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THE FIDELITY OF KLENTAQ POLYMERASE DIFFERS FROM TAQ POLYMERASE: A REPORT FROM A STUDY OF CYSTATHIONINE BETA SYNTHASE (CBS) GENE POLYMORPHISM

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ABSTRACT

Introduction: A study was carried out primarily to find out the distribution and frequency of the presence of CBS gene c.833 T>C polymorphism in patients of preeclampsia and in healthy subjects. This study was undertaken secondary to the original study and aims to find out whether any difference in fidelity exists among Taq polymerase and KlenTaq polymerase, so far as CBS gene polymorphism is concerned.

Method: In this case control study, DNAwas isolated from twenty patients presenting with preeclampsia (cases) and twenty healthy individuals (controls). ARMS-PCR of the DNA samples was performed for detection of CBS gene c.833T□C mutation. Taq polymerase was used to amplify the PCR products, which was followed by submarine gel electrophoresis in 3% agarose gel and staining by ethidium bromide. The same procedure was repeated using KlenTaq polymerase in place of Taq polymerase.

Results: When observed under UV light, no band was revealed when Taq polymerase was used. But when KlenTaq polymerase was used, all the DNA samples, both from cases and controls, revealed 174 bp product of normal (TT) allele of CBS gene and 126 bp product, which served as control for PCR reaction. No mutant band was observed.

Conclusion: Study revealed that KlenTaq polymerase and not ordinary Taq polymerase is needed to obtain PCR products of CBS gene showing that fidelity varies between these two types of Taq polymerase.

KEYWORDS : Taq polymerase, KlenTaq polymerase, fidelity, CBS gene

INTRODUCTION

A case control study was planned to study the role of H2S and any possible association of polymorphism in CBS genes in the patients of Pre-eclampsia. The exact etiology and pathogenesis of preeclampsia are unknown, but there is growing evidence, that multiple factors that can be involved in the aetio-pathogenesis may be, imbalance in angiogenic growth factors,[1,2] generalized inflammation and progressive endothelial damage,[3]etc. among a many molecule that may be involved, H_2S had drawn our interest. H_2S together with NO and CO, is now perceived to be a significant biological gaso-transmitter. There is increasing evidence of H_2S having key roles in many important signaling and homeostasis mechanisms, like role in maintaining the insulin level,relaxation of vascular smooth muscle by activation of K^+ ATP channels and inhibition of smooth muscle cell proliferation via mitogenactivated protein kinase signaling pathway.[4]These effects are important for maintaining normal blood pressure.[5,6] H_2S has also shown cyto-protective effects in cases of cellular damage induced by lethal hypoxia or reperfusion injury.[7,8] In regard to angiogenic effect of H_2S, evidence suggests that H_2S promotes angiogenesis via promoting PI3K/Akt or mitogen activated protein kinase / extracellular signalregulated kinase signaling pathways.[9] H_2S also shows antioxidant activity by direct scavenging of reactive oxygen speciesand reactive nitrogen species.[10]

Among different enzymes that are involved in synthesis of H2S in human cells, Cystathionine Beta Synthase (CBS) is one of the most important. A large number of mutations in differentregions of the human CBS gene havebeen found till date. The polymorphisms and mutations on the CBS gene have also been found to be associated with many human diseases, likeHomocysteinuria,[11]Ischemic Brain disease[12]and Down Syndrome.[13] Among different polymorphisms, 833T>C has been found to be associated with Homocysteinuria[14] and Pre-eclampsia[15] by certain studies.

With these prior knowledge and evidences, it was hypothesized that, because of its anti-inflammatory, pro-angiogenic, vasodilator and anti-oxidative characteristics, dysregulation of H_2S metabolism might play a role in pathogenesis of preeclampsia. The role of various polymorphisms of CBS gene with the H_2S dysregulation (if any), was also considered to be studied. Moreover, there is very little information in these regards, in the Indian population, that we could find. Thus the study was primarily aimed to shed some light in this matter.

We initially aimed to carry out a study to find out the distribution and frequency of the presence of CBS gene c.833 T>C polymorphism in patients of preeclampsia and in healthy subjects. But, even after repeating the process carefully, no PCR product was obtained using Taq polymerase enzyme as the standard PCR process.

Then in an effort to troubleshoot, as we studied further, we came with an idea of trying KlenTaq polymerase instead of Taq Polymerase.

Taq Polymerase, the thermostable DNA polymerase named after *Thermus aquaticus*, is the enzyme of choice for most of the routine PCR applications. However, Taq polymerase does not have 3'-5' exonuclease activity so it lacks proofreading capacity and is unable to correct mismatch nucleotide. This reduces the specificity of its activity.Compared to Taq DNA polymerase, KlenTaq polymerase, which is an exonuclease deficient derivative of Taq DNA polymerase is known to be approximately twice as thermostable and displays only half the error rate of Taq DNA polymerase. This is because KlenTaq lacks the first 280 amino acids of native Taq polymerase that contains the 5'-3' exonuclease domain. This deletion makes KlenTaq slightly more thermo-stable and has slightly greater fidelity than full length Taq. Like Taq polymerase, KlenTaq has no inherent 3'-5' exonuclease activity.[16]

So, it was decided to use KlenTaq polymerase in place of Taq Polymerase and to find out whether any difference in fidelity exists among these two enzymes or not.

METHODS

This case control study was undertaken in the Department of Biochemistry of Nil Ratan Sircar Medical College and Hospital, Kolkata, West Bengal, during the period from July 2016 to June 2017. Blood samples of 20 patients suffering from preeclampsia and 20 normal pregnant women attending (Out Patient Department) OPD of Obstetrics &Gynecology, NRS Medical College & Hospital, were collected. The study was approved by Institutional Ethics Committee of NRS Medical College & Hospital, Kolkata.

The cases and controls were selected through following inclusion and exclusion criteria:-

Inclusion Criteria for Cases -

1) Patients of preeclampsia diagnosed and confirmed by clinico-biochemical parameters.
2) Pre-eclamptic women whose Blood Pressure was normal during

first 20 weeks ofgestation
3) No previous history of hypertension

Exclusion Criteria for cases:
1) Patients having seizure disorders, eclampsia, disseminated intravascularcoagulation (DIC), pregnancy induced hypertension (PIH) and pre-existinghypertension were excluded from this study
2) Patients with history of repeated abortion were excluded
3) Histories of renal failure, diabetes mellitus, alcoholism, hepatic dysfunction were also excluded from the study.

Inclusion criteria for controls -
1) Twenty samples were collected from normal pregnant women attendingOPD of Obstetrics &Gynecology of N.R.SMedical College & Hospital, Kolkata forpre-natal check-up.
2) Gestational age more than 20 weeks,
3) No previous history of hypertension, abortion.

Approximately 3 ml blood was collected in EDTA vial for DNA separation, which was done from each EDTA blood sample on the next day of its collection. The kit used for DNA extraction is QIAamp Blood Mini Kit.[17] Prior separation of leukocytes was not necessary. This kit isolates DNA by the principle of adsorption chromatographic method in which DNA molecules binds with silica in a specific salt and certain pH condition. This is also called Spin Column-based Nucleic Acid extraction method which is a solid phase extraction method as DNA binds with solid phase of the silica under certain condition.

Absorbance of DNA samples at 260 nm and 280 nm were taken in spectrophotometer. A ratio of absorbance at 260 nm: 280 nm of within a range of 1.75 to 2 indicated that DNA samples were not contaminated with RNA or protein. Concentration of the DNA samples was measured using the following equation: 50 x A260 = concentration of DNA sample (µg/ml). The integrity of DNA samples was checked by electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Then ARMS PCR was carried out[18] in a total volume of 50 µl of DNA reaction mixture containing 5µl of DNA (150-200 ng of DNA), 1 µl (10 pmols) of each of the reconstituted primers (3 primers: forward normal primer, forward mutant primer and reverse primer), 1 µl of Taq polymerase, 1µl of dNTP (10 µM), 5 µl of Taq buffer and 35 µl of millipore water were mixed to form the PCR mixture, in a micro-eppendorf under sterile condition. The mixtures in the micro-eppendorfs are then loaded in the thermocycler.

30 cycles of PCR were performed as follows: 5 minutes at 94°C, then 30 cycles of [1 minute at 94°C, 1 minute at 63°C, 1 minute at 72°C], then 7 minutes at 72°C. After completion of all these procedures, the amplicons thus prepared were stored at -20°C and further processed by submarine agarose gel electrophoresis. DNA ladder (of the range 100 - 1000 bp) was also prepared (10 µl DNA ladder and 2µl of loading buffer) and run by electrophoresis. After completion of run, the gel was visualized under UV light in gel documentation system for the particular bands and photographs were taken.

The PCR process was repeated exactly in the same manner but using the KlenTaq polymerase[19] in place of Taq polymerase.

RESULTS

20 Pre-eclampsia patients and 20 age and sex match control subjects were selected for this study through the pre determined inclusion and exclusion criteria. All these patients were followed up until the completion of the study.

To avoid any selection bias on demographic or social characteristics of the study participants, participants were selected randomly from the Obstetrics Out Patient Departments using uniform clinical selection criteria.

The figure 1 shows the electrophoretogram of the PCR products when Taq Polymerase was used in the samples of both cases and controls. It is clearly evident from these pictures that apart from the DNA ladders used, there are no other bands visible in both the electrophoretograms, indicating failure of the PCR process to amplify the gene segment.

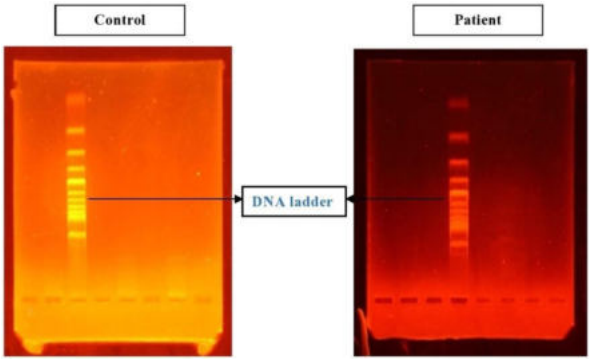

Fig 1: Gel doc images of PCR products done with Taq Polymerase in Healthy Controlsand Patients of Preeclampsia.

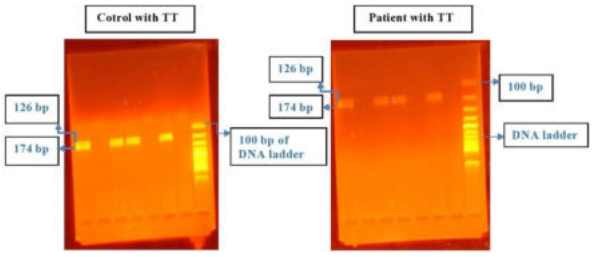

Fig 2: Gel doc images of PCR products done with KlenTaq Polymerase in Healthy Controls and Patients of Preeclampsia.

As seen in the figure 2, however, when KlenTaq Polymerase was used, it is clearly visible, that PCR is successful as seen from the clear bands visible in the samples from both cases and controls. The bands showing 126 and 174 base pair(bp) PCR products, as delineated, comparing with the corresponding bands in the DNA ladder, indicate, all the samples had TT polymorphic form in the CBS gene.

DISCUSSION

Use of the thermostable Taq enables PCR to run at high temperature and eliminates the need of adding new enzyme to each round of thermocycling. However, Taq lacks a proofreading domain, so it has a very high mutation rate especially when mismatch repair is concerned. Scientists were in search of a solution to this problem. In 1992, Barnes WM [16]published a paper where he urged that the fidelity of Taq polymerase in catalyzing PCR can be improved by an N-terminal deletion. This truncated polymerase was called KlenTaq polymerase. It was named after Klenow fragment. PCR tests show that KlenTaq polymerase has improved fidelity (about 25%) and thermostability (about 2 degrees) relative to wild-type Taq, and it gives higher yields of amplicon. It was found to amplify difficult structure or GC-rich templates. It was also found to amplify up to 5 kb genomic targets and up to 20 kb on less complex targets, such as lambda DNA. The increased thermostability allows higher temperature conditions to disrupt difficult secondary structures.[20] Different studies have demonstrated other advantages of KlenTaq polymerase. It was found to tolerate broad range of magnesium concentrations.[21] Moreover, we used klenTaq DNA polymerase from Merck. This polymerase mix contained Taq polymerase containing an N-terminal deletion combined with a small amount of a proofreading polymerase to improve sequence fidelity and length of amplified DNA.[22]

In the context of our study, the specific 833T>C mutation, that was being investigated, was tried to be identified without using a restriction enzyme, but directly using an additional "forward mutant" primer. The amplification signal with the normal (N) allele-specific primer pair is 174 bp which demonstrates the presence of T in position 833, whereas, the amplification signal with the mutant (M) allele-specific primer pair, should be of 242 bp length. Such difference in amplicon can be identified by detection of the 833T>C mutation. There is a very minute difference in the sequence of the "Forward Normal" (5'-CCTGAAGCCGCGCCCTCTGCAGATAAT-3') and "Forward Mutant" (5'-CCTGAAGCCGCGCCCTCTGCAGATAAC-3') primers. Detection of such minute difference in the two forward primers and amplification according to that difference requires a very high level of fidelity, which was found to be shown only by the KlenTaqpolymerase, and not with the usual Taq polymerase. This explains why PCR products were not observed when Taq polymerase

was used. But with use of KlenTaq polymerase in place of Taq polymerase PCR productscould be observed.

Although this study was primarily aimed at studying the association of CBS gene polymorphism in Pre-eclampsia patients, the trouble-shooting in the process revealed this curious and interesting result. But this study has a limitation of a very small sample size. A study with a larger sample size is needed to be followed up. This difference in results with Taq Polymerase and KlenTaq Polymerase is also relevant only in cases where the PCR needs to be high fidelity in distinguishingvery minute differences in primers, as in case of this study.[16] So this result is not generalisable to PCR of most of the usual gene amplification processes through PCR.

CONCLUSION

This study revealed that KlenTaq polymerase and not ordinary Taq polymerase is needed to obtain PCR products of CBS gene. The fidelity of KlenTaq DNA polymerase has been found to be better than usual Taq polymerase in this case.

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