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Evaluation of applicability of *CETP* gene mutation as the predictor of cardiovascular disease in Bangladeshi population

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Abstract

Cholesterol ester transfer protein (CETP) is a hydrophobic glycoprotein that mediates the transfer of cholesteryl ester from HDL in exchange for triglycerides in apolipoprotein B100 containing lipoproteins. Several studies showed genetic mutation in different populations in the CETP gene contributes to the manifestation of cardiovascular diseases (CVD) in that population. Exon 15 is the hotspot of CETP gene mutation. Therefore, the aim of the present study is to search for genetic mutation of CETP gene at exon 15 in the Bangladeshi population. In this study, total of 200 subjects including 100 CVD patients were recruited. Blood samples were drawn from the participants for the assessment of total cholesterol, LDL, HDL and triglycerides. Socio-demographic analyses were carried out among the CVD patients and control. DNA was extracted from the blood sample of selected patients and control individuals. Exon 15 of the CETP gene was amplified through PCR and was subjected to Sanger sequencing. Multiple sequence alignment was carried out to screen mutations. Sequence analysis revealed no mutation at exon 15 of the CETP gene. Thus, mutation of CETP in exon 15 did not provide any significant information for the prediction of CVD in the Bangladeshi population.

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Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide and a major contributor to disability and fatality. The prevalence of CVD is rapidly increasing from 271 million in 1990 to 523 million in 2020 (Roth *et al.*, 2020). Individuals from the Indian subcontinent are at 3 to 4 times higher risk of CVD than white Americans, 6 times higher than Chinese, and around 20 times higher than Japanese (Nag & Ghosh, 2013). Similar to the other part of the world CVDs are being considered as an exceedingly influential cause of mortality and morbidity in Bangladesh (Al Mamun *et al.*, 2016).

The reason and causal factors for CVD related mortality and morbidity in different parts of the world vary significantly due to biological, environmental, ethnicity, diet, dyslipidaemia and genetic background. Dyslipidaemia or imbalance of lipid profile is one of the leading causes of CDV. The abnormality of HDL-C and LDL-C level is greatly influenced by genetic factors which ranges from 40–60% (Qasim & Rader, 2006). Cholesterol ester transfer protein **(**CETP**)** deliver the cholesterol ester from HDL to LDL or apo B containing lipoprotein and decrease the cardiovascular risk (Maïga *et al.*, 2014). Some studies conclude that mutation in CETP alter the TG to HDL ratio which enhance the Cardiovascular disease in Roma and Hungarian population (Piko *et al.*, 2020). In 1994 a group of Japanese researcher reported a missense mutation (D442G) in exon 15 of CETP gene, showing a dominant effect on the CETP activity and HDL-cholesterol level (Sakai *et al.*, 1995), (Zhong *et al.*, 1996).

CVD is a multi-factorial disease. CETP is primarily secreted by the liver and circulates in plasma, bound to high-density lipoprotein (HDL). CETP mediates the transfer of cholesteryl ester. As a result, CETP mutations may affect HDL cholesterol levels and thus the risk of CVD. In many countries or ethnic population of the world, mutated or polymorphic CETP is correlated to CVD and they use CETP inhibitor to prevent the CVD. In Bangladesh, no research has been conducted on the genetic variation related to CVD though many other countries have adopted their population-based prognosis system to tackle this disease. But we still depend on biochemical diagnosis which is quite impossible before the disease manifestation. Since CETP gene mutation is considered as the predictor for CVD and used in early prognosis, proper measures can be taken such as counselling for lifestyle modification in some countries. To do that comprehensive data of the CETP gene mutation in a population is essential. Therefore, the current study was undertaken to check the incidence of CVD with the sociodemographic facts along with genetic mutations, and this is a pioneer study of molecular forecasting practice for Bangladeshi CVD patients. The purpose of the current study is to investigate the correlation of CETP to lipid profile and to find out any mutation prevail in exon 15 of CETP gene.

Materials and methods

Study population

The study was conducted during the period of August 2017 to January 2019. A total 200 subjects were selected including 100 CVD patients from northern regions of Bangladesh who were admitted at the Rajshahi Medical College Hospital, Popular Diagnostic Center and Royal Diagnostic Centre & Hospital, Rajshahi-6205. 100 healthy volunteers from Rajshahi participated in the study.

Selection criteria

The study population were selected based on several criteria. Subjects were included based on their known dyslipidemia condition and secondary conditions associated to CAD. Healthy subjects with normal lipid profile were included in this study. They were non-smokers, non-alcoholic, non-pregnant, sedentary and had stable body weight for at least 3 months before the study. Patients with serious comorbid disease (e.g, cancer, infection, major surgery, malabsorption, hepatic and renal disease or edema etc.) and history of using drugs such as glucocorticoids, Fibric acid derivatives, Corticosteroids, Protease inhibitors, Retinoid were excluded. Among the 100 patients 25

were selected for genetic analysis based on the lowest HDL-C level. A structured questioner was designed and later on filled for collecting various socio demographic data from the study subjects.

Collection of blood samples

Blood samples were drawn from participants who have fasted at least 9 hours before venipuncture. Written consent was taken from the participants before collecting blood and other sociodemographic data for using in the current research. A total of 3ml blood was collected and from that 1.5 ml was taken in a K_2 EDTA tube and 1.5 ml was kept in a glass tube for serum preparation. Proper precautions were taken into account at the time of collecting blood from the study subjects.

Blood serum separation and lipid profiling

The samples were incubated at room temperature to allow clotting. Centrifugation for 10 min at 4,000 rpm was done in a refrigerated centrifuge. The supernatant (serum) was carefully aspirated and was transferred into a fresh micro-centrifuge tube. Lipid profiling was conducted using a reagent kit obtained from Human Diagnostic Worldwide, Germany in a Microlab-300 clinical chemistry analyzer.

DNA extraction and PCR

Based on lipid profile data, 25 patients and 25 controls were chosen for genetic analysis. DNA was isolated following manufacturer's guideline of the TIANamp blood DNA kit, China. CETP gene sequence was retrieved from the NCBI database (Accession no.: NG_008952.1) and one pair (5'-GCCAGGCAAACTCTGCTCTT-3' and 5'-CCTTCTGCTACAAGCCCCAT-3') of primers were designed. As this study was aimed for sophisticated mutation screening, GoTaq® G2 Hot Start Green Master Mix was used which contains polymerase enzyme with proofreading activity to avoid generation of any mutation during PCR amplification.

The PCR product was cleaned up using Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) according to their working manual.

DNA sequencing

In this study, the cycle sequencing PCR was performed using a BigDye Chain Terminator version 3.1 Cycle Sequencing Kit following manufacturer's guide. The thermal cycling profile included (a)initial denaturation at 94°C for 1 minute, (b) cyclic denaturation at 94°C for 10 seconds and annealing at 58°C for 5 seconds and extension at 60°C for 4 minutes; and (c) final extension at 60°C for 10 minutes for 25 cycles. Purification of the product was performed using a BigDye X Terminator® Purification Kit. Finally, sequencing was performed on the ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, USA).

Mutation screening and bioinformatics analysis

Multiple sequence alignment was performed using DNAMAN software version 4.15. All the data sequence was taken in FASTA format and loaded in DNAMAN for multiple sequence alignment output as graphics. To identify any mutation present in the obtained sequences, they were aligned with the native CETP reference gene sequence retrieved from NCBI (Accession no.: NG_008952.1).

Ethics approval

All procedures were in accordance with the ethical standards and the protocol had been approved (Memo No: 110/320/IAMEBBC/IBSc) by the Institutional Ethics Committee for Experimentations on animal, human, microbes and living natural sources, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Results

Analysis of serum lipid profile and demographic parameters

A total of 200 participants (100 healthy control with no CVD records and 100 CVD patients) were included in the study for lipid profiling and demographic analysis. The findings of biochemical and demographic analysis of the study subjects are represented in figure 1. Among the 100 patients, the male patient number was 64 and the female was 36, which indicate the presence of gender variation in

CVD manifestation. The prevalence of family history, hypertension and diabetes were present in high percentage were 25%, 36% and 28% respectively. Multiple risk factors were found to be present at a time for the patients. Smoking prevalence was 20% but not as a single risk factor, 6% smoker had a strong family history of CVD, 8% had Hypertension and 6% had Diabetes. Only 2% patients showed Chronic Kidney Disorders. The assessment of total cholesterol, LDL-C, HDL-C and triglycerides result of lipid profile for the selected subjects for molecular study is shown in Table 1. The mean of total serum cholesterol (mean ±SD) was 220.16±32.67 (mg/dl) in patients and 173.52±63.34 (mg/dl) in control. Mean triglycerides level was 170.84±64.72 (mg/dl) in patients and 122.88±54.35 (mg/dl) in control. LDL-C was 121.08±69.50 (mg/dl) in patients and 95.08±27.43 (mg/dl) in controls, whereas HDL-C was 39.32±21.25 (mg/dl) in patients and 56.00±16.38 (mg/dl) in control.

Table 1. Analysis of Biochemical Parameters.

Variables	Control	CVD patients
Total cholesterol	173.52±63.34	220.16±32.67
HDL-C	56.00±16.38	39.32±21.25
LDL-C	95.08±27.43	121.08±69.50
Triglycerides	122.88±54.35	170.84±64.72

DNA sequencing for exon 15 of CETP gene

DNA sequencing was carried out for exon 15 of CETP gene for molecular analysis. Chromatograms were analyzed in Chromas Pro software. The chromatogram showed outstanding sequencing quality. All the sequences were retrieved from the Chromas Pro Software in FASTA format for further analysis.

Multiple sequence alignment and mutation screening

Multiple sequence alignment of the DNA sequences for exon 15 of CETP gene was performed using DNAMAN software. The results of DNA sequence alignment are shown in figure 2. After screening, no mutations were found in exon 15 of CETP gene. However, interestingly, a G>A mutation was found within intron 15 at nucleotide position 26640 of CETP gene for 12 patients (ID: 1,5-8,11-17).

Discussion

CETP gene is located on chromosome 16 encompassing 16 exons and 25 kb. CETP is a member of the lipopolysaccharide binding protein gene family (Armitage *et al.*, 2019). The gene locus (16q22) is highly polymorphic. CETP originates in the liver and intestine having molecular mass about 69,000 Dalton (Dullaart & Sluiter, 2008). It is consisting of 476 amino acids. It is a key plasma protein which influences circulating levels of HDLC by mediating the transfer of esterified cholesterol from HDL to apoB-containing particles in exchanges for triglycerides (Barter *et al.*, 2003). When comparing biochemical parameter, we found difference between CVD patient and control group. Total cholesterol, LDL-C, HDL-C and triglycerides result of lipid profile for the selected subjects showed variance e.g., HDL-C was 39.32±21.25 (mg/dl) in patients and 56.00±16.38 (mg/dl) in control. Similar type of results was found in Chinese population (Wang *et al.* 2013). High Triglycerides and low level of HDL Cholesterol was found in CVD patients in American Indian (Lee *et al.*, 2017; Thompson *et al.*, 2005). It is established that HDL cholesterol is cardiovascular disease protective in respect of age and sex. From Framingham study it is proved that high level of HDL-C is cardio-protective (Ali *et al.*, 2012). So scientists tried to intervene CVD by using CETP inhibitor (Armitage *et al.*, 2019).

However, later it was found that although CETP inhibitor increases the HDL Cholesterol it may not be able to stop CVD progression. Another study showed that even after taking 100mg of Anacetrapib (a CETP inhibitor) 84% of study subjects manifested CVD (Bowman *et al.*, 2017).

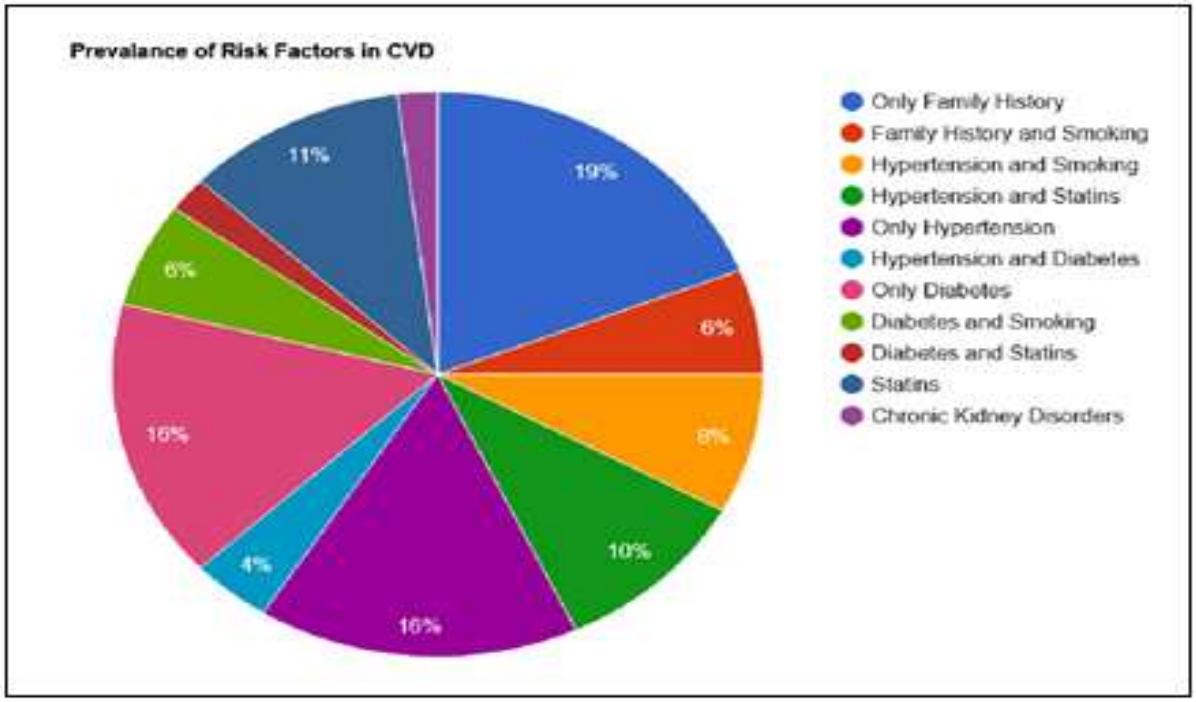

Fig. 1. Risk Factors prevalence of CVD, percentage of Family History, Hypertension, Diabetes, Smoking, Statins and Chronic Kidney Disorders.

In 1993 first mutation in Japanese population was identified as D442G (Takahashi *et al.*, 1993). Another mutation in exon 15 of CETP gene (R451Q) associated with low serum total cholesterol was found in women while high CETP activity found only in men. A study carried over hyper-alpha-lipoproteinic Japanese found that a notable amount (≈29%) of CETP mutation (D442G) in exon 15 (Sakai *et al.*, 1995). implicated high level of HDL but R451Q of exon 15 in Danish population implicated low HDL-Cholesterol (Agerholm-Larsen *et al.*, 2000).

Thus, all the studies show that genetic factor greatly influence the dyslipidemia which is one of the major cause of CVD. In different study carried over in different ethnicity, found mutations of this protein. American whites and African blacks population with two SNPs (rs1968905 and rs289740) with HDL-C were identified (Pirim *et al.*, 2016).

In Chinese population is has been predicted that CETP rs708272 may be associated with the risk of coronary atherosclerosis (Wang *et al.*, 2013). Therefore, in the current study, mutation prone region of CETP gene exon 15 were sequenced from 50 individuals which includes 25 confirmed CVD patient and 25 healthy controls. However, no mutation was found at exon 15 of CETP gene among selected CVD patients. Interestingly, we found a G>A mutation in intron 15 at nucleotide position 26640 of CETP gene for 12 patients among 25.

Although, the role of intronic mutations are unclear, but several studies show that the intron plays vital in regulation of a gene (Jung *et al.*, 2021; Kumari *et al.*, 2022). Thus, this mutation also may play role in miss-regulation of CETP gene. Maybe in near future they will be investigated thoroughly for the better understanding of molecular mechanisms of lipid metabolism. Because of our financial strain and lack of infrastructure, this study was carried out only on limited subjects. However, to obtain a comprehensive idea for the whole population we further need to increase our number of subjects or individuals for CETP gene sequencing. We will also need to compare other exonic mutation hotspot region of CETP gene to correlate the mutations with clinical manifestation of CVD. Further study should be focused on whole CETP gene with an increase of sample size including populations from all over the Bangladesh.

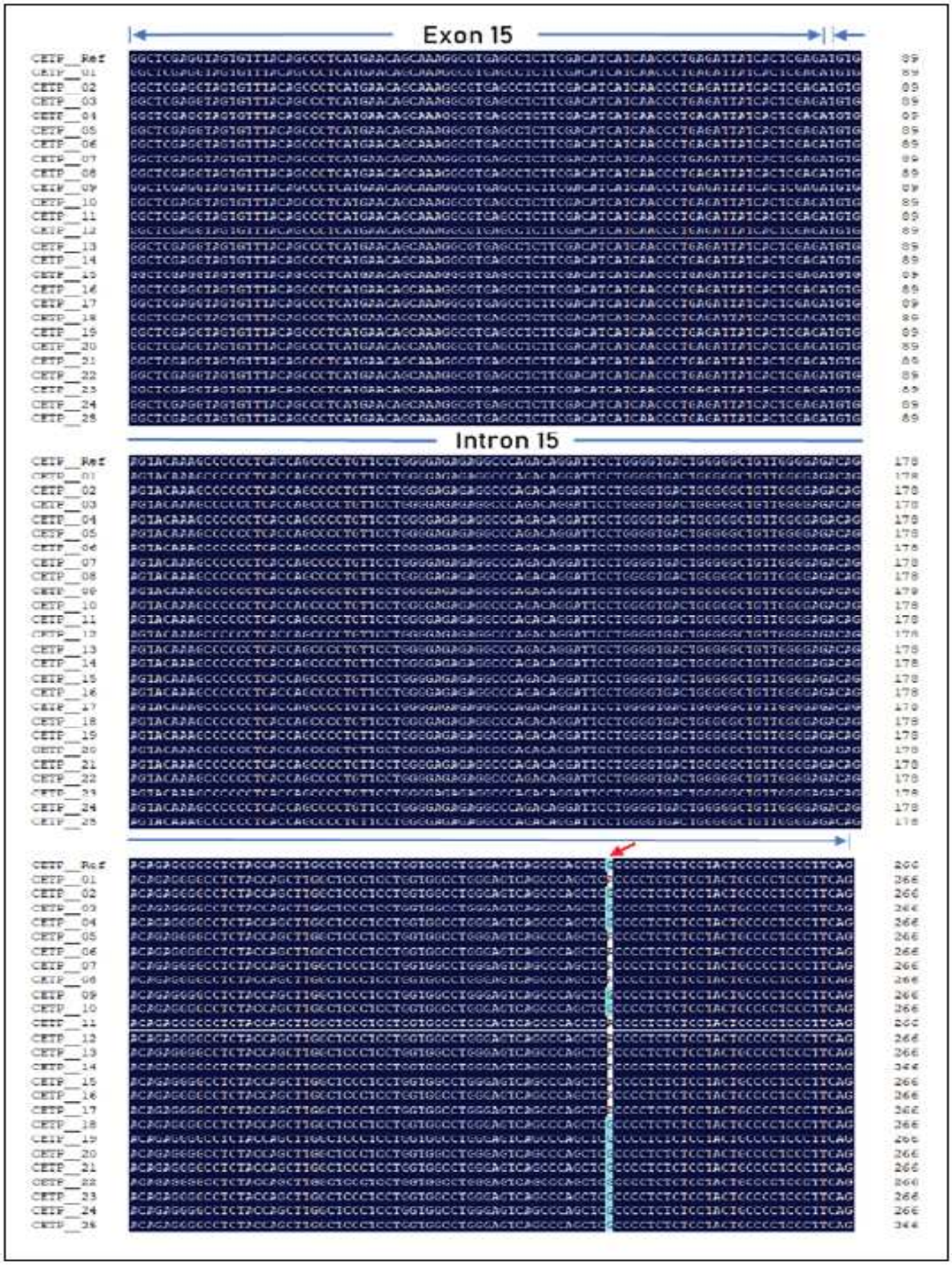

Fig. 2. Multiple Sequence Alignment of partial sequence of CETP gene from 25 patients. No change is detected in exon 15 showing 100% identity with the reference sequence. Red marked region indicates G>A mutation at nucleotide position 26640 in intron 15 of CETP gene.

Conclusion

In the current study, no mutation was detected at exon 15 of CETP gene in the study population. However, we detected a mutation in intronic region, its role is not clear. Therefore, at this point of time CETP gene mutation cannot be correlated with

manifestation of dyslipidemia or CVD and thus, should not be used for genetic prognosis of CVD in Bangladeshi population. Genetic analyses of larger number of samples are required to identify mutation on exon 15 of CETP gene and also mutations in other exons should be looked through to link it to dyslipidemia. Epigenetic or environmental factors may also be possibly responsible for the development of CVD in Bangladeshi population.

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