

Microsatellite Instability and Loss of Heterozygosity at DNA Mismatch Repair Gene Loci Occurs During Hepatic Carcinogenesis

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DNA mismatch repair is an important mechanism involved in maintaining the fidelity of genomic DNA. Defective DNA mismatch repair is implicated in a variety of gastrointestinal and other tumors; however, its role in hepatocellular carcinoma (HCC) has not been assessed. Formalin-fixed, paraffin-embedded archival pathology tissues from 46 primary liver tumors were studied by microdissection and microsatellite analysis of extracted DNA to assess the degree of microsatellite instability, a marker of defective mismatch repair, and to determine the extent and timing of allelic loss of two DNA mismatch repair genes, human Mut S homologue-2 (hMSH2) and human Mut L homologue-1 (hMLH1), and the tumor suppressor genes adenomatous polyposis coli gene (APC), p53, and DPC4. Microsatellite instability was detected in 16 of the tumors (34.8%). Loss of heterozygosity at microsatellites linked to the DNA mismatch repair genes, hMSH2 and/or hMLH1, was found in 9 cases (19.6%), usually in association with microsatellite instability. Importantly, the pattern of allelic loss was uniform in 8 of these 9 tumors, suggesting that clonal loss had occurred. Moreover, loss at these loci also occurred in nonmalignant tissue adjacent to 4 of these tumors, where it was associated with marked allelic heterogeneity. There was relatively infrequent loss of APC, p53, or DPC4 loci that appeared unrelated to loss of hMSH2 or hMLH1 gene loci. Loss of heterozygosity at hMSH2 and/or

hMLH1 gene loci, and the associated microsatellite instability in premalignant hepatic tissues suggests a possible causal role in hepatic carcinogenesis in a subset of hepatomas. (HEPATOLOGY 1998;28:90-97.)

Hepatocellular cancer (HCC) is a major cause of cancer-related deaths worldwide.¹ Outside areas with significant dietary exposure to aflatoxin B₁, the genetic events in hepatic carcinogenesis are poorly understood. Recently, there has been interest in the role of DNA mismatch repair system in a variety of sporadic malignancies and the familial cancer syndrome, hereditary nonpolyposis colon cancer.²⁻¹¹ The DNA mismatch repair system is a group of proteins that repair short DNA heteroduplex loops and nucleotide base mismatches that occur during DNA synthesis.¹²⁻¹⁴ Of the mismatch repair genes, human Mut S homologue-2 (hMSH2) and human Mut L homologue-1 (hMLH1) are most frequently affected by germline mutations in hereditary nonpolyposis colon cancer.¹⁵ There is evidence that the functional capacity of both alleles of hMLH1 or hMSH2 in a cell must be lost to inactivate mismatch repair.^{7,16-18} This is similar to the mechanism seen with classic tumor suppressor genes such as adenomatous polyposis coli gene (APC), p53, and DPC4, which play a key role in colorectal carcinogenesis and are inactivated by either loss of the region of the chromosome carrying the gene, referred to as loss of heterozygosity (LOH) and/or by mutations.¹⁹

Short variable number tandem repeats called microsatellites are pleomorphic oligonucleotide (<6 bp) repeat sequences found throughout the genome. A common microsatellite repeat motif is [CA], and there are estimated to be 100,000 CA microsatellites scattered throughout the human genome.^{20,21} The number of repeats in a microsatellite varies, and alleles of differing size are stably inherited characteristics. Additionally, the chromosomal location of many microsatellites is known. This combination of variable size and accurate mapping in relationship to genes of interest make microsatellites ideal to study LOH. Microsatellites can also be used to detect defective DNA mismatch repair. The DNA mismatch repair system acts to repair slippage that occurs by strand misalignment during DNA synthesis. Failure to do this results in the addition or deletion of one or more repeat units and a consequent change in microsatellite length.²² This can be detected as a "band shift" in autoradiographs of amplified DNA containing the mutated microsatellite. Tissues with defective DNA mismatch repair demonstrate multiple band shifts or microsatellite instability and are said to have a "mutator phenotype," which, in hereditary nonpolyposis colon cancer at least, allows the accumulation of mutations in

Abbreviations: HCC, hepatocellular carcinoma; hMSH2, human Mut S homologue-2; hMLH1, human Mut L homologue-1; APC, adenomatous polyposis coli gene; DPC4, deleted in pancreatic cancer; LOH, loss of heterozygosity; PCR, polymerase chain reaction; hPMS2, human post-meiotic segregation gene-2; O⁶-MT, O⁶-methylguanine methyltransferase.

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tumor suppressor genes and oncogenes.²³⁻²⁵ In colorectal cancer, there appears to be an inverse relationship between defective DNA mismatch repair and LOH at other tumor suppressor genes such as p53.²⁶

We hypothesized that inactivation of the DNA mismatch repair system might be mechanistically involved in the evolution of HCC. We have recently demonstrated that archival pathological tissues are excellent sources for the study of genetic events involved in colorectal carcinogenesis.²⁷ The use of microdissection and microsatellite allelotyping from multiple areas in individual tumors allows detection of LOH and microsatellite instability from pathologically defined tissues with a high degree of precision. The aim of this study was to use this technique to determine the extent and time of onset of microsatellite instability in premalignant and malignant hepatic tissues, to determine the role and timing of LOH of hMLH1 and hMSH2 gene loci in the evolution of microsatellite instability, and to assess the interaction between microsatellite instability and LOH at the APC, p53, and DPC4 gene loci.

MATERIALS AND METHODS

Paraffin-embedded specimens of HCC were obtained from the Department of Pathology, University of Michigan Medical Center, and the Ann Arbor Veterans Affairs Medical Center. All tissue was reviewed and graded²⁸ by one pathologist (H.D.A.) who was blinded to the results of the genetic analyses. Forty-five HCCs and one adenoma from 44 patients were studied, including 28 surgical resection specimens, 11 liver transplant explants, and 7 needle biopsies. Two patients had fibrolamellar HCCs resected twice. In both cases, the initial resection preceded the second by 2 years, and the initial resection margins were clear of tumor.

Twenty-seven patients had cirrhosis. This was caused by alcohol abuse alone in 8, alcohol combined with chronic hepatitis C in 4, alcohol and chronic hepatitis B in 1, chronic hepatitis B in 4, chronic hepatitis C in 3, and 1 patient each had Alagille's syndrome, α_1 -antitrypsin deficiency, primary biliary cirrhosis, and primary sclerosing cholangitis. Three patients had cryptogenic cirrhosis and these 3, along with 7 other patients with cirrhosis and 9 patients without cirrhosis, had HCC diagnosed before the availability of hepatitis C antibody testing. Four patients had insufficient nonmalignant liver tissue available to make or exclude a diagnosis of cirrhosis. The remaining 13 patients included 3 with chronic hepatitis (1 each with chronic hepatitis B and C, the other with no viral serological testing), 2 patients with steatosis or steatohepatitis caused by alcohol, and 8 with histologically normal liver. The 8 patients whose nonmalignant liver was histologically normal had no recognized risk factors for HCC, although only 6 had hepatitis B serology and only 1 had hepatitis C serology available. Details of patient age and sex, liver histology, tumor size, and serum α -fetoprotein are presented in Table 1.

DNA was extracted from microdissected 5- μ m sections of formalin-fixed, paraffin-embedded tissues using previously reported techniques.²⁷ One section was stained with hematoxylin-eosin. This reference slide was used to select areas for microdissection. An unstained adjacent section was mounted on a glass slide, partially deparaffinized by immersion in 20% xylene (diluted in absolute ethanol) for 5 seconds, lightly stained with hematoxylin-eosin, and superimposed upon the reference hematoxylin-eosin-stained sections. Pathologically identifiable target microdomains were removed using a scalpel blade and a dissecting microscope (Fig. 1). With experience, tissues could be removed by hand from either side of the transition between nonmalignant and malignant liver tissue. Up to nine areas, or microdomains, were microdissected from malignant tissue in each HCC (median 3; total 169 domains). Multiple

TABLE 1. Clinical and Histological Details of Patients With HCC

	All Patients (\pm SD)	LOH Mismatch Repair Gene Loci (\pm SD)	No LOH Mismatch Repair Gene Loci (\pm SD)	P
n	44	8	36	
Age	52.1 (17.8)	54.8 (15.8)	51.0 (18.4)	NS
Sex (Male/Female)	26/18	3/5	23/13	NS
Cirrhosis (Yes/No)	27/13*	5/3	22/10*	NS
Fibrolamellar variant	8	1	7	NS
HCC Size (cm)	8.2 (6.3)	15.1 (8.7)	6.9 (4.9)	.04
Serum α -fetoprotein†	44,804 (157,973)	517 (643)	54,276 (173,129)	.11
Chronic viral hepatitis	14	3	11	NS

*Four patients had insufficient nonmalignant liver tissue available to make or exclude a diagnosis of cirrhosis.

†Normal value for α -fetoprotein is <3.8 IU/ μ L.

domains were also studied from nonmalignant tissue (1 to 5 microdomains per case; median, 2; total 90 areas) to provide reference tissue for genetic informativity and to test for LOH in premalignant tissue. Each microdomain studied was histologically uniform and varied in size from 1.5 mm to 13 mm in diameter. The number and size of areas studied depended on both the amount of tissue available and histological diversity within the tumor.

The microdissected tissue was deparaffinized and desiccated before treatment with 15 μ L of Genereleaser (BioVentures Inc., Murfreesboro, TN) according to the manufacturer's instructions. The samples were then made up to a final volume of 40 μ L with 25 μ L of digestion buffer (50 mmol/L Tris-HCl [pH 8.0]) containing 400 μ g/mL proteinase K (Sigma, St. Louis, MO). Samples were incubated for at least 72 hours at 55°C, and residual proteinase K was inactivated by incubating at 95°C for 10 minutes.

The study strategy was to examine all areas with 16 microsatellites linked to hMSH2, hMLH1, APC, p53, and DPC4 and to correlate the pathological features with LOH at these loci. hMSH2 lies between D2S119 and D2S123 in a <1.1-MB region bounded by CA-5 and D2S391.¹⁸ hMSH2 also maps closely to CA-21, although the precise location of this marker is not known.⁷ These five microsatellites were used to study hMSH2. D3S1611 is located in an intron of hMLH1, while the gene is <1 cm centromeric to D3S1561. D3S1277 lies between D3S1561 and D3S1611, and these three microsatellites and D3S1029 were used to study hMLH1.^{29,30} An *Alu* polymorphism in intron 1 of the p53 gene and D17S261 were used to study LOH at p53.^{31,32} LOH at the APC gene was studied with D5S346, located 30 to 70 kb downstream from APC and D5S107, which is also located on 5q.^{33,34} LOH near DPC4 was examined using D18S35, D18S46, and D18S65. Each microdissection provided enough DNA for 13 polymerase chain reactions (PCRs), and DNA from two separate microdissections were required to test all 16 microsatellites.

DNA was amplified by PCR using 3 μ L of template DNA solution (from the tissue), 0.125 pmol of 5' [³²P] end-labeled forward primer, 0.125 pmol of the nonlabeled reverse primer, 200 μ mol/L each of dATP, dTTP, dCTP, and dGTP, and 0.25 units of Taq DNA polymerase (Gibco, Gaithersburg, MD) in a final reaction volume of 5 mL. PCR was performed for 35 to 40 cycles using optimal annealing temperatures determined for each set of primers, ranging from 54°C to 60°C. The amplified products were detected by autoradiography after electrophoresis in an 8% polyacrylamide gel containing 7.5 mol/L urea, 44.5 mmol/L Tris-borate (pH 8.3), and 1 mmol/L ethylenediaminetetraacetic acid.

The gels were scanned at >300 dpi and analyzed using NIH Image 1.57 (public domain software) to calculate the relative intensity of bands from each microdomain. Informative normal tissues were defined as those with two amplified bands on the

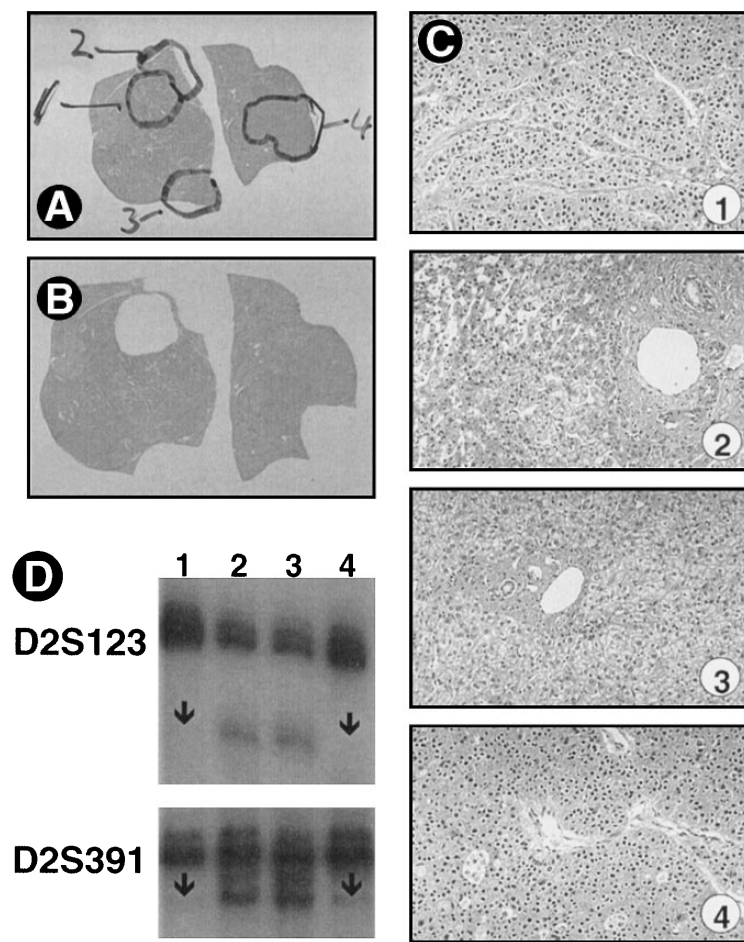


FIG. 1. Microdissection and determination of LOH (Case 34). (A) Reference slide marked for microdissection. (B) Adjacent section stained after microdissection. (C) Histology of microdomains marked 1 to 4 on reference slide: microdomain 1, Grade 2 trabecular HCC; microdomain 2, normal liver; microdomain 3, normal liver; microdomain 4, Grade 2 trabecular HCC. (D) PCR product for hMSH2-linked microsatellites are listed. Each lane corresponds to amplified DNA from the microdomain of the same number in (A). Vertical black arrows indicate bands with $\geq 70\%$ reduction in relative intensity compared with nonmalignant tissue (lanes 2 and 3). This HCC had LOH of hMSH2 gene loci.

autoradiographs, usually separated by 2 to 8 base pairs. LOH was suspected when the normal tissue was informative and there was a reduction in relative intensity of $\geq 70\%$ of one allele. Areas with $\geq 70\%$ reduction of relative intensity of one allele in the initial PCR were restudied so that LOH could be confirmed with at least two separate PCR amplifications. DNA for these two PCRs was usually from different microdissections. An HCC had LOH if any microdomain fulfilled criteria for diagnosis. A band shift was defined as the appearance of a new band on the autoradiograph, usually an increase or decrease in size of 2 to 8 base pairs, representing a novel PCR product. If there was more than one nonmalignant microdomain studied and LOH or band shifts appeared to be present in one or more of these areas, multiple PCR from multiple sections was performed to determine the consensus pattern of bands.

Unfortunately, there is no gold standard for the diagnosis of microsatellite instability with respect to the number of band shifts required. The number of band shifts detected will depend on the DNA mismatch repair status, number and type of microsatellites studied, and the number of areas studied. A variable number of microdomains was microdissected for each case. To provide a measure of the degree of microsatellite instability for each HCC, the average number of band shifts/microdomain of HCC studied was calculated for each tumor. The total number of band shifts from the first PCR amplification of all microsatellites was used as the numerator, and the number of areas of HCC studied for a case was used as the denominator. An average of ≥ 1.0 band shift per malignant microdomain was considered evidence of defective DNA mismatch repair.^{9,22,23,35}

Statistics for discrete variables were calculated by the χ^2 test or Fisher's Exact test and for continuous variables by the Student's *t* test assuming unequal variances. The study had the approval of both hospitals' Institutional Review Boards.

RESULTS

LOH of microsatellites linked to mismatch repair genes occurred in 9 of 46 (19.6%) tumors studied (Table 2). Two of these were fibrolamellar HCCs resected from the same patient 2 years apart, and they had identical patterns of microsatellite loss. All 9 had LOH at microsatellites linked to hMLH1, and 6 also had LOH at hMSH2 gene loci. LOH of mismatch repair gene loci was unrelated to the underlying liver pathology. The clinical correlates of LOH of mismatch repair gene loci are presented in Table 1, while Figs. 1 and 2 show the typical appearance of autoradiographs of PCR-amplified microsatellites.

In 26 cases, more than one nonmalignant microdomain was studied. Unexpectedly and of particular importance, LOH of hMSH2 and/or hMLH1 gene loci occurred in the nonmalignant liver of 4 (15.4%) of these 26 cases, always in association with LOH of hMSH2 and/or hMLH1 gene loci in adjacent HCC. The histology of these four areas included two areas of cirrhotic but non-neoplastic liver, a dysplastic nodule, and an area with chronic hepatitis (no viral serology available) adjacent to the hepatic adenoma. In 2 of these 4 cases, the chromosome lost in nonmalignant tissue was the same as that lost in the HCC.

Within each HCC, not all microdomains fulfilled criteria for LOH; however, the pattern of allelic loss was uniform throughout multiple microdomains in each cancer, with a single exception. In this HCC (case 9), two of seven malignant microdomains had evidence of loss of alleles from

TABLE 2. Pattern of LOH* of hMSH2, hMLH1, APC, p53, and DPC4 Linked Microsatellites

Case	Histology of Areas With LOH	hMSH2	hMLH1	APC	p53	DPC4
2	HCC					*
5	HCC				*	
9	HCC		*			
	Normal liver		*			
10†	HCC	*	*			
14†	HCC	*	*			
18	HCC				*	
19	HCC				*	
20	HCC	*	*			
26	HCC		*			
	Cirrhotic liver	*				
31	HCC				*	
33	HCC		*	*	*	
34	HCC	*	*			
39	HCC			*		*
41	HCC	*	*			
	Cirrhotic liver	*				
43	HCC				*	
46	Adenoma		*			
	Chronic hepatitis	*	*	*		
Total LOH (%)		6 (13.0%)	9 (19.6%)	3 (6.5%)	6 (13.0%)	2 (4.4%)

*LOH of a gene loci. Cases not shown had no LOH detected.

†Fibrolamellar HCCs resected from same patient 2 years apart. Initial resection margins were clear with no clinical evidence of residual disease at time of first operation.

one chromosome, while the other microdomains had evidence of loss of the alleles from the other chromosome. The picture in nonmalignant tissue with LOH of mismatch repair genes was chaotic by comparison with allelic heterogeneity occurring between adjacent microdomains from the same section and DNA extracted from the same area, but from a serial section.

LOH of tumor suppressor genes occurred in two cases with LOH of mismatch repair gene loci, and seven with intact mismatch repair genes (Table 3). LOH of the APC gene loci appeared more frequently in tumors with LOH of hMSH2 and/or hMLH1 gene loci ($P = .03$) than in HCCs without LOH at these loci, although the numbers are small in both groups. An additional four tumors with LOH of mismatch repair gene loci had probable LOH of APC that could not be confirmed because of insufficient tissue. However, there was no significant difference between HCCs with or without LOH at mismatch repair gene loci with respect to LOH at p53, DPC4, or at any combination of tumor suppressor genes.

Sixteen cases (34.8% of HCCs) had evidence of defective DNA mismatch repair with ≥ 1.0 band shifts per malignant microdomain. In the tumors with LOH of hMSH2 and/or hMLH1 gene loci, the range for the number of band shifts per microdomain of HCC studied was 0.77 to 2.33 (Fig. 3). Eight of these nine had > 1.0 band shift per microdomain. For HCCs without LOH of mismatch repair gene loci, the average was < 1.0 ($P < .0001$ when groups with and without LOH of mismatch repair gene loci were compared). Having noted this, 8 of the 37 cases without LOH of mismatch repair gene loci had ≥ 1.0 band shift per malignant microdomain.

Microsatellite instability was also found in nonmalignant liver tissue in 12 cases. Four of these 12 cases had LOH of DNA mismatch repair gene loci in nonmalignant liver tissue,

and in nonmalignant areas they had between two and four band shifts in the 16 microsatellites studied. A further 3 of these 12 cases had LOH of mismatch repair gene loci in the HCC, but not in the nonmalignant tissue. These three cases each had nonmalignant areas with two band shifts. None of the remaining 5 cases had LOH of mismatch repair loci in the cancer or adjacent nonmalignant tissue. Three of these 5 cases had one band shift, a regenerative nodule had two band shifts, and a dysplastic nodule had four band shifts present.

DISCUSSION

Microsatellite instability is a marker of defective DNA mismatch repair and was present in one third of our HCCs (34.8%). Microsatellite instability was strongly linked to LOH of DNA mismatch repair gene loci. The appearance of microsatellite instability in our HCCs with LOH of mismatch repair gene loci suggests that mismatch repair function has been compromised. LOH of hMLH1 and/or hMSH2 gene loci also occurred in nonmalignant liver tissue adjacent to HCC in 4 of the 9 tumors with LOH of these loci. The likelihood that LOH of mismatch repair gene loci in nonmalignant liver and adjacent to HCCs are unrelated is $P < .0005$.

If criteria for microsatellite instability similar to ours are used, microsatellite instability is more prevalent in HCCs than in other sporadic malignancies (where it typically affects $< 25\%$ of cases).³⁶ The degree of microsatellite instability we detected in association with LOH of mismatch repair gene loci (0.77 to 2.33 band shifts/area of HCC) was not as great as that seen in hereditary nonpolyposis colon cancer cancers,²³ in which both copies of relevant genes are inactivated.^{37,38} In malignancies from patients with hereditary nonpolyposis colon cancer, values of ≥ 4 band shifts per area of cancer would be expected using our markers. However, the degree of microsatellite instability we detected in HCCs is similar to that seen in some sporadic colorectal cancers.^{36,39}

It was not possible to determine whether LOH of hMSH2 or hMLH1 gene loci preceded the onset of microsatellite instability, although clearly both events may occur before malignant transformation. However, it is likely that LOH of mismatch repair gene loci in HCC usually occurs before the emergence of the malignant clone. If LOH of mismatch repair genes occurred after malignant transformation, it would be expected to have a heterogeneous distribution throughout the tumor, with LOH randomly affecting either parental chromosome. However, in 8 of 9 cases, there was a uniform pattern of LOH in different regions of the tumor, indicating that the same chromosome was lost throughout the cancer. By comparison, the pattern of hMSH2 and hMLH1 loss in premalignant liver tissue was chaotic. This highly variable pattern of mismatch repair gene loss detected in some premalignant liver tissues is probably caused by the presence of multiple tiny clones within the premalignant tissues, and our findings are analogous to the disordered pattern of allelic loss of p53-linked microsatellites in colonic adenomas with high-grade dysplasia and the stable pattern of p53 loss found in the cancer arising from these adenomas.²⁷ These data suggest that there may be selective pressure for LOH of hMLH1 and/or hMSH2 during malignant transformation in a subset of HCCs.

Detecting LOH in cirrhotic liver and particularly in noncirrhotic liver was somewhat unexpected. Since the commence-

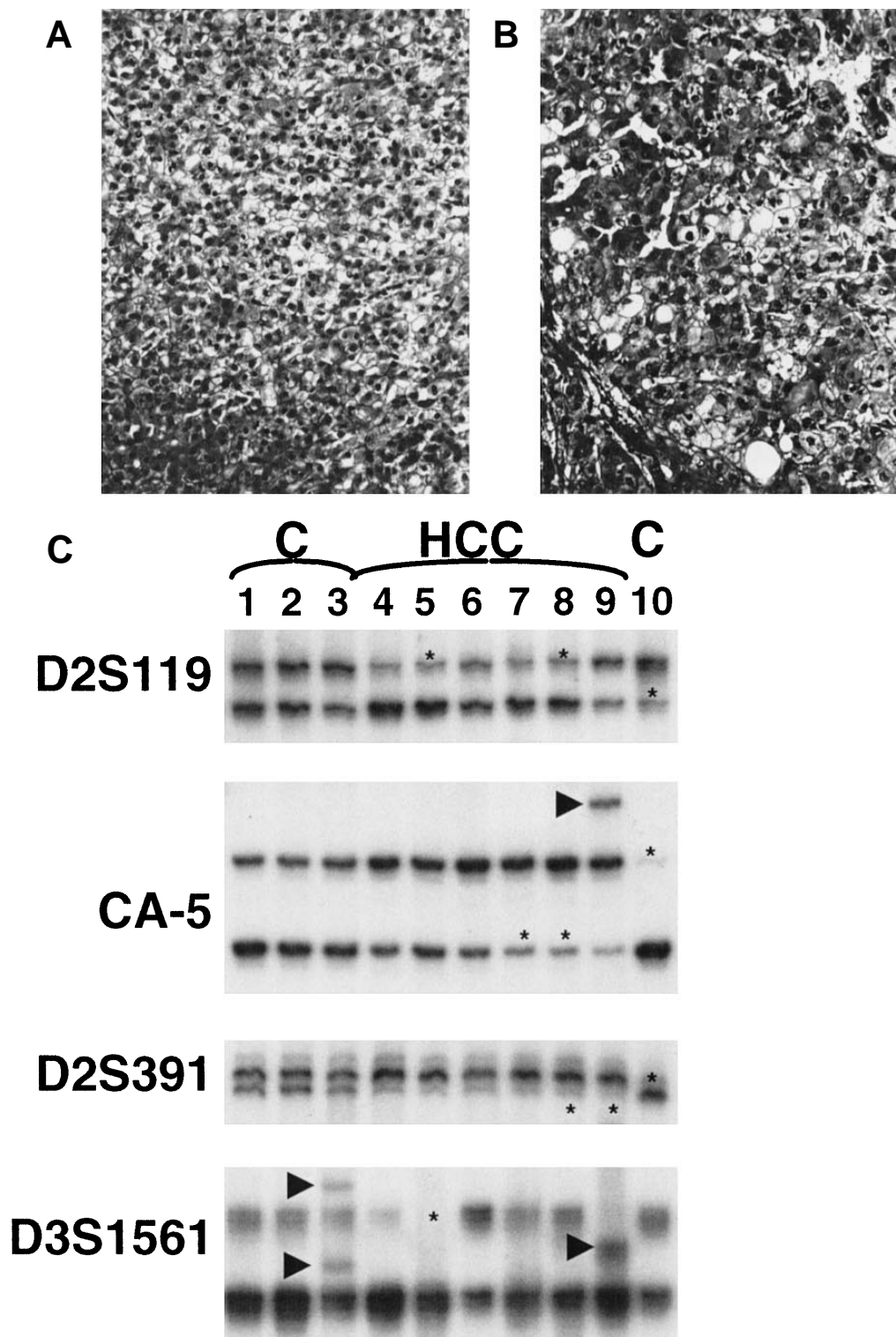


FIG. 2. Interpretation of LOH and band shifts: * $\geq 70\%$ reduction in relative band intensity; black arrow: band shift. Case 41 was from a 56-year-old woman with cirrhosis caused by α_1 -antitrypsin deficiency. (A) Cirrhotic nodule adjacent to HCC (original magnification $\times 165$) (DNA from this area gave results seen below in [C], lane 10). (B) Photomicrograph of HCC (original magnification $\times 165$). (C) PCR product. Lanes 1 to 3 and lane 10 are from cirrhotic liver, while lanes 4 to 9 are from the HCC. Compared with cirrhotic liver in lanes 1 to 3, there is a $\geq 70\%$ reduction in relative band intensity of the larger allele of the hMSH2-linked microsatellite D2S119 in lanes 5 and 8, from the HCC, and in lane 10 from a cirrhotic nodule adjacent to the HCC. For CA-5 (linked to hMSH2), there is LOH in lanes 7, 8, and 10, while lane 9 shows a band shift, and, as discussed in the text, cannot be considered to show LOH. D2S391 is also linked to hMSH2 and shows loss in lanes 8, 9, and 10. For all these microsatellites, the allele lost in the HCC was the opposite allele to that lost in lane 10, from a cirrhotic nodule. This indicates that LOH affected different chromosomes in the HCC and in microdomain 10. D3S1561, linked to hMLH1, shows evidence of LOH in the HCC, lane 6. Lane 9 shows another band shift, while there is also evidence of band shifts in lane 3, from cirrhotic tissue.

TABLE 3. LOH of the Tumor Suppressor Gene Loci for APC, p53, and DPC4

	All Patients	LOH of Mismatch Repair Gene Loci	No LOH of Mismatch Repair Gene Loci	P
LOH of APC	3	2	1	.03
LOH of p53	6	1	5	NS
LOH of DCC	2	0	2	NS
LOH of APC and/or p53 and/or DCC	9	2	7	NS

ment of this study, two groups have reported LOH in cirrhotic liver, at 8p21.3-22⁴⁰ and the mannose-6-phosphate/insulin-like growth factor II receptor.⁴¹ The detection of LOH in an area of chronic hepatitis adjacent to a tumor in one of our cases is not without precedent, because LOH has recently been described in histologically normal tissues adjacent to breast cancers.⁴² Our findings add strength to previous observations that premalignant genetic changes can occur in cirrhotic liver.

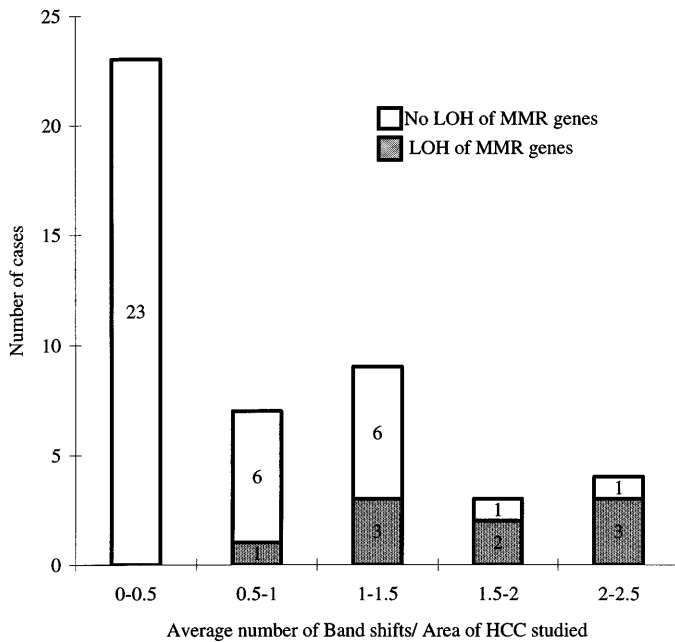


FIG. 3. Histogram of band shifts per domain of HCC. Band shifts per domain of HCC is an average calculated by dividing the total number of band shifts from the initial amplification of all 16 microsatellites for all malignant microdomains studied from an HCC, by the number of microdomains studied. LOH mismatch repair genes: the 9 tumors that fulfilled the criteria for diagnosis of LOH of hMSH2 and/or hMLH1 gene loci. No LOH of mismatch repair genes: remaining 37 HCCs. The presence of band shifts was significantly associated with LOH of mismatch repair gene loci ($P < .0001$), when the 9 tumors with LOH of mismatch repair gene loci were compared with 37 HCCs that did not reach criteria for diagnosis of LOH.

This study had a significant number of HCCs arising in noncirrhotic liver tissue. In large necropsy series, HCCs are present in approximately 17% of patients with cirrhosis, while for noncirrhotic patients, the prevalence is between 0.11% and 0.30%.^{43,44} Because of the relative prevalence of cirrhosis in these necropsy series, HCC is approximately three times more likely to be found in conjunction with cirrhosis than without cirrhosis, although clearly it does occur in both settings. Part of the reason for the relative excess of noncirrhotic patients in our study population is likely to be due to where tissues were obtained for study. Twenty-eight of our cases were from surgical resections and, as a consequence of selection criteria for surgery, less likely to have advanced liver disease.

A criticism of studying DNA extracted from microdissected formalin-fixed tissues is that the DNA concentration is very low (<10 ng/mL) and there is a risk of PCR amplification of one or other alleles because of limiting concentration of DNA template in the PCR reactions. This could give the false impression of LOH. We were concerned about this possibility and for this reason used criteria for LOH that were much stricter than previous studies.⁴⁵⁻⁴⁷ The uniform pattern of LOH of hMLH1 and/or hMSH2 gene loci throughout most affected HCCs and the fact that LOH events predominately involved mismatch repair genes rather than the tumor suppressor gene loci argues against amplification of one or other alleles due to low initial DNA concentration. PCR may amplify small alleles in favor of large alleles (so-called preferential amplification). If this occurred, it would give the impression of LOH affecting the larger microsatellite alleles.

However, as seen in Fig. 2, both the larger and smaller microsatellite alleles were affected by LOH, and preferential amplification seems a theoretical concern rather than a real issue.⁴⁸

It is possible that LOH of mismatch repair genes and defective DNA mismatch repair is more prevalent than we determined for several reasons. Although microdissection allows DNA extraction from a microdomain of "pure" malignant cells, admixture of nonmalignant connective tissue, hematopoietic and hepatic cells within and at the margins of malignant tissue can "dilute" LOH. Additionally, if microsatellite alleles vary in size by only two or four base pairs, bands and shadow bands can overlap, making quantitation of LOH difficult, while if a band shift is present, LOH cannot be diagnosed because the allele giving rise to the band shift cannot be determined with certainty. As a consequence of these technical issues, the initial microsatellite amplifications may have not correctly identified tumors with LOH.

There are two further reasons why this study may have underdiagnosed the prevalence of LOH of mismatch repair genes and microsatellite instability in HCC. A variety of mismatch repair genes have been identified in humans, and at least four—hMSH2, GTBP, hMLH1, and hPMS2—appear necessary for effective mismatch repair, while only loci known to be linked to hMSH2 and hMLH1 were studied, because these are the mismatch repair genes most frequently affected in hereditary nonpolyposis colon cancer.^{14,15} LOH could affect GTBP, hPMS2, and additional genetic loci that encode for proteins required for DNA mismatch repair and would not have been detected by this study. Second, all but one of the microsatellites studied were CA repeats. Cell lines with inactivating mutations in hMSH2, hMLH1, and hPMS2 show microsatellite instability for CA repeats. However, cell lines deficient in GTBP and another mismatch repair enzyme, DNA polymerase δ , do not and so functional loss of these proteins would not necessarily be detected by this study.^{14,49}

The association of LOH of DNA mismatch repair genes loci and microsatellite instability is unusual but has been previously reported in two circumstances. Microsatellite instability and LOH at the hMLH1 locus on 3p21—findings similar to this study—has been reported in non-small cell lung carcinomas,⁵⁰ while Parsons et al.⁵¹ described microsatellite instability in lymphocytes from some but not all patients with hereditary nonpolyposis colon cancer caused by mutations of DNA mismatch repair genes. They showed heterogeneity of microsatellite changes in these lymphocytes and that these changes were only apparent when DNA from a small number of cells was analyzed (as in our study). They were able to show deficient DNA mismatch repair activity in extracts from these cells, even though they could not show mutation in the remaining intact copy of the mismatch repair gene, or mutations in other known mismatch repair genes or evidence of inhibition of repair by the mutated gene product in mixing experiments. This implies an effect of haploinsufficiency but does not explain why this is not more widely seen.

More difficult to explain is why loss should affect two mismatch repair gene loci. Two groups have shown that when microsatellite instability is present, DNA repair genes themselves become targets for inactivation.^{52,53} Cells with a mutator phenotype may have a selective growth advantage if other DNA repair systems are inactivated. A similar series of events may be occurring in HCCs. Although numbers are small, all HCCs with LOH of hMSH2 gene loci also had LOH

of hMLH1 loci. Once LOH of one of these gene loci occurs, there may be selective pressure on loss of other DNA repair genes with progressive impairment of components of the mismatch repair system.

We can only speculate on the growth advantage related to the loss of mismatch repair competency in HCCs. DNA mismatch repair has been shown to interact with other DNA repair systems such as O⁶-methylguanine methyltransferase (O⁶-MT).⁵⁴ O⁶-methylguanine is the major mutagen produced by DNA alkylation⁵⁴ and is not directly cytotoxic, but results in G-to-A transitions at affected bases. O⁶-MT removes the abnormal methyl group. Cell lines deficient in O⁶-MT must inactivate mismatch repair to proliferate, presumably because mismatch repair proficient cells can detect O⁶-methylguanine and induce cell cycle arrest at the G2 checkpoint.⁵⁵ O⁶-MT activity is reduced in cirrhotic liver,⁵⁶ and it is possible that loss of mismatch repair function in O⁶-MT-deficient tissues may offer a selective growth advantage to an evolving premalignant clone. Another potential growth advantage related to microsatellite instability is that cells with defective DNA mismatch repair have a mutable phenotype and do not "require" LOH to inactivate tumor suppressor genes that limit malignant growth.

In summary, there appears to be a selective pressure for LOH of the DNA mismatch repair genes, hMLH1 and hMSH2, during the evolution of a subset of HCCs. This loss appears to occur before the emergence of the malignant clone and is accompanied by microsatellite instability, suggesting a role in hepatic carcinogenesis. Taken together, our findings confirm the malignant potential of cirrhotic liver and demonstrate that significant genomic injury can be present in histologically normal liver. The factors that predispose to LOH of mismatch repair genes and microsatellite instability, the relationship of these changes to malignant transformation, and their clinical significance are not clear and warrant further study.

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