Possible association of *ABCB1*:c.3435T>C polymorphism with high-density-lipoprotein-cholesterol response to statin treatment - a pilot study

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Abstract

The gene product ABCB1 (formerly MDR1 or P-glycoprotein) is hypothesized to be involved in cholesterol cellular trafficking, redistribution and intestinal re-absorption. Carriers of the *ABCB1*:3435T allele have previously been associated with decreases in ABCB1 mRNA and protein concentrations and have been correlated with changes in serum lipid concentrations. The aim of this study was to investigate possible association between the *ABCB1*:3435T>C polymorphism and changes in lipids in patients following statin treatment. Outpatients (n=130) were examined: 43 men (33%), 87 women (67%): treated with atorvastatin or simvastatin (all patients with equivalent dose of 20 or 40 mg/d simvastatin). Blood was taken for *ABCB1*:3435T>C genotyping, and before and after statin treatment for lipid concentration determination (total cholesterol, high-density-lipoprotein-cholesterol (HDL-C), triglycerides). Changes (D) in lipid parameters, calculated as differences between measurements before and after treatment, were analyzed with multiple regression adjustments: gender, diabetes, age, body mass index, equivalent statin dose, length of treatment. Univariate and multivariate analyses showed significant differences in DHDL-C (univariate p=0.029; multivariate p=0.036) and %DHDL-C (univariate p=0.021; multivariate p=0.023) between patients with TT (-0.05 ± 0.13 g/l; -6.8% ± 20%; respectively) and CC+CT genotypes (0.004 ± 0.15 g/l; 4.1 ± 26%; respectively). Reduction of HDL-C in homozygous *ABCB1*:3435TT patients suggests this genotype could be associated with a reduction in the benefits of statin treatment.

KEY WORDS: ABCB1 transporter, HMG-CoA reductase inhibitors, P-glycoprotein

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INTRODUCTION

Statins (HMG-CoA reductase inhibitors) result in lower LDL-Cholesterol (LDL-C) and TriGlycerides (TG) and are effective at reducing atherosclerosis and cardiovascular risks in clinical practice [1-3] and outpatient care [4-6]. Statin responses vary, however, with polymorphisms in, for example, the *ABCB1* gene [7-10]. ABCB1 (formerly: multidrug resistance gene MDR1; protein: P-glycoprotein) is involved in cellular drug excretion [11], but ABCB1 has other functions: cholesterol redistribution [12]; intestinal cholesterol re-absorption [13]; regulation of cholesterol cellular trafficking; and cholesterol redistribution in cholesterol-rich microdomains of the cell membrane [14,15].

Although only one single nucleotide polymorphism, *ABCB1*:c.3435T>C (rs1045642; exon 26; Ile1145Ile), is analyzed in the present study, this has been the subject of considerable research. The T allele of this synonymous polymorphism has previously been found to be associated with decreased mRNA and protein concentrations of ABCB1 [16,17]. The c.3435T>C polymorphism changes substrate specificity [15,18], and the c.3435T allele results in reduced ABCB1 expression in mononuclear cells in response to lipopolysaccharide-induced inflammation [19]. Additionally, c.3435T>C has been associated with altered responses to Cyclosporine A in liver-transplant recipients [20], prediction of immune recovery after initiation of retroviral treatment [8], and imatinib response in chronic myeloid leukemia [21]. Note that some studies did not control for multiple testing (see Table 4 in [22]) and so

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should be viewed with caution - but a notable exception is the study by Hung et al. [23]. Hung et al. studied multiple drug resistance in epileptic patients [23], and found that certain haplotypes which included c.3435T>C conferred drug resistance. Lastly, c.3435T>C has been associated with changes in the pharmacokinetics of the acid forms of Simvastatin and Atorvastatin [24].

The unknown mechanism, by which this synonymous SNP might cause the above-mentioned changes, or whether this is due to linkage to another SNP, has been debated. However, it has been suggested that c.3435T>C and 2677G.A/T contribute independently to gene expression (using quantitative trait loci; [25]), and that the mechanism of action of c.3435T>C might involve changes in ribosome stalling, which can change protein structure and function by altering protein folding [26]. Recently it has been found, in stable epithelial monolayers, that "silent" polymorphisms including c.3435T>C result in changes to ABCB1 folding, resulting in longer recycling times, and significantly change ABCB1 function with changes in response to P-gp inhibitors (that normally, for example, block efflux of rhodamine 123 or mitoxantrone).

It should also be noted that gender differences in lipid responses to statins, with associations with haplotypes which include *ABCB1*:3435T>C, have been previously suggested, most notably by Becker et al. [27], Rodrigues et al. [28], and Kajinami et al. [28], but the cause of such different lipid/haplotype effects at present is far from clear.

The aim of this study was to assess the relationship between *ABCB1*:3435T>C gene polymorphism and the effectiveness of statin lipid-lowering treatments in out-patients from Western Pomerania, using multivariate analysis with adjustments which included gender.

MATERIALS AND METHODS

Patients

Outpatients (n=130) were examined: 43 men (33%), 84 women (67%): treated orally with atorvastatin (10-20 mg = approximately 0.14 to 0.28 mg/kg body mass of calcium salt per day) or simvastatin (20-40 mg = approximately 0.28 to 0.56 mg/kg body mass of free form per day). Note that equivalent dose ranges for the two drugs were the same, (equivalent dose 20 mg/d (65 patients) or 40 mg/d (65 patients)) because Atorvastatin has twice the effect of simvastatin as shown by Jones et al. [30]. All patients gave informed, written consent to participate in the study, which was approved by the Bioethics Committee at the Pomeranian Medical University, Szczecin, Poland.

Inclusion criteria: age >18 years old and the presence of a lipid disorder. Data obtained: body mass, height, body mass index (BMI; BMI = body mass (kg)/(height (m))2), presence of concomitant diseases such as diabetes, hypertension, ischemic heart disease (note cardiology patients were subscribed beta-blockers, diuretics, angiotensin-converting-enzyme inhibitors, calcium channel blockers, nitrate, which are substrates/inhibitors of ABCB1, as discussed later). Exclusion criteria: thyroid disease (hyperthyroidism or hypothyroidism), smoking, or if, after extensive interview, patients had not complied fully with instructions, including a diet low in fat.

Procedures

Arm vein blood samples (5 ml) were taken twice for determination of lipid concentrations, before (time 1), and after (time 2) statin treatment: total-Cholesterol (Ch); High-Density Lipoprotein-Cholesterol (HDL-C); TriGlycerides (TG). DNA isolation and genotyping were carried out from blood taken at time 1.

Biochemistry

Blood samples (3 ml) were analyzed using a Pentra 400 (Horiba Medical, Montpellier, France): on-board assays were carried out according to the manufacturer's instructions (published online 30.06.2010): Triglycerides: "ABX Pentra Triglycerides CP" (document 103a00272ken). Total-Cholesterol: "ABX Pentra Cholesterol CP" (a93a00142ken). HDL-C: "ABX Pentra HDL Direct CP" (a93a00152len). (HDL-C sampling was omitted from 4 males and 6 females).

Lipid parameters before (time 1) and after statin treatment (time 2) were measured; absolute (D = time 2 - time 1) or percentage (% = 100*(time 2 - time 1)/time 1) differences were calculated: Cholesterol: Ch1, Ch2, DCh, DCh%; High-Density-Lipoprotein-Cholesterol: HDL-C1, HDL-C2, DHDL-C, DHDL-C%; Triglycerides: TG1, TG2, DTG, DTG%.

DNA Isolation

Genomic DNA was extracted from K_3EDTA-anticoagulated blood (0.15 ml) using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany); polymerase chain reaction used primers flanking the NM_000927.4:c.3435T>C (rs1045642) polymorphic region of ABCB1: 5'-TGTTTTCAGCTGCT TGATGG-3' sense, 5'-AAGGCATGTATGTTGGCCTC-3' antisense primers (TIB Molbiol, Berlin, Germany), yielding a 197 bp product; used 10 microliter total volume: 20 ng template DNA, 4 pM each primer, 1x PCR Master Mix (MBI Fermentas, Vilnius, Lithuania); initial denaturation 94°C, 5 min; then 36 cycles: denaturation 94°C, 20 s, annealing 59°C, 40 s, extension 72°C, 40 s; final extension 72°C, 8 min 40 s; using a Mastercycler gradient thermocycler (Eppendorf, Poznan, Poland). For restriction, product (8.5 microliter) was incubated (37°C, 12 h) with 5 U *Mbo*I (MBI Fermentas); products were separated using 3% agarose gel electrophoresis with ethidium

bromide, photographed with ultraviolet light. If present, 3435C allele was cleaved giving: 158 bp and 39 bp fragments; 3435T allele was not cleaved: 197 bp.

Statistical Analysis

Divergence from Hardy-Weinberg equilibrium was tested using a chi-squared test. Parameters were compared between genotype groups using Kruskal-Wallis or Mann-Whitney tests (numerical data) or chi-squared test (qualitative data). Wilcoxon signed-rank test was used to assess significance of differences between two measurements. Multivariate analysis using General Linear Model (GLM) was performed with adjustments for gender, diabetes, age, BMI, equivalent dose and logarithmically transformed length of treatment. All statistical analyses were performed using Statistica (version 10, StatSoft Inc., Tulsa, Oklahoma, USA). Critical significance level was set at $p = 0.05$.

RESULTS

The genotype distribution of *ABCB1*:3435T>C conformed to Hardy-Weinberg equilibrium. Clinical/statin treatment characteristics are given in Table 1.

Univariate analysis of lipid parameters gave T recessive (i.e. CC+CT vs. TT) significant genotype differences for HDL-C1, DHDL-C and DHDL-C%. While on average patients with CC+CT genotypes showed a percentage increase in HDL-C after statin treatment (+3.8%, calculated from CC and CT data together: +0.4%, CC; +5.5%, CT; Table 2), patients with genotype TT showed an average percentage decrease (-6.8%; Table 2). In the multivariate analysis with adjustments for gender, diabetes, age, BMI, equivalent dose and length of treatment this result remained significant: DHDL-C ($p=0.036$), DHDL-C% ($p=0.023$). High BMI was the second independent factor associated with HDL-C decrease during treatment. In univariate analysis HDL-C1 was significantly higher in TT than in CC+CT patients but this difference lost significance in multivariate analysis, where only male gender and presence of diabetes were independent factors associated with lower baseline HDL-C.

Low-density-lipoprotein-cholesterol concentrations were estimated using the Friedewald formula [31], but no significant associations with *ABCB1* genotype were found (data not shown). This means that the associations between *ABCB1* genotype and Ch2, DCh and DCh% (significant only by multivariate analysis) might well be connected to associations between the *ABCB1* genotype and HDL-C mentioned above, and are not discussed further. No significant differences were found with triglyceride concentrations.

DISCUSSION

Statins usually increase mean HDL-C concentrations very slightly: for example in the Collaborative Atorvastatin Diabetes Study (CARDS) study (2838 randomized patients; 68% men; 40 to 75 years of age; one year of treatment), an increase in mean HDL-C of 1.6% was found [32]. It is speculated, however, that this increase in HDL-C depends on initial concentrations, and with low initial HDL-C concentrations (<4 g/l), the mean increase in HDL-C due to statin treatment was found to range from 4.8% to 16% [33]. If baseline HDL-C concentrations of individuals were higher the increase was found to be less e.g. 1-2% or non-significant (from four studies listed in [33]). (Note that Ballantyne et al. [34] reported a net decrease in ApoA-I of 1.4% following 18 to 24 weeks of Atorvastatin treatment.) In line with the latter studies, in our study mean HDL-C baseline concentrations were >4 g/l for all groups and, overall, no significant change in HDL-C following statin treatment was found (-0.013 g/L; Table 2; Wilcoxon signed-rank test: $p=0.27$).

In those studies in which a slight increase has been found in average HDL-C concentrations, it has been hypothesized that this possibly involves a reduced rate of cholesteryl-ester transfer protein (CETP) activity and therefore reduced flow of cholesterol from HDL, and also an increased formation of

TABLE 1. Clinical characteristics of patients stratified according to *ABCB1*:3435T>C genotype.

Characteristic	[C];[C] (n=25)	[C];[T] (n=65)	[T];[T] (n=40)	All individuals (n=130)	p values*:
	Mean value (s.d. or range)				Kruskal-Wallis test
Age (y)	63.5 (10.7)	65.8 (8.9)	65.6 (8.9)	65.3 (9.2)	0.72
BMI (kg/m^2)	27.4 (4.4)	27.9 (4.2)	26.8 (4.2)	27.5 (4.2)	0.38
Duration of treatment (months)	19 (3 to 74)	15 (2 to 83)	11 (3 to 49)	14 (2 to 83)	0.28
	Number (percentage)				χ^2 test
Coronary heart disease	6 (24)	18 (28)	7 (18)	31 (24)	0.49
Arterial hypertension	20 (80)	53 (82)	27 (68)	100 (77)	0.23
Diabetes	2 (8)	17 (26)	3 (8)	22 (17)	**0.019**
Patients using Simvastatin rather than Atorvastatin	10 (40)	33 (51)	24 (60)	67 (52)	0.29
Patients using higher (40 mg/d) simvastatin equivalent dose	17 (68)	32 (49)	16 (40)	65 (50)	0.088

*Significant difference ($p < 0.05$) in bold.

TABLE 2. Lipid parameters in patients according to *ABCB1*:3435T>C genotype.

Measurement	Time code	Lipid concentration, g/l or % (mean (s.d.)) Genotype [C];[C] (n=25)	[C];[T] (n=65)	[T];[T] (n=40)	All (n=130)	$P_{RE}1$ CC+CT vs. TT unadjusted	$P_{DO}1$ TT+CT vs. CC unadjusted	$P_{RE}2$ CC+CT vs. TT adjusted	$P_{DO}2$ TT+CT vs. CC adjusted
Ch, total Cholesterol	1	2.57 (0.4)	2.58 (0.44)	2.67 (0.44)	2.61 (0.43)	0.324	0.941	0.499	0.206
	2	1.81 (0.27)	1.84 (0.42)	1.74 (0.24)	1.80 (0.35)	0.340	0.455	**0.029**	0.973
	D	-0.76 (0.39)	-0.74 (0.47)	-0.93 (0.46)	-0.80 (0.46)	0.099	0.784	**0.026**	0.280
	%D	-28 (13)	-28 (16)	-33 (12)	-30 (14)	0.096	0.553	**0.026**	0.364
TG, Triglycerides	1	1.63 (0.64)	1.58 (0.74)	1.61 (0.85)	1.60 (0.75)	0.685	0.434	0.570	0.920
	2	1.35 (0.74)	1.29 (0.53)	1.18 (0.44)	1.27 (0.55)	0.397	0.988	0.233	0.567
	D	-0.28 (0.72)	-0.28 (0.78)	-0.43 (0.83)	-0.33 (0.78)	0.383	0.862	0.161	0.612
	%D	-13 (40)	-7 (45)	-14 (46)	-10 (44)	0.359	0.743	0.413	0.730
HDL-C, High-Density Lipoprotein-Cholesterol	1	0.58 (0.16)	0.56 (0.14)	0.62 (0.13)	0.58 (0.14)	**0.039**	0.718	0.223	0.907
	2	0.57 (0.14)	0.57 (0.15)	0.57 (0.13)	0.57 (0.14)	0.986	0.911	0.313	0.875
	D	-0.01 (0.12)	0.01 (0.16)	-0.05 (0.13)	-0.01 (0.14)	**0.029**	0.618	**0.036**	0.967
	%D	+0.4 (19)	+5.5 (29)	-6.8 (20)	+0.7 (30)	**0.021**	0.561	**0.023**	0.888

Time code: 1, before, and 2, after, statin treatment; D, absolute change between times 1 and 2; %D, percentage change.
$P_{RE}1$, $P_{DO}1$: unadjusted p values for T recessive (RE) and T (DO) dominant modes of inheritance. Significant differences ($p < 0.05$, Mann-Whitney test) in bold.
$P_{RE}2$, $P_{DO}2$: adjusted p values for T recessive (RE) and T dominant (DO) modes of inheritance. General linear model was used with the fowllowing 7 independent variables: ABCB1:3435T>C genotype, gender, diabetes, age, body mass index, equivalent dose and logarithmically transformed length of treatment. Significant differences ($p < 0.05$) in bold.

HDL precursor particles (i.e. hepatic synthesis of apoA-I) [33].

The results of our study showed, however, that changes in HDL-C concentrations following statin treatment was genotype dependent. Patients with CC or CT genotypes showed no significant changes (p=0.70), whereas patients with TT genotype showed on average an 7% decrease in HLD-C concentrations (p=0.017).

The protein ABCB1 is known to be a multi-drug efflux protein, and therefore one might expect that its role with statins might be to reduce statin effects by increasing efflux of statins from cells. However, it is known that low ABCB1 expression in peripheral mononuclear cells correlates with a lower LDL-C response to statins [15] i.e. exactly the opposite. We can suppose that other physiological roles for ABCB1 are of importance in relation to lipid metabolism responses to statins, and Mizutani et al. [35] summarize possible roles including: cholesterol esterification, possibly by sphingomyelin translocation regulation i.e. that increased ABCB1 expression is likely to increase cholesterol esterification.

As mentioned earlier, the *ABCB1*:3435T allele is associated with decreased ABCB1 mRNA and protein concentrations [17], and additionally the T allele (as part of a haplotype) is associated with greater AUC (area under the curve) for both statins and a longer half-life for Atorvastatin [24] which would reduce ABCB1 expression further [15]. We can therefore speculate that these mechanisms result in a fall in HDL-C concentrations to levels at which the opposing statin CETP reduction (which tends to conserve HDL-C) has little effect.

As on average HDL-C concentrations fell by 7% in TT patients, this might indicate reduced benefits of this type of treatment for these patients. This is especially true considering that *ABCB1* haplotypes including 3435T>C have been associated with risk for coronary artery disease [15], and that a reduction in risk of death of 0.8% has been found for every 1% rise in HDL-C (Scandinavian Simvastatin Survival Study [36]). Additionally, increased risk of recurrent stroke or death in ischemic stroke patients on atorvastatin therapy was recently found to be less likely with a CC genotype [37].

Limitations of this pilot study should be considered: (1) small sample size; (2) inclusion of patients taking medications for diabetes or coronary heart disease e.g. angiotensin-converting-enzyme inhibitors, beta-blockers and diuretics are substrates for ABCB1; calcium channel blockers are inhibitors of ABCB1 [14]. (3) Inclusion of two types of statin: Atorvastatin and Simvastatin and dose 20 or 40 dose equivalents.

CONCLUSION

Despite the limitations this study provides further evidence that the effects of statins are dependent on *ABCB1*:3435T>C genotype and these effects should be further investigated as TT homozygote patients might have reduced benefits from statin treatment. Additionally, if these results are confirmed this might provide further evidence that ABCB1 is directly involved in cholesterol metabolism.

DECLARATION OF INTEREST

The authors state that there are no conflicts of interest.

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