



Genetic variants at the 9p21 locus contribute to atherosclerosis through modulation of ANRIL and *CDKN2A/B*[☆]

Ada Congrains^a, Kei Kamide^a, Ryousuke Oguro^a, Osamu Yasuda^b, Keishi Miyata^{c,d}, Eiichiro Yamamoto^e, Tatsuo Kawai^a, Hiroshi Kusunoki^f, Hiroko Yamamoto^a, Yasushi Takeya^a, Koichi Yamamoto^a, Miyuki Onishi^a, Ken Sugimoto^a, Tomohiro Katsuya^f, Nobuhisa Awata^g, Kazunori Ikebe^h, Yasuyuki Gondoⁱ, Yuichi Oike^c, Mitsuru Ohishi^{a,*}, Hiromi Rakugi^a

^a Department of Geriatric Medicine and Nephrology, Osaka University Graduate School of Medicine, Japan

^b Department of Cardiovascular Clinical and Translational Research, Kumamoto University Hospital, Japan

^c Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Japan

^d Immunology, Allergy and Vascular Medicine, Graduate School of Medical Sciences, Kumamoto University, Japan

^e Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, Japan

^f Clinical Gene Therapy, Osaka University Graduate School of Medicine, Japan

^g Department of Cardiology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Japan

^h Department of Prosthodontics and Oral Rehabilitation, Osaka University Graduate School of Dentistry, Japan

ⁱ Department of Clinical Thanatology and Geriatric Behavioral Science, Osaka University, Graduate School of Human Sciences, Japan

ARTICLE INFO

Article history:

Received 13 September 2011

Received in revised form 31 October 2011

Accepted 11 November 2011

Available online 19 November 2011

Keywords:

Chromosome 9p21

ANRIL

CDKN2A

CDKN2B

Atherosclerosis

ABSTRACT

Genome-wide association studies (GWAS) have identified genetic variants contributing to the risk of cardiovascular disease (CVD) at the chromosome 9p21 locus. The CVD-associated region is adjacent to the two cyclin dependent kinase inhibitors (*CDKN2A* and *2B*) and the last exons of the non-coding RNA, ANRIL. It is still not clear which of or how these transcripts are involved in the pathogenesis of atherosclerosis. **Objective:** We assessed the hypothesis that 9p21 locus polymorphisms influence the expression of the transcripts in the region (ANRIL, *CDKN2A/B*) and that these transcripts contribute to atherogenesis through the modulation of proliferation in VSMC.

Methods: We genotyped 18 SNPs ($r^2 < 0.8$ and $MAF > 0.05$) across the region of interest: *CDKN2A/B* and ANRIL, encompassing the CVD-associated region. RNA and DNA were extracted from the blood of 57 volunteers (69–72 years old). Carotid ultrasound was performed in 56 subjects. *CDKN2A/B* and ANRIL (exons 1–2 and 17–18) expression was measured employing RT-PCR. Gene expression and cell growth were evaluated in cultured VSMC after the siRNA-mediated knock-down of ANRIL.

Results: The risk alleles for atherosclerosis-related phenotypes were consistently associated with a lower expression of ANRIL when evaluating exons 1–2. Common carotid artery stenosis was associated with a significantly lower ($P < 0.01$) expression of ANRIL (exons 1–2). ANRIL knock-down in VSMC caused significant variation in expression of *CDKN2A/B* ($P < 0.05$) and reduction of cell growth ($P < 0.05$) *in vitro*.

Conclusion: Disease-associated SNPs at the 9p21 locus predominantly affect the expression of ANRIL. Overall, our results suggest that several CVD-associated SNPs in the 9p21 locus affect the expression of ANRIL, which, in turn modulate cell growth, possibly via *CDKN2A/B* regulation.

© 2011 Elsevier Ireland Ltd. All rights reserved.

Genome-wide association studies (GWAS) have identified genetic variants contributing to the risk of cardiovascular disease

(CVD) at the chromosome 9p21 locus [1,2]. The CVD-associated regions are adjacent to the last exons of a long noncoding RNA (lncRNA), ANRIL (also known as CDKN2BAS). Two cyclin-dependent kinases inhibitors, *CDKN2A* and *CDKN2B* (encoding p15^{INK4B}, p16^{INK4A}, and p14^{ARF}) lie close to the association spot (~100 kb from the associated SNPs). These kinase inhibitors are key players in cell proliferation and are appealing candidates to mediate these associations.

CDKN2A/B are known to be repressed by Polycomb proteins during cell growth and then activated during senescence [3]. There is strong evidence to support the role of ANRIL in the regulation of

[☆] This study was supported in part by grants-in-aid from the Program for Promotion of Fundamental Studies in National Institute of Biomedical Innovation of Japan (HR: 22-2-5), the Ministry of Education, Culture, Sports, Science, and Technology of Japan (KK: 22510211) and NOVARTIS Foundation for Gerontological Research (KK).

* Corresponding author at: Department of Geriatric Medicine and Nephrology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka (B6), Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3852; fax: +81 6 6879 3859.

E-mail address: ohishi@geriat.med.osaka-u.ac.jp (M. Ohishi).

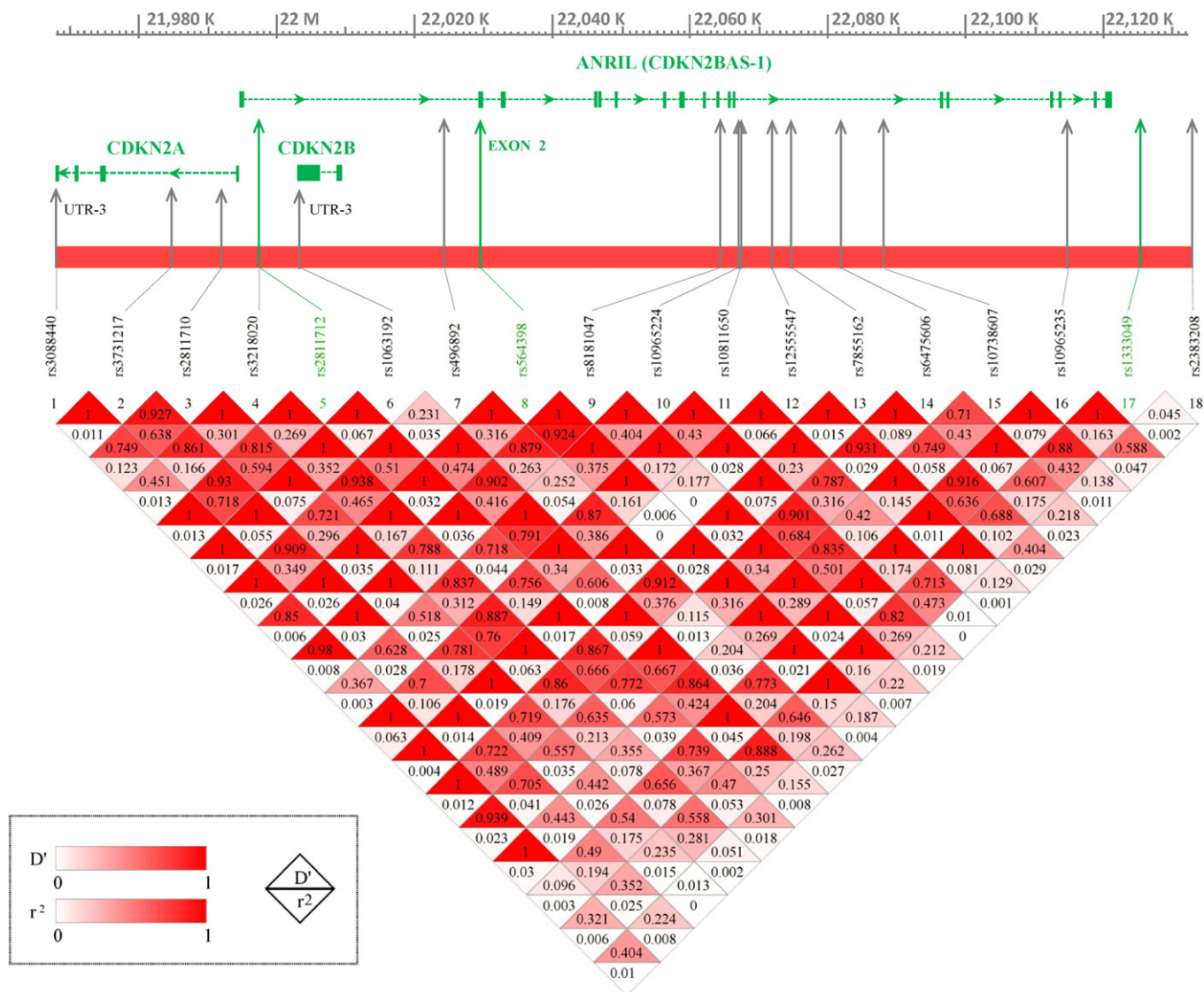


Fig. 1. Linkage disequilibrium (LD) and transcriptional map of the 9p21 locus.

the *CDKN2A/B* locus through histone modification [4,5]. ANRIL has been proposed to regulate senescence at the *CDKN2A* locus, and it showed a senescence-dependant role in proliferation [4]. The transcript complexes with Chromobox 7 (CBX7 subunit of Polycomb repressive complex 1) to silence p16^{INK4A} (*CDKN2A*) [4] and it is crucial in the recruitment of Polycomb repressive complex 2 (PRC2) to silence p15^{INKB} (*CDKN2B*) [5].

We investigated the association between single nucleotide polymorphisms (SNPs) in the region with expression of ANRIL as well as the genes encoded in this locus and explored the possible role of this non-coding RNA in *CDKN2A/B* regulation in vascular smooth muscle cells (VSMCs), which are directly involved in the pathogenesis of atherosclerosis.

The chromosome 9p21 region is particularly abundant in disease-associated SNPs, some phenotypes associated with the locus are connected with vascular disease such as atherosclerotic stroke, CVD, myocardial infarction, and more indirectly intracranial aneurism and type-2 diabetes [1,2,6,7], but, surprisingly, also with other conditions of more distant etiologies like melanoma [8], cutaneous nevi [9], leukemia [10], periodontitis [11,12] and Alzheimer's disease [13,14], endometriosis [15] and more recently with glaucoma [16,17]. Here, we provide evidence of the influence

of several SNPs in the region upon the expression of these transcripts, ultimately linking them with human atherosclerosis, and a role of ANRIL in regulating *CDKN2A/B* expression in aortic VSMC and proliferation.

1. Methods

1.1. Study sample

For the genetic association study between the SNPs: rs1333049, rs564398 and rs2811712 (see SNPs in green in Fig. 1), and CAD, patients with documented evidence of angiographically diagnosed coronary stenosis (including acute myocardial infarction, angina pectoris and, silent myocardial ischemia) (subjects characteristics in Supplementary Table 1 and Supplementary Methods).

For peripheral blood RNA expression analysis, we obtained whole blood samples from 57 volunteers (69–72 years old) from Asago City, Hyogo Prefecture, Japan (sample characteristics in Supplementary Table 2) who were participants in "SONIC" (Septuagenarians, Octogenarians, Nonagenarians Investigation with Centenarians) Study. Carotid ultrasonography using GE LOGIQ Book X-P was performed to measure the intima-media

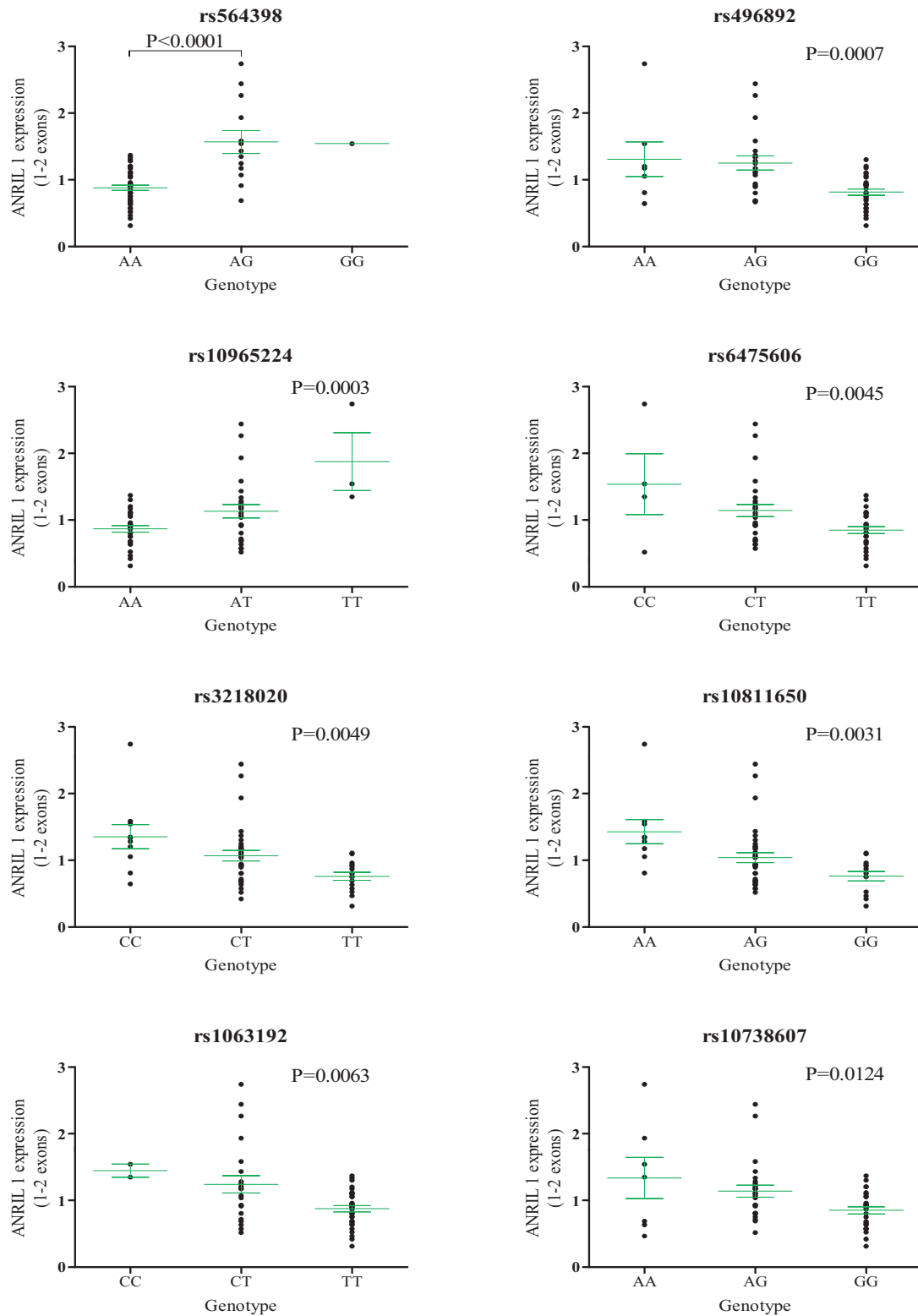


Fig. 2. ANRIL (exons 1–2) expression among genotypes in peripheral blood.

thickness (IMT) of the common carotid artery (CCA) for the evaluation of human atherosclerosis in 56 SONIC subjects (details in [Supplementary Methods](#)). We defined the criteria for CCA atherosclerosis as maximal-IMT \geq 1.1 mm. All participants gave written informed consent to participate in the genetic analyses and in all other procedures associated with the study. The ethics committee of Osaka University approved the study protocol.

1.2. Genotyping

All SNPs were genotyped with the standard TaqMan PCR method using commercial TaqMan Assays (rs numbers of genotyped SNPs are shown in [Supplementary Table 3](#); see [Supplementary Methods](#)). We selected three SNPs (rs1333049, rs564398, and rs2811712) for genotyping of subjects with coronary artery disease. Rs1333049 is

Table 1
SNPs associated with expression of 9p21 transcripts.

SNP	Disease association (risk allele)	Lower expression allele ^a	ANOVA P-value
ANRIL (1–2 exons) expression			
rs496892	Atherosclerotic stroke (G) [7] and periodontitis [12]	G	0.0007
rs10738607		G	0.0124
rs10811650		G	0.0031
rs564398	Atherosclerotic stroke (A) [7], coronary artery calcification [31] (A) and diabetes [19] (A)	A	<0.0001
rs3218020		T	0.0049
rs6475606	Coronary Heart Disease (T) [32]	T	0.0045
rs10965224		A	0.0003
rs1063192	Myocardial infarction in Chinese (T) [30], open angle glaucoma (C) [17]	T	0.0063
ANRIL (17–18 exons) expression			
rs2811712 (AA vs. GG+AG)	Severe physical impairment in the elder (A) [20]	GG+AG	0.0426
rs10965235 (AC vs. CC) ^b	Endometriosis (GWA) (C) [15]	AC	0.0275
rs3731217 (GG+GT vs. TT)	Acute lymphoblastic leukemia [10]	GG+GT	0.0097
rs7855162 (CC+CT vs. TT)		CC+CT	0.0286
CDKN2A expression			
rs10965235 (AA+AG vs. GG)	Endometriosis (GWA) (C) [15]	AA+AG	0.0414
CDKN2B expression			
rs2383208 (AA vs. AG+GG)	Diabetes in Japanese (A) [33]	AG+GG	0.0317
rs10738607		^c	0.0451

^a Allele associated with lower expression of 9p21 transcripts.

^b Only one subject had AA genotype, see Supplementary Fig. 3.

^c AG genotype was associated with increased expression of CDKN2B, see Supplementary Table 4.

the leading CAD-associated SNP [1]. Rs564398 is the SNP showing the strongest association with ANRIL expression in our analysis, as well as in a previous study [18], and has been associated with atherosclerotic stroke [7] and diabetes [19]. Rs2811712 was associated with frailty [20] and, due to its proximity with the CAD-associated locus, we hypothesized a possible association with CAD.

For expression analysis, 18 tag SNPs were selected for genotyping ($r^2 < 0.8$ and $MAF > 0.05$) using Haploview software in the region of interest: *CDKN2A/B* and ANRIL, and encompassing the CAD-associated region. After genotyping, SNP rs8181047 was in tight linkage disequilibrium ($r^2 > 0.9$) with rs564398 in our sample, and it was excluded from further analysis (see Fig. 1). The tag SNPs and relevant associations are presented in Supplementary Table 3.

1.3. Cell culture

The culture media was Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and antibiotics 1% (v/v). For siRNA transfection, VSMC were incubated in DMEM supplemented with 10% FBS only, as recommended by the transfection agent manufacturer (see details in Supplementary Methods).

1.4. ANRIL silencing

Custom silencer select siRNA[®]s (Ambion) was designed to target ANRIL (NCBI Reference Sequence: NR_003529.3) in its exon 19 (see Fig. 4B and Supplementary Methods). Universal negative control siRNA #1 (Ambion) was used as a control for all the experiments. Lipofectamine RNAiMax was purchased from Invitrogen and used as a transfection reagent according to the manufacturer's instructions (details in supplementary Methods). The cells were incubated for 48 h before RNA extraction for RT-PCR evaluation and 72 h before the proliferation assay.

1.5. RNA isolation and real time PCR

See Supplementary Methods.

1.6. Cell proliferation assay

Aortic VSMC were cultured in 96-well plates and cell proliferation was measured using a cell proliferation colorimetric assay (CCK-8, Dojindo) according to the manufacturer's instructions. Absorbance was measured 72 h after treatment with siRNA using a microplate reader (Multi-Spectrophotometer, Viento, Dainippon) at a wavelength of 450 nm.

1.7. Immunostaining in mouse atherosclerotic plaque

C57BL/6Jcl wild type and APOE^{-/-} mice (C57BL/6Jcl background) fed a normal diet, were used to obtain normal and atherosclerotic aortic sections at the age of 24 weeks (see Supplementary Methods).

1.8. Statistical analysis

Expression and genotype association results were analyzed using JMP software (version 4, SAS Institute Inc.) and Prism 5 for Windows (version 5.02, GraphPad Software Inc.). The results were analyzed using analysis of variance (ANOVA) and the *t*-test. *P*-values lower than 0.05 were considered significant.

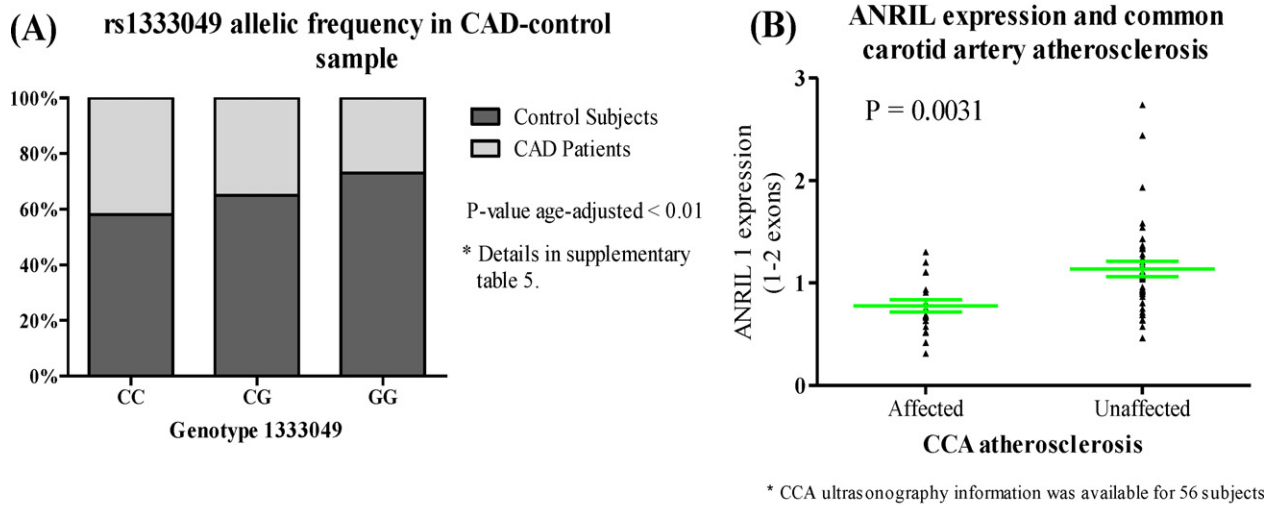
2. Results

2.1. Confirmation of 9p21 association in the Japanese sample

We assessed the possible association of the three SNPs: rs1333049, rs564398, and rs2811712 with CAD. We confirmed the rs1333049 association with CAD in our sample (Controls CC:101; CG:224; GG:122; Cases CC:72; CG:116; GG:43; age adjusted *P*-value < 0.01) (Fig. 2A); however, we were not able to detect any significant association of CAD with either rs564398 or rs2811712 (see Supplementary Table 4).

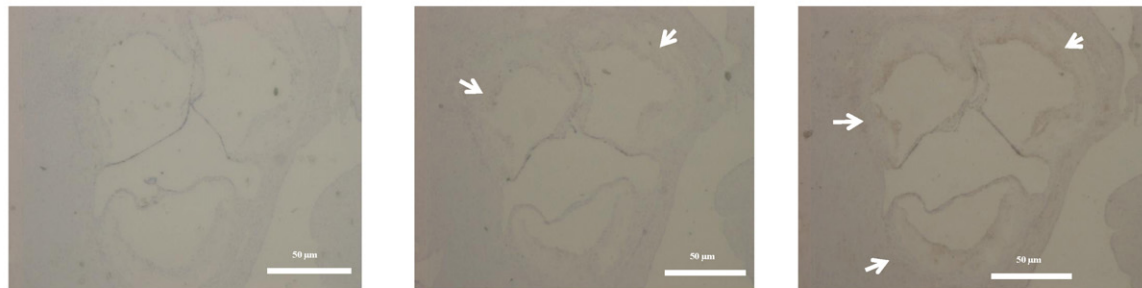
2.2. Expression analysis of CDKN2A/B and ANRIL in blood

The linkage disequilibrium (LD) map for the SNPs genotyped in this study and a transcriptional representation of the locus are



(C) Immunostaining

Atherosclerotic Aorta Section in APOE KO Mice

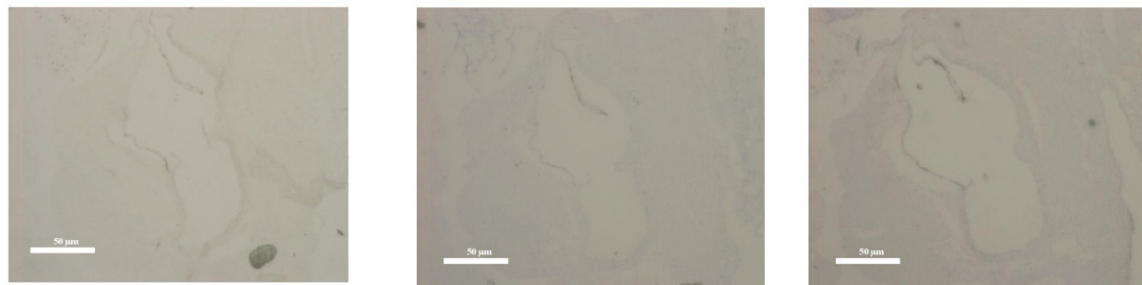


(-) control

CDKN2A

CDKN2B

Aorta Section in Wild-Type Mice



(-) control

CDKN2A

CDKN2B

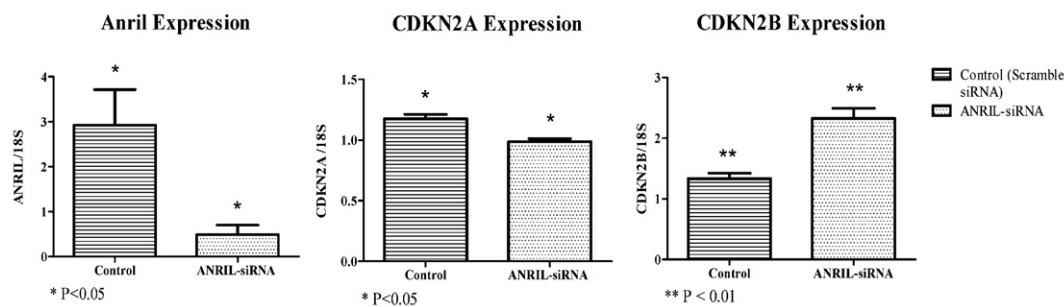
Fig. 3. (A) Association study results: the figure shows the allelic frequency of rs1333049 in our Japanese CAD-control sample and confirms the association of the SNP with coronary artery disease. (B) Comparative expression of ANRIL in subjects from SONIC Study with and without CCA atherosclerosis. (C) Immunostaining of atherosclerotic and normal aortic sections with antibodies against CDKN2A, B and negative control.

presented in Fig. 1. Expression levels of *CDKN2A*, *CDKN2B*, and ANRIL (with the exception of ANRIL in exons 17–18 with *CDKN2B*) were positively correlated (correlation plots, r and P -values shown in Supplementary Fig. 1), consistent with previous reports suggesting that the expression of these genes is co-regulated [18,21]. Common carotid artery atherosclerosis, a known predictor of ischemic stroke [22], was associated with a significantly lower expression of ANRIL (exons 1–2) (P -value < 0.01) (Fig. 2B).

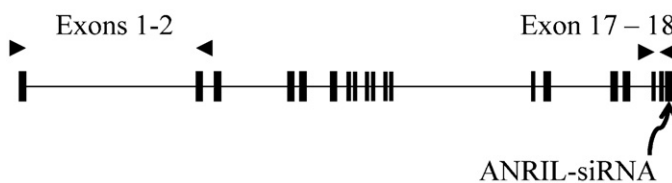
Several SNPs showed an effect in the expression of the 9p21 locus transcripts as shown in Table 1 and Supplementary Figs. 3–5;

and the most significant effects detected in this study affected ANRIL (exons 1–2) expression as shown in Fig. 3. Remarkably most of the risk alleles for atherosclerosis-related phenotypes (all except rs1333049, which did not reach significance) were associated with a lower expression of the transcript, at least when evaluating the exons 1–2 (see Fig. 3). The leading CAD-associated SNP, rs1333049 was not significantly associated with the expression of either of the transcripts but when grouped risk allele versus others, the P -value was 0.0653 (Supplementary Fig. 5), and again, the risk allele seemed to be associated with a lower expression of the first exons of ANRIL.

(A) ANRIL knock down efficiency (left), CDKN2A (center) and CDKN2B (right) expression after ANRIL Knock down



(B) RT-PCR primers and ANRIL-siRNA target



(C) ANRIL knock down effect in proliferation of VSMC

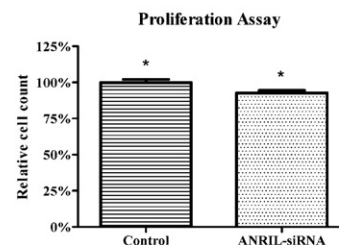


Fig. 4. (A) Anril Knock-down efficiency and effect of ANRIL knock-down on CDKN2A and B expression. (B) Transcriptional representation of ANRIL and RT-PCR primers and siRNA target location. (C) Cell proliferation results: relative cell counts of VSMCs following ANRIL-siRNA treatment.

2.3. Evidence of ANRIL-mediated regulation of CDKN2A/B expression

We performed siRNA knock-down of ANRIL in VSMC, as described in Section 1, and examined the expression of CDKN2A/B. The knock-down efficiency was confirmed by RT-PCR and ANRIL expression is presented in Fig. 4A and Supplementary Fig. 6. We noted a marked increase in the expression of CDKN2B and, interestingly, a slight but significant decrease in CDKN2A expression as shown in Fig. 4A.

2.4. ANRIL affects proliferation in VSMC

We measured proliferation in VSMC 72 h after siRNA treatment. ANRIL knock-down caused a reduced viable cell count in VSMC consistent with the over-expression of CDKN2B. The relative cell counts are shown in Fig. 4C.

2.5. CDKN2A/B expressed in mouse atherosclerotic plaque

Since our results linked the expression of ANRIL with risk alleles at the atherosclerosis-associated 9p21 locus, and also suggested that ANRIL is involved in the regulation of CDKN2A/B, we considered relevant to investigate the expression of these genes in atherosclerotic plaque *in vivo*. We detected the presence of both CDKN2A and CDKN2B in mouse aortic plaque on immunostaining, which supports our hypothesis that CDKN2A/B genes at least partially mediate the genetic susceptibility to atherosclerosis found at the locus 9p21 (Fig. 3C).

3. Discussion

Since the GWAs have revealed a CAD association at the 9p21 locus, researchers have hypothesized a potential regulatory role

of the associated SNPs in the expression of ANRIL, CDKN2A, and CDKN2B. However, publications demonstrating a correlation between the risk genotypes and the expression of the transcripts in the locus have reported contradictory results [18,23,24], highlighting the need to replicate such studies to clarify true associations. Probably, this lack of consistency is due to the splicing complexity of ANRIL and characteristics specific to the sample (ethnicity, age, health status, etc.). Since CDKN2A is a known marker of aging [25] and increases in expression with age in several tissues of rodents and humans [26] and ANRIL has been connected with cell senescence [4], we believe the very narrow age range (69–72 years old) of our subjects maximizes the detection of possible genotype effects. Here, we showed that several SNPs in the region affect the expression of the locus, particularly in the case of previously disease-associated SNPs. CDKN2A/B were prime candidates in the association of these SNPs, however, most of the significant associations found in this report pointed at ANRIL rather than CDKN2A/B. Despite the limitation of this study regarding the number of subjects, we were able to reproduce previously reported associations, as in the case of rs564398 and rs496892 and their effect on ANRIL expression [18]. Remarkably, the vascular disease related risk alleles seem to be consistently associated with the under-expression of the first exons of ANRIL at least in the peripheral blood. The CCA atherosclerosis phenotype was also correlated with reduced expression of ANRIL, with both results pointing to a possible protective role of ANRIL in the progression of atherosclerosis.

ANRIL is antisense to CDKN2B (also known as p15), a situation that typically implies an inhibitory effect in the sense sequence expression. Here, we established a correlation between the expression of ANRIL and CDKN2A/B in human aortic VSMC. The effect of ANRIL in CDKN2B expression identified in this report is consistent with the previously reported role of ANRIL in the repression of the locus CDKN2A/B [4]. Accordingly, in the situation of the

absence or reduction of ANRIL (by knock-down), the repression of the locus would be impaired and *CDKN2B* would be up-regulated. On the other hand, the PR complex hypothesis does not explain the reduction of *CDKN2A* following ANRIL silencing which seems to be operated by another mechanism. Even though the role of ANRIL in the repression of the locus *CDKN2A/B* through Polycomb proteins has been highlighted in recent publications, the specific role of each splicing variant of ANRIL as well as the totality of possible functions of the transcripts are still poorly understood. A clue to the wide spectrum of ANRIL functions is provided by Sato et al. [27], where a subset of genes involved in the regulation of the nucleus and chromatin architecture were up-regulated after the over-expression of a novel splicing variant of ANRIL. We believe that the repression of *CDKN2A* presented in this report is due to mechanisms that may be specific to VSMCs or to the splicing variants targeted in the experiment. Additionally, the reduction in proliferation caused by ANRIL knock down is consistent with the increased expression of anti-proliferative gene *CDKN2B*. Although, the deletion of the CAD risk region in a mouse model led to an exacerbated proliferation in SMC [28], which seems inconsistent with the results presented here, another report showed that inhibition of ANRIL causes reduced proliferation in human fibroblasts [4]. ANRIL does not have a homologous transcript described in mice, and ANRIL functions could be specific to humans.

These findings emphasize the importance of ANRIL in cell proliferation and regulation of the locus *CDKN2A/B* in a cell line directly involved in the pathogenesis of atherosclerosis. Overall, our results suggest that several SNPs in 9p21 locus affect the expression of ANRIL, which is further in control of the regulation of *CDKN2A/B* and cell growth. Cell proliferation mediates the progression of atherosclerosis and is also directly or indirectly involved in the pathogenesis of diseases associated with this locus.

In our study, expression of exons 17–18 was strongly reduced confirming ANRIL-siRNA efficiency, however, expression of exons 1–2 did not show significant reduction (Supplementary Fig. 6) suggesting the existence of novel splicing variants at least in VSMC (possibly comprising the last exons but lacking the first ones). Further characterization of all ANRIL splicing variants and their functions is needed for a better understanding of their role in cell physiology, atherosclerosis, and ultimately human health. However, our results point at ANRIL as a potential major player in human vascular disease.

Acknowledgements

We would like to express our gratitude to Ms. Yuki Imaizumi and Ms. Takako Baba for their continuous support in this investigation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.11.017.

References

- [1] The Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3000 shared controls. *Nature* 2007;447:661–78.
- [2] Broadbent HM, Peden JF, Lorkowski S, et al. Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked, SNPs in the ANRIL locus on chromosome 9p. *Hum Mol Genet* 2008;17:806–14.
- [3] Gill J, Peters G. Regulation of the INK4b–ARF–INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol* 2006;7:667–77.
- [4] Yap KL, Li S, Muñoz-Cabello AM, et al. Molecular Interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by Polycomb CBX7 in transcriptional silencing of INK4a. *Mol Cell* 2010;38:662–74.
- [5] Kotake Y, Nakagawa T, Kitagawa K, et al. Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15INK4B tumor suppressor gene. *Oncogene* 2011;30:1956–62.
- [6] Helgadottir A, Thorleifsson G, Magnusson KP, et al. The same sequence variant on 9p21 associates with myocardial infarction, abdominal aortic aneurysm and intracranial aneurysm. *Nat Genet* 2008;40:217–24.
- [7] Gschwendtner A, Bevan S, Cole JW, et al. International stroke genetics consortium, sequence variants on chromosome 9p21.3 confer risk for atherosclerotic stroke. *Ann Neurol* 2009;65:531–9.
- [8] Yang XR, Liang X, Pfeiffer RM, et al. Associations of 9p21 variants with cutaneous malignant melanoma, nevi, and pigmentation phenotypes in melanoma-prone families with and without *CDKN2A* mutations. *Familial Cancer* 2010;9:625–33.
- [9] Falchi M, Bataille V, Hayward NK, et al. Genome-wide association study identifies variants at 9p21 and 22q13 associated with development of cutaneous nevi. *Nat Genet* 2009;41:915–9.
- [10] Sherborne AL, Hosking FJ, Prasad RB, et al. Variation in *CDKN2A* at 9p21.3 influences childhood acute lymphoblastic leukemia risk. *Nat Genet* 2010;42:492–4.
- [11] Schaefer AS, Richter GM, Groessner-Schreiber B, et al. Identification of a shared genetic susceptibility locus for coronary heart disease and periodontitis. *PLoS Genet* 2009;5:e1000378.
- [12] Ernst FD, Uhr K, Teumer A, et al. Replication of the association of chromosomal region 9p21.3 with generalized aggressive periodontitis (gAgP) using an independent case–control cohort. *BMC Med Genet* 2010;11:119.
- [13] Yu JT, Yu Y, Zhang W, et al. Single nucleotide polymorphism rs1333049 on chromosome 9p21.3 is associated with Alzheimer's disease in Han Chinese. *Clin Chim Acta* 2010;411:1204–7.
- [14] Emanuele E, Lista S, Ghidoni R, et al. Chromosome 9p21.3 genotype is associated with vascular dementia and Alzheimer's disease. *Neurobiol Aging* 2011;32:1231–5.
- [15] Uno S, Zembutsu H, Hirasawa A, et al. A genome-wide association study identifies genetic variants in the *CDKN2BAS* locus associated with endometriosis in Japanese. A genome-wide association study identifies genetic variants in the *CDKN2BAS* locus associated with endometriosis in Japanese. *Nat Genet* 2010;42:707–10.
- [16] Burdon KP, Macgregor S, Hewitt AW, et al. Genome-wide association study identifies susceptibility loci for open angle glaucoma at *TMCO1* and *CDKN2BAS1*. *Nat Genet* 2011;43:574–8.
- [17] Ramdas WD, van Koolwijk LM, Lemij HG, et al. Common genetic variants associated with open-angle glaucoma. *Hum Mol Genet* 2011;20:2464–71.
- [18] Cunnington MS, Koref MS, Mayosi BM, Burn J, Keavney B. Chromosome 9p21 SNPs associated with multiple disease phenotypes correlate with ANRIL expression. *PLoS Genet* 2010;6:e1000899.
- [19] Cugino D, Gianfagna F, Santimone I, et al. Type 2 diabetes and polymorphisms on chromosome 9p21: a meta-analysis. *Nutr Metab Cardiovasc Dis NMCD* 2011;(April), doi:10.1016/j.numecd.2010.11.010 [Epub ahead of print].
- [20] Melzer D, Frayling TM, Murray A, et al. A common variant of the p16INK4a genetic region is associated with physical function in older people. *Mech Ageing Dev* 2007;128:370–7.
- [21] Pasmant E, Laurendeau I, Héron D, Vidaud M, Vidaud D, Bièche I. Characterization of a germ-line deletion. Including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense noncoding RNA whose expression colusters with ARF. *Cancer Res* 2007;67:3963.
- [22] Alexandrov AV. Cerebrovascular ultrasound in stroke prevention and treatment. Blackwell Publishing; 2004. p. 161–9 [chapter 9].
- [23] Jarinova O, Stewart AFR, Roberts R, et al. Functional analysis of the chromosome 9p21.3 coronary artery disease risk locus. *Arterioscler Thromb Vasc Biol* 2009, doi:10.1161/atvbaha.109.189522.
- [24] Holdt LM, Beutner F, Scholz M, et al. ANRIL expression is associated with atherosclerosis risk at chromosome 9p21. *Arterioscler Thromb Vasc Biol* 2010, doi:10.1161/atvbaha.109.196832.
- [25] Liu Y, Sanoff HK, Cho H, et al. Expression of p16INK4a in peripheral blood T-cells is a biomarker of human aging. *Ageing Cell* 2009;4:439–48.
- [26] Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. *Cell* 2006;127:265–75.
- [27] Sato K, Nakagawa H, Tajima A, Yoshida K, Inoue I. ANRIL is implicated in the regulation of nucleus and potential transcriptional target of E2F1. *Oncol Rep* 2010;24:701–7.
- [28] Visel A, Zhu Y, May D, et al. Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice. *Nature* 2010;464:409–12.
- [29] Chen J, Li D, Wei C, et al. Aurora-A and p16 polymorphisms contribute to an earlier age at diagnosis of pancreatic cancer in Caucasians. *Clin Cancer Res* 2007;13:3100–4.
- [30] Yang XC, Zhang Q, Chen ML, et al. MTAP and *CDKN2B* genes are associated with myocardial infarction in Chinese Hans. *Clin Biochem* 2009;42:1071–5.
- [31] Pechlivanis S, Scherag A, Mühleisen TW, et al. Coronary artery calcification and its relationship to validated genetic variants for diabetes mellitus assessed in the Heinz Nixdorf recall cohort. *Arterioscler Thromb Vasc Biol* 2010;30:1867–72.
- [32] McPherson R, Pertsemlidis A, Kavaslar N, et al. A common allele on chromosome 9 associated with coronary heart disease. *Science* 2007;316:1488–91.
- [33] Takeuchi F, Serizawa M, Yamamoto K, et al. Confirmation of multiple risk loci and genetic impacts by a genome-wide association study of type 2 diabetes in the Japanese population. *Diabetes* 2009;58:1690–9.