Mutational screening in the LDLR gene among patients presenting familial hypercholesterolemia in the Southeast of Brazil

G.A. Molfetta1,2, D.L. Zanette1,3, J.E. Santos4 and W.A. Silva Jr.1,2,3

1Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
2Centro de Medicina Genômica, Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
3Instituto Nacional de Ciência e Tecnologia em Células-Tronco e Terapia Celular, Fundação HemoCentro de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
4Departamento de Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: W.A. Silva Jr.
E-mail: wilsonjr@usp.br

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ABSTRACT. Familial hypercholesterolemia (FH) is a dominant, autosomal disease characterized by high LDL levels in blood plasma, and is caused by a defect in the gene encoding the LDL receptor (LDLR). The clinical diagnosis is based on personal and familial history, physical examination findings, and measures of high LDL cholesterol concentrations. LDLR is a cell-surface glycoprotein that controls the level of blood plasma cholesterol and triglyceride by LDLR-mediated endocytosis. Here we sequenced the entire LDLR gene-coding region to screen for mutations in 32 patients diagnosed with FH, and we have found 20 mutations including synonymous, missense, and intronic
mutations. Six of them were characterized as pathogenic mutations (D178Y, C184Y, S326C, C681X, IVS7+10G>C, and IVS11-10G>A). We have also found one intronic mutation not described so far (IVS11-63C>A). Our study corroborates the broad spectrum of mutations distributed along the entire LDLR gene, and we suggest that the genes APOB and PCSK9 should also be screened for mutations when considering the diagnosis of FH. It is already known that different types of mutations are directly associated with the phenotype heterogeneity presented by patients. Considering that Brazilian population is highly admixed, it is important to determine the geographic spectrum of LDLR mutations to provide information on the prognosis and treatment of each FH patient.

Key words: Familial hypercholesterolemia; LDLR gene; DNA sequencing

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by i) elevations in the levels of plasma low-density lipoprotein (LDL) cholesterol; ii) tendon xanthomas, and iii) premature coronary heart disease (Hobbs et al., 1990). The LDR receptor (LDLR) is a cell-surface transmembrane protein that facilitates the uptake of plasma LDL primarily into hepatocytes, where it is further catabolized (Diakou et al., 2011). A genetic defect caused by mutations in the LDLR gene results in malfunctioning or insufficient numbers of LDLR molecules, which account for the reduced clearance of LDL molecules from the plasma via the receptor-mediated pathway (Diakou et al., 2011).

However, mutations in three other genes regulating sterol and lipoprotein pathways may lead to similar phenotypes: Apo B-100, PCSK9, and ARH (Raal and Santos, 2012). Although mutations in these other genes lead to an increase in cholesterol levels, changes in the LDLR gene has shown to be the main cause of FH (Goldstein and Brown, 2001).

FH is a common inherited disease with heterozygotic and homozygotic frequencies of 1/500 and 1/10^6, respectively (Varret et al., 1998). The early identification and treatment of FH probands and their affected relatives with effective lipid-lowering drugs are important as they reduce the risk of future coronary artery disease. Therefore, the molecular characterization of the LDLR gene appears to be a useful tool for the correct diagnosis of FH since it provides an unequivocal result.

The study of the molecular defects of the LDLR in Brazil was initiated by Figueiredo et al. (1992) and Alberto et al. (1999). These researchers identified the molecular basis of FH in 16 families. They found a family in which the defect was a deletion of 4 kb involving exons 13 and 14 of the LDLR gene. Also, they observed that 14 families were carriers of Lebanese allele, a nonsense mutation in exon 14 that results in the production of a truncated protein without functional activity. The Lebanese allele is the most common molecular defect identified in patients with FH in Brazil. Due to the great diversity of Brazilian population, the spectrum of mutations in the LDLR gene may be broad, and the molecular basis of the FH in Brazil has not been characterized so far. According to data from the World Health Organization (1999), it is estimated that about 320,000 individuals are heterozygous for FH in
Brazil. The aim of this study was to carry out the molecular characterization of patients with a clinical diagnosis of FH in Brazil and to describe the mutation heterogeneity.

PATIENTS AND METHODS

A total of 32 patients with a clinical diagnosis of FH from the Nutrition Unit of the Medical School of Ribeirão Preto, University of São Paulo, participated in the study and had signed for the consented blood taken at our Institution’s Ethics Committee.

DNA extraction and PCR

Genomic DNA was extracted from peripheral blood lymphocytes through the Wizard Genomic DNA Purification kit (Promega), according to the manufacturer’s instructions. The entire LDLR gene-coding region was amplified with the specific sense and anti-sense primers that flanked each intron/exon (Table S1). All PCRs were standardized with the following reactants for each reaction: 2.5 mM of each primer, 2.5 mM dNTPs, 2.5 μL 1X buffer (Biotools), 1 U Taq polymerase (Biotools); 200 ng genomic DNA, and 14 μL distilled water for a 25-μL final volume.

DNA sequencing

DNA fragments amplified by PCR were subjected to direct sequencing in an automatic capillary sequencing system ABI Prism 377 DNA Sequencer® (Life Technologies), using the ABI Prism Big Dye™ Terminator Cycle Sequencing Ready Reaction® kit, following the manufacturer’s instructions. The sequencing results were analyzed through FinchTV version 1.4.0 (Geospiza Inc., 2004-2006) and Polyphred (Geospiza Inc.) programs; the sequences obtained were compared to the reference from the GenBank (NM_000527).

RESULTS AND DISCUSSION

A total of 20 mutations (Tables 1 and 2) were identified among the 32 patients with clinical diagnosis of FH enrolled in this study. Of the 20 mutations, eight of them were found within the exon region (Table 1) and 12 were found within the intron region (Table 2). The most frequent mutation causing FH among our cohort was the putative splicing mutation IVS7+10G>C (42%), followed by the mutations C681X (9%), D178Y (4%), and S326C (2%) (Table 3).

<table>
<thead>
<tr>
<th>Exons</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>dbSNP ID</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>c.625G&gt;T</td>
<td>D178Y</td>
<td>-</td>
<td>Humphries et al., 2006</td>
</tr>
<tr>
<td>4</td>
<td>c.644G&gt;A</td>
<td>C184Y</td>
<td>rs121908039</td>
<td>Lee et al., 1998</td>
</tr>
<tr>
<td>7</td>
<td>c.1070C&gt;G</td>
<td>S326C</td>
<td>-</td>
<td>van de Kerkhof et al., 2003</td>
</tr>
<tr>
<td>12</td>
<td>c.1818C&gt;T</td>
<td>L575L</td>
<td>rs1799898</td>
<td>Boright et al., 1998</td>
</tr>
<tr>
<td>12</td>
<td>c.1866T&gt;C</td>
<td>N591N</td>
<td>rs688</td>
<td>Boright et al., 1998</td>
</tr>
<tr>
<td>13</td>
<td>c.2052C&gt;G</td>
<td>V653V</td>
<td>rs5925</td>
<td>Lentesdorf et al., 1989</td>
</tr>
<tr>
<td>14</td>
<td>c.2330C&gt;A</td>
<td>G681X</td>
<td>rs121908031</td>
<td>Lehman et al., 1997</td>
</tr>
<tr>
<td>15</td>
<td>c.2325G&gt;A</td>
<td>R744R</td>
<td>rs5927</td>
<td>Boright et al., 1998</td>
</tr>
</tbody>
</table>

Table 1. Mutations localized in the coding region of the LDLR gene.
The articles of Figueiredo et al. (1992) and Alberto et al. (1999) are considered the first studies that reported the molecular characterization of FH caused by a deficiency of the LDLR gene in Brazil. These studies reported two mutations as the cause of FH in their patients: a 4-kb deletion involving exons 13 and 14 of the LDLR gene and the mutation C681X that creates a stop codon and the production of a truncated protein or a protein partially translated. We have detected the same C681X mutation in 21% of our patients. Lehrman et al. (1987) described the mutation C681X as producing a shortened receptor protein lacking three domains: the region of clustered $\theta$-linked carbohydrates, the membrane-spanning region, and the cytoplasmic tail. The truncated protein retains only two domains: a whole ligand-binding region and a partial epidermal growth factor precursor homology region. The high frequency of this mutation among Lebanese and Syrian subjects may be attributed to a founder effect, and this mutation is also known as the Lebanese allele because of the high incidence among those populations (Lehrman et al., 1987).

The third higher frequency mutation was the D178Y causing FH in 8% of the patients. This mutation was reported by Humphries et al. (2006) as pathogenic, causing the FH with high concentrations of serum cholesterol, LDLC, HDLC, and triglyceride in addition to the typical clinical features of xanthoma, corneal arcus, and xanthelasma.

The mutation S326C was found in 3% of patients here evaluated and was already described as the cause of FH by van de Kerkhof et al. (2003). This mutation was detected for the first time in a Brazilian patient presenting two mutations: C681X and the mutation S326C, confirming the diagnosis of homozygous FH.

We identified mutations spread along the entire LDLR gene emphasizing the higher presence of mutations in intron 7 and exons 4 and 14. These results agree with some data reported in the literature where exons 4, 10, 13, and 14 were described to bear the majority of the mutations in the LDLR gene (Schmidt and Kostner, 2000; Humphries et al., 2006).

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Table 2. Mutations localized in the intron region of the LDLR gene.

<table>
<thead>
<tr>
<th>Introns</th>
<th>Mutation</th>
<th>dbSNP ID</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>IVS6+16G&gt;A</td>
<td>rs13306513</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>IVS11-63C&gt;A</td>
<td>-</td>
<td>Described in this study</td>
</tr>
<tr>
<td>12</td>
<td>IVS12-199G&gt;T</td>
<td>rs2569468</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>IVS16+48C&gt;T</td>
<td>rs13306501</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>IVS7+106G&gt;C</td>
<td>rs12710290</td>
<td>Dedoussis et al., 2004</td>
</tr>
<tr>
<td>11</td>
<td>IVS11-100G&gt;A</td>
<td>rs724852</td>
<td>Cenario et al., 1998</td>
</tr>
<tr>
<td>16</td>
<td>IVS16-74G&gt;A</td>
<td>rs2738466</td>
<td>Amsellem et al., 2002</td>
</tr>
<tr>
<td>16</td>
<td>IVS16-116G&gt;C</td>
<td>rs2304182</td>
<td>Amsellem et al., 2002</td>
</tr>
<tr>
<td>15</td>
<td>IVS15-53G&gt;A</td>
<td>rs6415503</td>
<td>Amsellem et al., 2002</td>
</tr>
<tr>
<td>17</td>
<td>IVS17-42G&gt;A</td>
<td>rs6415504</td>
<td>Amsellem et al., 2002</td>
</tr>
<tr>
<td>18</td>
<td>*5G&gt;A</td>
<td>rs141558</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. LDLR pathogenic mutations in Brazilian patients with FH.

<table>
<thead>
<tr>
<th>Region</th>
<th>Mutations*</th>
<th>Allele frequency</th>
<th>Patient frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon</td>
<td></td>
<td>Nucleotide position</td>
<td>Amino acid position</td>
</tr>
<tr>
<td>4</td>
<td>c.625G&gt;T</td>
<td>D178Y</td>
<td>Missense</td>
</tr>
<tr>
<td>7</td>
<td>c.1079C&gt;G</td>
<td>S326C</td>
<td>Missense</td>
</tr>
<tr>
<td>Intron</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>IVS7+106C&gt;T</td>
<td>-</td>
<td>Splicing</td>
</tr>
</tbody>
</table>
Regarding the mutations localized close to the splicing sites, mutations IVS7+10G>C and IVS11-10G>A are characterized by altering the mRNA processing donor and acceptor sites, respectively (Cenarro et al., 1998; Dedoussis et al., 2004). Mutation IVS7+10G>C was the most frequent cause of FH in our patients (59%) while mutation IVS11-10G>A presented the lowest frequency among our patients (3%).

Dedoussis et al. (2004) described the potential new splice site mutation IVS7+10G>C (c.1060+10G>C). Regarding the pathogenic effect of this splicing mutation, Amsellem et al. (2002) described the same position intron mutation c.1060+10G>A, which could disrupt splicing by its computer estimated potential to create aberrant donor splicing site in close vicinity to original site. These authors say that the IVS7+10 variant could be a minor variant partially reducing LDLR expression by the competition of the aberrant donor splice with the original donor site. Accordingly, heterozygous carriers of this variant expressed a mild phenotype of FH.

Despite these observations, to the best of our knowledge, the actual effect of these mutations on LDLR splicing was not confirmed so far. Here we speculate whether the same causing disease splicing mechanism could be observed by the mutation IVS7+10G>C as they are localized at the same position.

Regarding other synonymous mutations that we have found, Dementieva et al. (2012) have already reported that L575L and R744R in the LDLR gene did not show a significant relationship with coronary artery disease.

Furthermore, one intron mutation is reported for the first time in this study. Table 3 shows more details about the SNPs that were found among our sample of patients presenting FH. Figure 1 demonstrates all the 20 LDLR gene mutations detected in this study.

Figure 1. Schema showing the position of each mutation identified in this study. In regular font are the mutations already described in the literature and in black are the mutations described for the first time in our study.

Salazar et al. (2000) also defined the molecular basis of a group of FH patients living in the city of São Paulo. All of their patients are of European origin. They identified 15 mutations in the LDLR gene located in different exons. Of the 15 mutations, seven were described for the first time in a Brazilian population; five of them were missense mutations, and two of
them were frameshift mutations. Interestingly, none of the mutations described by Salazar et al. (2000) was identified among our patients. We can point out one reason for this result: we suggest that the most likely situation is that the groups are of different ethnical origin. We do not have the race self-information of our patients; on the other hand, we know that our cohort of patients is quite heterogenic as they come from various cities in the Northeast region of the state. Corroborating these findings, Komarova et al. (2013) have already demonstrated that each megapolis has its own FH mutation spectrum sharing only small part of mutations with other populations.

Based on our data and on the previous data from Figueiredo et al. (1992) and Alberto et al. (1999), we are encouraged to affirm that mutations IVS7+10G>C and C681X may be a cause of FH in the Southeast Brazil and should be at first investigated when considering the clinical hypothesis of FH in this region. Furthermore, if considering an expanded panel of mutations, six mutations (Table 3) should be screened for the FH diagnosis among patients in this same region.

If common LDLR mutations are known in a population, selected panels of mutations can be used as the first strategy. On the other hand, in a population where no founder effect was detected or in a population where we find a great genetic heterogeneity, the gold-standard approach remains on the full sequencing of the LDLR-coding region followed by the search of APOB and PCSK9 gene defects (Izar et al., 2010). A recent study using the next-generation sequencing (NGS) to screen for a mutation in the three genes LDLR, APOB, and PCSK9 say that the NGS methodology could provide one rapid route to increasing the present low percentage of FH cases with a genetic diagnosis (Norsworthy et al., 2014). Regarding the seven patients who showed no mutations, the presence of the FH may probably be associated with other genes as APOB and PCSK9, as mutations in these two other genes cause patients presenting a similar phenotype to the ones with mutations in LDLR (Austin et al., 2004; Slimani et al., 2012).

At least but not last, there is an influence of the presence and type of the LDLR mutation on the lipid profile and the response to lipid-lowering therapy (Santos et al., 2014). These observations are of fundamental importance to characterize the molecular basis of FH in Brazil and its treatment as it reveals that FH may be associated with different genes besides the LDLR gene, and targeted pharmacological treatment should be performed in patients carrying an LDLR mutation.

We can summarize that of the mutations already described as associated with FH, six were identified in our study: IVS7+10C>G, C681X, D178Y, S326C, C184Y, and IVS11-10G>A. Four of them are situated in the coding region, exon 4 (D178Y and C184Y), exon 7 (S326C), and exon 14 (C681X). The two remaining mutations are located in introns 7 (IVS7+10C>G) and 11 (IVS11-10G>A), and are associated with FH because they alter the processing of DNA. The most frequent among our patients was the IVS7+10C>G, representing 59% of patients followed by the mutation C681X representing 21% of the patients. We also describe that the genes APOB and PCSK9 should be screened together with the LDLR gene. The NGS is a good approach for the molecular diagnosis of FH as this disease is caused by a distinct set of genes involved in the phenotype and showing a high prevalence in the community.

The present study contributes to a better knowledge of the genetic basis of FH in Brazil. It is already known that different types of mutations are directly associated with the phenotype heterogeneity presented by patients. Considering that the Brazilian population is
highly admixed, it is important to determine the geographic spectrum of LDLR mutations to provide information on the prognosis and treatment of each patient with high cholesterol levels or under lowering cholesterol therapy.

**Conflicts of interest**

The authors declare no conflict of interest.

**REFERENCES**


**Supplementary material**

*Table S1.* List of primers used for sequencing the promoter and coding regions of the LDLR gene.