Concerted Nonsyntenic Allelic Losses in Hyperplastic Hepatocellular Carcinoma as Determined by a High-Resolution Allelotype

Valérie Boige, Pierre Laurent-Puig, Pierre Fouchet, Jean François Fléjou, Geneviève Monges, Pierre Bedossa, Paulette Bioulac-Sage, Frédérique Capron, Anette Schmitz, Sylviane Olschwang, and Gilles Thomas

Service des Maladies du Foie et de l'Appareil Digestif [V. B.] and Laboratoire d'Anatomie-Pathologique, 78 Avenue du Général Leclerc, Hôpital de Bicêtre, 94270 Le Kremlin Bicêtre [P. B.]; Institut Curie, Laboratoire de Génétique des Tumeurs, [P. L. P., S. O., G. T.], 26 Rue d'Ulm, 75231 Paris Cedex 05; Laboratoire de Cancérologie Expérimentale, Commissariat à l'Energie Atomique, 60 Avenue Division Leclerc, 92265 Fontenay-aux-Roses [P. F., A. S.]; Laboratoire d'Anatomopathologie, Hôpital Beaujon, 100 Boulevard du Général Leclerc, 92110 Clichy [J. F. F.]; Laboratoire d'Anatomie-Pathologique, Centre Paoli-Calmettes, 232 Boulevard Sainte Marguerite, 13009 Marseille [G. M.]; Service d'Anatomie-Pathologique, Hôpital Pellegrin, Place Amélie-Raba-Léon, 33076 Bordeaux [P. B-S.]; and Laboratoire d'Anatomie-Pathologique, Hôpital Antoine Beclère, 157 Rue de la Porte de Trivaux, 92140 Clamart [F. C.], France

ABSTRACT

Although the occurrence of loss of genetic material in hepatocellular carcinoma (HCC) has been documented both by cytogenetic analysis and by monitoring of allelic losses, a global overview of the extent and frequency of deletion occurring throughout the genome is not yet available. To contribute to this information, DNAs extracted from flow-sorted aneuploid nuclei from HCC and matched normal DNAs were typed for 275 microsatellite loci that were distributed along the autosomes. An average of 190 (69%) informative loci per case were generated on 48 HCC. Complete loss of heterozygosity in the tumor DNA was observed for 15.6% of the typed loci. The chromosome segments that were most frequently affected by deletion were: 8p (60%), 17p (48%), 1p (44%), 4q (42%), 16p (40%), 16q (39%), 6q (35%), 9p (30%), and 13q (29%). On average, 8 of the 39 chromosome segments studied per tumor carried at least one locus that demonstrated loss of heterozygosity (i.e., the fractional allelic loss was 0.21). Groups of concerted nonsyntenic losses were observed for 16p and 1p and for 16p and 4q. The location of putative tumor suppressor genes on the most frequently deleted regions was confirmed and, in some cases, refined.

INTRODUCTION

With a worldwide annual incidence of 250,000 cases, HCC is among the three most common malignancies in the human population (1). It has been associated with several genetic alterations. Infrequent point mutations in K-ras, N-ras, and IGF2 receptor have been observed (2–4). Insertion of viral sequences from hepatitis B virus has on rare occasions been reported to activate neighboring genes (5–7). A mutation at codon 249 of the p53 gene has been observed in tumors of patients exposed to aflatoxin B1 (8, 9).

However, as initially suggested by karyotypic analysis and subsequently extended by monitoring allelic status, genetic alterations frequently involve the loss of material. Indeed, LOH has been reported for loci on chromosomes 1, 4, 5, 8, 10, 11, 13, 16, 17, and 22 (10–16). With the exception of p53, which is associated with LOH on 17p, none of the other losses has yet been associated with the inactivation of a specific TSG. For many chromosome segments, the LOH frequencies demonstrate large variation among published series. However, the most frequent losses appear to involve both arms of chromosome 16 and the short arms of chromosomes 17 and 8 (14, 17, 18).

Genome-wide allelotypes, which assay the frequency and extent of loss regions on all autosomal arms, have been performed for several tumor types. Their accuracy relies not only on the number of tumor samples studied but also on the number and informativeness of the polymorphic loci typed per tumor. Until recently, most genome-wide allelotypes had been obtained with a small number of loci, which were mostly RFLP loci analyzed by Southern blotting. This procedure has several drawbacks: it is time consuming, requires large amounts of DNA, and is only moderately informative because RFLP loci are frequently biallelic.

The development of a large number of microsatellite loci that can be typed by PCR has overcome these difficulties. Microsatellite loci are usually multiallelic, with a frequency of heterozygosity higher than 0.7. For their typing, microsatellite loci require small amounts of DNA that can be obtained from a few selected tumor cells.

Taking advantage of these technical advances, we have compared the allelic status of 275 polymorphic loci in 48 aneuploid HCCs and their matched normal DNA. To obtain a clear-cut pattern of allelic loss, potential contamination of the tumor fragments by nonneoplastic tissue was excluded by nuclei flow sorting. The resulting high-resolution allelotype identifies the chromosomal segments that are most frequently deleted and reveals nonrandom association between these alterations.

PATIENTS AND METHODS

Patients

Primary hepatocellular carcinoma and matched nontumor liver tissues were obtained from 100 patients during surgery (n = 97) or at autopsy (n = 3). The tissue samples were dissected and stored at —80°C until use. The mean age of the patients was 56 years (range, 27–74 years). The sex ratio (male:female) was 5:1.

Cell Sorting and DNA Extraction

Nuclei suspensions were obtained from frozen tumor samples. A fragment of approximately 100 mm3 was homogenized with a Dounce homogenizer and a B-pestle in a low ionic strength buffer (50 mm KC1/5 mm Hepes/10 mm MgSO4, pH 8) containing Hoechst 33258 (2.5 μg/ml; Riedel de Haen, Seelze, Germany) for DNA staining. Nucleus suspensions were filtered through a 45 μm nylon mesh.

DNA content measurements of the nucleus suspensions were performed with a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA) equipped with a Coherent laser 307 (Palo Alto, CA) tuned to excite the DNA fluorochrome and a B-pestle in a low ionic strength buffer (50 mm KC1/5 mm Hepes/10 mm MgSO4, pH 8) containing Hoechst 33258 (2.5 μg/ml; Riedel de Haen, Seelze, Germany) for DNA staining. Nucleus suspensions were filtered through a 45 μm nylon mesh.

DNA content measurements of the nucleus suspensions were performed with a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA) equipped with a Coherent laser 307 (Palo Alto, CA) tuned to excite the Hoechst 33258 in the UV range (358–361 nm; 0.5 W). The Hoechst 33258 fluorescence was collected with an LP400 and SP505 (Omega, Brattleboro, VT) filter combination. Hoechst 33258 fluorescence area and width parameters were used to exclude doublets of nuclei. Calibration was performed using chicken erythrocytes and calf thymocytes (Becton Dickinson, San Jose, CA).

Histograms of DNA content were performed on 25,000 nuclei per sample. PCR amplification was performed without a DNA extraction step on 300 hypervariable nuclei that had been sorted using ACDU software (Becton Dickinson, San Jose, CA) in each tube of a 96 PCR microtube array (Perkin-Elmer Corp.).
DNA Amplification and Gel Electrophoresis

Microsatellite Loci. Based on their frequency of heterozygosity, 275 microsatellite loci with an approximately even distribution on the 39 autosomal arms (the short arms of acrocentrics were not considered) were selected from the Genethon database (list available on request). The mean sex average genetic distance between two consecutive marker loci was 12.8 cm. Only 18 intervals were greater than 20 cm, and none reached 30 cm. They were used to determine LOH at each locus for 48 HCCs by comparing microsatellite patterns after PCR amplification from matched tumor and nontumor DNA of the same patient. Three or four microsatellite loci were amplified simultaneously in each reaction, and 83 MPCR were performed to amplify the 275 loci in tumor and nontumor DNA. In several cases, we checked that no allelic loss was detectable in normal reference tissues by comparing the genotyping data obtained on DNA extracted from lymphocytes and from nontumor liver tissues.

MPCR of microsatellite sequences was carried out as follows. The reaction was performed in a total volume of 20 μl containing 300 nuclei as template DNA in PCR buffer (10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM MgCl2/80 μM dNTPs/0.2 mM each appropriate primer) and using the following conditions of temperature: initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), and elongation at 72°C (90 s; the PCR9600 from Perkin-Elmer Corp. was used). Nontumor DNA was extracted using standardized methods, and 2 ng of DNA were used for each MPCR. This amount was approximately equivalent to that contained in 300 nuclei.

Amplified products (1 μl each) were electrophoresed in 6% polyacrylamide sequencing gels [7 M urea and 32% (v/v) formamide; acrylamide-bisacrylamide, 29:1] then briefly transferred by capillary blotting on a nylon membrane and hybridized with a 32P-3' end labeled (CA)12 oligonucleotide, which allows for the simultaneous detection of all amplified alleles. Each locus was scored either for its stability status, according to the absence or presence of mobility shifts (replication error), or for the LOH, according to the disappearance of one allelic band from the amplification product of the tumor DNA as compared to that of the nontumor DNA. Because the tumor nuclei had been specifically selected by flow sorting, the presence of an imbalance of intensity between the two allelic variants was not scored as an allelic loss.

RESULTS

Surgical fragments of tumors are contaminated to various degrees by nonneoplastic cells. Cryostat sectioning, in favorable cases, allows for the microdissection of the normal tissue component that is frequently found adjacent to the tumors. However, inflammatory or endothelial cells are commonly observed dispersed throughout the tumor mass, thus precluding their elimination by microdissection. To overcome this problem, the DNA content of nuclei prepared from nonneoplastic cells. Cryostat sectioning, in favorable cases, allows for the simultaneous detection of all amplified alleles. Each locus was scored either for its stability status, according to the absence or presence of mobility shifts (replication error), or for the LOH, according to the disappearance of one allelic band from the amplification product of the tumor DNA as compared to that of the nontumor DNA. Because the tumor nuclei had been specifically selected by flow sorting, the presence of an imbalance of intensity between the two allelic variants was not scored as an allelic loss.

A total of 275 microsatellite loci were assessed for each tumor, and an average of 190 (69%) informative loci per case were generated. This percentage was similar to that of the mean of marker heterozygosity (i.e., 70%).

All nonacrocentric autosomal chromosome arms were assessed at three or more loci for LOH. In total, 1853 of 1872 chromosome arms (99%) studied were informative for at least one microsatellite locus. A hemizygosity index was defined for each tumor as the ratio of the number of loci that demonstrated a LOH to the number of informative loci tested. This index ranged from 0 (two tumors demonstrated no allelic loss) to 0.40 (mean value, 0.156). Most tumors exhibited microsatellite instability at one locus or at a small number of loci. The ratio of the number of microsatellite loci that demonstrated a mobility shift in the tumor DNA to the number of tested loci ranged from 0 to 0.14, with a mean of 0.013 per tumor.

A second analysis of the LOH data, which took into account the synteny between loci located on the same chromosome segment, was performed. A chromosome arm qualified as deleted if at least one of the microsatellite loci located in this segment showed evidence of LOH. On average, tested chromosome arms were deleted in 20% of the cases. However, large variations were observed according to the specific chromosome arm (Fig. 2). The long arm of chromosome 2 was never deleted in our observations. In contrast, nine chromosome arms [frequency of deletion is shown in parentheses: 8p (60%; CL13q, 47–74), 17p (48%; CL19q, 35–63), 1p (44%; CL19q, 31–59), 4q (42%; CL13q, 29–57), 16p (40%; CL13q, 26–54), 16q (39%; CL19q, 28–55), 6q (35%; CL13q, 24–51), 9p (30%; CL19q, 19–44), and 13q (29%; CL13q, 19–44)] were deleted with a frequency higher than 29%. This latter value represents the 99.99% confidence upper limit for the overall rate of random chromosome loss in this series of tumors, suggesting that changes occurring more frequently than this baseline level are more likely to represent potentially causative genetic alteration rather than secondary events associated with generalized genomic instability. For the subsequent analysis, only these nine chromosome arms were taken into account.

As proposed by Vogelstein et al. (19), the FAL index was computed as the fraction of evaluable nonacrocentric autosomal arms demonstrating allelic deletion. The mean FAL index was evaluated to be 0.20 (range, 0–0.42; Fig. 2). The possible association between FAL index values and LOH at specific chromosome arms was investigated (Table 1). LOH was significantly associated with high FAL values for all arms tested, with the exception of 8p and 17p.

Correlation between LOH on different pairs of chromosome arms was also observed. Taking into account the number of tests performed (n = 36), the threshold of significance was taken at a level of 0.001 to keep the type I error at or under 5%. Under this condition, a significant association between LOH occurring on the short and long arms of chromosome 16 was observed. Interestingly, 12 of 19 tumors (63%) that showed LOH on chromosome 16p demonstrated LOH at all informative chromosome 16 loci, an observation compatible with the loss of the entire chromosome. Significant correlations between
the occurrence of LOH on 16p and that on 1p and 4q were also evident (Table 2). Thus, two significant groups of non-syntenic allelic losses were identified between chromosome arms 16p and 1p and between 16p and 4q. Although the threshold of significance ($P < 0.001$) was not reached, similar relationships between LOH occurring on 4q and

Table 2: Associations between LOH at specific chromosome arms

<table>
<thead>
<tr>
<th>Chromosome arm</th>
<th>1p</th>
<th>4q</th>
<th>6q</th>
<th>8p</th>
<th>9p</th>
<th>13q</th>
<th>16p</th>
<th>16q</th>
<th>17p</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Mean FAL index</td>
<td>0.14 ± 0.09</td>
<td>0.16 ± 0.11</td>
<td>0.27 ± 0.11</td>
<td>0.26 ± 0.10</td>
<td>0.25 ± 0.12</td>
<td>0.20 ± 0.13</td>
<td>0.28 ± 0.11</td>
<td>0.26 ± 0.12</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>$p^b$</td>
<td>0.0001</td>
<td>0.0025</td>
<td>0.025</td>
<td>0.007</td>
<td>NS$^e$</td>
<td>0.01</td>
<td>NS$^e$</td>
<td>0.0001</td>
<td>0.032</td>
</tr>
</tbody>
</table>

$^a$ FAL index calculated to include LOH at all sites excluding the chromosome under test.

$^b$ Based on Mann-Whitney U test.

$^c$ NS, not significant.

DISCUSSION

In their seminal description of the first allelotype, Vogelstein et al. (19) reported the use of 53 RFLP loci and a mean number of 25 typed
informative loci per tumor. In toto, information on an average of 25 autosomal chromosome arms was collected per tumor (i.e., 64%). Since this first publication, the alleloypotyping approach has been extended to a wide variety of tumors, including HCC (10). In HCC, LOH has been evaluated in 46 cases at 44 loci exhibiting RFLPs (average heterozygosity, 39%) located on 22 autosomal chromosomes.

With the development of microsatellite markers, both the number of loci that can be typed and their informativeness has considerably increased (20, 21). In our series, an informative locus was observed for 99% of the 39 chromosomes arms studied. This represents the most comprehensive coverage of the genome in any alleloypotype report to date.

LOH analysis of DNA extracted from tumor tissue is limited by the purity of the tumor specimens, which are inevitably contaminated to various degrees by normal cells. In the presence of such contamination, it may not be possible to decide whether an allelic imbalance is due to a trisomy or to a true hemizygosity. This difficulty may be more pronounced when PCR is used for genotyping. Microdissection of the tumor specimen has been used to enrich for tumor cells. However, such a procedure does not remove isolated stromal cells deeply buried in the tumor (22). An alternative and more radical approach to tumor DNA enrichment takes advantage of the frequent aberrant DNA index of tumor cell nuclei. The combined use of flow cytometric measurement of DNA index and sorting allows for the production of pure fractions of neoplastic nuclei (23). In the case of HCC, this procedure can be applied to aneuploid tumors, which make up 50–60% of HCCs (24, 25). In most cases, microdissected tumor fragments analyzed by flow cytometry revealed, in addition to the aneuploid component, a diploid fraction (frequently amounting to more than 20% of all nuclei) that may, in part, be contributed by stromal cells. The present series of tumors consists exclusively of aneuploid tumors, and thus, our conclusion pertains only to this group. Because increasing aneuploid DNA content correlates significantly with increasing allelic losses ($r = 0.40; p < 0.01$; data not shown), in diploid HCC, the frequencies of LOH might be smaller than those reported here.

The calculated mean FAL index in this series of 48 HCCs was similar to those calculated for a wide variety of tumors, including non-small cell lung carcinoma (26), colorectal tumors (19), esophageal squamous cell carcinoma (27), and pancreatic cancer (28). It is less than those reported for osteosarcoma (0.32; Ref. 29) and epithelial ovarian cancer (0.35; Ref. 30) and greater than those found in bladder cancer (0.11) and in situ ductal carcinoma of the breast (0.03; Refs. 20 and 31). However, the large SDs for the average FAL observed for each tumor type and differences in experimental setting for its determination preclude the conclusion that these differences reflect distinct mechanisms in tumor development.

In the present series, a classification of tumors according to the status of specific chromosome arms reveals that the average FAL index is usually highest in the group of tumors with the deleted segment. However, for 8p and 17p, this difference did not reach statistical significance, an observation suggesting their early involvement in tumorigenesis. For 8p, this hypothesis was reinforced by the observation of one HCC and one hepatocellular adenoma (data not shown) in which 8p was the only deleted chromosome segment. A high density of LOH information allows for the seeking of a correlation between nonsyntetic allelic losses. We found associations of losses between 16p, 4q, and 1p, indicating that the corresponding putative TSGs may cooperate in liver tumorigenesis.

Small regions of frequent deletion were identified in six chromosome arms (Table 3). In all six chromosomes, these deletions overlapped with those described previously in the literature. The smallest common region of deletion on chromosome 8p included the 600-kb region described by Fujiwara et al. (32, 33) as deleted in HCC, colorectal cancer, and non-small cell lung cancer. A putative TSG (PDGF-receptor β-like TSG) located in this region has been reported to be mutated in two HCCs (32, 33). The chromosome 13 deleted region contains the RB gene, which has been suggested to be involved in HCC progression (34, 35). The most frequently deleted region on 6q that is observed in this study is entirely included in larger regions pointed out by others as being frequently deleted in several tumor types (36–39). In human HCC, the observed reduced expression of M6P/IGFIR (40), a gene that maps in this region or in its vicinity, has led to the suggestion that it might play a tumor suppressor role. This hypothesis has been reinforced by the demonstration of LOH at this locus (41) associated with somatic point mutations in the remaining allele (4). The TSG TP53 is included in the SRCD of the short arm of chromosome 17. However, in developed countries, the large discrepancy between the high frequency of 17p LOH and the low incidence of TP53 gene mutation (42–44) is compatible with the involvement of a different TSG located on this chromosome segment. Ten tumors showed interstitial deletion at 4q, defining two regions of deletion on this chromosome arm. The first region lies between D4S406 and D4S424 and overlaps that identified by Buetow et al. (45). The second region, which is telomeric to the first, had not been described previously. Its presence is suggested by a single tumor that showed a deletion of 4q, which lies outside the first region. Such a deletion may have been generated by a random loss. In this study, the size of the minimal regions of deletion varies from 11 to 56 cM. Therefore, the sizes of these deletions remain too large to initiate positional cloning approaches in search of new TSGs. Further alleloypotyping of a large number of additional HCC tumors should allow the delineation of smaller regions, thus allowing one to narrow down the search for transcribed regions that would be potential target sequences of interest in such an approach.

**REFERENCES**


Concerted Nonsyntenic Allelic Losses in Hyperploid Hepatocellular Carcinoma as Determined by a High-Resolution Allelotype


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/10/1986

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.