International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Molecular assessment of Coronary Heart Disease(CHD) risk in obese and overweight subjects

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Jomy TJ and Lakshman Kumar B (2018) Molecular assessment of Coronary Heart Disease(CHD) risk in obese and overweight subjects, *Int. J. of. Life Sciences*, Special Issue, A11: 41-48.

ABSTRACT

Coronary artery disease is the most common type of heart disease for which obesity is known to be one of the causative factors. The present study was thus carried out to determine the distribution of SNP (single nucleotide polymorphism) rs3737787 in upstream stimulatory factor 1 (USF1) gene and SNP rs1130864 of C-reactive protein (CRP) gene in 25 obese and overweight blood samples (based on BMI) collected from Erode district, Tamil Nadu along with equal number of healthy samples. The methodology used for the study was PCR-SSCP followed by DNA sequencing. In the present study for the polymorphisms studied, both the controls and patients showed more or less similar distribution of genotypes and hence they could not be ascertained for coronary heart disease risk in the subjects studied. But for further confirmation, a higher sample size needs to be studied.

Keywords: Coronary artery disease, C-reactive protein, Upstream stimulating factor 1, SNP rs1130864, SNP rs3737787, BMI

INTRODUCTION

Obesity is a term used to describe body weight that is much greater than what is healthy. Adults with a body mass index (BMI, calculated as weight in kilograms divided by height in meters squared) between 25 kg/m^2 and 30 kg/m^2 are considered overweight. Adults with a BMI greater than or equal to 30 kg/m^2 are considered obese. Anyone who is more than 100 pounds overweight or who has a BMI greater than or equal to 40 kg/m^2 is considered morbidly obese.

Genetic factors play some part in the development of obesity. Children of obese parents are 10 times more likely to be obese than children with parents of normal weight (Leslie *et al*., 2007). The upstream transcription factor 1 (USF1) gene encoding USF1, a ubiquitously expressed transcription factor controlling some 40 genes (Naukkarinen *et al*., 2005).

The product of *USF1* regulates numerous genes of lipid and glucose metabolism (Choquette *et al*., 2007), and in large population cohorts specific alleles of *USF1* are associated with the risk of cardio vascular disease (CVD) and based on its function is an attractive candidate gene for CVD (Komulainen *et al*., 2006).

C-reactive protein (CRP) levels are associated with CHD in healthy subjects, both in a cross-sectional study in general practice (Mendall *et al*., 1996), and longitudinal-lly in the US Physicians Health Study (Ridker *et al*., 1997), the MONICA-Augsburg Cohort Study (Koenig *et al*., 1997), and the MRFIT Study (Kuller *et al*., 1996), where CRP levels predicted cardiovascular events or CHD mortality during a follow-up of between 2 and 17 years. The T-allele of SNP1444C>T (rs 1130864) has been reported to affect both baseline CRP and inflame-matory responses to experimental lipopolysaccharide-induced endotoxemia in healthy adults (Marsik *et al*., 2006). This allele is also associated with differential CRP responses in patients undergoing periodontal treatment and coronary artery bypass graft surgery (Brull *et al*., 2003). The present study was conducted to analyze previously reported SNPs of USF-1 and CRP in the population studied and check their association with the manifestation of the condition.

MATERIALS AND METHODS

Study participants

Twenty five blood samples were collected from Obese and overweight subjects and equal number of healthy normal controls from 'Deepa Micro Lab', Erode, Tamil Nadu. The ethical clearance for the work was obtained from Ethical committee, PSGIMSR (PSG Institute for Medical Sciences and Research) Coimbatore, Tamilnadu.

Genomic DNA extraction

One mL of whole blood was lysed with 3mL of chilled RBC lysis buffer, vortexed for 1 minute and centrifuged at 4000rpm for 5 minutes and the red supernatant was removed. This step was repeated twice to get white to pink pellet. To this 200µL of nuclei lysis buffer and 50µL of SDS were added. Followed by the addition of 3µL of proteinase K, the mixture was incubated at 65^{o}C for 2:30 hours. After this, 175µL of 5.3M Sodium chloride was added, centrifuged at 10,000rpm for 15 minutes, supernatant carefully siphoned off and transferred to a new 2 mL microcentrifuge tube. To this one mL of cold 100% ethanol was added and inverted ten times to precipitate the DNA. Thereafter the tube was centrifuged at 1500 rpm for 10 minutes, supernatant was removed and the pellet was resuspended in 75% alcohol. Centrifugation at 15000 rpm was performed to remove supernatant. The pellet was air dried and resuspended in 100-150µL of TE buffer and stored at -20^{0}C. The A_{260}/A_{280} values were checked to assess DNA purity through UV/Visible spectrophotometer.

PCR amplification and analyses of USF-1 (rs3737787) and CRP (rs1130864) +1444C>T SNPs

USF-1 (rs3737787) SNP was determined using the following primer pairs, forward and reverse primer sequences were as follows, forward 5'-GGCCTGCAGTGG TGTGAAA-3' and reverse 5-'TCCAGTATCCAGCATGGA GA CA-3'. CRP (rs1130864) +1444C>T polymorphism was assessed using previously reported primers and the sequence as follows, forward 5'-GTGTCTGGTCTGGGAGC TCGTTA-3' and reverse 5'CTTCTCAGCTCTTGCCTTATGA GT-3'. Thermal condi-tions for USF-1 consisted of Initial denaturation at 94^{o}C for 10 mins, 30 cycles of denature-tion at 94^{o}C for 1 min, Annealing at 62.1^{o}C for 45 Sec, extension at 72 for 1 min and final extension at 72^{o}C for 10 mins. Thermal conditions for amplification of CRP gene differed only in annealing which was 54^{o}C and the rest similar to thermal conditions of USF-1 gene.

Single Strand Conformational (SSCP) analysis of USF-1 and CRP

About 7µL of PCR amplicons (USF-1 / CRP) were taken and mixed with 15µL of loading dye. This mixture was denatured at 95^{o}C for 6 mins and immediately kept on ice to avoid renaturation and loaded on 10% PAGE. Silver staining (0.2% silver nitrate) method was used to stain DNA and viewed. The gel showing abnormal band pattern was confirmed by sequencing.

DNA sequencing

The abnormal bands observed in SSCP analysis were sequenced using an Automated DNA sequencer (ABI Prism, Chromous Biotech Pvt. Ltd, Bengaluru).

RESULTS

Based on the BMI, there were 18 obese and seven overweight subjects (Table 1).

PCR-SSCP analysis of USF-1 and CRP genes

PCR amplification of USF-1 gene resulted in 129 bp amplicon (Fig. 1). Of the 50 samples analyzed for USF-1

Table 1: Subjects recruited and categorized as obese, overweight or normal based on BMI

Samples	Weight in kg	Height in metre squared	BMI (in kg per metre square)	Category
S1	88	2.62	33.58	Obese
S2	70	1.96	35.71	Obese
S3	80	1.96	40.81	Obese
S4	60	2.4	25	Overweight
S5	75	2.25	3.33	Obese
S6	65	1.19	33.16	Obese
S7	55	2.01	27.36	Overweight
S8	45	2.4	18.75	Normal
S9	50	1.96	25.51	Overweight
S10	40	1.96	20.4	Normal
S11	43	2.04	21.07	Normal
S12	52	1.93	26.96	Overweight
S13	40	1.82	21.96	Normal
S14	45	1.74	29.88	Overweight
S15	84	2.62	32.06	Obese
S16	70	1.96	36.22	Obese
S17	90	2.62	34.35	Obese
S18	62	2.62	23.66	Normal
S19	63	2.52	25	Overweight
S20	59	2.59	22.77	Normal
S21	60	2.52	23.80	Normal
S22	71	1.96	36.22	Obese
S23	84	2.01	41.79	Obese
S24	65	2.04	31.86	Obese
S25	45	2.04	22.05	Normal
S26	48	2.25	21.33	Normal
S27	50	2.4	20.83	Normal
S28	61	1.96	31.12	Obese
S29	71	2.62	27.09	Overweight
S30	70	2.04	34.31	Obese
S31	48	2.04	23.52	Normal
S32	52	2.62	19.84	Normal
S33	58	2.52	23.01	Normal
S34	62	2.04	30.39	Obese
S35	63	2.62	24.04	Normal
S36	72	1.96	36.73	Obese
S37	93	2.52	36.90	Obese
S38	90	2.62	34.35	Obese
S39	80	2.04	39.21	Obese
S40	46	2.4	19.16	Normal
S41	42	1.96	21.42	Normal
S42	41	1.96	20.91	Normal
S43	45	2.04	22.05	Normal
S44	61	2.52	24.2	Normal
S45	51	2.4	21.25	Normal
S46	43	1.82	20.99	Normal
S47	52	1.74	23.5	Normal
S48	63	1.66	22.0	Normal
S49	60	1.80	21.0	Normal
S50	75	1.92	20.5	Normal

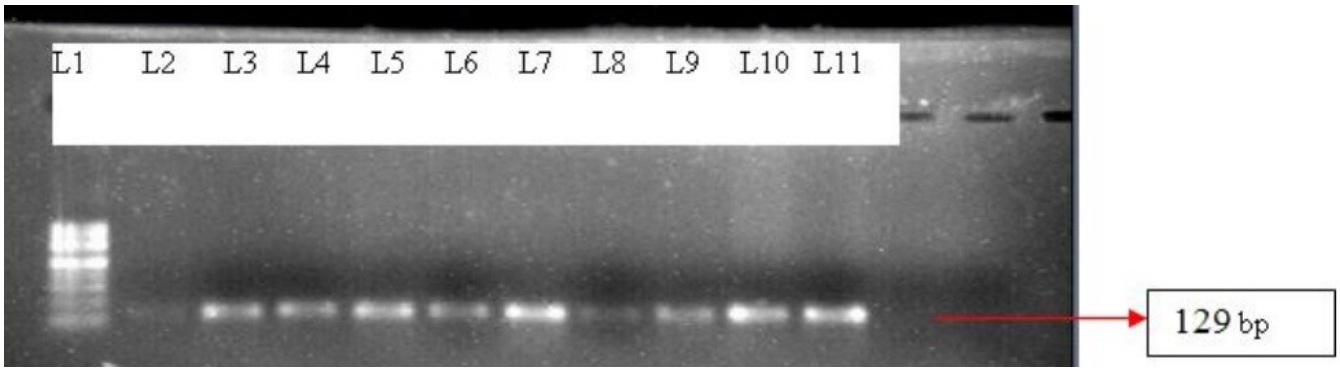

Figure 1: USF1 gene fragment amplified in obese and overweight samples

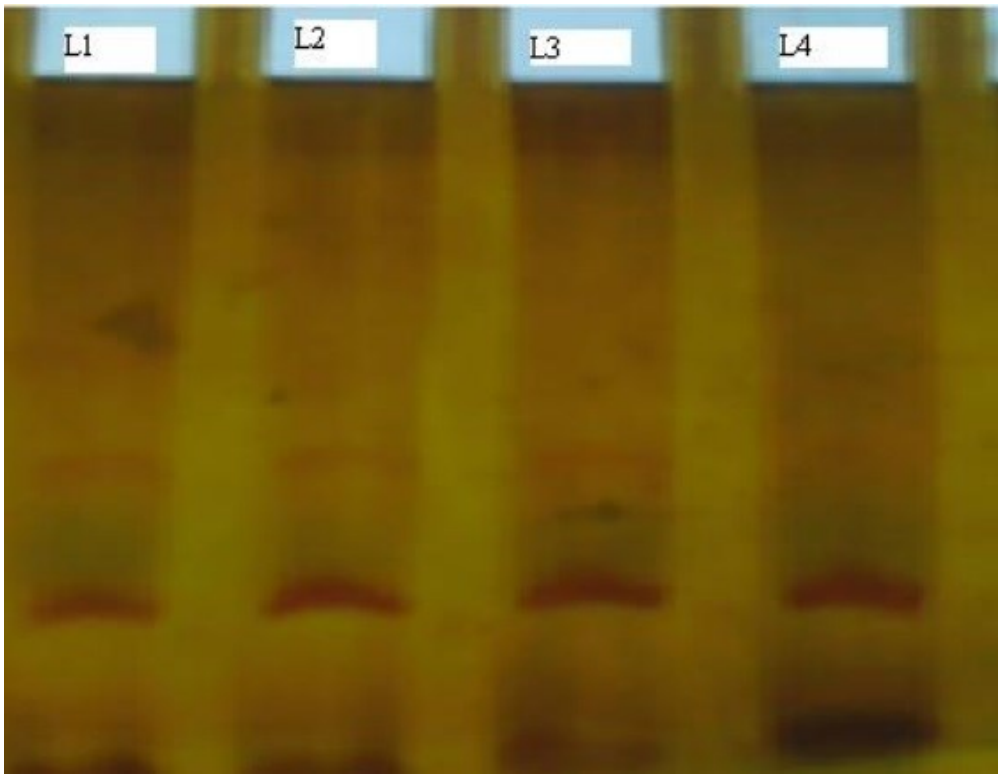

Figure 2: USF-1 gene PCR-SSCP abnormal band pattern in lane 4 (An obese patient)

Sequence ID: lcl|Query_37757 Length: 170 Number of Matches: 1

Range 1: 35 to 170 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
252 bits(136)	2e-72	136/136(100%)	0/136(0%)	Plus/Plus

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Query  1    GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGACGTGCACTGACAGCCTTGCCC  60
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Sbjct  35   GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGACGTGCACTGACAGCCTTGCCC  94

Query  61   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  154

Query  121  CATGCTGGATACTGGA  136
            ||||||||||||||||
Sbjct  155  CATGCTGGATACTGGA  170
```

Figure 3: Blast alignment of USF1 sequence in an obese subject showing the CC homozygote

Sequence ID: lcl|Query_39365 Length: 170 Number of Matches: 1

Range 1: 35 to 170 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
246 bits(133)	1e-70	135/136(99%)	0/136(0%)	Plus/Plus

```
Query  1    GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGATGTGCACTGACAGCCTTGCCC  60
            ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  35   GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGACGTGCACTGACAGCCTTGCCC  94

Query  61   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  154

Query  121  CATGCTGGATACTGGA  136
            ||||||||||||||||
Sbjct  155  CATGCTGGATACTGGA  170
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Figure 4: Blast alignment of USF1 sequence in an obese subject showing the CT heterozygote

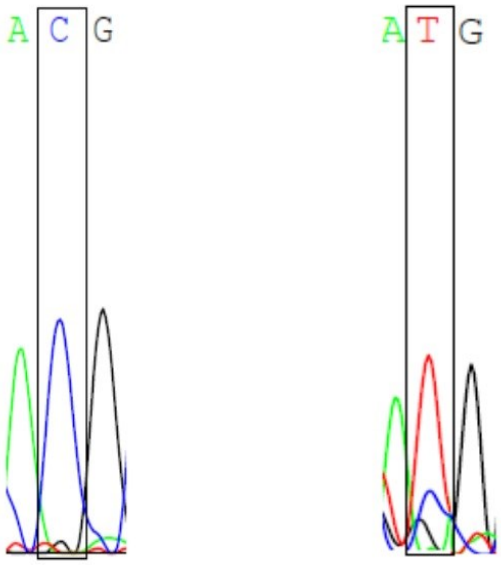

Figure 5: Homozygous CC and heterozygous CT change in USF1 gene as visualized on chromatogram present in obese subjects

SNPs through SSCP, three samples were found to posses abnormal bands and all of these were either obese or overweight (Fig.2). Blast analysis of the sequence is as given in figure 3 and figure 4. The predominant genotype observed among patients and controls was CC homozygous type. The heterozygous CT was the second genotype present in patients and controls as interpreted in the chromatogram (Fig. 5).

PCR amplification with CRP specific primers yielded a 195 bp amplicon (Fig. 6) and of the 50 samples analyzed by PCR-SSCP, two patients found to posses abnormal bands (Fig 7). Blast analysis is as shown in figure 8 and figure 9. Predominant genotype in SNP of CRP gene was found to be CC followed by CT as interpreted in the chromatogram (Fig.10). With respect to both USF-1 and CRP genes, the distribution of allele was more or less similar between obese or overweight patients and controls (Table 2). Hence, the current study could not associate any genotypes with the disease risk in patients.

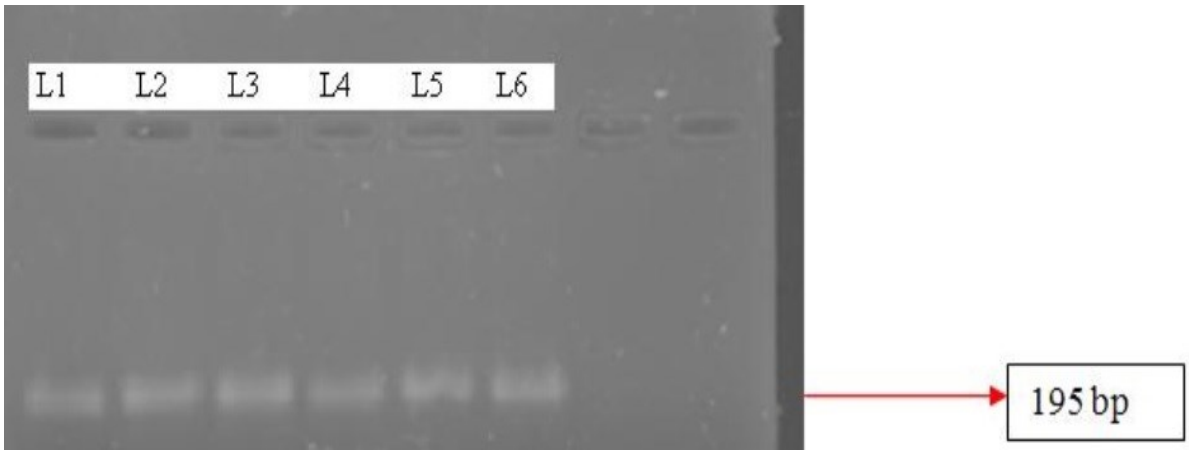

Figure 6: CRP gene fragment amplified in obese and overweight subjects

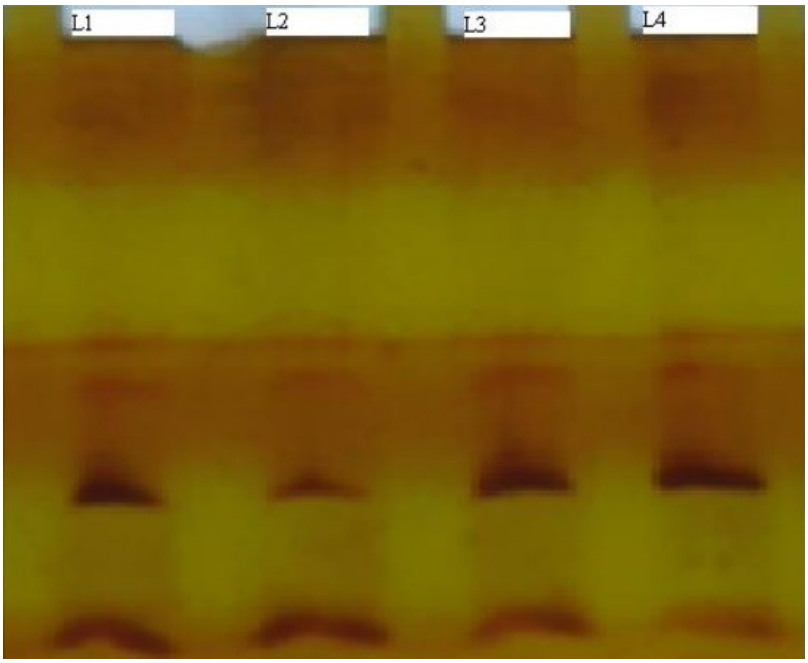

Figure 7: CRP gene SSCP abnormal band pattern in lane 2 (an obese patient)

Sequence ID: lcl|Query_57287 Length: 244 Number of Matches: 1

Range 1: 70 to 243 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
322 bits(174)	3e-93	174/174(100%)	0/174(0%)	Plus/Plus

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Query  1    TAACTATGCTGGGAAACGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  60
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Sbjct  70   TAACTATGCTGGGAAACGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  129

Query  61   ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  189

Query  121  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  243
```

Figure 8: Blast alignment of CRP sequence in a patient showing the CC homozygote

Download Graphics

Sequence ID: lcl|Query_180591 Length: 244 Number of Matches: 1

Range 1: 70 to 243 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
316 bits(171)	2e-91	173/174(99%)	0/174(0%)	Plus/Plus

```
Query  1    TAACTATGCTGGGAAATGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  60
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   TAACTATGCTGGGAAACGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  129

Query  61   ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  189

Query  121  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  243
```

Figure 9: Blast alignment of CRP sequence in an obese subject showing the CT heterozygote

Table 2: Genotypes of USF1 gene and CRP gene observed in subjects of present study with respect to the SNPs

S.no	SNP	CC	CT	TT	Allele frequency	
1	rs3737787	20 25	5 0	-	C=0.9,T=0.1	Patients Controls
2	rs1130864	21 24	4 1	-	C=0.9,T=0.1	Patients Controls

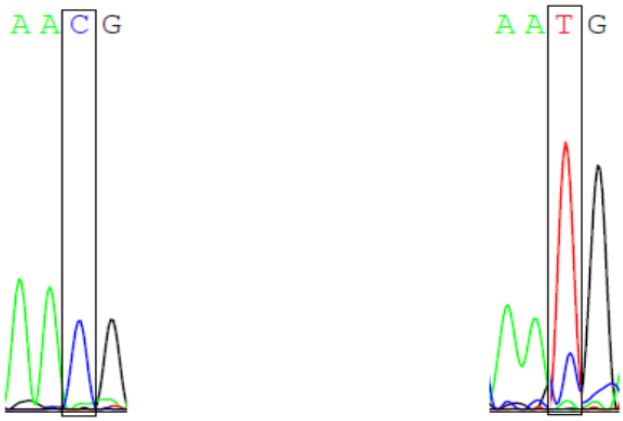

Figure 10: Homozygous CC and heterozygous CT change in CRP gene as visualized on chromatogram

DISCUSSION

Upstream transcription factor 1 (USF1) is a ubiquitously expressed transcription factor, and is a member of the basic helix-loop-helix leucine zipper family. The most commonly studied single nucleotide polymorphisms (SNPs) of USF-1 gene are rs3737787, rs2073653, rs2073655, rs2073658, rs251640, rs2516841, rs2516839, rs2774276 and rs2516837. Furthermore, rs3737787 is located in the promoter region (-789) of junctional adhesion molecule-1 (JAM1, also known as adjacent platelet F11 receptor, F11R), which was first discovered to be a surface protein on human platelets, and has been found to be associated with central obesity and systolic blood pressure in the Chinese population (Ong *et al*., 2008). Polymorphisms of the upstream transcription factor 1 (USF1) have been associated with familial combined hyperlipidemia and coronary heart disease (Pajukanta *et al*., 2004).

Obesity, age, gender, and diabetes are important factors that influence variation in blood levels of CRP. Increased serum CRP levels have been reported in subjects with obesity, metabolic syndrome, and type 2 diabetes (T2D), indicating that these individuals present a state of subclinical, low-grade inflammation that promotes the development of atherosclerosis mediated by a process of endothelial dysfunction, increasing the risk of ischemic heart disease (Hu *et al*., 2009). Several studies have reported an association between single-nucleotide polymorphisms (SNPs) in the CRP gene with variation in blood levels of CRP, or with coronary heart disease (CHD), diabetes, microangiopathic stroke, insulin resistance, metabolic syndrome, or hypertension (Szalai *et al*., 2005; Brull *et al*., 2003; Wolford *et al*., 2003).

In particular, polymorphisms in the CRP gene on chromosome 1 have consistently been associated with basal CRP levels in both men and women and with varying degrees of risk in the development of CHD (Miller, 2005). It has been shown that elevated serum CRP is a risk factor for CHD, and there is a relationship between increased serum levels of CRP with various CHD risk factors, particularly diabetes and hypertension. The effects of the SNPs on the variation in CRP levels have been reported in various populations around the world demonstrating that the effect of CRP SNPs on CRP occurs independent of ethnicity.

In conclusion, with respect to USF1 gene polymorphism rs3737787 and CRP polymorphism rs1130864 in the present study, both the controls and patients showed more or less equal distribution of genotypes and hence no genotype could be associated with coronary heart disease risk in the subjects studied. But in order to confirm the findings, a higher sample size needs to be studied.

Conflicts of interest: The authors stated that no conflicts of interest.

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