A SNP of NPC1L1 Affects Cholesterol Absorption in Japanese

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Aim: Ezetimibe is known to target Niemann-Pick Type C1 Like1 (NPC1L1), a key protein in intestinal cholesterol absorption, and thus to decrease serum LDL-cholesterol (LDL-C) levels. The response of serum LDL-C levels to ezetimibe was reported to differ among NPC1L1 haplotypes. We analyzed NPC1L1 genotypes in Japanese and investigated differences in markers of cholesterol synthesis/absorption among the genotypes.

Methods: Blood samples were collected from 42 adult volunteers to measure markers of cholesterol synthesis (lathosterol) and absorption (sitosterol and campesterol) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Based on a study by Hegele RA et al. in Canada, we selected three SNPs (1735 C>G, 25342 A>C and 27677 T>C (numbers relative to the transcription start site)) and analyzed them using PCR-RFLP.

Results: The frequencies of genotypes were as follows: 1735 C/G (46%) > C/C (35%) > G/G (19%), 25342 A/A (97%) > A/C (3%) > C/C (0%) and 27677 T/T (97%) > T/C (3%) > C/C (0%). Serum campesterol levels were significantly higher in the 1735 G/G group than 1735 C/G or C/C group, but lathosterol levels showed no significant differences between the genotypes.

Conclusion: Our results revealed differences in the frequency of the NPC1L1 polymorphism between Japanese and Canadians. In Japanese, the 1735 G/G group showed enhanced cholesterol absorption from the intestine, as compared to the 1735 C/G + C/C group.


Key words: NPC1L1, Genotype, Ezetimibe, Cholesterol absorption

Original Article

Introduction

In Japan, serum cholesterol levels have been increasing year by year¹,² and this is partly ascribable to increased dietary intake of cholesterol and saturated fatty acids. In particular, the cholesterol intake of young people is higher in Japan than in the USA³,⁴, implying that the incidence of atherosclerotic diseases resulting from dyslipidemia may well continue to rise in the future⁵.

About 1,200 mg of cholesterol is synthesized in the liver every day, while a comparable amount (200–300 mg/day from diet and 400–1,000 mg/day in bile) is absorbed from the small intestine⁶. Therefore, controlling the absorption as well as synthesis of cholesterol is important to regulate serum cholesterol levels, and the mechanism of cholesterol absorption has been studied extensively.

In the treatment of dyslipidemia, statins that inhibit the synthesis of cholesterol can lower LDL-cholesterol (LDL-C) levels significantly, the importance of which has been established by many large-scale clinical studies⁷. Also, statins can exert beneficial effects apparently independently of LDL-C⁸. Nonetheless, most clinical studies have found that statins cannot reduce the incidence of cardiovascular disease (CVD) events by more than 30%. In this regard, a cholesterol absorption inhibitor, ezetimibe, a drug with
a new mechanism of action, and has been receiving attention for its efficacy.

Ezetimibe lowers the serum LDL-C level by specifically inhibiting the function of Niemann-Pick Type C1 Like 1 (NPC1L1), a membrane protein in the brush border of epithelial cells in the small intestine that plays an important role in cholesterol absorption. However, there are inter-individual differences in the effectiveness of this drug.

Wang J et al. analyzed the NPC1L1 gene in ezetimibe-unresponsive patients and clarified the NPC1L1 genotypes. Hegele RA et al. investigated differences in concentrations of lipids in serum and the response of LDL-C to treatment with ezetimibe among various NPC1L1 genotypes, and reported significant differences in the percent decrease in LDL-C. These reports have suggested that the NPC1L1 genotype influences cholesterol absorption. Thus, genotyping might be useful for evaluating the risk of atherosclerotic disease and for selecting drugs to reduce LDL-C levels. We analyzed NPC1L1 genotypes in Japanese subjects and investigated differences in markers of cholesterol synthesis/absorption between the genotypes.

Subjects

One hundred and 42 healthy adults (64 males and 78 females) were recruited for this study. Informed consent was obtained from all of the participants using a consent form approved by the Teikyo University Institutional Review Board.

Methods

Serum Samples and Measurements of Serum Lipids

Blood samples were collected after fasting for 12 hours or more. Serum was separated and used to measure total cholesterol (TC), triglyceride (TG), and HDL-cholesterol (HDL-C) levels, and markers of cholesterol synthesis/absorption.

TC and TG levels were measured by an enzymatic method. HDL-C concentrations were measured by the selective dialysis method. LDL-C was calculated based on Friedewald's formula (LDL-C = TC - HDL-C - TG/5), applicable because no subject had a TG concentration that exceeded 400 mg/dL. Markers of cholesterol synthesis/absorption were determined as described later.

Identification of SNPs of NPC1L1 by the PCR-RFLP (Restriction Fragment Length Polymorphism) Method

Genomic DNA was purified from peripheral blood leukocytes. To genotype the 1735 C>G SNP in exon 2, the DNA fragment containing this region was amplified by PCR (using the primers 5'-GCT CAA CTT CCA GGG AGA CA-3' and 5'-AGC TTG TCA GAG AGG CTG G-3'). The amplified fragment was treated with the restriction enzyme Taq I. The PCR product carrying the 1735 C allele was digested into two fragments (211 bps and 170 bps), while that carrying 1735 G was not digested (381 bps).

For genotyping of the 27677 C>T SNP, DNA was amplified by PCR (5'-GAA GCT TGG GCT GTG AAC A-3' and 5'-CCA CTA TGG GAG CAG AGG AG-3'), and the PCR product was digested with Hpa I. The PCR product with the 27677 T allele was digested into two fragments (390 bps and 168 bps), while the product with the 27677 C allele was not digested (558 bps).

To genotype the 25342 A>C SNP in intron 18, DNA was amplified by PCR (5'-CCT GCC TGA CAC CTG GCT CTT A-3' and 5'-CCA GCA GAG CTG GCT CTT A-3') and the product was digested with the restriction enzyme Mse I. The primer 5'-CCT GCC TGA CAC CTG GCT CTT A-3' was designed by introducing a mismatch into the original primer sequence 5'-CCT GCC TGA CAC CTG GCT CTT A-3'. The G>T mismatch created a restriction site TTA, which could be recognized by Mse I in the PCR product with the 25342 A allele, but not that with the 25342 C allele. Thus, the PCR product bearing 25342 A was digested by Mse I to yield two fragments (125 bps and 21 bps), while that bearing 25342 C was not digested (146 bps).

Haplotyping

NPC1L1 haplotyping was performed as follows. According to the report by Hegele RA et al., 1735 C - 25342 A - 27677 T was designated as haplotype 2 and the other haplotypes were defined as haplotype X. Three subjects in whom combinations could not be identified were excluded, and the others were classified into the haplotype 2/2, 2/X, and X/X groups.

Determination of Sterol Concentrations

The concentrations in serum of sitosterol, campesterol and lathosterol were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described elsewhere. Briefly, 50 ng of coprostanol was added to 5 µL of serum as an internal standard, and alkaline hydrolysis in 1 N ethanolic KOH and derivatization to the picolinyl esters were carried out. Then the picolinyl ester derivatives of the sterols were quantified by LC-MS/MS. Coprostanol, a compound almost nonexistent in normal human...
serum, was used as an internal standard, because coprostanol and the sterols of interest to us should be lost to the same extent during the quantification process due to their structural similarity.

Statistical Analysis

Measured values were expressed as the mean ± standard deviation. Differences in mean values between two groups were analyzed with the t-test. Differences in the frequencies of genotypes were assessed using the χ² test. In all analyses, p < 0.05 was considered statistically significant. JMP software (SAS Institute Inc.) was used for the statistical analysis.

Results

Frequency of the NPC1L1 Genotypes

Table 1 shows the results of an analysis of 3 SNPs (1735 C>G, 25342 A>C and 27677 T>C) in 142 healthy adults (64 males and 78 females). The frequency of each genotype was as follows: for 1735 C>G, C/C (46%) > C/G (35%) > G/G (19%); for 25342 A>C, A/A (97%) > A/C (3%) > C/C (0%); and for 27677 T>C, T/T (97%) > T/C (3%) > C/C (0%).

Serum Lipids and NPC1L1 Genotypes

The frequencies of the 25342 A>C and 27677 T>C genotypes showed an extremely skewed distribution. Therefore, serum lipids were investigated to detect any differences between the 1735 C>G genotypes. As shown in Table 2, the levels of TC, TG, HDL-C and LDL-C showed no significant differences among the 1735 C/C, C/G, and G/G genotypes in 139 subjects for whom serum lipid data could be obtained.

Table 1. Frequency of each NPC1L1 genotype

<table>
<thead>
<tr>
<th>SNP</th>
<th>genotype</th>
<th>number ( ): female</th>
<th>frequency In Japanese</th>
<th>Hegele et al. 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1735 C&gt;G</td>
<td>C/C</td>
<td>50 (29)</td>
<td>0.35</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>C/G</td>
<td>65 (37)</td>
<td>0.46</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>27 (12)</td>
<td>0.19</td>
<td>0.05</td>
</tr>
<tr>
<td>25342 A&gt;C</td>
<td>A/A</td>
<td>138 (75)</td>
<td>0.97</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>4 (3)</td>
<td>0.03</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>0</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>27677 T&gt;C</td>
<td>T/T</td>
<td>138 (75)</td>
<td>0.97</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>4 (3)</td>
<td>0.03</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>0</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Three SNPs of NPC1L1 were determined by PCR-RFLP.
1) Hegele RA et al.: Lipid in Health and Disease, 2005 Aug 12, 4; 16

Table 2. Serum lipids for each NPC1L1 genotype

<table>
<thead>
<tr>
<th>1735 C/C</th>
<th>1735 C/G</th>
<th>1735 G/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 49)</td>
<td>(n = 64)</td>
<td>(n = 26)</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>187.0 ± 32.9</td>
<td>194.7 ± 36.3</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>71.2 ± 33.4</td>
<td>83.9 ± 53.7</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>67.1 ± 16.6</td>
<td>68.4 ± 16.5</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>105.6 ± 26.4</td>
<td>109.6 ± 32.4</td>
</tr>
</tbody>
</table>

The 1735 C>G SNP of NPC1L1 was identified as described in the text. Data are shown as the mean ± S.D.

NPC1L1 Haplotypes and Serum Lipids

Hegele RA et al. defined the most frequent combination 1735 C - 25342 A - 27677 T as haplotype 2 and all the other haplotypes as haplotype X. The frequencies of haplotype 2/2, 2/X and X/X were 35%, 45% and 20%, respectively, in 136 subjects for whom serum lipid levels were measured. Three subjects were excluded because it was impossible to determine their haplotypes. The levels of TC, TG, HDL-C and LDL-C showed no significant differences among these haplotypes.

NPC1L1 Haplotypes and Cholesterol Synthesis/Absorption Markers

Concentrations of lathosterol (a marker of cholesterol synthesis), and sitosterol and campesterol (markers of cholesterol absorption) were measured to assess the influence of NPC1L1 genotypes on cholesterol turnover.

One hundred and ten subjects were classified as carrying haplotype X/X, reported by Hegele RA et al. to be associated with a significantly higher percent decrease in LDL-C after ezetimibe treatment, compared to subjects with the other haplotypes (2/X + 2/2). The level of lathosterol (a cholesterol synthesis marker) showed no significant differences between the two groups (6.63 ± 3.46 µg/mL in X/X and 6.20 ± 4.17 µg/mL in 2/2 + 2/X). However, the concentration of campesterol (a cholesterol absorption marker) was significantly higher in the X/X group (5.32 ± 3.57 µg/mL in X/X and 4.14 ± 1.92 µg/mL in 2/2 + 2/X, p = 0.036), and the level of sitosterol also tended to be higher in the X/X group (4.25 ± 2.90 µg/mL in X/X and 3.48 ± 1.55 µg/mL in 2/2 + 2/X, p = 0.090). These results suggested that cholesterol absorption was enhanced in the haplotype X/X group.

NPC1L1 Genotypes and Cholesterol Synthesis/Absorption Markers

Since there were only 4 subjects with genotypes other than 25342 A/A or 27677 T/T, haplotype 2/2,
Table 3. Biomarkers of cholesterol synthesis and absorption in subjects with genotype 1735 C/C + C/G and genotype 1735 G/G

<table>
<thead>
<tr>
<th>Genotype</th>
<th>1735 C/C + C/G</th>
<th>1735 G/G</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=91)</td>
<td>(n=22)</td>
<td></td>
</tr>
<tr>
<td>Lathosterol (µg/mL)</td>
<td>6.28±4.16</td>
<td>6.63±3.46</td>
<td>0.717</td>
</tr>
<tr>
<td>Sitosterol (µg/mL)</td>
<td>3.47±1.54</td>
<td>4.25±2.90</td>
<td>0.084</td>
</tr>
<tr>
<td>Campesterol (µg/mL)</td>
<td>4.14±1.90</td>
<td>5.32±3.57</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Biomarkers were measured by LC-MS/MS as described in the text. Differences between two groups were analyzed with a t-test. Data are means ± S.D.

2/X or X/X turned out to be determined by the 1735 G>C genotype in most subjects. Therefore, markers of cholesterol synthesis/absorption were compared between the 1735 G/G and 1735 C/C + C/G groups. As shown in Table 3, the campesterol level was significantly higher in the 1735 G/G group than in the 1735 C/C + C/G group (p=0.033) and the sitosterol level tended to be higher (p=0.084). The levels of lathosterol showed no significant differences between the two groups.

Discussion

In Japan, serum cholesterol levels have elevated with an increase of cholesterol intake due to westernization of the diet, and therefore it has become important to decrease the absorption of cholesterol from food. The incidence of cardiovascular events was reported to be higher in persons with increased cholesterol absorption even when there was little change in LDL-C levels. Enhanced cholesterol absorption is a risk factor for atherosclerosis, and attention has focused on controlling cholesterol absorption via NPC1L1. Hegele RA et al. reported that the percent decrease in LDL-C achieved by ezetimibe therapy differed between haplotypes of the NPC1L1 gene. Since ezetimibe is a specific inhibitor of the absorption of cholesterol from the small intestine, we proposed that the genotype had an influence on cholesterol absorption.

First, we examined genotypes and haplotypes of NPC1L1 in Japanese volunteers. The frequency of haplotype X/X, reported by Hegele RA et al. as associated with a significantly higher percent decrease of LDL-C after ezetimibe therapy, was approximately 12.8% in Canada, but was higher (20%) in Japan. This suggested there to be more high responders to ezetimibe in Japan than in Canada.

We next investigated specific markers of cholesterol synthesis and absorption. The level of lathosterol in serum (a marker of cholesterol synthesis) did not differ significantly among NPC1L1 genotypes or NPC1L1 haplotypes. However, the concentration of campesterol (a marker of cholesterol absorption) was significantly higher in the haplotype X/X group. These results suggested that the level of cholesterol absorption was indeed elevated in individuals with haplotype X/X.

Since the haplotypes were determined almost completely by differences of 1735 C>G in the Japanese population, we next compared the 1735 G/G and 1735 C/C + C/G groups. We found that the levels of campesterol showed a significant difference between the two groups. Thus, cholesterol absorption seemed to be enhanced when 1735 C>G was G/G in Japanese.

It is possible that NPC1L1 genotypes other than those dealt with here also contribute to inter-individual differences in intestinal cholesterol absorption and the response to ezetimibe. Recently, several studies have focused on NPC1L1 genotypes in different ethnic groups. For instance, Chen CW et al. reported that the −762 T>C (relative to the translation start site) polymorphism was associated with higher TC and LDL-C levels in a Chinese population, possibly because of the higher NPC1L1 expression in those with the −762 C than −762 T allele. We analyzed this polymorphism as well. The frequency of the −762 T>C genotypes was as follows: T/C (46%) > T/T (36%) > C/C (18%). In our analysis, serum campesterol and sitosterol concentrations as well as TC, TG and LDL-C levels did not differ significantly among the −762 T/T, T/C and C/C genotypes (data not shown). Perhaps the −762 T>C polymorphism is a less critical determinant of cholesterol absorption in Japanese, for some unknown reason.

In conclusion, we revealed that Japanese subjects with the 1735 G/G SNP of NPC1L1 exhibited enhanced cholesterol absorption. While those carrying 1735 G/G could have increased risk of atherosclerosis, they might respond better to ezetimibe than the carriers of the other SNPs due to their high cholesterol absorption. Therefore, analysis of this single SNP alone could be useful for evaluating the risk of atherosclerosis and predicting the efficacy of ezetimibe. The number of subjects in this study was not very large (n=142), so we are planning to perform more studies on a larger scale.

Acknowledgements

We thank Ms. Kazue Murata and Ms. Mineko Fujita for excellent technical assistance.
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