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DNA Methylation Analysis of the Insulin-like Growth Factor-1 (IGF1) Gene in Swedish Men with Normal Glucose Tolerance and Type 2 Diabetes

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Abstract

Objective: Recent genetic studies have demonstrated that Single Nucleotide Polymorphism (SNP) rs35767(C/T) in the IGF1 gene promoter is associated with insulin resistance and serum IGF-I levels and thereby implicated that IGF1 has genetic effect in Type 2 Diabetes (T2D). The present study aimed to investigate the alteration of DNA methylation levels of the IGF1 gene in T2D.

Subjects and methods: A total of 688 Swedish men with Normal Glucose Tolerance (NGT) or T2D were selected from Stockholm Diabetes Prevention Program. DNA methylation levels at rs35767 SNP-CpG site and other three CpG sites (P1-P3) in the IGF1 gene promoter region were analyzed with PyroMark Assays and bisulfite pyrosequencing. Fasting serum IGF-I levels were measured with an in-house radio-immunoassay.

Results: DNA methylation levels at CpG site P3 of the IGF1 gene promoter were increased in T2D patients compared with NGT subjects (84.8% vs. 74.2%, P<0.001), while serum IGF-I levels were lower in T2D than that in NGT subjects (152 μg/l vs 169 μg/l, P=0.029). In SNP rs35767(C/T), the carriers with CC genotype had higher DNA methylation levels at SNP-CpG site compared with the carriers with CT and TT genotypes in both NGT and T2D.

Conclusions: The present study provides the first evidence that increased DNA methylation levels of the IGF1 gene and decreased serum IGF-I protein concentration are associated with T2D, and suggests that DNA methylation in the IGF1 gene may interact with SNP rs35767 (C/T) in the gene promoter region.

Keywords: DNA methylation; Insulin; T2D

Introduction

Insulin-Like Growth Factor-I (IGF-I) is a circulating growth factor mainly produced in the liver [1]. Endocrine Growth Hormone (GH) is the principal hormone to stimulate the liver production of IGF-I [2]. High portal insulin levels make the liver GH sensitive and increase IGF-1 levels. Low portal insulin levels or hepatic insulin resistance, on the other hand, reduces the sensitivity for GH of the liver and lower serum IGF-1 levels [3]. IGF-1 effectuates a negative feedback loop on GH levels, whereas GH is a well-known counter-regulatory hormone for the actions of insulin [4]. IGF-I shares structure homology with insulin and plays an insulin-like role in the regulation of glucose homeostasis [5]. Clinical investigation has demonstrated that the administration of recombined human IGF-I protein may increase the insulin sensitivity in patients with type 2 diabetes (T2D) [6,7].

T2D is a complex metabolic disorder, characterized by impaired insulin secretion and/or insulin resistance influenced by genetic and non-genetic factors. Human IGF1 gene is located on chromosome 12q23.2. In the recent years, genetic studies have demonstrated that SNP rs35767 (C/T) in the IGF1 gene promoter region is associated with fasting insulin and insulin resistance [8,9]. The carriers with T allele had higher serum IGF-I levels than the C allele carriers [10,11]. Therefore, the IGF1 gene may have genetic effect in the development of T2D. However, no epigenetic study of the IGF1 gene in T2D has been reported yet. DNA methylation usually occurs at a cytosine followed by guanine (CpG) and DNA methylation levels are often analyzed in clusters of CpG sites in the gene promoter [12,13]. Accumulating data has demonstrated that analysis of DNA methylation alteration in the susceptibility genes for T2D, predicted by genetic studies, may provide further information to understand the epigenetic effects of the genes in the disease [14-16]. Furthermore, a recent study has implicated that the analysis of DNA methylation in whole blood samples is worthwhile for DNA methylation studies in the metabolic disorders [17].

There are several questions concerning epigenetic effects of the IGF1 gene in T2D. First, is there any CpG site in the IGF1 gene promoter, which may influence DNA methylation levels of the gene? Second, does DNA methylation display patterns at SNP-CpG of rs35767 (C/T) in the IGF1 gene relate to T2D since the minor allele T of this polymorphism removes a CpG site? Third, is DNA methylation alteration of the IGF1 gene related to serum IGF-I variation and associated with T2D? To address these questions, in present study we designed the assays to analyze IGF1 DNA methylation levels in Normal Glucose Tolerance (NGT) subjects and T2D patients.

Subjects and Methods

Subjects

A total of 688 unrelated Swedish men including subjects with normal glucose tolerance (NGT) (n=511) and patients with T2D (n=177) were selected from Stockholm Diabetes Prevention Program (SDPP) follow-up study [18]. Among 177 T2D patients, 95 were newly diagnosed and 82 had diabetes for a mean duration of three years. Oral glucose tolerance test were performed in all participants to this program. The diagnosis of diabetes was according to the World Health Organization criteria (WHO 1998) [19]. In SDPP baseline survey (around ten years before), none of these 688 subjects had diabetes. All individuals were included in the genotyping

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experiments. The information regarding Family History of Diabetes (FHD) was recorded according questionnaires. FHD was defined as having at least one fist-degree relative or at least two second-degree relatives with diabetes.

To avoid the influence caused by age difference, we selected the age-matched NGT subjects (n=242) and T2D patients (n=164, 89 were newly diagnosed and 75 were with anti-diabetes treatments) for DNA methylation analysis. Clinical parameters of individuals included in the genetic and epigenetic studies are presented in Table 1a and b, respectively. Informed consent from all subjects was received and the study was approved by the local ethics committee.

SNP rs35767 genotyping

Genomic DNA was extracted from peripheral blood using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). Genotyping experiments of SNP rs35767 (C/T) in the IGF1 gene were performed with TaqMan allelic discrimination using ABI 7300 system. SNP genotyping assay (ID: C_799146_10) was purchased from Life Technologies (Carlsbad, California, USA). For genotyping quality controls, the cases and controls were distributed randomly, and negative controls (universal mixture blanks) were included in each PCR plate. More than 20% samples were duplicated for genotyping experiments.

DNA methylation analyses

In addition to SNP-CpG site of rs35767, there are three CpG sites (P1-P3) in the IGF1 gene promoter region (Figure 1). Three PyroMark assays were designed using the PyroMark Assay design software

A. In genotyping study

	NGT	T2D	NGT *vs.* T2D *P*-value (FHD-/ FHD +)
N (FHD-/+)	511 (402/109)	177 (41/136)	
Age (years)	57 (57-58)/57 (56-58)	60 (58-61)/ 58 (57-59)	0.003/ <0.001
BMI (kg/m^2)	25.8 (25.5-26.0)/26.0 (25.4-26.7)	29.4 (27.9-30.8)/30.1 (29.3-30.9)	<0.001/ <0.001
f-Glucose (mmol/l)	5.0 (4.9-5.0)/5.1 (5.0-5.2)	7.0 (6.5-7.5)/8.2 (7.7-8.6)	<0.001/ <0.001
2h-Glucose (mmol/l)	4.9 (4.8-5.0)/5.2 (5.0-5.5)	13.4 (12.5-14.4)/14.0 (13.1-15.0)	<0.001/ <0.001
f-Insulin (pmol/l)*	92.7 (89.1-96.4)/99.0 (91.2-107.4)	150.3 (122.9-183.6)/ 167.3 (154.5-181.1)	<0.001/ <0.001
2h-Insulin (pmol/l)*	291.2 (276.7-306.5) /325.9 (292.5-363.3)	496.8 (405.3-609.1)/ 535.8 (468.8-612.5)	<0.001/ <0.001
HOMA-IR	3.20 (3.06-3.34)/3.51 (2.16-3.85)	8.28 (6.55-10.01)/9.67 (8.73-10.60)	<0.001/ <0.001
SBP (mmHg)	133 (131-134)/135 (131-139)	145 (140-151)/145 (142-148)	<0.001/ <0.001
DBP (mmHg)	82 (81-82)/82 (81-85)	88 (85-92)/87 (86-89)	<0.001/ <0.001

B. In DNA methylation analysis

	NGT	T2D	NGT *vs.* T2D *P*-value (FHD-/ FHD +)
N (FHD-/+)	242 (178/64)	164 (38/126)	
Age (years)	58 (57-58)/57 (56-59)	60 (59-61)/ 58 (57-59)	0.010/ 0.739
BMI (kg/m^2)	26.3 (26.0-26.6)/25.6 (25.1-26.1)	29.0 (27.6-30.3)/ 30.1 (29.3-30.9)	<0.001/ <0.001
f-Glucose (mmol/l)	5.0 (4.9-5.1)/5.0 (4.9-5.1)	6.9 (6.4-7.4)/ 8.2 (7.7-8.7)	<0.001/ <0.001
2h-Glucose (mmol/l)	4.9 (4.7-5.1)/5.2 (4.9-5.4)	13.4 (12.4-14.4)/ 14.1 (13.1-15.1)	<0.001/ <0.001
f-Insulin (pmol/l)*	96.6 (91.0-102.5)/97.0 (87.8-107.2)	149.0 (120.1-184.7)/ 167.8 (154.3-182.5)	<0.001/ <0.001
2h-Insulin (pmol/l)*	307.0 (283.1-332.9)/ 332.9 (290.0-382.1)	512.5 (413.9-634.7)/ 537.9 (467.0-619.6)	<0.001/ <0.001
HOMA-IR	3.35 (3.13-3.57)/3.35 (2.96-3.73)	8.19 (6.34-10.05)/ 9.80 (8.81-10.79)	<0.001/ <0.001
SBP (mmHg)	136 (134-138)/130 (130-139)	145 (140-151)/145 (142-148)	<0.001/ <0.001
DBP (mmHg)	82 (82-84)/84 (81-86)	88 (84-92)/ 87 (86-89)	0.004/ 0.017

Data were expressed as means (95% CI) for normally distributed variables and as geometric means (95% CI) for *non-normally distributed variables; FHD: Family history of diabetes; NGT: Normal glucose tolerance; T2D: Type 2 diabetes; BMI: Body mass index; IR: Insulin resistance index; SBP and DBP: Systolic and Diastolic blood pressures.

Table 1: Clinical parameters of Swedish men.

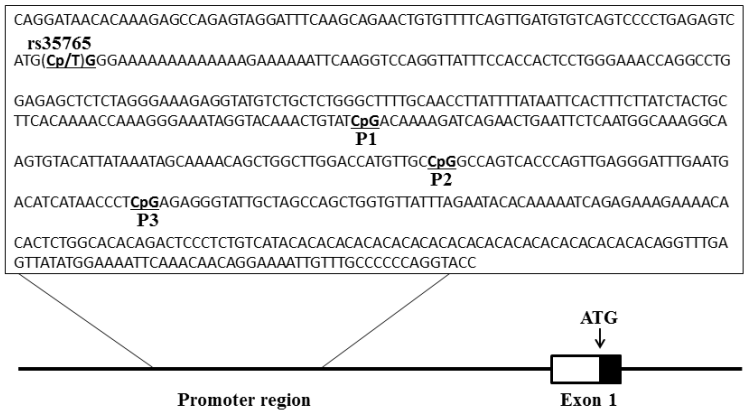

Figure 1: Location of SNP rs35767 and CpG sites (P1-P3) in the *IGF1* gene promoter region.

2.0 (Qiagen, Hilden, Germany) and sequencing information of all primers in the designed assays is represented in Table 2. A bisulfite pyrosequencing protocol was used for DNA methylation analysis [20]. Initially, the bisulfite conversion of 500 ng genomic DNA was performed using EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. The methylation levels of these CpG sites were subsequently detected using PyroMark Gold 96 Reagent Kit (Qiagen) and PyroMark Q96 ID pyrosequencing system (Biotage, Uppsala, Sweden). PyroQ-CpG software (Biotage) was used for methylation analysis. Un-methylated bisulfite converted and unconverted DNA samples (Qiagen) were used to monitor the conversion efficiency of the bisulfite treatment and the accuracy of the methylation analysis.

Serum IGF-I levels measurement

Fasting serum levels of IGF-I were measure using an in-house radio-immunoassay after acid-ethanol extraction and cryoprecipitation. Des (1-3)-IGF-I was used as a tracer to minimize interference by IGFBPs as described previously [21]. The intra- and inter- assay coefficient variation were 4% and 11% respectively. Levels of IGF-I were measured in serum samples from 68% (n=471) of all included individuals.

Statistical analyses

All data were presented as means/geometric means (if not normal distributed) with 95% Confidence Interval (CI). Non-normal distributed data were log-transformed to give a normal distribution before analysis. Comparison tests of continuous variables were performed using an unpaired t-test or one-way ANOVA followed with Turkey's post hoc test. It is known that total IGF-I concentration declines with increased age in adults [22,23]. In the present study, all analyses regarding serum IGF-I levels were adjusted for age. IGF-I SD score was used to describe the deviation from age-adjusted mean levels of IGF-I and it was calculated with the equation [(log10IGF-Iobserved+0.00693*age)-2.581]/0.120 as previously described [23]. P-values less than 0.05 were considered as significant. All analyses were analyzed using PASW program (SPSS20.0. Chicago, IL, USA).

Assay ID	Primer	Sequence
CpG-SNP	PCR forward primer	5'-AAGAGTTAGAGTAGGATTTTAAGTAGAAT-3'
	PCR reverse primer	5'-CTAAAAAACTCTCCAAACCTAATTTC-3'*
	Pyroseq primer	5'-TAGTTGATGTGTTAGTTTTTTG-3'
CpG site P1	PCR forward primer	5'-AAATTAAAGGGAAATAGGTATAAATTGT-3'
	PCR reverse primer	5'-TAACACCAACTAACTAACAATACCC-3'*
	Pyroseq primer	5'-GGGAAATAGGTATAAATTGTAT-3'
CpG sites P2 and P3	PCR forward primer	5'-AAATTAAAGGGAAATAGGTATAAATTGT-3'
	PCR reverse primer	5'-TAACACCAACTAACTAACAATACCC-3'*
	Pyroseq primer	5'-ATAGTTGGTTTGGATTATGTTGT-3'

*All reverse primers were labeled with biotin.

Table 2: PyroMark assays designed for DNA methylation analysis of the *IGF1* gene.

Results

Analyses of the IGF1 gene promoter DNA methylation and serum IGF-I levels

We first analyzed DNA methylation levels at three CpG sites in the IGF1 gene promoter region. Compared with NGT subjects, T2D patients had significantly increased DNA methylation levels at CpG site P3 (84.8% vs. 74.2%, P<0.001). No difference of DNA methylation levels at the CpG sites P1 and P2 between NGT and T2D was observed (Figure 2a). Furthermore, newly diagnosed and treated T2D patients had similar methylation levels at three CpG sites. No correlation between IGF1 DNA methylation levels and ages was observed.

Circulating levels of IGF-I and mean IGF-I SD score were found to be lower in T2D patients compared with NGT subjects (152 μg/l vs 169 μg/l, P=0.029 adjusted for age, Figure 2b) (0.01 vs. 0.28, P=0.011). Newly diagnosed and treated T2D patients had similar circulating IGF-I levels. We further categorized all subjects according to FHD. Compared to NGT subjects, T2D patients had lower circulating IGF-I levels (150 μg/l vs. 183 μg/l, P<0.001 adjusted for age) among the individuals with FHD, but not in the group without FHD (155 μg/l in T2D vs. 160 μg/l in NGT, P=0.839 adjusted for age). In NGT subjects, the individuals with FHD had higher serum IGF-I levels than those without FHD (183 μg/l vs. 160 μg/l, P<0.001 adjusted for age). Meanwhile, the DNA methylation levels at CpG site P3 were decreased in both groups with or without FHD.

Epigenotypic analyses according to SNP rs35767

We then analyzed DNA methylation levels at SNP-CpG site according to the genotypes of rs35767 (C/T). In this SNP, the allele T removes a CpG site in the IGF1 promoter region. We found that the carriers with the CC, CT and TT genotypes of rs35767 among NGT subjects had decreasing DNA methylation levels from 93.1%, 47.6%

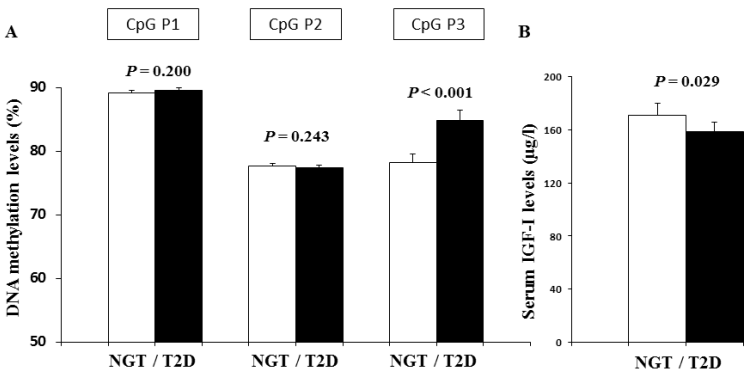

Figure 2: IGF1 DNA methylation and serum levels in Swedish men with normal glucose tolerance and type 2 diabetes
A. T2D patients had significantly increased DNA methylation levels at CpG site P3 (84.8% *vs.* 74.2%, *P*<0.001) compared with NGT subjects. But no difference of DNA methylation levels at the CpG sites P1 and P2 between NGT and T2D was observed. **B**. Circulating levels of IGF-I were lower in T2D patients compared with NGT subjects (152 μg/l vs 169 μg/l, *P*=0.029 adjusted for age). Data were expressed as mean (DNA methylation levels) / geometric mean (serum IGF-I levels) and 95% CI.

to 1.5% (P<0.001, Figure 3a). In comparison with NGT, similar distribution of DNA methylation levels in the carriers with CC, CT and TT genotypes was seen among T2D patients (93.2%, 46.4% and 1.3%, P<0.001) (Figure 3a). There was no difference of the methylation levels in any of the genotyping groups between NGT and T2D.

There was no difference of serum IGF-I levels among three genotyping groups in neither NGT subjects (CC: 161 μg/l vs. CT: 168 μg/l vs. TT: 188 μg/l, P=0.340 adjusted for age), nor T2D patients (CC: 150 μg/l vs. CT: 155 μg/l vs. TT: 149 μg/l, P=0.871 adjusted for age) (Figure 3b). There was no association between SNP rs35767 and fasting insulin, fasting glucose or HOMA-IR index in neither NGT subjects nor T2D.

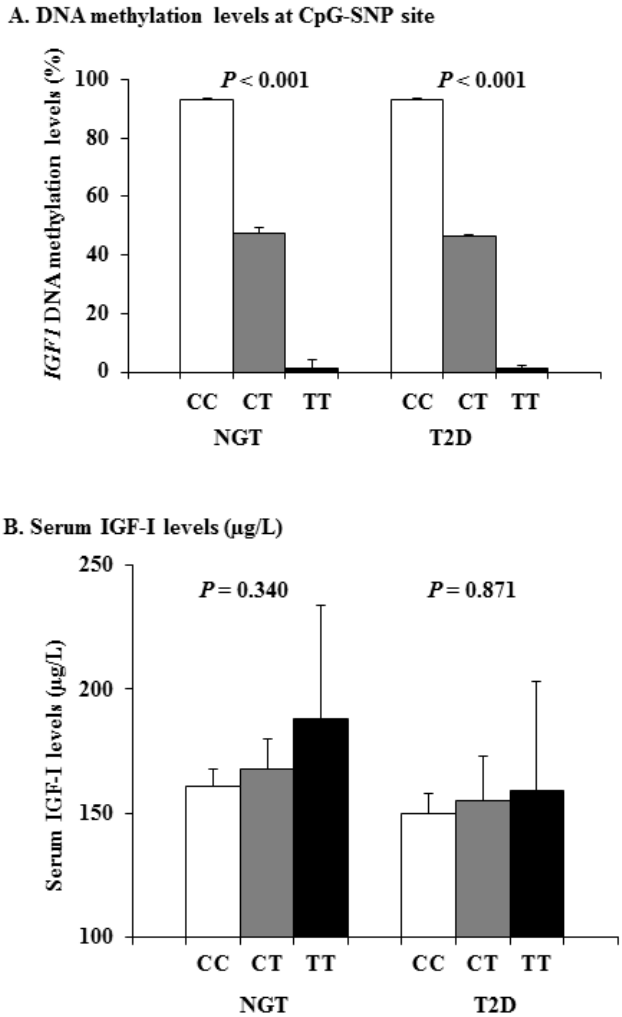

Figure 3: IGF1 DNA methylation and serum levels in Swedish men with normal glucose tolerance and type 2 diabetes according to the genotypes of SNP rs35767

A. The carriers with the CC, CT and TT genotypes of rs35767 had decreasing DNA methylation levels among NGT subjects from 93.1%, 47.6% to 1.5% (P<0.001), which is similar among T2D patients (93.2%, 46.4% and 1.3%, P<0.001). **B**. There was no statistical significance but a tendency of increased IGF-I levels in T allele carriers in NGT subjects (CC: 161 μg/l *vs.* CT: 168 μg/l *vs.* TT: 188 μg/l, P=0.340 adjusted for age). Meanwhile, the circulating IGF-I levels were similar among three genotyping groups in T2D patients (CC: 150 μg/l *vs.* CT: 155 μg/l *vs.* TT: 149 μg/l, P=0.871 adjusted for age). Data were expressed as mean (DNA methylation levels) / geometric mean (serum IGF-I levels) and 95%CI.

Discussion

In present study, we analyzed DNA methylation levels of the IGF1 gene in Swedish men with NGT or T2D. Data indicated that increased DNA methylation levels at CpG P3 site in the IGF1 gene promoter were associated with T2D. Furthermore, the T allele carriers of SNP rs35767 (C/T) had lower DNA methylation levels at SNP-CpG site, which may be associated with increased pattern of IGF-I serum levels.

Yakar et al. [24] previously suggested that lower serum IGF-I levels might result in over expression of growth hormone by insufficient negative feedback and consequently decrease insulin sensitivity. In the present study, serum IGF-I levels in T2D patients were found to be lower than that in NGT subjects. In general, increased DNA methylation in the gene promoter may block the gene transcription and silence gene transcription [12,13]. Our data showed increased IGF1 DNA methylation levels in T2D and it might explain the reduction of serum IGF-I concentration. Quantitative genetic analyses have demonstrated that genetic influences are more important than environmental influences for IGF-I levels [25]. In our study, the NGT subjects with FHD had significant higher serum IGF-I levels than those without FHD, indicating the genetic influences on circulating IGF-I. It was demonstrated that high circulating IGF-I levels were associated with reduced risk of development of impaired glucose tolerance [26]. The elevated serum IGF-I in those NGT subjects with FHD may have the protective effect against development of glucose intolerance.

SNP rs35767(C/T) is 1.2kb upstream of the IGF1 gene, and it has been found to be associated with fasting insulin and insulin sensitivity by large scale meta-analysis. The T allele may have a protective effect in T2D [8,9]. DNA methylation typically occurs in a CpG dinucleotide context. The T allele of SNP rs35767 (C/T) removes a CpG site which may influence the methylation levels and subsequently affect the gene expression. Previous studies have shown that the T allele carriers at rs35767 had increased circulating IGF-I levels [10,11]. In the present study, data clearly demonstrated the repressed DNA methylation levels at rs35767 SNP-CpG site in T carriers. However, although the mean difference of serum IGF-I among three genotyping groups of SNP rs35767 was similar as previous reports [10,11], we were not able to see the increased serum IGF-I levels in T allele carries in the present study, probably due to the limited sample size or differences of gender and ages. Dayeh et al. [16] have recently identified a multiple of T2D related CpG-SNPs were associated with differential DNA methylation in the human islets, and also demonstrated the differential DNA methylation affect the gene expression or transcriptional splicing and further contributed to the impaired insulin secretion. However, SNP rs35767 in the IGF1 gene was not included in Dayeh's study. The differential DNA methylation levels at rs35767 may be associated with altered circulating IGF-I and subsequently contribute to insulin resistance.

In the present study, there is a limitation that we have no human liver tissue available for tissue specific DNA methylation and gene expression analyses of the IGF1 gene. Although Dick et al. [17] have recently suggested that the analysis of whole blood DNA methylation can reflect the changes in relevant tissues for a phenotype, further investigation of IGF1 DNA methylation and mRNA expression changes with liver tissue samples is necessary for better understanding the epigenetic effects of the gene in T2D.

In conclusion, the present study provides the first evidence that increased DNA methylation levels of the IGF1 gene and decreased serum IGF-I protein concentration are associated with T2D, and suggests that DNA methylation in the IGF1 gene may interact with SNP rs35767 (C/T) in the gene promoter region.

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Author contributions

TG, HFG and KB designed the study. TG conducted experiments. AH and CGÖ collected subjects. TG and HFG analyzed data. TG, HFG and KB wrote the manuscript. All contributed to data interpretation, discussion and commented the manuscript.

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