Plasminogen activator inhibitor-1 promoter sequence variations in idiopathic osteonecrosis of head of femur

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Background & objectives: Primary or idiopathic osteonecrosis of femur head (ONFH) is the second most commonly observed cause among Indian patients suffering from ischemic ONFH. Although a number of genetic polymorphisms have been associated with idiopathic ONFH pathogenesis in Korean and Chinese populations, there are no studies in the Indian population. This is an exploratory study designed to implicate in promoter sequence polymorphisms of a critical fibrinolytic system regulator, plasminogen activator inhibitor-1 (PAI-1) gene, in cases of idiopathic osteonecrosis. Promoter sequence variations can affect expression levels of PAI-1 gene and may disrupt the coagulation/fibrinolytic equilibrium, which may finally culminate into osteonecrosis. Hence, the aim of the study was to investigate the role of single-nucleotide polymorphisms (SNPs) in the promoter region of PAI-1 gene and osteonecrosis development.

Methods: Two SNPs of the PAI-1 gene (rs2227631, -844 G/A; rs1799889, -675 4G/5G) were genotyped in 25 patients diagnosed with idiopathic ONFH and 25 control subjects, using direct sequencing. Subsequently, association analyses were performed for the genotyped SNPs.

Results: Both the rs2227631 and rs1799889 genotype and allele frequencies of PAI-1 gene showed an insignificant association with osteonecrosis risk (P=0.717, 0.149). Haplotype frequencies of rs2227631 and rs1799889 were also calculated in patients having idiopathic ONFH and controls. Although the distribution of haplotype GA-4G 4G was found to be the highest among the cases, it was not significantly different when compared with the controls.

Interpretation & conclusions: Our findings demonstrate that the minor alleles of promoter region sequences of the PAI-1 gene do not contribute to an increase in ONFH predisposition. However, this is a preliminary study and its findings should be considered as suggestive for studies to be done in a larger sample size.

Key words ONFH - osteonecrosis of femur head - plasminogen activator inhibitor-1 - polymorphism
the various other factors responsible for ONFH, for example, corticosteroid therapy (37.3%), chronic alcohol (20.1%) and trauma (15.3%)⁶. The disease mainly affects men aged between 20 and 40 yr, which emphasises the importance of early diagnosis of the disease.

Although the cause of idiopathic ONFH is poorly understood, the process is a final outcome of compromise to the already precarious blood supply of the head of the femur. Comparing this impairment with the myocardial vessels interruption, Chandler⁴ also termed the disease as ‘coronary disease of the hip’. Since a decrease in the blood flow is a potential risk factor for arterial or venous thrombi, abnormalities in the coagulation system might play a pivotal role in the development of idiopathic ONFH. Many previous studies have suggested that genetic polymorphisms in factor V, prothrombin, methylenetetrahydrofolate reductase and plasminogen activator inhibitor-1 (PAI-1) genes, leading to intravascular coagulation disorders, may predispose an individual to ONFH⁵-⁷.

PAI-1 gene is one of the most important regulators for the balance between coagulation and fibrinolytic systems and has been reported to closely correlate with ONFH susceptibility⁶,8-12. The PAI-1 gene also known as endothelial PAI-1 belongs to SERPINE1 family¹³ and is located on chromosome 7 (7q21.3-q22) in humans¹⁴. Its main function entails regulating plasma PAI-1 antigen levels which inhibit tissue plasminogen activator and urokinase plasminogen activator, both of which are activators of plasminogen, and hence reduce fibrin degradation. This subsequently leads to disseminated intravascular coagulation which causes formation of thrombotic clogs in the femoral head, finally culminating into non-traumatic osteonecrosis¹⁵.

The transcriptional regulation of PAI-1 gene is extremely complex, and many transcription factor binding sites for PAI-1 are found in the 5’ and 3’ untranslated regions (UTR) of the gene¹⁶. Single nucleotide polymorphisms (SNPs) within the 5’ UTR have been shown to result differences in PAI-1 antigen expression in various pathological conditions such as cancer, rheumatoid arthritis, stroke and other atherothrombotic events¹⁶-¹⁹. A common transcription-altering insertion/deletion single-nucleotide polymorphism (SNP; rs1799889) of four or five guanine (4G/5G) nucleotides, that is 675 bp upstream of the transcription start site of PAI-1 gene, has been reported previously to regulate PAI-1 levels, such that homozygous or heterozygous carriage of the 4G allele is associated with higher PAI-1 levels²⁰. In addition to rs1799889, another SNP in the promoter region of PAI-1 gene, rs2227631 (-844 G/A), is potentially implicated in PAI-1 regulation. It is found to be in tight linkage disequilibrium with the widely reported 4G/5G polymorphism in the promoter region of the PAI-1 gene²¹.

Glueck et al²², for the first time, showed association between SNP in PAI-1 gene and idiopathic ONFH in twins. Significantly, upregulated expression levels of PAI-1 protein were detected in the sera of patients with idiopathic ONFH using enzyme linked immunosorbent assay by Tan et al²³. Many recent studies have also suggested significant association between sequence variations in the PAI-1 gene¹¹,²³ and the susceptibility of ONFH, but the debate still remains inconclusive.

More often, the studies focussing on association between PAI-1 gene polymorphism and ONFH have been conducted in Chinese or Korean population, but there are no such studies from India. Hence, the aim of this study is to determine the presence of promoter gene sequence variations in PAI-1 gene (SNP-rs2227631, -844 G/A; rs1799889, -675 4G/5G) and development of non-traumatic idiopathic ONFH.

Material & Methods
A total of 25 (17 males, 8 females; mean age 39.3 ± 4.7) north Indian patients, 20-60 yr of age and either sex, presenting to the orthopaedic department of All India Institute of Medical Science (AIIMS), New Delhi, India, between March 2016 and March 2017, and diagnosed with idiopathic ONFH were recruited for the study. Since this is the first study pertaining to the pathogenesis of idiopathic ONFH in north Indian population, no reference of anticipated odds ratio (OR) was available in the literature, and hence, sample size calculation for the study was done using a simple formula $n=\log \beta/\log p$, where $n$ is the sample size (in numbers), $\beta$ is the probability of committing a Type II error (usually 0.05) and $p$ represents the proportion of the individuals in the population who are not infected with the disease. Considering the north Indian population ONFH epidemiology data², around 21.3 per cent (~21%) of ONFH patients had an idiopathic cause. Hence, $n=\log 0.05/\log 0.79 = 13$ or more and hence, considering all constraints and feasible parameters, 25 controls and 25 patients were enrolled in the study.
The study was performed as a controlled laboratory study in the Laboratory of Molecular Reproduction and Genetics, department of Anatomy, AIIMS, New Delhi after procuring approval from the Institutional Ethics Committee and the departmental Review Board. The patients were evaluated radiologically with anteroposterior and lateral pelvic radiographs and magnetic resonance images (MRIs), and a confirmed diagnosis of ONFH was made on the basis of radiographs and MRI findings. Patients with a demonstrable history of direct trauma to the hip joint or with the possibility of a combination of overt causes, such as alcoholism (consumption of more than 400 ml/wk was considered as alcohol-induced ONFH), cigarette smoking (active chain smokers were excluded), steroid usage (even a single dose of steroid was accountable for exclusion), infections, marrow infiltrating diseases, coagulation defects and other autoimmune diseases were excluded. Control group consisted of 25 (19 males, 6 females; mean age 40.9 ± 5.1) healthy asymptomatic individuals, preferably those who accompanied the patients, who had no history of any complaints relating to hip joint or any chronic disease, such as hypertension or diabetes, or coagulation-related disorders. Control subjects were matched with patients with regard to age and gender. A structured questionnaire was used to obtain information on familial and medical history as well as medications (specifically steroid intake), occupation and other environmental and lifestyle factors. Detailed history regarding personal habits such as smoking specifically the number of cigarettes usually smoked per day, tobacco consumption and alcohol use over the last 10 yr, was obtained before taking the sample from both patients and controls. Blood samples were collected from all cases and controls. Written informed consent was obtained from both cases and controls.

**Genomic DNA extraction from blood, PCR amplification, DNA sequencing and mutation analysis:** Five millilitres of venous blood was drawn under aseptic precautions and collected in EDTA tubes. DNA was extracted using Miller’s method from blood. Optical density was measured using NanoDrop spectrophotometer (Thermo Fischer Scientific, Massachusetts, USA), and quality of the DNA procured was assessed in 0.8 per cent agarose gel. The promoter region of PAI-1 gene was amplified using previously published primer set. The reaction was performed in a 25 µl volume containing 0.2 µl of 20 mM stock solution of each primer, 200 ng of genomic DNA, 0.25 µl of Taq polymerase, 0.2 µl of each deoxynucleotide triphosphate and 2.5 µl of 10x PCR buffer (with 25 mM MgCl₂) (Thermo Fisher Scientific, MA, USA). The annealing temperature was determined as 62°C by gradient PCR (C1000 Series Touch Thermal Cylers, Bio-Rad, CA, USA). The PCR products were analyzed on a 1.8 per cent agarose gel containing ethidium bromide (0.10 µg/ml) (Supplementary Figure). Amplified PCR products were purified using a gel/PCR DNA fragments extraction kit (Geneaid Biotech. Ltd., Sijih City, Taiwan) following the manufacturer’s protocol. Purified PCR products were sent for sequencing to Eurofins Genomics Pvt Limited (Bangalore, India). All sequence variants were compared to the Human Genome Reference Sequence provided by the National Center for Biotechnology Information, using ClustalW2 (multiple sequence alignment program for DNA; European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK). All PCR products that harboured nucleotide variation were sequenced bidirectionally.

**Statistical analysis:** Deviations in genotype frequency were tested from Hardy–Weinberg equilibrium at each polymorphic variant. The statistical analysis was carried out using IBM SPSS (Statistical Package for Social Sciences) statistical version 20 (SPSS, Chicago, IL, USA). The analysis included frequency table, association of variables based on Chi-square test and if any cell frequency was <5, then Yates corrections was used for 2×2 contingency table or method pooling. Fisher’s exact test (for higher order than 2×2 table), ORs and 95 per cent confidence intervals were used to estimate the relative risks of ONFH patients associated with the presence of different PAI-1 promoter region genotypes. Two-tailed level of significance (P≤0.01 and P≤0.05) was used to evaluate the significance of all the tests.

**Results**

To identify whether PAI-1 SNPs were involved in the susceptibility to ONFH, two SNPs (rs2227631, -844 G/A and rs1799889, -675 4G/5G) (Figs 1 and 2) in the promoter region were genotyped in 25 idiopathic ONFH and 25 control subjects (Table I).

Allelic frequencies of PAI-1 genotype G/G and its polymorphism G/A and A/A at rs2227631 were found to be seven (28%), 13 (52%) and five (20%) in controls and five (20%), 13 (52%) and seven (28%)
As the frequencies were found to be nearly equal in cases and controls, they could not deduce any statistical significance with the disease ($P > 0.05$) (Fig. 3).

Presence of homozygous 4G/4G, 5G/5G and heterozygous 4G/5G PAI-1 genotype at rs1799889 was found in 18 (72%), four (16%) and three (12%) controls and 17 (68%), eight (32%) and no patients
with idiopathic osteonecrosis, respectively. The polymorphism was not found to have significant association with ONFH ($P > 0.05$) (Fig. 4).

Both the rs2227631 and rs1799889 genotype and allele frequencies of PAI-1 gene were insignificantly associated with ONFH risk ($P=0.717, 0.149$), which suggests that the minor alleles of promoter region sequences probably do not contribute to an increase in ONFH risk and hence no role in ONFH predisposition (Table II). The sample size was, however, too small to be further subdivided according to gender.

The haplotype frequencies of rs2227631 and rs1799889 in ONFH patients and controls were also calculated. The proportionate analysis between cases and controls for a particular haplotype frequency was assessed, using $z$-proportionate test. However, the distribution of none of the haplotypes was significantly different between the ONFH patients and controls ($P > 0.05$) (Table III).

### Discussion

Idiopathic ONFH has been reported to be associated with a variety of conditions. Previously, many studies have suggested association of idiopathic ONFH with heritable thrombophilia (an increased tendency for intravascular thrombosis) or hypofibrinolysis (a reduced ability to lyse thrombi)$^{6,22}$. Hypofibrinolysis caused by high levels of PAI, transmitted as an autosomal dominant trait, has been cited as a major cause of idiopathic osteonecrosis by Glueck et al$^{9}$.

This preliminary study determined the contribution of PAI-1 gene promoter region SNPs to idiopathic ONFH. Sequence variations in PAI-1 gene are associated with altered or dysregulated PAI-1 antigen levels in the plasma which may promote ONFH by inhibiting fibrinolysis. Although the previous studies confirmed that PAI-1 gene polymorphism is an important biomarker in the prediction of idiopathic ONFH, in this preliminary study, due to small sample size, significant association between PAI-1 gene promoter polymorphism could not be found with idiopathic ONFH.

Many studies have reported an increased PAI-1 serum levels in ONFH patients compared to controls$^{15,23}$, which may be a result of genetic polymorphisms. Muñoz-Valle et al$^{25}$ reported varying PAI-1 plasma

### Table I. Frequencies of plasminogen activator inhibitor-1 PAI-1 promoter region polymorphisms (n=50)

| Position         | Location  | Genotype | Heterozygosity |
|------------------|-----------|----------|----------------|
| g-844 G/A (rs2227631) | Promoter  | GG       | 0.52           |
|                  |           | GA       |                |
|                  |           | AA       |                |
|                  |           | N        |                |
| g-675 4G/5G (rs1799889) | Promoter  | 4G4G     | 0.06           |
|                  |           | 4G5G     |                |
|                  |           | 5G5G     |                |

**Fig. 3.** Frequency of PAI-1 polymorphism at g-844G/A (rs2227631) in cases and controls.

**Fig. 4.** Frequency of PAI-1 polymorphism at g-675 4G/5G (rs1799889) in cases and controls.
levels with 4G/5G SNP (rs1799889). They showed higher PAI-1 plasma levels in the 4G/4G homozygous carriers and hence increased susceptibility to ONFH as compared to 4G/5G and 5G/5G. A meta-analysis of PAI-1 4G/5G polymorphisms (five studies with 419 cases of ONFH and 969 controls) showed 4G/4G genotype to be a significant risk factor for predicting ONFH.

Another group, Asano et al. studied 31 Japanese patients with post-renal transplant ONFH and found four patients with 5G/5G, 11 with 4G/5G and 16 with 4G/4G. However, analysis revealed no significant differences in the incidence of ONFH among these patients \( (P=0.49)^{12} \), which are similar to our findings, wherein of 25 Indian patients diagnosed with idiopathic ONFH, 17 patients had 4G/4G, no patient was heterozygous for 4G/5G and 8 patients had 5G/5G genotype as compared to 18, 3 and 4 controls, respectively, which were again not found to reveal any significant difference statistically \( (P=0.149) \).

Kim et al.\(^{11} \) found that the risk effects of rs2227631 and rs11178 in the idiopathic subgroup and of these SNPs in men were significantly associated with ONFH. Hence, they showed that rs2227631 is an essential SNP involved in the regulation of PAI-1 gene expression in ONFH. Moreover, epidemiologic analysis of their study showed that idiopathic ONFH is more common than steroid induced and is gender biased. Zhang et al.\(^{27} \) significantly associated SNP rs2227631 with steroid-induced ONFH group in co-dominant \( (P=0.04) \) and recessive models \( (P=0.02) \). However, our study results and could not prove any significant association between rs2227631 and ONFH. Haplotype frequencies of rs2227631 and rs1799889 were also calculated in idiopathic ONFH patients and controls. Although the distribution of haplotype GA-4G/4G was found to be the highest among the cases, it was not significantly different when compared with the controls.

The main limitation of this study was the small sample size. In this study, the focus was on genetic susceptibility to idiopathic ONFH risk because of its increased incidence and delayed diagnosis. While a few polymorphisms (frequencies of mutant homozygous 5G/5G and heterozygous 4G/5G) were found to be considerably different in cases than controls, but the differences were not significant \( (P=0.149) \) (Table II). Hence, to firmly establish the
relationship, a larger sample size is needed. Further studies considering functional regulation of PAI-1, for example, correlation of PAI-1 gene SNPs with plasma PAI-1 antigen levels and other coagulation factor profiles, are recommended.

Overall, this study for the first time identified an insignificant association between SNPs of the PAI-1 gene (rs2227631, -844 G/A; rs1799889, -675 4G/5G) in idiopathic ONFH in the Indian population. The results of this association study suggest that there exists an interplay between various factors regulating the coagulation system, besides PAI-1 gene and ONFH.

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Conflicts of Interest: None.

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Supplementary Figure. 1.8 per cent agarose gel, showing PCR products of 628 bp on GeneDireX 100 bp DNA ladder H3 RTU (ready-to-use) (P1-7, cases; M, ladder; C1-7, controls).