Indian Journal of Experimental Biology
Vol. 61, July 2023, pp. 517-525
DOI: 10.56042/ijeb.v61i07.2212

Hypermethylation and reduced expression of lipoprotein metabolism genes *ABCG1*, *LIPC* and *PLTP* in Obese and Diabetic subjects: Potential risk factors for Coronary artery disease

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Received 30 January 2023; Revised 11 May 2023

Increased lipid levels in the body can cause a plethora of complications and the lipoprotein metabolism genes such as ATP binding cassette transporter G1 (*ABCG1*), hepatic lipase (*LIPC*) and phospholipid transfer protein **(***PLTP*) have a key role in the efflux of cholesterol from peripheral tissues to the liver. Altered methylation and expression of these genes in obese individuals and patients with diabetes mellitus can increase the risk of coronary artery disease (CAD). Here, we investigated the relationship of promoter methylation and expression of these lipoprotein metabolism genes ABCG1, LIPC and PLTP with the risk of CAD in diabetes and obese subjects. The study group consisting of 574 subjects, including 207 angiographically confirmed CAD patients, 100 diabetic patients and 82 obesity subjects without CAD and 185 healthy controls. DNA methylation status of *ABCG1*, *LIPC* and *PLTP* gene loci was determined by methylation-specific PCR and gene expression was analysed by real-time PCR. In obese individuals, the hypermethylation of *ABCG1* (OR = 3.83, 95% CI = 2.11–6.96, P =0.0001) and *PLTP* (OR = 1.88, 95% CI = 1.07–3.28, P = 0.02 versus control) and reduced expression of *ABCG1* (0.4-fold) and *PLTP* (decreased by 0.0025-fold) were observed. Hypermethylation of *ABCG1* (OR = 1.71, 95% CI = 1.14–2.55, P = 0.01) and *LIPC* (OR = 1.58, 95% CI = 1.06–2.36, P = 0.02) genes was significantly higher in CAD patients when compared to healthy controls, whereas reduced expression of these genes by 0.77- and 0.82-fold was observed. The hypermethylation of *LIPC* was observed in CAD subjects with higher cholesterol and LDL (low density lipoproteins) levels (OR = 2.2, 95% CI = 1.18–4.09, P = 0.01; OR = 2.08, 95% CI = 1.12–4.22, P = 0.02, respectively). CAD subjects with diabetes (n=118) showed hypermethylation of *LIPC* (OR = 2.1, 95% CI = 1.2–3.67, P = 0.01) and *PLTP* (OR = 2.01, 95% CI = 1.13–3.59, P = 0.01). Hypermethylation of *ABCG1* (OR = 1.91, 95% CI = 1.09–3.36, P = 0.03) and *LIPC* (OR = 2.0, 95% CI = 1.12–3.55, P = 0.02) genes were associated with lifestyle habit such as cigarette smoking. The results suggest that obese individuals are at a risk of CAD through alteration of lipoprotein homeostasis. Lifestyle modifications such as reduction in BMI and cessation of cigarette smoking might reduce the risk of CAD.

Keywords: ATP binding cassette transporter G1 (*ABCG1*), Coronary artery disease (CAD), Diabetes, Hepatic lipase (*LIPC*), DNA methylation, Obesity, Phospholipid transfer protein **(***PLTP*), Smoking

Coronary artery disease (CAD) is a complex, multi-factorial disease, is the leading cause of morbidity and mortality globally, including in developing countries like India[1,2]. Factors such as age, family history, diabetes mellitus, dyslipidaemia, hypertension, obesity, smoking and unhealthy diet are associated with an increased risk of CAD[3]. In addition, lipid over-accumulation is also a good predictor of CAD risk[4].

Several candidate genes including ATP binding cassette transporter G1 (*ABCG1*), hepatic lipase (*LIPC*) and phospholipid transfer protein (*PLTP* have been shown to influence the risk of CAD[5]. Previous studies have shown that several human diseases, including atherosclerosis and cancer, are caused by abnormal DNA methylation[6,7]. DNA methylation is the most recognized epigenetic modifications linked to transcriptional splicing and is important for gene regulation which acts through the disruption of the binding of transcription factors of candidate genes that contributing to the development of CAD[8].

The *ABCG1* gene is a transmembrane cholesterol transporter that mediates cholesterol efflux to HDL and plays an important role in macrophage sterol homeostasis, reverse cholesterol transport and atherosclerosis[9]. *ABCG1* is ubiquitously expressed in lipid-loaded macrophages. Upon differentiation,

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macrophages upregulate the expression of *ABCG1* and scavenger receptors (SRs) which in turn have the ability to take up modified lipoproteins and regulate the cellular lipid metabolism. Influx and efflux imbalance of modified LDLs promotes the macrophages to become lipid-laden foam cells, thus increasing the risk of CAD[10]. Though the numerous polymorphisms have been reported in *ABCG1* gene, none of the SNPs have been found to be associated with lipid metabolism. Interestingly, several studies have reported a strong association between the DNA methylation of *ABCG1* gene with circulating triglycerides and HDL-C levels[11-13]. In CAD subjects, downregulation of the *ABCG1* gene expression indicates its association with early atherosclerosis[14]. In another study, the promoter hypermethylation was observed in patients with chronic heart disease[15].

The human LIPC gene encodes hepatic lipase (HL), an enzyme involved in metabolism and regulation of lipoprotein[16]. It catalyses the hydrolysis of triglycerides and phospholipids from plasma lipoproteins, thus remodelling very low-density lipoprotein (VLDL) remnants, LDL and HDL[17]. Hepatic lipase activity is also inversely related to the buoyancy and size of both LDL and HDL particles. Independent of its lipolytic function, HL also plays a role in the hepatic uptake of remnants, HDL and LDL particles[17,18]. Low HL activity has been related to high triglyceride-rich LDL and HDL concentrations and the accumulation of remnant lipoproteins in addition to triglyceride-rich LDL and HDL which accounts for the premature CAD in HL deficiency[19].

PLTP plays several key roles in HDL metabolism including the transfer of surface phospholipids, cholesterol from triglyceride-rich lipoproteins, such as

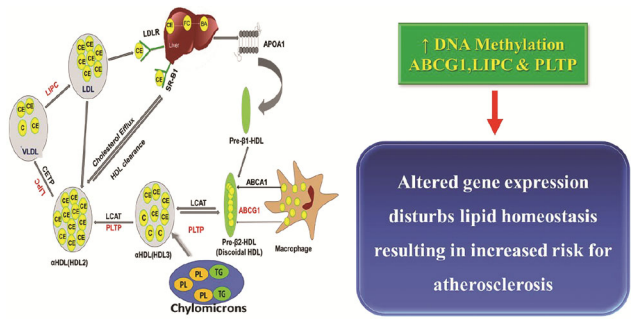

Fig. 1 — Role of *ABCG1*, *LIPC* and *PLTP* genes in cholesterol efflux. [*ABCG1*, *LIPC* and *PLTP* genes are involved in the reverse cholesterol transport pathway, and play a major role in the efflux of cholesterol from peripheral tissues to the liver through a variety of mechanisms]

chylomicrons, VLDL particles to HDL during intravascular lipolysis, remodelling of HDL particles, facilitation of HDL-mediated efflux of phospholipids and cholesterol and modulation of cholesteryl ester transfer protein (CETP) activity[20,21]. The role of *ABCG1*, *LIPC* and *PLTP* genes in cholesterol efflux is depicted in Fig. 1.

Methylation of CpG islands, in the promoter region of the genes, is one of the major mechanisms through which the gene downregulation takes place[22]. Previous studies have shown that DNA-promoted methylation of certain genes which in turn are responsible for predisposition of CAD[23-26]. Diabetes and obesity are known risk factors that are associated with CAD and there are limited reports on the study of epigenetic alterations and expression of *ABCG1*, *LIPC* and *PLTP* in diabetes as well as obese subjects. Hence, in this study, we investigated the relationship of promoter methylation and expression of *ABCG1*, *LIPC* and *PLTP* genes with the risk of CAD in diabetes and obese subjects.

Materials and Methods

In this retrospective case-control study, a total of 574 subjects were recruited, including 207 angiographically documented CAD patients without and with diabetes, 100 diabetic subjects (fasting blood glucose >126 mg/dL), 82 obese subjects (BMI >30 kg/m^2) and 185 age-, gender- and ethnicity-matched healthy controls from Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India. Healthy control subjects were hospital staff and voluntary blood donors who were screened for dyslipidaemia, liver and kidney functions and with no family history of CAD. Patients with non-atherosclerotic myocardial ischemia, pregnant/nursing women, and patients with type 1 diabetes mellitus, ketoacidosis and infections were excluded from the study. Baseline characteristics of all the patients and control subjects were recorded (Table 1). Data on gender, age, body mass index, smoking, alcohol consumption, tobacco consumption, hypertension and diabetes were collected from the self-designed questionnaire. The data of serum total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels were obtained from medical records. The study was approved by the Institutional Ethics Committee of Nizam's Institute of Medical Sciences, Hyderabad (IEC) (EC/NIMS/1578/2015). Informed consent was obtained from all the subjects.

After 12 hours of overnight fasting, 5 mL of blood was collected in EDTA vacutainer and used for RNA and/or DNA isolation. The DNA isolation was performed from the whole blood leukocytes using the phenol-chloroform method. Quality and quantity of purified DNA were assessed by a NanoDrop Spectrophotometer (NanoDrop 2000c). The EpiTect Bisulfide Kit (Qiagen, Germany) was used to treat approximately 2 µg of extracted DNA by sodium bisulfide during which the unmethylated cytosines converted into the corresponding uracil, while the methylated cytosines remained unchanged in their positions. The converted DNA was stored at –20ºC until analyzed.

Methylation-specific PCR

The methylation-specific PCR (MSP) was used to assess the methylation status of *ABCG1*, *LIPC* and *PLTP* genes in study subjects and control. Primers specific for the methylated and unmethylated status of three genes were designed using a meth DB software. The sequence of primers and PCR conditions are given in Table 2. For amplification of the target sequence, MSP analysis was done in a final volume of 10 µL comprising 5 µL hot start master mix (EpiTect

Table 1 — Demographic and clinical characteristics of subjects studied

Variables	CAD (n=207)	Diabetes (n=100)	Obesity (n=82)	Controls (n=185)
Age (yrs)	53.79±11.64	53.27±11.39	36.79±9.19	52.25±12.15
Gender				
Male (%)	123 (59.42)	61 (61)	58 (70.73)	100 (54.05)
Female (%)	84 (40.57)	39 (39)	24 (29.26)	85 (49.94)
BMI (kg/m^2)	25.40±5.13	24.51±2.68	32.90±2.41***	22.34±4.93
Smoking				
Current (%)	111 (53.62)	28 (28)	38 (46.34)	82 (44.32)
Never (%)	96 (46.37)	72 (72)	44 (53.65)	103 (55.67)
Alcohol intake (%)	15.8	37:6	37.50	Nil
Diabetes (%)	57:43	100	Nil	Nil
Hypertension (%)	47.8	28:72	14.6	Nil
Lipid profile				
TC (mg/dL)	199.81±106.39*	159.1±42.42	158.64±43.99	172.2±10.6
TG (mg/dL)	188.73±107.05*	168.13±95.04*	176.59±99.64*	137.9±20.1
HDL (mg/dL)	37.95±16.49	43.42±12.88	42.59±13.58	38.5±2.3
LDL (mg/dL)	91.43±49.67	82.43±36.03	81.14±37.51	102±8.9

[Continuous and categorical characteristics are given as mean (SD) and percentage, respectively. BMI, body mass index; TC, Total cholesterol; TG, Triglycerides; HDL, High-density lipoprotein cholesterol; LDL, Low-density lipoprotein cholesterol. ***P <0.001 *vs*. control]

Table 2 — Methylation-specific PCR and Quantitative real-time PCR primers and conditions

Gene	Primer sequence	Product size(bp)	PCR conditions
ABCG1 MF	5'TTTAAGTAGTTGGGATTATAGGCGT3'	120	95°C 5 min, 95°C 45 s, 59.3°C 45 s, 72°C 45s, 72°C 5 min, 42 cycles.
ABCG1 MR	5'GAATCACGAAATCAAAAAATCG3'		
ABCG1 UF	5'TTTAAGTAGTTGGGATTATAGGTGT3'	120	
ABCG1 UR	5'CAAATCACAAAATCAAAAAATCAAA3'		
LIPC MF	5'TTTTTTGAGTTGTTCGTTATTTTACG3'	176	95°C 5 min, 95°C45 s, 60°C 45 s, 72°C 45s, 72°C 5 min, 42 cycles.
LIPC MR	5'CTACCTAAAACCCCCATATTAACGT3'		
LIPC UF	5'TTTTTGAGTTGTTTGTTATTTTATGT3'	176	
LIPC UR	5'TACCTAAAACCCCCATATTAACATT3'		
PLTP MF	5'GGTTATTGTACGGGGTTTTAAGTC3'	205	95°C 5 min, 95°C 45 s, 57.4°C 45 s, 72°C 45s, 72°C 5 min, 42 cycles.
PLTP MR	5'TAAACATACCCTTATTCTCCTCGC3'		
PLTP UF	5'GGTTATTGTATGGGGTTTTAAGTT3'	205	
PLTP UR	5'CACTAAACATACCCTTATTCTCCTCAC3'		
ABCG1 F	5'AGAGATGACGGAGCCCAAGT3'	193	-
ABCG1 R	5'CGAATAGGAAAGGTCCCTGA3'		-
LIPC F	5'ATGATGGGGGCTACATCAAC3'	154	-
LIPC R	5'TTGAAGGTTCCCCCGAAG3'		-
PLTP F	5'CCATTTGGAAGAAGAGCTCAA3'	176	-
PLTP R	5'CCGTGGATTATCATCACCAGA3'		-
β-ACTIN F	5'TGCTATCCCTGTACGCCTCT3'	219	-
β-ACTIN R	5'CTCCTTAATGTCACGCACGA3'	219	-

[MF, Methylation Forward; MR, Methylation Reverse; UF, Unmethylated Forward; UR, Unmethylated Reverse Real-time PCR Primers]

MSP Kit, Qiagen, Germany), 0.4 µL of each primer and 3 µL of bisulfide converted DNA. The amplified PCR products were checked on 2.5% agarose gels with ethidium bromide staining for methylation index.

Real-time PCR (qPCR)

Total RNA was isolated from whole blood using Trizol reagent from an RNAisoPlus kit (Clontech, DSS Takara Bio India Pvt Ltd). A total of 2 µg RNA was used for reverse transcription (Clontech, DSS Takara Bio India Pvt Ltd). qPCR was carried out using SYBR super mix (Thermofisher). The primers used for qPCR are given in Table 2. All qPCR reactions were carried out in triplicate with template-free negative control performed in parallel. Relative mRNA levels were normalized with the housekeeping gene β-actin for target expression using the ΔΔCt method.

Statistical analysis

Statistical analysis of the study data was analysed using Graph Pad prism software. All the data is presented as mean ± SD. Student t-test was performed to assess whether the mean difference between the two given groups is statistically significant. Analysis of variance (ANOVA) was used if there were more than two groups. Fisher exact test was used to calculate odds ratio (OR) and 95% confidence interval (CI) based on the distribution of categorical variable in two groups. Value of P <0.05 was considered statistically significant.

Results

Analysis of methylation and expression pattern of *ABCG1*, *LIPC* and *PLTP* in patients with CAD

As shown in Table 3, increased methylation of *ABCG1* and *LIPC* genes in the CAD group when compared with the controls (OR: 1.71, 95% CI: 1.14-2.55, P = 0.01 and OR: 1.58, 95% CI: 1.06-2.36, P = 0.02, respectively) was observed. There was an insignificant increase in methylation of *PLTP* gene in CAD cases. On the other hand, decreased expression was observed in *ABCG1* and *LIPC* genes when compared with controls (fold change of 0.77 and 0.82). Further, we also segregated the methylated and unmethylated samples in CAD cases and found a significant decrease in the expression of methylated samples compared to unmethylated samples of *ABCG1* and *LIPC* genes (fold-change of 0.62 *vs*. 6.04, P = 0.0012 and 0.88 *vs*. 3.01, P = 0.008, respectively).

Analysis of methylation and expression pattern of *ABCG1*, *LIPC* and *PLTP* in patients with diabetes

As compared to the control group, significant hypermethylation of the *PLPT* gene in the diabetic group (OR: 1.73, 95% CI: 1.03–2.89, P = 0.04) and decreased expression of the *PLTP* (fold change 0.002) was observed (Table 3). However, we could not find any relationship with the methylation of *ABCG1* and *LIPC* in the diabetic group when compared to controls (OR: 1.48, 95% CI: 0.91-2.43, P = 0.13 and OR: 1.51, 95% CI: 0.91-2.48, P = 0.1, respectively). Further in

Table 3 — Methylation pattern in CAD *vs*. Controls

	ABCG1		*LIPC*		*PLTP*	
	CAD (n=207)	Controls (n=185)	CAD (n=207)	Controls (n=185)	CAD (n=207)	Controls (n=185)
Methylation	127 (61.35%)	89 (48.10%)	122 (58.93%)	88 (47.56%)	133 (64.25%)	104 (56.21%)
Unmethylation	80 (38.64%)	96 (51.89%)	85 (41.06%)	97 (52.43%)	74 (35.74%)	81 (43.78%)
Odds ratio	1.71		1.58		1.39	
95% CI	1.14-2.55		1.06-2.36		0.93-2.10	
p value	0.01		0.02		0.12	
Methylation pattern in diabetes *vs*. Controls						
	diabetes (n=100)	Controls (n=185)	diabetes (n=100)	Controls (n=185)	diabetes (n=100)	Controls (n=185)
Methylation	58 (58%)	89 (48.10%)	63 (63%)	98 (52.97%)	69 (69%)	104 (56.21%)
Unmethylation	42 (42%)	96 (51.89%)	37 (37%)	87 (47.02%)	31 (31%)	81 (43.78%)
Odds ratio	1.48		1.51		1.73	
95% CI	0.911-2.43		0.91-2.48		1.03-2.89	
p value	0.13		0.1		0.04	
Methylation pattern in obesity *vs*. controls						
	obesity (n=82)	Controls (n=185)	obesity (n=82)	Controls (n=185)	obesity (n=82)	Controls (n=185)
Methylation	64 (78.04%)	89 (48.10%)	52 (63.41%)	97 (52.43%)	58 (70.73%)	104 (56.21%)
Unmethylation	18 (21.95%)	96 (51.89%)	30 (36.58%)	88 (47.56%)	24 (29.26%)	81 (43.78%)
Odds ratio	3.83		1.57		1.88	
95% CI	2.11-6.96		0.92-2.68		1.07-3.28	
p value	0.0001		0.1		0.02	

[*P <0.05 statistically significant]

the diabetic group, a significant decrease in the expression of *LIPC* and *PLTP* genes in methylated samples was observed when compared to unmethylated samples (fold change of 0.64 *vs*. 1.98, P = 0.0001 and 0.715 *vs*. 1.56, P = 0.02).

Analysis of methylation and expression pattern of *ABCG1*, *LIPC* and *PLTP* in Obesity

We found a significant hypermethylation of *ABCG1* and *PLTP* genes in the obesity group when compared with the controls (OR: 3.83, 95% CI: 2.11-6.96, P = 0.0001 and OR: 1.88, 95% CI: 1.07-3.28, P = 0.02, respectively). Compared to the control group, a reduced expression of the *LIPC* gene was observed in the obesity group (fold change of 0.011) and the same was also seen in methylated samples (fold change of 0.874 *vs*. 3.016, P = 0.008) (Table 3).

Association of methylation with clinical phenotypes

Further, the data were segregated based on the clinical phenotypes of CAD and found increased methylation of *LIPC* and *PLTP* genes in CAD with diabetes (OR: 2.1, 95% CI: 1.2-3.67, P = 0.01 and OR: 2.01, 95% CI: 1.13-3.59, P = 0.01) and without diabetes (OR: 1.89, 95% CI: 1.04-3.45, P = 0.03 and OR: 1.81, 95% CI: 1.00-3.29, P = 0.05) (Table 4). In case of CAD with obesity and without obesity, the hypermethylation of *PLTP* gene (OR: 2.07, 95% CI: 1.10-3.90, P = 0.02) was observed. Whereas in obesity *vs*. CAD without obesity, we observed a significant hypermethylation of all three genes in the obesity group (OR: 1.88, 95% CI: 1.01-3.52, P = 0.05 OR: 3.08, 95% CI: 1.71-5.54, P = 0.0001 and OR: 2.32, 95% CI: 1.30-4.12, P = 0.005). Additionally, it was seen that, the hypermethylation of *ABCG1* and *LIPC* genes were significantly associated with the smoking (OR: 1.91, 95% CI: 1.09-3.36, P = 0.03 and OR: 2.0, 95% CI: 1.12-3.55, P = 0.02, respectively) (Table 4). Hypermethylation of *LIPC* was associated with increased cholesterol and LDL-C (OR: 2.2, 95% CI: 1.18-4.09, P = 0.01 and OR: 2.18, 95% CI: 1.12-4.22, P = 0.02) and hypermethylation of *ABCG1* was associated with decreased HDL-C (OR: 1.99, 95% CI: 1.09-3.63, P = 0.02) (Table 5)

Table 4 — Association of methylation with clinical phenotypes

Parameters	*ABCG1*		*LIPC*		*PLTP*	
	Methylated	Unmethlated	Methylated	Unmethlated	Methylated	Unmethlated
CAD with hypertension (n=119)	54 (45.37%)	53 (60.22%)	68 (57.14%)	43 (48.86%)	66 (66.66%)	66 (61.11%)
CAD without hypertension (n=88)	45 (37.81%)	35 (39.77%)	51 (42.85%)	45 (51.13%)	33 (33.33%)	42 (38.88%)
Odds ratio	0.57		1.39		1.27	
95% CI	0.32-1.07		0.8-2.42		0.72-2.24	
p value	0.06		0.26		0.46	
CAD with smoking (n=111)	75 (67.56%)	36 (32.43%)	79 (71.17%)	42 (37.83%)	70 (63.06%)	41 (36.93%)
CAD without smoking (n=97)	50 (51.54%)	46 (47.42%)	53 (55.20%)	43 (44.79%)	63 (64.58%)	34 (35.41%)
Odds ratio	1.91		2		0.93	
95% CI	1.09-3.36		1.12-3.55		0.53-1.65	
p value	0.03		0.02		0.88	
CAD with diabetes (118)	73 (61.86%)	45 (38.13%)	72 (61.01%)	46 (38.98%)	84 (71.18%)	34 (28.81%)
CAD without diabetes (89)	54 (60.67%)	35 (39.32%)	38 (42.69%)	51 (57.30%)	49 (55.05%)	40 (44.94%)
Odds ratio	1.05		2.1		2.01	
95% CI	0.59-1.84		1.2-3.67		1.13-3.59	
p value	0.8		0.01		0.01	
Obesity (n=60)	31 (51.66%)	29 (48.33%)	35 (58.33%)	25 (41.66%)	41 (68.33%)	19 (31.66%)
CAD without obesity (n=147)	96 (65.30%)	51 (34.69%)	68 (46.25%)	79 (53.74%)	75 (51.02%)	72 (48.97%)
Odds ratio	0.56		1.62		2.07	
95% CI	0.3-1.04		0.88-2.98		1.10-3.90	
p value	0.08		0.12		0.03	
Diabetes (n=100)	58 (58%)	42 (42%)	63 (63%)	37 (37%)	69 (69%)	31 (31%)
CAD without diabetes(n=89)	54 (60.17%)	35 (39.32%)	46 (51.68%)	43 (48.31%)	49 (55.05%)	40 (44.94%)
Odds ratio	0.89		1.89		1.81	
95% CI	0.5 - 1.60		1.04-3.45		1.00 - 3.29	
p value	0.7		0.03		0.05	
Obesity (n=82)	64 (78.04%)	18 (21.95%)	60 (73.17%)	22 (26.82%)	58 (70.73%)	24 (29.26%)
CAD without obesity (n=147)	96 (65.30%)	51 (34.69%)	69 (46.93%)	78 (53.06%)	75 (51.02%)	72 (48.97%)
Odds ratio	1.88		3.08		2.32	
95% CI	1.01- 3.52		1.71-5.54		1.30-4.12	
p value	0.05		0.0001		0.005	

[**P* <0.05 statistically significant]

Table 5 — Association of methylation with lipid profile

Parameters	*ABCG1*		*LIPC*		*PLTP*	
	Methylated	Unmethlated	Methylated	Unmethlated	Methylated	Unmethlated
CAD with >200 cholesterol (n=66)	39 (59.09%)	27 (40.90%)	46 (69.69%)	20 (30.30%)	90 (63.82%)	51 (36.17%)
CAD with <200 cholesterol (n=141)	88 (62.41%)	53 (37.58%)	72 (51.06%)	69 (48.93%)	42 (63.63%)	24 (36.36%)
Odds ratio	0.8		2.2		1	
95% CI	0.47 - 1.58		1.18-4.09		0.54-1.85	
p value	0.75		0.01		1	
CAD with <40 HDL (n=86)	63 (73.25%)	23 (26.74%)	67 (55.37%)	54 (44.62%)	75 (61.98%)	46 (38.01%)
CAD with >40 HDL (n=121)	70 (57.85%)	51 (42.14%)	51 (59.30%)	35 (40.69%)	57 (66.27%)	29 (33.72%)
Odds ratio	1.99		0.85		0.82	
95% CI	1.09 - 3.63		0.48-1.49		0.46-1.47	
p value	0.02		0.66		0.55	
CAD with >200 Triglycerides (n=108)	63 (58.33%)	45 (41.66%)	57 (52.77%)	51 (47.22%)	51 (47.22%)	57 (52.77%)
CAD with <200 Triglycerides (n=99)	55 (55.55%)	44 (44.44%)	53 (54.08%)	45 (45.91%)	46 (46.46%)	53 (53.53%)
Odds ratio	1.12		0.94		1.03	
95% CI	0.64-1.94		0.54-1.64		0.59-1.78	
p value	0.77		0.88		1	
CAD with >130(n=57)	36 (63.15%)	21 (36.84%)	36 (63.15%)	21 (36.84%)	36 (63.15%)	21 (36.84%)
CAD with <130 (n=150)	91 (60.66%)	59 (39.33%)	91 (60.66%)	59 (39.33%)	91 (60.66%)	59 (39.33%)
Odds ratio	1.11		2.18		1.14	
95% CI	0.59-2.08		1.12-4.22		0.61-2.11	
p value	0.75		0.02		0.75	

[*P <0.05 statistically significant]

Discussion

Patients with type 2 diabetes and obesity have an increased risk for cardiovascular morbidity and mortality[27]. Several studies have shown that epigenetics plays a significant role in complex diseases and provides mechanisms whereby environmental factors can influence CAD, type 2 diabetes and obesity[28,29]. Epigenetics thus in turn may explain some of the missing heritability, and because of its effects on regulation, it provides a functional role for some of the intergenic loci associated with the diseases.

ABCG1 is a cholesterol transporter that plays a crucial role in cellular lipid homeostasis. In our study, a significantly higher promoter methylation of *ABCG1* gene was found in the CAD group as compared to the controls. This showed that the *ABCG1* gene promoter hypermethylation can increase the risk of CAD[15]. In another study, higher DNA methylation and decreased expression of *ABCG1* in familial hypercholesterolemia (FH) were demonstrated[5]. We found hypermethylation of *ABCG1* resulting in significantly lower LDL-C in men and higher triglyceride levels in women, whereas no conclusive result was observed with HDL-C levels indicating that DNA methylation could contribute independently to plasma lipid variability in FH[5]. In the present study, we showed that promoter methylation of *ABCG1* is significantly increased in CAD cases and a negative correlation between the *ABCG1* hypermethylation and the *ABCG1* expression levels were also observed (data not shown). Integrating gene expression data revealed an association between the cg06500161 methylation and lipid levels which may be partly mediated by *ABCG1* expression[30]. DNA methylation at this CpG site was also found to be elevated in myocardial infarction subjects compared to healthy individuals[31]. Interestingly, DNA methylation at cg06500161 in blood has been shown to be correlated negatively to *ABCG1* expression in blood[30]. Altogether, *ABCG1* gene methylation might play a key role because CpG (cg06500161) located in this gene is associated with both HDL-C and triglyceride levels[32]. In line with these results, we also have found a significant hypermethylation of *ABCG1* resulting in decreased HDL levels. Atherosclerosis has been reported to increase when associated with obesity, insulin resistance, and type 2 diabetes[33]. In the previous studies, reduced *ABCG1* expression and cholesterol efflux was found in patients with type 2 diabetes[34,35]. In this study, we observed increased *ABCG1* promoter methylation with obesity, but not with diabetes mellitus. Additionally, we also have found that reduced expression in both diabetes and obesity

groups. These results clearly explain that the methylation at the promoter region causes gene inactivation and silencing resulting in the gene repression. This leads to impaired efflux and causes increased intracellular cholesterol accumulation leading to vascular disease in patients with obesity and type 2 diabetes[34].

LIPC is a known risk factor for CAD[36]. Previous studies have shown that *LIPC* DNA methylation was higher in CAD patients compared with patients without a previous history of CAD[5]. Higher methylation levels of the *APOA5* and *LIPC* gene promoters has been shown to increase the risk for CAD[37]. The small DNA methylation changes at isolated CpG sites make large differences at gene-expression levels over time that predispose to the late-onset disease such as CVD (Cardio-vascular diseases)[38]. In this present study, we found hyper-methylation of *LIPC* is associated with the CAD risk. Though the percentage of methylation of *LIPC* was higher in diabetes and obese subjects, but we could not find significant association when compared to controls. However, the expression levels were significantly lower in both the groups. Obesity is a major public health problem and a risk factor for type 2 diabetes, hypertension and cardiovascular disease[39]. It has been suggested that elevated hepatic lipase activity would be associated with premature CAD, mediated by central obesity[38].

The *PLTP* activity is closely related to obesity, diabetes mellitus and insulin resistance[40-42]. Although the mechanism of these associations remains poorly defined, it is likely related to the putative role of *PLTP* in the development of atherosclerosis, a common underlying factor in each of these disease states[43]. The strong, inverse relationship between HDL levels and atherosclerosis risk, *PLTP* might influence the atherosclerosis through its effects on HDL metabolism. *PLTP* is highly expressed and regulated in macrophage cells and this suggests its potential involvement in lipid efflux[21].

One study found that a higher *PLTP* DNA methylation is associated with smaller HDL particles, as well as lower concentrations of HDL-phospholipid and HDL-C in men and also demonstrated a decreased in *PLTP* expression[5]. These results suggest that *PLTP*-mediated lipid transport decreases when *PLTP* DNA methylation increases[5]. In our present study, we observed increased methylation of *PLTP* in CAD and obesity subjects. We also found a reduced expression of *PLTP* in diabetes subjects as compared to healthy controls.

Conclusion

Our study shows the hypermethylation and reduced expression of *ABCG1*, *LIPC* and *PLTP* in diabetes and obesity individuals, which may be the potential risk factors for CAD. Smoking and lipid alterations are likely to be the important factors in influencing DNA methylation. Epigenetic modulation has a key role in gene transcription regulation, especially by disrupting the binding of transcription factors and influencing gene expression patterns. Hence, measuring the methylation and expression of these genes in diabetes and obese subjects may be useful in predicting the future risk for CAD. Lifestyle modifications such as reduction in BMI through exercise and cessation of cigarette smoking may reduce the risk for CAD.

Acknowledgement

The first author BI acknowledges award of Senior Research Fellowship from the Indian Council of Medical Research (ICMR), New Delhi, India and Department of Health Research (DHR), New Delhi, India.

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