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A New PCR-RFLP Method for Diagnosing PNPLA3 rs738409 Polymorphism

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Summary

Background:
PNPLA3 rs738409 (C10109G) polymorphism has been identified as a predisposingfactor for nonalcoholic fatty liver disease, fibrosis, alcoholic cirrhosis and elevation of serumalanine transaminase in the human. However, no simple method for its assay is available. Hence, this research work was undertaken to develop PCR-RFLP method for the assay of PNPLA3 rs738409.
Methods:
PCR primers were designed using NCBI primer Blast and restriction endonuclease was identified using NEB Cutter 2 software. PCR-RFLP based genotyping was confirmed by sequencing both strands of PCR amplicons of a normal homozygote, a heterozygote and a mutant homozygote.
Results:
Amplification of genomic DNA with specific primers demonstrated 333 bp fragment. PCR-RFLP of DNA from a normal homozygote demonstrated 200 and 133 bp fragments, of a heterozygote 333, 200 and 133 bp fragments and of a mutant homozygote 333 bp fragment. Sequencing of DNA from a normal homozygote, a heterozygote and a mutant homozygote demonstrated C, C/G and G with sense primer and G, C/G and C with anti sense primer, respectively.
Conclusion:
A simple PCR-RFLP method for the assay of PNPLA3 rs738409 has been developed and its validity confirmed by DNA sequencing. This shall facilitate genotyping of PNPLA3 rs738409.

Introduction

Homo sapiens PNPLA3 is located at chromosome 22q13.31. It is 23,830 bp long and contains 9 exons. It is transcribed in to 2,805 nt long mRNA which is translated in to patatin like phospholipase domain containing 3 or adiponutrin of 481 amino acid residues with a molecular weight of 52.86 kDa (Baulande S et al, 2001). The membrane bound multifunctional protein present in adipocytes and hepatocytes demonstrates various enzyme activities (EC 3.1.1.3) like triglyceride hydrolase, calcium independent phospholipase A2 μ and acylglycerol O-acyltransferase, thus having both lipolytic and lipogenic activities (Jenkins et al, 2004). A genome wide association study demonstrated the association of PNPLA3 genetic polymorphism with nonalcoholic fatty liver disease in Hispanics, European Americans and African Americans (Romeo et al, 2008). SNP responsible for this was rs738409 (PNPLA3, C10109G). This transversion in exon-3 causes a nonsynonomous change from isoleucine to methionine at codon 148 in the catalytic domain. This change decreases the binding affinity of triglycerides at the active site, hence attenuating its lipolytic activity (He et al, 2010). Thus mutant homozygotes (PNPLA3, G10109G) are predisposed to accumulation of fat in the liver. Meta analysis of the 16 studies reported between 2008 and 2011 has established a strong association of this polymorphism with nonalcoholic fatty liver disease in Hispanics, Asians, African Americans and Caucasians (Sookian et al, 2011). Mutant homozygotes (PNPLA3, G10109G) also demonstrated increased serum alanine transferase activity in different ethnic groups in this meta analysis. This polymorphism is also associated with alcoholic cirrhosis in Mestizos (Tian et al, 2010) and Germans (Stickel et al, 2011), steatosis in chronic hepatitis C Italian patients (Valenti et al, 2011) and fibrosis in Germans (Krawczyk et al, 2011). Most studies on genotyping for PNPLA3 rs738409 have employed TaqMan chemistry (Valenti et al, 2010) or microarrays (Tian et al, 2010).

Material and Methods

Due to the importance of this polymorphism, there is a need to develop a simple method for its assay which can be used in the research laboratories not having ultra modern facilities. Hence, a simple PCR-RFLP based method, which is well accepted and routinely used in the research laboratories, has been developed for the assay of PNPLA3 rs738409. Five mL blood was collected from 25 healthy volunteers after obtaining the written consent. Genomic DNA was isolated (Daly et al, 1996). PCR primers were designed with NCBI Primer Blast and restriction enzyme was selected using NEBCutter 2 software.

SNP rs738408 is located at 10112 and causes C to T transition, thereby changing codon CCC to CCT. This does not change proline at position 149. BtsCI recognition site is from 10104 to 10110 and it cleaves sense strand between 10105 and 10106 and anti sense strand between 10103 and 10104, thus leaving 3'-TT and 3'-AA overhangs on the sense and anti sense strands, respectively. Thus the presence of SNP rs 738408 did not interfere in the assay of SNP rs738409. The latter is present in the recognition site of BtsCI and thus results in the loss of restriction site for BtsCI in the mutant allele. There is no other known polymorphism in the recognition sequence of BtsCI. Hence the loss or gain of BtsCI site was solely due to rs738409 polymorphism. The sequence of sense primer was 5'- TGGGCCTGAAGTCCGAGGGT-3' (9906 to 9925, 20 bp) and that of anti sense primer was 5'- CCGACACCAGTGCCCTGCAG-3' (10238 to 10219, 20 bp). PCR mixture consisted of 1X PCR buffer (Tris-HCl 10 mM, KCl 50 mM, MgCl2 2 mM), 0.2 mM each dNTP, 1 µM each primer, 1 µg genomic DNA and 1.5 U Taq DNA polymerase in a total volume of 50 µL (Sambrook et al, 2001). Initial denaturation was performed at 94^{0}C for 2 min. Subsequent cycles, each consisting of denaturation at 94^{0}C for 30 sec, annealing at 66°C for 30 sec and synthesis at 72°C for 30 sec was carried out. Final synthesis was carried out at 72°C for 5 min. PCR was carried out in Mastercycler ep (Eppendorf AG, Germany) thermocycler for 35 cycles. PCR product was obtained which was digested with 4U of BtsCI at 50°C for 4 hr in 15 µL reaction mixture containing 10 µL PCR product and 1X NEBuffer 4. Digestion was stopped by incubating at 0°C. Digested PCR product was electrophoresed in 2g % agarose gel and was visualized using Alphaimager HP (Alpha Innotech, USA).

Result and discussion

As predicted from the designed primers, an amplicon of 333 bp was obtained on the amplification of genomic DNA of a healthy control (Figure 1, UC). DNA amplified and cleaved with BtsCI from a normal homozygote (PNPLA3, C10109C) demonstrated 200 and 133 bp fragments (Figure 1, C/C). DNA amplified and cleaved with BtsCI from a heterozygote (PNPLA3, C10109G) demonstrated 333, 200 and 133 bp fragments (Figure 1, C/G). DNA amplified and cleaved with BtsCI from a mutant homozygote (PNPLA3, G10109G) demonstrated 333 fragment (Figure 1, G/G). The observed pattern of restriction fragment analysis exactly matched the predicted pattern based on the knowledge of DNA sequence of the amplicon, presence of SNP rs738409 as well as the recognition sequence of BtsCI. Thus a simple PCR-RFLP based assay for PNPLA3 rs738409 has been developed. To elevate any doubt on the validity of PCR-RFLP method developed in this study, both strands of DNA isolated and amplified from a normal homozygote, a heterozygote and a mutant homozygote were sequenced using sense and anti sense primers in 3130xl DNA sequencer (16 capillaries), Applied Biosystems, USA. Sequence of DNA isolated a normal homozygote using sense primer demonstrated C at 10109 (See Supplemental data Figure 1) and using anti sense strand demonstrated G at 10109. Expectedly sequence of DNA isolated from a heterozygote using sense as well as anti sense primer demonstrated both C and G at 10109. Theoretically C to G mutation shall reverse the sequence at 10109 resulting G in sense strand and C in anti sense strand. This was exactly observed on sequencing DNA from a mutant homozygote using sense and anti sense primer (See Supplemental data Figure 1). DNA sequencing of both strands from a normal homozygote, a heterozygote and a mutant homozygote clearly validated the PCR-RFLP method developed for the assay of PNPLA3 rs738409. This study was in accordance with Helsinki declaration of clinical studies involving human subjects. The study was approved by the Institute Ethics Committee of PGIMER, Chandigarh.

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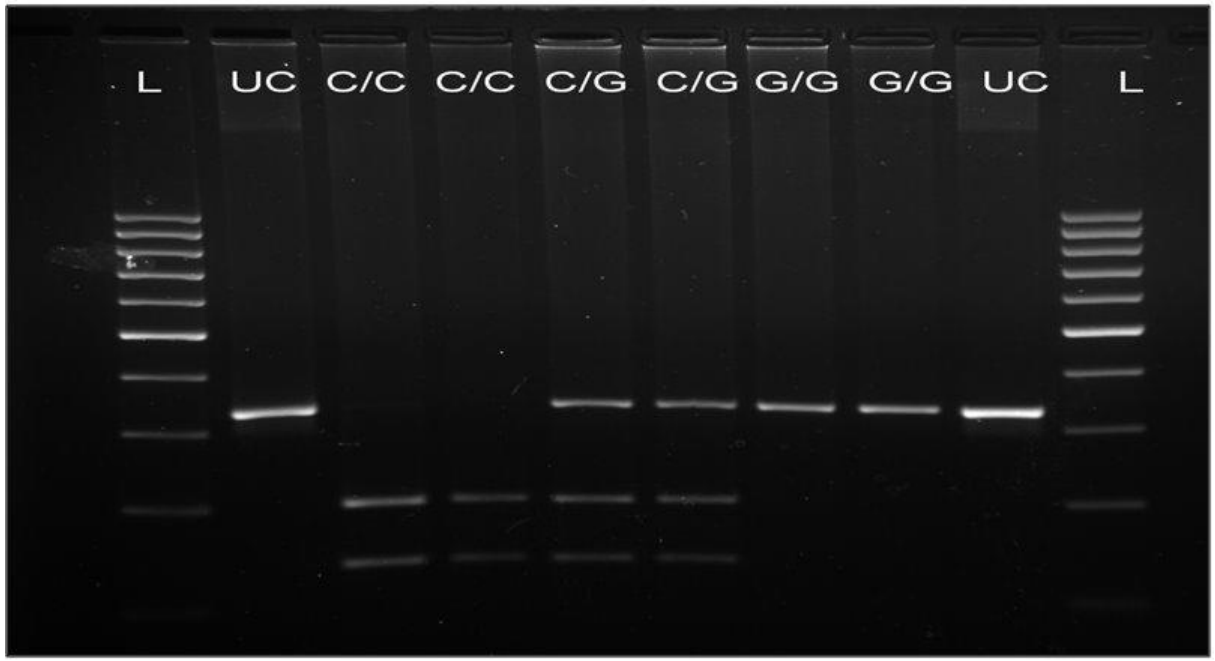
Illustrations

Illustration 1

Lanes 3 and 4 demonstrated 200 and 133 bp fragments, thus represented normal homozygotes. Lanes 5 and 6 demonstrated 333, 200 and 133 bp fragments, thus represented heterozygotes. Lanes 7 and 8 demonstrated 333 bp fragment, thus represented mutant homozygotes. L represents 100 bp DNA ladder and UC represents undigested 333 bp PCR product.

Figure 1
Genotyping for *PNPLA3 (C10109G) by PCR-RFLP*



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