Loss of heterozygosity on chromosomes 1, 2, 8, 9 and 17 in cerebral atherosclerotic plaques

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ABSTRACT: ***Objective.*** **Atherosclerosis is a fibroproliferative disease which has been attributed to several factors including genetic and molecular alterations. Initial studies have shown genetic alterations at the microsatellite level in the DNA of atherosclerotic plaques. Extending our initial findings, we performed a microsatellite analysis on cerebral atherosclerotic plaques.**

Methods. **Twenty-seven cerebral atherosclerotic plaques were assessed for loss of heterozygosity (LOH) and microsatellite instability (MI) using 25 microsatellite markers located on chromosomes 2, 8, 9 and 17. DNA was extracted from the vessels as well as the respective blood from each patient and subjected to polymerase chain reaction.**

Results. **Our analyses revealed that specific loci on chromosomes 2, 8, 9 and 17 exhibited a significant incidence of LOH. Forty-six percent of the specimens showed loss of heterozygosity at 2p13-p21, 48% exhibited LOH at 8p12-q11.2, while allelic imbalance was detected in 47% of the cases. The LOH incidence was 39%, 31% and 27% at 17q21, 9q31-34 and 17p13, respectively. Genetic alterations were detected at a higher rate as compared to the corresponding alterations observed in plaques from other vessels.**

Discussion. **This is the first microsatellite analysis using atherosclerotic plaques obtained from cerebral vessels. Our results indicate an elevated mutational rate on specific chromosomal loci, suggesting a potential implication of these regions in atherogenesis. (Int J Biol Markers, 2001; 16: 167-71)**

Key words: Atherosclerosis, Cerebrovascular disorders, Gene expression, Mapping

INTRODUCTION

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. It is the principal cause of heart attack, stroke, and gangrene of the extremities in Western countries and the underlying cause of about 50% of all deaths (1). In atherosclerosis, excessive accumulation of cells in the intima is believed to be the major cause of disease progression (2, 3). This accumulation has been attributed to increased migration and/or proliferation of smooth muscle cells, monocytes/macrophages and T lymphocytes (2, 4).

A great deal has been accomplished in clarifying the causes and effects of intimal proliferation in atherosclerosis but corresponding information on the death of the tissue in the lesions and the general mechanism of the disease remains obscure. Previous studies have revealed numerous risk factors for atherosclerosis, both genetic and environmental. Among the genetic factors, growth factors and cytokines such as PDGF, bFGF, IL-1, TNF-α and TGF-β (5-9) have been implicated in cell recruitment and migration, cell proliferation and the control of lipid and protein synthesis. However, several lines of evidence also indicate that DNA alterations occur and may contribute significantly to the development of the disease. These include activation of oncogenes conferring a transforming potential on smooth muscle cells (10-12). Furthermore, the monoclonal origin of the atherosclerotic plaques suggests the presence of cell clones with a proliferative advantage (13). Moreover, genes responsible for apoptosis have been described to be activated in human atherosclerotic lesions (14, 15).

Initial studies on repetitive DNA have revealed alterations in the microsatellite sequences (16-18) or a minisatellite locus of the H-*ras* proto-oncogene in atherosclerotic plaques of aorta (19). These studies have described loss of heterozygosity (LOH) detected by allelotype analyses in atherosclerotic lesions of the aorta. These findings show similarities in carcinogenesis and athero-

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genesis at the molecular level, indicating that inactivation of tumor suppressor genes located at specific regions is required for atherosclerotic plaque formation.

LOH analysis was performed in 27 atherosclerotic plaques, using 25 highly polymorphic markers focusing on chromosomes 1, 2, 8, 9 and 17. The selection of dinucleotide microsatellite markers was based on previous studies focused on specific chromosomal regions (16-18). This is the first microsatellite analysis using atherosclerotic plaques obtained from cerebral vessels. Our data revealed that specific loci on chromosomes 2, 8, 9 and 17 showed a considerable incidence of LOH. Genetic alterations were more frequent than corresponding alterations observed in plaques from other vessels (16-18). Our results indicate an elevated mutational rate, suggesting that these chromosomal regions are involved in the development or progression of atherosclerotic plaques.

MATERIALS AND METHODS

Specimens and DNA extraction

Twenty-seven atherosclerotic lesions (in 13 males and 14 females) from autopsies were obtained from the Laboratory of Forensic Science, Heraklion, Crete. Non-calcified atherosclerotic lesions were selected measuring approximately 0.5 cm in diameter. Calcified specimens and specimens with a significant fibrous component were excluded from the study. All specimens were obtained from brain vessels and contained foam cells as the main component. The tissue specimens were immediately frozen after excision and stored at –80°C until DNA extraction. In all cases blood was collected and used as the source for normal DNA. DNA from frozen tissues and peripheral blood lymphocytes was extracted using standard methods (20). The present study conformed to the principles outlined in the declaration of Helsinki (21).

PCR amplification and microsatellite analyses

Twenty-five highly polymorphic markers (Research Genetics, Inc, USA) were used for microsatellite analysis of the specimens. Micosatellite markers were located on chromosome arms 1p, 2p, 8p, 9p, 9q, 17p and 17q (Table I). PCR analysis was performed as previously described (20). Seven μL of the PCR product was electrophoresed in a 10% polyacrylamide gel and silver stained. Gels were scanned and the intensity of the bands corresponding to the microsatellite alleles was quantitated by an image analysis system (Adobe PhotoShop 5.0). The analyses were performed twice and the results were highly reproducible. Comparison of the electrophoretic patterns of the amplified marker segments between normal and pathological tissues was performed. LOH was defined as an at least 50% decrease in intensity of one allele relative to the other as determined after comparison of tumor and normal DNA (22).

Sensitivity assay

To assess the sensitivity of our method we prepared samples of various pathological (P) to normal (N) ratios from a tumor specimen with known LOH for a given microsatellite marker. The analysis showed that LOH was detectable in 1:16 dilution (P/N).

RESULTS

The incidence of LOH in atherosclerotic plaques of brain vessels was investigated on 10 chromosomal loci, using 25 different microsatellite markers. The results of

TABLE I - RESULTS OF MICROSATELLITE ANALYSES ON ATHEROSCLEROTIC PLAQUES

Microsatellite marker	Chromosome location	Loss of heterozygosity (%)
CRP	1q21-q23	-
APOA2	1q21-q23	2/22 (9)
D2S160	2q13-q14	7/15 (47)
D2S171	2p24-p21	-
D2S177	2p16	-
D2S337	2p15	5/19 (26)
PLAT	8p12-p11	4/19 (21)
PLAT2	8p12-p11	3/17 (18)
D8S133	8p21.3-q11.1	3/22 (14)
D8S137	8p21.3-q11.1	5/18 (28)
D8S7	8p23	3/16 (19)
ANK1	8p12-p11.2	4/20 (20)
D9S50	9p21	4/17 (24)
D9S270	9p21	3/17 (18)
D9S144	9pter-p22	2/19 (11)
D9S59	9q31-q33	4/20 (20)
D9S109	9q31	3/20 (15)
D9S112	9q31-q34	4/21 (19)
D17S578	17pter-p13.1	1/19 (5)
TP53	17p13.1	3/19 (16)
D17S515	17p13	4/20 (20)
D17S250	17q11.2-q12	1/15 (6)
THRA1	17q11.2-q12	4/22 (18)
D17S855	17q21	6/26 (23)
D17S579	17q21.1-q21.3	4/19 (21)

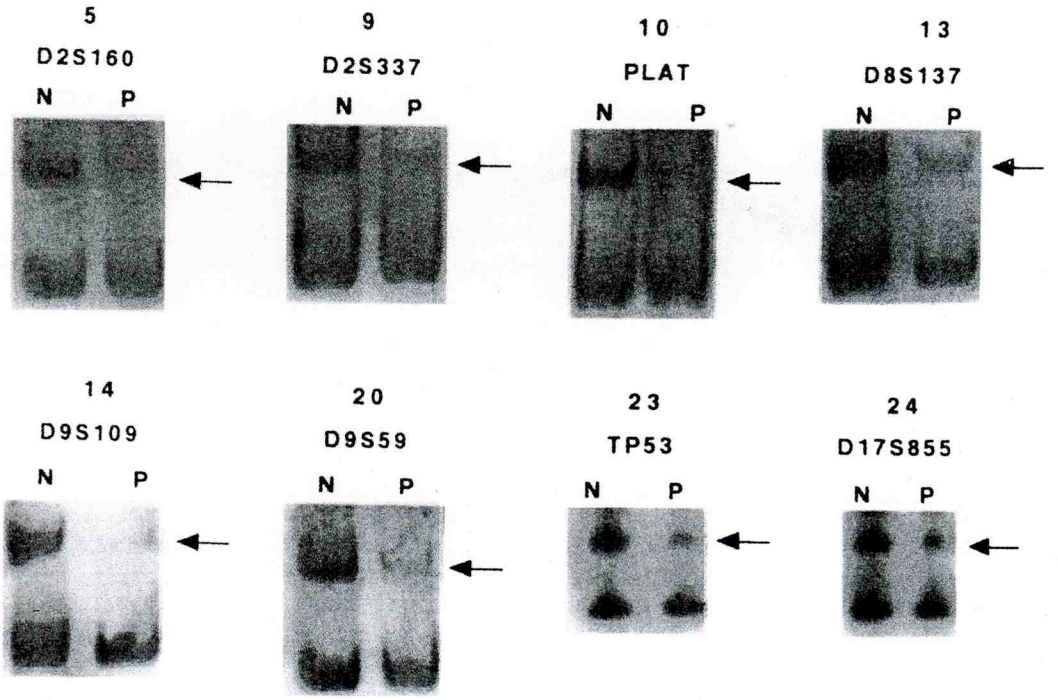

Fig. 1 - *Representative examples of specimens displaying LOH. Arrows indicate the presence of the deleted allele. N: normal DNA; P: pathological DNA. The patient number is given above the microsatellite markers.*

microsatellite analyses as well as the location of each marker are presented in Table I while the rates of allelic deletions per chromosomal locus are shown in Table II. The incidence of LOH varied among the chromosome arms examined. Representative examples of specimens displaying LOH are shown in Figure 1. Locus 2p21-p25 exhibited no evidence of LOH whereas region 2p13-p21 displayed a high incidence of LOH (46%). Similar levels of LOH were detected for the chromosome arms 8p12-q11.2 and 8p11.2-p21.1, namely 48% and 47%, respectively. A considerable incidence of LOH was also detected at regions 9p21 and 9q31-34, namely 24% and 31%, respectively. Allelic imbalance was investigated in three loci of chromosome 17, one on the short and two on the long arm of the chromosome. The analyses revealed LOH in 27% of the specimens at 17p13 and 19% at 17q12. Locus 17q21 exhibited considerable genetic alterations: 39% of the atherosclerotic plaques showed allelic deletions.

The distribution of allelic imbalances varied among the samples. The vast majority of LOH-positive cases (11 samples) were affected in two microsatellite markers while five samples were affected in three markers. Four specimens displayed LOH in four markers, three samples in one marker and two samples in five markers. Two specimens showed extensive allelic deletions presenting LOH in six and seven markers each.

The generation of a novel allele (microsatellite instability, MI) was observed only in two samples for the marker APOA2, which is located at the apolipoprotein A-II gene, and for the marker D9S270. These two samples exhibited no further alterations in the remaining microsatellite markers tested, suggesting the absence of a "true" mutator phenotype characterized by a high incidence of affected markers for the MI-positive cases. The manifestation of instability was due to the generation of a single novel allele and not to a "ladder" pattern as originally described in hereditary non-polyposis colorectal cancer (23-25).

TABLE II - INCIDENCE OF LOH PER CHROMOSOMAL LOCUS IN ATHEROSCLEROTIC PLAQUES OF THE CEREBRAL VESSELS

	Locus	Loss of heterozygosity (%)
Chromosome 1	1q21-q23	2/25 (4)
Chromosome 2	2p21-p25	-
	2p13-p21	10/22 (46)
Chromosome 8	8p12-q11.2	13/27 (48)
	8p11.2-p21.1	7/15 (47)
Chromosome 9	9p21	6/25 (24)
	9q31-q34	8/26 (31)
Chromosome 17	17p13	7/26 (27)
	17q11.2-q12	5/26 (19)
	17q21	10/26 (39)

DISCUSSION

Recent data exist on molecular alterations in atherosclerotic plaques, including the activation of oncogenes

(10-12, 26, 27) and the abberant expression of growth factors (5-9). Furthermore, genes related to apoptosis have been shown to be implicated in the development of the disease (14, 15). Previous studies have provided preliminary data concerning the microsatellite status of atherosclerotic lesions (16-18). We extended the microsatellite analyses to investigate the incidence of loss of heterozygosity in cerebral atherosclerotic plaques in specific chromosomal arms. Originally, LOH was reported in the development of human tumors and represents a manifestation of the recessive behavior of tumor suppressor genes (TSGs) (28); therefore, the identification of genetic loci with a high incidence of LOH indicates the location of genes involved in the disease.

The present findings suggest that the deletion of TSGs is also detectable in atherosclerotic plaques and is probably associated with the disease. LOH was found in 46% at 2p13-p21; by contrast, locus 2p21-25 remained unaffected. The mismatch repair gene h*MSH2* is located in the near vicinity of 2p13-p21, suggesting a possible defect of the DNA repair system in atherosclerotic plaques. Allelic deletions were observed in 48% and 47% for 8p12-q11.2 and 8p11.2-p21.1, respectively. The allelic imbalance observed in chromosome 8 may be due to the *myc* oncogene which has been shown to be amplified (29) and which is consistent with the *myc* overexpression observed in atherosclerotic plaques (11). Genetic loci examined on chromosome 9 displayed LOH in 24% at 9p21 and 31% at 9q31-q34. Chromosome 9 includes several genes involved in cell cycle regulation such as $p16^{INK4}$ (30, 31) and deletions in the one allele may be the first hit for the disruption of their function. Allelic loss was detected at the *p53* locus (17p13) in 27% of the specimens. *p53* is a cell cycle regulator involved in DNA repair, DNA synthesis, cell differentiation, and apoptosis (32). Inhibition of the *p53* gene results in growth of human aortic vascular smooth muscle cells (33), while the absence of p53 accelerates atherosclerosis by increasing cell proliferation *in vivo* (34). Thus, genetic alterations detected at the *p53* locus may represent inactivation of the *p53* gene, resulting in failure of the pivotal roles of the gene in cell cycle progression, DNA repair, and apoptosis.

A high incidence of LOH (39%) was found at 17q21, suggesting that important TSGs for the development or the progression of atheroscerosis may be located in this chromosomal region. Deletions at 17q21, where the BRCA1 gene is located, occur frequently in a variety of neoplasms (35-37).

Atherosclerotic plaques are characterized by the accumulation of lipids and the aberrant proliferation of smooth muscle cells, suggesting that atherosclerosis bears a similarity to neoplasia and should thus be considered as a benign neoplastic lesion (38). In the present study we report the existence of an essential malignant feature: the incidence of loss of heterozygosity in the cells of atherosclerotic plaques. LOH is strongly associated with the development of human tumors and characterizes the lack of normal control during cell proliferation. The incidence of allelic deletions constitutes strong evidence that there may be transformed cells in the atherosclerotic plaques. The detection of LOH in atherosclerosis may also reveal the inactivation of specific "atherogenesis suppressor genes". However, allelic loss in these chromosomal arms has already been described in a variety of human tumors, providing evidence for the pleiotropic effects of the tumor suppressor genes in human diseases (16).

In summary, loss of heterozygosity is a detectable phenomenon in a significant number of specific chromosomal regions, suggesting the presence of putative tumor suppressor genes. The inactivation of these genes may provide a proliferative advantage to the cells carrying these mutations, giving rise to the development or promoting the progression of athereosclerotic plaques. Future studies are required for the fine mapping of the chromosomal loci that were found to be highly affected in order to identify the genes implicated in atherogenesis.

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