



Trade Science Inc.

ISSN : 0974 - 7427

Volume 6 Issue 6

BioCHEMISTRY

An Indian Journal

Regular Paper

BCAIJ, 6(6), 2012 [193-198]

Analysis of nonsynonymous single nucleotide polymorphism rs1468384 of Niemann-Pick C1-Like 1 gene and its association with serum cholesterol levels in Nepalese population

K.P.Singh¹, U.Timilsina^{2*}, H.K.Tamang³, P.Yadav⁴, B.Gautam⁴, S.Shrestha⁴, I.Shrestha⁴, S.Khadka⁴

¹Department of Biochemistry, Maharajgunj Medical Campus (TUTH), Kathmandu, (NEPAL)

²Department of Biotechnology, College for Professional Studies, Kathmandu, (NEPAL)

³Department of Biochemistry, Kantipur Dental College Teaching Hospital, Kathmandu, (NEPAL)

⁴Department of Medical Laboratory Sciences, Nobel College, Kathmandu, (NEPAL)

E-mail : timilsinau@gmail.com

Received: 16th September, 2012 ; Accepted: 10th October, 2012

ABSTRACT

Background: Niemann-Pick C1-Like 1 protein is a newly identified sterol influx transporter actively involved in the cholesterol homeostasis pathway. Nonsynonymous Single Nucleotide Polymorphism rs1468384 (G→A transition) shows decrease in the stability of the protein as analysed in silico by MuPro software. Related data on allelic frequency and the effect of this polymorphism on the serum cholesterol levels in Nepalese population are not available. This study aims to detect rs1468384 in Nepalese population using the Polymerase Chain Reaction – Restriction Fragment Length Polymorphism technique, determine the allelic frequency of this polymorphism and to correlate it with the serum cholesterol level. **Materials and Methods:** A total number of 74 healthy human subjects were randomly selected within Kathmandu valley. DNA was isolated from blood leukocytes. Polymerase Chain Reaction – Restriction Fragment Length Polymorphism technique was used to identify the genotypes. Lipid profile analysis was performed using cholesterol oxidase peroxidase (CHOD-PAP) method (systemic reagent of Humastar 600, Human, Germany). **Results:** Genotype distribution was in accordance with Hardy-Weinberg Equilibrium ($X^2=2.0233$, $df=1$). 'G' allele frequency ($p=0.8581$) was higher than the mutated 'A' allele ($q=0.1419$) in Nepalese population. Genotype frequency distribution among Gender was not statistically significant (p value =0.427). Statistically significant differences in Total Cholesterol Level and LDL Cholesterol Level between genotypes were observed (Total Cholesterol Level "Genotype GG" 160.7 ± 30.3 mg/dl; "Genotype AG" 145.1 ± 22.5 mg/dl, p value =0.019 and LDL Cholesterol Level "Genotype GG" 99.9 ± 28.6 mg/dl; "Genotype AG" 84.7 ± 19.3 mg/dl, p value =0.011). **Conclusion:** Relatively higher prevalence of rs1468384 polymorphism among Nepalese population was found in this study. The study showed a strong association between this polymorphism and the serum cholesterol levels.

© 2012 Trade Science Inc. - INDIA

KEYWORDS

Niemann-Pick C1-Like 1 protein;
Nonsynonymous single nucleotide polymorphism;
Polymerase chain reaction-restriction fragment length polymorphism.

Regular Paper

INTRODUCTION

The identification of ezetimibe as a potent selective inhibitor of intestinal cholesterol uptake and absorption in animals and humans confirmed that the intestinal cholesterol uptake process is mediated by a specific transporter protein called as Niemann-Pick C1 like 1 (NPC1L1)^[1,2]. It is a homolog of Niemann-Pick C1 (NPC1) protein whose mutations cause Niemann-Pick disease type C1^[3,4]. The NPC1L1 gene spans ~29 kb of human chromosome 7p13, containing 20 exons with an unusually large exon 2 of 1,526 bp and a small exon 14 of 56 bp.^[5,6] The full-length cDNA encodes a 1,359 amino-acid protein. However, the predominant mRNA transcript skips exon 15 and produces a protein of 1,332 amino acids^[6-8]. The protein has been characterized by the presence of a signal peptide, 13 putative transmembrane regions, a conserved NPC1 domain and a sterol sensing domain (SSD)^[9]. In humans, NPC1L1 is highly expressed in the apical membrane of enterocytes and the canalicular membrane of hepatocytes^[5,10]. In the lumen of the small intestine, unesterified free cholesterol (FC) from dietary intake and biliary secretion is solubilized in mixed micelles containing bile acids (BA) and phospholipids (PL). FC is then taken up into enterocytes by the apically localized NPC1L1 protein^[5]. In the liver, cholesterol can be synthesized locally or taken up by hepatocytes from circulating lipoproteins. A large amount of FC is converted to bile acids for hepatobiliary secretion. NPC1L1 localized at the apical membrane of hepatocytes counterbalances the function of ATP-binding cassette (ABC) transporters G5 and G8 (ABCG5/ABCG8) by transporting newly secreted biliary cholesterol back into hepatocytes, thereby preventing excessive loss of endogenous cholesterol^[10]. Thus this protein is actively involved in the cholesterol homeostasis pathway.

Association of genetic variation in NPC1L1 with cholesterol absorption and low-density lipoprotein cholesterol has been studied a number of times. Cohen and colleagues^[11] showed that variation in human NPC1L1 gene sequences contributes to reduced intestinal cholesterol absorption efficiencies and LDL-C levels. Other sequence variants are identified in NPC1L1 which are also associated with alterations in sterol absorption, plasma LDL-C levels, or responses to ezetimibe^[12-14]. Nonsynonymous Single Nucleotide Polymorphism

(nsSNP) rs1468384 of Niemann-Pick C1-Like 1 Gene is the result of a nucleotide change G to A at position 2993 of the cDNA sequence in exon 2, and it results in the substitution of isoleucine for methionine at amino acid 510 of the NPC1L1 protein (also called M510I polymorphism). This polymorphism shows decrease in the stability of the protein as analysed in silico by MuPro and StructureSNP softwares^[14]. Genotyping as well as the association of this SNP with serum cholesterol levels has not been performed in Nepalese population till date as per our knowledge.

In this study we have identified the nsSNP rs1468384 by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) technique and determined the allelic frequency of this polymorphism in Nepalese subjects by genotype counting. Given the significant role of the NPC1L1 gene in cholesterol homeostasis, our goal was also been to examine potential association of this SNP with serum cholesterol levels in Nepalese population.

MATERIALS AND METHODS

5 ml of fasting venous blood were withdrawn from 74 healthy subjects within the age group of 20-60 years, randomly selected within Kathmandu valley with informed consent. The acceptance criteria were normal blood pressure and devoid of any clinically significant disorders while the rejection criteria were diabetes, hypercholesterolemia, alcoholism, heavy smoking, recent surgery and treatment with cholesterol reducing drugs. The collected whole blood samples were centrifuged; serum and cells separations were done. The serum and cells were stored at -20°C till further processing. Lipid profile analysis was done from separated serum by fully automated chemistry analyzer (Humastar 600, Human) at Razam Medical Centre Pvt. Ltd. Chabahil, Kathmandu, Nepal while the remaining molecular analysis were performed at Department of Biotechnology, College for Professional Studies, Maitidevi, Kathmandu, Nepal.

DNA from blood cells was isolated with the DNAsure[®] blood mini-kit from Genetix, India. Each extracted DNA samples were electrophorised on 1% agarose gel for an hour at 80-90 V on Tris Acetate EDTA (TAE) buffer system. Gels were stained with ethidium bromide (EtBr) and the DNA bands were observed under UV trans-illuminator. DNA extractions

showing a single prominent band were processed for PCR amplification.

The PCR reaction was optimized for 200 ng of DNA. The primer pair selected was according to Praveen P. Balgir et al.^[14] precisely amplifying the M510I specific Exon2 fragment of NPC1L1 gene in the DNA sample. A 25 μ L PCR mixture was optimized containing 1.5 mM MgCl₂ (Fermentas), 0.4 μ M of each primer (Eurofins), 200 μ M of each deoxynucleotide triphosphate (Fermentas), 1.0 Unit of Taq polymerase (Genetix), and buffer concentration of 16mM (NH₄)₂SO₄ and 67 mM Tris- HCl, pH 8.8. A two step PCR cycles were optimized, with first step with initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 61.5°C for 45 seconds and extension at 72°C for 1 minute. This is followed by a final extension at 72°C for 5 minutes and a 4°C hold. Each PCR amplified products were electrophorized on 2% agarose gel along with 100 bp DNA ladder, run for 60 minutes at 80-90 V on TAE buffer system, stained with EtBr and observed under UV trans-illuminator. PCR products showing a single prominent band of 437bp were processed for restriction digestion.

Five units of *BclI* (New England Biolabs) was

added to 10 μ L of PCR product and incubated at 37°C for 3 hours followed by heat inactivation at 65°C for 20 minutes. Whole volume (15 μ L) of restriction digested products were loaded for gel electrophoresis on 3 % agarose gel along with 100 bp DNA ladder and run for 60 minutes at 80-90 V on TAE buffer system. The gels were stained with EtBr and the separated DNA bands were observed under UV trans-illuminator.

Serum Total Cholesterol (TC), Serum Triglycerides (TG) and High Density Lipoprotein (HDL) Cholesterol were measured by using CHOD-PAP method. Low Density Lipoprotein (LDL) Cholesterol was estimated using Friedewald's formula^[15].

Genotype distribution and Allelic frequency were calculated using PopGene. S²(version 1.00) software. All the statistical analysis were done using IBM SPSS Statistics (version 19) software. All tests of statistical significance were two sided with 95% confidence intervals (CI).

RESULTS

Among the total 74 subjects under study, 30 subjects were males while 44 subjects were females. Their mean age was 34.93 \pm 10.11 years (males : 34.93 \pm

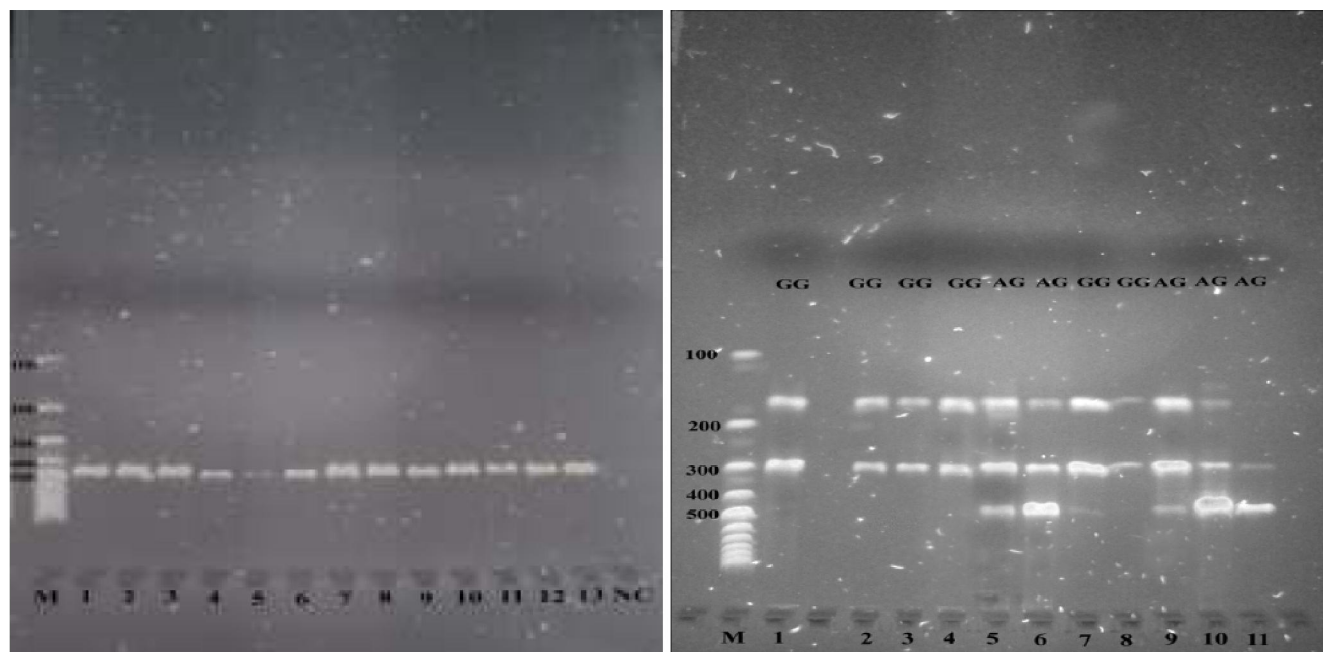


Figure 1 : Left - A 2 % agarose gel stained with EtBr showing M510I specific NPC1L1 PCR product of size 437 bp Right – A 3 % Agarose gel stained with EtBr showing the M510I genotype (Lane M = DNA ladder, Lane NC= Negative Control, Lane 1-13 = samples, GG-individual homozygous for the G allele with regard to the polymorphism rs1468384, AG- individual heterozygous for the polymorphism, demonstrating the presence of both alleles)

Regular Paper

10.57 and females: 34.93 ± 9.93 years).

After digestion of the 437 bp fragment obtained by PCR amplification, the three possible genotypes were distinguishable: homozygous AA (437 bp), heterozygous GA (437, 278 and 159 bp), and homozygous GG (278 and 159 bp).

TABLE 1 shows the distribution of genotypes of nsSNP rs1468384 within the study population. Genotype count for homozygous GG allotype was the highest while a complete absence of homozygous AA allotype was found. Genotype distribution was in accordance with Hardy-Weinberg Equilibrium ($X^2=2.02$, $DF=1$). Genotype frequency distribution among Gender was not statistically significant as analysed by Chi-square test ($X^2=0.62$, $DF=1$, p value = 0.427). 'G' allele frequency ($p=0.8581$) was found to be high in Nepalese population compared to that of 'A' allele ($q=0.1419$).

Mean Serum Total Cholesterol, Serum HDL Cholesterol, Serum Triglyceride and Serum LDL Cholesterol for the total study population were found to be 156.36 ± 29.11 , 38.64 ± 8.47 , 110.24 ± 51.89 and 95.66 ± 27.12 mg/dl respectively. TABLE 2 shows the distribution of serum cholesterol levels within the gender, no statistically significant differences were found

TABLE 1 : Genotype distribution of nsSNP rs1468384 in Nepalese population

Allotypes	AG	GG	AA
Band Pattern	437	-	437
	278	278	-
	159	159	-
Allotype Frequency			
Male	7	23	
Female	14	30	
Total Number of Samples		74	

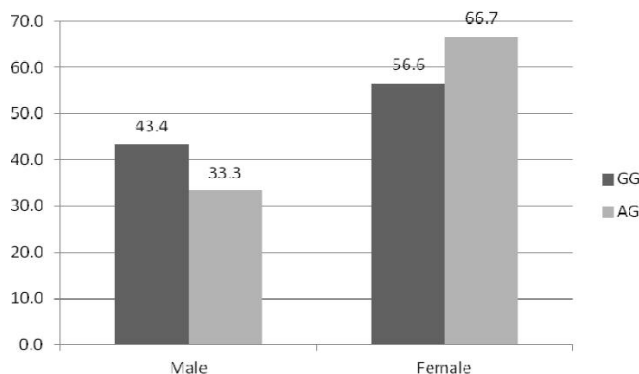


Figure 2 : Percentage distribution of genotype among gender

for individual parameters. Statistically significant difference in Serum Total Cholesterol Level between genotypes was observed (mean difference = 15.60 ± 7.79 mg/dl) and difference also observed for the Serum LDL Cholesterol Level (mean difference = 15.25 ± 9.29 mg/dl). TABLE 3 shows the genotypic distribution of Serum Cholesterol Levels.

TABLE 2: Distribution of serum cholesterol levels (mg/dl) within gender

	Gender	Frequency	Mean	SD	t-test (P value)
Serum Total Cholesterol	Male	30	155.86	24.71	0.89
	Female	44	156.70	32.04	
Serum HDL Cholesterol	Male	30	37.70	9.38	0.44
	Female	44	39.29	7.83	
Serum Triglyceride	Male	30	107.56	37.41	0.69
	Female	44	112.06	60.16	
Serum LDL Cholesterol	Male	30	96.65	21.84	0.78
	Female	44	94.99	30.42	

TABLE 3 : Genotype distribution of serum cholesterol levels (mg/dl)

	Genotype	Frequency	Mean	SD	t-test (P value)
Serum Total Cholesterol	GG	53	160.79	30.38	0.019*
	AG	21	145.19	22.59	
Serum HDL	GG	53	37.81	7.41	0.253
	AG	21	40.76	10.61	
Serum LDL	GG	53	99.99	28.65	0.011*
	AG	21	84.74	19.36	
Serum Triglycerides	GG	53	114.92	53.21	0.201
	AG	21	98.42	47.56	

*Statistically significant P value < 0.05

DISCUSSION

The purpose of this study was to determine the allelic frequency of nsSNP rs1468384 at the NPC1L1 gene and to evaluate its association with serum cholesterol levels in Nepalese population of healthy subjects. The allele frequencies obtained for the Nepalese population, as well as those reported for other populations in the NCBI's SNP Database^[16] and Praveen et al., 2009^[14] are presented in TABLE 4. The 'G' allele in Indian population showed the least frequency of 0.46 and Caucasian population sampled the maximum at 1.00. For 'A' allele it was 0.54 in Indian population while 'A'

allele was absent in Caucasian samples. The 'A' allele frequency in Nepalese population was found to be 0.1419 which was second highest after Indian population. Our findings were in favor with Praveen et al., 2009^[14] indicating that nsSNP rs1468384 polymorphism is common in South Asian populations.

The NPC1L1 transporter has been shown to be critical for cholesterol absorption and is the site of the

TABLE 4 : Genetic frequencies of nsSNP rs1468384 in Nepalese and other populations

Sample group	Total no. of Chromosomes (2n)	G allele frequency	G allele %	A allele frequency	A allele %
African American	78	0.99	99	0.01	1
Caucasian	80	1.00	100	0.00	0
Asian	60	1.00	100	0.00	0
Japanese	86	0.98	98	0.02	2
Indian	300	0.46	46	0.54	54
Nepalese (present study)	148	0.85	85	0.14	14

action of ezetimibe, a cholesterol lowering agent that inhibits cholesterol absorption^[5,17]. In this study, we documented that heterozygous allele carriers at M510I at the NPC1L1 locus had lower TC and LDL-C levels compared to its common allele homozygous counterpart. Again our findings were in favor with Praveen et al., 2009^[14] indicating that this mutation has an implication on the function of NPC1L1 gene as cholesterol transporter and may affect its role in the intestinal uptake of cholesterol. It is important to note that the design of this study does not allow us to draw definitive conclusions about the exact mechanisms that may lead to genotype-related differences in serum cholesterol levels. However, there are several plausible explanations for our findings. The M510I polymorphism results in the substitution of isoleucine for methionine at 510 amino acid position of NPC1L1 protein. The presence of isoleucine may reduce the number of disulfide bonds formed by methionine. As disulfide bonds play role in the structure of protein by forming covalent linkages within the molecule, this change may bring some conformational change leading to destabilization of the protein as predicted by MuPro web server. StSNP webserver based analysis showed that rs1468384 sequence region of NPC1L1 may also act as a molecular chaperon binding site and may play a potential role in the folding of the protein. Thus a change of amino acid

in this important protein region may change the folding of protein. As the protein is actively involved in cholesterol homeostasis pathway, any disruption in its structure will influence its function of cellular uptake of cholesterol^[18]. By inhibiting cholesterol absorption, less cholesterol would ultimately be delivered to the liver. This would also lead to an up-regulation of LDL receptors and increased clearance of LDL thereby lowering the serum LDL cholesterol concentration^[19]. Since high levels of blood cholesterol are associated with atherosclerotic coronary heart disease, by lowering blood cholesterol levels, M510I polymorphism in NPC1L1 should have beneficial effects on this disease. As the inhibition of NPC1L1 or NPC1L1-dependent intestinal cholesterol absorption has become a potential preventative and therapeutic approach for metabolic diseases such as nonalcoholic fatty liver disease, insulin resistance, type 2 diabetes, and central obesity^[20], a natural variant of NPC1L1 owing to this nsSNP rs1468384 associated with lowered blood cholesterol levels may be a boon to individuals possessing it.

The limitations of our study include the fact that our cohort size is small restricting generalization of our findings. However, the reliability and biological importance of this association should motivate further research to verify and extend these findings.

CONCLUSION

The study of genotype frequency distribution for the nsSNP rs1468384 and its association with serum cholesterol levels in Nepalese population for the first time will definitely serve as a major achievement in understanding some complex disease states as atherosclerosis resulting due to any interruption in the cholesterol pathway. In our study, we used PCR-RFLP method to determine the genotype frequency distribution which was reliable, rapid and cost-effective method. The Genotype frequency was in accordance with Hardy-Weinberg Equilibrium which suggested the suitability of sample size for the genotypic assays as well as the homogeneity of the concerned population. This study showed that the frequency of 'A' allele ($q=0.1419$) was higher in Nepalese population compared to Caucasians and Orientals. Serum total cholesterol and serum LDL cholesterol levels were found to be lower in subjects having minor allele of

Regular Paper

nsSNP rs1468384 NPC1L1 suggesting that this SNP helps in lowering blood lipid levels in such subjects. Further it can be concluded that subjects having this SNP will be benefited by having low risk of lipid related disorders such as atherosclerosis, hypertension, insulin resistance, and obesity.

ACKNOWLEDGEMENT

We would like to thank management of College for Professional Studies, Razam Medical Centre and Nobel College, Kathmandu, Nepal for their assistance in performing this study.

REFERENCES

- [1] J.Clader; The discovery of ezetimibe: a view from outside the receptor. *J.Med.Chem.*, **47**, 1-9 (2004).
- [2] T.Sudhop, D.Lutjohann, A.Kodal; Inhibition of cholesterol absorption by ezetimibe in humans. *Circulation*, **106**, 1943-8 (2002).
- [3] E.D.Carstea, J.A.Morris, K.G.Coleman, S.K.Loftus, D.Zhang; Niemann-pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science*, **277**, 228-31 (1997).
- [4] S.K.Loftus, J.A.Morris, E.D.Carstea, J.Z.Gu, C.Cummings; Murine model of niemann-pick C disease: mutation in a cholesterol homeostasis gene. *Science*, **277**, 232-5 (1997).
- [5] S.W.Altmann, H.R.Jr.Davis, L.J.Zhu, X.Yao, L.M.Hoos; Niemann-pick C1 like 1 protein is critical for intestinal cholesterol absorption. *Science*, **303**, 1201-4 (2004).
- [6] J.P.Davies, B.Levy, Y.A.Ioannou; Evidence for a niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. *Genomics*, **65**, 137-45 (2000).
- [7] L.Yu, S.Bharadwaj, J.M.Brown, Y.Ma, W.Du; Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake. *J.Biol.Chem.*, **281**, 6616-24 (2006).
- [8] Y.Yamanashi, T.Takada, H.Suzuki; Niemann-pick C1-like 1 overexpression facilitates ezetimibe-sensitive cholesterol and β -sitosterol uptake in CaCo-2 cells. *J.Pharmacol Exp.Ther.*, **320**, 559-64 (2007).
- [9] J.Wang, B.B.Chu, L.Ge, B.L.Li, Y.Yan, B.L.Song; Membrane topology of human NPC1L1, a key protein in enterohepatic cholesterol absorption. *J.Lipid Res.*, **50**, 1653-62 (2009).
- [10] R.E.Temel, W.Tang, Y.Ma, L.L.Rudel, M.C.Willingham, Y.A.Ioannou, J.P.Davies, L.M.Nilsson, L.J.Yu; Hepatic niemann-pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe. *Clin.Invest.*, **117**(7), 1968-78 (2007).
- [11] J.C.Cohen, A.Pertsemlidis, S.Fahmi, S.Esmail, G.L.Vega; Multiple rare variants in NPC1L1 associated with reduced sterol absorption and plasma low-density lipoprotein levels. *Proc.Natl.Acad.Sci.*, **103**, 1810-5 (2006).
- [12] E.Polisecki, I.Peter, J.S.Simon, R.A.Hegele, M.Robertson; Genetic variation at the NPC1L1 gene locus, plasma lipoproteins, and heart disease risk in the elderly. *J.Lipid Res.*, **51**, 1201-7 (2010).
- [13] J.S.Simon, M.C.Karnoub, D.J.Devlin, M.G.Arreaza, P.Qiu; Sequence variation in NPC1L1 and association with improved LDL-cholesterol lowering in response to ezetimibe treatment. *Genomics*, **86**, 648-56 (2005).
- [14] Praveen P.Balgir, Divya Khanna, Gurlovleen Kaur; Analysis of nsSNP rs1468384 of NPC1L1 and its association with plasma cholesterol levels in general population The Internet Journal of Cardiovascular Research [Internet]. Available from ISPUB with full text: <http://www.ispub.com/journal/the-internet-journal-of-cardiovascular-research>, **6**(2), (2009).
- [15] W.T.Friedewald, R.I.Lavy, D.S.Fredrickson; Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of preparative ultracentrifuge. *Clin.Chem.*, **18**, 499-502 (1972).
- [16] http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1468384
- [17] H.R.Jr.Davis, S.W.Altmann; Niemann-pick C1 like 1 (NPC1L1) an intestinal sterol transporter. *Biochim.Biophys.Acta.*, **1791**, 679-683 (2009).
- [18] Praveen P.Balgir, Divya Khanna. *In silico* sequence variation analysis of niemann pick C1 like 1 (NPC1L1) gene and its association with cholesterol binding and protein structure stability. *Indian J.Biotechnol.*, **9**, 192-202 (2010).
- [19] J.Shepherd; The role of the exogenous pathway in hypercholesterolaemia. *Eur.Heart J.*, **3**(Suppl E), E2-E5 (2001).
- [20] Jenna L.Bettters, Liqing Yu; NPC1L1 and cholesterol transport. *FEBS Lett.*, **584**(13), 2740-2747, July 2 (2010).