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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CKD
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Bam HI

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 2000 bp (few cases), 1500 bp, between 700–600 bp and the last one near 100 bp. 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.

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 personalized 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.
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 acts 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 6007 C > T (rs17563) of BMP4 is the main factor responsible of LVH incorporates blood pressure, duration of hypertension, age, obesity, diet, and pharmacologic treatment [16,17]. 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]. 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].

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’ TACTAGGACCATGTTGCTTACGACT tm=60; GC=44%; length=25; Reverse primer: TCAGGGCACCCACCATCCT tm=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 μl. The thermocycling procedure consisted of initial denaturation at 95 °C for 3 minutes, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The PCR products were analysed 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 Nebcutter2 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 μl sample with 5 μl 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 45–60 min 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/fisher2×3.html). Odds ratios were calculated with a 95% confidence interval limit using online medcalc calculator (https://www.medcalc.org/calc/odds_ratio.php). P < 0.05 was considered statistically significant.

Table 1

<table>
<thead>
<tr>
<th>Agarose gel lane no.</th>
<th>Patient groups</th>
<th>Restriction enzyme</th>
<th>Disease pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (n=50)</td>
<td>BamHI</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Group 1 (n=50)</td>
<td>BamHI</td>
<td>LVH without</td>
</tr>
<tr>
<td>3</td>
<td>Group 2 (n=50)</td>
<td>BamHI</td>
<td>Diabetic CKD</td>
</tr>
<tr>
<td>4</td>
<td>Group 3 (n=50)</td>
<td>BamHI</td>
<td>LVH and diabetic CKD</td>
</tr>
</tbody>
</table>
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 \textit{Hind III} 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 2200 bp which remained unrestricted when digested with both \textit{Bam HI} and \textit{Hind III}. The gene from patient having LVH without CKD when digested with \textit{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 1500 bp, between 700–600 bp and the last one near 100 bp (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 \textit{Hind III}. A similar fragmentation was not visualized for sample from patient having diabetic CKD without LVH when digested with \textit{Hind III}. But no such fragments were noted for the samples from the same patients when digested with \textit{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 6007 C > T polymorphism of BMP4 gene in cases compared to controls (Fig. 2).

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

### Table 2

Restriction digestion of PCR products of BMP4 gene in cases and controls with \textit{HindIII} restriction enzymes (Refer Fig. 2) and details of samples loaded in the agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Agarose Gel Lane No.</th>
<th>Patient Groups</th>
<th>Restriction Enzyme</th>
<th>Disease pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (n=50)</td>
<td>\textit{HindIII}</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Group 1 (n=50)</td>
<td>\textit{HindIII}</td>
<td>LVH without diabetic CKD</td>
</tr>
<tr>
<td>3</td>
<td>Group 2 (n=50)</td>
<td>\textit{HindIII}</td>
<td>LVH and diabetic CKD</td>
</tr>
<tr>
<td>4</td>
<td>Group 3 (n=50)</td>
<td>\textit{HindIII}</td>
<td>Diabetic CKD without LVH</td>
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<tr>
<td>5</td>
<td>UNLOADED</td>
<td>\textit{HindIII}</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Group 3 (n=50)</td>
<td>\textit{HindIII}</td>
<td>LVH and diabetic CKD</td>
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<td>\textit{HindIII}</td>
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<tr>
<td>9</td>
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<td>\textit{HindIII}</td>
<td>Diabetic CKD without LVH</td>
</tr>
<tr>
<td>10</td>
<td>UNLOADED</td>
<td>\textit{HindIII}</td>
<td>LVH and diabetic CKD</td>
</tr>
<tr>
<td>11</td>
<td>Group 1 (n=50)</td>
<td>\textit{HindIII}</td>
<td>LVH without diabetic CKD</td>
</tr>
<tr>
<td>12</td>
<td>UNLOADED</td>
<td>\textit{HindIII}</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Reference Ladder (DNA Marker – 100 bp)</td>
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</tbody>
</table>
Determination of Association between BMP4 and LVH is crucial, as:

- The Bone Morphogenetic Protein 4 (BMP4) is identified as a marker of LVH development in diabetic/non-diabetic CKD patients. In conclusion, this polymorphism can be used as a molecular marker for LVH development in diabetic/non-diabetic CKD patients.

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References


