Somatic Mutation of Mitochondrial DNA in Cancerous and Noncancerous Liver Tissue in Individuals with Hepatocellular Carcinoma

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Abstract

Unlike other types of cancer, hepatocellular carcinoma (HCC) is usually preceded by chronic inflammation caused by viral infection. The mutation of mitochondrial DNA (mtDNA) in hepatocarcinogenesis associated with viral infection was investigated. Compared with control liver tissue, the frequency of mtDNA mutations was markedly increased in both noncancerous and cancerous liver specimens from individuals with HCC. The accumulation of mtDNA mutations in HCC tissue reflected the degree of malignancy. The frequency of mtDNA mutations in HCC tissue was also greater than that described previously for other types of tumors. These observations suggest that the repeated destruction and regeneration of liver tissue associated with chronic viral hepatitis lead to the accumulation of mtDNA mutations. The genetic instability that results in the high rate of mtDNA mutation in cancerous liver tissue is also consistent with the multicentric hepatocarcinogenesis detected clinically.

Introduction

Most cases of chronic hepatitis are caused by hepatitis viruses. Persistent infection with hepatitis B virus or hepatitis C virus also ultimately results in the development of HCC. Thus, unlike other types of cancer, HCC is usually preceded by chronic infection for 20–40 years. The development of HCC is rapid, however, in individuals with viral hepatitis and high activities of alanine aminotransferase in plasma (1). Treatment of hepatitis patients with IFN reduces the incidence of HCC (2, 3). Normalization of the plasma activity of alanine amino transferase by IFN treatment reduces the rate of hepatocarcinogenesis, even if the hepatitis virus is not eliminated (4). Chronic inflammation induced by hepatitis viruses thus plays an important role in hepatocarcinogenesis.

The extent of oxidative stress is increased in the liver of individuals infected with hepatitis viruses (5, 6), and reactive oxygen species are important inducers of DNA mutations (7). Mutations accumulate to a greater extent in mtDNA than in nuclear DNA, in part because mitochondrial lack histones and DNA repair systems (8). The human mitochondrial genome comprises a 16.5-kb circular double-stranded DNA molecule that encodes 13 polypeptides of the respiratory chain, 22 transfer RNAs, and 2 rRNAs required for protein synthesis. Expression of the entire mitochondrial genome is necessary for the maintenance of mitochondrial functions, suggesting that small changes in the sequence of mtDNA might result in profound impairment of such functions. Mutations in mtDNA are associated with mitochondrial myopathy, encephalopathy, and diabetes mellitus (9).

Although it is unclear whether mutations in mtDNA underlie carcinogenesis, several such mutations have been detected in human colorectal (10), neck, urinary bladder (11), and thyroid (12) cancers.

We hypothesized that the increase in oxidative stress associated with carcinogenesis in individuals with chronic hepatitis might result in mutation of mtDNA. We therefore sequenced the mitochondrial genome of cancerous and noncancerous regions of the liver of individuals with HCC and compared the sites and frequencies of mutations with those detected in normal liver.

Materials and Methods

Tissue Specimens. We obtained 149 liver specimens with informed consent from 78 consecutive Japanese individuals who underwent surgery at Osaka City University Medical School. These specimens included 59 HCC samples and 59 corresponding samples of noncancerous regions of the liver of individuals with chronic hepatitis C virus infection; 12 HCC samples and 12 corresponding samples of noncancerous regions of the liver of individuals with chronic hepatitis B virus infection; and 7 control liver samples from individuals without viral infection but with liver disease other than HCC (including hepatolithiasis and colorectal carcinoma with liver metastasis). HCC was diagnosed histologically as well differentiated, moderately differentiated, or poorly differentiated in 7, 32, and 32 individuals, respectively. This study was performed in accordance with the Helsinki Declaration of 1975 (1983 revision) and was approved by the ethics committee of Osaka City University Medical School.

PCR Amplification. Liver specimens were frozen, microdissected with a cryostat, and digested with proteinase K (0.1 mg/ml) in the presence of 1% SDS. DNA was extracted with the use of phenol-chloroform and ethanol precipitation as described previously (13). Each DNA sample (50 ng) was subjected to amplification by PCR with the use of overlapping sets of primers to screen the entire mitochondrial genome. PCR (an initial incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min) was performed in a final volume of 50 μl with a GeneAmp PCR system 9600 (Perkin-Elmer).

The generation of large PCR products excluded the possibility of nuclear pseudogenes complicating the analysis (14). In addition, the primers were selected to avoid such coamplification by analysis with cell lines devoid of mtDNA.

Sequence Analysis. Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems). The entire sequence of mtDNA was examined for 1 control subject and 2 individuals with HCC, and the sequence of the displacement (D)-loop (nucleotides 100–600) was examined for the remaining 6 control subjects and 69 individuals with HCC. All mutations were confirmed by repeated analysis of DNA extracted from the specimens.

Results

The entire mitochondrial genomes of two poorly differentiated HCC specimens, the corresponding noncancerous tissue, and one control liver specimen were amplified by PCR and sequenced manually. The mtDNA mutations detected are indicated in Fig. 1. Com-
pared with a mtDNA sequence deposited in GenBank (accession no. J01415), the mtDNA sequence obtained from the liver specimen of our control subject contained three single-base mutations, all of which were located in the D-loop. The mtDNA sequences obtained from the two cancerous tissue specimens contained 67 and 77 mutations, half or more of which were also apparent in the mtDNA obtained from the paired noncancerous tissue specimens. Consistent with previous observations (10–12), most of the mutations detected in the present study were homoplasmic.

The greatest frequency of mtDNA mutations was apparent in the D-loop, especially in the region between nucleotides 100 and 600. All mtDNA samples contained a G→A transition at nucleotide 263, a T→C transition at nucleotide 489, and C insertion between nucleotides 311 and 312. We then compared the number of mutations in the D-loop region (between nucleotides 100 and 600), excluding the three mutations common to all samples, among specimens of control liver, noncancerous tissue from individuals with HCC, and well-differentiated, moderately differentiated, and poorly differentiated HCC (Fig. 2). For the 7 control specimens, the median number of mutations in the D-loop was 0 (25th and 75th percentiles: 0 and 1, respectively).

Discussion

Our data show that the number of mtDNA mutations in HCC tissue (~70 sites/tumor) is substantially greater than that detected previously for other types of cancer (10–12). Furthermore, the number of mtDNA mutations in noncancerous liver tissue from individuals with HCC (~40 sites) was shown to be markedly greater than that in control liver specimens.

Most of the identified mutations in mtDNA were homoplasmic, although hepatocytes contain hundreds of mitochondria and each mitochondrion contains 1–10 genomes (15). Mitochondria of tumor cells proliferate selectively when such cells are fused with normal cells (10, 16), possibly because certain mutant mtDNA molecules exhibit a replicative advantage. The D-loop region of mtDNA is important for both replication and expression of the mitochondrial genome because it contains the leading-strand origin of replication and the promoters of transcription (17). Thus, mutations in this region might affect the rate of DNA replication by modifying the binding affinity of important trans-acting factors. Mitochondria that undergo rapid replication appear to acquire DNA damage more readily, resulting in an accumulation of mutations, than do those maintained under resting conditions. Our data now suggest that mtDNA mutations accumulate during the neoplastic transformation of hepatocytes. Mitochondrial DNA harboring certain mutations might generate abnormal RNAs or proteins, the latter of which may promote leakage of electrons from the mitochondrial electron transport chain. The amounts of endogenously produced reactive oxygen species, such as superoxide and related free radicals, might thus be increased in cells with mutant mtDNA. The resulting oxidative modification of DNA therefore may contribute to the early stages of hepatocarcinogenesis. Although inflammatory cells may infiltrate HCC tissue, the number of such cells is small compared with the number of cancer cells. Moreover, the density of mitochondria in hepatocytes and HCC cells is much greater than that in inflammatory cells. Thus, the possible contribution of contaminating inflammatory cells to the extent of mtDNA mutation observed in HCC tissue is negligible. Furthermore, for some individuals with HCC, mtDNA preparations from liver tissue were compared with those from paired blood samples; almost no mutations were found in blood samples excluding the three common mutations. In contrast to the single-nucleotide mutations observed in the present study, large deletions have been described previously in mtDNA from certain types of tumors (18–20). Despite attempts to
Given the clonal nature and large number of copies of mtDNA, mutation of the mitochondrial genome in noncancerous regions of the liver of individuals with HCC is indicative of genomic instability and likely contributes to hepatocarcinogenesis.

References


14. Parfait, B., Rustin, P., Munnich, A., and Rotig, A. Co-amplification of nuclear and mitochondrial DNA indicates that the mutated mtDNA had become dominant in both tissue and hepatocytes in noncancerous regions of the liver. Fig. 2. Frequency distributions for the number of mutations in the D-loop of mtDNA of control liver and paired noncancerous and cancerous liver specimens from individuals with HCC. The number of mutations in the D-loop (nucleotides 100–600), with the exception of the three mutations common to all subjects, was compared among control liver (n = 7) as well as noncancerous and cancerous liver tissue from individuals with well-differentiated (n = 7), moderately differentiated (n = 32), or poorly differentiated (n = 32) HCC.


detect deletions in mtDNA with the use of multiple primers for PCR analysis, none was apparent in either cancerous or noncancerous tissue from individuals with HCC. Our results thus suggest that hepatocytes and HCC cells with large deletions in their mtDNA might be eliminated, and that cells without such deletions undergo continuous proliferation.

The high frequency of mtDNA mutations in noncancerous liver tissue of individuals with HCC suggests that hepatocytes in such tissue may already have undergone the initial stage of malignant transformation in chronic inflammation induced by virus infection. The observation that most of the mutations detected were homoplasmic indicates that the mutated mtDNA had become dominant in both HCC tissue and hepatocytes in noncancerous regions of the liver.

Fig. 2. Frequency distributions for the number of mutations in the D-loop of mtDNA of control liver and paired noncancerous and cancerous liver specimens from individuals with HCC. The number of mutations in the D-loop (nucleotides 100–600), with the exception of the three mutations common to all subjects, was compared among control liver (n = 7) as well as noncancerous and cancerous liver tissue from individuals with well-differentiated (n = 7), moderately differentiated (n = 32), or poorly differentiated (n = 32) HCC.
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