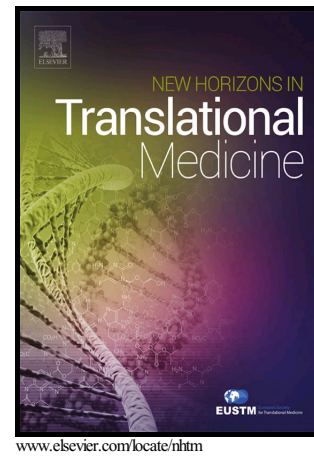


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# Association of Bone Morphogenic Protein 4 Gene Polymorphism and Left Ventricle Hypertrophy in Diabetic Chronic Kidney Disease Patients: A Pilot Study

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**Abstract**

**Background:** The Bone Morphogenetic Protein 4 (BMP4) is identified to play a significant role in cardiac remodelling; gene polymorphism and its resulting associations with Left Ventricular Hypertrophy (LVH) in diabetic Chronic Kidney Disease (CKD) patients of this protein are yet to be established.

**Aim:** To analyse the association between BMP4 gene polymorphism and LVH in diabetic CKD patients.

**Materials and Methods:** Isolation of DNA from whole blood samples of 50 patients each; patients diagnosed LVH with diabetic CKD and also from LVH patients without diabetic CKD, diabetic CKD without LVH and also normal patients as control were extracted. The gene of interest (BMP4 gene) purified from various samples digested using zero-cutter restriction endonucleases (*Hind III* and *Bam HI*) by employing the Restriction Fragment Length Polymorphism (RFLP) technique. The restriction has been analysed using 1 % agarose gel Electrophoresis.

**Results:** The gene from patient having LVH without diabetic CKD when digested with *Hind III* showed fragmentation, more specifically, it presented three/four fragments which were at a comparable distance corresponding with the following size reference markers at 2000bp(few cases), 1500bp, between 700bp to 600bp and the last one near 100bp. This fragmentation pattern was repeated identically for the gene from blood sample of patient having LVH with diabetic CKD which was also digested with *Hind III*. A similar fragmentation was not visualized for sample from patient having diabetic CKD without LVH when digested with *Hind III*. But no such fragments were noted for the samples from the same patients when digested with *Bam HI*.

**Conclusion:** BMP4 gene polymorphism has been confirmed in patients having LVH regardless of the presence or absence of diabetic CKD along with it.

Keywords: BMP4; LVH; CKD; Polymorphism; *Hind III*; *Bam HI*

### Focal Points

- **Benchside:**

Left ventricular (LV) hypertrophy is a strong autonomous predictor of increased cardiovascular morbidity and mortality in clinical and population-based samples. Thus understanding the correlation of LVH with BMP4 gene is necessitated to provide alternate therapeutics strategy at genome level. Eventually, genetic investigations provide high assurance for future prevention, early intervention and treatment of this major public health issue.

- **Bedside:**

Determination of BMP4 Polymorphism would raise a new drug development target using single nucleotide polymorphism. They also serve as molecular marker for next generation therapeutics of personalised medicine at genome level.

- **Community:**

The patient's therapeutic quality would be high due target specific approach with the understanding of personalized medicine. This can prevent unwanted treatment that can guide way to side-effects in the system.

- **Governments:**

Funding bodies should continue to acknowledge the importance that the BMP4 gene plays a role in LVH, which can be a causative agent for many other cardio-disorders. Overtime, this will help in benefiting patients and healthcare institutions.

## 1. Introduction

BMP4 (Bone Morphogenetic Protein 4) gene encodes protein known as BMP4 protein which is a member from the BMP family which is a part of the transforming growth factor beta superfamily and directing osteoblast separation and bone arrangement (1-4). BMP4 actuates cardiomyocyte separation and advances cardiomyocyte apoptosis after ischemia reperfusion injury affected myocardial infarction [5, 6]. The relationship amongst BMP4 and heart rebuilding is as of late reported. BMP4 is communicated in human and mouse hearts and recombinant BMP4 ensures grown-up mouse cardiomyocytes against hypoxia-reoxygenation injury [7]. BMP4 impels cardiomyocyte hypertrophy and apoptosis through expanding NADPH oxidase 4 expression and responsive oxygen species-subordinate pathways [8]. Hereditary variations in BMP4, as single

nucleotide polymorphisms (SNPs), may bring about a subjective or quantitative change in the nearby generation of BMP4 or in its adequacy by means of its related receptor [9]. Although numerous mutations inside BMP4 prompting different phenotypes have been accounted for [10], the single nucleotide polymorphism of 6007C > T (rs17563) of BMP4 is the main distinguished polymorphism in the coding region [11].

Left Ventricular Hypertrophy (LVH) is the extension and thickening of the walls of the left ventricle and it is one of the real inconveniences of hypertension, which is viewed as an autonomous risk variable for cardiovascular morbidity and mortality [12-15]. The established risk factors responsible of LVH incorporates blood pressure, duration of hypertension, age, obesity, diet, and pharmacologic treatment [16,17]. In addition, an expansive assemblage of confirmation demonstrates that the individual hereditary foundation inclines the LVH frequency [18-20]. Nonetheless, to date, there is no study in regards to the relationship between BMP4 quality polymorphism in diabetic chronic kidney disease and LVH.

## **2. Materials and Methods**

A total of 200 patients were enrolled in the present study. The patients were diagnosed LVH with diabetic CKD (N= 50), diabetic CKD without LVH (N=50), LVH without diabetics (N=50) and 50 patients without diseases considered as control were recruited from a private nephrology outpatient clinic in Tiruchirappalli, India from December 2015 to April 2016. A complete medical history was obtained from all subjects and the study protocol was approved by the local hospital ethics committee, Tiruchirappalli, India. All patients provided an informed written consent.

### **2.1 Genotyping of BMP4 gene**

5 ml of blood samples were obtained from each diagnosed patients having LVH, with diabetic CKD and also from patients with LVH, without diabetic CKD; without LVH, with diabetic CKD and also control samples were obtained from a normal individual, free of both LVH and diabetic CKD. From the obtained blood samples DNA was extracted by use of

MEDOX whole blood DNA extraction kit into separate tubes and labelled accordingly (Medox Biotech Pvt. Ltd., Chennai, India Catalog. No: MX-1135-02). Primarily, the BMP4 gene was identified along with its sequence; this was accomplished by referring to the NCBI website's gene section. Primers were designed for the gene of interest after it was removed of signal peptides. This was done using the New DNA software provided by England Biolabs. Primer was designed keeping in mind the optimum GC content: Forward primer: 5'→3' TACTAAGGACCATTGGCTTACGACT  $t_m= 60$ ; GC= 44%; length= 25; Reverse primer: TCAGCGGCACCCACCATCCCT  $t_m= 65$ ; GC= 65%; length= 20. The extracted DNA from each tube was run individually in a PCR machine along with the pre-designed primers and a MEDOX PCR core kit. Reactions were performed in a total volume of 25 $\mu$ l. The thermocycling procedure consisted of initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on 1% agarose gel. The amplified gene of interest was purified with the MEDOX PCR purification kit from each of the obtained blood samples.

## 2.2 Testing for gene polymorphism

Purified genes of interest from various samples which were appropriately labelled were independently digested using zero-cutter restriction endonucleases. Using the Ncb cutter2 online software, the zero-cutters were identified for the ideal gene of BMP4; they were identified to be *Hind III* and *Bam HI*. The digested genes of various samples (5  $\mu$ l sample with 5  $\mu$ l loading dye) were run in individual wells (Table 1) of an Agarose gel electrophoresis kit in reference to the control obtained from the normal individual and also a reference fragment length ladder. The electrophoresis kit was run for 45 minutes to 60 minutes with 100volts on the standard 1% Agarose gel. After the electrophoresis was finished, the gel was analysed for differences using U.V. Trans-illuminator and image was processed using Gel-Dock.

## 2.3 Statistical analysis:

All statistical analyses were performed using online statistical software. Allele and genotypic frequency was calculated by direct gene counting method. Comparison of the different allele and genotype was done using chi-square test (Hardy Weinberg Allele 2 calculator & <http://vassarstats.net/fisher2x3.html>). Odds ratios were calculated with a 95% confidence interval

limit using online medcalc calculator ([https://www.medcalc.org/calc/odds\\_ratio.php](https://www.medcalc.org/calc/odds_ratio.php)).  $P < 0.05$  was considered statistically significant.

### 3. Results

The analysis of the Agarose gel showed increased number of fragments of the loaded gene of interest for the blood samples obtained from patients having LVH, with diabetic CKD and also from patients having LVH, without diabetic CKD when digested with *Hind III* as compared to the gene of the blood sample from a normal individual free of both LVH and CKD, which was digested with the same enzyme. The control showed a size of about 2200bp which remained unrestricted when digested with both *Bam HI* and *Hind III*. The gene from patient having LVH without CKD when digested with *Hind III* showed fragmentation, more specifically, it presented **three/four** fragments which were at a comparable distance corresponding with the following size reference markers at 1500bp, between 700bp to 600bp and the last one near 100bp (Fig 1). This fragmentation pattern was repeated identically for the gene from blood sample of patient having LVH with diabetic CKD which was also digested with *Hind III*. A similar fragmentation was not visualized for sample from patient having diabetic CKD without LVH when digested with *Hind III*. But no such fragments were noted for the samples from the same patients when digested with *Bam HI*. This indicates the presence of polymorphism in BMP4 gene in LVH groups irrespective of the presence/absence of diabetes. The genotypes and allele distribution of BMP4 gene in four study groups has been represented in Table 2. In control study group, the BMP4 gene was distributed as CC 32(64%), CT 11(22%), and TT 7(14%). The group containing LVH without diabetic CKD has a genotype distribution as CC 21 (42%), CT 11(22%), and TT 18(36%). The group containing Diabetic CKD without LVH has a genotype distribution as CC 24(48%), CT 18(36%), and TT 8(16%). The group containing LVH with diabetic CKD has a genotype distribution as CC 16(32%), CT 14(28%), and TT 20(40%). Comparison between genotypic (CC and TT) and allelic (C and T) frequency distribution in the study groups were represented in Table 3. The comparison study shows significant difference between genotypic (CC and TT) and allelic (C and T) frequency distribution in LVH groups irrespective of presence of diabetes. The statistical analysis of allelic frequencies distribution (C

and T) was carried out using chi square test. Equal distribution of two alleles in the study population (null hypothesis) could be rejected with bias for T allele. This confirms the presence of 6007C > T polymorphism of BMP4 gene in cases compared to controls.

#### 4. Discussion

Several studies about BMP4 in cardiovascular system have inferred that BMP4 might be involved in pathological cardiac hypertrophy, for example, BMP4 stimulates ROS production through NADPH oxidases in endothelium, exaggerates cardiac ischemia-reperfusion injury by promoting cardiomyocytes apoptosis [6]. BMP4 was involved in valvular interstitial cell activation in human myxomatous mitral valve prolapse [21]. BMP4 induces cardiomyocyte hypertrophy and apoptosis through increasing nadph oxidase 4 expression and reactive oxygen species-dependent pathways [8]. BMP4 is expressed in pathological cardiac hypertrophy models and BMP4-mediated cardiomyocyte hypertrophy [22]. In this study, we found that the BMP4 inhibition by si-RNA technique significantly blunt the AngII induced the hypertrophy of cardiomyocyte in vivo, supporting the role of BMP4 in the regulation of hypertrophic response of cardiomyocytes. LVH is found in 75% of patients treated with hemodialysis. Detection of patients with high risk for development of left ventricular hypertrophy and application of suitable therapy to achieve target values of risk factors can also lead to regression of left ventricular hypertrophy, reduced cardiovascular morbidity and mortality rates and improved quality of life in patients treated with regular haemodialysis [13].

In the present study, we explored the association of bone morphogenic protein 4 (BMP4) gene and left ventricle hypertrophy (LVH) in diabetic chronic kidney disease patients (CKD). We found that the 6007 C>T polymorphism of the BMP4 gene was associated with risk responsible to develop LVH irrespective of comorbidity conditions. The earlier study [23] also confirmed the same kind of result which strengthens our result. BMP4 is a member of bone morphogenic proteins (BMP) superfamily which plays an important role in regulating osteoblast differentiation and bone formation. Since it is a mechanosensitive and proinflammatory gene, it



has an ability to induce endothelial cell apoptosis, endothelium dysfunction, cardiomyocyte differentiation and promotes cardiomyocyte apoptosis after ischemia-reperfusion injury-induced myocardial infarction. Since Single nucleotide polymorphisms (SNPs) of 6007C>T is the only polymorphism of BMP4 identified in coding region [5,6,11]. This polymorphism interferes with BMP4 gene expression and production because of change in amino acid residue from valine to alanine at residue 152 [24]. The allele and genomic distribution in our data suggest the significant correlation between the BMP4 gene and LVH (P = 0.028). The data also interprets that, presence of LVH can lead to high degree of Diabetic CKD (P = 0.0025). The odd ratio of the allelic distribution being greater than one implies that T allele is responsible for the disease and change in mRNA structure. In this study, we found that the genetic variants of BMP4 (6007C>T polymorphism) was significantly associated with the incidence of LVH in diabetic CKD patients. In conclusion, this polymorphism can be used as molecular marker of LVH development in diabetic/non-diabetic CKD patients.

### Executive Summary

- The Bone Morphogenetic Protein 4 (BMP4) is identified to play a significant role in cardiac remodelling; gene polymorphism and its resulting associations with Left Ventricular Hypertrophy (LVH) in diabetic Chronic Kidney Disease (CKD) patients of this protein are yet to be established.
- Determination of Association between BMP4 and LVH is crucial, as they serve as a main risk factor for development of cardiovascular morbidity and mortality
- DNA Isolation from whole blood samples of 50 patients each (patients diagnosed LVH with and without diabetic CKD, diabetic CKD without LVH and normal patients (control)) were extracted. Restriction Fragment Length Polymorphism (RFLP) technique was employed.
- BMP4 gene polymorphism has been confirmed in patients having LVH regardless of the presence or absence of diabetic CKD along with it.
- This polymorphism can be used as molecular marker of LVH development in diabetic/non-diabetic CKD patients

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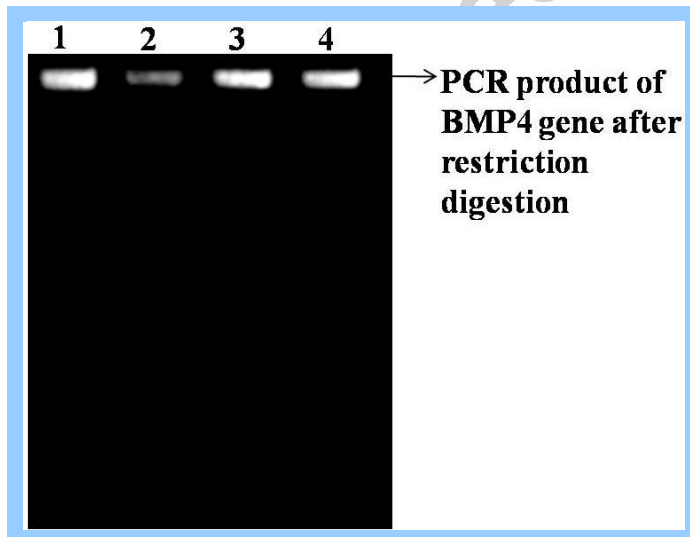
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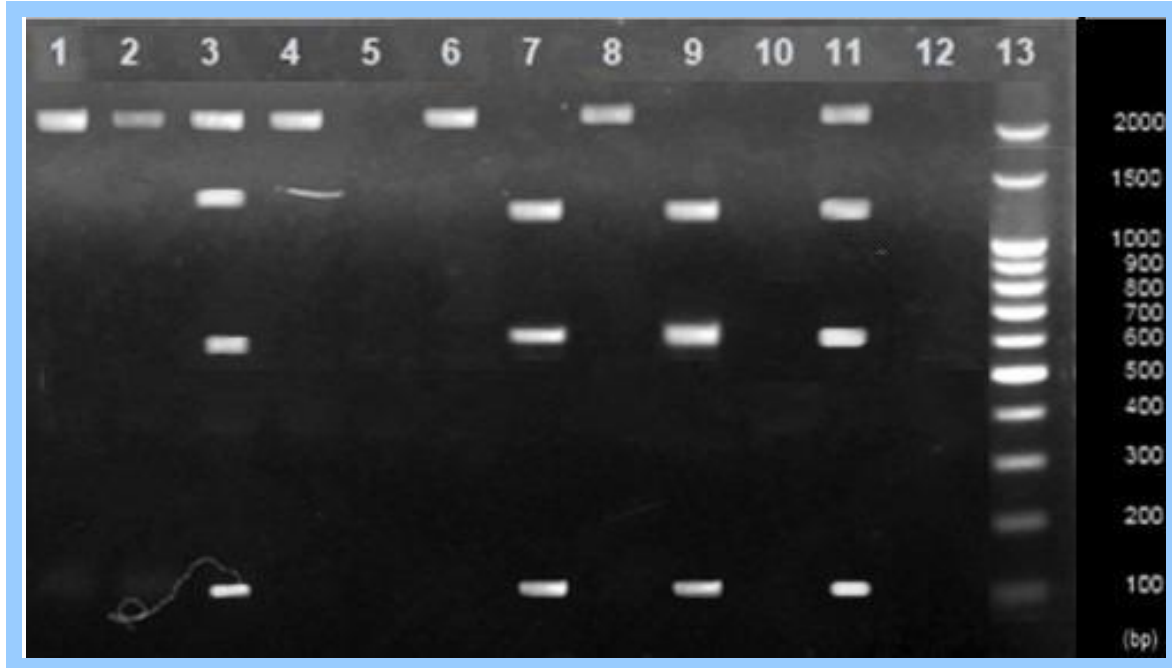
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**Fig.1. Restriction digestion of PCR products of BMP4 gene in cases and controls for detection of C/T polymorphism using *Bam*HI restriction enzyme. Lane 1 - control (Normal patients), Lane 2- patient group with LVH and without diabetic CKD, Lane 3 – Patient group with Diabetic CKD without LVH, Lane 4 – patient group with LVH and diabetic CKD.**



**Fig.2. Restriction digestion of PCR products of BMP4 gene from various patients groups (Refer Table 2) using *HindIII* restriction enzymes. Lane 1 – control group and Lane 13 – Marker (100bp ladder).**



**Table 1. Restriction digestion of PCR products of BMP4 gene in cases and controls with *BamHI* restriction enzymes (Refer fig 1) and details of samples loaded in the 1% agarose gel electrophoresis**

Agarose Gel Lane No.	Patient Groups	Restriction Enzyme	Disease pattern
1	Control (n=50)	BamHI	Normal
2	Group 1 (n=50)	BamHI	LVH without diabetic CKD
3	Group 2 (n=50)	BamHI	Diabetic CKD without LVH
4	Group 3 (n=50)	BamHI	LVH and diabetic CKD

**Table 2. Restriction digestion of PCR products of BMP4 gene in cases and controls with HindIII restriction enzymes (Refer fig 2) and details of samples loaded in the agarose gel electrophoresis**

Agarose Gel Lane No.	Patient Groups	Restriction Enzyme	Disease pattern
1	Control (n=50)	HindIII	Normal
2	Group 1 (n=50)	HindIII	LVH without diabetic CKD
3	Group 3 (n=50)	HindIII	LVH and diabetic CKD
4	Group 2 (n=50)	HindIII	Diabetic CKD without LVH
5	UNLOADED		
6	Group 3 (n=50)	HindIII	LVH and diabetic CKD
7	Group 1 (n=50)	HindIII	LVH without diabetic CKD
8	Group 2 (n=50)	HindIII	Diabetic CKD without LVH
9	Group 3 (n=50)	HindIII	LVH and diabetic CKD
10	UNLOADED		
11	Group 1 (n=50)	HindIII	LVH without diabetic CKD
12	UNLOADED		
13	Reference Ladder (DNA Marker – 100bp)		

**Table 3: Distribution of BMP4 genotypes and allele frequencies in study groups**

Study group	CC	CT	TT	C	T	X <sup>2</sup>	P value
control	32	11	7	0.75	0.25	8.54	0.0035
LVH without diabetic CKD	21	11	18	0.53	0.47	15.59	0.0001
Diabetic CKD without LVH	24	18	8	0.66	0.34	1.96	0.1618

LVH and diabetic CKD	16	14	20	0.46	0.54	9.52	0.002
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**Table 4: Comparison of genotype and allelic frequency distribution in study groups.**

Study groups	Chi-square value	Degree of freedom	P-value	Odds Ratio	95% confidence interval
Control vs LVH without diabetic CKD	7.12	2	0.028	2.62	1.13-6.09
Control vs Diabetic CKD without LVH	2.9	2	0.2345	1.46	0.61-3.46
Control vs LVH and diabetic CKD	11.95	2	0.0025	3.34	1.43-7.75