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Analysis of *Niemann-Pick C1-like 1* (*NPC1L1*) genetic polymorphisms and haplotypes in Chinese Han population

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Niemann-Pick C1-like 1 (NPC1L1) protein is the key transporter responsible for dietary cholesterol absorption. Recent studies indicated that several functional polymorphisms of *NPC1L1* were associated with coronary heart disease (CHD) and response to ezetimibe therapy. The aim of the present study was to analyze the allele frequency and haplotype distribution of *NPC1L1* polymorphisms in Chinese Hans and to compare them with those of other ethnic populations reported before. Blood samples were collected from 424 unrelated Chinese Hans (246 males and 178 females). Ten *NPC1L1* polymorphisms (-762T > C, -133A > G, -18C > A, 1721C > T, 1735C > G, 1764T > C, 1767G > A, 27677T > C, 25342A > C and 28650A > G) were genotyped by direct sequencing or polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. Among the variants, the minor allele frequency of -762T > C and 1735C > G were 35.0% and 37.0%, respectively. Furthermore, these two polymorphisms were highly linked with a *D'* value of 0.80. The observed frequencies of two major haplotypes were 59.1% for T^{-762}/C^{1735} and 30.1% for C^{-762}/G^{1735}, respectively. The frequencies of the rest variants were extremely low (1.8% for-133G, 1.5% for -18A, 0.9% for 1721T and only 0.2% for 27677C allele, respectively) or even not detected (1764T > C, 1767G > A, 25342A > C and 28650A > G) in our study population. Comparison with other ethnic populations revealed a remarkable genetic variability in the incidences of *NPC1L1* polymorphisms. The frequencies of *NPC1L1* polymorphisms in Chinese Hans are comparable to Japanese population but totally different from Caucasians, African-Americans and Hispanic individuals. This is the first study to report the ethnic difference in the frequencies of *NPC1L1* functional polymorphisms in detail. -762T > C and 1735C > G are two prevalent *NPC1L1* variants which need further studies to explore their clinical impact on CHD prevalence and response to ezetimibe therapy in Chinese Hans.

1. Introduction

Epidemiological studies have indicated that elevated plasma concentrations of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are closely associated with coronary heart disease (CHD) risk (Sharrett et al. 2001). Whole body Cholesterol homeostasis is tightly controlled by balancing dietary cholesterol absorption, *de vivo* cholesterol synthesis and bile acid secretion. Moreover, it is believed that profound inter-individual difference in dietary cholesterol absorption efficacy is closely related to the LDL-C level in humans (Gylling et al. 2002; Miettinen et al. 1989). Recent studies indicate that Niemann-Pick C1-like 1 (NPC1L1) protein, a sterol transporter highly expressed in the proximal intestine, is identified as the essential protein for dietary cholesterol absorption (Altmann et al. 2004; Davis et al. 2004). In addition, studies found that NPC1L1 is the molecular target of ezetimibe, a cholesterol absorption inhibitor demonstrated to reduce LDL-C level as monotherapy or combined with statins in clinic (Davis et al. 2007; Garcia-Calvo et al. 2005). To date, a large number of genetic polymorphisms in *NPC1L1* gene have been reported. Growing evidence illustrated that functional genetic mutations of *NPC1L1* gene contributed to the variability of lipid profiles, CHD risk and response to ezetimibe therapy.

Cohen et al. (2006) identified multiple rare sequence variants in *NPC1L1* gene which were associated with high or low cholesterol absorption. Furthermore, these variants were proven to influence plasma LDL-C levels. 1735C > G is a synonymous mutation (Leu272→Leu) in exon2, however studies indicated that 1735G/G homozygotes showed an increased cholesterol absorption ratio in a Japanese population, and similar results were found from an Italian population as well (Lupattelli et al. 2013; Maeda et al. 2010). Additionally, it is favorable to predict ezetimibe efficiency and CHD onset. Hegele et al. (2005)

Table 1: Allele frequencies and genotype distributions of *NPC1L1* gene in 424 Chinese Hans

SNP	db SNP	Position	Amino-acid change	Genotype	Frequency: No.(%)	Allele	Frequency: (%)
-762T > C	rs2073547	promoter	NA	TT	175 (41.3)	T	65.0
				TC	201 (47.4)	C	35.0
				CC	48 (11.3)		
-133A > G	rs17655652	promoter	NA	AA	409 (96.5)	A	98.2
				AG	15 (3.5)	G	1.8
				GG	0 (0)		
-18C > A	rs41279633	promoter	NA	CC	411 (96.9)	C	98.5
				CA	13 (3.1)	A	1.5
				AA	0 (0)		
1721C > T	rs114969055	exon2	A267A	CC	416 (98.1)	C	99.1
				CT	8 (1.9)	T	0.9
				TT	0 (0)		
1735C > G	rs2072183	exon2	L272L	CC	163 (38.4)	C	63.0
				CG	208 (49.1)	G	37.0
				GG	53 (12.5)		
1764C > T	rs115565879	exon2	P282L	CC	424 (100)	C	100
				CT	0 (0)	T	0
				TT	0 (0)		
1767G > A	rs150833705	exon2	G283D	GG	424 (100)	G	100
				GA	0 (0)	A	0
				AA	0 (0)		
27677T > C	rs217434	exon20	V1296V	TT	422 (99.5)	T	99.8
				TC	2 (0.5)	C	0.2
				CC	0 (0)		
25342A > C	rs217428	intron18	NA	AA	424 (100)	A	100
				AC	0 (0)	C	0
				CC	0 (0)		
28650A > G	rs3187907	3'-UTR	NA	AA	424 (100)	A	100
				AG	0 (0)	G	0
				GG	0 (0)		

SNP: single nucleotide polymorphism; 3'-UTR: 3'-untraslation region; NA: no available; *Pearson Chi-square test for genotype distribution

reported that dyslipidemia subjects without a rare haplotype of 1735C-25342A-27677T had a better response to ezetimibe treatment than subjects carrying at least one copy of the haplotype (35.9% vs. 23.6% decrease in LDL-C level). In addition, Polisecki et al. (2010) indicated that mutant homozygous carriers with 1735C > G as well as -18C > A, 27677T > C and 28650A > G variants had a higher TC and LDL-C levels and correspondingly increased CHD risk. In other studies, Simon et al. (2005) indicated that several *NPC1L1* variants (-133A > G, -18C > A and 1735C > G) are associated with an improvement in response to ezetimibe therapy. On the other hand, it was revealed that homozygous carriers with -133A-(-18C) haplotype represented a significantly lower cholesterol absorption/synthesis markers compared with all other haplotypes in an autosomal dominant hypercholesterolemia group (Martin et al. 2010). Moreover, it was reported that -133A > G variant associated with a elevation of ApoA1 levels after ezetimibe monotherapy (Zsíros et al. 2014).

Chen et al.(2009) sequenced *NPC1L1* promoter and coding regions in a small-scale sample of 50 Chinese and found that -762T > C and 1735C > G polymorphisms were two common variants. They reported that -762C allele had a 3.5-fold higher promoter activity compared with that of -762T allele, and further study indicated that subjects with -762T > C variant had a higher plasma TC and LDL-C levels than the non-carries. For 1735C > G mutation. Miao et al. (2012) performed the study to illustrate that it influenced serum baseline cholesterol profiles in Chinese Hans and Mulao.

To date, a number of studies for *NPC1L1* genetic polymorphisms were done in Caucasians, Hispanics, African-Americans and Japanese and illustrated a remarkable difference of allele frequency in *NPC1L1* gene among different ethnic groups (Kashiwabara et al. 2014; Simon et al. 2005). However, data is very limited for the Chinese population. Given the importance role of *NPC1L1* genetic polymorphisms in the plasma cholesterol profiles, CHD prevalence and response to ezetimibe therapy, it is necessary to perform a detailed analysis for allele frequencies of *NPC1L1* polymorphisms in Chinese population. Therefore, on the basis of their functional significance studied previously, we screened ten genetic variants in total (-762T > C, -133A > G,-18C > A,1721C > T, 1735C > G, 1764T > C, 1767G > A, 27677T > C, 25342A > C and 28650A > G), to investigate their allele frequencies and haplotype distributions in Chinese Han population, and then compare with those of other ethnic groups reported before.

2. Investigations and results

2.1. Allele and genotype frequencies of ten NPC1L1 genetic polymorphisms in Chinese Hans

In the present study, we analyzed ten genetic polymorphisms of *NPC1L1* gene in 424 unrelated Chinese Han subjects. Genotype distribution and allele frequency of *NPC1L1* polymorphisms in Chinese Hans are presented in Table 1. All the allele frequencies and genotype distributions of *NPC1L1* variants in the study population met the Hardy–Weinberg equilibrium when tested by Chi-square test ($P > 0.05$ for all). The genotype distributions of -762T > C and 1735C > G in Chinese Hans were TT: 41.3%, TC: 47.4%, CC: 11.3%; and CC: 38.4%, CG: 49.1%, and GG: 12.5%, respectively. Both -762T > C and 1735C > G had a high frequency of the minor allele, which was 35.0% for -762C allele

Table 2: Haplotype analysis of *NPC1L1* -762T > C and 1735C > G genetic polymorphisms in 424 Chinese Hans

SNP	Allele	Frequency(%)	Haplotype	Frequency (%)
-762T > C	T	65.0%	T^{-762}/C^{1735}	59.1
	C	35.0%	C^{-762}/G^{1735}	30.1
1735C > G	C	63.0%	T^{-762}/G^{1735}	5.9
	G	37.0%	C^{-762}/C^{1735}	4.9

SNP: single nucleotide polymorphism;

and 37.0% for 1735G allele, respectively. On the other hand, the allele frequencies of -133G, -18A, 1721T and 27677C variants were extremely low, where it was 1.8%, 1.5%, 0.9% and only 0.2%, respectively. In the case of 1764C > T 1767G > A 25342 A > C and 28650A > G, no variations were ever found in our study population.

2.2. Haplotype distribution of NPC1L1 genetic polymorphisms in Chinese Han population

For haplotype analysis, since the frequencies of the other eight variants were not higher than 2%, we constructed haplotypes with only two *NPC1L1* mutations, -762T > C and 1735C > G. There was a strong linkage disequilibrium between these two polymorphisms (*D'* value = 0.80). As listed in Table 2. The estimated frequencies of four haplotypes were 59.1% for T^{-762}/C^{1735}, 30.1% for C^{-762}/G^{1735}, 5.9% for T^{-762}/G^{1735} and 4.9% for C^{-762}/C^{1735}, respectively. Two major haplotypes, T^{-762}/C^{1735} and C^{-762}/G^{1735}, represented 90% haplotype in Chinese Han population.

2.3. Comparison of allele frequencies of seven NPC1L1 genetic polymorphisms among Chinese Hans and other ethnic groups reported before

NPC1L1 allele frequencies among different ethnic groups are shown in Table 3, which revealed a significant ethnic difference in allele frequencies of *NPC1L1* polymorphisms. So far as the *NPC1L1* polymorphisms listed in Table 3, it is clearly indicated that -762T > C and 1735C > G are the only two prevalent *NPC1L1* variants in our study population. The frequencies of -762T > C and 1735C > G are a little lower than those of Japanese, while they are much higher than those of Caucasians, African-Americans and Hispanics. The incidence of -133A > G,-18C > A, 27677T > C mutations are markedly low in Chinese Hans, however they are more common in Caucasians and less common in African-Americans and Hispanics. For 25343A > C and 28650A > G, they are totally monomorphic in Chinese Hans.

3. Discussion

In our present study, we provided a detailed characterization of allele and genotype frequencies of *NPC1L1* genetic polymorphisms in Chinese Han population and then compared them with those of other different ethnic groups reported before. The genetic variability of *NPC1L1* genetic mutations among different ethnic populations is remarkable and summarized in Table 3. Generally speaking, the allele frequencies of *NPC1L1* polymorphisms in Chinese Hans corresponds, in most cases, to Japanese but is significantly different from Caucasians, African-Americans and Hispanic individuals. As a result, Chinese Hans would exhibit a different CHD incidence and response profile to ezetimibe due to such significant ethnic differences.

Table 3: Comparison of allele frequencies for seven *NPC1L1* genetic polymorphisms in Chinese Hans and other ethnic groups

SNP	MAF(%)	Ethnic population (N)	Reference
-762T > C	35.0	Chinese Hans (424)	Present study
	41.0	Japanese (142)	Meada et al. (2010)
	0.3*	Caucasian (198)	Simon et al. (2005)
	8.3*#	Hispanic (78)	Simon et al. (2005)
	3.1*#	African American (99)	Simon et al. (2005)
-133A > G	1.8	Chinese Hans (424)	Present study
	29.9*	Caucasian (198)	Simon et al. (2005)
	24.6*	Spanish (274)	Martín et al.2010)
	18.6*	Hispanic (78)	Simon et al. (2005)
	9.2*#	African American (99)	Simon et al. (2005)
-18C > A	1.5	Chinese Hans (424)	Present study
	18.1*	Caucasian (198)	Simon et al. (2005)
	15.1*	Spanish (274)	Martin et al.2010)
	7.5*#	Hispanic (78)	Simon et al. (2005)
	6.0*#	African American (99)	Simon et al. (2005)
1735C > G	37.0	Chinese Hans (424)	Present study
	41.9	Japanese (142)	Meada et al. (2010)
	21.9*	Caucasian (198)	Simon et al. (2005)
	24.8*	Canadian (101)	Hegele et al. (2005)
	23.6*	Spanish (274)	Martıń et al.2010)
	28.3*	Hispanic (78)	Simon et al. (2005)
	17.9*	African American (99)	Simon et al. (2005)
27677T > C	0.2	Chinese Hans (424)	Present study
	1.1*	Japanese (142)	Meada et al. (2010)
	19.5*	Caucasian (198)	Simon et al. (2005)
	19.8*	Canadian (101)	Hegele et al. (2005)
	17.1*	Hispanic (78)	Simon et al. (2005)
	13.0*	African American (99)	Simon et al. (2005)
25342A > C	0.0	Chinese Hans (424)	Present study
	1.1*	Japanese (142)	Meada et al. (2010)
	24.2*	Caucasian (198)	Simon et al. (2005)
	28.7*	Canadian (101)	Hegele et al. (2005)
	30.5*	Hispanic (78)	Simon et al. (2005)
	8.0*#	African American (99)	Simon et al. (2005)
28650A > G	0.0	Chinese Hans (424)	Present study
	4.9*	Caucasian (198)	Simon et al. (2005)
	16.5*#	Spanish (274)	Martıń et al.2010)
	3.8*	Hispanic (78)	Simon et al. (2005)
	3.5*	African American (99)	Simon et al. (2005)

* Pearson Chi-square test compared with Chinese Hans, $P < 0.05$
# Pearson Chi-square test compared with Caucasian, $P < 0.05$
SNP: single nucleotide polymorphism; MAF: minor allele frequency;

As shown in Table 3, -762T > C genetic polymorphism is much more common in oriental people than in other different ethnic populations. The observed frequency of -762C allele was 35.0% in Chinese Hans in our study, in accordance with previous results (Chen et al. 2009). It still has a highest frequency of 41.0% in Japanese subjects (Meada et al. 2010). However, it shows significantly lower frequencies in Hispanics (8.3%) as well as African-Americans (3.1%) and is extremely rare in Caucasians (0.3%), (Simon et al. 2005). According to our results, the observed frequency of 1735C > G variant was 37.0%, which was similar to previous results (Chen et al. 2009; Miao et al. 2012). It shows a highest incidence of 41.9% in Japanese population as well (Meada et al. 2010). It is another prevalent *NPC1L1* genetic polymorphism in Asians besides the -762T > C variant. The frequency of 1735C > G was lower in the other ethnic groups, which were 17.9% in African-Americans, 21.9% to 24.8% in white people and 28.0% in Hispanics (Hegele et al. 2005; Martin

Table 4: Primer sequences, annealing temperatures and genetic analysis for *NPC1L1* gene amplified products

SNP	Primers(5'to 3')	Annealing temperature °C	Product size (bp)	Restriction enzyme	Digested products (bp)
-762T > C	F:AGTGAGAACGCAAGGTAG R:GGAGTGACGCAGTGACAAA	53	318	NIaIII	TT:206 + 112 TC:318 + 206 + 112 CC:318
-133A > G -18C > A	F: TCGGTCGCATTGGGAAGT R:CAGTAACGCTCGCCTGGTA	57	392	NA	NA
1721C > T 1735C > G 1764C > T 1767G > A	F:GCTCAACTTCCAGGGAGA R:CCTCTCTGACAAGCT	55	381	NA	NA
27677T > C	F:GAAGCTTGGGCTGTGAACA R:CTCCTCTGCTCCCATAGTGG	57	558	HpaI	TT:390 + 168 TC:558 + 390 + 168 CC:558
25343A > C	F:CACATTACCCGCTCCTTT R:TGTTCATAGTCCTGGCTTC	55	499	NA	NA
28650A > G	F:AATGGTGTTGGGTTTGGT R:GAGGAGGAAGCGAGGGTA	53	300	NIaIII	AA:184 + 92 + 26 AG:300 + 184 + 92 + 26 GG:274 + 26

SNP: single nucleotide polymorphism; NA: no available

et al. 2010; Simon et al. 2005). Among the variants we detected, only -762T > C and 1735C > G are the two prevalent *NPC1L1* polymorphisms in Chinese Hans. Previous studies indicated that both -762T > C and 1735C > G were gain-of-function *NPC1L1* variants associated with increased cholesterol absorption and improvement in ezetimibe therapy (Chen et al. 2009; Meada et al. 2010; Polisecki et al. 2010). Significantly higher frequencies of these two variants suggested that Chinese Hans may suffer from an enhanced CHD risk but also could benefit more from ezetimibe therapy. Furthermore, linkage disequilibrium analysis found 1735C > G and -762T > C were highly linked with a *D'* value of 0.80 in our study, in agreement with Chen et al. (2009). Therefore, it maybe more valuable to demonstrate the association between the haplotypes and ezetimibe efficiency or CHD risk in Chinese population in the further study.

On the other hand, the frequencies of -133A > G and -18C > A, the other two variants in the *NPC1L1* promoter region, were markedly low in Chinese Hans (1.8% and 1.5%, respectively). Interestingly, both -133A > G and -18C > A polymorphisms were commonly detected in the other ethnic populations. -133A > C mutation had a highest occurrence of 29.9% in Caucasians, which was a little higher than that of Spanish (24.6%) but much higher than those of Hispanic individuals (18.6%) and African-Americas (9.2%), respectively (Martin et al. 2010; Simon et al. 2005). For -18C > A variant, it was more common in Caucasians (18.1%) and Spanish (15.1%) compared with the Hispanic group (7.5%) and African-Americans (6.0%), respectively (Martıń et al. 2010; Simon et al. 2005).

27677T > C genetic mutation in exon 20 was more common in the other ethnic groups than in Chinese and Japanese populations. The frequency of 27677T > C had no significant difference among Caucasians, Hispanics and African-Americans, which were 19.5%, 17.1% and 13.0%, respectively (Hegele et al. 2005; Simon et al. 2005). However, it was detected at an extremely low frequency of 0.2% in our study population and it was very rare in Japanese as well with a frequency of 1.1% (Meada et al. 2010). In the present study, none of 25342A > C and 28650A > G genetic polymorphisms were found in our study population. 25343A > C polymorphism occurred at highest frequency of 30.5% in Hispanic subjects, followed by 24.2-28.7% in Caucasians but had a markedly lower incidence of 8.0% in African-Americans (Hegele et al. 2005; Simon et al. 2005). For 28650A > G mutation, it was less present in Caucasians (4.9%), Hispanics (3.8%) and African-Americans (3.5%), with an exception of a highest frequency of 16.5% in Spanish people (Martin et al. 2010; Simon et al. 2005). Although these variants displayed significant clinical impact in Caucasians, African-Americans and Hispanics, they would be less valuable to predict ezetimibe efficiency or CHD risk in Chinese Hans due to their extremely low frequencies.

To our knowledge, this is the first report the accurate frequency of 1721T allele in Chinese Han individuals (0.9%). It is another synonymous mutation in exon 2, and further studies are needed to confirm its function. Meanwhile, data from other ethnic populations are not available to carry out the comparison so we did not list it in Table 3. 1764C > T and 1767G > A are two non-synonymous and rare mutations identified from subjects who had high cholesterol absorption reported by Cohen et al. (2006). Although Chen et al. (2009) reported both 1764C > T and 1767G > A occurred at a frequency of 1% in a small-scale sample of 50 Chinese, they were monomorphic in our study population. Additionally, they were totally rare in other ethnic populations as reported by Cohen *et al.* (2006) so we did not list them in Table 3 either.

In summary, our present study revealed that there are remarkable genetic variabilities of *NPC1L1* functional polymorphisms among different ethnic populations. -762T > C and 1735C > G are two prevalent variants in Chinese Hans and they are highly linked to each other. Therefore, it is necessary to carry out further studies to explore their impact on ezetimibe efficiency and CHD risk in Chinese Hans.

4. Experimental

4.1. Study population

A total of 424 Chinese Hans volunteers (male: 246; female: 178) aged from 38 to 56 years old were enrolled in our present study. None of them had obvious liver kidney or cardiovascular diseases based on the questionnaires. All the participants signed the informed consent before blood samples were collected. Genomic DNA samples were extracted from peripheral blood leukocytes by Wizard Genomic DNA Purification Kit (Promega, USA) following the protocol. The study was approved by the Ethnic Committee of Institute of Clinical Pharmacology, Centre South University (No.CTXY-090011) and Chinese Clinical Trial Registry (ChiCTR-RCC-12002859).

4.2. Genetic analysis

The genotyping work was carried out by direct sequencing assay for -133A > G, -18C > A, 1721C > T, 1735C > G, 1764C > T, 1767G > A, 25342A > C polymophisms. On the othe hand, PCR-RFLP methods were

used for -762T > C, 27677T > C and 28650A > G genetic analysis. -133A > G and -18C > A mutations shared the same primer pairs. 1721C > T 1735C > G 1764C > T and 1767G > A were amplified using the same pair of primers. Briefly, the PCR amplification reactions were generated in a total volume of 25 μL including 100 ng gDNA as template, 2.5U Taq DNA polymerase (TakaRa, Japan), 10* PCR buffer 2.5 μL (TakaRa, Japan), 2.5 μM dNTP mixture (TakaRa, Japan) and 0.2 μM each primer (Biosence, China). The PCR cycles were as follows: 5 min at 94 °C, followed by 35cycles of 30 s at 94 °C, 30 s at each annealing temperature and 30 s at 72 °C. Final extension was for 5 min at 72 °C. Once the amplification reactions were done, the amplified products were sequenced by ABI 3700 using the same primers as amplification ones and compared with reference sequence, otherwise they were digested by respective restriction enzymes (NEB, USA) following the manufacturer's instructions. The PCR-RFLP genotyping results of -762T > C, 27677T > C and 28650A > G were then conformed by direct sequencing. The PCR product lengths, primer pairs, various annealing temperatures, restriction enzymes and digested products length were listed in Table 4.

4.3. Statistical analysis

Statistical analysis of data was performed by SPSS 18.0 version software package for windows (SPSS Inc., Chicago, IL, USA). Hardy–Weinberg equilibria of genotype and haplotype were tested with Chi-square test. Linkage disequilibrium (LD) and haplotype analysis were performed by SHEsis online software (http://analysis2.bio-x.cn/myAnalysis.php). Comparisons of allele frequencies among different ethnic groups were tested Chi-square test. In all the analysis, A P value < 0.05 was considered to be statistical significance.

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