Original Article

# Association of 4-basepair G-to-A transition in the 5′-untranslated region of ANKH gene with selected patients of primary knee osteoarthritis: A cross sectional study

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## Abstract

**Method:** A cohort study was carried out for a year to evaluate the presence of G-to-A transition in 5′-untranslated region of ankylosis human (*ANKH*) gene in Indian Khatri patients (closely resembling Europeans of primary knee osteoarthritis (OA), residing in Lucknow, India. **Results:** In the total participants, 25 were Khatri primary knee OA patients (cases) residing in Lucknow and 101 were random blood donors' samples (controls) collected from a blood bank. All were studied for the abovementioned mutation using real-time polymerase chain reaction (RT-PCR). GG genotype was present in 72.3% of controls and 76% of Khatri knee OA patients. The studied G-to-A mutation was found to be positive in 24.8% of controls and 16% of cases, odds ratio (95% confidence interval) being 0.6 (0.19–1.98, $P = 0.42$). The frequency of AA (D) genotype found around 3% (cases) and 8% (controls) with $P$ value of 0.70. The combined frequency of both homozygous and heterozygous mutation (GA and AA) in the studied population was 28 (27.7%) in controls and 6 (24%) in cases with the odds ratio (OD) ratio of 0.82 (0.29–2.27, $P = 0.70$). No significant differences were observed at both genotype and allelic level in the distribution of *ANKH-4* G-to-A gene polymorphism in studied subjects. **Conclusion:** This study did not show any significant G to A mutation in the studied subjects.

**Keywords:** Ankylosis human, chondrocalcinosis, genotype, Khatri, OA, osteoarthritis

## Introduction

Osteoarthritis (OA) as many other diseases is multifactorial in origin. This may partly explain why OA does not behave in the same way in its clinical presentation in every individual. Chondrocalcinosis (CC) has been shown to be frequently associated with calcium pyrophosphate dihydrate (CPPD) and basic calcium phosphate (BCP) crystal deposition in synovial fluid and articular cartilage as shown in the study by Derfus *et al.*[1] In one of the studies, CPPD and/or BCP crystals were found in the joint fluid in more than 50% of subjects at the time of total knee arthroplasty for OA.[1] Aging of cartilage which can be idiopathic, associated with metabolic conditions and in patients with OA, there is sustained elevation of extracellular inorganic pyrophosphate (PPi) in the joint, which stimulates

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**Received:** 18-06-2019 **Revised:** 19-06-2019 **Accepted:** 02-08-2019

Access this article online

Quick Response Code:

Website: www.jfmpc.com

DOI: 10.4103/jfmpc.jfmpc_471_19



**How to cite this article:** Kumar P, Sharma A, Das S, Srivastava R, Gupta N. Association of 4-basepair G-to-A transition in the 5′-untranslated region of ANKH gene with selected patients of primary knee osteoarthritis: A cross sectional study. J Family Med Prim Care 2019;8:2937-41.

deposition of CPPD crystals in articular cartilage. Levels of extracellular PPi are affected by transport of intracellular PPi to the exterior which involves ankylosis human (ANKH) protein, which is a transmembrane protein that regulates the bidirectional movement of the PPi between the cell and the extracellular space.[2] Expression of wild-type *ANKH* is increased in OA and chondrocalcinotic cartilage.[3] CC can be sporadic or familial. Familial CC can be caused by various mutations in *ANKH* gene. However, mutation of G-to-A transition in the 5'-untranslated region (5' UTR) 4-basepair (bp) upstream of the *ANKH* gene start codon has been associated with sporadic CC in Europeans.[4] Since we frequently encounter sporadic CC in OA, it was proposed to study the presence of G-to-A transition in 5'UTR region in the patients of primary OA in Khatri community residing in Lucknow, who most closely resemble Europeans.

## Method and Materials

It was a cross sectional study to study the presence of G-to-A transition in 5'UTR region of *ANKH* gene in selected patients of primary knee OA.

Study place: Department of Rheumatology and Transfusion medicine, KING George Medical University (KGMU), Lucknow.

Study population: All Khatri patients of primary knee OA attending out patient department (OPD) of Department of Rheumatology for management of OA. Healthy control subjects were blood donors, not receiving any medications, recruited from a blood bank.

## Inclusion criteria

A) All Khatri patients fulfilling the American College of Rheumatology (ACR) criteria for knee OA and age >50 years were taken as study subjects.

ACR criteria for knee OA taken were

a) Knee pain plus
b) One of the following
   1. Age >50 years
   2. Crepitus
   3. Stiffness <30 min
c) Plus osteophytes.

B) Healthy control subjects were blood donors, not receiving any medications, recruited from a blood bank.

## Exclusion criteria

1. Patients with hypercalcemia and hemochromatosis
2. Inflammatory arthritis, e.g., rheumatoid arthritis and psoriatic arthritis
3. History of knee trauma
4. Knee prosthesis
5. Secondary knee OA
6. Body mass index (BMI) >30 kg/m$^2$.

Clearance was taken from Institutional review board and ethical committee. Twenty-five Khatri patients of knee OA were taken as study subjects and hundred-and-one healthy controls (samples of random blood donors collected from a blood bank) were enrolled in the study. The study was funded by an internal grant from the Department of Rheumatology, KGMU.

A detailed history and physical examination was done. Investigations including X-rays of both the knees in lateral and anteroposterior view in the standing position and synovial fluid examination for CPPD crystals were done in patients presenting with synovial effusion as a part of routine care. We analyzed the studied mutation using RT-PCR. We used customized TaqMan single-nucleotide polymorphisms genotyping assay manufactured by ABI, Life technologies. The forward primer used in our study was CGCGGCAGCAGATGTG, while the backward primer used was GGGCCAGTAGTGCGTGAG. Reporter 1 (ATTTCACCATAGTCCCCGCC), which was a wild variety and VIC attached to it, gave green fluorescence while reporter 2 (TTCACCATAGTTCCCGCC), which was mutant variety and FAM dye attached to it, gave blue fluorescence on the detection of G > A transition.

## Statistical analysis

The statistical analysis was performed by SPSS 16.0 software. Hardy–Weinberg equilibrium was analyzed in Haploview (version 3.32, 2005). Dominant and recessive models were used for frequency analysis of various genotypes in males and females. Regression analysis was performed using SPSS software version 16.0 (SPSS, Chicago, IL, USA).

# Results

Mean and median ages of our patients were 58.68 ± 4.59 and 59 years, respectively. Mean and median weights of our patients were 61.84 ± 4.74 and 60 Kg, respectively. Mean and median BMIs of our patients were 23.85 ± 1.33 and 23.70 kg/m$^2$, respectively.

According to the median age, out of 25 patients, 16 (64%) patients were ≤59 years and 9 (36%) patients were >59 years. Out of total cases, 8 (32%) were male and 17 (68%) were female.

A) RT-PCR allelic discrimination plot for *ANKH* (4-G > A) gene polymorphism in studied subjects is shown in Figure 1.

The threshold value for amplification is mentioned in red.

B) Genotype and allelic association of G > A *ANKH* gene polymorphism in primary OA patients (cases) and controls is shown in Table 1 and Figure 2.

We found the studied mutation (G > A) in 25 controls out of 101 and 4 cases in our studied subjects out of 25. The distribution of *ANKH* G > A transition is shown in Table 2. The observed genotype frequencies of all the studied polymorphisms in controls were in accordance with Hardy–Weinberg equilibrium ($P > 0.05$). No significant differences were observed at both genotype

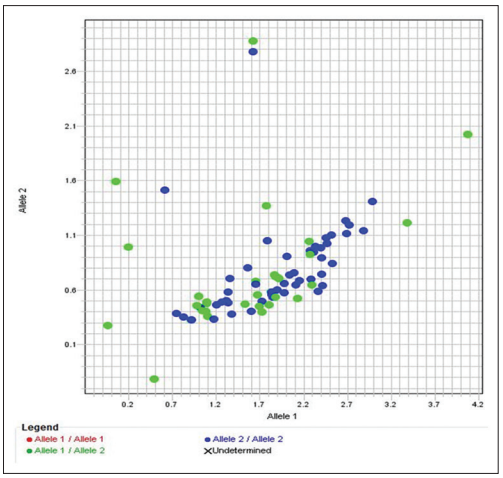


**Figure 1:** RT-PCR allelic discrimination plot for *ANKH* (4 G > A) gene polymorphism in studied subjects. RT-PCR amplification plot (Log graph)

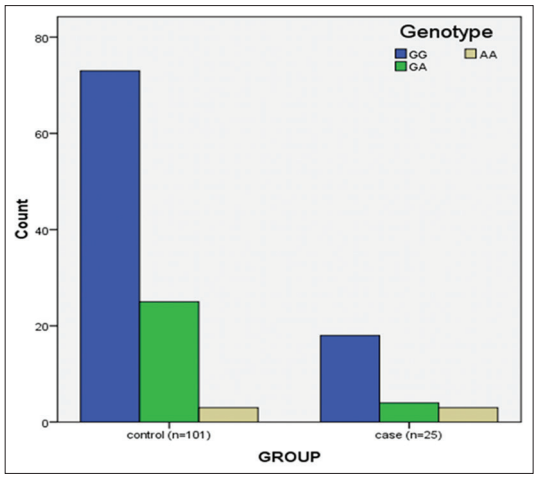


**Figure 2:** Distribution of genotype in control and case

and allelic level in the distribution of *ANKH-4* G > A gene polymorphism in cases.

C) Gender-wise distribution of genotype frequency in primary OA patients

The cases were stratified in males and females. We found a higher frequency of all three genotypes in females compared to male patients [Table 2]. These conflicting results may partly be due to the inadequate number of males compared to female OA patients. The frequency analysis was divided into two models, i.e. recessive and dominant models [Tables 3-5]. In the recessive model, wild and heterozygous mixed up and correlated with a variant type, whereas in the dominant model, we correlated wild-type allele frequency with heterozygous and variant pool. The dominant model was significantly associated with the risk of primary OA.

D) Dominant and recessive model

We analyzed genotype frequency according to gender and divided into two different models, i.e. dominant and recessive models. We checked the frequency distribution of genotype with two different combinations of genotypes. In the dominant model, wild-type GG was compared with heterozygous and variant genotype, whereas in the recessive model, variant genotype was compared with heterozygous and wild genotype combinations. The genotype frequency distribution in males and females was found significantly associated.

D) Median age-wise distribution of genotype frequency in primary OA patients:

We compared the genotype frequency according to median age and found that patients with ≤59 years age have higher G >A transition frequency [Table 4]; however, the data was not significant.

**Table 1: Genotype frequency of 4 G>A *ANKH* gene polymorphism in study groups**

| Genotype/Allele | Control (*n*=101) | Case (*n*=25) | OR (95% CI) | *P* |
|---|---|---|---|---|
| *ANKH-4 G*>A transition | | | | |
| GG | 73 (72.3%) | 19 (76%) | Reference | |
| GA | 25 (24.8%) | 4 (16%) | 0.6 (0.19-1.98) | 0.42 |
| AA (D) | 3 (3%) | 2 (8%) | 2.5 (0.3-16.43) | 0.32 |
| GA + AA | 28 (27.7%) | 6 (24%) | 0.82 (0.29-2.27) | 0.70 |
| AA (R) | 3 (3%) | 2 (8%) | 2.84 (0.45-17.99) | 0.26 |
| G | 171 (84.7%) | 42 (84%) | Reference | |
| A | 31 (15.3%) | 8 (16%) | 1.05 (0.45-2.45) | 0.91 |

OR=Odds Ratio; CI=Confidence Interval; D=Dominant, R=Recessive

**Table 2: Genotype distribution of *ANKH* gene polymorphism in cases according to gender**

| Gender | Genotype frequency | | | *P* |
|---|---|---|---|---|
| | GG *n* (%) | GA *n* (%) | AA *n* (%) | |
| Male | 8 (32%) | 0 (0%) | 0 (0%) | 0.102 |
| Female | 10 (40%) | 4 (16%) | 3 (12%) | |

**Table 3: Recessive and dominant model for genotype frequency**

| Gender | Genotype frequency (recessive model) | | *P* |
|---|---|---|---|
| | GG + GA *n* (%) | AA *n* (%) | |
| Male | 8 (32%) | 0 (0%) | 0.205 |
| Female | 14 (56%) | 3 (12%) | |
| | **Genotype frequency (dominant model)** | | |
| | **GG + GA *n* (%)** | **AA *n* (%)** | |
| Male | 8 (32%) | 0 (0%) | 0.032* |
| Female | 10 (40%) | 7 (28%) | |

**P*-value<0.05 is significant. *P*-vaule calculated by Chi-square test

## Discussion

Till date, only one study has shown the presence of G-to-A *ANKH* gene mutation in 5'UTR region, which is a gain of function mutation, in sporadic CC among Europeans patients.[4]

**Table 4: Genotype distribution of *ANKH* gene polymorphism in cases according to median age**

| Median age (years) | Genotype frequency | | | *P* |
|---|---|---|---|---|
| | GG *n* (%) | GA *n* (%) | AA *n* (%) | |
| ≤59 | 9 (56.2%) | 4 (25.0%) | 3 (18.8%) | 0.065 |
| >59 | 9 (100%) | 0 (0%) | 0 (0%) | |

**Table 5: Dominant model for genotype frequency**

| Gender | Genotype frequency | | *P*-value |
|---|---|---|---|
| | GG *n* (%) | GA + AA *n* (%) | |
| Male | 8 (32%) | 0 (0%) | 0.032* |
| Female | 10 (40%) | 7 (28%) | |

**P*-value <0.05 is significant

CPPD crystals commonly affect knee joints in patients of primary knee OA. One of the studies has even validated the association between knee OA and sporadic CC at the same joint.[5] *ANKH* gene mutation is well documented in Europeans with sporadic CC.[4] We could not find even a single study in the literature on *ANKH* gene mutation in primary knee OA. Hence, this study was conducted to assess the frequency of this mutation in patients with primary knee OA without any regards to the presence of CC.

European race is thought to inhabit central and south Asia. Aryans who inhabit north India are considered to be European. Khatris is a caste from the northern Indian subcontinent and can be considered European. Khatris in India are mostly from the Punjab region. Thus, we tested whether Khatri OA patients, who closely resemble Europeans in Indian population, harbor the studied mutation in *ANKH*. We chose to study 5'UTR region because the location of the mutation in the 5'UTR is just after the start codon in exon 1 or right in front of the stop codon in the last exon of *ANKH*. This has the potential to increase *ANKH* RNA ribosomal binding or stability, possibly explaining the increase in protein expression.[4] We used RT-PCR to detect the predefined mutation. This method had been used earlier in other studies for studying the *ANKH* gene mutation.[6] PCR appears to be an adequate method for studying the present mutation. We decided to choose the sample size of 25 Khatri patients of primary knee OA because in the previous study,[4] almost 4% of patients with clinically identified sporadic CC were homozygous for the similar mutation, which was 8-fold more common than in normal controls. Therefore, we postulated that in the sample size of 25 patients, at least one should come positive for the studied mutation. Blood donors were taken as control subjects, four times the number of cases, as was used in the previous study.[4] Moreover, this was just a pilot study where we only looked for the presence or absence of point mutation at one particular locus of *ANKH* gene. There may be other loci of the gene that might contribute to genetic risk in primary knee OA. Nevertheless, the findings of this study should be confirmed in a larger study. To the best of our knowledge, this was the first study to examine the genetic contribution of *ANKH* gene in primary knee OA in a specified Indian community, closely resembling Europeans, in whom this sporadic gene mutation has been found to be associated with CC.

We found the studied mutation in 4 cases and 25 controls, as was described in the earlier study in sporadic CC,[4] but the association was statistically insignificant. In our study, we could not appreciate any significant difference at both genotype and allelic level in the distribution of *ANKH-4* G >A gene polymorphism in studied subjects.

At present, there are very few studies on the genetic contribution of various genes in OA. In a study by Adrian Pendleton *et al.*, a positive association was found between *ANKH* gene mutation and CC.[6] Although a very rare occurrence, a familial predisposition of *ANKH* gene mutation is reported from several countries and different ethnic groups.[7-10]

Limitations of our study: First, the sample size was quite small to detect this mutation. Second, this is a hospital-based study carried out in a small number of specified populations. Therefore, the study population may not resemble a community-based sample. Third, we did not check the synovial fluid for CPPD crystal in each patient of knee OA though we could not find any significant G >A transition in our studied group, which might be attributed to the fact that studied mutation might not be present in Khatri group. Another appropriate reason can be that this particular mutation might not be present in all Indian subgroups being defined under European race. One of the shortcomings of our study is that we did not include all other castes that were classified under the European race. We restricted our study to one particular caste, which might not be a true representation of European race.

## Conclusion

We carried out the evaluation of G-to-A transition in the *ANKH* gene in 5' UTR region, 4-bp upstream of start codon, in Khatri patients of primary knee OA. However, in our study, we could not find any significant mutation in the studied subjects. The results of our study need to be confirmed in a further large study because of certain limitations. Interestingly, being the first study of its kind to look into *ANKH* gene mutation in primary knee OA, it has opened new doors for further research in this field. Physicians should keep in mind that CC is common among patients of OA and keep it as a differential diagnosis.

## Financial support and sponsorship

Nil.

## Conflicts of interest

There are no conflicts of interest.

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