Original Article

RANKL Gene Polymorphism as a Potential Biomarker to Identify Acute Charcot Foot Among Indian Population With Type 2 Diabetes: A Preliminary Report

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Abstract

Studies addressing the link between gene polymorphism and Charcot neuropathic osteoarthopathy (CN) have been limited to analyse osteoprotegerin gene. Aim is to understand the association of RANKL gene variants on the susceptibility of diabetic neuropathy and CN and to measure the serum levels of sRANKL among Indian population with type 2 diabetes. 77 subjects (48 males: 29 females) were recruited and divided into 3 groups. Group 1 Control: normal glucose tolerance (NGT). Group 2: Type 2 diabetes mellitus and neuropathy (DPN). Group 3: Established type 2 diabetes mellitus, DPN, and CN. Subjects were genotyped for RANKL SNP 693 C/G and 643 C/T using polymerase chain reaction-restriction fragment length polymorphism. sRANKL levels were measured using ELISA (enzyme-linked immunosorbent assay). The serum levels of sRANKL were significantly different between the 3 groups. In RANKL -643 C/T the frequency of "CT" genotype and the minor allele "T" was greater among the DPN and CN group compared with the NGT. Further statistical analysis found a significant difference in genotypic frequencies between DPN and NGT subjects with CT genotype. In RANK L -693 C/G the frequency of homozygote mutant "GG" and the minor allele "G" was greater among the DPN and CN group compared with the NGT. Significant differences in genomic frequencies were observed among "GG" genotype. RANKL -643 C/T was significantly associated with DPN alone while -693 C/G was significantly associated with both DPN and CN. Thus, the study suggests RANKL polymorphism might be considered as an independent risk factor for the development of CN.

Keywords

neuropathy, osteoarthopathy, RANKL, gene polymorphism

Charcot neuropathic osteoarthopathy (CN) is a long-term continuing condition of bones, joints, and soft tissues, which occur most often in the region of the foot and ankle as an outcome of peripheral neuropathy. CN is indicated by a confined inflammatory action in the prior phases and systematic progress of bone depletion, joint displacement, and firm disfigurement.[1] A data mining approach to see the association of CN with other disorders using the International Classification of Disease, Revision 9, diagnosis code done at the University of Michigan, revealed that 87.2% of CN subjects had type 2 diabetes preceding CN.[2] The acute phase relates inflamed CN and the chronic phase relates to stable CN.[3] On an average 0.4% to 13% of diabetes subjects develop this defect, which frequently goes unidentified in the early phase when it is difficult to distinguish between acute osteomyelitis and CN.[4] Previous studies showed that the mortality proportion is 28.3% in CN population.[5] Management of CN is clinically challenging because of the specific mechanism of disease development, which is still obscure. Elevated osteoclastic activity is marked for enormous and unfounded bone turnover.[6] The role of RANKL system in acute CN pathogenesis is

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proven by the fact that the same RANK/RANKL/OPG system is also involved in the development of medial arterial calcification, which could be the reason for combining the basic hypothesis for the rudimentary pathogenesis supporting the inconsistent osteolysis and medial arterial calcification seen in CN and similar disease conditions.[7] It is shown that RANKL mediates osteolysis in CN by activating monocytes to differentiate into osteoclast.[8] This RANKL gene has been linked with Charcot foot predisposition in candidate gene association studies. Single nucleotide polymorphism (SNP) identified in the promoter and 5 untranslated region of the RANKL gene has shown a correlation with bone mineral density.[9,10] The functional part of these polymorphisms has not been enlightened. Studies have observed an uplifted transcriptional action of those SNPs that may correspond to elevated RANKL protein rate that culminates in bone metabolism asymmetry.[11]

Diabetes accelerates peripheral neuropathy, which may induce foot ulceration and is also associated with substantial morbidity, mortality, and socioeconomic concern.[12] RANKL diabetic neuropathy is also associated with increased incidence of calcification of arterial wall in diabetes.[13] Identifying genetic polymorphism will help in earlier prediction of this complication among the Indian population with type 2 diabetes. There are limited data related to genes associated with DPN and CN. In this preliminary report, we aimed to investigate the association between the RANKL 693 C>G and 643 C>T gene polymorphisms and serum sRANKL levels among the study population.

Materials and Methods

Study Population

A total of 77 (48 males and 29 females) subjects were recruited for this study. The subjects were divided into 3 groups. Group 1: 26 subjects with normal glucose tolerance (Controls), Group 2: 25 type 2 diabetes mellitus subjects with neuropathy (DPN). Group 3: 26 type 2 diabetes subjects with neuropathy and Charcot foot (CN). The study subjects were selected from the outpatient department of a tertiary care center for diabetes. Subjects were classified into groups based on the occurrence of clinical features and characteristics of CN, DPN, and diabetes. Diagnosis of diabetes was based on the World Health Organization criteria (WHO report; 2006). Eligibility for CN group was primarily based on the history and clinical examination of the foot. The ethics committee of the institute approved the study (No. IEC-005/07/2018), and all the participants gave written informed consent. The study was carried out in accordance with the Declaration of Helsinki ethical principles on human studies, and all the experiments were conducted in accordance with the approved guidelines and regulation.

Inclusion and Exclusion Criteria

Subjects with fasting plasma glucose (FPG) <5.6 μmol/L (100 mg/dL) and 2-hour post glucose (PG) value <7.8 μmol/L (140 mg/dL) during an oral glucose tolerance test formed the NGT group. Type 2 diabetes was defined when FPG level was 7.0 μmol/L (126 mg/dL) and/or 2-hour PG level was ≥11.1 μmol/L (200 mg/dL). All the subjects included in this study were unrelated individuals of Indian origin aged 30 to 65 years. Patients diagnosed with other potential causes of damage to the peripheral nervous system, such as vitamin B_{12} deficiency, use of neurotoxic drugs, hereditary neuropathy, abuse of alcohol, or heavy metal poisoning were excluded from the study.

Diagnosis of DPN and CN

Type 2 diabetic subjects with a vibration perception threshold ≥25 using biothesiometer were considered abnormal and diagnosed as DPN. Patients were classified as having CN primarily based on history and clinical examination. Both dorsal and plantar surfaces of the foot were examined for swelling, erythema, increase in temperature, and any musculoskeletal deformity, which was later on confirmed by X-rays (Figure 1).

Sample Collection

After fasting for 12 hours, from each subject 2 to 3 mL of blood was collected from the cubital vein into ethylenediaminetetraacetic acid containing tubes (BD Vacutainer k_2 EDTA) and another tube without the anticoagulant from each patient. After collecting the blood, the plasma was separated and the tube without anticoagulant was left undisturbed in room temperature and the blood was allowed to clot, and serum was separated by centrifugation at 2500*g* for 10 minutes.

Anthropometric Measurements and Biochemical Analysis

Anthropometric and demographic details like age, weight, height, and duration of diabetes were recorded for all the study subjects. Biochemical analysis was done using fully automated biochemistry analyzer using commercial kits (Diasys). Estimation of glucose was by glucose oxidase peroxidase method, serum cholesterol by cholesterol oxidase peroxidase amidopyrine method, serum triglycerides (TGL) by glycerol phosphate oxidase peroxidase amidopyrine method, high-density lipoprotein cholesterol by direct method with polyethylene glycol pretreated enzymes, and creatinine by Jaffe's method. The intra- and interassay coefficients of variation for the biochemical assays ranged between 4.5% and 6%.

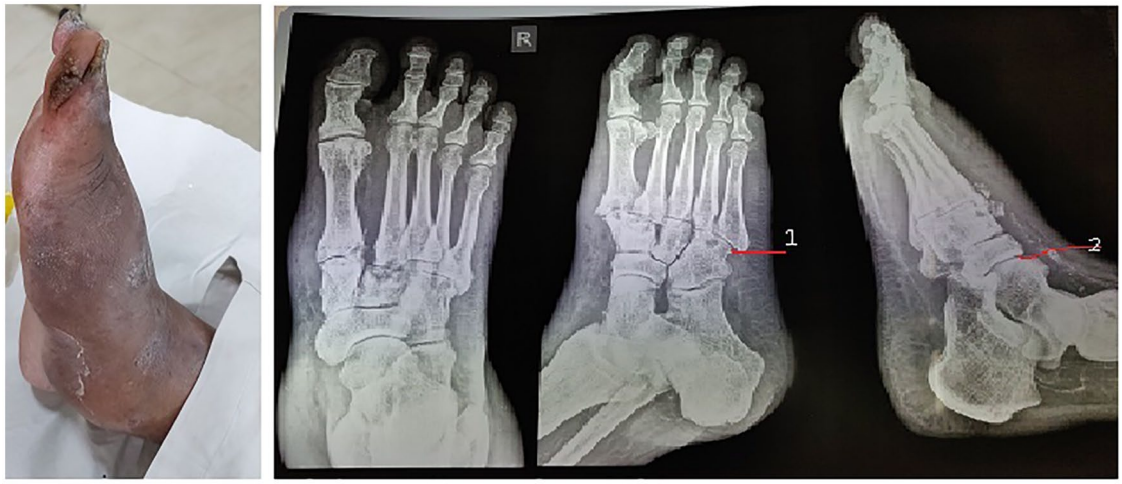

Figure 1. Image of acute Charcot neuropathic osteoarthopathy. (A) Clinical image. (B) X-Ray image—(1) Altered trabecular pattern over the tarsal bones and bone resorption involving all tarsal bones and (2) osteophytes formation.

Table 1. Primer Sequences, Amplicon Sizes, and the Restriction Enzymes Used in the Study.

	Gene Symbol	SNP Position	Reference SNP Number	Primer Sequences	PCR Product Size (bp)	Restriction Enzyme	Expected Band Patterns (bp)
1	RANKL	643 C/T	rs9533156	F: 5′TGGTCAGCAACTTCCTTCTG3′ R: 5′GACATTCCTCCTGCATCCAT3′	299	Tspr1 (65°C)	299, 193, and 106
2	RANKL	693 C/G	rs9533155	F: 5′TGGTCAGCAACTTCCTTCTG3′ R: 5′GACATTCCTCCTGCATCCAT3′	299	BseD1 (55°C)	159, 140, and 299

Abbreviations: SNP, single nucleotide polymorphism; PCR, polymerase chain reaction.

Glycated hemoglobin A1c was estimated by high-pressure liquid chromatography (Bio-Rad). All the biochemical parameters were assayed in National Accreditation Board for Laboratories accredited laboratories. sRANKL levels in the blood serum were measured using enzyme linked immunosorbent assay (ELISA) method. sRANKL kit available commercially (elabscience) were used for analysis as per manufacturer's protocol. The detection limit was 0.1 ng/mL. The intra- and interassay coefficients of variation for the assays were less than 5%.

SNP Genotyping

Genomic DNA was isolated using QIAmp DNA blood mini kit (Qiagen). The DNA at 260 nm/280 nm concentration was assayed using Nano Drop Spectrophotometer. Genotypes for RANKL 693 C/G (rs9533155) and 643 C/T (rs9533156) SNPs were determined by PCR followed by restriction fragment length polymorphism. The primer sequences, anplicon sizes, and the restriction enzymes used are shown in Table 1.

The PCR amplification of the related regions were carried out in 25 µL mixture containing 12.5 µL takara PCR master mix (Takara, Germany), 1.5 µL of each set of specific primers, 7.5 µL molecular grade water, and 2 µL DNA samples. The PCR condition were 95°C for 5 minutes followed by 30 cycles at 95°C for 1 minute, at 58.5°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension was carried out at 72°C for 7 minutes in an automated thermal cycler (Biorad T100). Restriction digestion was done using Tspr1 enzyme, and 10 µL of PCR products were used. The determination of the restriction enzyme, specific for these 2 polymorphisms, were predicted by New England Biosciences cutter version 2 software. The amplified products were analyzed by electrophoresis on 2% agarose gel (Figures 2).

Data Validation

Nearly 10% of the samples that showed heterozygous genotypes were chosen and the samples were genotyped for the same assay for the second time.

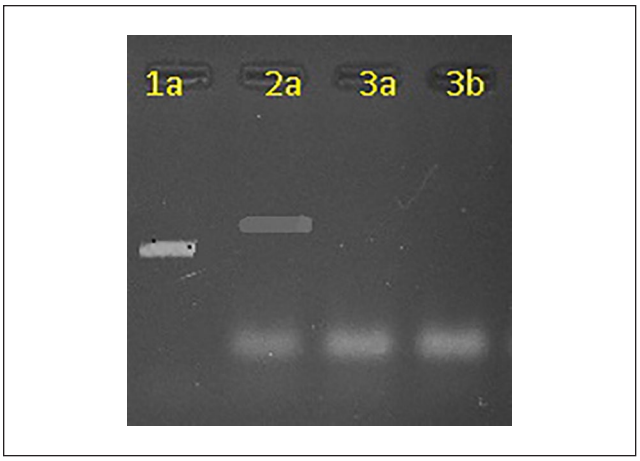

Figure 2. Agarose gel images of Bsdr1 digested products. (1a) Mutated homozygote GG, (2a) heterozygote GC, (3a and 3b) wild type CC.

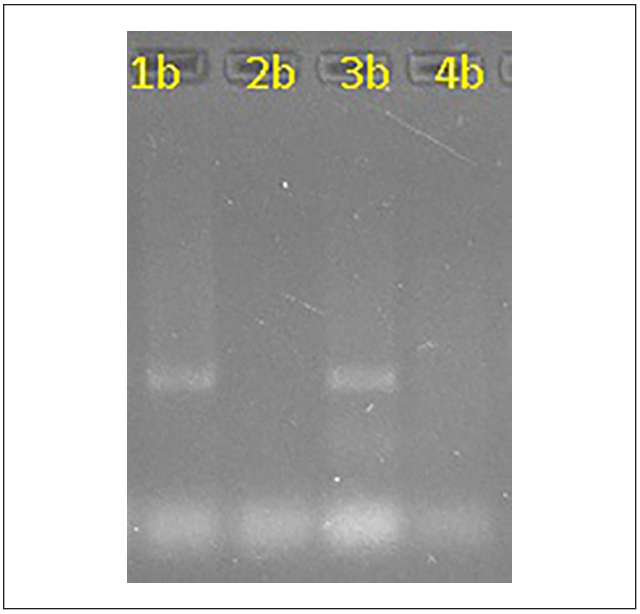

Figure 3. Agarose gel images of Tspr1 digested products. (1b) Wild type CC, (2b and 4b) mutated homozygote TT, (3b) heterozygote CT.

Statistical Analysis

Statistical calculations were performed using software SPSS for windows (Version 20.0; SPSS, Chicago, IL). Normally distributed data are presented as mean ± SD. The Hardy-Weinberg equilibrium was tested with the χ^2 test. Genotype distribution and allele frequencies were compared between groups using a χ^2 of independence with 2×2 contingency and Z statistics. One-way ANOVA (analysis of variance) was used to determine statistical significance between groups. The OR (odds ratio) with 95% CIs (confidence intervals) were calculated using Medcalcs statistical software version 18. A P value of $<.05$ was considered as statistically significant.

Results

Clinical and Biochemical Characteristics of the Study Groups

Table 2 shows the clinical characteristics and serum biomarker levels of the study subjects. The DM subjects (DPN and CN) were significantly older and had higher systolic blood pressure, FPG, postprandial plasma glucose, and glycated hemoglobin A1c. Low-density lipoprotein cholesterol levels were significantly lower in DPN and CN groups compared with NGT subjects, since most of them were on statin therapy. The serum levels of sRANKL were elevated in DPN and CN groups as compared to NGT group. One-way ANOVA showed that the sRANKL levels were statistically significant between the 3 groups ($P = .008$).

Genetic Association of RANKL SNPs With Disease Phenotype-DN and CN

Tables 3 to 6 summarize the genotype and allele frequencies of 643 C/T and 693 C/G RANKL SNPs among the study groups. The genotypic distribution of these SNPs was found to be in Hardy-Weinberg equilibrium. The allelic frequencies as well as the genotypic distribution of the RANKL 643 C/T and 693 C/G polymorphism differed significantly between DPN, CN, and NGT. In both the polymorphisms "CC" genotype was more frequent among NGT subjects as compared with DPN and CN (Tables 3 and 4). In addition, for RANKL 643 C/T, the homozygous genotype "TT" of the minor allele as well as the heterozygous genotype "CT" were more frequent in the DPN (4% and 64%) and CN (15.4% and 23.1%) compared with the NGT (0% and 11.5%; Tables 3 and 4). Similarly, for RANKL 693 C/G, the homozygous genotype "GG" of the minor allele as well as the heterozygous genotype "GC" were more frequent in the DPN (28% and 40%) and CN (61.5% and 15.4%) compared with the NGT(3% and 0%; Tables 3 and 4). The frequency of the common homozygote genotype "CC" (wild type) found among the control group (NGT) is considered as the reference genotype to find out the association of these polymorphisms with DPN and CN. There was no significant difference in the genotypic frequencies for RANKL (643 C/T and 693 C/G) polymorphism between DPN and CN subjects (Table 5).

There was a significant difference in the genotypic frequencies for RANKL (643 C/T) polymorphism between DPN and NGT subjects. OR was calculated in comparison

Table 2. Clinical Characteristics and Serum Biomarker Levels in the Study Groups[a].

Variables	Group 1: Controls (n = 26)	Group 2: DPN (n = 25)	Group 3: CN (n = 26)	P^b
Gender (male–female)	14:12	16:9	18:8	—
Age (years)	48.5 ± 8.9	56 ± 6.3	55.3 ± 7.2	**<.001**
Body mass index (kg/m^2)	27.9 ± 3.9	27.7 ± 6.1	27.5 ± 4.0	.934
Systolic BP (mm Hg)	120.8 ± 11.9	132.8 ± 19.4	131.9 ± 16.0	.014
Diastolic BP (mm Hg)	79.2 ± 7.4	81.9 ± 8.5	80.4 ± 7.2	.456
Fasting plasma glucose (mg/dL)	91.9 ± 7.6	182.1 ± 90.35	156.3 ± 55.11	**<.001**
2-Hour post glucose (mg/dL)	100.7 ± 19.4	279.6 ± 97.9	237 ± 82.4	**<.001**
Glycated hemoglobin (%)	5.4 ± 0.21	8.74 ± 1.2	8.41 ±1.8	**<.001**
Total serum cholesterol (mg/dL)	195.6 ± 55.8	161.5 ± 42.6	173.1 ± 52.2	.067
Serum triglycerides (mg/dL)	120.4 ± 55.2	175.5 ± 50	161.2 ± 50	.306
HDL cholesterol (mg/dL)	50.2 ± 11.1	43.9 ± 11.8	41.4 ± 10.5	.026
LDL cholesterol (mg/dL)	114.3 ± 38.4	89.5 ± 25.3	100.7 ± 36.4	.044
VLDL Cholesterol (mg/dL)	31.2 ± 13.7	28.1 ± 21.5	31.7 ± 17.6	.756
Urea (mg/dL)	23.3 ± 12	32.8 ± 22.1	28.1 ± 12.9	.168
eGFR (mL/min)	76.9 ± 19.7	67.7 ± 20.7	69.6 ± 18.3	.274
Serum biomarker				
sRANKL (ng/mL)	5.12 ± 3.1	7.4 ± 4.1	8.9 ± 5.3	**.008**

Abbreviations: DPN, diabetes mellitus subjects with neuropathy; CN, Charcot neuropathic; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; eGFR, estimated glomerular filtration rate.
[a]All data are reported as mean ± SD, unless specified.
[b]$P < .05$ is considered as significant. $P < .001$ is considered as highly significant.

Table 3. Genotypic Frequencies of RANKL (-643 C/T and -693 C/G) SNPs Among NGT and DPN[a].

	NGT (n = 26)	DPN (n =25)	OR (95% CI)	*P*
RANKL 643 C/T				
CC	23 (88.46%)	8 (32%)	Ref	Ref
CT	3 (11.54%)	16 (64 %)	**15.33** (3.52-66.84)	**.003**
TT	0	1 (4%)	—	—
RANKL 693 C/G				
CC	23 (88.46%)	8 (32%)	Ref	Ref
GC	0	10 (40%)	—	—
GG	3 (11.54%)	7 (28%)	**6.708** (1.39-32.36)	**.0178**

Abbreviations: SNP, single nucleotide polymorphism; NGT, normal glucose tolerance; DPN, diabetes mellitus subjects with neuropathy.
[a]Values are number (%).

Table 4. Genotypic Frequencies of RANKL (-643 C/T and -693 C/G) SNPs Among NGT and CN[a].

	NGT (n = 26)	CN (n = 26)	OR (95% CI)	*P*
RANKL 643 C/T				
CC	23 (88.46%)	16 (61.5 %)	Ref	Ref
CT	3 (11.54%)	6 (23.1%)	2.875 (0.625-13.22)	.1759
TT	0	4 (15.4%)	—	—
RANKL 693 C/G				
CC	23 (88.46%)	6 (23.1%)	Ref	Ref
GC	0	4 (15.4%)	—	**—**
GG	3 (11.54%)	16 (61.5%)	**20.44** (4.44-94.01)	**.0001**

Abbreviations: SNP, single nucleotide polymorphism; NGT, normal glucose tolerance; CN, Charcot neuropathic; OR, odds ratio; CI, confidence interval.
[a]Values are n (%).

with the most common homozygote genotype observed among the subjects. Significant difference was found in genotypic frequencies for RANKL 643 C/T polymorphism between DPN and NGT subjects with "CT" genotype (Table 3); however, no significant genotypic difference was found for TT genotype. This clearly indicates the association of this variant with DPN. However, there was no significance in "CT" genotype and "TT" genotype among CN and NGT (Table 4).

Interestingly, significant differences in genomic frequencies were observed for RANKL (693 C/G) polymorphism between DPN and healthy controls (Table 3). Similarly significant differences in genomic frequencies for same polymorphism was observed between CN and NGT (Table 4). In 643 C/T polymorphism of RANKL gene greater frequency of "T" allele among the DPN and CN groups as compared with NGT group (Table 6). Similarly in 693 C/G polymorphism of RANKL gene there was greater frequency of "G"

Table 5. Genotypic Frequencies of RANKL (-643 C/T and -693 C/G) SNPs Among DPN and CN[a].

	DPN (n = 25)	CN (n = 26)	OR (95% CI)	*P*
RANKL 643 C/T				
CC	8 (32%)	16 (61.5%)	Ref	Ref
CT	16 (64%)	6 (23.1%)	0.1875 (0.053-0.664)	.0095
TT	1 (4%)	4 (15.4%)	2.0 (0.191-20.97)	.5632
RANKL 693 C/G				
CC	8 (32%)	6 (23.1%)	Ref	Ref
GC	10 (40%)	4 (15.4%)	0.533 (0.11-2.56)	.4326
GG	7 (28%)	16 (61.5%)	3.0476 (0.765-12.13)	.114

Abbreviations: SNP, single nucleotide polymorphism; DPN, diabetes mellitus subjects with neuropathy; CN, Charcot neuropathic; OR, odds ratio; CI, confidence interval.
[a]Values are n (%).

Table 6. Allelic Frequencies of RANKL (-643 C/T and -693 C/G) SNP'S in NGT, DPN, and CN[a].

	NGT (n = 26)	Neuropathy (n = 25)	Charcot Osteoarthopathy (n = 26)
RANKL 643 C/T			
C	49 (94.23%)	32 (64%)	38 (73.1%)
T	3 (5.77%)	18 (36%)	14 (26.9%)
RANKL 693 C/G			
C	46 (88.46%)	26 (52%)	16 (30.77%)
G	6 (11.54%)	24 (48%)	36 (69.23%)

Abbreviations: SNP, single nucleotide polymorphism; NGT, normal glucose tolerance; DPN, diabetes mellitus subjects with neuropathy; CN, Charcot neuropathic.
[a]Values are n (%).

allele among the DPN and CN groups as compared with NGT group (Table 6). The increase in GC and GG genotypes for DPN and CN subjects may indicate the importance of the variant alleles in the disease conditions. This polymorphism may therefore, play an important role in the pathogenesis of CN.

Discussion

CN disease is not absolute vascular or endothelial disease but rather an inflammatory condition that targets bones and joints. RANKL, the member of tumor necrosis factor-α, has been identified to affect the immune system and control bone regeneration and remodelling.[14] It is the final effector for osteoclastogenesis sequence variation in the promoter region that could affect the transcription of the gene and levels of RANKL protein.[14] The current study highlights findings in Indian subjects with type 2 diabetes. sRANKL levels in the serum of DPN and CN subjects were significantly elevated compared with the control subjects. This is in agreement with the previous studies.[7,13] In conjunction with our results, this association marks RANKL as an essential factor of bone metabolism. The action of RANKL mediated osteoclastic resorption in acute CN is supported by several studies.[7,14] The RANKL/RANK/OPG signaling pathway has a crucial role in bone remodeling regulation.[15] Our preliminary data deliver the initial key in implementing analysis of RANKL expression in serum to diagnose acute CN. We hypothesize that the prospective study might pave way to use the RANKL levels as a correlative tool that can be employed to get quantifiable factor, that may assist the clinicians to keep track of disease progression in subjects with acute CN. Further studies on other ethnic populations are warranted to assess a temporal relation between RANKL levels and incidence of Charcot foot among diabetic neuropathy subjects.

We have studied 2 SNPs in RANKL gene and found a significant genotypic difference among DPN and CN compared with the control subjects. In both the polymorphisms, "CC," which is a wild genotype, was more frequent among control subjects compared with DPN and CN subjects.

In RANKL 643 C/T there was a significant difference in the genotypic frequencies between DPN and NGT subjects for CT genotype; however, no significant genotypic difference was found for TT genotype in comparison with the control group. No significant association of the genotype CT and TT was found between CN and controls. This clearly indicates the association of this variant with DPN alone. Similar genotypic studies were seen among other osteo-related disease conditions. A study found allelic frequencies of 643 C/T to be significantly different between ankylosing spondylitis patients and normal controls.[16] The results of the recent study indicated that CT genotype of RANKL 643 C/T polymorphism was significantly associated with peri-implantitis, and may be considered as a genetic determinant for peri-implantitis.[17] These results are in agreement with another study which also showed the association with CN and DPN.[13] In RANKL 693 C/G polymorphism interestingly significant differences in genomic frequencies were observed between DPN and NGT and also between CN and NGT subjects for both GC and GG genotypes. GG genotypic frequency was found to be highly significant among CN in comparison with NGT group. Another recent study in Tunisian postmenopausal women with osteoporosis did not find a significant association of 693 C/G with the above-mentioned disease condition.[18] The prevalence of this polymorphism was studied in DPN, CN, and diabetes subjects without DPN and CN alone, and it showed almost same distribution among all the subjects and formed discrete cluster in the hierarchical clustering

analysis.[13] This gives us a precise picture that RANKL 693 C/G is strongly associated with CN, a long-term complication of DPN. In CN there is neither fever nor any major biological inflammatory syndrome and this clinical presentation frequently bring about unreliable or deferred diagnosis, culminating in development to chronic phase with irreversible deformation. Initial diagnosis of CN can rescue a lasting period of distress of the patient, high hospital costs, and ultimately amputation.

In conclusion, to our knowledge, this is the first study showing a significant association between the SNP of the RANKL gene and the susceptibility of CN in Indian Population with type 2 diabetes. We note that this mutation is significantly associated with DPN and CN, confirming its involvement in the complication of diabetes leading to elevated RANKL levels, thereby increased osteoclastogenesis. Hence, this polymorphism may, play a vital role in the pathogenesis of CN. This preliminary report limits to the small sample size but further prospective study with large sample size may pave way to further elucidate these findings. This will serve as a basic research platform for further research to know the mechanism or the functionality of the variant of RANKL gene.

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Declaration of Conflicting Interests

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