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No association of two functional polymorphisms in human ALOX15 with myocardial infarction

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1. Introduction

A wealth of information emerged about the involvement of the 12/15-lipoxygenases in atherosclerosis with several of its mechanisms ascribed to pro-atherosclerotic pathways (for review see Ref. [1]). The reticulocyte-type 12/15-lipoxygenase directly oxygenates linoleic acid esters on LDL particles leading to the formation of oxLDL and to foam cell formation [2–6]. On the other hand, there is compelling evidence for an anti-inflammatory and anti-atherosclerotic effect of the 12/15-lipoxygenases through the generation of lipid mediators involved in the resolution of inflammation [1]. The 12/15-lipoxygenase is involved in the metabolism of at least four classes of such pro-resolution lipid mediators, including the production of 15-HETE and the lipoxins from arachidonic acid [7], of 13-HODE from linoleic acid [8,9] and of the resolvins from DHA [10].

Animal models of atherosclerosis did not solve the question of whether the 12/15-lipoxygenase activity is pro- or anti-atherogenic because two different animal models showed contrasting results [11–15]. The discrepancies observed between different animal

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models have been explained by the different positional selectivities of the mammalian 12- and 15-lipoxygenase iso-enzymes which oxidize arachidonic acid at the carbon atoms 12 and 15 and which have different expression patterns [1].

In light of these contradicting reports and the equivocal results in two animal models of atherosclerosis [11–15], it will be essential to dissect the effect of the human reticulocyte 15-lipoxygenase (ALOX15) on human atherosclerosis for cardiovascular risk assessment in humans. Since functionally relevant polymorphisms in the ALOX15 gene could elucidate whether altered ALOX15 activity does influence disease progression in humans, we recently screened the ALOX15 gene for polymorphisms and detected 11 variations of which five defined all major haplotypes with frequencies >0.01 [16]. One of the polymorphisms, a C to T substitution at position c.−292 in the ALOX15 promoter, created a novel transcription factor binding site for the myeloid specific transcription factor SPI1 [16]. Binding of SPI1 to the c.−292T promoter increased ALOX15 transcription and activity in macrophages from heterozygous c.−292C > T carriers [16,17]. This functional polymorphism showed a trend to be atheroprotective in a small case–control study for coronary artery disease (CAD) [17]. In addition, a recent study showed evidence for another rare polymorphism in ALOX15 to be associated with CAD in two independent samples [18]. This T560M ALOX15 polymorphism with a minor allele frequency of 1.2% in Caucasians was shown to have 20 times reduced enzyme activity and was associated with an increased risk for CAD. Hence, both studies investigating the association with either an activating or an inactivating polymorphism in human ALOX15 suggest that the physiological ALOX15 expression does not accelerate atherosclerosis but protects from atherosclerosis.

In this study, we investigated whether any of the functional polymorphisms or a haplotype of the ALOX15 gene is associated with myocardial infarction (MI) in the MONIKA/KORA cohort S3 and in the KORA B study, a large case–control study including 518 cases and 2111 controls.

2. Materials and methods

2.1. Study population

Cases were derived from the KORA B study on patients with myocardial infarction (MI). In 1996, a total of 589 patients with history of sporadic MI prior to the age of 60 years were identified through the Augsburg, Germany, MONIKA MI register and invited to the study centre. The diagnosis of MI was established according to the MONIKA diagnostic criteria. MI patients were studied by physical examination, blood testing, echocardiography, and a detailed standardized interview. Controls were defined as study participants without MI from the population-based KORA study S3, which has been conducted during the years 1994/1995 in the region of Augsburg. Study participants were selected based on local registries and invited to undergo medical examination in the KORA study centre [19].

2.2. Genetic analysis

Genotyping was performed as previously described [20,21]. Briefly, genomic DNA was extracted from whole blood by standard salting-out method. High quality DNA for genotyping was obtained from 518 MI cases and 2111 controls. Genotyping of SNPs c.−635A > G (rs2255888), c.−186G > C (rs2664593), c.337 + 8C > T (rs11078528), c.1693C > T (rs34210653, T560M) and c.2076 + 200C > T (rs11568131) in ALOX15 was done at the Genotyping Unit of the Gene Discovery Core Facility at the Innsbruck Medical University, Austria, using a 5′ nuclease allelic discrimination (Taqman) assay in all subjects with sufficient amount and quality of DNA [21]. The MassArray system (Sequenom, San Diego, CA) was used for genotyping of c.−292C > T (rs11568070) whereby a base-specific extension reaction was performed with Thermosequenase (Amersham, Piscataway, NJ) and this reaction was dispensed onto a 384-format Spectro-Chip (Sequenom) [20]. A matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF) spectrometer, model Bruker Autoflex (Sequenom), was used for data acquisition. Genotyping calls were made in real time with MassArray RT software (Sequenom). Calling rates were above 95% for all SNPs and did not differ between cases and controls for an individual assay.

2.3. Statistical analysis

Differences in study characteristics were tested either through non-parametric Mann–Whitney *U* tests for continuous variables and χ^2 tests or Fisher's exact test for categorical variables. SNP association analysis was performed under the assumption of an additive genetic effect model. Association with MI was analyzed through logistic regression models, association with CRP plasma levels through linear regression (on the log scale for CRP). All association analyses were adjusted for age and sex. Haplotype estimation was performed using "haplo.em" from R library "haplo.stats" [22] stratified by case–control status and only for individuals with complete genotype information in all SNPs. A likelihood ratio test to test for differences in haplotype frequency estimates between cases and controls was applied. For haplotype analysis, the expected number of copies of all haplotypes with frequency ≥1% were calculated and coded as covariates. Haplotypes with probabilities <1% were collected into a group of rare haplotypes. All haplotype variables (including the group of rare haplotypes) were modelled against the group of subjects with two copies of the most frequent haplotypes (reference). All haplotype analyses were adjusted for sex and age alone or additionally for waist, HDL-C and hypertension. Significance was defined for *p*-values below 0.05.

3. Results

To investigate the influence of ALOX15 on atherosclerosis, we analyzed the association of six polymorphisms including the functional polymorphisms c.−292C > T and T560M in ALOX15 with MI in the MONIKA/KORA case–control sub-study including 518 Caucasian cases and 2111 healthy controls. The characteristics of the controls and cases are shown in Table 1. No significant association between the detected genetic variants in the ALOX15 gene and MI was observed (Table 2) but the rare functional polymorphism T560M leading to reduced ALOX15 enzymatic activity showed a trend to increase the risk of MI (OR 1.7, CI: 0.96–3.01, $p = 0.06$). A similar trend for an association was observed when the analysis of the T560M polymorphism was restricted to men only (OR = 1.9, CI: 1.0–3.7, $p = 0.05$).

Haplotype analysis of the ALOX15 gene in the MONIKA/KORA cohort revealed nine previously defined haplotypes (Table 3) with similar frequencies to the ones found in a Swiss study population [16]. Since the T560M polymorphism was only observed in rare haplotypes with a frequency below 1% in the entire population and no further contribution to haplotype association analysis was expected from this SNP, we excluded this SNP from haplotype estimation, in order to increase the number of individuals with comprehensive genotype information. Logistic regression analysis adjusted for age and sex showed a significant association of haplotypes 4 and 8 with MI ($p_{haplo.4} = 0.02$, $p_{haplo.8} = 0.03$). After the additional adjustment for waist, hypertension, and HDL-C only haplotype 4 remained significantly associated with MI while the association for haplotype 8 became borderline significant ($p_{haplo.4} = 0.03$, $p_{haplo.8} = 0.05$,

Table 1
Characteristics of controls and cases.

	Controls ($n = 2111$)	Cases ($n = 518$)	p-Value
Age (years)	56.0 (51.0; 62.0)[a]	58.0 (54.0; 61.0)	0.0002
Male (%)	49.3	87.6	$<10^{-20}$
Hx of diabetes (%)[b]	6.3	16.5	$<10^{-13}$
Hx of hypertension (%)[b]	50.7	64.5	1.8×10^{-8}
Lipid lowering medication	4.4	41.7	$<10^{-20}$
Smoking			2.2×10^{-16}
Non-smokers	50.9	12.5	
Ex-smokers	29.4	65.1	
Current smokers	19.8	22.0	
Waist (cm)	93.0 (84.0; 100.9)	101.0 (94.5; 108.0)	$<10^{-20}$
Hip (cm)	104.0 (99.5; 109.0)	102.0 (98.0; 107.0)	7.3×10^{-7}
Alcohol (g/day)	5.7 (0; 24.6)	8.7 (0; 25.7)	0.1720
Cholesterol (mg/dl)	238.6 (212.1; 266.7)	235.6 (206.8; 268.7)	0.1541
HDL-C (mg/dl)	51.0 (42.0; 62.4)	48.0 (40.5; 58.6)	4.0×10^{-5}
LDL-C (mg/dl)	148.0 (122.9; 176.0)	139.7 (112.7; 168.4)	8.3×10^{-6}
Total C/HDL-C ratio	4.7 (3.7; 5.8)	4.9 (4.0; 5.8)	0.0120
CRP (mg/l)	1.6 (0.8; 3.2)	2.2 (1.1; 3.9)	9.4×10^{-9}

[a] Median (interquartile range).
[b] Hx of [. . .] = history of [. . .].

Table 2
Exact logistic regression models predicting case-status (MI).[a]

Variation	MAF (%) genotypes		Odds-ratio[a]	95% CI	p-Value
	Controls ($n = 2111$)	Cases ($n = 518$)			
c.−635A > G rs2255888	25.3 1176/794/135	23.4 308/174/34	0.96	0.81–1.14	0.63
c.−292C > T rs11568070	1.7 2038/69/1	1.7 501/16/1	1.04	0.58–1.76	0.89
c.−186G > C rs2664593	22.1 1240/700/101	21.4 310/155/28	0.98	0.82–1.18	0.84
c.337 + 8C > T rs11078528	27.3 1112/841/154	29.0 264/205/47	1.07	0.91–1.26	0.41
c.1693C > T rs34210653	1.3 1976/51/1	2.0 458/19/0	1.73	0.43–2.30	0.06
c.2076 + 200C > T rs11568131	16.5 1417/546/60	14.5 359/116/13	0.88	0.71–1.08	0.21

[a] Age and sex adjusted using an additive genetic effect model.

data not shown). However, no overall significant difference between haplotype frequencies in cases and controls was found ($p = 0.23$).

To explore whether these haplotypes modulate the anti-inflammatory role of ALOX15, we performed linear regression analyses to investigate the influence of the ALOX15 haplotypes on CRP levels. No haplotype and no individual polymorphism were associated with CRP levels without or with adjustment for case status, age, sex, waist, hypertension, lipid lowering drugs, and HDL-C (data not shown).

Table 3
Predicted haplotypes and their frequencies in the study[a] and results from logistic regression models predicting case-status (MI).[b]

Haplotype	Alleles[a]	Frequency in		OR	95% CI	p-Value
		Controls ($n = 1997$)	Cases ($n = 486$)			
14	ACG**T**C	0.254	0.278	–	–	–
10	ACGCC	0.243	0.254	1.002	0.811–1.237	0.98
8	AC**C**CC	0.176	0.175	1.305	1.027–1.656	0.03
5	**G**CG**CT**	0.119	0.112	1.205	0.920–1.571	0.17
11	**G**CGCC	0.111	0.094	1.080	0.805–1.442	0.60
4	ACG**CT**	0.022	0.012	0.408	0.180–0.826	0.02
1	AC**CCT**	0.019	0.013	0.705	0.328–1.389	0.34
12	A**T**GCC	0.016	0.016	1.078	0.581–1.903	0.80
9	**GCC**CC	0.016	0.009	0	0–0	0.96
Rare[c]		0.013	0.016	5.999	2.884–12.635	<0.001

[a] Haplotypes formed by the alleles of the ALOX15 polymorphisms c.−635A > G, c.−292C > T, c.−186G > C, c.337 + 8C > T, c.2076 + 200C > T. Rare alleles are printed in bold.
[b] Adjusted for age and sex.
[c] Haplotypes with frequencies < 0.01 were pooled in a group as rare haplotypes.

4. Discussion

ALOX15 was previously shown to have contrary effects on atherosclerosis in different animal models, which complicated the interpretation of the role of the enzyme in human atherosclerosis. We and others previously identified two rare functional polymorphisms in ALOX15, which led to increased (c.−292C > T) [16] and reduced (T560M) [18] enzyme activity, respectively. While the activating c.−292C > T polymorphism showed a trend towards an atheroprotective effect in a case–control study for CAD, the inactivating T560M polymorphism was associated with a significantly increased risk for CAD, indicating that ALOX15 is anti-inflammatory and anti-atherogenic in humans [17,18]. In the present study, we investigated for the first time the role of ALOX15 on the emergence of MI and show that the functional polymorphisms are not associated with MI in a larger Caucasian case–control sample, suggesting that ALOX15 does not play a major role during later stages of atherosclerosis involving atherothrombotic events.

Such a divergent role of ALOX15 during different stages of atherogenesis would be supported by the temporal and regiospecific expression of ALOX15 and by the expression of its specific linoleic acid metabolite in the arterial wall. In rabbit atherosclerotic lesions, the expression of ALOX15 was 10 times higher in early lesions than in more advanced lesions [23] and the specific ALOX15 metabolite 13(S)-hydro(per)oxy-octadecadienoic acid (13-HpODE) was abundant in early human atherosclerotic lesions but was not during later stages of plaque development [8,9]. In line with this, a recent human genetic study suggested a role for ALOX15 only during early atherosclerotic lesion development. This study investigated the association of three ALOX15 promoter polymorphisms with carotid plaques and found an association of a certain promoter haplotype only in the subgroup of the younger stratum, while this haplotype was not associated with the intermediate phenotype in the older subgroup [24]. Therefore, it is conceivable that in humans ALOX15 may play an anti-inflammatory role in the initiation of atherosclerosis but to a lesser extend during later stages of atherogenesis, although ALOX15 expression during human atherosclerosis is under debate [1].

There is another possible interpretation of this negative finding. There could be an effect of these rare polymorphisms with an OR of 1.5 or less, which our study could not detect because it was underpowered (39%). We had a power of 87% to detect an effect of OR = 2.0 for a SNP with MAF comparable to c.−292C > T and T560M (MAF = 1.4%). An indication for a lack of power of the study could be the observed trend for an association of the T560M polymorphism with MI with a similar risk increase (OR 1.7) as previously reported for CAD in the CARDIA and the ADVANCE studies [18]. This raises the question why the second functional promoter polymorphism, c.−292C > T, would not show a similar trend for an association with MI. This discrepancy may derive from the tissue specific influence of the two functional polymorphisms on ALOX15 activity. While the T560M polymorphism will result in reduced ALOX15 activity in all cell types [18], the c.−292C > T promoter polymorphism only leads to increased ALOX15 expression in myeloid cells [16]. There is indication that endothelial expression of ALOX15 will result in the formation of arachidonic acid metabolites leading to vasodilation [25–27], which would only be influenced by the T560M polymorphism and not by the c.−292C > T promoter polymorphism. Therefore, the trend of the T560M polymorphism for an association with MI might indicate that endothelial expression of ALOX15 could be involved during the later atherosclerotic events leading to MI. On the other side both of these findings could derive from chance.

To get additional information on whether this M560 ALOX15 allele is associated with CAD, we investigated its association with angiographically documented CAD in a smaller case–control study involving 498 participants [17,28,29]. Again, the T560M polymorphism showed a trend to be associated with increased risk for angiographically defined CAD in this small study (OR 3.30 [CI: 0.68–16.0]; $p = 0.14$) potentially indicating some role as a risk marker for CAD (data not shown).

The inactivating M560 allele was not linked with a specific haplotype but was found on four different haplotypes including the three frequent haplotypes 8, 10 and 14 while being absent on haplotype 4. These results show that none of the functional polymorphisms in ALOX15 do account for the association of haplotype 4 with MI in the MONIKA/KORA case–control study, leaving the possibility of the presence of another atheroprotective mutation linked with this haplotype or of a chance finding.

In summary, we show that the two functional polymorphisms in ALOX15 are not associated with MI in a large case–control study which argues that ALOX15 seems less involved during the later stages of atherosclerosis than eventually during early lesion development. Indeed, these activating and inactivating polymorphisms in the ALOX15 gene seem inversely associated with CAD and suggest an anti-inflammatory role for ALOX15 during early lesion development.

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