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Research paper

Functional characterization of two single nucleotide polymorphisms of acyl-coenzyme A:cholesterol acyltransferase 2

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ABSTRACT

Background: Acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) plays a critical role in the formation of cholesteryl esters from cholesterol and fatty acids, and is a potential target for treating hypercholesterolemia. We recently reported the significant effects of two human *ACAT2* gene polymorphisms, 41A>G ($Glu^{14}Gly$, rs9658625) and 734C>T ($Thr^{254}Ile$, rs2272296), on plasma lipid levels and coronary artery disease susceptibility in a case–control association study. In the present study, we evaluated the possible biological influence of the two polymorphism using two approaches.

Methods: In the first approach, the functional impact of the two polymorphisms was predicted *in-silico* using available web-based software, and in the second approach, the varying functions of the two polymorphisms were characterized in *in vitro* experiments, using ACAT2-deficient AC-29 cells.

Results: Our results show that the enzymatic activity of mutant $Glu^{14}Gly$ is approximately two times higher than wildtype, and that this increase is primarily due to the increased expression and/or stability of the mutant ACAT2 protein.

Conclusions: These results suggest that the genetic variation at $Glu^{14}Gly$ is functionally important and may contribute to ACAT2 protein expression and stability.

1. Introduction

Increased serum cholesterol level is indicative of hyperlipidemia and is one of the important risk factor for the development of atherosclerosis. Acyl CoA:cholesterol O-acyltransferase 2 (ACAT2) is an integral membrane enzyme that functions in the esterification of free cholesterol during cholesterol absorption in the enterocytes and in the secretion of apoB-containing lipoproteins in hepatocytes (Chang et al., 2009; Nguyen et al., 2012). The deletion of inhibition of intestinal or hepatic ACAT2 has consistently been shown to be atheroprotective in mouse models (Buhman et al., 2000; Willner et al., 2003; Lee et al., 2004; Bell et al., 2006, 2007; Zhang et al., 2012). Therefore, ACAT2 has been a pharmaceutical target for treatment of hypercholesterolemia (Rudel et al., 2005).

In a previous study, we identified genetic variations in ACAT2 and explored the effects of the polymorphisms on plasma lipid levels and coronary artery disease (CAD) susceptibility among the Singaporean population (He et al., 2005). The $Glu^{14}Gly$(41A>G, rs9658625) and $Thr^{254}Ile$(734C>T, rs2272296) single nucleotide polymorphisms (SNPs) were found to have significant associations with lipid levels and CAD risk (He et al., 2005). In the current study, we further evaluated the possible biological influence of the two polymorphisms through two approaches. In the first approach, the functional impact of the two polymorphisms was predicted *in-silico* using available web-based software. In the second approach, the varying functions of the two polymorphisms were characterized in *in vitro* experiments, using ACAT2-deficient AC-29 cells (Cadigan et al., 1988).

Abbreviations: ACAT2, acyl-coenzyme A:cholesterol acyltransferase 2; SNP, single nucleotide polymorphism; CAD, coronary artery disease; nsSNPs, non-synonymous polymorphisms; PSIC, position-specific-independent count; CHO, Chinese hamster ovary; FBS, fetal bovine serum; DIC, differential infraction contrast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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2. Materials and methods

2.1. Prediction of functional impact of the two polymorphisms: in-silico web-based software

The *in-silico* investigation of function was based on a variety of algorithms, including substitution matrices, multiple sequence alignments, and PolyPhen, as listed below, to predict the possible effects of the two non-synonymous polymorphisms (nsSNPs).

Two substitution matrix scores, GRANTHAM (Grantham, 1974) and BLOSUM62 (Henikoff and Henikoff, 1992), were used to assess the impact of amino acid changes and their potential deleterious effects on protein function and stability. GRANTHAM matrix is based on the formula that identifies the chemical factors, individually correlating with evolutionary exchangeability of protein residues in order to establish the difference in amino acids while the BLOSUM62 substitution matrix is based on amino acid pairs in blocks of aligned protein segments.

Variations involving the same amino acid are given the same weight, irrespective of their positions in the protein. Amino acid changes with GRANTHAM scores of more than 100 are considered radical changes and are increasingly associated with the disease (Balasubramanian et al., 2005). BLOSUM62 matrix defines amino acid substitution as conservative or non-conservative changes. Conservative changes are those that have a positive or neutral value in the matrix, whereas non-conservative changes are those that have a negative score. An amino acid change with a value less than −1 is indicative of a disease-causing mutation.

Multiple sequence alignment was used to identify conserved amino acid positions. Five mammalian ACAT2 protein sequences from human (NP003569), monkey (O77759), mouse (NP666176), rat (NP714950), and dog (XP543637) were selected for alignment using CLUSTAL W version 1.82. All orthologs were at least 65% identical to the human ACAT2 protein sequence.

To evaluate the evolutionary conservation of these two polymorphic sites, a position-specific phylogenetic approach, Polymorphism Phenotyping (PolyPhen) (http://genetics.bwh.harvard.edu/pph/), was employed to identify functionally important nsSNPs. PolyPhen is based on the analysis of profile scores and various structural parameters and is designed to discriminate between disease-causing mutations and neutral substitutions (Sunyaev et al., 2001). The profile score, known as position-specific-independent count (PSIC), is a logarithmic ratio of the probability of a given amino acid occurring at a particular site to the probability of this amino acid occurring at any site (background frequency). A large PSIC difference between allelic variants indicates that the substitution is rarely or never observed in the protein family and probably affects protein function (Sunyaev et al., 2001). A variant is predicted to be damaging if the PSIC is more than 1.5 (Ramensky et al., 2002).

We used I-TASSER to predict the tertiary structures of wildtype and mutant ACAT2 (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al., 2010). The accuracies of the predicted models were assessed by the confidence score (C-score) and template score (TM-score) (Roy et al., 2010; Yang et al., 2014).

2.2. Characterization of functional variation: in vitro, using ACAT2-deficient AC-29 cells

The *in vitro* functional analysis was performed by measuring the expression levels of the various polymorphic isoforms of ACAT2 and their relative enzymatic activities.

2.2.1. Cell line, vector, and reagents

The AC-29 cell line, an ACAT-deficient CHO cell mutant (Liang et al., 2004), was kindly provided by Dr TY Chang's lab (Department of Biochemistry, Dartmouth Medical School, USA). The vectors, pRS426GP and pCR3.1/*ACAT2*, were obtained from Dr Yang (Department of Biochemistry, National University of Singapore). The pcDNA3.1/His expression vector containing the Xpress epitope, monoclonal antibody against Xpress, Geneticin (G-418 sulfate), Ham's F-12, DMEM/F-12 medium, and fetal bovine serum (FBS) were purchased from Invitrogen (California, US). Antipain, chymostatin, leupeptin, FBS, and Nile red, were purchased from Sigma (St. Louis, US). The [9,10(n)-^{3}H] oleic acid and anti-mouse Ig were from Amersham Biosciences (Hillerød, Denmark).

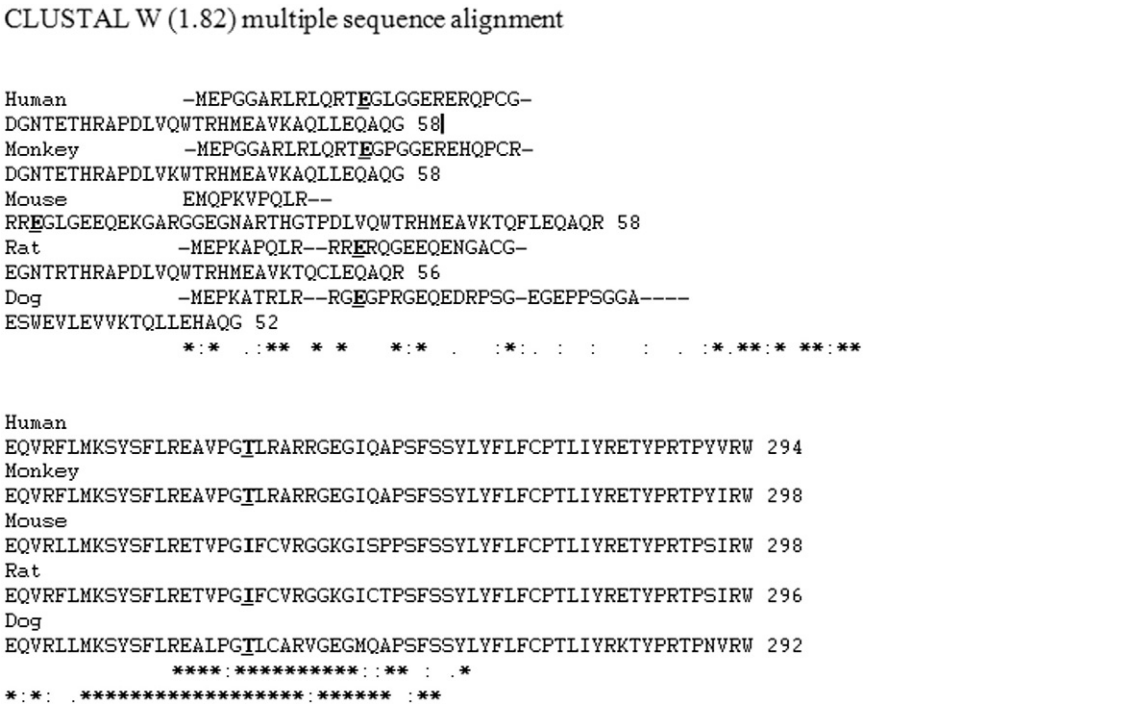

Fig. 1. Multiple alignments of ACAT2 protein orthologs from *Homo sapiens* (NP003569), Africa green monkey (O77759), *Mus musculus* (NP666176), *Rattus norvegicus* (NP714950) and *Canis familiaris* (XP543637) using CLUSTAL W ver.1.82. Bold underlined letters are non-synonymous SNPs studied in the current study (E: Gly^{14}Glu; T: Thr^{254}Ile). "*": identical residues; ":": conserved residues; ".": semi-conserved.