MOLECULAR BASIS OF FAMILIAL HYPERCHOLESTEROLEMIA: AN INDIAN EXPERIENCE

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by a multitude of low-density lipoprotein (LDL) receptor gene mutations. The LDL receptor is a cell surface trans-membrane protein that mediates the uptake & lysosomal degradation of plasma LDL, thereby providing cholesterol to cells. Affected individuals have elevated plasma levels of LDL, which causes premature coronary atherosclerosis. FH has an estimated worldwide prevalence of 0.2%. In some subpopulations there is an increased frequency of FH and specific LDL receptor mutations are found to be common due to 'founder gene effect'. Overall, more than 300 naturally occurring LDL receptor mutations have been described. To date upto ten LDL receptor gene mutations have been identified in Indians in South Africa, suggesting increased incidence of FH among Indians. Most mutations have occurred at CpG dinucleotide, a mutational hotspot in human genetic disease. In our study in 25 hypercholesterolemic subjects we have identified two novel insertion mutations in two patients. But the mutations underlying FH are still undefined in the majority of cases. Mutational heterogeneity on the other-hand has complicated disease diagnosis at DNA level. These findings warrant application of a generalized mutation screening method in search for new LDL receptor gene defects.

KEY WORDS: Familial hypercholesterolemia (FH), LDL receptor, Mutations, Atherosclerosis, DNA diagnosis.

Most diseases in adulthood are a result of complex interactions between genetic and environmental factors. Coronary heart disease (CHD) is one of the largest killers in the Western population. In India too, the prevalence of CHD is on the rise. Moreover, the disease is seen in the younger population, which is a cause of great concern. Thus it becomes imperative to identify the risk factors leading to premature heart diseases. The genetic evidence of CHD is rapidly emerging. Familial Hypercholesterolemia (FH), though not the only cause of CHD is certainly one of its major correctable causes. FH as a disease was first described in 1938 by Carl Muller as an "inborn error of metabolism"

that produces high blood-cholesterol levels and myocardial infarctions in young people. Goldstein & Brown began their work in 1972 in an attempt to understand the genetic basis of FH. A series of studies since 1973 led to the award of a Nobel Prize in 1985 to Brown and Goldstein for their discovery of LDL receptor and its exact role in FH (1). These receptors specifically bind LDL-cholesterol and carry it into the cell by receptor mediated endocytosis. FH occurs clinically in two forms: heterozygous and homozygous. FH heterozygotes, inherit one mutant LDL receptor allele, manifest a two-to-three fold elevation in plasma LDL-cholesterol and typically develop premature coronary heart disease after age 35. FH homozygotes, inherit two mutant LDL receptor alleles, are rare, have six to eight fold elevation in plasma concentrations of LDL-

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cholesterol and often die of myocardial infarctions during the first two decades of life. It is also seen that some phenotypic FH homozygotes inherit two identical mutant LDL receptor alleles (true homozygotes) while others inherit two different mutant alleles (compound heterozygotes) (2).

The LDL Receptor Pathway

The LDL receptor is a cell surface glycoprotein synthesized in the rough endoplasmic reticulum (ER) as a precursor that contains high-mannose N-linked carbohydrate chains and the core sugar (N-acetylgalactosamine) of the O-linked chains (3). The O-linked core sugars are added before the mannose residues of the N-linked chains are trimmed (while the receptor is still in the endoglycosidase H-sensitive stage) either in the ER or in the transitional zone between ER and Golgi apparatus. The receptor migrates during SDS-PAGE as a single band corresponding to an apparent MW of 120 kDa (3). Within 30 minutes after its synthesis, the mobility decreases and the apparent MW increases from 120 kDa to 160 kDa. This change is coincident with the conversion of the high-mannose N-linked oligosaccharide chains to the complex endoglycosidase H-resistant form. At the same time, each O-linked chain is elongated by the addition of one galactose and one/two sialic acid residues (3). About 45 minutes after synthesis, LDL receptor appears on the cell surface and gathers in the coated pits. Within 3-5 minutes of the formation, the coated pits invaginate to form coated endocytic vesicles. Very quickly, the clathrin coat dissociates, and multiple endocytic vesicles then fuse to create large sacs called endosomes or receptosomes. The pH of the endosomes falls below 6.5 because of the ATP-driven proton pumps in the membrane. At this pH, the LDL dissociates from the receptor and returns to the cell surface (1). Once it reaches the surface, the receptor binds another LDL particle and initiates another cycle of endocytosis.

The LDL Receptor Structure

The human LDL receptor complementary DNA (cDNA) and gene has been cloned and characterized (4, 5). The amino acid sequence that was deduced from the nucleotide sequence of the cDNA, has provided insight into the structural domains of the

LDL receptor. The human LDL receptor mRNA is 5.3 kilobases (kb) in length and encodes a protein of 860 amino acids (4). The LDL receptor gene, located on the distal short arm of chromosome 19 (p13.1 - p 13.3), spans 45 kb, and is comprised of 18 exons and 17 introns. There is a striking correlation between the exons in the gene and the functional domain of the protein (5). Exon 1 encodes the short 5' untranslated region plus the signal sequence of the protein. Exon 2-6 encodes the ligand-binding domain. Exon 7-14 encodes a region that shares sequence identity to the human EGF precursor gene. This domain contains three 40-amino acid, cysteine-rich growth factor repeats (designated A, B and C). The repeats A and B (encoded by exon 7 and 8) are separated from repeat C (encoded by exon 14) by five copies of a 40-60 amino acid repeat (encoded by exon 9-13). Each of these five repeats contain a conserved motif, Tyr-Trp-Thr-Asp (YWTD). Exon 15 encodes the O-linked chains of 58-amino acids. Exon 16 and the 5' end of exon 17 encode the membrane-spanning region. The remainder of exon 17 and 5' end of exon 18 encode the cytoplasmic domain. Transcription of the LDL receptor gene is regulated by feedback suppression mediated by the end product, intracellular cholesterol. The 5'-flanking region of the LDL receptor gene contains most, if not all, of the cis-acting DNA sequences responsible for the sterol-regulated expression of the gene in animal cells (6).

Genetic Defects In LDL Receptor

The mutations in the LDL receptor gene in FH patients have helped to delineate the crucial steps of receptor-mediated endocytosis. More than 150 mutations in the LDL receptor gene have been characterized at molecular level (7). Functionally, the LDL receptor mutations have been classified into 5 classes based on biosynthetic and functional studies of fibroblast cell strains derived from skin biopsy specimens.

Class 1 mutants include "null alleles" that fail to produce immunoprecipitable LDL receptor protein. The most frequent type of class 1 mutations are nonsense and frameshift mutations, and these are randomly distributed among the exons. e.g. FH French Canadian -1 (8), FH Denver - 1 (9).

Class 2 mutations are the most common at the LDL receptor locus (7). Class 2 alleles are the "transport-defective alleles" which encode proteins that are blocked, either completely (Class 2A) or partially (Class 2B) in transport between the ER and the Golgi apparatus. Most of the class 2 defects cluster in the exons that encode the ligand-binding domain and in the EGF precursor homology domain. e.g. FH French Canadian - 3 (10), FH Afrikaner-1 (FH Maine) (11). Mutations in the EGF precursor homology domain are mostly located in one of the three growth factor repeats (A, B and C). This is the most highly conserved domain in the receptor protein (12), and its structure is easily disrupted if only a single amino acid is changed. All missense mutations in the C repeat produce a class 2 phenotype. e.g. FH French Canadian - 2 (10), FH Lebanese (13) and FH Gujarat (14). The other half of Class 2 mutations in the EGF precursor homology domain are located in the spacer region between the B and C growth factor repeats, which contains the Tyr-Trp-Thr-Asp (YWTD) motifs. e.g. FH Cincinnati - 4 (7).

Class 3 mutants include the "binding-defective alleles" which encode proteins that are synthesized and transported to the cell surface, but fail to bind LDL. Most class 3 mutations are caused by in-frame insertions or deletions in the ligand binding or EGF precursor homology domains. e.g. FH French Canadian - 5 (10), FH Paris -1 (15).

Class 4 mutations encode receptors that move to the cell surface and bind LDL normally, but are unable to cluster in clathrin-coated pits and thus do not internalize LDL ("internalization-defective alleles"). The mutations have been divided into two groups based on whether the mutations involve the cytoplasmic domain alone [Class 4A] or together with adjacent membrane-spanning region [Class 4B] (16). The most informative class 4A mutation is the J. D. allele (FH Bari allele) in which single base-pair change substitutes a cysteine for tyrosine at position 807 in the cytoplasmic domain (17). The second subclass of internalization - defective alleles (Class 4B) produce truncated receptors that lack the membrane-spanning domain as well as the cytoplasmic tail. The deletion mutations - FH Rochester (18), FH Helsinki (19) have this phenotype.

Class 5 mutations encode "recycling-defective alleles" that fail to discharge the ligand in the endosome and thus do not recycle to the cell surface. The class 5 mutations cluster in the 5' end of the EGF precursor homology domain, especially in the first YWTD repeat (Tyr-Trp-Thr-Asp). This domain mediates the acid-dependent dissociation of the receptor and ligand in the endosome, an event that is essential for receptor recycling (20). If the ligand is not released from the receptor in the endosome, the receptor-ligand complex is degraded, and the receptor fails to be recycled to the cell surface. FH Afrikaner-2 (11), FH Algeria-1 (7) and FH Osaka-2 (21) have this phenotype.

Alu repeats are the predominant "middle repetitive" DNA sequences in the mammalian genome. Alu sequences are distributed throughout all chromosomes occurring mostly in intergenic region and introns, but occasionally in the 3'-untranslated regions of mRNAs as in the LDL receptor (6). The large number of Alu repeats in the human genomes, coupled with their capacity to be transcribed into RNA, has led to the idea that these sequences might serve as sites for genome rearrangements (22). This hypothesis has received its strongest support from the almost invariant finding of Alu sequence at the function of large rearrangements in the LDL receptor gene (16). e.g. FH Rochester (18), FH Osaka-2 (21), FH Paris -1 (15). Number of Alu sequences in this gene is twofold more frequent than the average region of the genome. This twofold increase may be significant. It seems likely that the number of rearrangements in a region of DNA may rise in geometric fashion with the number of Alu sequence, each of which may act as a hot spot for recombination (22). A few possible mutations result from a cytosine (C) to thymidine (T) transition at a CpG dinucleotide, a frequent cause of mutations in the human genome (23). At the LDL receptor locus, ~15 percent of the point mutations are C to T substitution at the CpG dimers.

In most populations of the world, the frequency of heterozygous FH is ~0.2%, and a plethora of different mutations are responsible for the disease. However, in certain populations of the world a small number of mutations predominate due to founder

effects. These populations include Ashkenazi Jews of Lithuanian descent, Afrikaners, French Canadians, Christian Lebanese, Druze, Sephardic Jews, and Finns (10, 11, 13, 19). In some of these populations, the frequency of FH is much higher than 0.2%.

Clinical Diagnosis

The clinical diagnosis of FH in the homozygote usually causes no difficulty. The finding of plasma cholesterol level exceeding 650 mg/dl in a non-jaundiced child is virtually pathognomonic (2). Diagnosis of heterozygous FH begins with the documentation of an elevated plasma level of LDL. In most cases the finding of hypercholesterolemia without hypertriglyceridemia is sufficient to establish that the LDL cholesterol level is elevated. Having documented an elevated LDL level, the physician must then determine whether the cause of the elevation is the heterozygous form of FH. This distinction is of crucial importance because most individuals with type 2 lipoprotein pattern (WHO classification of lipoproteinemia; Fredrickson type) have polygenic hypercholesterolemia, which is derived from a combination of environmental and multiple poorly understood genetic factors (24). Therefore, wherever possible, the criteria reported by the scientific steering committee of the Simon Broome Register Group (25), even described by Durrington (24) should be adopted for the diagnosis of FH. Definite FH is defined as, a) cholesterol level

above 260 mg/dl (6.7 mmol/l) in children under 16 or 290 mg/dl (7.5 mmol/l) in an adult or LDL level above 190 mg/dl (4.9 mmol/l) in adults PLUS, b) tendon xanthoma in patient or in first or second degree relative. Possible FH is defined as, a) cholesterol level above 260 mg/dl (6.7 mmol/l) in children under 16 or 290 mg/dl (7.5 mmol/l) in adults or LDL level above 190 mg/dl (4.9 mmol/l) in adults PLUS ONE OF (b) or (c); b) familial history of myocardial infarction below age of 50 in second degree relative or below age of 60 in first degree relative, c) family history of raised cholesterol levels above 290 mg/dl (7.5 mmol/l) in first or second-degree relative.

Diagnostic Relevance

In the French Canadian, Christian Lebanese, Finnish, Afrikaner, and Ashkenazi Jewish populations due to 'founder gene effect', it is now feasible to screen for FH at the DNA level and thereby to detect the mutation responsible for majority of FH.

In India to the best of our knowledge no studies on LDL receptor gene mutations have been published so far. A few mutations nevertheless, have been described among Indians in South Africa (14, 26, 27, 28) suggesting relatively high frequency of FH among Indians. The mutations described to date in FH patients in Indian immigrants of South Africa are listed in Table 1.

Table 1. Low-density lipoprotein receptor gene mutations identified in Indians with familial hypercholesterolemia in South Africa (27).

Name	N	Region of origin	Haplotype	Reference Nos.
M21L	1	Unknown	S-St+A+N+	28
D69Y*	1	Unknown	St+A+	26
E119K*	1	Unknown	St+A+	26
D200Y*	1	Unknown	S+St+A-N-	19
E207K*	2	Maharashtra	S-St+A+N+	10
C371X	1	Unknown	S-St+A+N+	28
N384K	1	Surat	S-St+A+N+	27
P664L*	9	Gujarat	St+A+N+	14
S765C	1	Unknown	S+St+A-N-	27

* Mutation at CpG dimers

N, Number of "unrelated" families with the mutation

S, restriction enzyme Sma I; St, Stu I; A, Ava II; N, Nco I

We screened 25 hypercholesterolemic patients with clinical features of FH for previously reported point mutations: W66G, E207K, E387K, and P664L in exons 3, 4, 9, and 14 of the LDL receptor gene by PCR-based restriction enzyme analysis and gel electrophoresis.

The W66G mutation is caused by T → G transversion at nucleotide 259 in exon 3. The amino acid substitution (Try → Gly) is located in the second ligand binding repeat and interferes with either the binding of receptor to its ligand (class 3) or the turnover of the LDL receptor protein whereby it gets degraded rapidly and thus fails to be recycled (class 5) (10). This mutation was originally identified among the French Canadians, designated as FH French Canadian – 4 and accounted for 7% of the French population.

The E207K mutation is caused by G → A transition at nucleotide 682 in exon 4. The E207K mutation results in the production of a LDL receptor protein of class 2 phenotype. The amino acid substitution (Glu → Lys) results in a change of basic for an acidic amino acid residue, which somehow interfere with the folding of the LDL receptor protein such that it is not transported out of the ER at a normal rate (10). The mutation was originally identified among the French Canadians, designated as FH French Canadian – 3 and accounts for 2% of their population. Recently, this mutation was identified in Asian immigrants from Maharashtra residing in South Africa (27). Two families were found to have this mutation, which have previously been identified in genetically distinct populations and/or on different chromosomal haplotypes (7, 10).

The E387K mutation is caused by G → A transition at nucleotide 1222 in exon 9. This missense mutation results in a class 5 phenotype which occurs in the EGF precursor homology domain, especially in the first YWTD repeat. This domain mediates acid-dependent dissociation of the receptor and ligand in the endosome. If the ligand is not released from the receptor in the endosome, the receptor - ligand complex is degraded and the receptor fails to be recycled to the cell surface (7,

29). This mutation was identified in an immigrant family from Gujarat residing in South Africa and characterized later (29).

The P664L mutation is caused by C → T transition at nucleotide position 2054 in exon 14. Proline 664 occurs in the growth factor – repeat C, a disulfide and proline rich region of the EGF-precursor homology domain of the LDL receptor protein. Due to the large number of proline residues, an amino acid that caused fixed kinks in polypeptide chains, it is likely that this region is of irregular structure and the difference in amino acid sequence between it and the other two growth factor like repeats A and B, which resemble more closely the sequence found in the EGF precursor itself, suggests that it is folded differently from them (5). The mutation of proline to leucine might be expected to have a marked effect on the folding of the protein as it would release the constraint on the flexibility of the chain caused by the rigidity of peptide bonds adjacent to proline which in turn could account for slow processing and decreased stability of the receptor (14). This mutation was originally identified in an FH subject, a Zambian who originated from Gujarat (30). Subsequently this mutation was identified in four families of the same racial and geographical origin (14). The frequent identification of this mutation in Indian immigrants of South Africa suggested that this mutation might be very common in this population.

In all the 25 DNA samples analyzed from heterogenous group of Indian hypercholesterolemic patients with clinical feature of FH and equal number of normolipemic controls, we were unable to detect the presence of these four known point mutations in the four different coding regions of the LDL receptor gene (31). The absence of the analyzed known point mutations thought to be common among Indian subjects therefore, suggested that the mutations causing hypercholesterolemia in these patients may be different than those included in the study or that they may occur in other coding regions of the LDL receptor gene. In order to further elucidate the genetic basis of FH, simplified heteroduplex analysis (HDA) was performed to detect other known or unknown

mutations in the exons 3, 4, 9 and 14 of the LDL receptor gene (32). Eventually, we detected 2 patients showing mutation in exon 3 and exon 4, which on automated sequencing revealed the presence of novel insertion mutations (33,34,35).

Recent study by Kotze et al (27) has indicated CpG dinucleotides as mutational hotspot among South African Indians. However, due to the absence of the transition mutations analyzed and the presence of two novel mutations, mutational heterogeneity among Indians is predicted. The molecular basis or bases for the remaining cases is (are) not known. The possibility that some of these patients may have familial defective apolipoprotein B-100 (FDB), a genetic disorder that is clinically indistinguishable from FH, associated with hypercholesterolemia and caused by mutations in the LDL receptor ligand, apolipoprotein B-100 gene (36) has not been explored. Mutation in this gene has been associated with FH in Caucasian population in Europe, including the French (37) and North Americans (36) with a frequency of 1% among FH individuals (38).

Molecular detection and identification of mutant LDL receptor alleles is important from several aspects. First, it provides the only definitive diagnosis of FH. Second, the correct estimation of the prevalence of FH in a specific population is only possible by screening for the actual molecular defects. Third, the exclusion or a diagnosis of FH in children based on plasma cholesterol levels carries the risk of mislabeling since mutant genes are not always fully expressed in this age group. Fourth, identification of common LDL receptor gene defects would facilitate development of rapid molecular diagnostic techniques. Fifth, characterization of the different mutant alleles and their intracellular trafficking are of major scientific interest and have important implications in other research fields. Lastly, a definitive molecular diagnosis of FH will be required prior to gene therapy, a treatment modality that may soon be available for this disease (39). Since studies among Indians settled in Africa suggests a high frequency of FH among Indian sub-groups, there exists a need to

look out for common mutations among different ethnic groups, which has remained culturally isolated over years. Identification of identical genetic defects would facilitate rapid molecular diagnosis and thus early therapeutic intervention. However, the mutational heterogeneity of the LDL receptor gene complicates disease diagnosis at the DNA level. Therefore, in the routine search for gene defects, it is essential to develop a strategy based on methods that are sensitive, reliable, easy, inexpensive and hence applicable to routine laboratory work. The Simplified HDA method applied in our study is found to be satisfactory and is being currently applied to a larger group of patients as a rapid initial method of screening for detecting known or unknown mutation in the LDL receptor-gene along with a combined heteroduplex-single strand conformation polymorphism (HDX-SSCP) technique described by Kotze et al (40). These findings thus warrant for an application of a generalized molecular screening technique to large group of patients to search for new LDL receptor gene defects.

The genes involved in hyperlipidemia have been the main targets for treatment and management of CHD. This review has emphasized the screening for LDL receptor gene mutations that causes hypercholesterolemia, which is certainly a major cause of premature CHD. However, in a multifactorial disease such as CHF, defects in single gene would not possibly account for all cases. We must therefore, recognize the potential of other newer candidate markers for CHD. In our laboratory we are involved in identification of, polymorphisms in the apo E gene (41) that influence the plasma lipid concentrations; mutations in the apo B-100 gene causing elevated plasma LDL levels; polymorphisms in the angiotensin converting enzyme (ACE) gene (42) and angiotensin II type I receptor (AT1R) gene (43); mutations in methylenetetrahydrofolate reductase (MTHFR) gene (44) and cystathionine β -synthase (CBS) gene that results in hyperhomocysteinemia, and plasma lipoprotein(a) or Lp(a) (41) a highly polymorphic lipoprotein that may function as a bridge between the fields of atherosclerosis and thrombosis. Such studies will have broader utility in the primary prevention of CHD.

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