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Gene Expression Levels of Elastin and Fibulin-5 According to Differences Between Carotid Plaque Regions

EMRE SIVRIKOZ[1], ÖZLEM TIMIRCI-KAHRAMAN[2], ARZU ERGEN[2], ÜMIT ZEYBEK[2], MURAT AKSOY[3], FATIH YANAR[4], TURGAY İSBIR[5], MEHMET KURTOĞLU[4]

[1]*Gaziosmanpasa Taksim Training and Research Hospital, Department of Surgery, Istanbul, Turkey;*
[2]*Istanbul University, Institute of Experimental Medicine, Department of Molecular Medicine, Istanbul, Turkey;*
[3]*Liv Hospital, Bahcesehir University, Department of Surgery, Istanbul, Turkey;*
[4]*Istanbul School of Medicine, Istanbul University Department of Surgery, Istanbul, Turkey;*
[5]*Yeditepe University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey*

Abstract. *Aim: The purpose of this study was to investigate the gene expression levels of elastin and fibulin-5 according to differences between carotid plaque regions and to correlate it with clinical features of plaque destabilization. Materials and Methods: The study included 44 endarterectomy specimens available from operated symptomatic carotid artery stenoses. The specimens were separated according to anatomic location: internal carotid artery (ICA), external carotid artery (ECA) and common carotid artery (CCA), and then stored in liquid nitrogen. The amounts of cDNA for elastin and fibulin-5 were determined by Quantitative real-time PCR (Q-RT-PCR). Target gene copy numbers were normalized using hypoxanthine-guanine phosphoribosyltransferase (HPRT1) gene. The delta-delta CT method was applied for relative quantification. Results: Q-RT-PCR data showed that relative fibulin-5 gene expression was increased in ICA plaque regions when compared to CCA regions but not reaching significance ($p=0.061$). At the same time, no differences were observed in elastin mRNA level between different anatomic plaque regions ($p>0.05$). Moreover, elastin and fibulin-5 mRNA expression and clinical parameters were compared in ICA plaques versus CCA and ECA regions, respectively. Up-regulation of elastin and fibulin-5 mRNA levels in ICA were strongly correlated with family history of cardiovascular disease when compared to CCA ($p<0.05$). Up-regulation of fibulin-5 in ICA was significantly associated with diabetes, and elevated triglycerides and very low density lipoprotein (VLDL) when compared to ECA ($p<0.05$). Conclusion: The clinical significance is the differences between the proximal and distal regions of the lesion, associated with the ICA, CCA and ECA respectively, with increased fibulin-5 in the ICA region.*

Correspondence to: Professor Dr. Mehmet Kurtoglu, Istanbul University, Faculty of Medicine, Department of Surgery, Capa, 34390, Istanbul, Turkey. Tel: +90 2124442000, Fax: +90 21244432280, e-mail: metlevkurt@superonline.com

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Carotid artery disease is described by decreased patency of the carotid arteries, which is commonly caused by atherosclerosis, potentially leading to ischemic stroke (1). Recent literature suggests that carotid plaque itself should be re-classified referring to the point of maximum stenosis. Documentation of microscopic pathology implies that the region of plaque proximal (upstream) to the maximal point of stenosis features severe inflammatory characteristics of an unstable lesion. However, the distal region was shown to feature characteristics of a stable lesion (2). Development of atherosclerotic carotid plaque is a dynamic and complex process that involves various events, for instance, proliferation of smooth muscle cells, macrophage and lymphocyte migration, formation of new blood vessels, and remodeling of the extracellular matrix. In recent years, many studies have suggested that the different phases of atherosclerosis may be mediated through the action of metalloproteinases and other matricellular proteins (3-6).

Elastin and fibulin-5 are extracellular membrane proteins actively involved in matrix stabilization. Their expression was shown to be increased at atherosclerosis-resistant regions of the aorta, and they were shown to be degraded by metalloproteinase activity at thoracic aortic dissections (7, 8). Vascular shear stress was reported to be associated with atherosclerotic activity. One of the proposed mechanisms was due to alteration of the extracellular matrix composition (8-10).

Elastin is one of the most important components of elastic fibers and vascular extracellular structures, and was found to have decrements and structural alterations in vessel walls in response to atherosclerosis (11). Elastin assembly is a

Table I. *Demographic and clinical characteristics of the study group.*

Parameter	Total (n=44)
Gender (F/M)	15/29
Age (years)	68±10
Smoking (%)	50
Alcohol (%)	22.7
Hypertension (%)	68.2
Diabetes (%)	45.5
Triglycerides (mg/dl)	136±57
Total cholesterol (mg/dl)	183±56
HDL-cholesterol (mg/dl)	41±11
LDL-cholesterol (mg/dl)	115±50
VLDL-cholesterol (mg/dl)	27±12
Homocysteine (μmol/L)	12±3
C-Reactive protein (mg/L)	12±19
Platelets (per μL)	241,000±103,000
Prothrombin time (sec)	15±16
PTT (sec)	93±25
APTT (sec)	27±15
Heart rate (bpm)	79±6
Diastolic blood pressure (mmHg)	79±9
Systolic blood pressure (mmHg)	132±17

HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: very low density lipoprotein; PT: prothrombin time; APTT: activated partial thromboplastin time. Values are reported as the mean±standard deviation or the number of patients as a percentage of the total group.

delicate process, that can be affected by several other proteins and factors. Its role is being increasingly appreciated in the pathogenesis of atherosclerosis and other diseases (12). While the molecular interactions between elastin and other microfibrils during elastogenesis have been investigated, the mechanisms of elastic fiber formation remain unclear (13). Recently, Basu *et al*. showed that the ratio of elastin to collagen expression is higher in carotid artery and the aorta compared to that in femoral artery and vein (14).

Fibulin-5, also known as embryonic vascular growth factor (EGF-)like repeat-containing protein and developmental arteries and neural crest EGF-like protein, is a calcium-binding extracellular matrix protein shown to play a critical role in the assembly of elastic fibers (15, 16). Fibulin-5 appears to be essential for the polymerization of elastin (15-17). Furthermore, fibulin-5 interacts with the elastic fiber molecules tropoelastin and fibrillin-1 and therefore was said to be important for elastic fiber formation (15, 18, 20). Aside from its elastogenic function, fibulin-5 plays multiple roles in various cellular processes (21). Recent studies showed that fibulin-5 is expressed in developing arteries, as well as atherosclerotic arteries (21).

It is yet unknown whether elastin and fibulin-5 are affected during carotid atherosclerosis. In the present study, we aimed to investigate the relation of these genes with carotid atherosclerotic activity with regard to the anatomic divisions of the plaque.

Table II. *The relative gene expressions for elastin and fibulin-5 in internal carotid artery (ICA) and common carotid artery (CCA) plaque locations.*

Gene	ICA (n=44)	CCA (n=44)	*p*-Value
Elastin	−0.375±2.949	−1.048±2.739	0.124
Fibulin-5	−3.250±3.545	−4.089±3.371	0.061

Values are reported as mean±SD, *p*-values less than 0.05 denote statistical significance.

Materials and Methods

Patients and specimen. The study population consisted of 44 patients operated at the Peripheral Vascular Surgery Service, Istanbul School of Medicine with symptomatic carotid stenoses ≥70%. Those with asymptomatic stenoses or stenoses <70% were treated medically. Carotid artery plaques were obtained immediately after endarterectomy. All operations were performed with standard surgical techniques and minimal manipulation of the specimen. After obtaining Institutional Review Board approval (number:2741/10.21.2008), specimens were collected prospectively. Following endarterectomy, the plaque was divided *ex vivo* into internal carotid artery (ICA), external carotid artery (ECA) and common carotid artery (CCA) parts. These were then stored immediately frozen in liquid nitrogen to prevent nucleic acid and protein breakdown.

RNA isolation and cDNA synthesis. Total RNA for cDNA synthesis was extracted from tissues. Total RNA was extracted by using commercial Magnapure Compact RNA isolation Kit (ROCHE Diagnostics, GmbH, Roche Applied Science, Manheim, Germany). RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Synthesis of cDNAs was performed on a Thermal Cycler device (Applied Biosystems GeneAmp PCR System 9700, Life Technologies, Foster City, USA) using Transcriptor First-Strand cDNA Synthesis kit (ROCHE).

Quantitative real-time PCR (Q-RT-PCR). First strands of the cDNA samples were synthesized using Reverse Transcriptase-PCR . The PCR assays were carried out in a LightCycler 1.5 (ROCHE) device using LightCycler TaqMan master kit (ROCHE) and specific primer and probe sequences.

The primers and probes were designed using www.universalprobelibrary.com. The gene-specific primers and probes for elastin and fibulin-5 used in quantitative PCR were as follows: forward primer for elastin: 5'CAGCTAAATACGGTGCTGCTG3, reverse primer; 5'AATCCGAAGCCAGGTCTTG3', and probe 5'-FAM-TGGAGGAG-3'-dark quencher; forward primer for fibulin-5: 5'CTGCCCTCCAGGCTACATC3', reverse primer 5' CCTGTGCT CACATTCGTTGA3', and probe 5'-FAM-GCTGGGATG-3'-dark quencher. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with

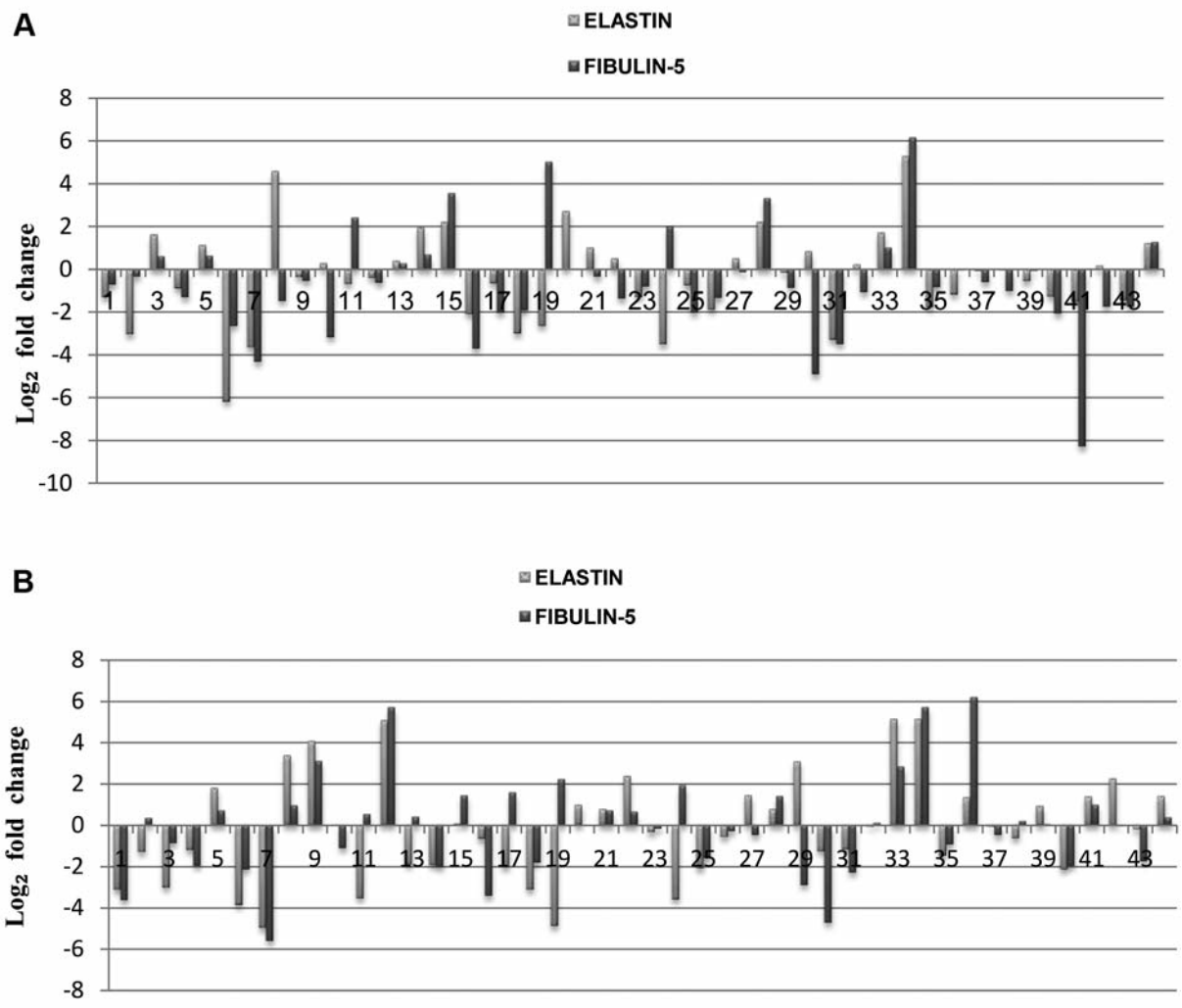

Figure 1. *Relative gene expression levels of elastin and fibulin-5 in plaques in ICA compared to CCA regions (A) and ICA compared to ECA regions (B).*

oligonucleotide primers and probes specific for the constitutively expressed gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) and normalized. *HPRT1* primers and probe were as follows: forward primer: 5'TGACCTTGATTTATTTTGCATACC3', reverse primer: 5' CGACAAGACGTTCAGTCCT3', and probe 5'-FAM-GCTGAGGA-3'-dark quencher.

The cycle number was determined as being within the linear amplification range from a linear amplification curve. The copy numbers of samples were obtained after quantitative amplification and target gene C_T values were normalized to the respective C_T values obtained for *HPRT1* due to previously designed relative quantification method (22). Differences in expression levels are denoted as log-transformed ratios to show fold change

Statistical analysis. Statistical analysis were performed using the SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to examine significance of differences between two groups and Chi-square and Fisher's exact tests was used to compare expression by demographic information. Values are given as the mean±standard deviation (SD). *p*-Values less than 0.05 denoted statistical significance..

Results

The study population consisted of 44 patients with symptomatic carotid stenosis treated with carotid endarterectomy. Demographic data are shown in Table I.

Q-RT-PCR was used to assess the mRNA level of elastin and fibulin-5 genes with regard to anatomical region of plaque. The expressions of our target genes are shown in Figure 1. Elastin and fibulin-5 overexpression was found in 18 and 13 ICA plaques *vs*. CCA plaques, on the other hand, decreased elastin and fibulin-5 expression was found in 26 and 31 ICA plaques *vs*. CCA plaques, respectively. Fibulin-5

Table III. *Correlation of elastin and fibulin-5 gene expression with clinical parameters of patients with plaque located in internal carotid artery (ICA) and common carotid artery (CCA).*

Parameters	Elastin expression ICA *vs*. CCA (n=44)		Fibulin-5 expression ICA *vs*. CCA (n=44)		*p*-Value	
	Down-regulation (n=26)	Up-regulation (n=18)	Down-regulation (n=31)	Up-regulation (n=13)	Elastin expression	Fibulin-5 expression
Demographics and co-morbidities						
Female gender (%)	34.6	66.7	35.5	30.8	0.930	1.000
Age >55 years (%)	73.1	94.4	77.4	92.3	0.115	0.402
Smoking (%)	50.0	50.0	48.4	53.8	1.000	0.741
Alcohol (%)	26.9	16.7	19.4	30.8	0.489	0.449
Hypertension (%)	65.4	72.2	74.2	53.8	0.632	0.186
Diabetes (%)	46.2	44.4	38.7	61.5	0.911	0.165
Family history of cardiovascular disease (%)	15.4	44.4	16.1	53.8	0.045*	0.010*
Hypercholesterolemia (%)	38.5	38.9	38.7	38.5	0.977	0.988
Laboratory values						
Triglycerides (mg/dl)	132.71±58.92	140.56±54.92	136.91±58.59	132.75±52.68	0.688	0.858
Total cholesterol (mg/dl)	183.7±60.74	182.83±52.06	182.01±57.38	187.75±56.39	0.965	0.805
HDL-Cholesterol (mg/dl)	42.52±12.69	39.98±9.77	41.41±11.46	41.37±11.98	0.527	0.993
LDL-Cholesterol (mg/dl)	115.21±53.35	115.82±46.86	114.29±50.07	119.32±52.31	0.972	0.807
VLDL-Cholesterol (mg/dl)	27.38±12.6	27.23±10.82	27.39±12.11	27.05±11.02	0.970	0.943
Homocysteine (μmol/L)	11.81±4.23	11.26±2.43	11.34±3.89	11.95±2.42	0.726	0.711
C-Reactive protein (mg/dl)	12.51±21.09	11.47±15.38	14.17±21.6	6.56±5.88	0.873	0.283
Platelets (per μL)	244922.39±97544.52	236960.87±115171.69	245977.93±103095.32	227250±110182.64	0.817	0.641
Prothrombin time (sec)	16.54±21.12	12.12±1.09	15.49±18.74	12.39±1.27	0.396	0.589

Values are reported as mean±SD and or as percentages, *Statistically significant.

mRNA expression was higher in ICA plaques *versus* CCA but this did not reach statistical significance (p=0.061) (Table II). When we compared ICA and ECA plaque, fibulin-5 expression was not significantly different in these regions (p=0.569). Elastin and fibulin-5 overexpression was indicated in 19 and 23 ICA plaques *vs*. ECA plaques, nevertheless, decreased elastin and fibulin-5 expression was found in 25 and 21 ICA plaques *vs*. ECA plaques, respectively.

We also compared fibulin-5 expression between CCA and ECA regions. There were no significant differences between these two regions (p=0.231). At the same time, no differences were observed between elastin mRNA levels in plaques from different anatomical regions (p>0.05).

Additionally, we investigated associations between elastin and fibulin-5 gene expression levels and clinical parameters in ICA compared to CCA plaque. Up-regulation of elastin and fibulin-5 gene expression was strongly correlated with family history of cardiovascular disease (elastin: p=0.045 and fibulin-5: p=0.010) (Table III). When ICA was compared to ECA, the up-regulation of fibulin-5 expression was correlated with diabetes (p=0.032), and high levels of triglycerides (p=0.007) and VLDL-cholesterol (p=0.031) (Table IV).

Discussion

Ischemic strokes are frequently caused by plaque rupture and embolization resulting from unstable carotid atherosclerotic lesions (23). The pathological mechanisms responsible for plaque instability are increased inflammation, angiogenesis and vessel wall remodeling (23-26). Several studies have shown an increased number of macrophages in symptomatic internal carotid artery plaques (27-29).

Arteries close to the heart may have adapted to high pulsatile blood pressure by increasing their medial thickness and their elastin content, which ultimately gives the artery an increased level of compliance and stability, as shown in *in situ* models (10). The elastin:collagen ratio and matrix metalloproteinases play a crucial role in vascular remodeling and in the development of atherosclerosis (10). Stretch, rather than the shear stress produced by flow, may influence the development of the elastic–hyperplastic layer at the apical walls of the aorta (and its branch dividers such as the ICA), which is followed by a inhibition of smooth muscle cell growth and atherogenic gene expression by elastin, resulting in the construction of an atherosclerosis-resistant structure (8).

Table IV. *Correlation of elastin and fibulin-5 gene expression levels with clinical parameters of patients with carotid artery ICA plaque compared to ECA plaque locations.*

Parameters	Elastin expression ICA *vs*. ECA (n:44)		Fibulin-5 expression ICA *vs*. ECA (n:44)		*p*-Value[1]	*p*-Value[2]
	Down-regulation (n=25)	Up-regulation (n=19)	Down-regulation (n=21)	Up-regulation (n=23)		
Demographics and co-morbidities						
Female gender (%)	32	63.2	28.6	39.1	0.737	0.460
Age (>55) (%)	80.8	83.3	81	82.6	1.000	1.000
Smoking (%)	48	52.6	57.1	43.5	0.761	0.365
Alcohol (%)	24	21.1	19	26.1	1.000	0.724
Hypertension (%)	68	68.4	66.7	69.6	0.976	0.837
Diabetes (%)	40	52.9	28.6	39.1	0.405	*0.032
Family history of cardiovascular disease (%)	20	36.8	19	34.8	0.214	0.318
Hypercholesterolemia (%)	36	42.11	38.1	39.1	0.680	0.944
Laboratory values						
Triglycerides±SD (mg/dl)	126.4±55.67	147.96±57.27	112.02±43.97	162.76±58.26	0.262	*0.007
Total Cholesterol±SD (mg/dl)	173.94±51.55	194.46±61.41	166.91±54.75	200.7±54.27	0.290	0.076
HDL-Cholesterol±SD (mg/dl)	41.38±12.12	41.42±10.93	42.57±12.26	40.23±10.73	0.993	0.558
LDL-Cholesterol±SD (mg/dl)	107.66±41.93	124.27±57.6	101.8±44.83	129.15±52.13	0.340	0.111
VLDL-Cholesterol±SD (mg/dl)	25.8±11.52	29.21±12.07	23.37±11.39	31.73±10.75	0.394	*0.031
Homosistein±SD	11.5±3.97	11.63±2.52	11.86±4.06	11.2±2.72	0.935	0.668
C-reactive protein±SD	16.43±24.3	7.04±7.24	18.8±26.53	6.96±7.14	0.114	0.098
Platelets±SD	245879±116554.18	236191.23±87575.67	258216.9±122279.8	224223.73±79421.75	0.777	0.313
Prothrombin Time±SD	17.22±22.09	11.87±1.17	12.37±3.53	16.54±21.44	0.293	0.420

n:Number of individuals, Values are reported as mean±SD and numbers with percentages, *p-values less than 0.05 denoted statistical significance. [1]p=Elastin expression, [2]p=Fibulin-5 expression.

Recently, carotid plaque composition was assessed *in vivo* referring to the point of maximum stenosis. Unstable plaque phenotype was shown in proximal (upstream) areas of the carotid atherosclerotic plaque, whereas there was a stable plaque phenotype in distal (downstream) parts (2). At the same time, plaque develops most significantly in the proximal portion of the ICA (the area known as the carotid bulb) and plaque at this site is the most common cause of stroke (30). Atherosclerotic carotid plaque has been studied extensively to correlate contents with lesion characteristics regarding microscopic as well as genetic features. Various proteins were shown to be associated with certain lesions (31). Elastin and fibulin-5 are cell-membrane proteins composing the extracellular matrix. Elastin is a main component of elastic fibers that provides elasticity to various tissues such as ligaments, arterial walls and skin (32). Fibulin-5 is an elastin-binding protein essential for elastic fiber development, as shown *in vivo* (29). The elastin content was shown to increase vascular compliance in arteries. Recent studies of transgenic mice and some inherited human diseases focused on the biological function of fibulin-5 (33, 34). Studies have demonstrated differential gene expression of fibulin-5 in the neointima after carotid artery ligation, and in activated endothelial cells of atherosclerotic plaques (14, 35). Furthermore, elevated fibulin-5 was associated with increased thickness of the internal and external elastic laminae and thus was suggested to alter vascular tone (36).

Since the point of maximum stenosis almost certainly resides at the ICA, we aimed to re-classify the carotid plaque anatomically and consistently. Therefore, in the present study, the endarterectomy specimens were divided into anatomic ICA, CCA and ECA parts following tissue harvest. We hypothesized that ICA contents would reflect the atherogenic load compared to CCA and ECA counterparts. To our knowledge, this is the first study that provides a gene-expression analysis based on the anatomical divisions of atherosclerotic carotid plaque. We observed that fibulin-5 mRNA expression was higher in ICA plaques *versus* those of the CCA regions, although not significantly (p=0.061).

In conclusion, we suggest that the clinical significance is the differences between the proximal and distal regions of the lesion, namely increased fibulin-5 in the ICA region. Specification of cellular and molecular mechanisms that denote the presence of vulnerable plaques could be useful as diagnostic biomarkers for patients with stenosis of the ICA. This differential expression pattern needs further evaluation, with extended protein and gene panels.

Conflicts of Interest

The Authors declare that no competing interests exist with regard to this study.

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