Project Overview

Title: Study of miRNome to identify new molecular causes of Familial Hypercholesterolemia

Type of Research: Basic Research

Keywords: Familial Hypercholesterolemia, LDLR characterization, miRNome

Abstract:

**Background and rationale.** Familial Hypercholesterolemia (FH) is a severe dyslipidaemia consisting in dramatically increased levels of LDL and total cholesterol leading to premature atherosclerosis. FH is a monogenic disease caused by mutations in the genes encoding for the LDL receptor (LDLR) or in the gene encoding the only one apolipoprotein of LDL, the apolipoprotein B (APOB), or in the gene encoding a protease regulating LDLR levels on cell membrane (PCSK9). In around 20% of patients the genetic cause has not been identified. We hypothesize that the dyslipidemic profile could be caused by additional mechanisms including regulation of expression due to abnormal presence/sequence of regulatory miRNAs.

**Objectives.** We propose to characterize the LDLR status and to study the miRNome of patients without mutations in the traditionally associated genes. Aim 1: Functional characterization and expression of LDLR. Aim 2: Identification of miRNA alterations.

**Methods and research strategy.** Patients without mutations in LDLR, APOB, PCSK9 genes will be characterized assaying the functional activity and the expression (mRNA and protein) of LDLR. This characterization will allow to obtain different populations of patients with: 1. normal LDLR activity; 2. decreased activity with decreased expression of LDLR; 3. decreased activity with normal LDLR expression. The miRNome analysis will be performed in order to identify: 1. altered levels of miRNAs with respect to healthy controls and 2. variants in the miRNA’s sequence that could lead to altered binding to mRNAs. Results will be analysed in relation to the previous evaluation of activity/expression of LDLR, as example, miRNAs binding.

**Expected results.** Results will allow to identify new players in the pathogenesis of FH that will be useful to 1. identify new molecular marker of FH and improve the diagnosis; 2. identify new target to improve the therapeutic approach of affected patients.

Scientific Strategy

**Background**

Dyslipidemias are a heterogeneous group of diseases characterized by the alteration of the different lipid particles. Hyperlipidemias are the most clinically relevant dyslipidemias because the high levels of lipoproteins are associated with cardiovascular diseases (CVD). Autosomal dominant Familial Hypercholesterolemia (FH) is a severe dyslipidaemia consisting in dramatically increased levels of LDL and total cholesterol leading to tendon xanthomas, xanthelasma, corneal arcus, premature atherosclerosis and a dramatical increased of CVD prevalence (1, 2). FH is a monogenic disease caused by mutations in the gene encoding for the LDL receptor (LDLR) or in the gene encoding the only one apolipoprotein of LDL, the apolipoprotein B (APOB), or in the gene encoding a protease regulating LDLR levels on cell membrane (PCSK9). In around 20% of patients the genetic cause has not been identified (1) and the research is now focused on the identification of the molecular cause of FH, e.g., the hypothesis of causative genes not yet identified or polygenic base (3, 4).

Recent epidemiological studies revealed a possible prevalence of the disease at the heterozygote status of 1:200 and highlighted that a great portion of patients is still underdiagnosed (5).

Although the diagnosis can be made by a clinical and biochemical evaluation, genetic diagnosis is essential to confirm the diagnosis. By the genetic approach it is possible to verify the mutation status of patients and to distinguish between heterozygous, compound heterozygous or homozygous patients, the last two groups with worse clinical features than the first. The genetic identification of homozygous or compound heterozygous patients is essential to administrate a more powerful therapy, like plasma aphaeresis combined with lipid lowering drugs.
An early diagnosis allows an adequate therapy before the appearance of the disease first clinical signs preventing the cardiovascular outcomes, whereas most patients are often identified when an acute event happens (6). A cascade screening applied in the relatives of a genetically diagnosed patient allows the identification of young patients before the development of phenotypic alterations.

Epidemiological data suggest that cholesterol levels are largely variable among patients with a mutation at heterozygous status (7). In some case heterozygous patients even show cholesterol levels similar to unaffected subjects.

Current research on the identification of new genes indicates the complexity of FH genetics (3, 8) suggesting the need to search for other molecular mechanisms affecting the pathogenesis of this dyslipidaemia (9, 10). Since a modest decrease of LDLR activity could lead to FH, genetic mechanisms different from the presence of a mutation could be hypothesized.

Rationale

The genetic mechanisms underlying FH still have to be completely revealed. We hypothesize that the presence of a mutation could not be the only one cause of the disease and that the dyslipidemic profile could be caused by additional mechanisms including regulation of expression due abnormal presence of regulatory miRNAs or by unexpected pathways. Another possible mechanism is the presence of a mutation in DNA regions encoding for miRNAs leading to miRNAs with an increased ability to bind the mRNA of LDLR and of other proteins involved in its activity.

We propose an innovative approach to identify the pathways involved in FH using the study of the miRNome in patients without mutations in the traditionally associated genes. To date this analysis has never been performed in FH patients. The integrated study of miRNome in patients without mutations can help: 1. identification of variants in miRNA sequences that could alter their binding to target mRNA; and 2. the identification of new pathways involved in FH.

The identification of new molecular causes of FH is important to make possible the molecular diagnosis in a wide number of patients allowing to perform the cascade screening in the relatives of studied patients.

Objectives

Aim 1: Functional characterization and expression of LDLR

After a screening of FH-associated genes (LDLR, APOB, PCSK9) a subset of FH patients without mutations will be studied. These patients will be characterized assaying the functional activity and the expression (mRNA and protein) of LDLR. Since we observed that a modest decrease (around 20-30%) of LDLR activity causes the FH phenotype (11, 12), the functional characterization will be applied to evaluate in toto the complexity of the receptor activity including accessory or regulatory proteins.

The consecutive evaluation of LDLR expression at mRNA and protein levels will allow to obtain different populations of patients with: 1. normal LDLR activity; 2. decreased activity with decreased expression of LDLR; 3. decreased activity with normal LDLR expression.

Aim 2: Identification of miRNA alterations.

In a subset of 20 patients without mutations, the miRNome analysis will be performed in order to identify: 1) altered levels of miRNAs with respect to 10 healthy controls and 2) variants in the miRNA’s sequence that could lead to altered binding to mRNAs. Genes targeted by altered miRNAs will be predicted by bioinformatics tools and their expression will be studied.

Results will be analysed in relation to the previous evaluation of activity/expression of LDLR.

Preliminary Results

Patient's recruitment has been performed at the "Centro per l'Aterosclerosi e le Malattie Dismetaboliche", Dipartimento di Medicina Clinica e Chirurgia, Università degli Studi di Napoli Federico II. The clinical diagnosis of FH has been based on clinical criteria.

To date, samples from 400 FH patients have already been collected and genetically screened, indicating
around 20% of FH patients without mutations in LDLR (11), PCSK9 and in the exons of APOB gene encoding the binding region. Mutation screening has been performed by direct sequencing of the coding regions, exon-intron junctions and promoter of the genes associated with autosomal dominant FH (LDLR, APOB, PCSK9). MLPA has been used to detect large rearrangements of LDLR. Also 40 normolipidemic subjects genetically screened have been collected to perform comparisons of LDLR activity/expression and miRNA analysis.

As to functional characterization, we have developed an assay on T-lymphocytes and we have demonstrated that the complete separation of FH patients from controls can be achieved (12). We also set up the method for the quantification of LDLR amounts on lymphocyte surface by an antibody-based assay (13). Viable cells from FH patients to be studied have been stored in liquid nitrogen and can be used to perform functional studies and miRNA extraction.

Research Plan

**Experimental Plan Aim 1**

After the selection of a subset of FH without mutations in traditional genes, the first step of the project consists in the characterization of the LDLR status by quantifying its activity and its expression.

To this aim, the total LDLR activity will be evaluated by measuring binding and uptake of fluorescently labelled LDL (DiI-LDL) in ionomycin plus PMA stimulated T-lymphocytes isolated from patients or controls. For 48 hours before analysis, cells will be incubated in a medium with a lipoprotein deficient serum to up-regulate lipoprotein receptors, and will be incubated for 3 hours at 37°C with DiI-LDL. Fluorescence intensities will be measured with a FACSCanto (Becton Dickinson) flow cytometer. The receptor activity will be calculated as the ratio between the median fluorescence intensity of cells from patients and the median fluorescence intensity of control cells. This method allows to quantify the total LDLR activity, allowing to detect all possible defects in LDLR pathway, e. g. class 1 (synthesis), class 2 (expression in membrane), class 3 (binding), class 4 (endocytosis) and class 5 (recycle) defects.

Analysis of receptor expression will be evaluated through real time PCR on cDNA obtained by a reverse transcription step of total RNA extracted from peripheral cells of patients without mutations and with low receptor activity. Real time PCR will be performed using TaqMan methodology, using a probe spanning exon-exon junctions.

The levels of receptor proteins will be evaluated by flow cytometry using an antibody directed against LDLR incubated with patient's cells for 1h at 4°C. Cells will be then incubated with a secondary antibody and their fluorescence will be detected by flow cytometry. Results will be normalized for those obtained in healthy controls. With this method we will able to identify defects belonging to the classes 1 and 2 allowing to distinguish them from the other defect classes.

The dual evaluation of expression and activity of LDLR could help to better focus on the mechanism leading to hypercholesterolemia. Patients with low LDLR activity or expression will be studied through miRNome analysis. At the end of this characterization we will be able to identify different groups of patients: 1. normal LDLR activity (no defects in LDLR); 2. decreased activity with decreased expression of LDLR (defect in LDLR pathway due to decreased LDLR expression); 3. decreased activity with normal LDLR expression (defect in LDLR due to other regulatory/accessory proteins). Since the action of miRNAs can lead to degradation of mRNA or to its silencing, the contemporary evaluation of mRNA and protein is necessary to reveal action mechanism of miRNAs.

**Experimental Plan Aim 2**

The study of miRNAs will include the whole miRNome through the isolation of small RNAs and high throughput sequencing (miRNA-Seq) that allows to quantify miRNA levels and to identify variants in their sequence.

The extraction of small RNAs from peripheral leukocytes of previously characterized patients will be performed by mirVana miRNA Isolation Kit (Life Technologies). To study the miRNome performed, the TruSeq Small RNA Sample Preparation Kit (Agilent) will be used as capture method of small RNAs from total RNA and MiSeq system (Agilent) will be used for sequencing.

Data from patients will be compared with data from controls to identify 1. miRNAs with different levels and 2.
miRNAs with difference in nucleotide sequence. The miRNAs with different levels between patients and controls or with sequence variants will be further studied searching for their target genes and to search for relationship between these genes and LDLR metabolism.

The comparison of miRNA levels will be also performed among the 3 above-mentioned groups of patients identified by the functional/expression evaluation of LDLR. This comparison will allow to better identify miRNAs related to the different mechanism leading to the FH phenotype. Also the identification of relevant genes targeted by miRNAs with altered expression or carrying sequence variants will take into account the function and expression of LDLR.

The expression levels of the identified target genes involved in the lipid metabolism will be measured in patients and controls to reveal differences. The expression of target genes will be evaluated at RNA level by real time PCR and at protein level by western blot analysis.

IPA platform (Ingenuity® Systems Ltd.) will be used for the identification of functions and canonical pathways differing between several populations of patients/controls as well as to analyze the networks based on molecular interrelations of altered genes/proteins.

To confirm the role of identified miRNAs, in vitro experiments with transfected miRNAs will allow to detect the direct action of the miRNAs on the protein expression. To this aim hepatic cell lines will be transfected with a construct carrying a specific miRNA or its scrambled version and the expression of protein encoded by the targeted genes will be evaluated by western blot.

**Statistical analysis**

Statistical analysis, performed by PASW v. 18.0 (SPSS Inc.) and by MedCalc, will include:

- normality test to verify the distribution type of continuous variables
- comparison of mean values: T-test or ANOVA for parametric distributions; Mann-Whitney or Kruskal-Wallis for non parametric distributions
- comparison of frequencies for categorical data (Chi-square test)
- correlation analysis (Pearson or Spearman)
- multivariate linear regression and multivariate logistic regression.

**Expected outcomes**

This project aims to disclose genetic basis of FH, the most common genetic dyslipidemia, and solve some of their molecular mechanisms allowing to create new therapeutic strategies. After the exclusion of known gene defects, functional assays are essential to characterize status of LDLR focusing additional studies on them. We expect that functional characterization evaluating in toto LDLR could be a rapid method to identify defects in lipid metabolism without screening many different genes. We will be able to identify additional pathways by the study of miRNome in patients with a decreased expression/activity of lipoprotein receptors.

Altogether these results will allow to identify new players in the pathogenesis of FH that will be useful to 1. identify new molecular marker of FH and improve the diagnosis; 2. identify new target to improve the therapeutic approach of affected patients. The identification of new molecular marker is important to make possible the genetic diagnosis in a wide number of patients allowing to perform the cascade screening in the relatives of studied patients.

**Significance and Innovation**

This project is focused on the identification of molecular causes of FH, a dyslipidemia strictly causing cardiovascular diseases. Since the genetic diagnosis does not allow to identify mutations in all patients, we propose a combined approach of functional characterization and miRNome analysis in patients suffering from FH.

This study is very innovative because:

- this would be the first study of miRNome in FH patients;
- we propose the identification of molecular pathways by the combination of results from the two different approaches: functional characterization and miRNA study.
The knowledge of molecular causes of FH will improve the identification of new disease markers, allowing a better identification of affected patients allowing the early diagnosis also in their relatives which is useful to prevent cardiovascular complications. New pathways could be useful to develop innovative therapies directed against newly identified targets.

**Risk analysis, possible problems and solutions**

Possible critical aspects could be related to the high number of candidate genes/protein resulting from the pathway identification. The adopted study design can help in overcome this possible problem, in fact, the decision to study patients characterized in advance for the activity/expression of LDLR can help to address the possible involved pathways, e.g. if a decreased expression of LDLR is observed, a pathway involving transcription factors could be looked as a good candidate to be further investigated.

**Time planning**

Months 1-3: functional characterization and expression evaluation of LDLR in patients without mutations and in controls.

Months 4-6: miRNA sequencing experiments.

Months 7-8: analysis of miRNA sequencing data; identification of targeted genes and of relevant pathway.

Months 9-12: expression analysis of identified target genes; statistical analysis of results; write of scientific papers and reports.

**Cited Literature**

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