YAKUT MEDICAL JOURNAL 2(46)2014

Methylation Status of the Cell Cycle Control-Associated Genes in the Carotid Artery Tissue of Atherosclerotic patients

Maria S. Nazarenko, Anton V. Markov, Iuliia A. Koroleva, Igor N. Lebedev, Alexey A. Sleptsov, Alexey V. Frolov, Olga L. Barbarash, Valery P. Puzyrev

ABSTRACT

Objective: We tested the hypothesis that the aberrant methylation of the promoters and first exons of *CDKN2A* (*p16INK4a*, *p14ARF*), *CDKN2B* (*p15INK4b*) and *RB1* genes was associated with carotid atherosclerosis.

Methods and results: The DNA methylation status of these cell cycle control-associated genes was analysed in 120 samples of carotid arteries using two different techniques: methylation-sensitive polymerase chain reaction (MS-PCR) and methylation-specific PCR (MSP-PCR). DNA methylation was not detected in advanced atherosclerotic plaques or nearby macroscopically intact tissues of the vascular wall from the same patients.

Conclusion: The methylation status of *CDKN2A*, *CDKN2B* and *RB1* genes does not appear to be a marker of human carotid atherosclerosis.

Keywords: DNA methylation, atherosclerosis, *CDKN2A*, *CDKN2B, RB1*.

INTRODUCTION

Atherosclerosis and associated ischaemic events remain a major cause of morbidity and mortality worldwide, including Russia. In the last years, insights into the molecular mechanisms of disease pathogenesis have progressed considerably.

The 9p21 locus is currently considered the most robust genetic marker of atherosclerotic vascular disease [10]. Disease-associated SNPs are located in proximity to *CDKN2A* (coding for the cyclin-dependent kinase inhibitor p16INK4a and its alternative reading frame transcript variant p14ARF in humans and p19ARF in mice) and *CDKN2B* (coding for the CDK inhibitor p15INK4b). These genes are involved in regulating the cell cycle and apoptosis. Deletions and aberrant DNA methylation of the *INK4b-ARF-INK4a* locus are frequent events in tumours. Recent studies have demonstrated that the loss of cell cycle control-associated proteins, such as p19ARF and Rb in gene-targeted animal models, are related to atherosclerotic lesion progression coinciding with changes in cellular composition [4, 7].

It is becoming clear that the inappropriate epigenetic regulation of the 9p21 locus via a large antisense ncRNA named ANRIL also contributes to atherosclerosis [10]. It is possible that this mechanism occurs not in isolation but in close relation to other epigenetic modifications, such as DNA methylation. Aberrant methylation in promoters leads to the transcriptional inactivation of genes involved in the cell cycle regulatory p15INK4b-p16INK4a-cyclin D/CDK4-RB1-mediated pathway (RB1 pathway) in human malignancies. To our knowledge, the methylation status of these genes has never been investigated in atherosclerosis.

In this study, the methylation status of the promoters and first exons of *CDKN2A* (*p16INK4a*, *p14ARF*), *CDKN2B* (*p15INK4b*) and *RB1* genes was evaluated in carotid artery samples of patients with atherosclerosis.

MATERIALS AND METHODS

The study group included 60 asymptomatic/symptomatic patients (men, age 62.3±6.7 years, mean±S.E.) diagnosed with >70% carotid artery stenosis (NASCET criteria) referred to the Research Institute for Complex Problems of Cardiovascular Diseases for the surgical treatment of severe carotid artery stenosis. Twenty-one patients (35%) presented with an ischaemic cerebral event history before surgery. All cases were diagnosed as having coronary heart disease and hypertension. A total of 26 patients (43.3%) had peripheral artery disease. Fifty-three men (88.3%) had hyperlipidaemia. Diabetes mellitus was found in 50% of patients. Cardiovascular risk factors and disease history were recorded at the time of surgery. This study was approved by the local ethics committee, and written informed consent was obtained from all patients.

Vascular samples were collected from advanced atherosclerotic plaques and nearby macroscopically intact tissues from the same patients. Immediately after endarterectomy, the samples were examined by a pathologist, carefully cleaned of calcifications, fatty deposits, and thrombotic material, and washed with a sterile physiological saline solution. All samples, which consisted of intima and the inner media, were fixed in liquid nitrogen and stored at -80°C until used for molecular analysis.

Genomic DNA was purified using standard proteinase K digestion and phenol/chloroform extraction methods.

The methylation status of the promoters of *CDKN2A* (*p16INK4a*, *p14ARF*) and *RB1* genes was determined with methylation-sensitive polymerase chain reaction (MS-PCR), as previously described [1]. Genomic DNA samples were digested with *Hpa*II (Fermentas, Lithuania) before PCR. A 351-bp fragment of the *CDKN2A* (*p16INK4a*) promoter containing 4

*Hpa*II sites, a 283-bp fragment of the *CDKN2A* (*p14ARF*) promoter containing 6 *Hpa*II sites, and a 239-bp fragment of the *RB1* promoter containing 4 *Hpa*II sites were amplified using PCR. Fragments of *EXT2* exon 8 (253 bp) and microsatellite D9S145 (144 bp) were used as internal PCR controls.

In addition to MS-PCR, another approach, methylation-specific PCR (MSP-PCR), was used to analyse the methylation status of exon 1 of *CDKN2A* (*p16INK4a*, *p14ARF*) and *CDKN2B* (*p15INK4b*). The sodium bisulfite conversion of DNA was performed using an EZ DNA Methylation Kit (Zymo Research, United States). Bisulfite-modified DNA was amplified with PCR using two primer sets specific for methylated sequences and two primer sets specific for unmethylated sequences, as described by Herman et al. (1996) [8] and Amatya et al. (2004) [2]. In total, twenty-three CpG dinucleotides from exon 1 of *CDKN2A* (*p16INK4a*, *p14ARF*) and *CDKN2B* (*p15INK4b*) were investigated using MSP-PCR.

RESULTS AND DISCUSSION

The methylation of *CDKN2A*, *CDKN2B* and *RB1* was analysed in 120 carotid artery samples of atherosclerotic patients using two different techniques: MS-PCR and MSP-PCR. No PCR band was observed in the *Hpa*II-digested DNA samples from atherosclerotic plaques (APs) or the macroscopically intact tissue (IT) of carotid arteries (fig. 1). In the experiments using MSP-PCR, all arteries exhibited only unmethylated alleles (fig. 2).

Atherosclerosis is a common disease in which cell proliferation plays an important role. This biological process underlies lesion evolution at all stages, from establishment to plaque complications [6]. The unique roles of *CDKN2A* (*p16INK4a*, *p14ARF*), *CDKN2B* (*p15INK4b*) and *RB1* in cell proliferation suggest a possible role of these genes in atherogenesis.

Numerous studies have underscored the importance of DNA methylation changes in atherosclerosis [3, 13]. However, only a few studies to date have reported DNA methylation changes in vascular tissues from patients with atherosclerosis using a candidate gene approach [9,11,12,14,15] and microarray-based genome-wide analysis [5].

The principal finding of the current study was that the promoters and/or first exons of *CDKN2A* (*p16INK4a*, *p14ARF*), *CDKN2B* (*p15INK4b*) and *RB1* genes were unmethylated in the atherosclerotic plaques and nearby macroscopically intact tissues of the carotid arteries from the same patients.

DNA methylation within gene promoters is suggested to have the highest functional relevance to gene expression control. Our findings are in agreement with those of other authors

who showed that *CDKN2A* (*p16INK4a*, *p14ARF*) and *CDKN2B* (*p15INK4b*) were expressed in the smooth muscle cells of coronary atherosclerotic plaques and normal human arteries [10].

There are several limitations of the current study. The DNA methylation profile may be affected by differences in the cell type composition in various arterial beds, which was not characterised in the present study. Laser microdissection with an appropriate quantitation method (e.g., pyrosequencing) would provide cell-specific information on DNA methylation.

CONCLUSION

Our study of patients with advanced carotid atherosclerosis provides evidence that the methylation status of *CDKN2A*, *CDKN2B* and *RB1* does not appear to be an important determinant of human carotid atherosclerosis.

Funding sources

This research was supported by a grant from the Russian Foundation of Basic Research (№ 10-04-00674a) and the Federal Program "Scientific and educational personnel of innovative Russia" (№ 8062).

REFERENCES

[1] Zemliakova V. V. Anomal'noe metilirovanie nekotoryh genov-supressorov pri sporadicheskom rake molochnoj zhelezy [Abnormal methylation of several tumor suppressor genes in sporadic breast cancer] / V. V. Zemliakova, A.I. Zhevlova, V. V. Strel'nikov [et al.] // Mol. Biol. (Mosk) – 2003. – V. 37. - № 4. - P. 696-703.

[2] Amatya V. J. Methylation of p14(ARF) gene in meningiomas and its correlation to the p53 expression and mutation / V. J. Amatya, Y. Takeshima, K. Inai // Mod. Pathol. - 2004. - V. 17. - № 6. - P. 705-710.

[3] Baccarelli A. Cardiovascular epigenetics: basic concepts and results from animal and human studies / A. Baccarelli, M. Rienstra, E. J. Benjamin // Circ. Cardiovasc. Genet. - 2010. - V. 3. - № 6. - P. 567-573.

[4] Boesten L. S. Macrophage retinoblastoma deficiency leads to enhanced atherosclerosis development in ApoE-deficient mice. / L. S. Boesten, A. S. Zadelaar, A. Nieuwkoop [et al.] // FASEB. J. - 2006. - V. 20. - № 7. - P. 953-955.

[5] Castillo-Diaz S. A. Extensive demethylation of normally hypermethylated CpG islands occurs in human atherosclerotic arteries / S. A. Castillo-Diaz, M. E. Garay-Sevilla,

M. A. Hernandez-Gonzalez [et al.] // Int. J. Mol. Med. - 2010. - V. 26. - № 5. - P. 691-700.

[6] Fuster J. J. Control of cell proliferation in atherosclerosis: insights from animal models and human studies / J. J. Fuster, P. Fernandez, H. Gonzalez-Navarro [et al.] // Cardiovasc. Res. - 2010. - V. 86. - № 2. - P. 254-264.

[7] González-Navarro H. p19(ARF) deficiency reduces macrophage and vascular smooth muscle cell apoptosis and aggravates atherosclerosis / H. Gonzalez-Navarro, Y. N. Abu Nabah [et al.] // J. Am. Coll. Cardiol. - 2010. - V. 55. - № 20. - P. 2258-2268.

[8] Herman J. G. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands / J. G. Herman, J. R. Graff, S. Myohanen [et al.] // Proc. Natl. Acad. Sci. U. S. A. - 1996. - V. 93. - № 18. - P. 9821-9826.

[9] Hiltunen M. O. DNA hypomethylation and methyltransferase expression in atherosclerotic lesions / M. O. Hiltunen, M. P. Turunen, T. P. Rutanen [et al.] // Vasc. Med. - 2002. - V. 7. - № 1. - P. 5-11.

[10] Holdt L. M. Recent studies of the human chromosome 9p21 locus, which is associated with atherosclerosis in human populations / L. M. Holdt, D. Teupser // Arterioscler. Thromb. Vasc. Biol. - 2012. - V. 32. - № 2. - P. 196-206.

[11] Kim J. Epigenetic changes in estrogen receptor beta gene in atherosclerotic cardiovascular tissues and in-vitro vascular senescence / J. Kim, J. Y. Kim, K. S. Song [et al.] // Biochim. Biophys. Acta. - 2007. - V. 1772. - № 1. - P. 72-80.

[12] Post W. S. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system / W. S. Post, P. J. Goldschmidt-Clermont, C. C. Wilhide [et al.] // Cardiovasc. Res. - 1999. - V. 43. - № 4. - P. 985-991.

[13] Turunen M. P. Epigenetics and atherosclerosis / M. P. Turunen, E. Aavik, S. Yla-Herttuala // Biochim. Biophys. Acta. - 2009. - V. 1790. - № 9. - P. 886-891.

[14] Zawadzki C. Tissue factor pathway inhibitor-2 gene methylation is associated with low expression in carotid atherosclerotic plaques / C. Zawadzki, N. Chatelain, M. Delestre [et al.] // Atherosclerosis. - 2009. - V. 204. - № 2. - P. e4-14.

[13] Zhu S. Inactivation of monocarboxylate transporter MCT3 by DNA methylation in atherosclerosis / S. Zhu, P. J. Goldschmidt-Clermont, C. Dong // Circulation. - 2005. - V. 112. - № 9. - P. 1353-1361.

The authors:

Maria S. Nazarenko, Ph.D.

Research Institute of Medical Genetics, Siberian Branch, Russian Academy of Medical Sciences

10 Ushaika Embankment, Tomsk, 634050, Russian Federation

Anton V. Markov
Research Institute of Medical Genetics, Siberian Branch, Russian Academy of Medical Sciences
10 Ushaika Embankment, Tomsk, 634050, Russian Federation

Iuliia A. Koroleva
Research Institute of Medical Genetics, Siberian Branch, Russian Academy of Medical Sciences
10 Ushaika Embankment, Tomsk, 634050, Russian Federation

Igor N. Lebedev, Ph.D.
Research Institute of Medical Genetics, Siberian Branch, Russian Academy of Medical Sciences
10 Ushaika Embankment, Tomsk, 634050, Russian Federation

Alexei A. Sleptcov
Research Institute of Medical Genetics, Siberian Branch, Russian Academy of Medical Sciences
10 Ushaika Embankment, Tomsk, 634050, Russian Federation

Alexey V. Frolov, Ph.D.
Research Institute for Complex Problems of Cardiovascular Diseases, Siberian Branch, Russian Academy of Medical Sciences
6 Sosnovy Blvd, Kemerovo, 650002, Russian Federation

Olga L. Barbarash, Ph.D., MD
Research Institute for Complex Problems of Cardiovascular Diseases, Siberian Branch, Russian Academy of Medical Sciences
6 Sosnovy Blvd, Kemerovo, 650002, Russian Federation

Valery P. Puzyrev Ph.D., MD
Research Institute of Medical Genetics, Siberian Branch, Russian Academy of Medical Sciences
10 Ushaika Embankment, Tomsk, 634050, Russian Federation