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Original Article

Genetics Section

Association between Single Nucleotide Polymorphisms of *SMAD3* and *BMP5* with the Risk of Knee Osteoarthritis

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ABSTRACT

Introduction: The role of genetic factors influencing osteoarthritis (OA) susceptibility is well documented and several candidate genes have been identified to be associated with it. Among these genes are Bone Morphogenetic Protein 5 (*BMP5*) and Smad family member 3 (*SMAD3)*, all involved in Transforming Growth Factor (TGF) signaling pathway. The knee is the commonly affected joint, and knee OA has an especially high prevalence in Asian population.

Aim: To investigate associations between Single Nucleotide Polymorphisms (SNPs) rs12901499 in *SMAD3* and rs921126 in the *BMP5* gene with knee OA susceptibility in and around Lucknow, Uttar Pradesh, India.

Materials and Methods: SNPs rs12901499 in *SMAD3* and rs921126 in *BMP5* were genotyped in patients with knee OA and age- sex matched OA-free controls from our population. A total of 450 patients with knee OA and 458 controls were enrolled in the study. Venous blood samples were obtained from all cases as well as controls for PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism). Data was collected and entered in excel sheets. Statistical analyses of the data were performed using statistical software package SPSS version 16.0. Chi-square, Student's t-test and logistic regression tests were used to analyse the data.

Results: GA and GG genotypes of both SNPs (rs12901499 and rs921126), and variant G, were associated with a significantly increased risk of knee OA. A significantly increased risk of knee OA was associated with the genotype GG and GA of rs12901499 ($p < 0.03$ and $p < 0.004$ respectively) and rs921126 ($p < 0.0001$ and $p<0.001$ respectively) compared with the AA genotype. In addition, those bearing at least one G allele (GG + GA) had a significantly increased risk of knee OA compared with those without the G allele (AA) in rs921126 ($p< 0.0001$). However, in rs12901499, significant association with the risk of knee OA was not found ($p<0.4$). On age and gender based stratification, the association between the risk of OA and rs921126 GG mutant compared with AA homozygotes was strong in both gender (adjusted OR= 2.93 for male and 2.25 for female) and in those aged >55 years (adjusted OR= 3.4), similarly in rs12901499, GG mutant compared with AA homozygote was strong in female (adjusted OR= 1.5) and in those aged >55 years (adjusted OR= 1.5).

Conclusion: The results showed that both in *SMAD3* rs12901499 and *BMP5* 921126, G allele is significantly associated with knee OA. A to G change and variant G genotype may contribute to knee OA risk in our study population of Lucknow.

Keywords: Allele, Body mass index, Deoxyribonucleic acid, Kellgren-lawrence grade

INTRODUCTION

Osteoarthritis is the most common degenerative arthritis, a type of arthritis that is caused by breakdown of articular cartilage of the joints [1]. The prevalence of OA is high and expected to increase in the coming years [2]. About 80% of population having radiographic evidence of OA by the age of 65 years, only 60% is symptomatic [3]. In India, no data is available on prevalence but it is estimated that more than 30-40% of our population suffer from OA beyond the age of 50 years [4]. OA is a multifactorial disease and involves many factors like age, sex, BMI and genetic, environmental factors [5-9]. The role of genetics in OA is established for long and several candidate genes are found to be associated with it [10-12]. *SMAD3* [13,14] and *BMP5* [15,16] gene are under investigation due to their involvement in (TGF-β) signaling pathway [17].

TGF-β has anabolic effects on chondrocytes especially via the Smad3 genes signaling which plays a pivotal role in the homeostasis of synovial joints [18]. A relationship between the genetic variants of TGF-β itself, TGF-β signalling and binding molecules, and OA, has been reported in humans [19]. In signalling pathway of TGF-β, phosphorylated Smad3 forms a complex with Smad4 which translocates to the nucleus to regulate gene expression and promote an anabolic phenotype in cartilage [20]. Valdes AM et al., studied the association of knee OA with 10 different SNPs. It was reported that four out of ten SNPs (rs266335, rs12901499, rs6494629, and rs2289263) were significantly associated with knee OA [13]. They also observed that the major allele G was found at a higher frequency among OA patients than among controls.

BMP5, a member of the TGF-β superfamily was found to be involved in synovial joint development and joint tissue homeostasis [21]. SNP 921126 of *BMP5* has shown significant association with hip osteoarthritis [16]. Based on these observations, we investigated the possible correlation between these SNPs of *SMAD3* (rs12901499) and *BMP5* (rs921126) with knee OA patients of North Indian population.

MATERIALS AND METHODS

This hospital-based case–control study was conducted in the Department of Orthopaedic Surgery, King George's Medical University (KGMU), Lucknow, Uttar Pradesh, India. A total of 450 consecutive patients diagnosed with knee OA between March 2010 and May 2015 were evaluated for inclusion.

The radiographic evaluation was performed on anteroposterior standing and lateral X-Ray views of the knee by a single investigator using the Kellgren–Lawrence (KL) score ranging between 0 and 4.

OA were classified into mild (K-L grade 2), moderate (K-L grade 3) severe (K-L grade 4) [22]. Only patients with radiographic OA, defined as KL score of ≥2, were included in the study. Persons suffering from other knee joint ailments like rheumatoid arthritis, gouty arthritis, septic arthritis, post traumatic or dysplasias were excluded.

A total of 458 age and sex-matched healthy volunteers were recruited from the same hospital during the same period. Selection criteria for the controls included no history of OA. Age, sex, weight, height, and Body Mass Index (BMI) were recorded for all study participants. Written informed consent was provided by all participants, and the study protocol was approved by the Ethics Committee of the KGMU, Lucknow, Uttar Pradesh, India.

Association of genotypes (rs12901499 and rs921126) of *SMAD3* and *BMP5* gene with OA knee was determined using standard chi-square test. Frequencies for the rs12901499 and rs921126 polymorphisms in the controls agreed with that expected according to the Hardy–Weinberg principle. Associations between the *SMAD3* or *BMP5* variants and OA risk were estimated by calculating the odds ratios (ORs) and 95% confidence intervals (CIs) using both univariate and multivariate logistic regression analyses with adjustments for age, sex, and BMI. Two sided tests were used for statistical analyses, and a p-value < 0.05 was considered to be statistically significant for comparison of continuous data of general characteristic of cases and controls, unpaired student t-test was performed. The statistical power of the study was calculated using formula "n=(r + 1/r) p (1-ϕ) (Zβ +Zα/2)2/ (p1 – p2)2" with proportion of Allele (A) exposed in controls (37%) and considering to detect Odd ratio of 2.0 and r =1. the calculated sample size was 150 in each case and controls. But, for increasing power of study we recruited 450 cases and 458 controls.

STATISTICAL ANALYSIS

All statistical analysis was performed with the SPSS software package (version 16.0 for windows; SPSS Chicago, IL).

DNA isolation and genotype analysis: Venous blood samples were obtained in ethylene diamine tetra-acetic acid anticoagulant (20g/l) for genomic DNA extraction. Samples were stored at -80 ºC until analysis. Isolated DNA was subsequently used for genotyping.

Detection of rs921126 and rs12901499 polymorphism of BMP5 and SMAD3 gene: Reaction was performed with 50–200 ng of genomic DNA, 0.2 µmol of each primer, 200 µmol of dNTP (Fermentas, USA), 1.2 mmol of $MgCl_2$, 1 unit of Taq polymerase (Fermentas, USA) and sterile MilliQ water in a total volume of 20 µl. Amplification was performed on GeneAmp PCR (Polymerase Chain Reaction) system 9700 (Applied Bio System) using the following specific PCR conditions for each polymorphism.

rs12901499 polymorphism: Initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58.6°C for 45 seconds and extension at 72°C for one minute. It was further followed by final extension at 72°C for 10 minutes. PCR reaction resulted in a 226 bp product. PCR products (10 µl) were digested with 10 U of MboII restriction enzyme (MBI Fermentas, Germany) to identify the presence of polymorphic sites in *SMAD3* gene. Digestion of 226bp PCR product of *SMAD3* gene with MboII restriction enzyme into two fragments of 176 bp and 50 bp indicated the presence of GG genotype and the presence of fragments of three sizes (226 bp, 176 bp and 50 bp) was indicative of AG genotype while the undigested 226 bp PCR fragment was indicative of AA genotype.

rs921126 polymorphism: Initial denaturation at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C or 45 seconds and extension at 72°C for one minute. It was further followed by final extension at 72°C for 10 minutes. PCR reaction resulted in a 794 bp product. PCR products (10 µl) were digested with 10 U of Dra1 restriction enzyme (MBI Fermentas, Germany) to identify the presence of polymorphic sites in *BMP5* gene. Digestion of 794 bp PCR product of *BMP5* gene with Dra1 restriction enzyme into two fragments of 424 bp and 360 bp indicated the presence of AA genotype and the presence of fragments of three sizes (794 bp, 424 bp and 360 bp) was indicative of the AG genotype while the undigested 794 bp PCR fragment was indicative of GG genotype.

RESULTS

A total of 958 subjects were evaluated. Of these, 450 had radiographic knee OA. In addition, 458 age- and sex- matched healthy controls were recruited. There were no significant differences in demographic characteristics between the two groups [Table/Fig-1].

The observed genotype frequencies for the rs12901499 in *SMAD3* and rs921126 in *BMP5* polymorphisms in the controls agreed with that expected according to the Hardy–Weinberg principle [23] (data not shown). The results of repeat genotyping of randomly selected samples were 100% concordant.

The genotype and allele distributions of the rs12901499 and rs921126 polymorphisms in patients with knee OA and healthy controls are shown in [Table/Fig-2]. The genotype distribution for rs12901499 andrs921126 was significantly different between the two groups (p<0.03 and p<0.001). After adjustment for age, sex, and BMI, a significantly increased risk of knee OA was associated with the genotype GG and GA of rs12901499 and rs921126 compared with the AA genotype. In addition, those bearing at least one G allele (GG + GA) had a significantly increased risk of knee OA compared with those without the G allele (AA) in rs921126. However, in rs12901499 were not significantly associated with the risk of knee OA.

Characteristics	Case(n=450)	Control(n=458)	p-value	95% CI
Age in years	54.56±9.30	53.93±8.55	0.265	-0.480 to 1.74
Height in m	159.27±8.60	160.12±8.04	0.106	-1.884 to 0.184
Weight in kg	63.64±9.15	63.91±8.31	0.62	-1.356 to 0.816
BMI in kg/m^2	25.44±3.24	24.99±4.29	0.06	-0.022 to 0.922

[Table/Fig-1]: General characteristics of cases and controls.
Data were as mean ± SD (SD: Standard Deviation)
Difference between groups was calculated using unpaired student t-test (p- value considered satisfactory significant).

Genotype	Case-450 (%)	Control-458(%)	OR (95% CI)	p-value
***SMAD3* gene**				
Comparison of genotype with OA				
AA(wt/wt)	165 (36.67)	158 (34.50)	Ref. group	
GA(wt/mt)	131(29.11)	198 (43.23)	0.63 (0.46 -0.86)	0.003
GG(mt/mt)	154 (34.22)	102 (22.27)	1.4 (1.03 - 2.01)	<0.03*
GA+GG(n=585)	285(48.72)	300(51.28)	0.9(0.69 to 1.19)	<0.4
Allele frequency				
A(wt)	461(51.22)	514(56.11)		
G(mt)	439(48.78)	402(43.89)	1.22 (1.01 - 1.5)	<0.03*
BMP5				
Comparison of genotype with OA				
AA(wt/wt)	142(31.56)	223(48.69)		
GA(wt/mt)	137(30.44)	129(28.17)	1.7 (1.2 to 2.3)	<0.001*
GG(mt/mt)	171(38.00)	106(23.14)	2.53 (1.83 to 3.49)	<0.0001*
GA+GG(n=543)	308(56.72)	235(43.28)	2.05(1.57 to 2.69)	<0.0001*
Allele frequency				
A (wt)	421(46.78)	575(62.77)		
G (mt)	479(53.22)	341(37.23)	1.92(1.59 to 2.31)	<0.0001*

[Table/Fig-2]: Comparison of genotypes (rs12901499 and rs921126) of *SMAD3* and *BMP5* gene between case and control.
Values are given as frequency. Differences were tested by Chi-square test. (* p-value <0.05 considered statistically significant)

When stratified by age, both young (<55 years) and old (>55 years) patients showed significant differences in genotype frequencies compared with controls (p<0.001 and p<0.0001) [Table/Fig-3].

Stratification analysis was performed to evaluate the potential association of genetic variants of *SMAD3* rs12901499 and *BMP5* rs921126 with knee OA risk in subgroups based on demographic characteristics. When stratified by age, both young (≤55 years) and old (>55 years) patients showed significant differences in genotype frequencies compared with controls (p<0.0001 and <0.04) and similarly in male and females (p<0.0001 and <0.03) [Table/Fig-3]. Likewise, in polymorphism rs9382564 both young (≤55 years) and old (>55 years) patients showed significant differences in genotype frequencies compared with controls (p<0.01 and <0.0001) and similarly in male and females (p<0.0001 and <0.0008) [Table/Fig-4].

When the association between G allele carriers and the risk of OA was evaluated using logistic regression analysis, GG homozygotes carried a 1.3 and 1.5 fold risks in the young and old patients group respectively. Likewise 1.3and 1.5-fold increased risk of OA compared with AA homozygotes in male and female group [Table/Fig-3].

Similarly, in the [Table/Fig-4], GG homozygotes carried a 1.91 and 3.4 fold increased risk of OA compared with AA homozygotes in the both age group and 2.93 and 2.25 fold increased in both gender groups. In addition, GA heterozygote carried a 1.6- and 1.7-fold increased risk of knee OA in young and old patients, respectively, compared with AA homozygotes. Likewise 1.59 and 1.76 fold increased risk of OA in male and female group [Table/Fig-4].

DISCUSSION

In this case control study, SNP rs921126 and rs12901499 were found associated with Knee OA. The result showed that SNP rs921126 polymorphic both (GG and GA) genotype of *BMP5* and variant G may contribute to the risk of knee OA and that this risk increased with age (>55) and in both gender.

To the best of our knowledge, this is the first report linking BMP SNP rs921126 with knee OA in North Indian population and first report with age, gender stratified association in any population. Further on this age, gender stratification, the association between risk of knee osteoarthritis and SNP rs921126, GG genotype compared with AA genotype was stronger in both genders and in those >55-year-old.

Many evidences are documented between OA pathogenesis and TGF-β signaling pathways [24,25]. *BMP5* is involved in the maintenance of synovial joint development and tissue homeostasis [21]. The SNPs in *BMP5* have been well documented. It was reported that SNP 921126 polymorphism in *BMP5* gene was associated with female hip OA [16]. In our case-control study we found that SNP 921126 was associated with Knee OA. Our results support the hypothesis that the SNP 921126 of *BMP5* gene might be a risk of OA. In the present study, the individuals carrying *BMP5* SNP 921126, the GG and GA or GG + GA genotypes had a higher risk of knee OA than those carrying the AA genotype. The G allele of *BMP5* SNP 921126 might be associated with the development of knee OA. This result is in consonant with a study by Wilkins JM et al., on female hip osteoarthritis [16].

Similarly, our study shows that *SMAD3* rs12901499 genetic variants, GG and GA genotype, were significantly associated with knee OA and variant G may contribute to the risk of knee OA. This result is similar to a study by Valdes AM et al., on knee and hip OA and in the Northeastern Chinese population between knee OA and *SMAD3* polymorphisms (rs12901499A/G and rs6494629T/C) [13], However Su SL et al., found no such associations [14]. Wu Q et al., suggested that Loss of *SMAD3* appears to enhance bone morphogenetic protein signaling in the articular chondrocytes, leading to hypertrophy and OA-like changes [26]. In addition Cherlet T et al., reported that *SMAD3* levels are lower in women than in men, which is consistent with other data showing that estrogens inhibit *SMAD3* transcriptional activity [27]. In addition, Valdes AM et al., found a significant association of SMAD3 intronic SNP with OA in both the genders [13]. Remarkable similarity in effect sizes between male and female subjects strengthened this finding of their study. This is consistent to our study in which gender stratified analysis found that there was an association between risk of knee osteoarthritis and SMAD3 SNP rs12901499, but none of the genotypes were strongly associated. However, the association of this polymorphism with age was also found and this finding is being reported for the first time.

		Cases				Controls			GG versus AA		GA versus AA	
Age group	n =450	AA	GA	GG	n=458	AA	GA	GG	Adjusted ratio	95% CI	Adjusted ratio	95% CI
≤55	n=205	66 (32.19)	59 (28.78)	80 (39.02)	n=216	58 (26.85)	107(49.54)	51(23.61)	1.3	0.8– 2.3	0.5	0.3 – 0.8
>55	n=245	99 (40.41)	72 (29.39)	74 (30.20)	n=242	100 (41.3)	91(37.60)	51(21.07)	1.5	0.9 – 2.3	0.8	0.5 – 1.2
Gender												
Male	n=220	80(36.36)	61(27.73)	79(35.91)	n=224	68(30.36)	106(47.32)	50(22.32)	1.3	0.8 – 2.2	0.51	0.3 – 0.8
Female	n=230	85(36.96)	70(30.43)	75(32.61)	n=234	90(38.46)	92(39.32)	52(22.22)	1.5	0.96 – 2.4	0.80	0.5 – 1.2

[Table/Fig-3]: Logistic regression analysis of rs12901499 in *SMAD3* genotype frequencies and risk of osteoarthritis (OA) in our population with OA of the knee and controls.
Data presented as n (%) of patients
OR odds ratio, CI confidence interval
Adjusted for the other covariate presented in this table and for body mass index using a logistic regression model for each stratum

		Cases				Controls			GG versus AA		GA versus AA	
Age group	n =450	AA	GA	GG	n=458	AA	GA	GG	Adjusted ratio	95% CI	Adjusted ratio	95% CI
≤55	n=205	67(32.68)	60(29.27)	78(38.05)	n=216	100(46.3)	55(25.46)	61(28.24)	1.91	1.21 – 3.01	1.62	1.0 – 2.6
>55	n=245	75(30.61)	77(31.43)	93(37.96)	n=242	123(50.83)	74(30.58)	45(18.60)	3.4	2.14 – 5.4	1.7	1.1 – 2.6
Gender												
Male	n=220	69(31.36)	70(31.82)	81(36.82)	n=224	110(49.11)	70(31.25)	44(19.64)	2.93	1.82 – 4.71	1.59	1.02 – 2.5
Female	n=230	73(31.74)	67(29.13)	90(39.13)	n=234	113(48.29)	59(25.21)	62(26.50)	2.25	1.45 – 3.48	1.76	1.11 – 2.78

[Table/Fig-4]: Logistic regression analysis of rs921126 in *BMP5* genotype frequencies and risk of osteoarthritis (OA) in our population with OA of the knee and controls.
Data presented as n (%) of patients
OR odds ratio, CI confidence interval
Adjusted for the other covariate presented in this table and for body mass index using a logistic regression model for each stratum

LIMITATION

Our study has certain limitations like a moderate sample size of 450 cases and 458 controls; therefore the results should be confirmed with larger sample size. Secondly only two *SMAD3* and *BMP5* SNPs were investigated; SNPs at other loci may also be associated with susceptibility to OA in TGF-β signaling pathway.

CONCLUSION

The present study was the first to show that the genotype distribution of the *BMP5* rs921126 was significantly associated with the risk of knee OA. Further, it validates the significant association of the *SMAD3* rs12901499 polymorphisms in our population. Following validation the effect of levels or functional roles of the rs921126 and rs12901499 polymorphism may help us in determining the aetiology of knee OA and thereby in formulating further research in its prevention and management.

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