

## ORIGINAL ARTICLE

# The g.–762T > C polymorphism of the *NPC1L1* gene is common in Chinese and contributes to a higher promoter activity and higher serum cholesterol levels

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Niemann–Pick type C1-like 1 (*NPC1L1*) protein is responsible for intestinal cholesterol absorption. The aim of the study was to identify genetic polymorphisms of the *NPC1L1* gene as well as their functional significance. The method involved screening of promoter and coding regions of the *NPC1L1* gene for genetic polymorphisms by direct DNA sequencing of genomic DNA from 50 individuals. Functional studies on promoter polymorphisms were performed using luciferase assay. Association between the polymorphisms and serum cholesterol levels were investigated in 224 individuals. The results showed that in total, 11 single nucleotide polymorphisms were identified. Among them, a promoter polymorphism, g.–762T > C, and a synonymous polymorphism, g.1679C > G, were common (34 and 36%, respectively). These two polymorphisms were highly linked ( $D'$  value = 0.7459,  $P$ -value < 0.00001). For the g.–762T > C promoter polymorphism, luciferase assay in HepG2 cell line demonstrated that the –762C allele had a significantly higher promoter activity than the –762T allele ( $1.30 \pm 0.22$  vs  $0.37 \pm 0.06$ , 3.5-fold,  $P < 0.05$ ). We also showed that the *NPC1L1* promoter activity was downregulated by cholesterol content in both genotypes. When association studies were performed, we found that –762C allele was associated with significantly higher serum total cholesterol and LDL-cholesterol content levels in a recessive model (LDL-cholesterol value =  $131.2 \pm 8.1$  vs  $116.4 \pm 2.2$  mg dl<sup>-1</sup>; total cholesterol value =  $214.7 \pm 9.0$  mg dl<sup>-1</sup> vs  $196.9 \pm 2.6$ ,  $P$ -value < 0.05,  $n = 224$ ). In conclusion, the C allele at –762 position of the *NPC1L1* gene was common in people of Chinese ethnicity. The –762C allele had a higher promoter activity and was associated with a higher serum total cholesterol and LDL-cholesterol level.

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## INTRODUCTION

Cardiovascular disease is a worldwide problem and hypercholesterolemia contributes to cardiovascular diseases by promoting atherosclerosis.<sup>1</sup> Cholesterol homeostasis in the human body is tightly regulated through dietary cholesterol absorption, biliary secretion and *de novo* synthesis. There are variations in serum cholesterol levels and inter-individual variations in cholesterol absorption rates might be a possible reason. It has been reported that cholesterol absorption is influenced by several factors, such as dietary composition, age and genetic factors.

Recently, the Niemann–Pick type C1-like 1 protein (*NPC1L1*) was identified as an intestinal cholesterol transporter.<sup>2–8</sup> It is also the molecule target for the new cholesterol-lowering agent, ezetimibe.<sup>9</sup> Human *NPC1L1* is expressed mainly in the intestine and liver and is responsible for transporting cholesterol from intestinal lumen to enterocytes. It is now believed that intestinal cholesterol absorption is regulated by two transporters, *NPC1L1* and *ABCG5/8*.<sup>10,11</sup> *ABCG5/8*

is a heterodimer transporter and may be responsible for selective reverse cholesterol transport from the enterocytes to the intestinal lumen. Several genetic variations in *ABCG5/8* have been identified to cause sitosterolemia, a rare disorder characterized by increased serum sitosterol levels.<sup>12–15</sup> However, there are a limited number of studies that focus on the effects of *NPC1L1* genetic variation. Therefore, the aim of this study was to identify genetic variations of *NPC1L1* and to investigate whether these variations are associated with variations in serum cholesterol levels.

## MATERIALS AND METHODS

### Study subjects

A total of 224 consecutive individuals undergoing health check-ups at the National Taiwan University hospital were included in the study. Serum levels of total cholesterol, triglyceride, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein-cholesterol were determined independently. The demographic data and serum cholesterol profiles are shown in Table 1.

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**Table 1 Demographic data and serum lipid levels of the study subjects (n=224)**

	Men	Women	Overall
N	112	112	224
Age (years)	63.3 ± 1.5	62.9 ± 1.5	63.1 ± 1.1
TG (mg dl <sup>-1</sup> )	129.7 ± 5.5	131.4 ± 7.5	130.6 ± 4.6
T-CHO (mg dl <sup>-1</sup> )	198.3 ± 3.3	198.7 ± 3.9	198.5 ± 2.6
LDL-C (mg dl <sup>-1</sup> )	121.8 ± 2.7	113.6 ± 3.3	117.7 ± 2.1
HDL-C (mg dl <sup>-1</sup> )	43.7 ± 1.0	53.2 ± 1.6	48.4 ± 1.0

Abbreviations: HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; T-CHO, total cholesterol; TG, triglyceride. The data are expressed as mean ± s.e.m. except for age. N value means healthy individuals.

### Detection of genetic polymorphisms and genotyping

We screened the promoter and coding regions of the *NPC1L1* gene for genetic polymorphisms by direct DNA sequencing of genomic DNA from 50 individuals. Total cellular DNA was isolated from peripheral blood lymphocytes using a PureGene genomic DNA purification kit (Gentra Systems Inc., Valencia, CA, USA). The promoter (from -971 to +1 bp) and coding regions of the *NPC1L1* gene were amplified using PCR. The base sequence of the primer pairs is shown in Table 2. PCR was performed with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. A final elongation step was performed at 72 °C for 10 min. DNA sequencing was performed using an automated sequence analyzer. Further genotyping was performed on the 224 individuals using PCR and direct DNA sequencing.

### Plasmid construction

The promoter region between -1405 and +52 bp of the human *NPC1L1* gene was amplified using human genomic DNA as the template. *KpnI* and *BglII* restriction sites were introduced into the forward and reverse primers, respectively, to facilitate subsequent cloning work. The forward primer was: 5'-CAGGTACCGGCTCATTCCCATTACCGACTGC (underline indicates *KpnI* cutting site) and the reverse primer was: 5'-GTAGATCTAGCGCAGGAG CAGGGCCACAGCAG (underline indicates *BglII* cutting site). The PCR product was then digested with *KpnI* and *BglII* enzymes and inserted into a luciferase gene control vector, pGL3 basic vector (Promega, Madison, WI, USA). All constructs were confirmed by DNA sequence.

### Cell culture and transient transfection

HepG2 cells were cultured in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 1.5 g l<sup>-1</sup> sodium bicarbonate, 100 μM non-essential amino acids, 1.0 mM sodium pyruvate and supplemented with 10% fetal bovine serum at 37 °C with 5% CO<sub>2</sub>.

For transfection studies, HepG2 cells were plated onto 96-well plate (8 × 10<sup>4</sup> cells per well) before 24-h transfection.<sup>16</sup> The cells were transiently co-transfected with 250 ng of pGL3 basic luciferase vector, pGL-762T or pGL-762C constructs, and 25 ng pRL-TK vector (*Renilla* luciferase-bearing vector) using jetPEI transfection reagent according to the manufacturer's instructions. In all transfections, pRL-TK vector (*Renilla* luciferase-bearing vector—Promega) served as an internal control to normalize the efficiency of transfection. After 24-h transfection, the cells were washed with phosphate-buffered saline solution and incubated for an additional 24 h in either fresh medium, 40 μM of lovastatin, 10 μg ml<sup>-1</sup> or 20 μg ml<sup>-1</sup> cholesterol (Sigma, St Louis, MO, USA), and 5 or 15 μM of ezetimibe (Schering-Plough/Merck, Kenilworth, NJ, USA).<sup>17</sup> Finally, luciferase assay was performed using the Dual-GloLuciferase Reporter Assay System according to the manufacturer's instructions (Promega).

### Statistical analysis

Data were expressed as mean ± s.e.m. Parametric data between groups were compared using the Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant. Linkage disequilibrium and haplotype analyses were

**Table 2 Base sequence of the primer pairs used in PCR**

	Primer sequence	Size (bp)
Promoter-exon1-(1)	Sense: AGGAGATGTTCCCAACAAGC Antisense: CAGGGTGGAGTGACGCAGT	423
Promoter-exon1-(2)	Sense: TTTGTCACTGCGTCACTCCA Antisense: ATCCACCCTCCCACTGTTT	422
Exon1-(3)	Sense: GTGAGTGGAGACCCTAGCACC Antisense: GCCTGTAACTGGAAACCC	488
Exon 2-(1)	Sense: TGCTCCATGCTCTTACCC Antisense: ACATTGATGAAGGGCTCTGATT	464
Exon 2-(2)	Sense: TGACGAATGTGGGAAGAACCC Antisense: AAGGGCAGAGCCATACACG	481
Exon 2-(3)	Sense: GGCCCGCAAGATCACAGGT Antisense: GCCAGGCTCCAAGAGGTGG	493
Exon 2-(4)	Sense: CAGCTAGGGGCTGGACAAC Antisense: AGCGAAGACAGAGCAGAGGAT	459
Exon 2-(5)	Sense: GCTCAACTCCAGGGAGACA Antisense: ACCACCGGGATGACAGATAG	486
Exon 2-(6)	Sense: TGAGTCCCAAGGTGACGACG Antisense: TGGTTGGTTCGGGAAGAAGG	496
Exon 2-(7)	Sense: TCACCATCTGCTTTGTGGG Antisense: TGTGCTTCGGGCCACCATA	493
Exon 2-(8)	Sense: CCAAGAATCTCAGCGGAATC Antisense: GCCAGAGCCGTAGGAATAGC	426
Exon 3	Sense: CTCCATAGTGTCTGGGTCT Antisense: GAAGATAAAGGAGATGGCAGA	378
Exon 4	Sense: AGCAGGAAGGGTCTGAGTATTT Antisense: ACCCGTTCTGACGAGCCAGCCACT	334
Exon 5	Sense: GGTGATTGTGATGGCTGTT Antisense: TGCTGTTCTGAGGTGCTGT	392
Exon 6	Sense: GGGCTTTAATGTGAGATGTG Antisense: AGAAGAGTGGGCAGTTGG	390
Exon 7	Sense: CCAGCCAGACCCTGGAAGAA Antisense: GGCAAGTGGTAGCCGACATT	436
Exon 8	Sense: ATTAGGCTTGCTGGGTTAGT Antisense: TGTAGGAAATTGGAGAATACATG	445
Exon 9	Sense: TGCTGCTCTGGGACTACCTT Antisense: GTCATGGGCAACATTCTGG	336
Exon 10	Sense: CTCATGCAGCAGGTCCCTT Antisense: CGAATGTGGCTTCACTCCC	284
Exon 11	Sense: GTGCATAGCGAACCAGAAAG Antisense: AGCAGGACAGGGATAGAAAC	333
Exon 12	Sense: CCCCTCTTCTGATTTCTATC Antisense: CTTTCTGTTGCTCAGTTTTC	338
Exon 13	Sense: CTGGTTGAGGCTGGAGTG Antisense: CAAGAAGGTGAGGCAAAA	410
Exon 14	Sense: AGGCAGGAGGAGGCTATCA Antisense: TGTGGAGCTGGGAACAAG	318
Exon 15	Sense: CCTGCTCTGAGATTGTATG Antisense: CGACACTCTGCCTCTATT	313
Exon 16	Sense: GACCTTTGCCCTTCCATC Antisense: CCTCCACCCTAACCTCCAGT	264
Exon 17	Sense: TGGCAGGATCACCATTGTG Antisense: CCCAAGGGTTCGAGGCTAC	413
Exon 18	Sense: CGGCCAATACTCCAGTT Antisense: GTTCAGAGCCAGGTGTCCAGG	354
Exon 19	Sense: TGCTGACACCTGGCTCTG Antisense: CCTGGCTTCTGACATCTGC	316
Exon 20	Sense: TGCCCTGGGTTTGTGTAC Antisense: CAGTGGTGTCTGCCTGGATA	467

performed using Powermarker software. For comparing groups of cells treated with increasing concentrations of ezetimibe, linear regression was calculated by GraphPad Prism software. Hardy–Weinberg equilibrium was assessed by the  $\chi^2$  test.

## RESULTS

### Identification of NPC1L1 polymorphisms

To detect genetic polymorphisms of the *NPC1L1* gene, genomic DNA from 50 individuals (100 chromosomes) were analyzed. In total, 11 single nucleotide polymorphisms (SNPs) were identified. Among these, five were in the *NPC1L1* coding region, with two causing amino-acid changes (P282T and A1238V). For the remaining six SNPs, four were intronic SNPs, and two located in the promoter region. The allele frequencies of the SNPs are shown in Table 3. Among the 11 SNPs, g.-762T>C (ss119336593) and g.1679C>G (rs2072183) were common and the frequencies of the minor allele were 34 and 36%, respectively. In addition, our results revealed that the two common SNPs, g.-762T>C and g.1679C>G, were highly linked ( $D'$  value=0.7459,  $P$ -value <0.00001) (Table 4).

### Promoter assay for NPC1L1 promoter SNP

As to the two common SNPs, g.-762T>C was in the promoter region and g.1679C>G was synonymous, we therefore performed luciferase assay to assess whether g.-762T>C contributed to variations in the promoter activity of the *NPC1L1* gene. We cloned two promoter constructs (pGL-762T and pGL-762C), and transient transfection into HepG2 cells was performed. In luciferase assays, both of the promoter constructs (pGL-762T and pGL-762C) showed a significant increase in promoter activity compared with the pGL3 basic vector without *NPC1L1* promoter region (3.1-fold,  $P$ -value=0.0278

**Table 3** The *NPC1L1* SNPs and their allele frequencies identified in 50 subjects

SNP	Amino-acid change	Frequency of minor allele (%)
g.-762 T>C	—	34
g.-133 A>G	—	2
g.1679 C>G	L272L	36
g.1707 C>A	P282T	1
g.1901 G>A	T346T	1
g.2294 G>A	P477P	2
g.4288 C>T	Intron	1
g.7320 C>T	Intron	2
g.20339 G>T	Intron	1
g.20423 C>T	Intron	1
g.25175 C>T	A1238V	1

Abbreviations: NPC1L1, Niemann–Pick type C1-like 1; SNP, single nucleotide polymorphism.

**Table 4** Allele frequencies, linkage disequilibrium and haplotype analysis for g.-762T>C and g.1679C>G

SNP	Allele	Allele frequency	Haplotype	Haplotype frequency
g.-762T>C	T	0.6585	T-762/C1679	0.5849
	C	0.3415	C-762/G1679	0.2903
g.1679C>G	C	0.6429	T-762/G1679	0.0669
	G	0.3571	C-762/C1679	0.0580

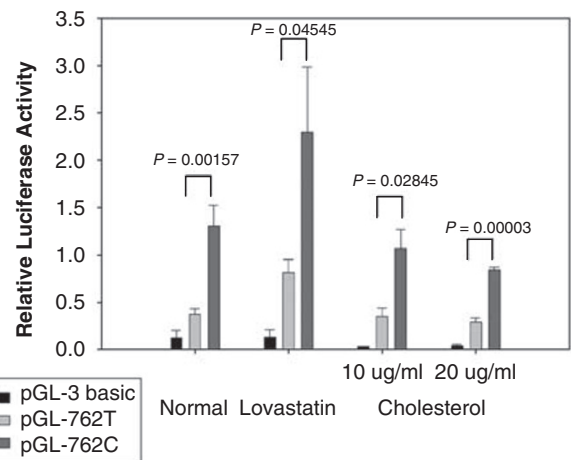
Abbreviation: SNP, single nucleotide polymorphism.  
Linkage disequilibrium (LD)  $D'$  value=0.7459,  $P$ <0.00001.

and 10.8-fold,  $P$ -value=0.0001, respectively) (Figure 1). In addition, the pGL-762C construct showed significantly higher promoter activity than that of pGL-762T construct (3.5-fold,  $P$ -value=0.00157), suggesting that the minor C allele may contribute to increase *NPC1L1* transcript production (Figure 1).

### Effect of added cholesterol, lovastatin and ezetimibe on the promoter activity of NPC1L1

We further investigated whether the -762T and -762C differed in their response to cholesterol, lovastatin (a cholesterol synthesis inhibitor) as well as ezetimibe (an intestinal cholesterol absorption inhibitor). When HepG2 cells were incubated with cholesterol at  $10 \mu\text{g ml}^{-1}$ , the promoter activity of constructs, pGL-762T and pGL-762C, decreased slightly (0.95- and 0.82-fold, respectively). Furthermore, when cells were treated with higher cholesterol contents ( $20 \mu\text{g ml}^{-1}$ ), the promoter activity of both pGL-762T and pGL-762C constructs declined significantly (0.78- and 0.65-fold, respectively). This result suggested that the inhibitory effect of cholesterol was dose dependent. In contrast, when lovastatin was added, the promoter activity showed a significant increase (2.17-fold for pGL-762T and 1.76-fold for pGL-762C), demonstrating that the promoter activity of *NPC1L1* was regulated by cholesterol level. However, when cells were treated with either lovastatin ( $40 \mu\text{M}$ ) or cholesterol ( $20 \mu\text{g ml}^{-1}$ ), the promoter activity of pGL-762C construct was still significantly higher than that of pGL-762T (2.8-fold,  $P$ -value=0.04545,  $n$ =5–6 for treatment lovastatin; 2.9-fold,  $P$ -value=0.00003,  $n$ =4–6 for treatment cholesterol) (Figure 1).

We further investigated the response of the two promoter constructs to ezetimibe. Although cells were incubated with  $5 \mu\text{M}$  of ezetimibe, the promoter activity of pGL-762T and pGL-762C dropped by 0.79- and 0.89-fold, respectively, respectively). At a higher concentration of ezetimibe ( $15 \mu\text{M}$ ), the promoter activity further declined (0.70- and 0.69-fold of basal level, respectively). These results demonstrated that the inhibitory effect of ezetimibe on the *NPC1L1* promoter activity was dose dependent ( $R^2$ =0.3683,  $P$ -value=0.0164 for pGL-762T and



**Figure 1** Effect of exogenous cholesterol or lovastatin on the Niemann–Pick type C1-like 1 (*NPC1L1*) promoter activity. pGL-762T and pGL-762C indicate -762T and -762C promoter constructs, respectively. pGL3 basic represents a negative control vector, which does not have a *NPC1L1* promoter sequence. HepG2 cells were transiently transfected with the promoter constructs. pGL-762C had a significantly higher promoter activity than pGL-762T ( $P$ <0.005). Supplementing with cholesterol (10 or  $20 \mu\text{g ml}^{-1}$ ) decreased *NPC1L1* promoter activity, while adding lovastatin ( $40 \mu\text{M}$ ) increased promoter activity. Data are means  $\pm$  s.e.m. ( $n$  ~ 3–6).

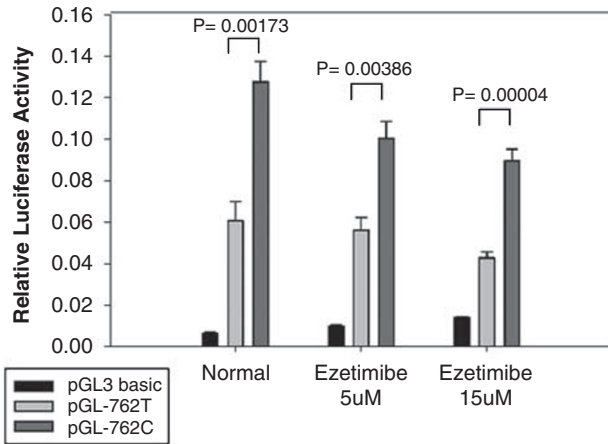
$R^2=0.3201$ ,  $P$ -value=0.035 for pGL-762C by linear regression). When comparing the two promoter constructs, the promoter activity of pGL-762C construct was significantly higher than that of pGL-762T at both ezetimibe concentrations (Figure 2).

#### Association between NPC1L1 polymorphisms and serum cholesterol levels

For analysis of association between NPC1L1 SNP and cholesterol levels, only SNPs with a frequency of >5% were used. Hardy-

Weinberg equilibrium was tested for g.-762T>C and g.1679C>G. Both did not significantly violate the Hardy-Weinberg equilibrium ( $P$ -value=0.0687 and 0.1834 in the  $\chi^2$  test). Tables 5 and 6 show the results of association analysis of serum cholesterol levels and genotypes for SNP g.-762T>C and g.1679C>G, respectively. This demonstrated that the genotype of g.-762T>C was significantly associated with serum total cholesterol and LDP-cholesterol level in a recessive genetic model ( $P$ -value=0.0477 and 0.0494,  $n=224$ ), but was not associated with triglyceride and high-density lipoprotein-cholesterol level. The minor allele (g.-762T>C) had a frequency of 34.1% ( $n=153/448$ ), and individuals with homozygous -762C allele had 9.0% higher serum total cholesterol and 12.7% higher serum LDL-C, respectively (total cholesterol value=214.7  $\pm$  9.0 vs 196.9  $\pm$  2.6 mg dl<sup>-1</sup>,  $P$ -value=0.0477; LDL-cholesterol value=131.2  $\pm$  8.1 vs 116.4  $\pm$  2.2 mg dl<sup>-1</sup>,  $P$ -value=0.0494) (Figure 3).

If we divided the patients into normocholesterolemic (total cholesterol <240 mg dl<sup>-1</sup>) and hypercholesterolemic (total cholesterol >240 mg dl<sup>-1</sup>) groups, the genotype distribution of g.-762T>C differed significantly between the two groups (Table 7). On the other hand, the genotype distribution of g.1679C>G did not differ significantly between the two groups (Table 7). We also analyzed the genotype distributions of both polymorphisms by using 200 mg dl<sup>-1</sup> as the cholesterol cutoff value. We found that there were no significant differences in the genotype distributions in both polymorphisms.



**Figure 2** Effect of ezetimibe on the Niemann-Pick type C1-like 1 (NPC1L1) promoter activity. HepG2 cells were transiently transfected with two promoter constructs, pGL-762T and pGL-762C. After 24 h, cells were incubated with ezetimibe for another 24 h. The promoter activities of both pGL-762T and pGL-762C were decreased by ezetimibe treatment. The promoter activity of pGL-762C remained significantly higher than that of pGL-762T. Data are means  $\pm$  s.e.m. ( $n \sim 4-7$ ).

#### DISCUSSION

The NPC1L1 gene encodes for the intestinal protein that is responsible for cholesterol absorption. This protein is also the target of the new cholesterol-lowering agent, ezetimibe. As diagnosis and treatment of hyperlipidemia are important clinical issues, there has been much research effort focusing on genetic polymorphisms of the NPC1L1 gene. It has been shown that genetic variations in NPC1L1 result in

**Table 5** Association between serum cholesterol levels and g.-762T>C polymorphism

SNP	Genotype			P-value		
	T/T	T/C	C/C	Dominant	Co-dominant	Recessive
<i>g.-762T&gt;C</i>						
Number (%)	91 (40.6)	113 (50.4)	20 (9.0)			
CHO	197.9 $\pm$ 4.1	196.2 $\pm$ 3.5	214.7 $\pm$ 9.0	0.8455	0.4629	0.0477*
LDL-C	119.4 $\pm$ 3.6	114.0 $\pm$ 2.7	131.2 $\pm$ 8.1	0.5116	0.9763	0.0494*
HDL-C	48.5 $\pm$ 1.6	47.4 $\pm$ 1.4	53.7 $\pm$ 3.2	0.9523	0.9985	0.0986

Abbreviations: CHO, serum cholesterol level; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SNP, single nucleotide polymorphism.

\* $P < 0.05$ .

Data were expressed as means  $\pm$  s.e.m.

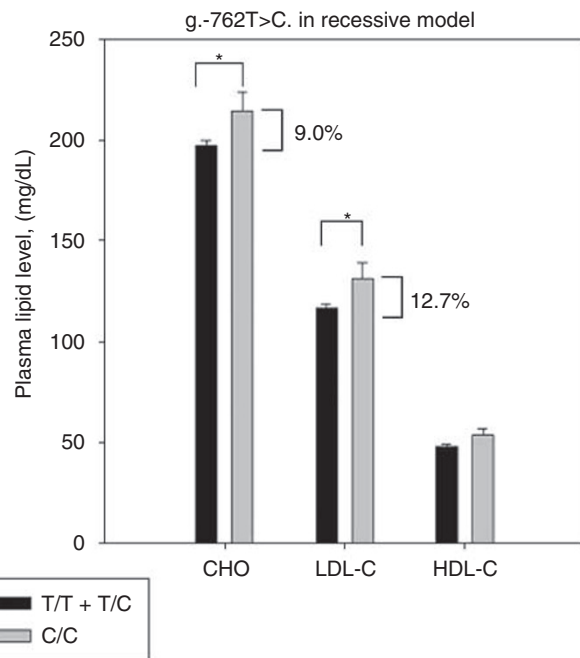
**Table 6** Association between serum cholesterol level and g.1679C>G polymorphism

SNP	Genotype			P-value		
	C/C	C/G	G/G	Dominant	Co-dominant	Recessive
<i>g.1679C&gt;G</i>						
Number (%)	88 (39.3)	112 (50)	24 (10.7)			
CHO	196.8 $\pm$ 4.1	198.3 $\pm$ 3.5	206.2 $\pm$ 9.0	0.5849	0.9763	0.2998
LDL-C	118.8 $\pm$ 3.6	116.6 $\pm$ 2.9	118.9 $\pm$ 6.7	0.6853	0.3728	0.8538
HDL-C	47.4 $\pm$ 1.4	49.0 $\pm$ 1.5	49.4 $\pm$ 2.6	0.4257	0.6852	0.7279

Abbreviations: CHO, serum cholesterol level; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SNP, single nucleotide polymorphism.

Data are expressed as mean  $\pm$  s.e.m.

differences in response to ezetimibe. Hegele *et al.*<sup>18</sup> reported that certain haplotypes (incorporating g.1679C>G, g.25286A>C and g.27621T>C) are associated with inter-individual variations in drug response. Simon *et al.*<sup>19</sup> also demonstrated that patients carrying g.-18C>A had better response to ezetimibe therapy. However, these variations did not influence baseline cholesterol levels. Cohen *et al.*,<sup>20</sup> on the other hand, demonstrated that rare non-synonymous SNPs of the *NPC1L1* gene were associated with baseline plasma LDL-cholesterol levels. However, the incidences of these SNPs were extremely low. In this study, we demonstrated that the allele frequencies of the *NPC1L1* polymorphisms in people of Chinese ethnicity were quite different from those reported in Western countries. For instance, g.-762T>C polymorphism was common in Chinese with a minor allele frequency of 34.1% in this, whereas its frequency is extremely low (<1%) in Caucasians as reported by Simon *et al.* On the other hand, SNP g.-18C>A is common in Western reports, but was absent in the 50 individuals of our study. For another common SNP, g.1679C>G (L272L), the minor allele frequency was similar (30–40%) among our study and previous reports.



**Figure 3** Serum cholesterol levels in patients with different Niemann–Pick type C1-like 1 (*NPC1L1*) genotypes. In a recessive model, homozygous carriers of the g.-762T>C minor allele had significantly higher serum total cholesterol (by 9.0%) and low-density lipoprotein (LDL)-cholesterol (by 12.7%) levels. \**P*-value <0.05.

Functional studies were performed for the promoter polymorphism (g.-762T>C) and we demonstrated that the minor allele contributed to a higher promoter activity. We also showed that the minor allele was associated with higher serum cholesterol levels. Theoretically, variations in *NPC1L1* transcript amount might be related to variations in baseline cholesterol levels. In *NPC1L1* knockout mice, intestinal cholesterol absorption decreased by 69%. In humans, Miettinen and Kesaniemi<sup>21</sup> reported that lower cholesterol absorption was associated with lower serum levels of cholesterol. It is therefore reasonable that variations in promoter activity might be related to changes in serum cholesterol levels.

Our functional analysis of the *NPC1L1*g.-762T>C polymorphism suggested that homozygous carriers of the minor allele of g.-762T>C might have more *NPC1L1* gene expression in enterocytes, leading to an increase in cholesterol absorption. We also demonstrated that *NPC1L1* promoter activity was regulated by cholesterol concentration, suggesting that *NPC1L1* promoter regulation was similar to other cholesterol metabolism genes, such as HMG-CoA reductase, LDL receptor and high-density lipoprotein receptor.<sup>22–24</sup> Besides, our results were consistent with data reported by other group, *NPC1L1* transcription activity was inhibited while cells were treated with ezetimibe.<sup>25</sup> In addition, it has been reported that *NPC1L1* promoter is regulated mainly by SREBP-2, a transcription factor-regulated cholesterol metabolism-related genes expression.<sup>17</sup> Both putative sterol regulatory elements and Ying Yang-1 (YY1)-binding sites are present in the promoter region of *NPC1L1*, suggesting that these transcription factors may regulate the *NPC1L1* gene expression.<sup>26</sup> Alrefai *et al.*<sup>17</sup> also demonstrate that there are two putative sterol regulatory elements (~-657 to -648 and ~-35 to -26 bp) within human *NPC1L1* promoters. YY1 has been proved to be a negative regulator of SREBP-dependent genes.<sup>27–29</sup> However, it is still unknown whether YY1 also participates in regulating *NPC1L1* expression. According to a report by Davies *et al.*, there are two putative YY1 conserved binding sites located in the promoter (~-771 to -752 bp) and coding region (~-10 to +11 bp) of the *NPC1L1* gene. The consensus sequence of the YY1 binding site is 5'-CCAT-3' or 5'-ACAT-3'. The g.-762T>C polymorphism is located in the YY1 conserved binding sites in the promoter region. The minor allele might cause a reduction of YY1's DNA-binding activity, reducing the transcription inhibition effect of YY1 and leading to an increase in *NPC1L1* transcripts.

In summary, our studies showed that SNP g.-762T>C is significantly associated with serum total cholesterol and LDL-cholesterol levels. This was consistent with our finding that the minor allele promoter construct (g.-762T>C) showed significantly higher promoter activity than the major allele construct. These results suggest that homozygous carriers of the minor allele of g.-762T>C might have more *NPC1L1* expression in enterocytes, and therefore leads to increased cholesterol absorption. However, it is still unclear

**Table 7** Genotype distributions of two common single nucleotide polymorphisms of the *NPC1L1* gene in normocholesterolemic and hypercholesterolemic patients

	g.-762T>C polymorphism				g.1679C>G polymorphism			
	TT	TC	CC	P	CC	CG	GG	P
CHO < 240 (%) (n=192)	79 (41.1)	100 (52.1)	13 (6.8)	0.02	77 (40.1)	97 (50.5)	18 (9.4)	0.28
CHO > 240 (%) (n=32)	12 (37.5)	13 (40.6)	7 (21.9)		11 (34.4)	15 (46.9)	6 (18.8)	

Abbreviations: CHO, serum cholesterol level; *NPC1L1*, Niemann–Pick type C1-like 1. Percentages of genotypes are shown in parentheses.

whether g.-762T>C is associated with hypercholesterolemia-related diseases (for example, atherosclerosis), and this needs to be further investigated.

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