with periodontitis in males while demonstrated a significant involvement in females, indicating its importance in the etiology of periodontitis in women. Due to modest sample size, replicative studies on large subjects from different ethnic groups are required to clarify the relationship between this polymorphism and periodontitis before considering it as a molecular marker.

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