Original Article

The Severity of Internal Carotid Artery Stenosis is Associated with the Cyclin-Dependent Kinase Inhibitor 2A Gene Expression

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***Aim*: The INK4b-ARF-INK4a locus in the chromosome 9p21 region is known to play an important role in the development of atherosclerosis. The INK4/ARF transcript $p16^{INK4a}$ inhibits the activity of the cyclin-dependent kinases CDK4/CDK6 and arrests cell-cycle progression. CDK inhibitors also regulate G1/S phase progression in vascular smooth muscle cells (VSMCs) and may modulate the early stages of atherosclerosis. Therefore, we aimed to study the expression of the INK4/ARF locus genes CDKN2A and CDKN2BAS in order to examine the $p16^{INK4a}$ protein expression and the level of cell proliferation in carotid plaques and saphenous tissue samples.**

***Methods*: A total of 50 patients (33 symptomatic subjects and 17 asymptomatic subjects) with carotid atherosclerosis (CA) were studied. The CDKN2A and CDKN2BAS gene expression levels were determined using quantitative real-time polymerase chain reaction (qRT-PCR). All tissue sections were also analyzed for the $p16^{INK4a}$ and proliferating cell nuclear antigen (PCNA) protein expression using immunohistochemistry (IHC).**

***Results*: The CDKN2A gene expression was significantly higher in the carotid plaques than in the saphenous tissues ($p=0.009$), whereas no such differences were observed in the CDKN2BAS transcripts ($p=0.157$). The carotid plaque CDKN2A mRNA levels were higher in the symptomatic patients than in the asymptomatic patients ($p=0.050$); this finding was also associated with the severity of internal carotid artery (ICA) stenosis ($p=0.034$). The $p16^{INK4a}$ immune (+) cell counts in the carotid plaques were higher in the symptomatic patients than in the asymptomatic patients ($p=0.056$), as was the cell proliferation index ($p=0.001$).**

***Conclusions*: An increased CDKN2A gene expression in carotid plaques may increase the severity of ICA stenosis, thus raising the risk of atherosclerosis and contributing to the development of symptoms. In addition, the $p16^{INK4a}$ expression is associated with carotid atherosclerosis in various patient subgroups.**

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Key words: Carotid atherosclerosis, CDKN2A, CDKN2BAS, Cell cycle, Gene expression

Introduction

Atherosclerosis, a condition that increases the risk of myocardial infarction (MI) and stroke, remains the leading cause of death in the Western world. In humans, the accumulation of atherosclerotic plaque within the intima of the vascular wall develops over many decades. This process involves the actions of inflammatory cells, lipids, smooth muscle cells (SMCs) and extracellular matrix (ECM) proteins[1)]. Carotid atherosclerosis (CA) is defined as atherosclerotic stenosis of the proximal internal carotid artery

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(ICA) and is one of the primary causes of stroke. In cases in which the degree of stenosis is ≥70%, carotid endarterectomy (CEA) is one of the most suitable surgical interventions and may be prophylactic against the onset of stroke[2-5]. Therefore, conducting biological examinations of CEA specimens may be useful for understanding the cellular and molecular mechanisms underlying CA.

Recent genome-wide association studies (GWAS) have indicated that the chromosome 9p21 INK4b-ARF-INK4a is a novel locus for susceptibility to coronary artery disease (CAD), MI and cardiac death[6-10], independent of traditional risk factors, including gender, age, obesity, smoking, hypertension and hyperlipidemia[11, 12]. The INK4/ARF locus has also been linked to other diseases, such as intracranial aneurism formation[13], type-2 diabetes[14, 15] and metabolic syndrome[16]. The SNPs most closely associated with cardiovascular diseases do not map within an annotated gene sequence. Therefore, neighboring cyclin-dependent kinase inhibitors (CKIs), including CDKN2A and CDKN2B, and methylthioadenosine phosphorylase (MTAP) genes were initially suggested to be candidate susceptibility genes for CAD and MI[17]. The two CKIs, CDKN2A/B, are key modulators of cell proliferation and senescence. There is thus the possibility that they mediate these cellular events under conditions of cardiovascular pathology. CDKN2A/B encode $p16^{INK4a}$ and $p15^{INK4b}$, respectively, which regulate cell cycle progression. A newly annotated, large antisense non-coding RNA gene, CDKN2BAS (also known as ANRIL, Antisense Non-coding RNA in the INK4 Locus), has also been identified, the functions of which remain somewhat unclear[18].

CDKN2BAS is a non-coding RNA expressed in tissues and cell types affected by atherosclerosis, such as endothelial cells, vascular SMCs and immune cells[14]. CDKN2BAS transcripts have been shown to regulate cell senescence and proliferation in various cell lines. Additionally, CDKN2BAS has been demonstrated to exert regulatory effects upon its neighbors, including CDKN2A/B via histone modification[19-21]. CDKN2BAS has been suggested to regulate senescence at the CDKN2A locus, with an associated senescence-dependent role in proliferation.

The molecular and cellular basis of the association between the 9p21 region and CA is not fully understood. Dysregulation of the cell cycle may promote pathologic vascular proliferation, which has the potential to accelerate the development of atherosclerosis. Furthermore, the INK4b-ARF-INK4a locus may influence the properties of blood vessels, resulting in susceptibility to a broad range of vascular diseases. To date, no clear associations between the CA-related gene and protein expression levels have been elucidated. We therefore decided to explore the possible role of the 9p21 region gene CDKN2BAS NR_003529.3 in CDKN2A-mediated regulation in vascular tissues and the development of CA symptoms. In order to shed light on the possible influence of the relative expression of the CDKN2BAS NR_003529.3 and CDKN2A genes on the pathophysiology of symptomatic and asymptomatic CA, we investigated the possible associations between the expression levels of candidate genes within the 9p21.3 locus and stiffness of the carotid arteries in elderly individuals. We also investigated the expression of the CDKN2A gene product, $p16^{INK4a}$, in symptomatic and asymptomatic plaques and saphenous tissue samples. We further calculated the cell proliferation index in the plaques and saphenous tissues by measuring the PCNA expression, an important marker of the progression of atherosclerosis. To our knowledge, no previous studies have examined the relationship between 9p21 region genes and symptomatic and asymptomatic subgroups of CA plaques using saphenous vein tissues.

Materials and Methods

Study Subjects

The study group consisted of 50 Turkish patients diagnosed with CA. A total of 33 symptomatic and 17 asymptomatic CA patients were compared with respect to the gene and protein expression levels. The potential subjects underwent cardiovascular examinations, and eligible patients were those with high-grade (≥70%) atherosclerotic stenosis of the carotid artery on ultrasound/angiography who had undergone CEA. Selected patients with ≥70% stenosis of the ICA who had experienced cerebrovascular episodes of stroke, TIA or amaurosis fugax prior to the carotid artery examination were classified as symptomatic. Selected patients with ICA stenosis ≥70% but lacking clinical symptoms were classified as asymptomatic. Patients with CA were selected from among those treated at the Istanbul University Cerrahpasa Medical Faculty, Heart and Vessel Surgery Department. The study protocol was approved by the Local Ethics Committee of Istanbul Medical Faculty, Istanbul, Turkey. All participants provided their written informed consent prior to participation. All subjects were from the same geographical area, with similar ethnic and socioeconomic backgrounds, as assessed based on their responses to a health questionnaire. All of the patients were Turkish and of middle-income status. The mean age of the symptomatic group was 66.52±8.12 years (range:

48-83), while that of the asymptomatic group was 65.65±7.83 years (range: 52-79). The exclusion criteria included autoimmune disease, severe kidney and/or hepatic disease, cancer or pregnancy.

Carotid Plaque and Saphenous Vein Tissue Collection

The carotid plaques and saphenous vein tissues were excised into sterile tubes at the time of CEA surgery. All plaques were divided into two sections: one section was immediately fixed for 16 hours in buffered formalin and later embedded in paraffin for the immunohistochemical examination, while the other was immediately stored at −80℃ until further processing for RNA extraction.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the plaques and saphenous specimens using a High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNase 1 treatment was also provided according to the manufacturer's instructions. The quantity of RNA was measured using a Nanodrop Spectrophotometer (ThermoScientific). The total RNA samples were stored at −80℃ until use. A total of 100 ng of total RNA was reverse transcribed using random hexamers as primers and reverse transcriptase with a total volume of 20 μL. The expression levels of the CDKN2A and CDKN2BAS genes in the carotid plaques and saphenous veins were determined according to quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 1.5® instrument (Roche Applied Biosystems™, California, USA) with Universal Probe Library (UPL) probes and a LightCycler® TaqMan® Master kit (Roche Diagnostics GmbH, Mannheim, Germany). The primers and probes chosen from Roche's UPL system were as follows: CDKN2A (Accession no: NM_000077.4) with UPL Probe #34; CDKN2BAS (Accession no: NR_00003529.3) with UPL Probe #44; and HPRT (Accession no: NM_000194.2) with UPL Probe #73. The CDKN2A and CDKN2BAS mRNA levels were normalized to that of HPRT (Accession no:NM_000194.2), which was used as an endogenous reference gene[22]. The amplicon lengths of CDKN2A, CDKN2BAS and HPRT were 132 nt, 67 nt and 102 nt, respectively. The primer sequences were as follows: *CDKN2A*, 5′-GTG GAC CTG GCT GAG GAG-3′ (forward) and 5′-CTT TCA ATC GGG GAT GTC TG-3′ (reverse); *CDKN2BAS*, 5′-TTG TTA GAA ACC AGG CTG CAC-3′ (forward) and 5′-TTC TCT CTT TCT GTG GTT TCT CAA T-3′ (reverse); *HPRT*, 5′-TGA CCT TGA TTT ATT TTG CAT ACC-3′ (forward) and 5′-CGA GCA AGA CGT TCA GTC CT-3′ (reverse). Five serial dilutions of the control sample were used to calculate the PCR efficiency. Reaction mixtures without a cDNA template were utilized as negative controls. Ct values of 40 were excluded from further mathematical calculations, and the experiments were repeated twice.

Immunohistochemistry for p16 and PCNA

Tissues

The atherosclerotic carotid plaques and saphenous vein tissues were removed and fixed in neutral-buffered 10% formalin, and 5-μm-thick paraffin-embedded sections were immunostained with $p16^{INK4a}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Proliferating Cell Nuclear Antigen (PCNA) (LabVision Corp, Fremont, CA, USA) antibodies using the streptavidin-biotin-peroxidase technique.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed using neutral-buffered, 10% formalin-fixed, paraffin-embedded specimens. The 5-μm sections were dewaxed in toluene and rehydrated in graded alcohol. The Histostain Plus Bulk Kit (Invitrogen Corp., USA) was used for immunoperoxidase staining. IHC was performed as outlined in the previously described protocol[23]. The sections were counterstained with Mayer's hematoxylin (Invitrogen Corp., USA) and mounted. In order to determine the specificity of immunostaining, a negative control was always used in which the primary antibody was omitted. Mouse IgG (Santa Cruz Biotechnology, Santa Cruz CA, USA) was used as a negative control[23, 24].

The immunostained $p16^{INK4a}$ and PCNA cells were counted using a Leica DM 2500 light microscope with a Leica DFC280 digital camera system (Leica Microsystems, Wetzlar, Germany), and the results were expressed as ($p16^{INK4a}$ or PCNA immunopositive cell number/total cell number) ×100. Cell counting was performed under 400× magnification, with two researchers examining different fields at different times in a single-blind fashion. At least 10 fields per slide were evaluated for every patient sample[23, 24].

Statistics

The statistical analyses were performed using Student's *t*-test to compare variables such as age, the serum glucose level, body mass index (BMI) and the plasma lipid levels between the symptomatic and asymptomatic groups. The data are expressed as the

Table 1a. Comparison of the CDKN2A and CDKN2BAS mRNA levels in the carotid plaques and saphenous tissues

mRNA expression level	Carotid Plaque	Saphenous Tissue	*p*
CDKN2A	(*n*=43)	(*n*=48)	0.009
	0.729 ± 0.10	0.381 ± 0.07	
CDKN2BAS	(*n*=21)	(*n*=17)	0.157
	0.0092 ± 0.0037	0.0031 ± 0.0006	

The *t*-test was performed. The data are presented as the mean ± SE.

Table 1b. Relative expression levels of the CDKN2A and CDKN2BAS genes in the carotid plaques and saphenous tissues determined using the REST 2009 software program

Gene	Type	mRNA expression*	Std.Error	95%C.I.	P(H1)	Result
HPRT	REF	1.00				
CDKN2A	TRG	1.498	0.879-2.524	0.557-4.496	<0.001	UP**
CDKN2BAS	TRG	0.822	0.293-1.848	0.116-5.318	0.177	

REST 2009 was used for the statistical analysis.
*The expression levels were normalized to that of the HPRT housekeeping gene.
**CDKN2A is upregulated in the carotid plaques (in comparison to that observed in the saphenous tissues).
REF, reference; TRG, target

mean ± standard deviation (SD). The chi-square test (χ^2) was used to assess categorical variables. A p value of <0.05 was considered to be statistically significant.

Ct values for the qRT-PCR analysis were calculated according to the $\Delta Ct = 2^{(Ct\ reference\ gene - Ct\ target\ gene)}$ formula in order to determine the relative expression level of the target gene. The results were analyzed using Student's *t*-test, and the data are presented as the mean ± standard error (SE).

A more sophisticated analysis of the qRT-PCR results was performed using the Relative Expression Software Tool, REST 2009 (Corbett Research Pty LtD and Michael Pfaffl, New South Wales, AU), which includes an algorithm for PCR efficiency, normalization and 95% confidence interval calculations[25]. The reaction efficiency was calculated using five different dilutions of the cDNA template. The software's hypothesis test, P(H1), determines the probability that the difference between two groups is due to chance. Therefore, a P(H1) of <0.05 indicates a significant difference between the two groups, with the probability that the difference is due to chance being less than 5%. The REST 2009 and Student's *t*-test results were consistent with each other.

The relative gene and protein expression levels were also compared using Spearman's non-parametric correlation test. All statistical analyses were performed using the SPSS Version 17.0 software package.

Results

Demographic Data of the Symptomatic and Asymptomatic Patients

The demographic and clinical characteristics of the symptomatic and asymptomatic subgroups are shown in **Supplemental Table 1a**. There were no significant differences in age, gender, BMI, smoking status or the levels of total cholesterol, HDL, LDL, triglycerides, fasting glucose, hematocrit, urea, creatine and ALT ($p>0.05$) between the subgroups. In contrast, a significant difference was noted in the AST levels between the symptomatic and asymptomatic patients ($p=0.014$). The severity of stenosis in the symptomatic and asymptomatic subgroups is shown in **Supplemental Table 1a**. A significant difference was found in the severity of ICA stenosis between the symptomatic and asymptomatic patients: the degree of ICA stenosis in the symptomatic patients was more severe than that observed in the asymptomatic patients ($\chi^2=11.022$; $p=0.001$). The pharmacological treatments used in the symptomatic and asymptomatic subgroups are shown in **Supplemental Table 1b**. The patient subgroups were compared according to the active use of treatment with aspirin, ACE-Is/ARBs, statins, antihypertensive drugs, oral antidiabetic drugs and insulin. No significant differences were noted between the medications of the symptomatic and asymptomatic CA patients (**Supplemental Table 1b**).

Table 2. CDKN2A and ANRIL mRNA levels in the symptomatic and asymptomatic CA patients

Tissue type/Gene	Symptomatic	Asymptomatic	p
Carotid	($n=33$)	($n=17$)	0.050
Plaque/CDKN2A	1.58 ± 0.30	0.75 ± 0.27	
Carotid	($n=15$)	($n=6$)	0.40
Plaque/ANRIL	0.0058 ± 0.0016	0.017 ± 0.0125	
Saphenous	($n=31$)	($n=17$)	0.77
Tissue/CDKN2A	0.36 ± 0.09	0.41 ± 0.12	
Saphenous	($n=12$)	($n=5$)	0.13
Tissue/ANRIL	0.0022 ± 0.0005	0.0052 ± 0.0015	

The t-test was performed. The data are presented as the mean ± SE.

Gene Expression Levels

qRT-PCR was used to determine the relative expression levels of the target genes by measuring the mRNA levels in the symptomatic and asymptomatic CA patients. The CDKN2A and CDKN2BAS mRNA levels in the carotid plaques and saphenous tissues are shown in **Table 1a**. The CDKN2A mRNA levels were significantly increased in the carotid plaques compared with those observed in the saphenous tissue samples ($t=2.68$, $p=0.009$), whereas the levels of CDKN-2BAS mRNA did not differ between the two types of specimens ($t=1.44$, $p=0.157$) (**Table 1a**). The relative expression levels of the CDKN2A and CDKN2BAS genes in the carotid plaques and saphenous tissues were also compared using the REST 2009 software program. The expression levels were normalized to that of the HPRT housekeeping gene. The relative CDKN2A mRNA levels were found to be significantly upregulated in the carotid plaques in comparison to that observed in the saphenous vein tissues ($p<0.001$). However no significant differences were observed in the CDKN2BAS relative mRNA levels between the carotid plaques and saphenous vein tissues ($p=0.177$). The REST 2009 software program and Student's t-test results were consistent with each other (**Table 1b**).

The symptomatic and asymptomatic subgroups of CA patients were also compared in terms of the CDKN2A and CDKN2BAS expression levels in the carotid plaques and saphenous tissues. In the carotid plaques of the symptomatic patients, the CDKN2A mRNA levels were significantly higher than those observed in the asymptomatic patients ($p=0.050$). However, there were no significant differences in the CDKN2BAS mRNA levels between the symptomatic and asymptomatic patients ($p=0.40$) (**Table 2**). In addition, no significant differences were observed in the CDKN2A mRNA expression levels between the saphenous vein tissues of the symptomatic and asymptomatic patients ($p=0.77$). In the saphenous vein tissues of the asymptomatic patients, the CDKN2BAS mRNA levels were found to be higher than those observed in the symptomatic patients; however, this trend did not reach statistical significance ($p=0.13$) (**Table 2**).

The severity of carotid artery stenosis is associated with an increased risk of cerebrovascular events. Such neurological symptoms are also associated with increased rates of stroke, transient ischemic attack (TIA), major and minor stroke, amaurosis fugax and reversible ischemic neurologic deficits (RIND). In addition, carotid stenosis is preferably treated with medical therapy in asymptomatic patients with $<80\%$ stenosis. Therefore, we separated the CA patients according to their clinical symptoms and the degree of ICA stenosis ($<80\%$ versus $\geq 80\%$).

The CDKN2A and CDKN2BAS mRNA expression levels of the CA patients were compared according to the severity of ICA stenosis. Interestingly, the severity of ICA stenosis was found to be associated with the expression of CDKN2A mRNA in the carotid plaques of the CA patients. The CDKN2A mRNA expression was also found to be significantly higher in the patients with ICA stenosis of $\geq 80\%$ ($t=-2.194$, $p=0.034$). However, there were no significant relationships between the CDKN2BAS expression level and the severity of ICA stenosis ($p=0.39$) (**Table 3**).

Immunostaining of $p16^{INK4a}$ and PCNA in the Human Vascular Tissues

We used immunohistochemical studies to confirm our gene expression findings. The (%)number of $p16^{INK4a}$ and PCNA immunopositive cells in the carotid plaques and saphenous tissue samples is shown in **Table 4**. Higher numbers of $p16^{INK4a}$ immunopositive cells were found in the symptomatic subgroup of CA patients than in the asymptomatic subgroup,

Table 3. CDKN2A and ANRIL (CDKN2BAS) mRNA expression levels in the CA patients according to the severity of ICA stenosis

mRNA expression level	ICA stenosis <80%	ICA stenosis ≥80%	*p*
CDKN2A	(*n*=19) 0.488±0.11	(*n*=24) 0.92±0.16	0.034
ANRIL	(*n*=9) 0.0129±0.008	(*n*=12) 0.0063±0.002	0.39

The *t*-test was performed. The data are presented as the mean ± SE.

Table 4. $p16^{INK4a}$ and PCNA immunopositive cell numbers (%) in the carotid plaques and saphenous tissue samples

$p16^{INK4a}$ expression	Symptomatic	Asymptomatic	*p*
Carotid plaque $p16^{INK4a}$ expression	16,40±2,82 (*n*=30)	9,49±2,09 (*n*=16)	0,056
Saphenous tissue $p16^{INK4a}$ expression	8,55±1,88 (*n*=25)	16,51±3,82 (*n*=12)	0,043
Cell proliferation index	Symptomatic	Asymptomatic	*p*
Carotid plaque PCNA expression	17,83±2,52 (*n*=30)	7,05±1,39 (*n*=17)	0,001
Saphenous tissue PCNA expression	3,76±1,52 (*n*=25)	1,00±0,91 (*n*=12)	0,23

The *t*-test was performed. The data are presented as the mean ± SE

although the difference was only just above the threshold of significance ($p=0.056$) (**Table 4**) (**Fig. 1A, 1D**). In the saphenous vein tissues, however, the numbers of $p16^{INK4a}$ immunopositive cells were found to be significantly higher in the asymptomatic subgroup than in the symptomatic subgroup ($p=0.043$) (**Table 4**) (**Fig. 1E-H**).

The number of $p16^{INK4a}$ immunopositive cells in the patients with ICA stenosis of ≥80% appeared to be increased in the carotid plaques (**Fig. 1B**). In addition, a decreased number of $p16^{INK4a}$ immunopositive cells was noted in the patients with ICA stenosis of <80%, as shown in **Fig. 1C**. However, this finding was not statistically significant ($p=0.23$).

The PCNA expression was found to be higher in the carotid plaques of the symptomatic patients than in those of the asymptomatic patients ($t=3.73$, $p=0.001$) (**Fig. 1I, 1L**) (**Table 4**). In addition, an increased number of PCNA immunopositive cells was observed in the patients with ICA stenosis of ≥80%, while a decreased number of PCNA immunopositive cells was detected in the patients with ICA stenosis of <80%, as shown in **Fig. 1J, 1K**. However, this finding did not reach a statistically significant level ($p=0.11$). Furthermore, PCNA immunopositive cells were observed in the saphenous vein tissues of both the symptomatic and asymptomatic CA patients, as shown in **Fig. 1M-P**. The number of PCNA immunopositive cells in the saphenous vein tissues of the symptomatic subgroup was higher than that observed in the asymptomatic subgroup among the CA patients. However, this finding was not statistically significant ($p=0.23$) (**Table 4**) (**Fig. 1M-P**).

Correlations Between the $p16^{INK4a}$ and PCNA Expression and that of CDKN2A and CDKN2BAS in the Carotid Plaques and Saphenous Tissue Samples

We next explored possible correlations between the gene and protein expression levels in the vascular tissues. We investigated the correlation between the $p16^{INK4a}$ and PCNA expression levels and the CDKN2A and CDKN2BAS NR_003529.3 mRNA expression levels in the carotid plaques. The level of CDKN2A mRNA was found to be significantly correlated with that of its product, $p16^{INK4a}$, in the carotid plaques ($r=0.54$, $p<0.001$) (**Fig. 2**). Additionally, the CDKN2A mRNA level was found to be positively correlated with that of the PCNA cell proliferation marker ($r=0.38$, $p=0.009$) (**Fig. 3**). We determined the CDKN2BAS expression in the plaque tissues and found positive correlations with the $p16^{INK4a}$ and PCNA expression levels ($r=0.30$, $p=0.21$; $r=0.33$, $p=0.16$, respectively). On the other hand, we observed an inverse correlation between the CDKN2A and CDKN2BAS mRNA levels ($r=-0.21$, $p=0.42$) in the

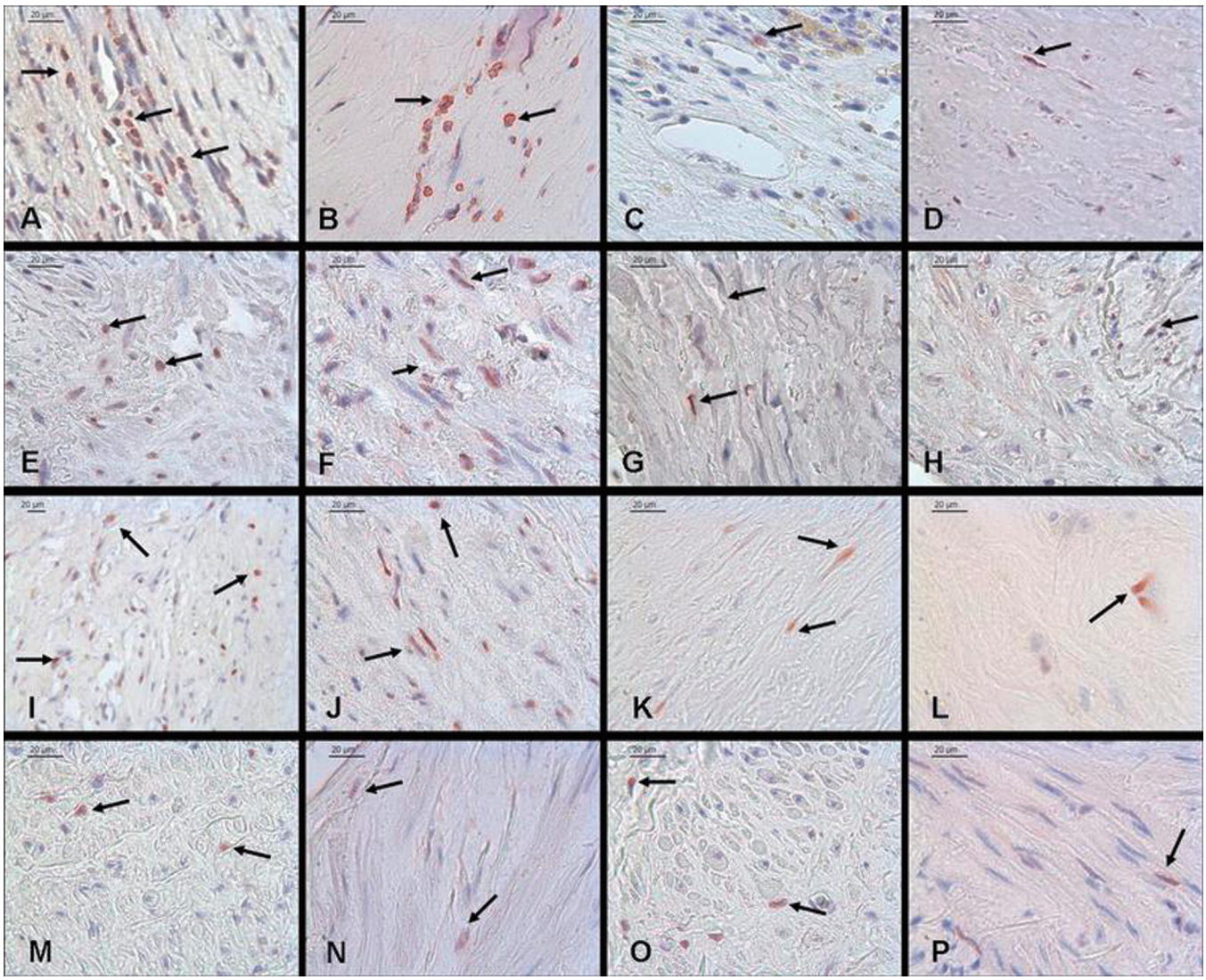


Fig. 1. Immunolocalization of $p16^{INK4a}$ and PCNA in the carotid plaques and saphenous vein tissues.

(A) $p16^{INK4a}$-positive cells in the carotid plaques of the symptomatic patients. (B) $p16^{INK4a}$-positive cells in the carotid plaques of the patients with ICA stenosis of ≥80%. (C) $p16^{INK4a}$-positive cells in the carotid plaques of the patients with ICA stenosis of <80%. (D) $p16^{INK4a}$-positive cells in the carotid plaques of the asymptomatic patients. (E, F) $p16^{INK4a}$-positive cells in the saphenous vein tissues of the asymptomatic patients. (G, H) $p16^{INK4a}$-positive cells in the saphenous vein tissues of the symptomatic patients. (I) PCNA-positive cells in the carotid plaques of the symptomatic patients. (J) PCNA-positive cells in the carotid plaques of the patients with ICA stenosis of ≥80%. (K) PCNA-positive cells in the carotid plaques of the patients with ICA stenosis of <80%. (L) PCNA-positive cells in the carotid plaques of the asymptomatic patients. (M, N) PCNA-positive cells in the saphenous vein tissues of the asymptomatic patients. (O, P) PCNA-positive cells in the saphenous vein tissues of the symptomatic patients. Counterstaining: Hematoxylin. (Bars: 20 μm).

carotid plaques, indicating that lesional tissue rich in CDKN2A mRNA contains little CDKN2BAS NR_003529.3 mRNA and vice versa. However, these results were not statistically significant. We also observed a positive correlation between the $p16^{INK4a}$ and PCNA expression levels ($r=0.41$, $p=0.005$) in the plaque tissues, suggesting that cell proliferation in plaque may be regulated by a functional CKI mechanism other than that involving $p16^{INK4a}$.

We also tested whether there were any correlations between the $p16^{INK4a}$ and PCNA expression levels and the CDKN2A and CDKN2BAS NR_003529.3 mRNA expression levels in the saphenous tissue samples. The CDKN2BAS NR_003529.3 expression level was not correlated with the CDKN2A mRNA level or the $p16^{INK4a}$ and PCNA protein levels. In addition, no

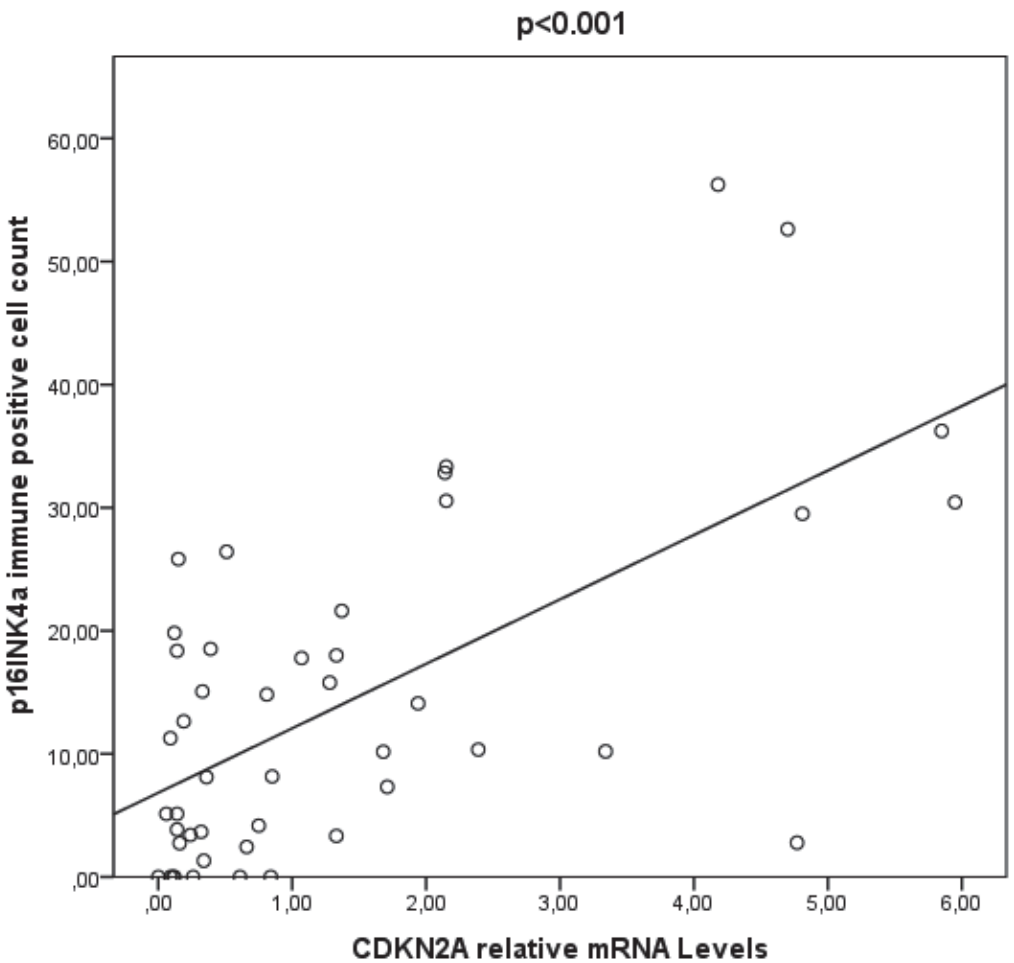


Fig. 2. Correlation between the CDKN2A mRNA expression and $p16^{INK4a}$ protein levels in the carotid plaques.

correlations were observed between the CDKN2A mRNA and PCNA expression levels in the saphenous tissue samples.

Discussion

Various studies have shown that the gene expressions and polymorphisms in the 9p21 locus may be associated with the development of CVD[26-28]. Genetic variation in this region has also been shown to be associated with severe premature atherosclerosis[29], angiographic CAD[30, 31] and the risk of progression of carotid atherosclerosis[32]. Variations in the 9p21 region can affect the expression of proximal genes. In the current study, we investigated the expression levels of the 9p21 genes CDKN2A and ANRIL and proteins $p16^{INK4a}$ and PCNA in human symptomatic and asymptomatic carotid atherosclerotic plaques and saphenous vein tissues and showed that the CDKN2A expression levels were significantly higher in the carotid plaques than in the saphenous tissues. We also showed for the first time that the CDKN2A mRNA expression is significantly higher in the plaques of symptomatic patients than in those of asymptomatic patients. In the current study, we did not observe any significant differences in clinical characteristics between the symptomatic and asymptomatic patients, except for the AST levels, which were higher in the asymptomatic patients than in the symptomatic patients. In addition, regarding active pharmacological treatment, the rate of statin use was higher in the asymptomatic subgroup than in the symptomatic subgroup. The higher frequency of statin use observed in the asymptomatic patients compared to the symptomatic patients may account for the higher AST levels in the asymptomatic subgroup. We also found that the severity of ICA stenosis was significantly associated with the CDKN2A mRNA level and that the CDKN2A expression may be associated

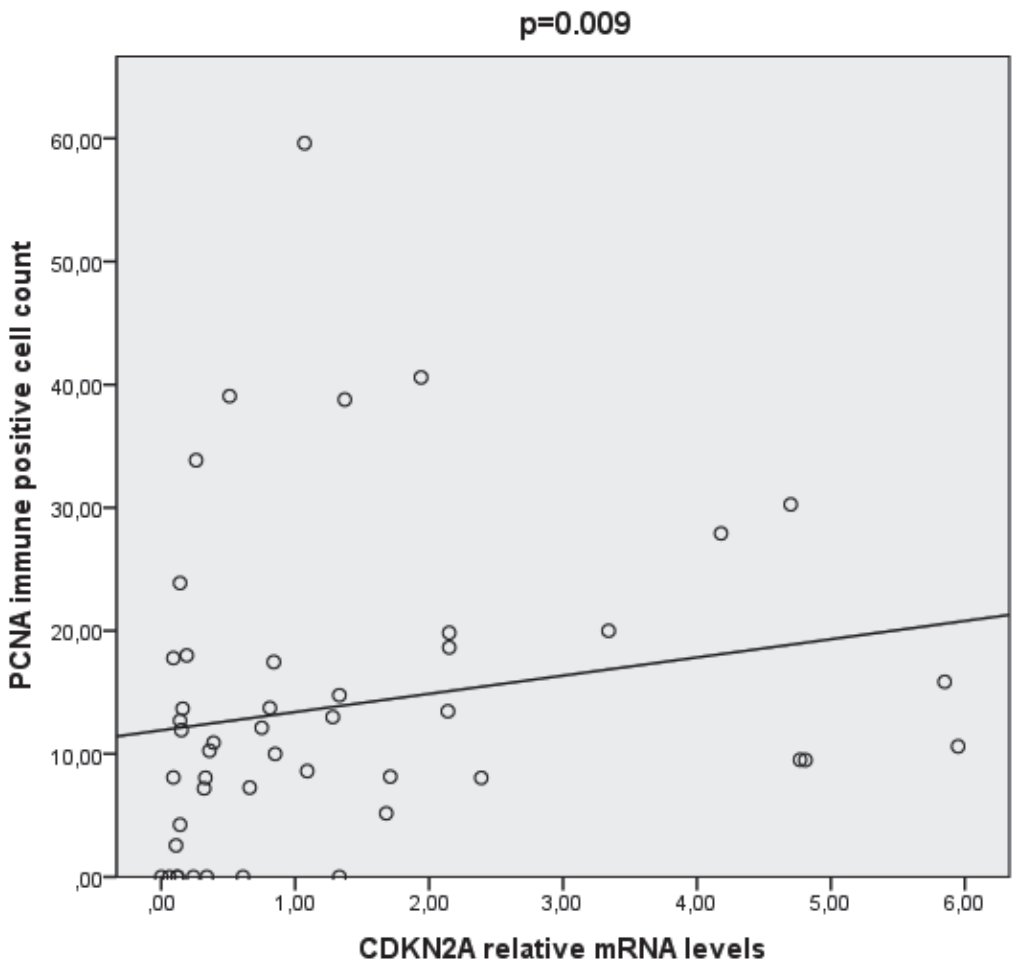


Fig. 3. Correlation between the CDKN2A mRNA expression and PCNA protein levels in the carotid plaques.

with plaque development. We determined the levels of ANRIL NR_003529 transcripts in the plaques and saphenous tissue samples and showed for the first time that ANRIL NR_003529 transcripts are expressed in saphenous tissue samples. The ANRIL NR_003529 expression was not found to be significantly associated with CA; however, this question is complicated by the existence of multiple splice variants of ANRIL.

To our knowledge, there are two published reports examining the vascular protein expression of genes adjacent to the chromosome 9p21 core region[27, 33]. Kalinina *et al*. demonstrated that the $p15^{INK4b}$ expression is restricted to macrophage-rich fibrofatty lesions and is absent in the SMCs within the fibrofatty lesions of the aorta[27]. Meanwhile, Holdt *et al*. assessed the expression of $p15^{INK4b}$ and $p16^{INK4a}$ in human coronary and thoracic atherosclerotic arteries and showed that these proteins are co-localized with macrophages and SMCs[33]. In the present study, we demonstrated the $p16^{INK4a}$ and PCNA protein expression in carotid plaques in comparison with that observed between CA subgroups. Among the carotid plaques, the $p16^{INK4a}$ protein expression was significantly increased in the symptomatic CA patients compared with that observed in the asymptomatic patients. The PCNA expression was also significantly increased in the CA plaques of the symptomatic patients. The functional relevance of increased $p16^{INK4a}$ and PCNA levels is perhaps represented by accelerated aging and the occlusion of symptomatic plaques compared to that observed in asymptomatic subjects. The increased PCNA expression observed in symptomatic plaques also suggests that cell proliferation in these tissues may be regulated by CKIs other than $p16^{INK4a}$.

We also evaluated saphenous vein grafts, as they are widely used in CEA surgery. Although arterial grafts display superior long-term patency, saphenous vein grafts continue to be used because the veins are

readily accessible, have an adequate length for accessing different vessels in the heart and exhibit the correct diameter for coronary and aortic anastomosis[34]. Cell proliferation is required for the arterial-vein graft to adjust to new hemodynamic conditions. However, an uncontrolled proliferative process can result in the occlusion of the vein graft or the development of atherosclerotic lesions[24]. Borin *et al*. reported that p21 is the main CKI, while the level of $p16^{INK4a}$ remains unchanged during rat vein arterialization[24]. Cardenas *et al*. demonstrated that the rate of venous thrombosis is increased in vascular injury models of $p16^{INK4a}$-overexpressing transgenic mice[35]. In the present study, the $p16^{INK4a}$ expression in the saphenous vein tissues was significantly decreased in the symptomatic subgroup of CA patients. This finding is associated with G1/S phase progression in the cell cycle of VSMCs and may indicate the regulation of VSMC proliferation in saphenous vein tissues, emphasizing the significance of the $p16^{INK4a}$ expression in vein grafts. However, although the PCNA expression was increased in this group, the difference was not statistically significant. This finding suggests that cell proliferation in symptomatic patients with a history of arterial-vein grafting may be increased compared to asymptomatic patients and that CKIs may be significant markers of vascular remodeling and vein arterialization to accommodate the hemodynamic stressors of high pressure and shear stress after CEA surgery.

We also assessed the associations between the CDKN2A, ANRIL, $p16^{INK4a}$ and PCNA expression levels and the severity of ICA stenosis. The CDKN2A expression was found to be significantly increased in the plaques associated with ICA stenosis of ≥ 80%, suggesting that the progression of atherosclerotic lesions proceeds aging. On the other hand, the ANRIL NR_003529 expression was decreased in the plaques associated with ICA stenosis of ≥ 80%, although this finding was not statistically significant. Nevertheless, this result indicates that the levels of CDKN2A and ANRIL NR_003529 transcripts are inversely correlated in carotid plaque tissues. Borin *et al*. demonstrated that apoptosis and cell proliferation are both involved in the remodeling process induced by arterialization of the rat jugular vein[24]. In the present study, we showed that cell proliferation is increased in plaques associated with ICA stenosis of ≥ 80%, although this finding was not statistically significant. Additionally, before arterialization, the PCNA expression in the saphenous veins was similar to that observed in the patients exhibiting < 80% and ≥ 80% ICA stenosis.

Recent studies have shown correlations between 9p21 SNPs and transcription at this locus[28, 33, 36, 37]; however, the results are conflicting. SNPs located at this locus have been shown to be associated with the ANRIL expression in a previous report[37]. In another study of healthy individuals, the relative copy numbers of CDKN2A transcripts were found to be decreased in the peripheral blood[36]. Meanwhile, Holdt *et al*. reported that the ANRIL expression was increased in CAD patients carrying the risk haplotype[28]. Another study by Holdt *et al*. also found the ANRIL DQ485454 transcription level to be inversely correlated with that of $p16^{INK4a}$, although the finding was not statistically significant. The authors also stated that the expression levels of ANRIL EU741058 and NR_003529 were not correlated with that of CDKN2A mRNA[33]. In the present study, we found that the level of ANRIL NR_003529 transcripts was inversely correlated with the CDKN2A expression; however, our results were also not statistically significant. This finding shows that the expression of $p16^{INK4a}$ is not affected by the expression of ANRIL NR_003529 transcripts, consistent with the results obtained by Holdt *et al*.[33] Additionally, many SNPs in the 9p21 region may interact with each other, thereby regulating the expression patterns of neighboring genes and influencing the gene expression.

Congrains *et al*. reported that the expression levels of CDKN2A, CDKN2B and ANRIL are positively correlated. These authors also found that common carotid artery atherosclerosis is associated with a significantly lower expression of ANRIL[21]. In contrast, the present study showed that the ANRIL NR_003529 and CDKN2A expression levels are negatively correlated. However, this finding did not reach statistical significance. Our results are not consistent with those of Congrains *et al*.; however, they studied the RNA expression in the peripheral blood, while we investigated the expression levels of CDKN2A and ANRIL in vascular tissues. These findings indicate that the regulation of these genes may be tissue-specific.

Conclusions

This study is the first study to examine the relationships between 9p21 locus genes and atherosclerosis in Turkish CA patients. For the first time, we measured the gene expression of CDKN2A and ANRIL and the protein expression of $p16^{INK4a}$ and PCNA in symptomatic and asymptomatic subgroups of CA patients using both atherosclerotic carotid plaques and saphenous vein tissues. In this study, we demonstrated the ANRIL gene NR_003529 transcript expression in saphenous vein tissues for the first time. We also showed that the CDKN2A gene expression is higher

in patients exhibiting severe ICA stenosis. These findings indicate that CDKN2A transcripts may affect the pathogenesis of CA and that the $p16^{INK4a}$ protein expression levels differ according to the CA subgroup. However, our results must be replicated and confirmed in future studies. This study may provide insight for future clinical and pharmacological studies of atherosclerosis research involving plaque rupture in CA patients.

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Conflicts of Interest

None.

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Supplemental Table 1a. Demographic and clinical characteristics of the symptomatic and asymptomatic patients

Parameter	Symptomatic ($n=33$)	Asymptomatic ($n=17$)	p
Age (years)	66.52 ± 8.12	65.65 ± 7.83	>0.05
Male/female (n, %)	25/8 (75.8, 24.2%)	13/4 (76.5, 23.5%)	>0.05
BMI (kg/m^2)	27.58 ± 3.61	28.23 ± 3.43	>0.05
Total-cholesterol (mg/dL)	196.21 ± 49.52	190.47 ± 36.55	>0.05
HDL cholesterol (mg/dL)	42.27 ± 9.32	48.76 ± 27.36	>0.05
LDL cholesterol (mg/dL)	122.91 ± 43.87	117.99 ± 34.23	>0.05
Triglycerides (mg/dL)	173.00 ± 101.66	133.76 ± 42.22	>0.05
Fasting glucose (mg/dL)	111.24 ± 37.55	112.65 ± 37.67	>0.05
Hematocrit (%)	39.31 ± 5.76	40.46 ± 4.47	>0.05
Urea	44.12 ± 24.12	39.71 ± 14.00	>0.05
Creatine (mg/L)	0.99 ± 0.32	0.91 ± 0.15	>0.05
ALT (U/L)	19.97 ± 10.84	24.12 ± 12.59	>0.05
AST (U/L)	18.33 ± 7.04	25.12 ± 11.76	0.014
Smoking status n (+/−), (%)	20/13 (60.6, 39.4)	7/10 (41.2, 58.8)	0.239
ICA Stenosis Severity n (%)			
<80%	9 (27.3)	13 (76.5)	
≥80%	24 (72.7)	4 (23.5)	0.001

The t-test and χ^2 test were performed. The data are presented as numbers (%) and the mean ± SD.
Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine transaminase; AST, aspartate aminotransferase; ICA, internal carotid artery

Supplemental Table 1b. Active pharmacological therapy in the symptomatic and asymptomatic CA patients

Active therapy	Symptomatic ($n=33$) n (%)	Asymptomatic ($n=17$) n (%)	p
Aspirin	22 (66.7)	12 (70.6)	0.778
ACE I/ARB	12 (36.4)	6 (35.3)	0.941
Statin	18 (54.5)	10 (58.8)	0.773
Anti-hypertensive drugs	15 (45.5)	9 (52.9)	0.616
Oral anti-diabetic drugs	4 (12.1)	2 (11.8)	0.971
Insulin	3 (9.1)	3 (17.6)	0.378

The χ^2 test was performed. The data are presented as numbers (%).
Abbreviations: ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker