Isolation of NotI Clusters Hypomethylated in HBV-integrated Hepatocellular Carcinomas by Two-Dimensional Electrophoresis

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(Received 11 March 1999; revised 26 May 1999)

Abstract

To examine genetic and epigenetic alterations associated with HBV integration in hepatocarcinogenesis, we compared genomic DNA profiles of primary hepatocellular carcinomas (HCCs) and cell lines that either contained or did not contain integrated HBV. To accomplish this, we carried out Restriction Landmark Genomic Scanning (RLGS), a two-dimensional system that displays 2000-3000 NotI landmark sites in a single gel electrophoresis experiment. We identified one NotI landmark spot that showed high signal intensity in HBV-integrated cell lines or in primary HCCs, but not in HCCs or tumor-cell lines free of HBV integration. Cloning of this spot revealed that it consisted of a NotI cluster sequence enriched with CpG dinucleotides. This sequence, hypomethylated in association with HBV integration, was found in the peri-centromeric region of human acrochromosomes. The results demonstrate that epigenetic changes at specific sequences in the genome occur in association with HBV integration during the process of hepatocarcinogenesis.

[The sequence data have been deposited in the EMBL database under accession number Y10751]

Key words: Restriction Landmark Genomic Scanning (RLGS); hepatocellular carcinoma (HCC); hepatitis B virus (HBV); methylation

1. Introduction

A new system for scanning the human genome by two-dimensional electrophoresis, called RLGS (Restriction Landmark Genomic Scanning), combined with micro-cloning methods to resolve NotI-containing DNA fragments (spots), was recently established in our research group.1,2 In brief, RLGS allows genomic DNAs to be surveyed at roughly 2000–3000 “landmark” sites in a single two-dimensional electrophoretic gel. Genomic DNA fragments cleaved by NotI and end-labeled with radioisotopes are then cleaved by two additional endonucleases, and separated by two-dimensional electrophoresis that displays at a glance thousands of NotI-containing landmarks for the genome.

The human genome contains approximately 4000 NotI sites; of those, 3500 are thought to lie in the CpG islands scattered throughout the genome.3 A large proportion of NotI sites present in the genome can be surveyed in a single RLGS analysis. Since the NotI spot pattern resolved by RLGS is consistent and reproducible, comparing RLGS profiles of genomic DNA from different sources enables one-to-one matching of spots and detection of multiple genetic or epigenetic changes in a single experiment. Alterations in the methylation status of individual CpG islands can also be effectively monitored by RLGS, because NotI is sensitive to methylation within its DNA-recognition sequence. Therefore the RLGS system and micro-cloning together serve as a powerful means of detecting and characterizing changes in methylation status.

Primary hepatocellular carcinoma (HCC) is one of the most frequent cancers among males throughout the world. As the hepatocytes go through repeated regeneration as a consequence of chronic hepatitis or liver cirrhosis, a variety of genetic and epigenetic changes are likely to take place. Epidemiological data associating hepatitis B virus (HBV) infection with HCC have been accumu-
Hypomethylated Not I Clusters in Human HCCs

2. Results

2.1. Identification and cloning of intensified Not I spot in genome DNA

Among the multiple spots resolved in RLGS analysis of human DNA cleaved by and labeled at Not I restriction sites, we found that one highly intensified spot appeared in cell line HB611, which contains transfected, integrated HBV, but not in its parent cell line, Huh6, which does not contain HBV (Fig. 1). This spot, spot “A”, was targeted for cloning. We had previously shown that this same spot was intensified in 81% of primary HCC tissues we examined.2

We cloned the Not I-cleaved DNA fragment representing the highly intensified spot by a method we developed recently.6 This genomic DNA fragment, designated DMHA-1, was 591 bp long and had a Not I site at one end and a Hinf I site at the other. Figure 2a shows the nucleotide sequence of DMHA-1 (Not I-Hinf I, 591 bp, EMBL accession No. Y10751) that has a GC content of 52% and is similar to the sequence of so-called “CpG DNA clones”. One such clone, 11e8 (GenBank accession No. Z36481), showed 88% identity with DMHA-1 DNA in an overlapping region of 289 bp.

2.2. Structure of genomic region surrounding DMHA-1

We screened and isolated three cosmid clones harboring the DMHA-1 locus. Sequencing and restriction mapping of one of the cosmids (cosA-1) together with the original DMHA-1 fragment revealed that the genomic insert of cosA-1 consisted of six Not I fragments, ranging from 6.5-Kb to 22.5-Kb in size (Fig. 2b). Restriction mapping indicated that Not I restriction sites were clustered around the DMHA-1 locus. Multiple tissue Northern blots, including HCC samples where the genomic changes occurred at the DMHA-1 locus, indicated that cosmid clone contained no transcribed sequences (data not shown).

2.3. Hypomethylation of DMHA-1 locus in HBV-integrated HCCs

We analyzed the HB611 cell line and primary HCCs by Southern blotting and confirmed earlier observations that the band produced by the DMHA-1 clone was highly in-
Figure 2. (a) Nucleotide sequences of DMHA-1 (Not I-Hinfl fragment, 591 bp). (b) Not I restriction map of cosmid cosA-1 insert. CosA-1 contains 6.5 Kb Not I fragment. The bold bar at the bottom indicates the position of the Not I-Hinfl sequence cloned from the 2D gel. The 2.5 Kb Not I-EcoRV (2.5 Kb NV) fragment used as probe in Southern blot analysis is indicated above the map. N: Not I, E: EcoRI, V: EcoRV.

HCCs than in the specimens free of HBV integration (Table 1). Among cancers from tissues other than liver, the change was observed only in two squamous cell carcinomas of the lung (Table 1).

2.4. Chromosomal mapping and tissue specificity

In situ hybridization of cosA-1 containing the DMHA-1 sequence as a probe gave fluorescence in situ hybridization (FISH) signals as follows: strong signals at the pericentromeric regions on acrocentric chromosomes, such as 13p13, 14p13, 15p13, 21p13 and 22p13; moderate signals on 14q11.1, 19q13.2, 20p11.1, and 22p11.1; and weak signals on 3q11.1-11.2 and 4p11.1-12, all of which are rich in satellite DNAs (Fig. 4). These results indicate the DMHA-1 belongs to a gene family consisting of homologous sequences scattered in the centromeric regions.

3. Discussion

We have demonstrated frequent hypomethylation of a centromeric sequence characterized by Not I cluster in primary HCCs and hepatoma cell line whose genome contained integrated HBV. In previous RLGS experiments involving primary HCCs, we had observed five spots that were often intensified in the tumor DNA as compared to their normal counterparts. One of them, spot “A”, was
Figure 3. Tumor-associated hypomethylation of CpG sequences demonstrated by Southern blots of DNAs from HCCs and normal counterparts probed by DMHA-1. (a) Each lane contains 5 μg of genomic DNAs from a HCC (C), which showed intensification of spot A by RLGS analysis and its normal counterpart DNA (N). An intensified band is indicated by an arrowhead; this band appeared only when genomic DNAs were digested with Not I/EcoRV, which is sensitive to methylation. (b) Methylation assay. The same DNAs were digested with Hpa II, Msp I, Hha I and Hae III. The samples digested with Hpa II and Hha I, enzymes that are sensitive to methylation of cytosine at CpG, show mobility shifts indicating that hypomethylation occurred in the HCC. (c) Southern blot analysis of DMHA-1 in genomic DNAs from hepatoma cell lines (Not I/EcoRV digests). Two HBV DNA integrated cell lines, HB611 and PLC/RPF/5 show hypomethylation. (d) Southern blot analysis of HepKANO hepatoma cell line (Not I/EcoRV digests). C, original HCC tissue; N, original normal liver tissue; HKCL2 0m, CL2 at initiation of culture; HKCL2 17m, CL2 after 17 months; HKCL1 20m, CL1 after 20 months; HKCL1 26m, CL1 after 26 months of culture. Each lane contains 5 μg of genomic DNA digested with Not I and EcoRV. An intensified 2.5-Kb band (arrowhead) in both HB611 and HepKANO CL after 26 months are noted. These bands indicate hypomethylation of CpG residues of DMHA-1 in HB611, and de novo hypomethylation in older HepKANO cells.
Table 1. Frequencies of hypomethylation of DMHA-1 sequence among primary tumors.

<table>
<thead>
<tr>
<th>Tumors</th>
<th>DMHA-1 hypomethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>0/5</td>
</tr>
<tr>
<td>Colon</td>
<td>0/6</td>
</tr>
<tr>
<td>Thyroid (Pap)</td>
<td>0/3</td>
</tr>
<tr>
<td>Kidney (RCC)</td>
<td>0/2</td>
</tr>
<tr>
<td>Lung (SCC)</td>
<td>2/2</td>
</tr>
<tr>
<td>Lung (Ad.ca)</td>
<td>0/2</td>
</tr>
<tr>
<td>HCC (HBV int+, HBsAg+)</td>
<td>6/8</td>
</tr>
<tr>
<td>HCC (HBV int-, HBsAg–)</td>
<td>4/21</td>
</tr>
</tbody>
</table>

The number of hypomethylation of the DMHA-1 Not I regions among cancers detected by Southern blot analysis digested with Not I/EcoRV; (RCC), Renal cell carcinoma. (SCC), Squamous cell carcinoma. (Ad.), Adenocarcinoma. + in parenthesis indicates the HBV DNA integrase positive, –, negative.

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Loss of methylation has been suggested as a means of gene activation; for instance, experimental data in rats have shown that proto-oncogenes such as c-myc, cHa-ras, cKi-ras, or c-fos tend to be hypomethylated in the cell overgrowth in regenerating liver and early nodules. Methylation is considered to suppress transcription of genes associated with CpG islands. For example, in cell lines cultured for long periods, most nonessential genes may have become methylated, leaving unmethylated only those whose expression is required for survival. At the chromatin level, methylated islands coincide with an altered structure in which methylated CpG sites are protected against nucleases.

At the chromatin level, methylated islands coincide with an altered structure in which methylated CpG sites are protected against nucleases. Chromatin might be expected to impede rather than facilitate the interaction of certain molecules with their target sites; indeed, DNA-binding proteins are excluded from sites that are assembled in chromatin in vitro. It is therefore significant that active promoters coincide with nucleosome-hypersensitive sites, which are probably stretches of DNA from which a nucleosome has been displaced. On the other hand, the hypomethylation we observed might affect the chromatin structure in such a way as to ensure aberrant transcription, by permitting transcription factors to bind to CpG sites.

Alternatively, hypomethylation could be interpreted as a result of an impaired normal methylation mechanism in already highly disordered cells. There is no evidence that inhibitors of methylation such as 5-azacytidine can directly activate naturally methylated CpG-deficient promoters. Methylation is not necessary for mitotic condensation, but once a chromosomal region is marked by methylation it apparently resists interphase decondensation. Binding of proteins to methylated CpG sites may make them inaccessible to transcriptional factors or other molecules that would maintain the chromosomes in condensed form. Thus a large, highly folded chromosomal domain can be converted both structurally and functionally by hypomethylation; this mechanism could presumably specify other large chromosomal domains during differentiation. Inactivity of genes and late replication in methylated chromosomal regions would be likely consequences of this chromosomal conformation. Therefore, decreases in methylation status are not random, and heterogeneity might parallel the heterogeneous biological properties. Chromosome unraveling associated with replication is transient; replicated DNA returns rapidly to a more condensed configuration. Therefore transient, dynamic conversion to an extended structure by hypomethylation, if it occurs during DNA replication, may provide a basis for the rapid replication characteristic of cancer cells or tumor cell lines. Since the change
we observed in the experiments reported here was associated with HBV-containing primary HCCs and hepatoma cell lines, the epigenetic event it reflects may prefer particular chromosomal arms in a tissue-specific manner. It is known, for example, that treatment with 5-azacytidine causes unfolding of centromeres with selectivity for some chromosomes.\textsuperscript{17,18}

Our sequence hypomethylated in association with HBV integration was found mainly in the pericentromeric region of human acrocentromeric chromosomes. This region is characterised by a major class of repetitive DNA sequences known as the alpha satellite DNA.\textsuperscript{19}-\textsuperscript{21} Cloning repetitive sequences including alpha satellites on selective chromosomes has been reported.\textsuperscript{22} The existence and maintenance of several discrete alphoid domains within a centromere suggest that this region can no longer be regarded as a single amorphous block of alpha satellite DNA in which two homologous chromosomes can pair and undergo unrestricted unequal crossing-over as is generally found?\textsuperscript{23} A possible biological role of centromere-specific, tandemly repeated sequences in the fission yeast, \textit{Saccharomyces pombe} is the centromere-mediated maintenance of sister chromatid attachment in mitosis.\textsuperscript{24} Although our clones did not contain the typical fundamental repeated alpha satellite monomer units of approximately 170 bp, they seem to share some common properties such as peri-centromeric localization of acrocentromeric chromosomes.

In conclusion, we have isolated a hypomethylated “hotspot” in hepatoma cells that is associated with integration of HBV DNA into the genomes of these cells. The hypomethylation was observed specifically in Cpg dinucleotides around Not I restriction sites located at the centromeres of several chromosomes. The changes in methylation status that closely correlate with HCC may be responsible for altering chromatin structures, leading the genes involved in carcinogenesis to be activated. However, the mechanism that controls methylation/ hypomethylation and the implied etiological relationship between tumorigenesis and methylation status remain to be clarified in future work.

4. Materials and Methods

4.1. Primary cancers and cell lines

Genomic DNAs extracted from surgically resected samples of primary cancers, and from HCC-derived cell lines were analyzed in these experiments. The primary cancers consisted of 41 HCCs, five gastric carcinomas, six colon cancers, three papillary thyroid carcinomas, two renal-cell carcinomas, two squamous cell carcinomas of lung, and two adenocarcinomas of the lung. The hepatoma cell lines were HepG2, Huh6, Huh7, PLC/RFP/5, and HepTABATA. The last named was derived from a patient whose serological tests were negative for HBV but positive for HCV antibody (Baba et al., unpublished data). Hep KANO clones 1 and 2 (CL1 and CL2) were previously described.\textsuperscript{25} A similar pattern of HBV integration in both of these cell lines suggests they have the same origin: chromosomal analysis revealed that CL1 contains a modal number of 34-45. HB611 was established by transfection of a tandemly arranged HBV genome into the Huh6 hepatoma cell line.\textsuperscript{26} Genomic DNAs from all these sources were analyzed by RLGS and/or Southern blotting using Not I and EcoRV as restriction enzymes.

4.2. RLGS, target-spot cloning, and cosmid preparation

Genomic DNAs were prepared from cell lines and from primary HCC tissues matched with adjacent noncancerous liver tissues. Ten micrograms of genomic DNA was used for each RLGS analysis. Details of RLGS and spot DNA cloning have been described previously.\textsuperscript{26} Cloned DNA from intensified spot A was named DMHA-1 and used as a probe for Southern analysis and for cosmid library screening. DNA inserts were isolated and radiolabeled by random priming to a specific activity of 10^6 cpm/mg of DNA and used to screen a human placenta cosmid library (Clontech, Inc.). A total of 4 x 10^5 recombinants were screened. After colony hybridization, the membranes were washed at 65°C with 2 SSC (1 SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7) containing 0.1% SDS (sodium dodecyl sulfate), followed by 0.1 SSC containing 0.1% SDS.

4.3. Fluorescence in situ hybridization

Chromosome spreads were obtained from phyto- magglutinin-stimulated blood lymphocytes of a healthy donor after thymidine synchronization and bromodeoxyuridine incorporation by the method of Takahashi et al.\textsuperscript{27,28} The probe DNA (DMHA-1) was labeled with biotin-16-dUTP (Boehringer Mannheim) by nick translation, and in situ hybridization was performed according to Lichter et al.\textsuperscript{29} in the presence of COT-1 DNA (GIBCO BRL, Gaithersburg, MD) as a competitor. Hybridized probe was detected with FITC-conjugated avidin (Boehringer Mannheim) without any amplification step. Chromosomes were counterstained with 0.2 µg/ml propidium iodide for R-banding. In this method, R-banded chromosomes simultaneously showed the corresponding G-banding pattern stained with Hoechst 33258. Fluorescence signals were visualized with a Zeiss Axioskop epifluorescence microscope equipped with a cooled Charge Coupled Device (CCD) camera (Photometrics, PXL1400). Image acquisition was performed on a Macintosh Quadra 840AV computer with the software program IPLabTM (Signal Analysis Co.). The images were pseudo-colored and merged using Adobe PhotoshopTM 2.5J (Adobe System Inc.). Hoechst, FITC, and propidium iodide images appeared in blue, green and red, respectively. The merged images were printed.
directly on a Fuji Pictrography 3000 from a Macintosh computer. The mapping results were based on observations of more than 20 pro-metaphase chromosomes.

References


