Frequent Multiplication of the Long Arm of Chromosome 8 in Hepatocellular Carcinoma

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ABSTRACT

Frequent allelic losses at loci on several chromosomes have been detected in human hepatocellular carcinomas, but other types of chromosomal abnormalities have not been characterized well. Using eight polymorphic DNA markers on chromosome 8, we examined 120 primary hepatocellular carcinomas for abnormalities in the copy number of these loci in tumor cells. A 2- to 6-fold increase in intensities of bands representing single alleles was observed in 32 of the 78 tumors that were informative for one or more of the markers, indicating an increase in copy number ("multiplication") of alleles on 8q. The regions that multiplied varied from almost the entire long arm to a distal segment of chromosome 8, but a terminal region distal to 8q24.11 was multiplied in all 32 cases. Similar multiplications on 8q were detected in nine of 56 colorectal carcinomas that were informative for one or more of the markers. Our study demonstrated that polymorphic DNA markers can be used to detect numerical abnormalities of chromosomal material in solid tumors in which cytogenetic analysis is technically difficult.

INTRODUCTION

Carcinogenesis is generally considered to be a multistep process that may involve cumulative mutations in oncogenes and tumor suppressor genes, as illustrated in a published genetic model for colorectal tumorigenesis (1). Any one of several mechanisms, such as point mutation, amplification of genes, chromosomal translocation, and loss or gain of chromosomal material can activate oncogenes or inactivate tumor suppressor genes. In HCCs, LOHs described at loci on chromosomes 4q, 8p, 11p, 13q, 16q, and 17p (2-6) imply the presence of putative tumor suppressor genes on these chromosomes and indicate that HCC, like colon cancer, may arise from a complex process. Little is known, however, about other chromosomal mechanisms that may be involved in hepatocarcinogenesis.

Cytogenetic analyses of primary cultured cells from solid tumors have often uncovered chromosomal abnormalities. For instance, karyotypic observations have identified translocations, partial trisomies, or losses of specific chromosomal regions as significant changes in renal cell carcinomas (7). In HCCs, however, cytogenetic data derived from primary cultured cells have been limited to a single case report (8), because of technical difficulties in chromosome preparation.

Recently the usefulness of polymorphic markers for detecting both LOH and "multiplications" (modest increases of chromosomal copy number) in solid tumor tissues was proven by careful comparative studies of karyotypic and RFLP analyses; partial trisomy of chromosome 5q in renal cell carcinomas (9) and trisomy of chromosome 7 in malignant mesotheliomas (10) were unambiguously demonstrated in autoradiographies of hybridization experiments. In these RFLP analyses, comparison of the four bands that represented both alleles in normal and tumor DNAs enabled visualization of subtle changes that had occurred in tumor DNAs.

We initiated a systematic RFLP analysis of paired DNAs from HCCs and their corresponding normal tissues to examine whether multiplication of chromosomal segments may take place during development of HCC. In this paper, we present results of RFLP analysis at loci on chromosome 8, and we demonstrate that multiplication of single alleles on part or all of the long arm of chromosome 8 has occurred in a large proportion of HCCs.

MATERIALS AND METHODS

Samples. Tumors and their corresponding noncancerous tissues were obtained during surgery from 120 patients with HCC and 99 patients with CRC. All tissues were dissected in the operation room, frozen immediately, and stored at -80°C until the DNA was isolated.

DNA Extraction and Southern Blotting. Frozen tissue samples were ground to a very fine powder in liquid nitrogen, suspended in lysis buffer, treated with proteinase K, and extracted by phenol-chloroform-isooamyl alcohol as described elsewhere (11). Five µg of each DNA sample were digested overnight with 10 times excess of restriction enzymes (Boehringer Mannheim) and fractionated by electrophoresis using a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed in order to visualize the actual amount of DNA present on each lane of the gel. The DNAs were then transferred to nylon membranes (Biodyne; Pall) in 0.1 N NaOH-0.1 M NaCl and fixed by UV cross-linking (11).

Probes and Hybridization. Of eight polymorphic markers used in this study, cosmide clones CHI-494 (D8S234), C18-277 (D8S194), C18-512 (D8S238), C18-340 (D8S223), C18-442 (D8S231), C18-212 (D8S188), and C18-134 (D8S177) were described previously (12); they had been physically localized on chromosome 8 by fluorescent in situ hybridization on R-band human prometaphase chromosomes. The chromosomal locations of these markers are shown in Tables 1 and 2. C18-512 was recently reassigned to 8q11.21-q11.22.* p380-8A, a polymorphic probe at the c-myc gene that detects a RFLP with three alleles (13), was kindly provided by Dr. Tsujimoto; the c-myc gene lies within band 8q24.12-q24.13 (14). A polymorphic restriction fragment of each cosmid was purified by agarose gel electrophoresis for use as a probe. Probes were labeled with [α-32P]dCTP by random-primer extension (15). Prehybridization, hybridization, and autoradiography were carried out as described elsewhere (11). The membranes were stripped in 0.1 N NaOH and repeatedly hybridized.

Determination of Allelic Dosage. Only the cases showing constitutional heterozygosity were used in the evaluation of allelic dosage. The signal intensity of the polymorphic alleles was quantified with a Hoefer GS-300 scanning densitometer; the peak areas corresponding to each hybridizing signal were calculated by electric integration using a GS 370 one-dimensional electrophoresis data system (Hoefer Scientific Instruments).

The difference in the amount of DNAs between paired normal and tumor DNA may result in an increase or decrease in signal intensities of both alleles in tumor DNA. Therefore, we measured the amount of DNA on each lane by ethidium bromide staining of the gel and compared that amount with the signals observed by control probes on other chromosomes. Information for the amount of DNA was taken into consideration when signal intensities for normal and tumor DNAs were compared. Some instances that required such adjustments were displayed (see Fig. 1, D to F). In these cases, overloading of tumor DNA was detected by measurement of DNA amounts and was taken into account in judging the multiplication of alleles. An analysis of allelic dosage

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; CRC, colorectal carcinoma.

4 M. Emi, Y. Fujiwara, and Y. Nakamura, unpublished data.
results consisted of the comparison of signal intensities of four bands, two derived from the alleles of the probed locus in constitutional DNA and the other two derived from the same pair of alleles in tumor DNA. The extent of multiplication of a given allele was calculated by dividing the ratio of the intensity of the multiplied allele to that of the normal allele in tumor DNA by the corresponding ratio measured in normal DNA; when the results exceeded 1.8, the allele was considered to be multiplied.

**Genotyping and Linkage Analysis.** Genotyping and linkage analysis were carried out on 40 Centre d’Etude du Polymorphism Humain (CEPH) reference families according to a procedure previously described (16).

### RESULTS

**Multiplication of 8q in HCC.** When paired normal and tumor DNAs from 120 patients with HCC were analyzed for abnormalities in the copy number of chromosome 8, the signal intensity in one allele was increased in 26 of 73 tumor DNAs, that were informative with a variable-number tandem repeat probe (CI8-134) at the telomeric band of 8q. The same panel of samples was subsequently examined with four additional polymorphic markers located on different segments of the long arm and with three markers on the proximal part of 8p. Representative autoradiographies and their densitometric profiles are presented in Fig. 1.

In HCC10, for example, probe p380-8A revealed an increased intensity of the larger allele in tumor DNA compared to its counterpart in normal DNA (Fig. 1A). Densitometric quantification showed that the larger allele was multiplied 4.4-fold in the tumor DNA. Probe CI8-134 also detected multiplication in the same tumor; the intensity of the smaller allele was increased 3.5-fold (Fig. 1B).

Three other probes on the long arm (CI8-212, CI8-340, and CI8-512) also detected multiplication of one allele in this tumor; however, no change in the intensity of alleles was observed with probes CI8-277 or CI8-494, both located in a proximal portion of the short arm. These data, which indicated that an entire long arm of one homologue of chromosome 8 was multiplied in this tumor, provided molecular evidence for partial polysomy. Fig. 1, D and E, shows examples for HCC15, in which CI8-134 detected a 3.3-fold increase in intensity of the larger allele after variations in signal intensities caused by overloading of tumor DNA were adjusted; however, a more proximal probe (CI8-212) revealed the same intensity on both alleles, indicating that multiplication in this tumor is limited to a distal part of the long arm. The frequencies of multiplication observed at each of the eight loci are shown in Table 1; 32 of 78 tumors informative for at least one locus revealed multiplication at one or more loci. Multiplication was found almost exclusively on the long arm.

The multiplication data for each of the 32 tumors are summarized schematically in Fig. 2. Among the 26 cases informative for at least one of three probes at the telomeric region (CI8-134, p380-8A, and CI8-212) and for at least one of four probes near the centromere (CI8-340, CI8-512, CI8-277, and CI8-494), seven cases showed multiplication at both telomeric and centromeric loci that suggested polysomy involving an almost entire long arm. Three tumors, HCC15, 108, and 148, showed multiplication only in the distal loci of 8q; these cases seemed to reflect partial polysomy limited to the distal 8q segment. The data presented in Fig. 2 indicate that a commonly multiplied region lies in a segment at 8q24.11-qter that includes the loci defined by p380-8A and CI8-134; the genetic distance of these two loci was estimated to be 21 cm by two-point linkage analysis of genotypes of 40 CEPH reference families (the maximum lod score of 4.36 at the recombination frequency of 0.21).

**Multiplication of 8q in CRC.** Ninety-nine CRC samples were analyzed for abnormalities in the dosage of chromosome 8 in the same manner. Examples of autoradiographies and densitometric profiles are presented in Fig. 1F. In Case 21, probe p380-8A detected increased intensity of a larger allele in tumor DNA compared to the same allele in normal DNA. Densitometric quantification showed that the allele was multiplied 4.9-fold. Frequencies of multiplication observed in CRCs at each of the eight marker loci on chromosome 8 are shown in Table 2. Multiplications were detected in 9 of 56 tumors informative with the probes p380-8A and CI8-134; these data are summarized schematically in Fig. 3. In two tumors, the probes detected multiplication of an almost entire long arm, and in five, partial polysomy distal to CI8-442 or CI8-212.

### DISCUSSION

Although losses of heterozygosity have been detected on several chromosomes in hepatocellular carcinomas, no information about other numerical abnormalities of chromosomes has been available for this type of tumor. In the present study, we demonstrated by means of RFLP analysis that multiplication of an entire arm or parts of chromosome 8q had existed in 32 of 78 HCCs.

LOH studies in human primary tumors have become more efficient because of an increase in available RFLP markers that enable
MULTIPLICATION OF 8q IN HEPATOCELLULAR CARCINOMAS

Fig. 2. Schematic representation of multiplications at loci on chromosome 8 in hepatocellular carcinomas. Case numbers are shown below, and the physical location of each marker on chromosome 8 (12) is indicated to the right of the idiogram. Closed circles, multiplication (+); open circles, multiplication (−); shaded circles, LOH.

Table 2 Multiplication at loci on 8q in colorectal cancer

<table>
<thead>
<tr>
<th>Probe</th>
<th>Locus</th>
<th>Location</th>
<th>No. of patients tested</th>
<th>Multiplication/informative case</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18-494</td>
<td>DSS234</td>
<td>8p11.23-p.11.22</td>
<td>96</td>
<td>2/45 (4)*</td>
</tr>
<tr>
<td>C18-277</td>
<td>DSS194</td>
<td>8p11.23-p11.21</td>
<td>86</td>
<td>1/40 (3)</td>
</tr>
<tr>
<td>C18-512</td>
<td>DSS238</td>
<td>8q11.21-11.22</td>
<td>93</td>
<td>0/50 (0)</td>
</tr>
<tr>
<td>C18-340</td>
<td>DSS223</td>
<td>8q11.23</td>
<td>99</td>
<td>1/25 (4)</td>
</tr>
<tr>
<td>C18-442</td>
<td>DSS231</td>
<td>8q22.2-22.3</td>
<td>74</td>
<td>0/23 (4)</td>
</tr>
<tr>
<td>C18-212</td>
<td>DSS188</td>
<td>8q24.11-24.12</td>
<td>78</td>
<td>3/25 (12)</td>
</tr>
<tr>
<td>C18-134</td>
<td>DSS177</td>
<td>8q24.23-q24.3</td>
<td>81</td>
<td>8/50 (16)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Fig. 3. Schematic representation of multiplication at loci on chromosome 8 in colorectal carcinomas. Case numbers are shown below, and the physical location of each marker on chromosome 8 is indicated to the right of the idiogram. Closed circles, multiplication (+); open circles, multiplication (−).

toxic-induced rat hepatoma (23), no amplification of c-myc in human primary HCCs has been demonstrated to date. Considering the rather modest increase of copy number detected in the present study, multiplication of this gene in HCCs may well have escaped detection in previous studies where nonpolymorphic DNA fragments were used as probes. Although Kinzler et al. (24) reported that the amplification unit containing the c-myc gene was 90 to 300 kilobases in their analysis of five cell lines derived from human cancers of the lung, brain, colon, and hematopoietic tissue, it is difficult to directly compare their results to those found here in primary HCCs, because these authors used cultured cell lines which had been passed repeatedly in

reliable detection of allelic loss, even when tumor DNAs are contaminated with DNA from normal cells. Multiplication of chromosomes is also examined in tumors contaminated with DNA from normal cells. It is obvious that the use of polymorphic DNA markers will greatly improve the sensitivity and accuracy of estimations of copy numbers, because the dosage of each allele can be compared with its counterpart.

In all 32 HCCs showing gains of chromosomal material on 8q, only one of any pair of homologous alleles was multiplied; the magnitude of multiplication of an allele ranged from 2- to 6-fold. The multiplied regions appeared to vary in size, but all were relatively large; multiplication involving almost the entire long arm was detected in seven tumors, and three others revealed multiplication of the distal part of 8q. The degrees of multiplication and the sizes of the regions involved indicate that the chromosomal changes in these tumors represent trisomy, tetrasomy, or polysomy of all or parts of 8q, rather than the "gene amplification" phenomenon represented by double-minutes or heterogeneously staining regions in which a minute segment of a chromosome containing a drug resistance gene or an oncogene, for example, is amplified up to several hundredfold.

Cytogenetic analyses have frequently detected trisomy or tetrasomy of 8q in acute nonlymphocytic leukemia, myelodysplastic syndrome, gastric cancer, and uveal melanoma (17–20). Isochromosomes of 8q have been observed in colon cancers and in lung cancers (21, 22). Frequent observation of gain of 8q in these hematopoietic malignancies and cancers, together with our findings in HCC and CRC, emphasizes the likelihood of etiological significance for this type of chromosomal aberration in carcinogenesis.

Plasmid 380-8A, a polymorphic marker for the c-myc gene located at 8q24, was used in the present study. One of the alleles of the c-myc gene was multiplied 2- to 6-fold in 44% of the informative HCCs tested. Although amplification of c-myc has been reported in an afla-
vitro and were preselected for the presence of a high degree of c-myc amplification (>50-fold).

In the tumors presenting multiplications of 8q in this study, c-myc might be the gene playing an important role during carcinogenesis. However, our findings indicated that the common region of multiplication spans a segment of 8q24.11 to 8qter, which is at least 21 CM long and probably contains several hundreds of unknown genes. Hence, we assume that the increase in the copy number of one or more of these genes confers a growth advantage during development and/or progression of tumors. Identification of such gene(s) and their relevance to carcinogenesis await future characterization of the 8q24.11-qter region. The same set of HCC samples described here had been subject to LOH analysis in our previous study that detected frequent LOH on chromosome 8p (13). Of the 32 HCC cases with 8q multiplications, 19 detected loss of 8p, raising the possibility that some of the combined changes may reflect creation of isochromosome 8q.

We examined the presence of 8q multiplications in CRCs as this type of tumor has also shown frequent LOH on chromosome 8p (13). The 8q amplification was observed at a lower frequency in CRCs compared to that in HCCs. Although we observed no multiplications with probes on chromosome 2q, 9q, 10p, and 20p, the significance of the 8q amplification in primary breast and colorectal tumors in which somes will facilitate similar investigations. In addition to the frequent multiplications of 8q described here, we have found multiplications on other chromosomes in HCCs. These data imply that hepatocarcinogenesis is truly a complex process, which involves not only losses but also multiplications of chromosomes as mechanisms for development and progression of tumors.

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