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Instability at the H-*ras* minisatellite in human atherosclerotic plaques

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Abstract

The development of atherosclerotic plaques is characterised by the accumulation of lipids and the proliferation of smooth muscle cells. At the subcellular level, the abnormal expression of cytokines and growth factors, as well as the presence of transforming oncogenes, has been recognised and associated with the disease. The aim of the present study was to investigate whether instability at a minisatellite region located downstream of the H-*ras* proto-oncogene possessing enhancer activity, is a detectable phenomenon in atherosclerotic plaques. Thirty specimens were analysed by polymerase chain reaction (PCR) in order to reveal alterations of the repetition number and by restriction fragment length polymorphism (RFLP) with *Bst*NI restriction endonuclease for the detection of point mutations within the 28 bp core repetitive element. No point mutations were found among the 30 cases tested; however, alterations of the repetition number of the core were detected in 5 (17%) cases. Our results suggest that instability at the H-*ras* minisatellite may be associated with development of the disease.

Keywords: H-*ras*; Minisatellite; Genetic instability; Atherosclerosis

1. Introduction

Molecular studies revealed that atherosclerotic plaques are characterised by the abnormal expression of cytokines and growth factors [1] and the acquisition of a transforming potential [2–4]. These observations, in association with the relatively high mutational rate (H. Kiaris et al., unpublished data), indicate that the atherosclerotic plaques possess similarities with neoplasia, and one earlier report suggested that they are similar to a benign neoplastic lesion [5].

Among oncogenes, the members of the *ras* family genes (H-, K- and N-*ras*) are involved in the development of a wide range of human tumours

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[6]. *Ras* genes are usually activated by point mutations and/or aberrant expression [7]. Recently, instability at the 3′ minisatellite region of the H-*ras* proto-oncogene has been reported and associated with aberrant expression of the H-*ras* transcript in head and neck tumours, indicating an additional mechanism of activation for the H-*ras* gene [8]. This minisatellite consists of a 28 bp core repeated 30–100 times (variable number tandem repeat, VNTR) and thus generating 4 main and several rare alleles. It has been suggested that the presence of rare VNTR alleles is associated with an increased risk for the development of cancer and that mutations within the 28 bp repetitive core alter the recognition sequence of particular *trans*-acting factors, disregulating the expression of the H-*ras* proto-oncogene [9].

Recent reports suggested that instability of the H-*ras* minisatellite occurs in tumour specimens [8] as well as in spontaneously aborted embryonic tissues [10]. In the present study, we investigated whether genomic instability at this VNTR region is a detectable phenomenon in atherosclerotic plaques. Our results suggest that atherosclerotic lesions exhibit alterations at the repetition number of the 28 bp core.

2. Materials and methods

2.1. Specimens and DNA extraction

Specimens were obtained from 30 autopsy cases ranging from 60 to 79 years of age from the Laboratory of the Public Forensic Pathology Service, Athens. The plaques were selected to be not calcified and measured around 0.5 cm in diameter. Histologically, all specimens contained foam cells as the main component. Calcified specimens and the specimens with significant fibrous components were excluded from the study. Twenty specimens were taken from the aorta and ten specimens from the basilar cerebral artery. Tissues were frozen in liquid nitrogen immediately after excision and stored until DNA extraction. Genomic DNA was extracted from the frozen tissues as previously described [8]. DNA samples were stored at 4°C.

2.2. PCR amplification of the VNTR

PCRs were performed in a 50 μl reaction volume containing 200 ng of genomic DNA, 1 μM of each primer (5′-GAGCTAGCAGGGCATGCCGC-3′ and 5′-AGCACGGTGTGGAAGGAGCC-3′) [11], 250 μM dNTPs, 10% dimethylsulphoxide (DMSO), 5 μM of 10 × buffer (670 mM Tris-HCl, pH 8.5; 166 mM ammonium sulphate; 67 mM magnesium chloride; 1.7 mg/ml bovine serum albumin (BSA); 10 mM β-mercaptoethanol and 1% (w/v) Triton X-100) and 1 U of *Taq* DNA polymerase.

2.3. Detection of VNTR instability

Alterations at the repetition number of the 28 bp core were detected by electrophoresing 10–15 μl of the PCR product on a 1% agarose gel, stained with ethidium bromide. Shifts in the mobility of the microsatellites were interpreted as positive for instability. Polymorphisms within the 28 bp core were detected by comparison of the restriction fragment length polymorphism (RFLP), after digestion with the restriction endonuclease *Bst*NI, as follows: 10 μl of the PCR product were ethanol precipitated, resuspended in ddH_2O, digested with 10–15 U of *Bst*NI in a buffer as recommended by the supplier, electrophoresed on a 10% polyacrylamide gel and visualised with silver staining.

3. Results

Thirty tissue sections obtained from atherosclerotic plaques were analysed for genetic instability at a 28 bp-core repetitive element, located at the 3′-end of the H-*ras* proto-oncogene. The analysis involved the detection of mutations affecting either the repetition number of the repetitive unit or altering the *Bst*NI restriction endonuclease-recognition site within the core element, generating a detectable RFLP. For each case, the electrophoretic pattern of the atherosclerotic lesion was compared with the corresponding pattern of the adjacent normal tissue and any apparent difference between them was scored as positive for

instability. All positive cases were repeated at least twice and the results were highly reproducible (Fig. 1).

Five (17%) among 30 cases exhibited the generation of novel VNTR alleles in the atherosclerotic tissue which were absent from the control normal tissue of the same patient and thus interpreted as positive for instability (Table 1). In 3 of these 5 positive cases, a ladder pattern was observed, indicating extensive destabilization of the VNTR alleles, while in the remaining two cases, instability was due to the generation of a single VNTR allele in the atherosclerotic tissue. No association was found between the presence or the type of instability (S or L as defined in Fig. 1) and the histology or the artery type.

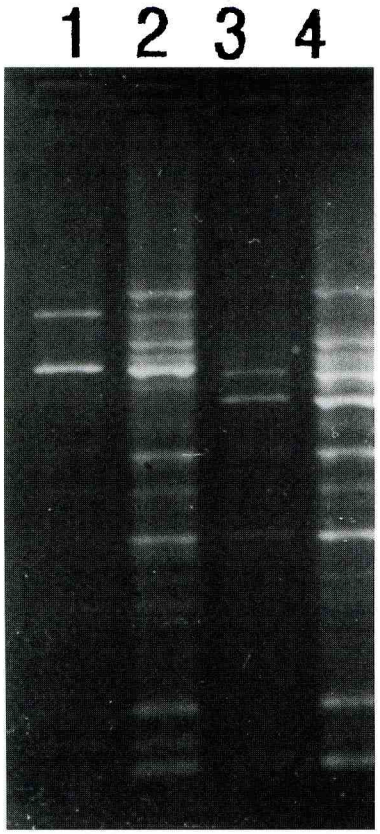

Fig. 1. Specimens exhibiting instability at the H-*ras* minisatellite. Lanes 1 and 3 correspond to normal and lanes 2 and 4 to atherosclerotic plaque respectively (cases 5 and 15). In both cases, a ladder pattern suggesting genetic heterogeneity was observed in the atherosclerotic plaque.

Table 1
Instability at the H-*ras* minisatellite in atherosclerotic plaques

Specimen no.	Age	Altered repetition no.	Pattern of instability[a]
1	65	–	–
2	70	–	–
3	63	–	–
4	61	–	–
5	72	+	L
6	64	–	–
7	62	+	S
8	76	–	–
9	69	–	–
10	71	+	L
11	79	–	–
12	64	–	–
13	68	–	–
14	71	–	–
15	67	+	L
16	60	–	–
17	78	–	–
18	75	–	–
19	67	–	–
20	68	–	–
21	73	–	–
22	78	–	–
23	75	–	–
24	64	+	S
25	69	–	–
26	75	–	–
27	76	–	–
28	71	–	–
29	78	–	–
30	62	–	–

[a]S indicates the generation of a single allele, while L indicates a ladder pattern in the atherosclerotic lesion.

The same panel of specimens was also analysed for point mutations within the 28 bp repetitive unit but all cases tested were negative for a detectable *Bst*NI polymorphism.

4. Discussion

Atherosclerosis represents a multifactorial process implicating the abnormal regulation of several molecules such as cytokines and growth factors [1]. Recent data showed that induction of

apoptosis is also involved in this process, at least in the advanced stages of the disease [12]. However, although the majority of the studies recognised several quantitative changes in these lesions, little is known about qualitative alterations capable of initiating this process. An exception is the detection of transforming oncogenes in the atherosclerotic plaque DNA [2–4] which, however, was not confirmed by other investigators [13].

In the present study, we demonstrated that alterations at the minisatellite region located downstream of the H-*ras* proto-oncogene occur in atherosclerotic plaque DNA and are possibly associated with the development of the disease. Exploring the significance of these findings, the role of this repetitive element in the regulation of the H-*ras* proto-oncogene should be considered. It possesses differential enhancer [11,14] activity and the rare alleles of the VNTR region predispose patients to an increased risk for the development of cancer [9]. Furthermore, destabilization of this region has been shown in tumours and associated with aberrant expression of the H-*ras* gene [8], as well as in aborted embryonic tissues, implicating this genetic event with the recurrent miscarriage of the embryo [10]. The 28 bp repeat binds transcription factors such as NF-κB, providing evidence for the molecular mechanism involved in the regulation of the H-*ras* proto-oncogene by the VNTR region [15]. This suggests that the destabilisation of the VNTR region may have considerable effect on the expression levels of the H-*ras* gene. H-*ras* overexpression increases the mitotic rate of the cells and, in the normal (wild-type) form, induces immortalisation, while in the mutant form induces transformation [16]. We may postulate that this particular genetic alteration is associated with the development of atherosclerotic plaques, probably in the early stages of the disease, which require the induction of the proliferative rate.

The destabilization of the VNTR region, as suggested by the present study, is limited to the alteration of the repetition number of the 28 bp core, while in the spontaneously aborted embryonic tissues in which a similar investigation was performed, mutations within the repetitive element are more frequent [10]. The absence of *Bst*NI polymorphisms within this VNTR does not guarantee the lack of mutations since this endonuclease recognises only 5 among 28 (18%) bases of the repetition core. However, if indeed the incidence of point mutations is very low, it might be argued that the mutational process in the VNTRs of H-*ras* in these two diseases is highlighted by alternative pathways. Furthermore, 3 cases among the 5 positive for instability exhibited a ladder pattern in the atherosclerotic tissue which is due to the generation of multiple VNTR alleles with increasing repetition number, indicating the presence of a heterogeneous population of cells as regards their VNTR allele length. This ladder pattern was not observed in our previous investigation on head and neck tumours and aborted embryonic tissues. It might be postulated that, although atherosclerotic plaques have a monoclonal origin [17], the accumulation of somatic mutations during the development of the disease due to an increased mutational rate results in the generation of a heterogeneous population of cells comprising the atherosclerotic tissue.

Summarising, we demonstrated that instability at the minisatellite region located downstream of the H-*ras* proto-oncogene is a detectable phenomenon in the development of atherosclerotic plaques which may be associated with the development of the disease.

References

[1] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801–809.
[2] Penn A, Garte SJ, Warren L, Nesta D, Mindich B. Transforming gene in human atherosclerotic plaque DNA. Proc Natl Acad Sci USA 1986;83:7951–7955.
[3] Parkes JL, Cardell RR, Hubbard D, Meltzer A, Penn A. Cultured human atherosclerotic plaque smooth muscle cells retain transforming potential and display enhanced expression of the *myc* oncogene. Am J Pathol 1991;138:765–775.
[4] Penn A, Hubbard F, Parkes JL. Transforming potential is detectable in atherosclerotic plaques of young animals. Arterioscler Thromb 1991;11:1053–1058.
[5] Vanni R, Cossu L, Licheri S. Atherosclerotic plaque as a benign tumour. Cancer Genet Cytogenet 1990;47:273–274.

[6] Kiaris H, Spandidos DA. Mutations of *ras* genes in human tumours. Int J Oncol 1995;7:413–422.
[7] Barbacid M. Ras genes. Annu Rev Biochem 1987;56:779–827.
[8] Kiaris H, Spandidos DA, Jones AS, Vaughan ED, Field JK. Mutations, expression and genomic instability of the H-*ras* proto-oncogene in squamous cell carcinoma of the head and neck. Br J Cancer 1995;72:123–128.
[9] Krontiris TG, Devlin B, Karp DD, Robert NJ, Risch N. An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. N Engl J Med 1993;329:517–523.
[10] Kiaris H, Ergazaki M, Spandidos DA. Instability at the H-*ras* minisatellite is associated with spontaneous abortion of the embryo. Biochem Biophys Res Commun 1995;214:788–792.
[11] Green M, Krontiris TG. Allelic variation of reporter gene activation by the HRAS1 minisatellite. Genomics 1993;17:429–434.
[12] Geng Y-J, Libby P. Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1β-converting enzyme. Am J Pathol 1995;147:251–266.
[13] Yew PR, Rajavashisth TB, Forrester J, Barath P, Lusis AJ. NIH3T3 transforming gene not a general feature of atherosclerotic plaque DNA. Biochem Biophys Res Commun 1989;165:1067–1071.
[14] Spandidos DA, Holmes L. Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-*ras* 1 gene. FEBS Lett 1987;218:41–46.
[15] Trepiccio WL, Krontiris TG. Binding of the NF-κB transcription factor at the HRAS1 minisatellite. Nucl Acids Res 1992;20:2427–2434.
[16] Spandidos DA, Wilkie NM. Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 1984;310:469–475.
[17] Benditt EP, Benditt JM. Evidence of a monoclonal origin of human atherosclerotic plaques. Proc Natl Acad Sci USA 1973;70:1756–1756.