

Endothelial Nitric Oxide Synthase (eNOS) Gene Expression determine the abundance of circulatory Endothelial Progenitor Cells (EPC) in Premature Coronary Artery Disease (PCAD) Patients

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Research note

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Abstract

Objectives: Several studies has reported a reduced circulatory level and impaired functionality of EPCs in coronary artery disease (CAD) patients and the role of endothelial nitric oxide Synthase (eNOS) in relation with reduced circulatory level of EPC and their impaired functionality has been revealed by in vitro and animal studies. However EPC's eNOS gene expression profile in in vivo condition in PCAD patients is yet to be revealed as the prevalence of CAD at young age is markedly increased in developing countries. Our previous study has already reported a significantly reduced circulatory level of EPC in PCAD patients compared to control subjects and in continuation of that finding, present study aimed to investigate the eNOS gene expression of EPC in same study subjects as well as to establish the association between EPC's eNOS gene expression and reduced circulatory level of EPC in PCAD patients.

Results: Reduced eNOS gene expression in EPC from PCAD patients compared to healthy controls were found ($0.998\pm 0.096/1.063\pm 0.107$) with a p-value of 0.002 and this difference was persistent even after adjusting for confounding factors ($p=0.002$). A positive correlation was found between eNOS gene expression and level of EPC in circulation ($r = 0.234$ and $p = 0.0664$); data from previous study.

Introduction

Bone marrow derived endothelial progenitor cells, take part in the process of neovascularization and re-endothelization in postnatal life [1]. EPC can be mobilized from bone marrow in response to ischemia or vascular trauma and repair damaged endothelium by differentiating into mature endothelial cells. EPC also exerts its paracrine effect and promote vascular growth by secreting angiogenic factors [2–4].

Endothelial nitric oxide Synthase, a heme containing enzyme catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide (NO), with involvement of tetrahydrobiopterin and NADPH as essential cofactors [5]. The NO synthesized by eNOS serves as an atheroprotective agent and mediates a variety of physiological roles in vivo, including neovascularization. However, emerging evidence from recent studies reported that the eNOS derived NO exerts additional functions by regulating mobilization of stem or progenitor cell.

However several studies have measured the eNOS gene expression, and suggested the role of eNOS in the depletion of EPC levels in cell culture assays and animal studies, but the role of eNOS gene expression has not been studied yet in premature coronary artery disease (PCAD) patient and that too in in vivo condition rather culturing the cells in in vitro. Our previous study already revealed a significantly reduced circulatory level of EPC in PCAD patients compared to healthy control [6] and in continuation of the previous data the present study estimated the EPC's eNOS gene expression in premature coronary artery disease patients in in vivo condition to explore whether eNOS gene expression profile is a determinant of the reduced circulatory EPC level in PCAD patients.

Methods

This is a case control study was carried out in which 50 subjects with age of ≤ 50 years of either sex and having evidence of CAD , recognized by the presence of either $>50\%$ stenosis in the left main coronary artery and fifty age matched non diabetic subjects with a normal coronary angiogram or without demonstrable ischemia on trade meal test (TMT) were recruited as controls from Department of Cardiology, All India Institute of Medical Sciences, New Delhi, India . The subjects with any kind of cancer, rheumatoid arthritis, pneumonia, nephropathy and cerebrovascular disease were excluded. The study protocol was approved by Institutional review board and written consent was obtained from all the participants prior to recruit as a study subject.

Endothelial progenitor cells were isolated from peripheral blood on the basis of cell surface antigens CD34+/KDR+ by Magnetic Activated Cell sorting (MACS) method. The intracellular eNOS gene expression was assessed by RT PCR method . The primer sequence for eNOS and GAPDH as reference gene used for RT-PCR were as follows: eNOS (forward, 5'-GGGGAGCCAAAAGGGTCATCATCT-3'; reverse, 5'-GAGGGGCCATCCACAGTCTTCT-3'), GAPDH (forward, 5'-GGGGAGCCAAAAGGGTCATCATCT-3'; reverse, 5'-GAGGGGCCATCCACAGTCTTCT-3'). Integrated density values were measured by using Image J software (NIH, Bethesda) for both eNOS and GAPDH bands in both cases and controls and the ratios were computed and compared.

Statistical analysis was done using STATA/SE, version 9.0. The distribution of eNOS gene expression data was not normal and hence data was log transformed, expressed as geometric mean with 95% confidence intervals (95% CI). Linear regression analysis was employed to adjust for confounding factors such as age, sex, BMI, smoking and medications. Correlation between EPC's eNOS gene expression and circulatory EPC level was established by Pearson correlation.

Results

The EPC's eNOS gene expression (eNOS/GAPDH ratio) in PCAD cases and healthy controls ranged from 0.725 to 1.19 and 0.726 to 1.337. eNOS gene expression was significantly lower in EPC from PCAD patients compared to controls and the difference was consistent even after adjustment for confounding factors like age, gender, BMI, and smoking, and medication use (Table 1).

Table 1
eNOS gene expression of EPC in cases and controls

	PCAD Mean (C.I) N = 50	Control Mean (C.I) N = 50	p values (\$)	p values (#)	p values (@)
eNOS expression* (eNOS/GAPDH)	0.998 (0.97–1.025)	1.063 (1.032–1.093)	0.002	0.002	0.019
*Data log transformed for analysis and expressed as mean with 95% confidence interval (C.I)					
\$Unadjusted for confounding factors. #Adjusted for age, sex, BMI and smoking. @Adjusted for age, sex, BMI, smoking and medication.					

A positive correlation between EPC's eNOS gene expression and circulatory level of EPC was observed ($r = 0.234$ and $p = 0.0664$.) and shown in fig 1. The circulatory EPC level in PCAD [0.018, CI = 0.013-0.023] and age matched controls [0.039, CI = 0.03-0.048], has been reported in our previous study (6). The EPC level were significantly higher in control subjects [$p = 0.001$].

Fig: 1. Correlation between EPC's eNOS gene expression and circulatory CD34+/KDR+ cells

Discussion

In our previous study, shortening of telomere length and reduced telomerase activity was directly associated with decreased EPC count in circulation in PCAD patient which may be implicated by accelerated cellular senescence [6]. Apart from the cellular senescence, an impaired mobilization of EPC from bone marrow may also lead to their depleted numbers in peripheral blood. Studies in animals have shown that eNOS play an important role in the mobilization of endothelial progenitor cells as well as EPC senescence [7, 8, 9]. In in vitro study eNOS transfected EPCs had increased functional capacity and migration compared to untransfected cells which further suggests the role of eNOS in the EPC migration and function [10]. A reduced circulatory EPC level in CAD patients might be the result of decreased eNOS gene expression as homing of EPC from bone marrow to the peripheral circulation is mediated via different cytokines including nitric oxide.

In conclusion, we have found a significant decreased eNOS gene expression in PCAD patients as compared to controls which might be a determinant of depleted circulating EPC level in young CAD patients and this in turn may indicate an impaired mobilization of EPC from bone marrow into the peripheral circulation. In addition of this, as the study determined the eNOS gene expression directly in EPC isolated from study subjects, the result more realistically reflect the in vivo condition compared to in vitro study.

Limitations

- The prevalence of coronary artery disease at young age is being increased gradually in developing countries and circulatory level of endothelial progenitor cell and its functionality directly reflect the disease severity in cardiovascular event. This study demonstrate that intracellular endothelial nitric oxide synthase enzyme is associated with reduced circulatory EPC level in PCAD patients which might be an indication of disease prognosis.
- However EPCs functionality should have been assessed in PCAD as well as its association with intracellular eNOS expression.
- Intracellular EPC's eNOS expression has been carried out in the present study using RTPCR with GAPDH as reference which is actually semiquantitative method, however for the assessment of relative fold change in gene expression, qPCR method is more accurate for quantitative gene expression.

- We also did not assess Intracellular EPC's eNOS protein level which would have helped to establish the association between intracellular eNOS level and circulatory EPC level.

Abbreviations

EPC, endothelial progenitor cell; eNOS, endothelial nitric oxide synthase; PCAD, premature coronary artery disease; NO, nitric oxide; CAD, coronary artery disease; MACS, magnetic activated cell sorting.

Declarations

Ethics approval and consent to participate: Ethics approval was obtained from ethical committee of All India Institute of Medical Sciences. Written signed consent from all the participants was obtained.

Availability of data and material: The dataset used in the current study are available from the corresponding author on reasonable request.

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Authors contribution: KV and LR conceived, designed and implemented the study. KV and AS performed the laboratory work, statistical data analysis and prepared the manuscript. AR and VKB was involved in the clinical assessment and recruitment of the study participants. RMP and SS gave their valuable inputs in statistical analysis and laboratory experiments. DP and KSR gave their intellectual input for the manuscript. All the authors read and approved the final manuscript.

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Figures

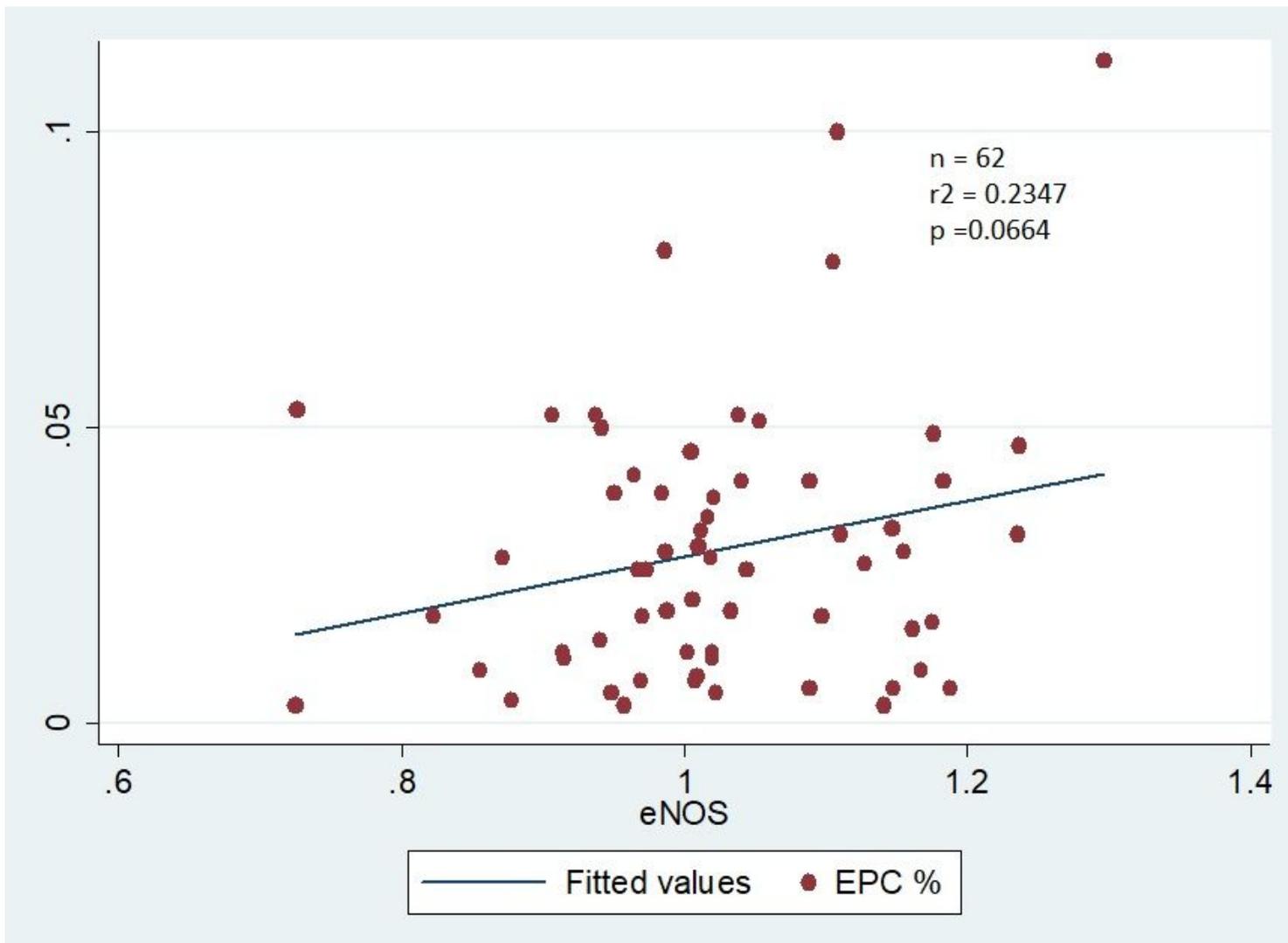


Figure 1

Correlation between EPC's eNOS gene expression and circulatory CD34+/KDR+ cells