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Loss of Heterozygosity and Microsatellite Instability in Human Atherosclerotic Plaques

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Several lines of evidence suggest that mutation events may be involved in the development of atherosclerosis. The aim of the present investigation was to perform an allelotype analysis in 30 atherosclerotic lesions in order to reveal any deletions involved in the development of the disease. Eighteen chromosomal arms were tested by one microsatellite marker located on each arm and allelic imbalance in at least one marker was observed in 7 (23%) cases. Furthermore, the analysis revealed the presence of microsatellite instability (MI) in 10 (33%) cases, suggesting that an increase in the mutation rate may be involved in the formation of the plaque. These results highlight the mutation concept for the atherogenesis and suggest that LOH and MI may be involved in the development of the disease. © 1996 Academic Press, Inc.

The accumulation of lipids and the proliferation of the smooth muscle cells represent a major histological feature of the atherosclerotic plaques. A key role in the development of this pathological entity has been attributed to particular cytokines and growth factors which trigger the biochemical changes that result in atherogenesis. This process is considered as a response to the injury of the arterial wall (1).

However, several lines of evidence also suggest that alterations at the DNA level occur and may contribute significantly to the development of the disease. These alterations include the presence of activated oncogenes which confer a transforming potential and may induce the proliferation of the smooth muscle cells (2–4). Furthermore, the monoclonal origin of the atherosclerotic plaques indicates the presence of cell clones with a proliferative advantage (5).

Recently we reported that an increase in the mutation rate also exists, affecting either microsatellite sequences (6) or a minisatellite region of DNA (7) located downstream of the H-*ras* proto-oncogene and which possesses enhancer activity (8). Thus, in addition to the presence of transforming oncogenes, similarities exist with tumorigenesis at the molecular level in the development of the atherosclerotic plaques (9). However, in the development of tumours the activation of oncogenes is not sufficient, the inactivation of oncosuppressor genes is also required. The latter is frequently achieved by the reduction to homozygosity of particular chromosomal regions which include the onco-suppressor gene(s) (10). The aim of the present investigation was to perform a loss of heterozygosity (LOH) analysis in 18 chromosomal arms in atherosclerotic plaques in order to investigate whether allelic deletions are a detectable phenomenon in atherogenesis.

Furthermore, the cases exhibiting microsatellite instability (MI) were also recorded providing evidence for the acquisition of an increased incidence of microsatellite somatic mutations (6,11,12).

Our results suggest that both LOH and MI are detectable phenomena in atherosclerotic

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plaques providing evidence for an additional factor contributing to the development of the disease.

MATERIALS AND METHODS

Specimens and DNA extraction. Thirty specimens (17 males and 13 females) from myocardial infraction autopsy cases, were obtained from the Laboratory of the Public Forensic Pathology Service, Athens. Non-calsified atherosclerotic lesions were selected measuring approx. 0.5 cm in diameter. All specimens contained foam cells as the main component while 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. The tissue specimens were frozen in liquid nitrogen immediately after excision and stored until DNA extraction.

Genomic DNA was extracted from the frozen tissues as previously described (13). DNA samples were stored at 4°C.

Microsatellite analysis. Eighteen microsatellite markers were used (Table I) (14). PCR reactions were performed in a 12.5 μl reaction volume containing approx. 100 ng of genomic DNA, 500 μM dNTPs, 10 pmol of each forward and reverse primer, 1.25 μl of 10X buffer (670 mM Tris-HCl pH 8.5, 166 mM ammonium sulphate, 67 mM $MgCl_2$, 1.7 mg/ml bovine serum albumin, 100 mM β-mercaptoethanol and 1% (w/v) Triton-X-100) and 0.3 U *Taq* polymerase. The reactions were denatured for 5 min at 95°C and the DNA was subsequently amplified for 28 cycles at 95°C, 58–60°C and 72°C each step. 5 μl of the PCR product was electrophoresed in a 10% polyacrylamide gel and silver stained. LOH was scored when a significant reduction in the intensity of one allele in the heterozygous specimens was observed in the plaque DNA. MI was scored when altered mobility or the generation of novel microsatellite alleles was observed in the plaque DNA. The analysis in the MI and the LOH positive cases was repeated at least twice and the results were highly reproducible.

RESULTS

The incidence of LOH was investigated on 18 chromosomal arms of 13 different chromosomes using one microsatellite marker for each arm. Seven out of 30 (23%) cases exhibited LOH on at least one marker tested (Table I, fig. 1).

The highest incidence of allelic imbalance was observed for the marker MYC on 8q (3/22, 14%) which is closely linked to the *myc* proto-oncogene. Furthermore, 12 out of the 18 (67%) markers used exhibited LOH in an incidence ranging between 4–14%.

The generation of novel microsatellite alleles was observed in 10 out of 30 (33%) cases (Table I, fig. 1). All MI-positive cases were affected in 1 to 3 among the 18 markers tested, suggesting the absence of a ''true'' mutator phenotype which is characterised by a high incidence of affected markers for the MI-positive cases (15–17).

The manifestation of instability was due to the generation of a single or just a few novel microsatellite alleles and not by a ''ladder'' pattern as originally described in HNPCC (15–17).

DISCUSSION

In the present study we performed an allelotype analysis in 30 atherosclerotic plaques, on 18 chromosomal arms. Seven out of 30 (23%) cases, exhibited LOH in at least one microsatellite marker. Allelic imbalance was found for the arms 1p, 1q, 2q, 3p, 5p, 8p, 8q, 10q, 11p, 17p, 17q and 18q while, for the chromosomal arms 2p, 4q, 7q, 9p, 9q and 12q, retention of heterozygosity was observed for all the informative specimens. The allelic imbalance observed for the MYC locus may be due to gene amplification (18) which is consistent with the *myc* overexpression observed in atherosclerotic plaques (3).

Originally LOH was reported in the development of human tumours and represents a manifestation of the recessive behaviour of the onco-suppressor genes (10,19). The present findings suggest that the deletion of onco-suppressor genes is also detectable in atherosclerotic plaques and is probably associated with the disease.

Although the incidence of LOH reported in the present investigation is not very high, this is the first report to our knowledge which demonstrates LOH in atherosclerotic lesions. The use of more markers in the regions exhibiting LOH, may increase the figures and provide evidence for the precise location of the genes involved. This may also reveal the inactivation

TABLE I
Loss of Heterozygosity and Microsatellite Instability in Human Atherosclerotic Plaques

Case No.	D5S111	ANKI	D9S54	D9S103	D17S578	D17S250	D18S34	TPO	D3S1234	D4S194	AMY2B	HRM	CRP	ILIA	MYC	D12S43	D10S109	D7S473
1	h	h	H	H	H	H	h	h	H	H	H	H	H	HS	h	H	H	H
2	h	h	H	h	h	H	h	h	H	H	H	H	H	H	h	H	H	H
3	h	h	H	H	H	H	h	h	h	H	H	H	H	H	H	H	H	H
4	h	H	h	H	h	H	h	H	h	h	H	hS	h	h	h	h	h	H
5	HS	H	HS	H	h	H	H	h	L	h	h	h	H	H	H	h	h	H
6	h	H	H	H	h	H	H	h	H	H	H	H	h	H	H	H	H	H
7	h	H	H	h	H	H	h	h	H	H	h	H	H	h	H	h	h	H
8	h	h	H	h	H	H	H	H	H	H	H	h	H	H	H	H	h	H
9	H	H	H	H	H	H	h	H	H	H	H	H	H	H	H	H	H	H
10	h	L	H	H	L	HS	H	hS	h	h	h	L	H	h	L	H	L	h
11	h	H	H	H	H	H	H	h	h	H	H	h	h	H	h	H	H	H
12	h	H	H	H	H	H	H	h	h	H	H	H	h	h	H	H	H	H
13	h	H	H	H	h	h	H	H	H	H	H	H	H	H	H	h	H	H
14	H	H	H	H	h	H	H	h	H	h	H	H	h	H	H	H	H	H
15	H	H	H	h	H	h	H	H	H	H	h	H	H	h	H	H	H	H
16	H	H	H	H	h	H	H	H	h	h	h	H	h	h	H	H	H	H
17	h	h	H	hS	h	H	h	h	h	h	h	h	H	h	H	h	h	h
18	H	h	H	h	h	H	H	H	H	h	H	h	H	H	H	h	H	h
19	H	H	H	H	h	H	H	H	HS	H	h	h	hS	H	H	h	H	h
20	H	h	H	h	H	H	H	H	H	H	H	H	h	H	h	H	H	h
21	H	h	h	h	HS	H	h	h	h	h	H	h	h	HS	h	h	h	h
22	H	H	H	H	h	H	H	h	H	H	h	h	H	H	H	H	H	H
23	h	h	H	H	H	h	H	H	h	h	h	h	H	H	L	h	H	H
24	h	h	H	h	H	h	L	H	LS	H	L	h	h	H	H	h	h	H
25	h	H	H	H	h	H	H	H	h	H	H	hS	H	H	h	H	h	H
26	L	H	H	H	h	H	H	H	h	h	H	H	H	L	H	H	L	H
27	H	h	H	h	H	H	H	H	H	H	H	H	h	H	H	h	H	H
28	h	H	H	h	H	L	h	H	h	H	H	h	H	H	L	H	H	h
29	H	h	H	h	h	H	L	h	H	h	h	H	L	h	H	h	H	H
30	h	H	H	h	H	H	HS	HS	h	H	H	H	h	H	h	h	HS	H

H, heterozygous; h, homozygous; L, LOH; S, MI.

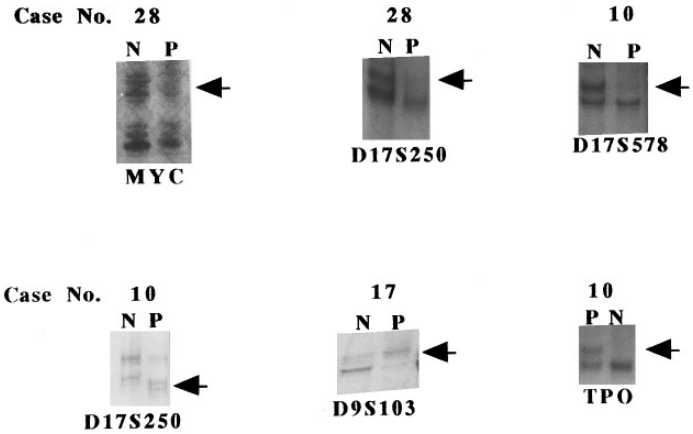

FIG. 1. Examples of specimens exhibiting LOH (upper panel) and MI (lower panel). Arrows indicate the position of the deleted allele (LOH) or the novel microsatellite alleles (MI) in the atherosclerotic lesion. N, normal; P, plaque.

of specific ''atherogenesis suppressor genes''. However, LOH in these chromosomal arms has already been reported in a variety of human tumours, providing evidence for the pleiotropic effects of the onco-suppressor genes in the human diseases.

The present analysis also exhibited MI in 10 out of 30 (33%) cases. This phenomenon was initially reported in hereditary non polyposis colorectal cancer (HNPCC) (15–17) and was later extended to almost all sporadic human tumours (11,12). Although the precise mechanism for MI remains obscure, it indicates an increase in the mutation rate which corresponds to the accumulation of somatic mutations. The demonstration of MI in human atherosclerotic plaques suggests that the development of this disease involves genomic destabilization which may affect genomic sequences important for the formation of the plaques. Cell clones with MI, probably acquire a proliferative advantage which makes this phenomenon detectable, consistent with the observation that the atherosclerotic plaques are monoclonal (5). Evidence for mutational events in plaques have been reported (2–4), recognising the presence of transforming oncogenes in atherosclerotic plaque DNA.

The present study demonstrated that MI and LOH are detectable phenomena in human atherosclerotic plaques. Both of these alterations are strongly associated with the development of human tumours, and characterise the lack of normal control during cell proliferation. Although atherosclerotic plaques do not represent neoplastic tissue, extensive muscle cell proliferation remains as a main feature of this histological entity. This together with other observations contributed to the hypothesis that plaques may represent a benign disease (9). Within this context and apart from the detection of activated oncogenes, the detection of allelic deletions (LOH) and the demonstration of microsatellite mutations (MI) provide clues for additional factors involved in atherogenesis that highlight the mutation concept for the development of the disease.

However, it might be argued that despite the accumulation of these alterations, which characterise malignant tumours, atherosclerotic plaques never give rise to cancer lesions, although such a genetic background may favour neoplastic transformation. A challenging explanation for this discrepancy could be derived from recent reports that recognised the induction of apoptosis in human atherosclerotic plaques (20). It might be argued that the normal cell death protects the tissue by the tumour development.

Summarising, we reported that LOH and MI occur in atherosclerotic plaques and may be involved in the development of this disease. Several questions may arise from these

observations, however, such genetic alterations may represent additional factors involved in atherogenesis.

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