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Original Research Article

A Molecular Analysis of Angiotensin Converting Enzyme (ACE) Gene Insertion/Deletion (I/D) Polymorphism in Toxaemia of Pregnancy and Normotensive Mothers

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ABSTRACT

Toxaemia of pregnancy is diagnosed in India around 6-10% of all deliveries and is associated with 22% of perinatal foetal deaths and cause 30% of maternal death. Angiotensin Converting Enzyme (ACE) plays a vital role in the Renin Aldosterone System (RAS) which regulates blood pressure by converting Angiotensin-I into a powerful vasoconstrictor Angiotensin-II. The aim of the present study was to explore out the ACE geneI/D polymorphism in cases of toxaemia of pregnancy and normotensive mothers in north India population. Study was done in 100 cases of Toxaemia of pregnancy and 100 controls (Normotensive) pregnant women who were admitted in department of obstetrics and gynecology, Rama Medical College Hospital and Research Centre, Kanpur. 5ml venous blood was collected in EDTA vials and processed for ACE gene I/D polymorphism withusing conventional polymerase chain reaction (PCR). A Deletion polymorphism (D allele) has found to be associated with elevated ACE activity leading to hypertension in pregnant women.On genotype analysis, the frequency of I/I, I/D and D/D polymorphism in controls group were found 40%,34% and 26% respectively. Among cases group, the frequency of I/I, I/D and D/D were recorded 16%,30% and 54% respectively. On comparison of the individual frequencies of the three genotypes, it was found that D/D genotype were more prone to develop disease with an odd's ratio of 5.192% (D/D vs I/I) and 2.353% (D/D vs I/D). On statistical comparison of D/D genotypes between the controls and cases, the cases showed a significant increase of frequency (p-value=0.0005), an odd's ratio of 3.341 was obtained against (I/D+I/I) polymorphism.

Keywords: Toxaemia of Pregnancy, Angiotensin Converting Enzyme (ACE), Insertion/Deletion polymorphism (I/D), polymerase chain reaction (PCR), significant p-value.

INTRODUCTION

Hypertensive disorders are creating complications during pregnancy (Toxaemia of pregnancy) which are common and forming deadly traits along with haemorrhage and infection. [1] Pre-eclampsia (PE) is a disease occurs during the pregnancy which is specified by the inception of hypertension and the presence of protein in the urine in large amount. [2] Pre-eclampsia is considered if one or more of the following criteria are present: Blood pressure140 mm Hg or higher systolic or 90 mm Hg or higher diastolic after 20 weeks of gestation in a woman with previously normal blood pressure. Proteinuria: 0.3g or more of protein in a 24-hours urine collection (usually correspond with 1+ or

greater on a urine dipstick test) known as mild preeclampsia. [1] When systolic blood pressure of 160 mm of Hg or higher or 110mm of Hg or higher diastolic on two occasions at least six hours apart in a woman on bed rest, the condition is known as severe preeclampsia. It is associated with proteinuria and oliguria, cerebral or visual disturbances, pulmonary oedema of cyanosis, epigastric pain or right upper quadrant pain, impaired liver function, thrombocytopenia and foetal growth restriction known as Eclampsia. [1]

Toxaemia of pregnancy is a key cause of large number of maternal deaths and thereof foetal deaths. Maternal hypertension (toxaemia of pregnancy) is diagnosed in 6-10% of all deliveries which is associated with 22% of perinatal foetal deaths and 30% of maternal death. [3]

Angiotensin converting enzyme (ACE) is a monomeric, membrane-bound, zinc and chloride dependent peptidyldipeptidase that catalyses the conversion of decapeptide angiotensin I to the octapeptide angiotensin II by removing a carboxy terminal dipeptide. ACE has been well known for a key part of renin-angiotensin system (RAS) that regulates blood pressure in pregnant women. [4] ACE plays a vital role in the RAS which regulates blood pressure. ACE gene High activity can contribute to hypertension because of its vasoconstriction effect. [5,6]

Angiotensin-converting enzyme (ACE) geneis located on chromosome 17q23 position. It is 21 kb in size and consists of 26 exons and 25 introns. [7] ACE gene insertion/deletion (I/D) polymorphism defined by the presence of an extra 287 base pair sequence inside intron 16 of the ACE gene. A deletion polymorphism (D allele) has been reported to be associated with elevated ACE activity in plasma and serum. [7] Some investigators have been reported a strong association between the ACE gene D and I allele polymorphismin pregnant women from various geographical regions. [7] Allele DD genotype is associated with higher levels of ACE genein serumand II genotype is concerned with its lower levels, ID genotype is associated with its intermediate levels of ACE gene in serum. Moreover, it has been assumed that I allele has a sequence similar to a silencer role, which might explain the reason; why the D allele is associated with increased risk of preeclampsia or PIH. [7]

MATERIALS AND METHODOLOGY

The present study was done in 200 pregnant mothers divided into two groups. 100 samples were taken from case (patients) suffered from Toxaemia of Pregnancy. Another 100 samples were collected from normal pregnant women as control (Normotensive). All the cases and controls samples taken from pregnant women who were admitted in the department of obstetrics and gynaecology, Rama Medical College, Hospital and Research Centre Kanpur (India).All the cases and controls pregnant women have filled written consent form for willing to give their samples for this study. Inclusion criteria: Antenatal mothers diagnosed with pregnancy induced hypertension with their blood pressure of 140/90mm of Hg or more in to case group. Beside this a standard questionnaires were prepared to get the past and present medical/surgical history of cases and controls. In questionnaires several parameters were taken such as history of renal, liver failure, seizures, mother who has the hypertensive disorder before the pregnancy and other medical problems. The permission has taken from the institution ethical committee prior to conduction of this study.

5ml venous blood was collected in EDTA vials and stored in -200C temperature till the processing of ACE gene I/D polymorphism with simple polymerase chain reaction (PCR).

Following Primers were used for the detection of ACE gene I/D polymorphism in this study:

Forward primer:5'-CTGGAGACCCCCATCCTTTCT-3' Tm- 59oC

Reverse primer:5'-GATGTCGCCATCACATTCGTCAGAT-3'. Tm-58oC

Primers were synthesized from Banglore Genei and reconstituted with sterile TE buffer (0.1mM) and further dilution was done with double distilled water as manufacturer's instruction. Primers final (working) concentration was 10 picomoles. The PCR conditions were 95oC for 3min, 35 cycles of 95oC for 30s, 52oC for 30s, 72oC for 1.20 min and final extension was at 72oC for 5min. Then PCR product was run with 1% agarose gel containing ethidium bromide. Genotype was determined by fragment size under UV light in gel documentation system (Bio-rad) and PCR product was directly submitted for gene sequencing to Chromous Biotech Pvt. Ltd (Bangaluru).

RESULTS

All the DNA samples (100 cases and 100 controls) were amplified and analyzed for I/D polymorphism of the ACE gene. In this study, D and I alleles were identified at 190 bp and 490 bp respectively, leading to the confirmation of the three types of polymorphism D/D,I/D and I/I in ACE gene.

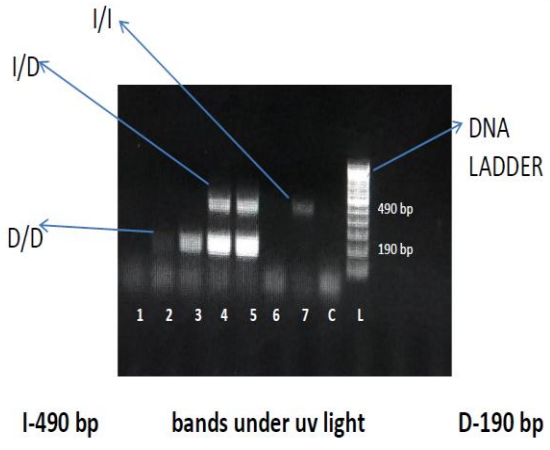

Fig 1, Agarose gel picture, Single band present at 190bp was represented as D homozygous while single band present at 490bp was denoted as I homozygous and presence of two bands denoted as heterozygous conditions.

With the use of genotype analysis the gene frequency for I/I, I/D and D/D polymorphism in controls group were found 40%, 34% and 26% respectively. Among case group, the frequency of I/I, I/D and D/D polymorphism were noted 16%, 30% and 54% respectively.

TABLE 1: DISTRIBUTION OF THE POLYMORPHISMS BETWEEN THE CONTROLS AND CASES.

Type Of ace Gene Polymorphism	Controls N=100	Cases N=100
I/I	40(40%)	16 (16%)
I/D	34(34%)	30(30%)
D/D	26(26%)	54(54%)

TABLE 2: DISTRIBUTION OF GENOTYPE BETWEEN CONTROLS AND CASES

Type of ACE polymorphisms	Controls N=100	Cases N=100
D/D	26(26%)	54(54%)
I/D +I/I	74(74%)	46(46%)

With the use of biostatistics Odd's ratio was calculated for cases/controls: 3.341.

TABLE 3: DISTRIBUTION OF GENOTYPE BETWEEN DD AND II GENOTYPE AMONG CONTROLS AND CASES.

Genotype	Controls	Cases	Odds ratio
D/D	26(26%)	54(54%)	5.192
I/I	40(40%)	16(16%)	

On comparison of the individual frequencies of the three genotypes, it was found that D/D genotype were 5 times more prone to develop Toxaemia of pregnancy than I/I genotype with odd's ratio 5.192.

TABLE 4: DISTRIBUTION OF GENOTYPE BETWEEN DD AND ID AMONG CONTROLS AND CASES.

Genotype	Controls	Cases	Odds ratio
D/D	26(26%)	54(54%)	2.353
I/D	34(34%)	30(30%)	

Similarly, with the comparison of the individual frequencies it was found that D/D genotype was 2 times more prone to produce Toxemia of pregnancy than I/D genotype with Odd's ratio 2.353.

First Submitted sequence

TACGCCAATTTTATTCCAGCTCTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATT
CAAACCCCTACCAGATCTGACGAATGTGATGGCCACATCAGGTTTACGCCAATTTTATTCCAGCTCTGAAATTCTCTGAGCTC
CCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGATCTGACGAATGTGATGGCCACAT
CACATTCGTCAGATCTGGTAGGGGTTTGAATGCCTTGAGCTCCAGCCCTTAGCTCACCTCTGCTTGTAGGGGGAGCTCAAAAA

ATTTCAAAGCTGGAACAAAATTGGCGAAACCACATAAAAGTGACTGTTTGGGACGCAGTCTAGAGAAATGGGAAAAAGGAT
GGGAGTGGTCTCCAGGA

Fig 2, the obtained ACE gene sequence of 490bp (upper) band.

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Query 246        CATCACATTCGTCAGATCTGGTAGGGGTTTGAATGCCTTGAGCTCCAGCCCTTAGCTCAC 303
                 | |||||||||||||||||||||||||| |||||| ||||||||||||||||| ||||||||||||
Sbjc 61630153    CATCACATTCGTCAGATCTGGTAGGGGTTTGAATGCCTTGAGCTCCAGCCCTTAGCTCAC 1630094

Query  304       CTCTGCTTGTAGGGGGAGCTCAAAAAATTTCAAAGCTGGAACAAAATTGGCGAAACCACA 361
                 || |||||||||| ||| |||||||| || |||||| |||||| | ||||||||||||||||||
Sbjc 61630093    CTCTGCTTGTAAGGGGAGCTCAGAGAATTTCAGAGCTGGAATAAAATTGGCGAAACCACA 61630034

Query  362       TAAAAGTGACTGTTTGGGCAGCAGGTCTAGAGAAATGGGAAAAAGGATGGGAGTGGCTCT 415
                 ||| | |||||||||| | |||||||| ||||||||||||||||| ||||||||||||||||||
Sbjc 61630033    TAAAAGTGACTGTATAGGCAGCAGGTCTAGAGAAATGGGAGAAAGGATGGGAGTGGCTCT 1629974

Query 416        CCAG 419
                 ||| |
Sbjct61629973    CCAG 61629970
```

Fig 3, Homology sequence alignment of 490bp (upper) band was indicated the Insertion (I) polymorphism of ACE gene in population. The red mark nucleotide has replaced by black colour nucleotide.

AGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGATCTGACGAATGTGATGGCCACATCAGGTTTCGCCATTTTAT
TCCAGCTCTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCA
GATCTGACAATGTGATGGCCACATCA

Fig 4, the obtained ACE gene sequence of 192bp (lower) band.

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                 ⇩
Query  1         GGTTTCGCCA-TTTTATTCCAGCTCTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTG  60
                 | |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjc 61630037    GGTTTCGCCAATTTTATTCCAGCTCTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTG 61630096

Query  61        AGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGATCTGAC-AATGTGATGGCC  120
                 |||||| ||| |||||||||||| |||||||| |||||||||||||||||| ||||||||||||
Sbjc 61630097    AGCTAAGGGCTGGAGCTCAAGGCATTCAAACCCCTACCAGATCTGACGAATGTGATGGCC1630156
                                                                    ⇧
Query 121        AC  122
                 | |
Sbjct  61630157  AC  61630158
```

Fig 5, Homology sequence alignment of 192bp (lower) band was indicated the Deletion (D) polymorphism of ACE gene in studied samples.

Statistical analysis with comparison of D/D genotypes between the controls and cases, the cases have showed a significant increase of frequency (p value=0.0005) and odd's ratio of 3.341 was obtained against (I/D+I/I) polymorphism. This has showed that cases with D/D genotypes are three times more prone for the development of Toxemia disorder. Similarly, statistical comparison of the individual frequencies among three genotypes, it was found that D/D genotype were found more prone to develop disease with an odd's ratio of 5.192% (D/D vs I/I) and 2.353(D/D vs I/D).

DISCUSSION

According to (Urban, 2015) it has been mentioned in his study by the analysis of 168 samples that preeclampsia toxaemia women genotype DD of the I/D polymorphism of the ACE gene has been associated with the risk of preeclampsia. [8] Rahimi et al., 2012 also recorded in their study of pre-eclamptic women they have mentioned the association between D/D genotype of ACE I/D polymorphism and risk of preeclampsia. [9] However, (Dimri, et al., 2011) in his study of north Indian women had noticed DD genotype of ACE gene I/D polymorphism appears to predispose to severe pre-eclampsia but not non-severe pre-eclampsia. [10] Correspondingly, (Chen, et al., 2011) in their meta analysis study had mentioned about the subgroup analysis by ethnicity which increased PIH risk was found among

both Asians and Causians. In this report it has been mentioned a significantly elevated risk among Indian and Asian countries population due to D allele of ACE gene polymorphism. [11] Although, Uma et al., 2010 in have not been precisely supported that association between the DD genotype of the ACE gene during the later onset of the disease. [12]

In the report of Biller et al., 2000 it has been recorded that the ACE genemay regulate the level of human serum ACE concentration. Human serum ACE is at the highest level of activity in DD genotype carriers in during the disorder of Taxemia. [13] Correspondingly, Bai et al., 2002 had been found the frequency of the D allele was higher in cases than that in the control group. It had also recorded in the study about the serum concentration of ACE was higher in the PIH group than that in the control group. [14] It had also mentioned by Kauret al., 2005 about the frequency of the DD genotype in the PIH group which had around 60%, while it was only 30% in the control group. The study had showed the statistical difference up to significant level. And the frequency of the D allele in patients with PIH (74%) had been found higher than that the control group (56%). And also has mentioned about the association of ACE I/D polymorphism with PE with OR of 3.35 for the DD genotype and 2.24 for D allele. [15] In another report presented by Mrozikiewicz et al., 2000 about the frequency of distribution of the D and I alleles of the ACE gene were inconsistent in cases and control groups, and had confirmed that the D allele may be a risk factor for PIH. [16]

The data generated by present study coincides with results of various co-workers in this field and showing a strong association of D/D genotype of ACE gene I/D polymorphism and toxaemia of pregnancy in the north Indian population. The main finding of this study was to explore out the D allele of the ACE I/D polymorphism which was may be major risk factor for toxaemia of pregnancy in the studied populations.

CONCLUSION

Based on the data generated by this study, it may conclude that D/D genotype were more prone to develop disease with an odd's ratio of 5.192% (D/D vs I/I) and 2.353(D/D vs I/D) respectively. This genotype polymorphism has a significant association between D allele of the ACE gene and increased risk for toxaemia of pregnancy in the north Indian population. Further researches with a larger dataset is needed for better results to prevent study bias. This study is novel and not available in literatures till date.

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