

9p21.3 risk locus is associated with first-ever myocardial infarction in an Austrian cohort

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Aims Atherosclerosis often presents as a complex systemic disease that is strongly influenced by lifestyle factors, but also by the genetic background. The sequence variant rs1333049 affects the expression of *ANRIL*, a noncoding RNA transcript playing a key role in the regulation of inflammatory processes. We thus aimed to replicate the predictive value of genetic information on this variant regarding the development of cardiovascular events in an Austrian high-risk cohort.

Methods Nine hundred and eighty-eight patients from an angiologic outpatient ward at a large University hospital were genotyped by means of the 5'-nuclease assay. Relative risk ratios were assessed for carriers of different alleles. Statistical independence of genetic information was evaluated in multivariable analysis including known risk markers.

Results In patients carrying the [G]-allele, metabolic parameters (serum low-density lipoprotein, total cholesterol) significantly decreased during the initial 6 months of the observation period ($P < 0.01$). Likewise, homozygous [C]-allele carriers were at a higher risk of

suffering myocardial infarction (relative risk = 2.681, 95% confidence interval 1.418–5.070). In contrast, we found no interaction between rs1333049 genotype and progression of carotid atherosclerosis or stroke.

Conclusions These results are in line with the previous findings, suggesting that genetic information on the rs1333049 variant might be a useful predictor of adverse cardiac events. Thus, we could successfully replicate the predictive value of the 9p21 risk allele in an Austrian cohort.

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Introduction

Atherosclerosis must be seen as a systemic disease that might be influenced by lifestyle factors including smoking, immobility or diet. Moreover, this disease tends to occur with an elevated frequency in some families, thus suggesting a hereditary component¹ driven by a considerable variance in protein/mediator function, which is mainly based on genetic polymorphisms.

In this context, *ANRIL*, a noncoding RNA (ncRNA), has been discussed as a potential repressor of arteriosclerosis.² This transcript acts as an activating antisense RNA for *CDKN2B* (*p15*), an inhibitor of cyclin-dependent kinases (CDK)4 and CDK6. Blocking of CDK4 and CDK6 is a major effect of transforming growth factor-(TGF)- β -mediated cell cycle arrest in endothelial, myeloid and lymphoid cells,³ which are involved in inflammatory processes as present in atherosclerosis.

In the literature, distinct splicing variants have been identified that differ in *CDKN2B* regulation.⁴ Whereas a long variant might facilitate the production of CDKN2A and CDKN2B, thus mediating a repressive effect on the

inflammatory system, no such effect can be proven for the short variant. There is evidence that the ratio of *ANRIL* splicing variants is balanced by the presence of certain single-nucleotide variants.

A single-nucleotide variant (NC_000009.11:g.22125503G > C, rs1333049) resulting in a guanine to cytosine change less than 5 kb downstream of the long *ANRIL* splice variant might be involved in the regulation of post-transcriptional modification of *ANRIL*. Hence, the [G];[G] genotype correlates with the presence of the long splicing variant of the antisense RNA, whereas individuals being homozygous for the [C]-allele express significantly higher amounts of short *ANRIL* transcripts.⁴

In genome-wide association studies,^{5,6} as well as in confirmatory replication trials,^{7–9} rs1333049 and the corresponding sequence variants¹⁰ have been identified as risk markers for coronary artery disease (CAD). In this regard, rs1333049 residing at the short arm of chromosome 9 (9p21.3)⁴ shows high linkage disequilibrium ($D' > 0.87$, $r^2 > 0.55$) with the neighboring variants, which build up a haplotype block strongly connected to CAD.⁹

Because the present cohort has been recruited at a large university hospital's angiologic outpatient ward, it can be assumed that these patients bear a higher cardiovascular risk. Moreover, an interaction between the rs1333049 sequence variant and the major cardiac events has not been replicated in an Austrian population to date. However, this study is intended to fill this important gap.

Methods

Study design

Between March 2002 and March 2003, 1268 consecutive neurologically asymptomatic patients who underwent carotid duplex sonography were prospectively enrolled at the Department of Angiology, Medical University of Vienna, in the Inflammation and Carotid Artery – Risk for Atherosclerosis Study (ICARAS), as reported previously.¹¹ The inclusion and exclusion criteria have been published previously.¹¹ Briefly, asymptomatic carotid artery disease, which was the primary inclusion criterion, was defined as the absence of transient ischemic attacks (TIAs), amaurosis fugax and stroke within the last 12 months, or the lack of residual symptoms. Sonographic assessment of carotid artery stenosis as well as standard laboratory tests was performed at baseline and at a 6 to 9-month follow-up examination as reported previously.¹¹ The occurrence of major cardiovascular events after study inclusion was observed until January 2006. Due to missing biomaterial or clinical information for genotyping analysis, 280 patients were excluded from the analysis, resulting in a total of 988 patients who were finally genotyped for the rs1333049 sequence variant. The study was approved by the local ethics committee (EC-No. 1933/2012) and has been performed in accordance with the ethical standards specified by the Declaration of Helsinki and its amendments.

Clinical definitions

Arterial hypertension was defined as a repetitive resting blood pressure above 140/90 mmHg and was assumed to be present in patients with antihypertensive medication. Hyperlipidemia was diagnosed in patients with a total serum cholesterol above 200 mg/dl or a low-density lipoprotein (LDL) cholesterol above 130 mg/dl, and was considered to be present in all patients taking lipid-lowering drugs. Diabetes mellitus was diagnosed as supposed by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus.¹² Peripheral artery disease was graded using Fontaine's classification system,¹³ and CAD was defined according to the classification by the Canadian Cardiovascular Society (CCS).¹⁴ Anamnestic myocardial infarction (MI) was defined according to Alpert *et al.*¹⁵ Stroke was defined by a neurological deficit persisting for more than 24 h and was evaluated according to the modified Rankin stroke scale.¹⁶ Progression of carotid artery atherosclerosis was defined as an increase in stenosis by at least one North

American Symptomatic Carotid Endarterectomy Trial angiographic degree.

Genotyping of rs1333049

DNA was isolated manually from EDTA-anticoagulated whole blood by means of spin-column-based nucleic acid purification. Genotyping was done on an ABI TaqMan 7900HT fast real-time thermocycler (Applied Biosystems, Rotkreuz, Switzerland) using the 5'-nuclease assay.¹⁷ In the present study, rs1333049 was analyzed in a total reaction volume of 5 µl using a fully tested, commercially available TaqMan SNP genotyping assay (Assay ID: C_1754666_10, Applied Biosystems) and TaqMan Universal Mastermix II without UNG (Applied Biosystems) according to the standard protocol supplied by the manufacturer. The results were interpreted using SDS 2.3 sequence detection software (Applied Biosystems).

Statistical analysis

Continuous data are given with respect to its distribution as mean and SD or median and interquartile range. For this purpose, the presence of normal distribution within the subgroups was assessed by Kolmogorov–Smirnov tests. Categorical data are presented as counts and percentage. Associations between the rs1333049 genotypes and the study end points as well as relative risks were estimated by contingency tables and Pearson's chi-square tests. Different distributions of the continuous variables were evaluated using analysis of variance (ANOVA) and Student's *t* tests, or Kruskal–Wallis tests and Mann–Wilcoxon *U* tests, respectively (if data do not show normal distribution). Multivariable models were calculated by means of binary logistic regression. The results were considered statistically significant at a *P* value less than 0.05, unless otherwise noted. All *P* values were interpreted two-sided unless otherwise stated. All statistical calculations are done using IBM SPSS 20.0 (IBM Corporation, Armonk, USA).

Results

Study characteristics

The characteristics of the study population are listed in Table 1. Baseline characteristics did not depend on the rs1333049 genotype. Genotyping was not successful in 64 cases; however, this group does not significantly differ from the analyzed study population regarding the indicator parameters age, sex, hyperlipidemia, diabetes mellitus, arterial hypertension, peripheral arterial disease, coronary arterial disease, nicotine consumption and history of stroke/MI (data not shown). Therefore, this lack of data was not seen as a study limitation. Allele frequencies were in the Hardy–Weinberg equilibrium ($\chi^2 = 0.05$, $P = 0.83$, $DF = 1$), but did significantly differ from the published reference Hap Map-CEU collectives ($\chi^2 = 14.918$, $P = 0.001$, $DF = 2^{18}$; $\chi^2 = 31.987$, $P = 1.1 \times 10^{-7}$, $DF = 2^{19}$).

Table 1 Baseline characteristics of the study cohort

		[G];[G]	[G];[C]	[C];[C]	
Genotype distribution		215 (23.3%)	458 (49.6%)	251 (27.1%)	
		Median (Interquartile range)			<i>p</i> -value
Age	Total	[G];[G]	[G];[C]	[C];[C]	
	68.0±10.8	68.4±11.0	68.0±11.0	67.8±10.3	0.841
		Counts (percentages within patients sharing the same genotype)			<i>p</i> -value
Sex		[G];[G]	[G];[C]	[C];[C]	
	Male	575 (62.2%)	127 (59.1%)	293 (64.0 %)	155 (61.8%)
	Female	349 (37.8%)	88 (40.9%)	165 (36.0%)	96 (38.2%)
Smokers		242 (26.2%)	59 (27.4%)	123 (26.9%)	60 (23.9%)
Peripheral artery disease		388 (42%)	88 (40.9%)	186 (40.6%)	114 (45.4%)
	I	226 (24.5%)	46 (21.4%)	107 (23.4%)	73 (29.1%)
	II	155 (16.8%)	41 (19.1%)	74 (16.2%)	40 (15.9%)
	III	7 (0.8%)	1 (0.5%)	5 (1.1%)	1 (0.4%)
Coronary artery disease		493 (53.4%)	118 (54.9%)	236 (51.5%)	139 (55.4%)
	I	282 (30.5%)	62 (28.8%)	138 (30.1%)	82 (32.7%)
	II	175 (18.9%)	50 (23.3%)	78 (17.0%)	47 (18.7%)
	III	28 (3%)	3 (1.4%)	19 (4.1%)	6 (2.4%)
Diabetes mellitus		204 (22.1%)	39 (18.1%)	106 (23.1%)	59 (23.5%)
	Type I	63 (6.8%)	11 (5.1%)	34 (7.4%)	18 (7.2%)
	Type II	141 (15.3%)	28 (13%)	72 (15.7%)	41 (16.3%)
Arterial		624 (67.5%)	133 (61.9%)	320 (69.9%)	171 (68.1%)
Hyperlipidemia		594 (64.3%)	141 (65.6%)	289 (63.1%)	164 (65.3%)
History of stroke		151 (16.3%)	34 (15.8%)	84 (18.3%)	33 (13.1%)
History of myocardial infarction		221 (23.9%)	52 (24.2%)	108 (23.6%)	61 (24.3%)

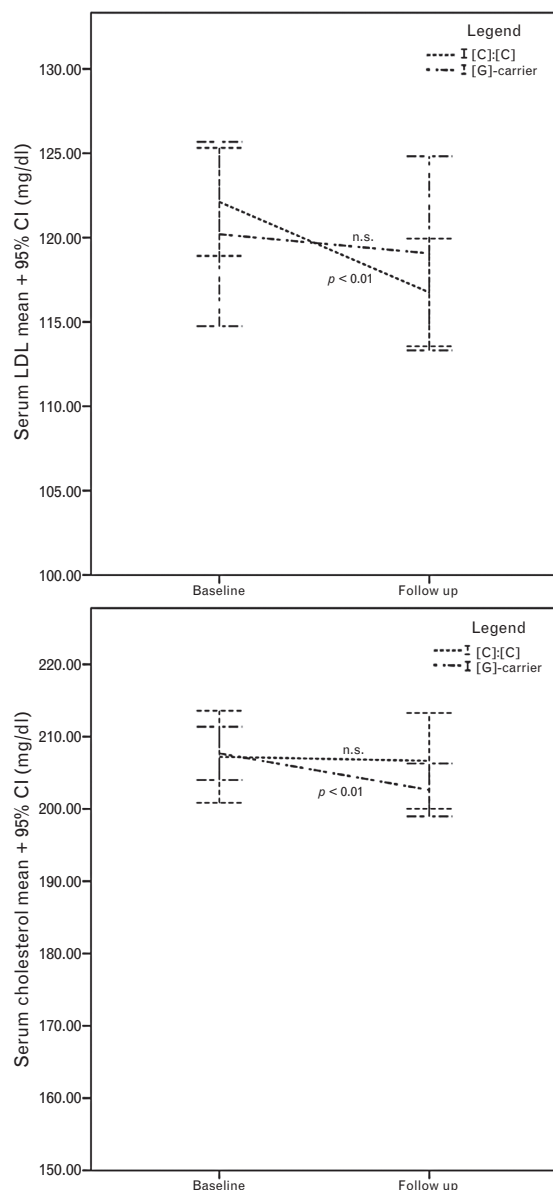
Metric data have been evaluated by analysis of variance (ANOVA), and categorical/rank-scaled data by chi-square tests.

Lipid markers

Genotype-specific differences in total serum cholesterol and LDL levels between baseline and follow-up were evaluated in patients with [C];[C] genotype and in carriers of the [G]-allele. Paired sample tests showed a significant decrease in both serum cholesterol (baseline 207.7±44.7 mg/dl, follow-up 202.6±44.4 mg/dl; $P=0.003$) and serum LDL (baseline 122.1±38.7 mg/dl, follow-up 116.7±38.5 mg/dl; $P=2.9 \times 10^{-4}$) in [G]-carriers, but not in [C];[C] individuals (cholesterol:

baseline 207.2±47.2 mg/dl, follow-up 206.6±49.3 mg/dl, $P=0.844$; LDL: baseline 120.2±4.3 mg/dl, follow-up 119.1±42.4 mg/dl, $P=0.605$) (see Fig. 1). When comparing the difference in follow-up and baseline serum cholesterol/LDL directly between [C];[C] individuals and [G]-carriers using a Mann–Whitney U test, the decreases in total cholesterol/LDL concentrations were significantly higher in carriers of the G-allele (cholesterol: Mann–Whitney $U=56289$, one-sided $P=0.048$; LDL: Mann–Whitney $U=54004.5$, one-sided $P=0.026$). There

Fig. 1



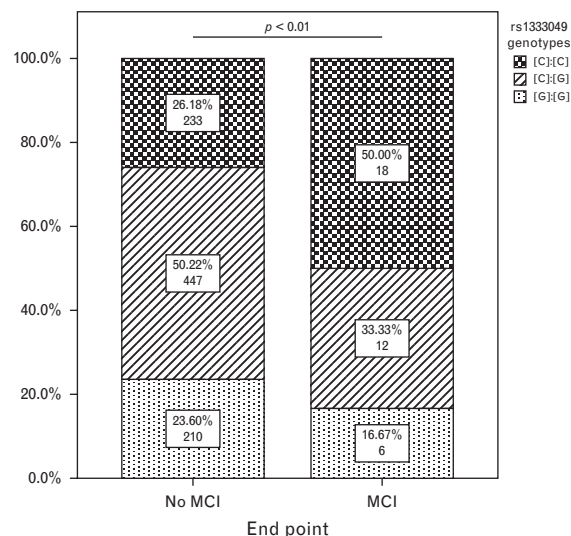
Differences in serum cholesterol/low-density lipoprotein concentrations between the baseline and follow-up were calculated for [C];[C] homozygous patients and carriers of the [G]-allele by paired-samples *t* tests. Whereas there was no difference in [C];[C]-individuals, both parameters decreased significantly in carriers of the [G]-allele (directional hypothesis: *P* values must be interpreted one-sided).

was no difference in the prevalence of hyperlipidemia ($\chi^2 = 0.166$, $P = 0.683$, $DF = 1$) or prescription of anti-hyperlipidemic therapy ($\chi^2 = 0.1$, $P = 0.752$, $DF = 1$) between both the groups.

Myocardial infarction

Within the study population, 36 (3.9%) individuals suffered a MI during the observational period; among those, 20 cases (2.8%) were considered as first-ever MIs. We

Fig. 2



Differences in the distribution of NC_000009.11:g.22125503G>C genotypes between patients experiencing myocardial infarction (MI) and MI-free participants were assessed by chi-square test. The test resulted in a statistically significant disparity between groups at *P* less than 0.01.

found a relation between the [C];[C] genotype and the incidence of MI after study inclusion ($\chi^2 = 9.873$, $P = 0.002$, $DF = 1$; see Fig. 2). When comparing the patients carrying this genotype with carriers of the [G]-allele ([G];[C] and [G];[G]), a relative risk of 2.681 [95% confidence interval (CI) 1.418–5.070] regarding the development of MI was calculated. Interestingly, this linkage was even more pronounced in patients without a diagnosis of CAD (relative risk for [C];[C]-carriers 3.988, 95% CI 1.292–12.31) than in patients with a medical history regarding this disease (relative risk for [C];[C]-carriers 2.155, 95% CI 0.989–4.694). Likewise, the genotype–phenotype association lost significance in patients with previous MI ($\chi^2 = 2.251$, $P = 0.134$). Thus, the relative risk for [C];[C] homozygous patients regarding the development of first-ever MI amounts to 3.3 (95% CI 1.389–7.838).

Hence, a binary logistic regression model ($\chi^2 = 40.603$, $P = 2.8 \times 10^{-5}$, $DF = 11$) was calculated providing genotype information, together with known atherosclerosis risk factors and biomarkers such as age, sex, nicotine consumption, hyperlipidemia, arterial hypertension, HbA1c, BMI, diagnosis of peripheral or CAD and previous insults for prediction of first-ever MI. rs1333049 presented as an independent predictor with an odds ratio (OR) of 3.846 (95% CI 1.613–9.171, $P = 0.002$) per [C]-allele. Apart from the carrier status of the rs1333049: [C]-allele, only a medical history of stroke significantly contributed to the model (OR 14.755, 95% CI 4.888–44.538, $P = 2 \times 10^{-6}$).

Neurologic markers

In contrast, progression of carotid artery atherosclerosis occurred to an equal extent across the genotypes ($\chi^2 = 2.825$, $P = 0.244$, $DF = 2$). Likewise, there was no statistically significant relation between the rs1333049 genotypes and the incidence of stroke, neither in the whole cohort (48 events, $\chi^2 = 1.121$, $P = 0.571$, $DF = 2$) nor in patients with ($P = 0.271$) and without ($P = 0.599$) a history of central nervous events.

Discussion

To our knowledge, this is the first study showing a high risk of future MI for individuals carrying the [C];[C] genotype in an Austrian cohort. Genotype distributions of the present cohort differed from published reference samples insofar as the [C];[C]-risk genotype was more frequent. This could be plausibly explained by the employed recruiting strategy; assignment to carotid duplex sonography is mostly indicated in cases of known atherosclerotic disease (CAD, peripheral artery disease), for clarification of carotid bruits and before major cardiac surgery.¹¹ Thus, the investigated cohort in total represents patients featuring a higher cardiovascular risk due to a manifest underlying atherosclerotic disease.

rs1333049 and myocardial infarction

In our study cohort, a statistically significant linkage between the [C];[C] genotype and MI has been found. This is in frame with previous findings from the study by Saleheen *et al.*,²⁰ who reported an OR of 1.12 (95% CI 1.04–1.2, $P = 0.002$) per [C]-allele regarding the development of first-ever MI. Moreover, Peng *et al.*²¹ assessed rs1333049 genotypes in 520 MI patients and 560 controls. The main outcome of the study was a different allele frequency between the groups. Whereas only 26.2% of the control participants possessed two copies of the [C]-allele, this combination has been found in 50% of the patients.

Furthermore, Hughes *et al.*²² integrated genetic information, including the rs1333049 risk-allele carrier status, into the existing cardiovascular risk scores. The authors reported that this might improve risk classification, especially in middle-aged men.

We could show that the investigated genotypes were of no predictive value in patients with a history of MI. Likewise, Peng *et al.*²¹ reported no association with the recurrence of MI and supposed that secondary preventive measures as alterations in lifestyle and cardioprotective medication after a first acute coronary event may attenuate the genetic effect on progression of the disease. Do *et al.*²³ described a statistical interaction between the sequence variant and dietary intake. The authors postulated that the detrimental effect of risk alleles on future MI could be mitigated by a diet often referred to as a 'prudent diet', which is rich in raw vegetables and fruits.

Farzaneh-Far *et al.*²⁴ could not find any linkage between genotypes and cardiovascular structure and function quantified by means of cardiac sonography and exercise performance tests in a cohort suffering from stable coronary heart disease. Dandona *et al.*²⁵ reported an association between rs1333049 and CAD severity, but not the incidence of MI, in a cohort of patients with CAD. This has been corroborated by the present data, since relative risk estimators for a future MI were not statistically significant in patients with diagnosed CAD. In this regard, Samani *et al.*²⁶ proposed that the ability to form stable atherosclerotic plaques might be reduced in carriers of the risk allele. However, the underlying mechanisms still remain unclear.

Affection of serum lipid concentrations

Serum LDL as well as total cholesterol concentrations showed a higher tendency to decrease between baseline and follow-up examinations in carriers of the [G]-allele. Congrains *et al.*² applied exon-specific small interfering RNA knock-down of ANRIL exon 1, which resulted in reduced cell proliferation and a 1.34-fold up-regulation of the peroxisome proliferator-activated receptor (PPAR)- δ . This nuclear receptor protein suppresses macrophage-mediated inflammation²⁷ and, interestingly, facilitates a less hazardous lipid profile by lowering of LDL levels via diminution of small and medium-sized LDL particles.²⁸ These findings strengthen the hypothesis that the molecular mechanisms by which the rs1333049 sequence variant influences the pathogenesis of atherosclerosis are located at multiple levels.

No association of rs1333049 and neurologic outcome

We could not find any association between carrier status of the rs1333049 [C]-allele and progression of carotid atherosclerosis ($P = 0.244$), previous insults ($P = 0.196$) or stroke incidence during the observation period ($P = 0.571$). This is consistent with the results from the study by Cunnington *et al.*,²⁹ who found that measures of intima-media thickness did not differ between the rs1333049 genotypes. In contrast, Karvanen *et al.*³⁰ reported slight associations between the [C]-allele and previous (OR 1.22, 95% CI 1.06–1.41) as well as incident stroke (OR 1.15, 95% CI 0.99–1.34). However, the latter finding did not seem to be statistically significant. Likewise, Smith *et al.* found only weak associations between ischemic stroke and the rs1333049 genotypes (OR 1.13, 95% CI 1.04–1.23) when mixing data of two Swedish studies recording incident and consecutive cases and controls. In fact, stroke events can stem from different reasons and might not necessarily be a direct result of atherosclerosis. Thus, cerebral ischemia due to cardioembolism or hemorrhage probably does not correlate with atherosclerosis risk alleles. In this regard, Chimowitz *et al.*³¹ described for their study cohort different prevalences of asymptomatic cardiovascular disease dependent on the cause of stroke.

In conclusion, the effect size of the 9p21 risk allele on the development and progression of neurologic events might be too small to be detected in our preselected high-risk cohort.

Limitations

Of course, the present study comes with several limitations that need to be acknowledged. The applicability of these findings in cohorts consisting of younger persons or individuals originating from other ethnicities, as well as in the basic population, still needs to be elucidated. Moreover, the applied recruitment strategy enrolling consecutive patients in a large hospital department contains a certain potential for biased selection.¹¹ In addition, a more extensive observation period would have improved the evaluation of the predictive value of the rs1333049 genotypes.

Conclusion

In conclusion, the present work replicates a potential relationship between rs1333049 [C];[C] and adverse cardiovascular events in a Middle-European cohort of patients under risk.

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