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eNOS T786C (rs2070744) Gene Polymorphism and Its Underlying Mechanism Associated with Coronary Heart Disease

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Abstract: The study aims to examine plasma nitric oxide concentration and its relationship to T-786C gene polymorphism levels in the development of coronary heart disease in young people. Reduced nitric oxide (NO) bioavailability and endothelial nitric oxide synthase (eNOS) T786C gene polymorphism have been identified as risk factors for the development of coronary heart disease. This cross-sectional study was conducted in SRM Medical College Hospital and Research Centre on 200 angiographically proven CHD subjects attending the Department of Cardiology and medicine and 100 controls from MHC in the age group of ≤ 45 years. Overnight fasting plasma samples were obtained to evaluate lipid profile and nitric oxide by Griess reaction in UV spectrophotometry utilizing the ELISA technique. Polymerase Chain Reaction and Restricted fragment length polymerase amplify the eNOS gene T-786C. As a result, NO levels in plasma were significantly lower in CHD patients than in controls. In addition, C allele carriers of the eNOS T786C polymorphism showed significantly lower mean plasma NO levels than T allele carriers ($P < 0.001$). Our findings suggest that a lower plasma NO level is related to an increased risk of CHD. Furthermore, the eNOS T786C polymorphism is a significant risk factor for CHD development by lowering NO levels in the plasma. However, the eNOS T786C polymorphism influences the severity of CHD.

Keywords: coronary heart disease, endothelial nitric oxide synthase, gene polymorphism, nitric oxide.

一氧化氮合酶吨786C（rs2070744）基因多态性及其与冠心病相关的潜在机制

摘要：该研究旨在检查血浆一氧化氮浓度及其与年轻人冠心病发展过程中吨-786C基因多态性水平的关系。一氧化氮生物利用度降低和内皮一氧化氮合酶吨786C基因多态性已被确定为发生冠心病的危险因素。这项横断面研究是在斯里拉马斯瓦米纪念医学院医院和研究中心进行的，研究对象为200名在心脏病学和医学系就诊的经血管造影证实的冠心病受试者和100名年龄≤45岁年龄组主要组织相容性复合体的对照。利用酶联免疫吸附测定技术，通过紫外分光光度法中的格里斯反应，获得隔夜空腹血浆样品以评估脂质谱和一氧化氮。聚合酶链式反应和限制片段长度聚合酶扩增一氧化氮合酶基因吨-786C。结果，冠心病患者血浆中的一氧化氮水平显着低于对照组。此外，一氧化氮合酶吨786C多态性的C等位基因携带者的平均血浆一氧化氮水平显着低于T等位基因携带者（磷<0.001）。我们的研究结果表明，较低的血浆一氧化氮水平与冠心病风险增加有关。此外，一氧化氮合酶吨786C多态性通过降低血浆中的一氧化氮水平是冠心病发展的重要危险因素。然而，一氧化氮合酶吨786C多态性影响冠心病的严重程度.

关键词：冠心病, 内皮型一氧化氮合酶, 基因多态性, 一氧化氮.

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1. Introduction

The endothelium is essential for controlling vascular tone and shape. Endothelial dysfunction contributes to atherosclerosis [1]. Endothelial cells produce nitric oxide (NO) with an essential enzyme. Endothelial Nitric Oxide synthase (eNOS) aids in the control of blood pressure and blood flow by decreasing the migration and proliferation of vascular smooth muscle cells, which reduces platelet adhesion, and neutrophils, which scavenge oxygen free radicals [2]. Endothelial dysfunction caused by increased generation or removal of reactive oxygen species and superoxide anion (O2•) results in reduced nitric oxide (NO) bioactivity [3].

2. Review of Literature

eNOS is the most common isoform of NO synthase, accounting for the bulk of nitric oxide synthesis. The eNOS genes are in charge of nitric oxide's endothelial and vascular actions [4]. Several single nucleotide polymorphisms in the eNOS gene have been found (T). A single-nucleotide polymorphism, T786C (rs2070744), at nucleotide position at 786 exons causes a conversion of (T-C) has been found in the promoter region of the eNOS gene and has been demonstrated to lower promoter activity by roughly 50%. As a result, it is possible to hypothesize that if the partial structure of eNOS is altered due to the eNOS T786C gene polymorphism, the enzyme's activity may be reduced [5]. That could result in decreased NO generation and improper regulation of vasodilatation and vasoconstriction, ultimately leading to CHD progression [6]. The study's primary goal is to identify the SNP of the eNOS gene polymorphism, which is a vital risk factor for developing CHD in young people. Several studies have found that the polymorphism of the T786C gene is a risk factor for the development of CHD [7-9]. Furthermore, little research has connected the association between intracellular NO production and eNOS gene polymorphism [10].

3. Methods

This cross-sectional study was done on individuals attending the Cardiology and Medicine OP unit at SRMMCH&RC in Chennai, Tamil Nadu, India, from June 2021 to Dec 2021. A total of 300 participants were included, 200 CHD subjects and 100 healthy people were chosen, and all of them were ≤45 years old. The control participants were selected from the Master's Health Check-up Programme and the medicine OP at SRM Medical College Hospital and Research Centre in Chennai, Tamil Nadu, India.

3.1. Inclusion Criteria

The CHD subjects were chosen based on confirmed coronary angiography greater than 50% stenosis, persistent chest discomfort for more than 30 minutes, raised ST > 0.1 mV on at least two adjacent ECG leads, and elevated creatine kinase (CK) to peak values at least 2-fold higher than the normal range.

The control group received an angiogram, and they proved no coronary vessel disease and no ECG evidence of CHD, smoking, hypertension, or DM.

3.2. Exclusion Criteria

Patients having a history of myocardial infarction, end-stage renal failure, or severe liver cirrhosis.

3.3. Ethical Clearance

Following clearance from the Institutional Ethical Committee, all CHD patients and controls were asked to sign a permission form. Height, weight, and BMI were all measured anthropometrically.

3.4. Measurement of Laboratory Parameters

After overnight fasting, the Patient's blood samples (5 ml) were taken in simple vacutainers under aseptic conditions during angiography. First, 2ml of blood was drawn to determine the lipid profile (Total cholesterol by Cholesterol Oxidase technique, Triglycerides by Glycerol Peroxidase method, HDL-C, and LDL-C by Direct method using Beckman Coulter Auto Analyzer) (AU480). Then, the remaining 3ml of blood was allowed to clot for 30 minutes before being centrifuged at 5000 RPM for 10 minutes to detect Nitric Oxide using (Griess Method) utilizing ELISA assay in UV spectrophotometry.

3.5. Measurement of Nitric Oxide

Serum NO was quantified using the Griess reagent as nitrite/nitrate in CHD patients and controls.

It is a two-step procedure. The initial stage in nitrate reductase is converting nitrate to nitrite. The addition of Griess Reagents in the second stage converts the nitrite into a deep purple azo molecule. The concentration of NO2- is entirely determined by this azo chromophore.

UV absorbance is measured at 540nm-Spectrophotometer.

3.6. Molecular Analysis (DNA Extraction)

In all CHD patients and controls, 1ml peripheral blood samples were collected for DNA isolation from whole human blood using the QIAGEN DNA Extraction Kit (Catalogue number: 51104). The extracted DNA is measured to determine the purity of the DNA in the sample. The extracted DNA is measured to determine the purity of the DNA in the sample. The integrity of extracted DNA is verified by 2% Agarose Gel Electrophoresis calculated by Ultra Violet (UV) Spectroscopy and kept at -20°C.

3.7. PCR and RFLP Analysis (Primer Sequence for eNOS)

Forward Primer – (5'-GTCTCTCAGCTTCCGTTTCTT-3')

Reverse Primer – (5'-CCTTGAGTCTGACATTAGGGTATC-3')

PCR amplification was carried out under a variety of heat cycling conditions, followed by:

- Denaturation at 95^{o}C for 2 min (30 cycles)
- Annealing at 58^{o}C for 30 seconds
- Elongation at 72^{o}C for 90 seconds

3.8. Restriction Enzyme Analysis

By using 1 µl of Msp1 (Biolabs) with 2 µl of 10x restriction buffer and 18 µl of RNAase free water, the PCR product (10 µl) was digested separately and should be incubated at 37^{o}C for 5 hours. The digested product was analyzed by 2% agarose gel in a 1×TAE buffer solution, and it was stained using SYBR Green dye. Under Ultra Violet light, the bands were observed to reveal the genotype for the single nucleotide polymorphism.

3.9. Statistical Analysis

The Statistical Package for the Social Sciences was used to analyze the data (SPSS 25).

For Qualitative and Quantitative variables, descriptive statistics were employed to assess percent, percentage, and range. The Chi-Square test was used to determine group association for qualitative variables. A student t-test was performed for quantitative variables. Pearson Correlation was used to examine two quantitative variables and the relationship between variables. P-values of less than 0.05 were deemed statistically significant.

4. Results

Among the 300 Participants, 200 CHD subjects with an average of 34.3 ± 4.5 years and 100 Healthy Control with an average age of 36.8 ± 2.7. The clinical and demographic data of the CHD patients and control participants revealed no significant differences in mean ages (P > 0.05). The majority of CHD and control subjects are in the age group ≤40 years. The mean levels of BMI, Waist Circumference, Waist Hip Ratio, and systolic blood pressure are higher in CHD subjects when compared to controls in the current research.

The study found that FBG, total cholesterol, triglycerides, and LDL-C levels are substantially higher in CHD subjects when compared to controls. However, the mean values of HDL-C did not differ substantially between the two groups. Furthermore, the mean level of serum NO in CHD individuals was significantly lower (12.97 ± 1.20) compared to controls (19.08 ± 4.74) which shows a significant difference (Fig. 1). All samples were amplified using an eNOS T-786C gene product. The eNOS T786C gene polymorphism is associated with a significant difference between CHD and control individuals (P < 0.05) (Table 1). The genotypic distribution of the eNOS T786C polymorphism in CHD patients and healthy controls was studied.

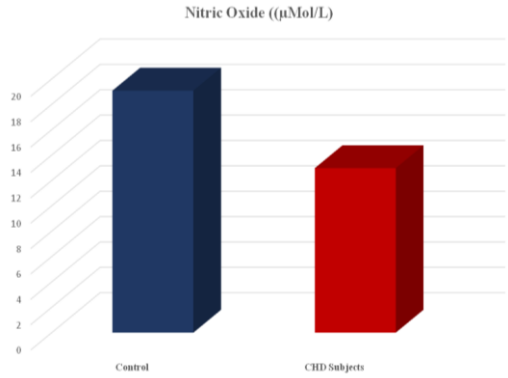

Fig. 1 Mean level of Nitric Oxide between control and CHD subjects

Table 1 Demographics and biochemical characteristics of the coronary heart disease subjects and healthy controls

Variables	Controls (n = 100)	CHD subjects (n = 200)	p-value
Mean age	36.8 ± 2.7	34.3 ± 4.5	0.2138
Height (cm)	170.5 ± 2.46	170.81 ± 3.44	0.4853
Weight (Kg)	65.09 ± 3.26	71.75 ± 5.73	< 0.001
BMI (Kg/m^2)	21.91± 0.37	24.47 ± 1.61	< 0.001
WC (Cm)	84.77 ± 3.26	90.51 ± 4.45	< 0.001
HC (Cm)	99.54 ± 3.11	98.8 ± 5.23	0.2475
W/H Ratio	0.84 ± 0.02	0.90 ± 0.04	< 0.001
Systolic Blood Pressure	117.5 ± 3.3	135.6 ± 4.2	< 0.001
Diastolic Blood Pressure	80 ± 1.2	74.3 ± 6.2	< 0.001
FBG (mg/dl)	90.24 ± 4.18	94.29 ± 6.98	< 0.001
Total cholesterol (mg/dl)	168.8 ± 16.3	219 ± 41.42	< 0.001
Triglyceride (mg/dl)	84.6 ± 30.5	159.7 ± 69	< 0.001
HDL-C (mg/dl)	46 ± 9	34 ± 7	< 0.001
LDL-C (mg/dl)	106.4 ± 12.59	189.4 ± 27.46	< 0.001
VLDL (mg/dl)	17.26 ± 8.77	28.06 ± 12.14	< 0.001
Nitric Oxide ((µMol/L)	19.08 ± 4.74	12.97 ± 1.20	< 0.001

Notes: BMI - body mass index, SBP - systolic blood pressure, DBP - diastolic blood pressure, LDL - low-density lipoprotein, FBG - fasting blood glucose, DM - diabetes mellitus, HT - hypertension

The distribution of heterozygote TC genotype (odds ratio (OR), 1.9; 95% confidence interval (CI), 1.13-3.21; P = 0.017) and mutant homozygote CC genotype (odds ratio (OR), 4; 95% confidence interval (CI), 1.34-10.44; P = 0.011) was significantly different between CHD patients and control subjects.

In addition, the C allele of the T786C polymorphism was significantly more common in CHD patients than in controls (odds ratio (OR), 2.15; 95% confidence interval (CI), 1.34-3.27; P = 0.001). The interaction between the eNOS T786C gene polymorphism and CHD risk was also studied using the dominant genetic model. The eNOS T786C gene polymorphism was found to enhance CHD risk in the dominant genetic model (OR, 2.25; 95% CI, 1.33-4.14; P=0.003) (Table 2, Fig. 2). In the presence of the C allele, digestion of the PCR product resulted in a single non-cleaved 248 bp fragment, but in the presence of the T allele, the 248 bp amplicon was cleaved into 163 bp and 85 bp fragments.

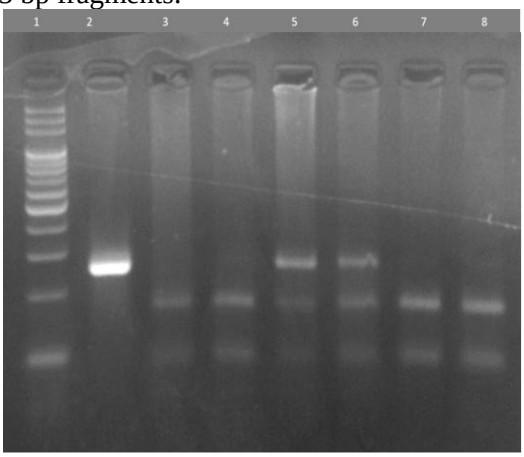

Fig. 2 RFLP analysis of eNOS T-786C gene polymorphism. Lane 1 - 100 bp ladder. Lane 2 represents the mutant homozygote genotype (786CC). Lanes 5 and 6 are heterozygote genotypes (786TC). Lanes 3, 4, 7, and 8 represent the wild type genotype (786TT)

Table 2 T-786C gene polymorphism between CHD and control group

T-786 C Genotype	Control Group n = 100	CHD Group n = 200	Relative Risk (Odds Ratio)	Confidence Interval (95%)	p-value
TC	29 (29)	80 (40)	1.9	1.13-3.21	0.017
CC	4 (4)	23 (11.5)	4	1.34-10.44	0.011
TT	67 (67)	97 (48.5)	-	-	-
Dominant Model					
CC + TC	33 (37)	103 (41.5)	2.25	1.33-4.14	0.003
Allele frequency					
T	163 (21.5)	274 (36.5)	-	-	-
C	37(78.5)	126 (63.5)	2.15	1.34-3.27	0.001

Notes: TT - wild type, TC – heterozygote, CC – homozygote, CHD - coronary heart disease, eNOS - endothelial nitric oxide synthase, OR - odds ratio, CI - confidence interval

5. Discussion

Mutations in the eNOS encoding gene may result in decreased nitric oxide output and coronary artery disease. By this conclusion, we revealed that reduced plasma NO concentrations and polymorphism of the eNOS T786C gene are important risk factors for developing CHD in young patients. The eNOS T786C gene polymorphism is associated with lower eNOS gene expression levels, which operate as a lowering factor for plasma NO concentration. In Table 1, the demographic profile shows the mean level of systolic blood pressure was higher in the CHD subject's 135.6±4.2 mm of Hg when compared to controls 117.5±3.3 mm of Hg. Furthermore, smokers had a significantly higher frequency of the eNOS T-786C variant than non-smokers in CHD patients. The synergistic relationship between eNOS and the (786TC) genotype and smoking leads to decreased NO production, which in turn attenuates endothelial dysfunction and raises the risk of CHD in young individuals. NO is a critical regulating molecule of cardiovascular function in health, and it plays a role in inhibiting atherogenesis and the development of atheromatous plaques [11]. A lack of Nitric Oxide generation leads to the development of CHD [12]. The plasma NO concentration was considerably lower in a subject with coronary heart disease, showing that the physiologic concentration is required to maintain the vascular wall's standard, vasodilatory, and non-inflammatory function [13]. Similar to our findings, Charakida et al. discovered that those with lower NOs concentrations are more prone to develop atherosclerosis [14]. NO production is crucial in pathophysiological conditions at the microvascular level. Several mechanisms, including increased vascular tone and enhanced angiotensin II actions, may accelerate the development of microvascular problems caused by a drop in NO levels [15]. In the current investigation, the distribution of genotype and allele frequencies involved in the polymorphism of the T786C eNOS gene differed considerably between CHD and control patients that were by some previously published studies [16].

Furthermore, this polymorphism's elevated risk of CHD demonstrated the significant connection of this genetic variation with the development and severity of CHD. Several investigations have found that the eNOS gene polymorphism may influence the activities of enzymes involved in the development of coronary heart disease [17]. However, other research contradicted our

findings that found the T786C polymorphism to be unrelated to the incidence of CHD [18-20]. The reasons for these inconsistencies may be due to a variety of factors such as differences in study design, selection criteria for CHD patients and control subjects, differences in sample size, several interactions between gene-gene and gene-environmental, as well as the ethnic background of the studied populations [21-22]. Nevertheless, the findings are consistent with prior research, including a meta-analysis study by Rai et al. and a case-control study by Salimi et al. [23-24]. Since plasma NO concentration is regulated by three distinct isoforms of NO synthase (NOS1, NOS2, NOS3), the current discovery offers evidence for the direct involvement of the eNOS T786C polymorphism in regulating plasma NO concentration by affecting eNOS gene expression levels. According to several studies, decreased NO bioavailability is the primary cause of endothelial dysfunction and atherosclerosis development [25-29].

The current study's findings in CHD individuals also revealed decreased NO bioavailability. This finding may be related to the higher C allele frequency in CHD individuals, which indicates a substantial reduction in eNOS gene promoter activity connected with eNOS T-786C allele. That is the primary reason for lowered eNOS gene expression levels and NO production compared to the control group.

5.1. Limitations

This study has a few limitations. First, more accurate measurement is needed to measure Nitric oxide levels. Secondly, this cross-sectional study design and the results are limited. These observations need further confirmation using a prospective study design. Also, the sample size was not large enough due to the high cost of genotyping.

6. Conclusion

Individuals with the mutant TC and CC genotype have an increased risk of developing CHD. Mutant "C" allele frequency was also high in CHD subjects. Thus, heterozygosity/homozygosity (TC/CC) for the T786C eNOS polymorphism has been associated with CHD. The possible mechanism that modifies and impacts eNOS (786 TC) mutation activity that contributes to the development of CHD in young patients is reduced NO production. It may be attributed to the decreased nitric oxide synthase activity in individuals with mutant "C" allele, which may lead to decreased nitric oxide synthesis. In future studies, the polymorphism in individuals with various genotypes for this polymorphism should be determined in large sample sizes to validate the above theory, which may assist in the development of innovative medicines for the treatment of CHD at a molecular level.

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