Detection of Mitochondrial DNA Alterations in the Serum of Hepatocellular Carcinoma Patients[1]

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ABSTRACT

Purpose: **Somatic mutations in mitochondrial DNA (mtDNA) have recently been detected in various cancers. These mutations could possibly be detected in serum because mtDNA has a higher copy number than nuclear DNA. Thus, we examined genetic alterations in the D-loop region of mtDNA in hepatocellular carcinoma (HCC) patients.**

Experimental Design: **Fifty patients with HCC were investigated in this study. Somatic mutations in the D-loop region of tumor mtDNA were screened by direct sequencing, and then the paired serum samples were investigated using mutation-specific mismatch ligation assay.**

Results: **Fifteen of 100 sequence variants that were detected in tumor mtDNA have not been recorded previously. True somatic mutations in the D-loop region were detected in 17 of 50 patients (34%). Subsequent screening for paired serum by mismatch ligation assay revealed that 5 of 15 paired serum samples (33%) contained the same mutations as primary tumors.**

Conclusions: **mtDNA mutation may be a novel tumor marker of HCC and may prove effective for detection of tumor DNA in the serum.**

INTRODUCTION

The incidence of HCC[3] has increased in the world for the last decade (1, 2). HCC can only be cured if diagnosed at an early stage, and methods to detect surgically resectable tumors could significantly reduce deaths from this disease. We previously examined colorectal cancer patients using mutation-specific mismatch ligation assay for genetic alterations in primary tumors and paired serum samples (3). Of 25 cases with K-*ras* and/or *p53* gene mutations in the tumor, 10 cases exhibited the same alterations in the matched serum DNA. This result indicated that genetic alterations present in the tumors of cancer patients can be detected in the serum of those same patients. Molecular biology studies have revealed that a variety of oncogenes such as c-*myc*, *cyclin D1*, and β-*catenin* and tumor suppressor genes such as *p16*, *p53*, and *Rb* are involved in the pathogenesis of HCC (4–7). These genetic alterations could also be tumor markers to detect tumor DNA in the serum of HCC patients (8).

In recent years, somatic mutations in the mitochondrial genome have been identified in various human cancers. Fliss *et al.* (9), for example, showed somatic mutations of mtDNA in bladder, head and neck, and lung cancers, most of which were observed in the D-loop region of mtDNA. The D-loop region regulates the transcription and replication of mtDNA, and alterations in this region might indeed alter the rate of mtDNA replication. This may be why various cancers frequently have mutations in the D-loop region. Therefore, we thought that alterations in the mitochondrial genome might also become a genetic marker of HCC.

In this study, we first examined somatic mutations in the D-loop region of mtDNA in HCC using direct sequencing, and then we attempted to detect those mutations in paired serum samples using sensitive mutation-specific mismatch ligation assay.

MATERIALS AND METHODS

Sample Collection and DNA Preparation. Fifty primary tumor and corresponding nontumorous liver tissue samples were collected at Nagoya University School of Medicine from patients with HCC during liver resection surgery. All primary tumors were diagnosed histologically. Serum samples were collected from the same patients before tumor resection. These collected samples were stored at −80°C until DNA extraction. DNA was prepared as described previously (3, 10).

Mutation Analysis for the D-loop Region of mtDNA. Extracted DNA (100 ng) was amplified using the step-down PCR method for the following investigation of the D-loop region of mtDNA (Fig. 1). The primers used were as follows: DloopS (sense), 5′-CGCACGGACTACAACCACGAC, and DloopAS (antisense), 5′-CTGTGGGGGGTGTCTTTGGGG. Step-down PCR was performed with a protocol consisting of 3 cycles of 95°C for 30 s and 68°C for 4.5 min; 3 cycles of 95°C for 30 s, 64°C for 1.5 min, and 70°C for 3.5 min; 3 cycles of 95°C for 30 s, 61°C for 1.5 min, and 70°C for 3.5 min; 30 cycles of 95°C for 30 s, 58°C for 1.5 min, and 70°C for 3.5 min; and a final extension at 70°C for 5 min. All PCR products were purified and sequenced directly with the AmpliCycle sequencing kit (Perkin-Elmer) as described previously (11).

Mismatch Ligation Assay. When mtDNA alterations were detected in HCC, the same alterations were sought in

Received 3/25/02; revised 6/3/02; accepted 6/3/02.

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[1] Supported in part by the Ministry of Education, Science, Sports and Culture, Japan.

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; mtDNA, mitochondrial DNA.

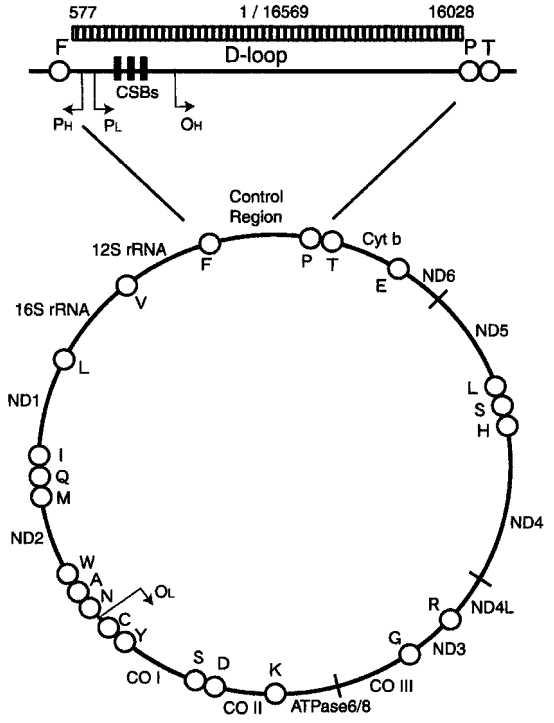

Fig. 1 Schematic map of the D-loop region in human mtDNA. Human mtDNA is a 16.5-kb circular double-stranded DNA molecule. The *striped bar* indicates the D-loop region (1.1 kb). *ND,* NADH dehydrogenase; *CO,* cytochrome *c* oxidase; *ATPase,* ATP synthase; *Cyt b,* cytochrome *b*; O_H/O_L, heavy/light-strand origin of replication; P_H/P_L, heavy/light-strand promoter; *CSBs,* conserved sequence blocks; *open circles* with an adjacent single letter indicate tRNA.

corresponding serum samples using mutation-specific mismatch ligation assay. Paired tumor and nontumorous mtDNA were used as a positive and a negative control, respectively. The mutation-specific oligomers used in the ligation assay were designed individually according to each mtDNA alteration. For example, the tumor of case 39 had a mutation at nucleotide position 414 (5′-TTTGGCGGTATGCACTTT to 5′-TTTGGCGGCATGCACTTT). To detect this alteration in the serum, a mutation-specific oligomer (5′-TTTGGCGGC), the adjacent ^{32}P-labeled oligomer (5′-ATGCACTTT), and the blocking oligomer (5′-GGCGGTATGCA) were synthesized, and the mismatch ligation assay was performed exactly as described previously (3).

RESULTS

We amplified an approximately 2.4-kb fragment of the mtDNA genome in 50 primary HCC and corresponding nontumorous liver tissues using step-down PCR and sequenced the D-loop region manually (Fig. 2). The sequences obtained were compared with those from a mitochondrial databank website.[4] Consequently, 15 of 100 sequence variants detected in mtDNA

[4] http://www.gen.emory.edu/mitomap.html.

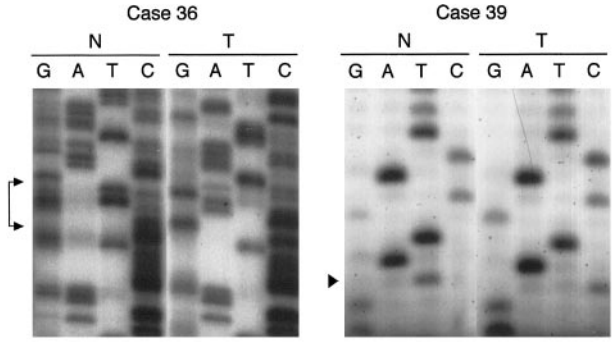

Fig. 2 Representative mutations in the D-loop region of mtDNA in HCC patients. The sequence of mtDNA was analyzed in primary tumors (*T*) and corresponding liver tissues (*N*). *Arrows* indicate the large deletion at nucleotide position 313 to 321 in case 36 and a T to C transition at nucleotide position 414 in case 39.

of the primary tumors were not recorded in this databank. We next compared the sequences of mtDNA obtained from primary tumors and corresponding nontumorous liver tissues and defined the somatic mutations in the primary tumors. By this sequence analysis, 19 true somatic mutations in 17 HCC cases (17 of 50 cases, 34%) were confirmed in the D-loop region of mtDNA (Table 1). Of the newly found 15 sequence variants, 9 were somatic mutations.

When compared with the clinicopathological profiles, there was no significant difference between the patients with and without mtDNA alterations with regard to age, tumor differentiation, hepatitis viral infection, or liver cirrhosis (data not shown).

We subsequently proceeded with mismatch ligation assay to detect tumor mtDNA in the serum samples. Preoperative serum samples were available for 15 of 17 patients whose tumors included mtDNA mutations. Of the 17 alterations in 15 cases examined by mismatch ligation assay, 5 (33%) could be detected in the serum (Fig. 3). To exclude the possibility that these alterations were derived from lymphocytic mtDNA, we also examined lymphocytic mtDNA from patients who showed the mutations in the corresponding tumor tissues and sera, and we found that no lymphocytes had the same mutations (data not shown). This result suggested that mtDNA alteration could be a novel tumor marker to detect tumor DNA in the serum of HCC patients.

DISCUSSION

Various studies have suggested that abnormalities of the mitochondrial genome are closely related to a wide variety of degenerative diseases, aging, and cancers (12). Large numbers of mtDNA mutations have been identified in several degenerative diseases of nerve and muscle (13, 14), and high copy numbers of specific point mutation were revealed in the control region of human fibroblast mtDNA from normal old, but not young, individuals (15). Somatic mutations in the mtDNA genome have also been detected in several cancers. Polyak *et al.* (16) screened the mtDNA genome of 10 colorectal cancer cell lines and found mutations in 7 of them. It might be possible that the ongoing evolution in tumor cells cultivated *in vitro* has a

Table 1 Summary of mtDNA alterations in HCC

Case	Nucleotide position	Type of mutation	Mutation in serum
1	307–356	50-bp deletions	−
2	310	T deletion	+
5	72	T to C	+
6	303–309	1-bp C insertion	−
7	303–309	1-bp C insertion	−
16	436	C insertion	−
17	303–309	1-bp C insertion	−
18	303–309	1-bp C insertion	−
20	303–309	1-bp C insertion	−
29	523	A deletion	+
32	94	G to A	−
	303–309	1-bp C deletion	−
34	60	T to C	−
	72	T to C	−
36	313–321	9-bp deletions	+
39	414	T to C	+
40	94	G to A	NA[a]
44	303–309	1-bp C insertion	−
48	303–309	1-bp C deletion	NA[a]

[a] NA, not available.

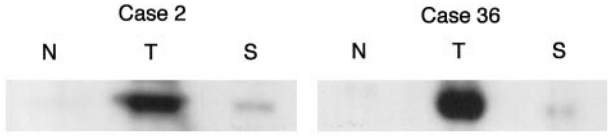

Fig. 3 Representative autoradiographs of mismatch ligation assay. The mutations in primary tumors of cases 2 and 36 have been identified by sequence analysis. The signals of mutated mtDNA were found in primary tumors (*T*) and paired serum samples (*S*), but there was no detectable signal in corresponding liver tissues (*N*).

stronger effect in the mtDNA than in the genomic DNA, resulting in a higher mutation rate of mtDNA in cell lines. Subsequently, Fliss *et al.* (9) detected somatic mutations of mtDNA in >40% of bladder, head and neck, and lung cancers. Moreover, most mutations were located in the D-loop region: 4 in 14 bladder cancers (29%), 3 in 13 head and neck cancers (23%), and 5 in 14 lung cancers (36%). On the other hand, the D-loop region is well-known as a hypervariable region where many kinds of polymorphisms are present. This and other observations suggested that the D-loop region might be a hot spot for the mutation in mtDNA (15, 17).

In the current study, we detected somatic mutations in the D-loop region of mtDNA in 17 of 50 (34%) HCCs by direct sequence analysis. Of the 19 mutations detected by sequencing, 11 were observed around nucleotide position 310, and 8 of these 11 mutations were 1-bp deletion/insertion of a mononucleotide repeat at nucleotide position 303 to 309. Recently, this homopolymeric C-stretch region has been reported to frequently harbor deletions or insertions in several primary tumors (18–20). Our observation in HCC is consistent with these reports.

The role of mtDNA mutation in tumor development and progression has not yet been solved. The increasing mitochondrial oxidative stress caused by changing the cellular capacities of reactive oxygen species may lead to the impairment of nuclear DNA, or the disruption of the apoptotic process induced by mitochondria may contribute to neoplastic transformation. We observed mtDNA alterations frequently around nucleotide position 310, a region that contributed to persistent RNA-DNA formation, leading to the initiation of mtDNA replication (21, 22). These observations suggest that some severe alterations in this region may play an important role for growth advantage in tumor cells. Further examinations are required to discover the functional significance of the mtDNA mutations.

We subsequently tried to detect tumor mtDNA in the serum of 15 HCC patients. We used a highly sensitive method, a mismatch ligation assay, to detect mtDNA alterations in the serum samples of HCC patients. We could identify the mtDNA mutations in 5 of 15 (33%) patients. In this study, we used mtDNA alteration as a tumor marker to detect tumor DNA in the serum because each mitochondria has around 10 copies of mtDNA in itself, and there are more than 100 mitochondrias in a cell (23). The high copy number of mtDNA may make it possible to detect the mutation at a site away from the primary tumor. In fact, Fliss *et al.* (9) frequently observed mtDNA mutations consistent with the primary tumors in the urine of bladder cancer patients and in the saliva of head and neck cancer patients by direct sequence analysis and mismatch ligation assay.

HCC is among the most common and fatal cancers in the world. Methods to detect surgically resectable tumors could significantly reduce the number of deaths from this disease. In this study, we found frequent mtDNA mutations in the D-loop region and demonstrated those mutations in paired serum samples of HCC patients. Therefore, patients at high risk for HCC could be screened for the presence of tumor cells in the serum by analysis of mtDNA mutations. Based on the method described in this study, however, one needs to know the exact mutation within the mtDNA. Therefore, these markers are limited to use for the follow-up of disease at present. Advances in technologies to permit rapid detection of an array of specific mutations would enhance the utility of this approach.

ACKNOWLEDGMENTS

We thank M. Taguchi and Y. Nishikawa for technical assistance.

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