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Chromosomal Alterations in Hepatocellular Nodules by Comparative Genomic Hybridization: High-Grade Dysplastic Nodules Represent Early Stages of Hepatocellular Carcinoma

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SUMMARY: Data from experimental hepatocarcinogenesis and recent studies in humans have suggested that the emergence of hepatocellular carcinoma (HCC) is a stepwise process. However, despite abundant experimental data, the precise molecular mechanisms and genetic alterations involved in human liver carcinogenesis are still unclear. Comparative genomic hybridization was used to analyze 26 hepatocellular nodules obtained from patients undergoing liver transplantation or surgical resection for HCC. According to the criteria proposed by the International Working Party, 16 nodules were classified as multiacinar regenerative nodules (MRN), 4 as low-grade dysplastic nodules (LG-DN), and 6 as high-grade dysplastic nodules (HG-DN). Our aim was to investigate the possible genetic differences between MRN, LG-DN, and HG-DN. The whole group of nodules showed only a few aberrations (mean 1.1/case), without any significant pattern. This finding is comparable to what happens in non-neoplastic tissue. On the contrary, in three of six HG-DN, we found deletions of 8p and gains of 1q. LG-DN and MRN did not show these chromosomal imbalances. These results confirm the important role of allelic losses on 8p as well as of gains of 1q in HCC. We conclude that the genes that are important in early stages of hepatocarcinogenesis are probably located on these chromosomal arms. (*Lab Invest 2002, 82:547–553*).

Hepatocellular carcinoma (HCC) is one of the most frequent neoplasias worldwide (Parkin et al, 1997), but the incidence of this tumor varies significantly in different geographical areas, with the highest incidence occurring in Sub-Saharan Africa and the Far East. The remarkable geographic variability of HCC is related to the variable prevalence of the environmental risk factors that play a critical role in liver carcinogenesis. Among these, hepatitis B virus and hepatitis C virus are by far the best-documented risk factors (Brechot et al, 1998; Moriya et al, 1998) implicated in the development of HCC. In Western countries and in Japan, most HCC develop from the morphologic setting of an active or inactive cirrhosis. There is growing evidence that liver cancerogenesis in cirrhosis is a multistep process. Because the prognosis of patients with HCC is extremely poor, the search for premalignant changes has gained an increasing interest in the last years. Early detection of small HCC is likely to represent the best way to achieve better therapeutic results.

However, morphologic and molecular features of premalignant hepatic lesions are far from being fully elucidated and uniformly accepted. Several lesions in the liver have been proposed to represent premalignant changes, but the exact nature of these possible HCC precursors is not understood. This uncertainty is reflected in the different classifications of preneoplastic lesions (Edmondson and Steiner, 1954; Eguchi et al, 1992; Ferrell et al, 1993; Furuya et al, 1988) and the continuous dispute on the various types and meanings of hepatic dysplasia (Anthony et al, 1973; Lee et al, 1997; Watanabe et al, 1983). In 1995 (IWP, 1995) an international study group proposed a classification of nodular hepatocellular lesions distinguishing regenerative lesions (multiacinar regenerative nodules; MRN) from neoplastic lesions. According to this classification, the neoplastic lesions encompass HCC and dysplastic nodules (DN), the latter including both low-grade (LG-DN) and high-grade (HG-DN) categories. However the same group acknowledged that a strict line cannot be drawn between premalignant and malignant lesions and stated that "the most certain way

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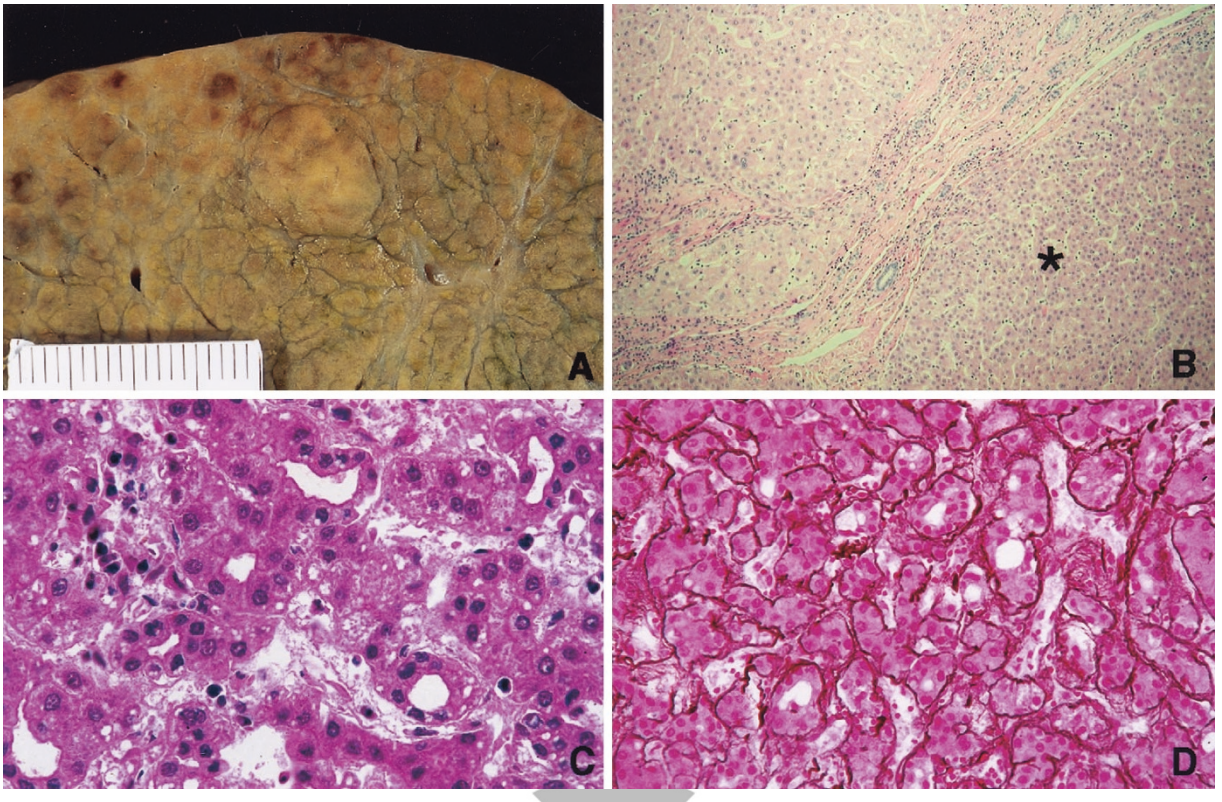

Figure 1.
Gross and microscopic characteristics of hepatocellular nodules. A, Macroscopic aspect of a dysplastic nodule. B, Low-grade dysplastic nodule (LG-DN; *asterisk*) in cirrhotic liver (top left). Hematoxylin and eosin, ×100. C, High-grade dysplastic nodule (HG-DN) displaying pseudoglandular pattern and high nuclear/cytoplasmic ratio. Hematoxylin and eosin, ×400. D, High-grade dysplastic nodule showing preserved reticulin pattern. Novotny, ×400.

to make a diagnosis of dysplastic nodule is application of molecular genetic techniques" (IWP, 1995).

Recent studies have indicated that at least in a subset of cases, MRN and DN are indeed monoclonal (Paradis et al, 1998) and that their clonal expansion may be initiated before the full development of cirrhosis (Theise, 1995, 1996). Results of other molecular studies (Maggioni et al, 2000; Roncalli et al, 1999; Zondervan et al, 2000) are in keeping with the concept of a multistep process in hepatic carcinogenesis from cirrhosis to well-developed HCC. Several studies have shown that the oncogenes cyclin D1, c-myc, and transforming growth factor-α (Nishida et al, 1994; Thorgeirsson et al, 1996) and the tumor suppressor genes p16 (MTS1), p53, and Rb (Biden et al, 1997; Lunn et al, 1997; Zhang et al, 1994) are involved at least in subsets of HCC. Possible locations of other genes that might be critical for HCC development and progression have been implicated by molecular cytogenetic studies showing frequent allelic losses at 1p, 4q, 6q, and 8p as well as frequent gains at 1q, 6p, 8q, and 17q in HCC (Chen et al, 2000; Guan et al, 2000; Kusano et al, 1999; Marchio et al, 1997, 2000; Nagai et al, 1997; Piao et al, 1998; Pineau et al, 1999; Tornillo et al, 2000; Wong et al, 1999). Little is known, however, about the genomic alterations in possible HCC precursor lesions.

The aim of this study was to characterize chromosomal changes occurring in MRN, LG-DN, and HG-DN by comparative genomic hybridization (CGH) and to compare these findings with those previously reported in a series of HCC (Tornillo et al, 2000).

Results

All CGH alterations that were found in hepatocellular nodules are shown in Figure 2. Taken all together, hepatocellular nodules showed few aberrations, with no preferential pattern (mean 1.1/case). These results were similar to those observed in four nonneoplastic, cirrhotic specimens (1/case). However three of six cases of HG-DN showed gain of 1q (minimal overlapping region 1q32-ter) and deletions of 8p. The LG-DN and MRN showed very few aberrations. 1q+ and 8p− were not seen in LG-DN and MRN nor in cirrhotic tissues. Furthermore, in the LG-DN and HG-DN coming from the same liver explant, 1q+ and 8p− were detected exclusively in the HG-DN. The difference in 1q+/8p− between HG-DN and LG-DN/MRN reached statistical significance ($p = 0.002$). A comparison of the present data with previous results (Tornillo et al, 2000) showed that the frequency of 1q+ and 8p− in HCC is comparable to that in HG-DN (Table 2).

Discussion

Cirrhotic livers of patients with HCC have been shown to have increased numbers of genetic alterations as

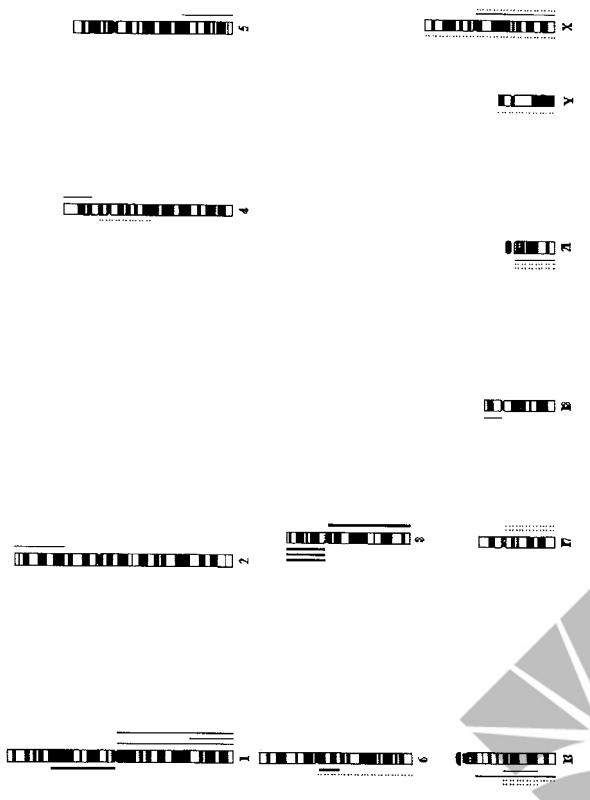

Figure 2.

Chromosomal alterations in 26 hepatocellular nodules. Each line represents a case. Deletions are displayed on the left and gains on the right of the chromosome ideogram. *Bold lines* represent HG-DN; straight lines represent LG-DN; *dotted lines* represent multiacinar regenerative nodules. Only chromosomes with at least one alteration are shown

compared with HCC-free cirrhosis (Roncalli et al, 2000). Cirrhosis has been considered in itself a preneoplastic condition (Okuda, 1992), while it has been shown that at least a subset of cirrhotic nodules may be monoclonal in origin (Aihara et al, 1994). Monoclonal neoplastic expansion could be initiated before the full development of cirrhosis (Theise, 1996). Therefore it could be speculated that cirrhosis progresses to cancer through a molecular pathway marked by sequential and additional genetic changes towards malignancy (Roncalli et al, 2000). There is no precise correspondence between genetic and morphologic alteration (IWP, 1995), so that the concept itself of hepatic dysplasia is still controversial. The original description of Anthony et al (1973) referred to what is today called "large cell dysplasia" (enlarged hepatocytes with nuclear pleomorphism and prominent nucleoli), a morphologic change that has been considered a reactive change (Lee et al, 1997; Su et al, 1997) or a sign of concurrent malignant neoplasia (Natarajan et al, 1997). On the other hand, a truly premalignant lesion seems to be represented by "small cell dysplasia" (Watanabe et al, 1983) (small hepatocytes with decreased, more basophilic cytoplasm and hyperchromatic nucleus), which shows phenotypic markers that are in favor of its precancerous nature (Su et al, 1997; Zhao et al, 1994a, 1994b).

In this study CGH was used to define molecular cytogenetic alterations in preneoplastic hepatic nodules. The comparison of the data with the results in non-neoplastic cirrhotic liver tissue and in HCC allowed us to define two distinct groups of preneoplastic lesions. MRN and LG-DN had very few alterations, similar to the background level found in cirrhotic tissue. The number of genomic alterations was clearly higher in HG-DN. In these nodules, the number and the type of chromosomal changes (1q+ and 8p−) were similar to those previously found in HCC by us and others (Kusano et al, 1999; Marchio et al, 1997, 2000; Tornillo et al, 2000; Wong et al, 1999). These observations provide strong evidence for the assumption that HG-DN are immediate HCC precursor lesions, with a high risk of progression, and therefore should be treated accordingly. The results are consistent with observations made in previous studies suggesting major biologic differences between LG-DN and HG-DN on the basis of vascular profile of the different types (Park et al, 1998; Roncalli et al, 1999; Terada and Nakanuma, 1995).

8p− is one of the major alterations in liver cancer (Chen et al, 2000; Guan et al, 2000; Kusano et al, 1999; Maggioni et al, 2000; Marchio et al, 1997, 2000; Tornillo et al, 2000; Wong et al, 1999). In many tumor types, microsatellite studies have detected at least three different regions of allelic loss on 8p, suggesting the presence of several tumor suppressor genes located on 8p21, 8p22, and 8p23 (Lerebours et al, 1999; Pineau et al, 1999; Wright et al, 1998). 8p has also been shown to be a site of preferential loss of heterozygosity in a series of hepatocellular nodules, with increasing frequency from cirrhosis to HCC (Maggioni et al, 2000). As in our study, Maggioni et al (2000) could not detect significant differences between HG-DN and HCC. Candidate tumor suppressor genes located on chromosome 8p may be FEZ1, a recently described cancer-related leucine zipper motif gene that showed altered expression in multiple tumor types (Ishii et al, 1999). 8p− has also been suggested to play a role in tumor progression in HCC (Boige et al, 1997; Guan et al, 2000) as well as in oral (Ishwad et al, 1999), renal (Schullerus et al, 1999), and breast carcinoma (Radford et al, 1995). 8p− is often found together with 8q+, a finding that is normally interpreted as an isochromosome 8q (Mertens et al, 1997). Marchio et al (2000) have recently reported a series of non-virus–associated HCC in which 30% of 8p− were not balanced by a symmetric gain of 8q. The observation that 8p− was only associated with 8q+ in one of three of our HG-DN would also be consistent with inactivation of a tumor suppressor gene on 8p− preceding overexpression of genes on 8q.

Recently Zondervan et al (2000) investigated 12 hepatic dysplastic areas adjacent to HCC by CGH without detecting 8p losses. However, the lower frequency of alteration found in this study may be a result of a high frequency of LG-DN in their set of nodules because no information was given on the degree of dysplasia in these DN.

Table 1. Clinico-Pathologic Characteristics of 26 Hepatocellular Nodules

	Patients	Nodules
Total	17	26
M/F	10/7	17/9
Mean age	49.3 (±4.3)	49
Mean size	1.3	1.3 (±0.2)
HBV	9	12
HCV	8	14
HCC	8	12
MRN (HCC+)[a]	12 (3)	16 (5)
LG-DN (HCC+)[a]	3 (1)	4 (2)
HG-DN (HCC+)[a]	5 (4)	6 (5)

In six patients more than one nodule was examined.

[a] HCC+, HCC was present in other areas of the resection specimen.

M, male; F, female; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; MRN, multiacinar regenerative nodules; LG-DN, low-grade dysplastic nodules; HG-DN, high-grade dysplastic nodules.

Gains of 1q have been described in many tumors (Mertens et al, 1997). Previous studies in bladder cancer have shown recurrent high-level amplification in the pericentromeric region of 1q, suggesting involvement of an as yet unknown oncogene in this area (Richter et al, 1997, 1999). Circumscribed high-level amplifications have not been found in HCC. In most cases low-level gains of entire 1q were seen (Guan et al, 2000; Kusano et al, 1999; Marchio et al, 1997, 2000; Tornillo et al, 2000; Wong et al, 1999, 2001). It is therefore possible that a simultaneous overexpression of multiple genes on 1q can provide a growth advantage to neoplastic liver cells. Recently Wong et al (2001) have reported that hypomethylation of band 1q12 is strictly related to aberrant 1q formation in HCC. Therefore the heterochromatin fragility could result in the clonal evolution of cells with extra copies of 1q and may confer proliferative advantages to these clones. However, it seems more possible that the actual target genes are still undiscovered, given the small fraction of known genes as compared with the estimated total number of about 30,000 genes (Venter et al, 2001).

In summary, the results of this study suggest that HG-DN are tightly related to HCC based on the molecular cytogenetic profile. 8p− and 1q+ are the most common alterations in HG-DN and HCC. Because none of these changes were found in LG-DN or cirrhotic liver tissue, it is possible that the molecular examination of these loci might be helpful in the difficult distinction between LG-DN and HG-DN in biopsy material.

Materials and Methods

Specimens

Twenty-six formalin-fixed, paraffin-embedded hepatocellular nodules from 21 patients were retrieved from the files of the Institutes of Pathology of Naples, Milan, and Basel. The clinicopathologic characteristics of the lesions are summarized in Table 1. Sixteen nodules were classified as MRN, four as LG-DN, and six as HG-DN. The hepatitis B virus+/hepatitis C virus+ ratio was three of three for HG-DN, one of three for LG-DN, and eight of eight for MRN. Moreover in one case, we were able to analyze a LG-DN and an HG-DN from the same liver explant. In addition, four specimens of cirrhotic, nonneoplastic tissue were examined as controls. MRN and dysplastic nodules were defined grossly and microscopically following the criteria and nomenclature of the International Working Party (IWP, 1995). All MRN were larger than 0.5 cm in diameter (mean, 1.2; range, 0.9 to 1.5 cm) and surrounded by a condensed rim of fibrous tissue. Portal structures were distributed throughout the lesion. Grossly, dysplastic nodules were lesions distinct from the surrounding liver parenchyma in size, color, texture, or degree of bulge beyond the cut surface of the surrounding liver (Fig. 1A). Histologically, dysplastic nodules were classified as low grade (showing normal architecture and mild cytologic atypia) (Fig. 1B) or high grade (containing architectural and severe cytologic atypia) (Fig. 1, C and D).

DNA Preparation

All nodule blocks were trimmed to enrich for tumor. Fifteen 10-μm thick sections were taken for DNA extraction. The first and the last sections were stained with hematoxylin and eosin. Nodules having an average tumor cell content of less than 75% in these sections were excluded. DNA extraction and labeling was as described (Kallioniemi et al, 1992). Sections were deparaffinized and suspended in DNA extraction buffer containing 0.5 mg/ml proteinase K. Additional proteinase K was added at 24 and 48 hours, for a total incubation time of 72 hours. One microgram of tumor DNA was nick translated by using a commercial kit (BioNick kit; Invitrogen, Carlsbad, California) and Spectrum Green-dUTPs (Vysis Inc., Downers Grove, Illinois) for direct labeling of tumor DNA. Spectrum Red-labeled normal reference DNA (Vysis) was used for cohybridization.

CGH and Digital Image Analysis

The hybridization mixture consisted of 200 ng of Spectrum Green-labeled tumor DNA, 200 ng of Spectrum Red-labeled normal reference DNA, and 20 μg of Cot-1 DNA (Invitrogen) dissolved in 10 μl of hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, pH 7.0). Hybridization was for 3 days at 37° C to normal metaphase spreads (Vysis). Posthybridization washes were as described (Kallioniemi et al, 1992). Digital images (4,6-diamidino-2-phenylindol, FITC, and Texas Red) were collected from four to six metaphases using a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, British Columbia, Canada) and a Sun workstation. The Vysis software program was used to calculate average green to red ratio profiles for each chromosome. At least four observations per autosome and two obser-

Table 2. Most Important Alterations Found in Hepatocellular Nodules and HCCs

	MRN	LG-DN	HG-DN	HCC[a]
N	16	4	6	41
Deletions	8 (0.5/case)	3 (0.7/case)	6 (1/case)	189 (4.6/case)
Gains	3 (0.2/case)	2 (0.5/case)	6 (1/case)	105 (2.5/case)
Total	11 (0.7/case)	5 (1.2/case)	12 (2/case)	296 (7.2/case)
1q+	0	0	3 (50%)	19 (46%)
8p−	0	0	3 (50%)	18 (44%)
4q−	1	0	0	16 (39%)
9p−	0	0	0	9 (22%)
6q−	0	1 (25%)	1 (16%)	15 (37%)
13q−	2	1 (25%)	1 (16%)	16 (39%)
6p+	0	0	0	8 (20%)
8q+	0	0	1 (16%)	17 (42%)
17q+	2	0	0	15 (37%)

[a] Data from Tornillo et al, 2000.
MRN, multiacinar regenerative nodules; LG-DN, low-grade dysplastic nodules; HG-DN, high-grade dysplastic nodules; HCC, hepatocellular carcinoma.

vations per sex chromosome were included in each analysis.

Controls and Threshold Definition

Each CGH experiment included a tumor cell line (Spectrum Green MPE-600; Vysis) with known aberrations (positive control) and a hybridization of two differentially labeled sex-mismatched normal DNAs to each other (negative control). Moreover, we performed CGH on nontumoral liver. Sex-mismatched normal controls were used to test the ability of each metaphase batch to allow for a linear relationship between fluorescence intensities and DNA sequence copy numbers. Metaphases were only used if the color ratio of sex-mismatched normal DNAs was $\leq$0.66 at the X chromosome. Thresholds used for definition of DNA sequence copy number gains and losses were based on the results of CGH analyses of formalin-fixed normal tissues. A gain of DNA sequences was assumed at chromosomal regions where the hybridization resulted in a tumor to normal ratio $>$1.20. Overrepresentations were considered amplifications when the fluorescence ratio values exceeded 1.5 in a subregion of a chromosome arm. A loss of DNA sequences was presumed at chromosomal regions where the tumor to normal ratio was $<$0.80. To define an aberration, it was additionally required that the first standard deviation was above (gain) or below (deletion) 1.00.

Statistical Analysis

Chromosomal alterations were compared between various groups by the χ^2 test with Fisher's correction for continuity.

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