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Rapid detection of BMP6 gene single nucleotide polymorphism by PCR-RFLP in sickle cell patients

S.Pandey[1]*, R.Ranjan[1], R.M.Mishra[2], Sweta Pandey[1], V.Shah[3], R.Saxena[1]

[1]Department of Hematology, AIIMS, New Delhi, (INDIA)

[2]Department of Environmental Biology, APS University Rewa, (INDIA)

[3]Department of Cardiac Bio-Chemistry, AIIMS, New Delhi, (INDIA)

E-mail: pandeysanjaybt@rediffmail.com

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ABSTRACT

We present here to the application of the PCR-RFLP technique for the determination of the BMP6 gene single nucleotide polymorphism and frequency of five BMP6 SNPs among Asian Indian sickle cell patients. Five known SNPs of BMP6 gene; rs73719353, rs73719341, rs73719318, rs73381662 and rs73381650 were determined using the Sfc1, BccI, HpyCH4V, ScrFI and AflIII restriction enzyme. BMP6 gene SNPs amplified using the PCR technique. Each amplified fragment is subsequently digested with the appropriate enzyme. However all individual among patient and control groups were wild type and frequency was zero. Protocols for PCR amplification and restriction digestion were standardized for 5 single nucleotide polymorphisms in BMP6 genes. Their presence can be detected and identified in the DNA samples and would further be used in their establishment as molecular markers in osteonecrosis afflicted sickle cell disease patients, for early diagnosis. This technique has the advantages of rapidity, safety, and cost-effectiveness.

KEYWORDS

SNPs;
PCR-RFLP;
Restriction digestion;
BMP6;
SCD.

INTRODUCTION

Bone morphogenetic protein 6(BMP6) is encoded by the BMP6 gene in humans and located on chromosome 6, (6p24-p23)[1-3]. The bone morphogenetic proteins are a family of secreted signaling molecules that can induce ectopic bone growth[4]. Case-control studies are beginning to define the relationships between single-nucleotide polymorphisms in candidate genes and the many sub-phenotypes of sickle cell anemia. A common theme emerging from these studies is that single-nucleotide polymorphisms in genes of the transforming growth factor-β/bone morphogenetic protein are associated with several sub phenotypes of sickle cell disease[5]. Osteonecrosis is a common sequel of sickle cell disease. Baldwin et al. suggested that the presence or absence of osteonecrosis in sickle cell patients is influenced by genetic variability in genes other than HBB that are expressed in either bone or the vasculature. They thus examined the potential association of osteonecrosis with single nucleotide polymorphisms (SNPs) in candidate genes of different functional classes, includ-

ing those involved in vascular function, informmation, oxidant stress, and endothelial cell biology[6]. BMPs, members of the transforming growth factor-B super family, are known to induce de novo cartilage and bone formation by stimulating endochrondal bone formation cascade via the Smad signal transduction pathway[7]. BMP6 is involved in inflammatory processes[8] and is important for bone formation[9] and in association with parathyroid hormone (PTH) and vitamin D[10]. Although genes that may play a significant role in the understanding the genetic risk factors for the development of sickle cell osteonecrosis may provide new insight into the pathogenesis of this disease and eventually provide opportunities for its treatment, which now is limited[11]. For example, regulating the activity of the TGF-β pathway to modulate its effects on bone may be possible[12-14]. To investigate the association between common SNPs of BMPs and osteonecrosis in SCD patients, a study need to be conducted with large sampling of patients suffering from SCD diagnosed with osteonecrosis[15, 16]. However in India none of the data available in the frequency and clinical association of BMP6 SNPs with sickle osteonecrosis. Thus our objective was the standardization of rapid and cost-effective method for identification of BMP6 SNPs and determination of the frequency in sickle cell patients.

MATERIAL & METHOD

Study subjects were sickle cell patients, attending out patients department; All India Institute of Medical Sciences, New Delhi. About 5 ml of blood collected in a 3.2% sodium citrate tube after taken signed consent form. Study was approved by institutional ethics committee. Osteonecrosis confirmation done by X-ray. Diagnosis of sickle cell patients and quantitative assessment of hemoglobin Hb F, Hb A, Hb A2 and Hb S done by high performance liquid chromatography (HPLC-Bio-Rad-Variant™ Bio Rad, CA, USA). Complete blood count and red cell indices were measured by automated cell analyzer (SYSMEX K-4500, Kobe Japan). DNA was extracted using Bioserve kit. Taq DNA polymerase and DNTPs were obtained from NEB, oligonucleotide were procured form Sigma Eldritch Company and used 25 pm concentration. 1.5U/µl Taq polymerase while 0.2 mm/µl DNTPs each and 1.5mm/µl $mgcl_2$ were used for 50 µl reaction. Amplification was performed using MJ thermo-cycler machine. A total of 35 cycle were performed using the following conditions; 94°C,1 min.; 60°C,1 min; and 72°C, 2 min. with an additional 5 min. extension at 72°C, in the final cycle.

TABLE 1 : Primer sequence and restriction digestion of BMP6 gene SNPs.

Gene	SNPs	Primer sequence	Restriction enzyme
BMP6	rs73719353	5'-GCTCCTTTGCA CTTCGCTGT-3' F 5'-AGGCTCTGCTG AGCTCCTAC-3' R	Sfc1
	rs73719341	5'-TGAACTTCCC ATTCCCCTCT-3' F 5' ATAAAATTAG CATTGATCCA 3' R	BccI
	rs73719318	5'- CAGGTGCT GTGCAACTTCTT-3'F 5'-AGAGGGCACC ATGGTTGCCT-3'R	HpyCH4V
	rs73381662	5'- CTGAGATTCAA TTAGGCCCA -3'F 5'-TAAAGAACAGC AAAAGTCTG-3'R	ScrFI
	rs73381650	5'-CACATAAAGA TTGCTGCATT-3'F 5'- TAGTAATCCT AAAAATGGGA-3'R	AflIII

TABLE 2 : SNPs genotype of BMP6 gene and restriction product size.

SNPs	Genotype	Product size	
		Total product size	Restriction fregments product size
rs73719353	GG-/-	500bp	340,140,20bp
	GT-/+		340,250,140,90,20bp
	TT+/+		250,140,90,20bp
rs73719341	GG-/-	400bp	212,188bp
	GA-/+		212,188,150,39bp
	AA+/+		212,150,38bp
rs73719318	CC-/-	296bp	207,89bp
	C/A-/+		207,89,63,26bp
	AA+/+		207,63,26bp
rs73381662	CC-/-	300bp	158,142 bp
	CG-/+		158,143,99,43bp
	GG+/+		158,99,43bp
rs73381650	AA-/-	350bp	298,52bp
	AG-/+		298,201,97,52bp
	GG+/+		201,97,52bp

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The PCR reactions protocol and program were common for all 5 SNPs. Restriction enzyme procured from NEB Company. Five Known Single nucleotide polymorphism (SNPs) of bone morphogenic protein-6 (BMP6) were selected from http://www.ncbi.nlm.nih.gov/pubmed/ and manually primers were designed for PCR-RFLP. Restriction enzymes were selected with the help of NEB cutter software. Appropriate restriction enzyme used according to the manuals of the manufacturer. Details of the SNPs primer sequences and SNPs genotype with restriction product size are given in TABLE 1 and TABLE 2.

RESULT

Study subjects were divided in 4 groups; 60 HbSS in group-1 (35 male and 25 female with mean age 11.3±7.6 years), 75 sickle β-thalassemia in group-2 (57 male and 18 female with mean age 12±8.3 years), 15 sickle cell hemoglobin D in group-3 (10 male and 5 female with mean age 6.53±4.35 years) and 152 controls in group- 4 (88 male and 64 female with mean age 11. 22±7.8 years). Gene of interest of all SNPs were amplified using simple PCR. A 500bp, 400bp, 296bp, 300bp and 350 bp PCR product size of rs73381650, rs73719341, rs73719318, rs73381662 and rs73719353 were visualized in 2% agarose gel respectively. The amplified PCR product put on restriction digestion with enzyme Sfc1, BccI, HpyCH4v, ScrFI and AflII (All from NEB) according to manufacturer protocol. Digested products were checked on 3% agarose gel. After restriction digestion of PCR product, the fragmented product size of rs73381650 SNP was found 250bp, 140bp, 90bp. The pattern of SNP genotype of all patients and control was TT (+/+) wild type and reference SNP alleles was G/T and ancestral allele was T[17]. After the restriction digestion of rs73719341 SNP, a 212bp and 188 bp product was found and genotype pattern was GG-/- in all patients and controls while reference SNP alleles was A/G and ancestral allele was G[17]. The restriction digestion of a rs73719318 SNP, a 207 bp and 89 bp product size was seen. Reference SNP alleles was A/C and ancestral alleles was C[17] while all the patients and control genotype pattern was CC-/-homozygous. With restriction digestion of rs73381662 SNP, a 158 bp and 142 bp products were visualized

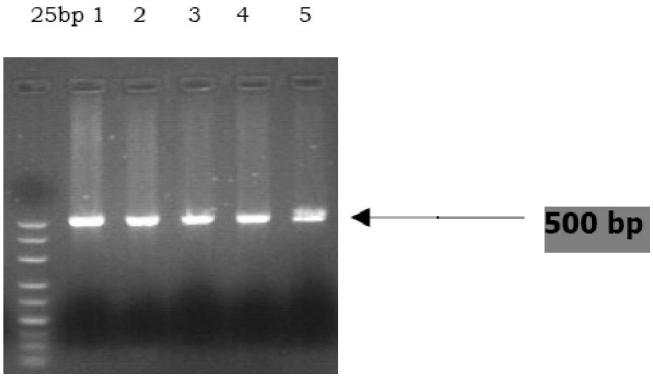

Figure 1A : Check gel of rs73719353

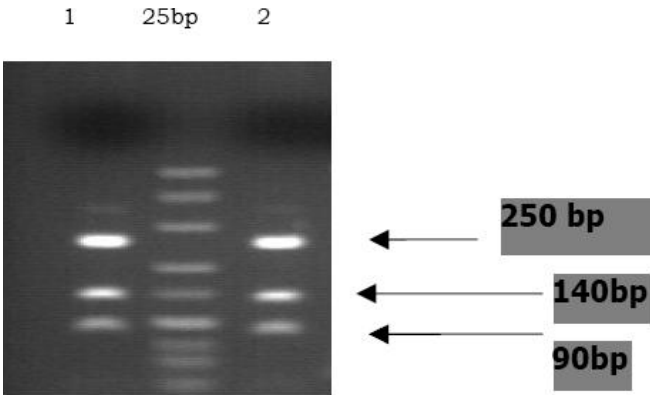

Figure 1B : Restriction digestion of rs73719353 with Sfc1

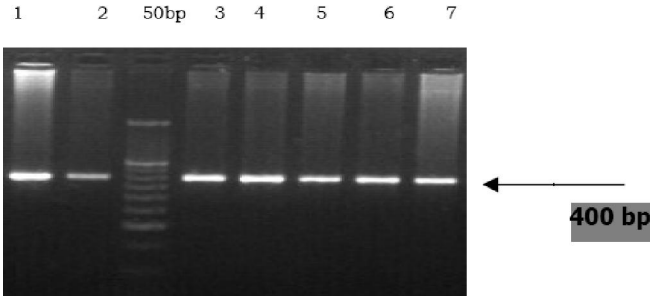

Figure 2A : Check gel of rs73719341

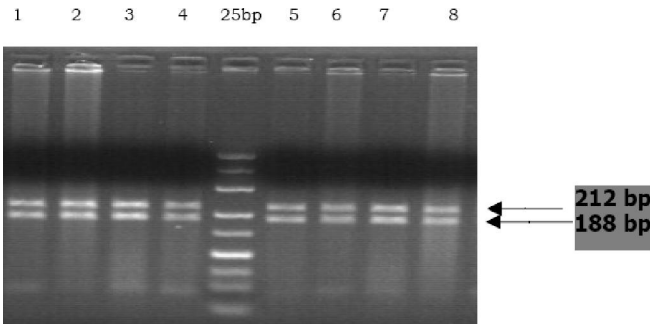

Figure 2B : Restriction digestion of rs73719341 with BccI

where patients and control genotype pattern was CC-/- homozygous while reference SNP alleles was C/G and ancestral alleles was C[17]. Restriction digestion of rs73381650 SNP, a 298 bp and 52 bp products were

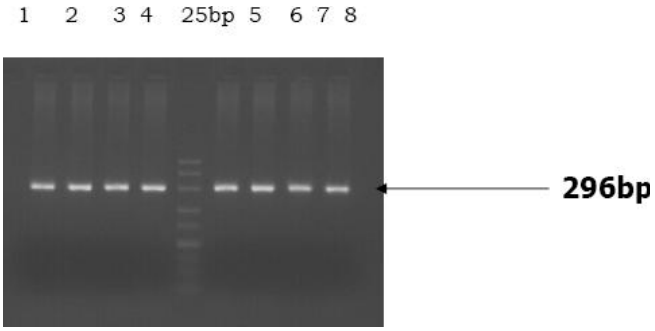

Figure 3A : Check gel of rs73719318

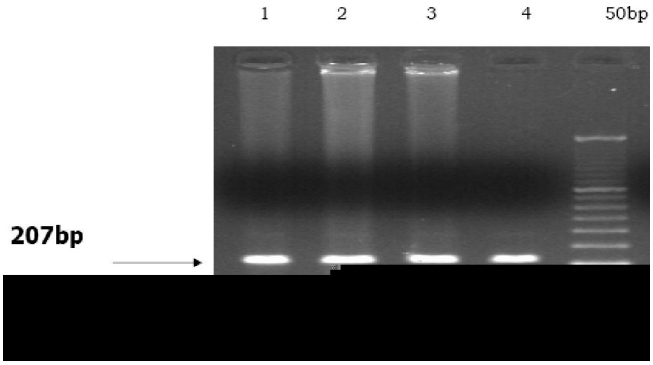

Figure 3B : Restriction digestion of rs73719318 with HpyCH4V

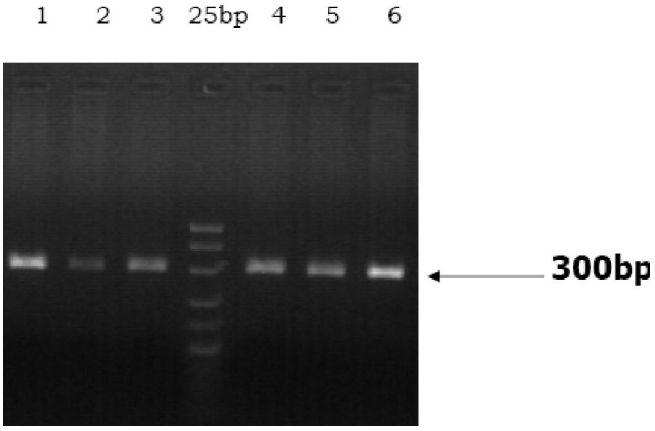

Figure 4A : Check gel of rs73381662

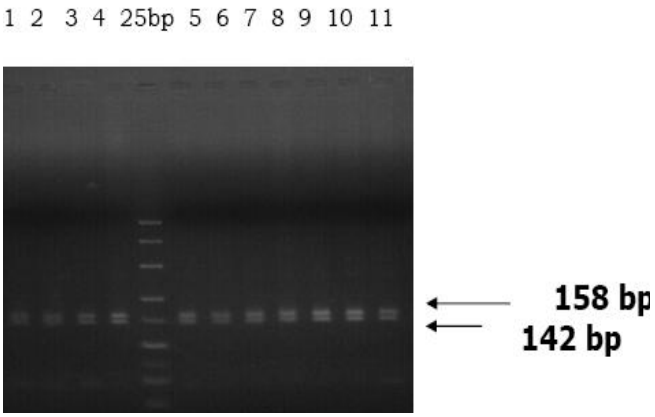

Figure 4B : Restriction digestion of rs73381662 with ScrFI

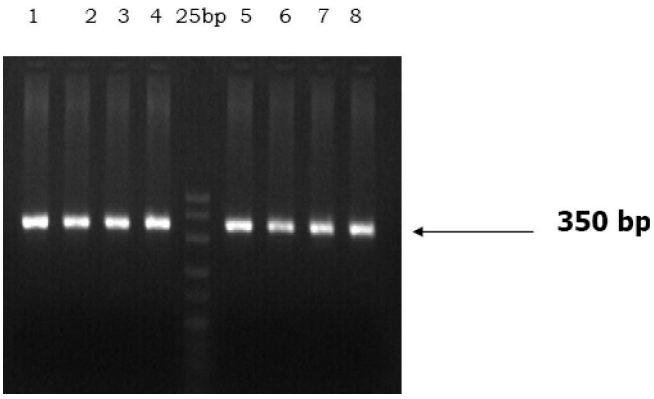

Figure 5A : Check gel of rs73381650

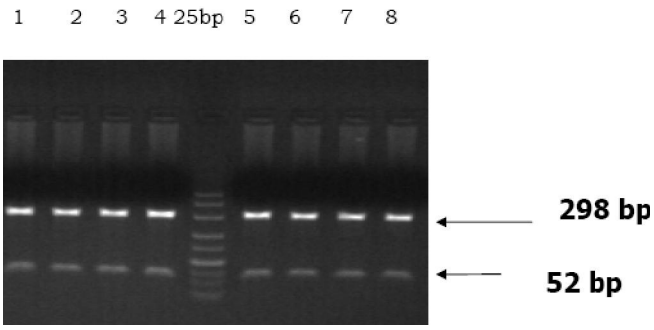

Figure 5B : Restriction digestion of rs73381650 with AflIII

seen with genotype of all patients and control AA-/- homozygous and reference SNP alleles was A/G and ancestral alleles was A[17]. A known 50 bp (Fermentas company) DNA ladder was used for the rs73719341 SNP amplified product and rs73719318 SNP restriction digested fragments. Remaining amplified and restriction digested fragments of SNPs were compared by 25 bp known DNA ladder of Bio Basic Inc. company. Gel picture of check gel and restriction digestion fragments of rs73381650, rs73719341, rs73719318, rs73381662 and rs73719353 are presented in Figure 1A-1B, 2A-2B, 3A-3B, 4A-4B and 5A-5B respectively.

DISCUSSION

Osteonecrosis is a significant complication associated with Sickle cell disease (SCD). Baldwin et al [6] suggested association of genes BMP6 with osteonecrosis in SCD. Thus with the objective to identify genetic polymorphisms associated with a risk for specific clinical outcomes in SCD,we had established standard PCR and restriction digestion protocols for detection of 5 specific single nucleotide polymorphisms (SNPs)

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in BMP6 genes. We had evaluated all the patient and control group for the above known SNPs and all were wild type. rs73719353, rs73719341 and rs73719318 clinical association of SNP and population diversity frequency data is unknown. rs73381662 and rs73381650 SNPs clinical association is unknown and population diversity detected in Sub-Saharan African and Average Het.+/- std err. was -1.000+/-0.[17] Seven patients were diagnosed with Osteonecrosis in HbSS while 6 patients were osteonecrotic in sickle β-thalassemia patients. Only one patient was osteonectic in HbSD patients. All patients were evaluated for above five SNPs but all were wild type. It suggests above SNPs absent in our population and does not play any role in sickle osteonecrosis. PCR-RFLP protocols can thus be used further for detection of other BMP6 SNPs in osteonecrosis afflicted SCD patients. Presence of frequency of other BMP6 SNPs in patients as compared to control samples will thus help in their frequency in the sickle osteonecrotic patients.

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