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Short communication

Analysis of Mitochondrial DNA Mutation in hepatoma

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Mitochondrial DNA (mtDNA) mutation was investigated in a hepatoma patient using a polymerase chain reaction (PCR) and an *in situ* hybridization technique. Biotin-labeled probes for the subunit III of cytochrome c oxidase revealed differences in the *in situ* hybridization. A PCR assay using biopsied and microdissected tissues showed that common deletion (4,977 bp) was more pronounced in the cancer region than in the normal parts of the same patient. These results suggest that mtDNA deletion might be associated with tumorigenesis in hepatoma.

Keywords: mtDNA deletion, Hepatoma, CoxIII, *In situ* hybridization, PCR

Introduction

Since Otto Warburg claimed (Warburg, 1956), there have been arguments for mitochondrial involvement in cancer cells (Wilkie *et al.*, 1975; Cotton and Rogers, 1993). Harman, who proposed a free radical theory for the aging (Harman, 1956), suggested mitochondria as the cause of free radicals (Harman, 1972). Later studies demonstrated free radical is related to pathology, including cancer (Bandy and Davison, 1990; Toyokuni *et al.*, 1995; Loft and Poulsen, 1996) and neuromuscular degenerative diseases (Beal, 1995).

Mitochondrion is an autonomous organelle that supplies most of the cellular energy via oxidative phosphorylation (OXPHOS) in animals. It has its own DNA (mtDNA) encoding genes that are necessary for respiratory components. The polypeptides of the mtDNA are all subunits of the mitochondrial energy-generating pathway, oxidative phosphorylation (OXPHOS). The mitochondrial mRNAs are translated within the mitochondrion using mtDNA encoded rRNAs and tRNAs on chloramphenicol-sensitive ribosomes. Inside the cell, electrons are transferred to oxygen by a respiratory chain that is located in the inner membrane of mitochondria. Throughout the process, free radicals are continuously generated by mitochondria (Kowaltowski and Vercesi, 1999). As mitochondria are inevitable targets of their own products, i.e. free radicals, mitochondrial DNA, membrane lipids and proteins, and resultant membrane structure should be altered. Again, any abnormality in mitochondria may lead to an altered oxidative phosphorylation and construct vicious cycle (Lenaz, 1998).

Mitochondrial DNA has a high mutation rate due to the damage from free radicals generated by the electron transfer and its limited capacity for repair (Ames and Gold, 1991). An increase in mtDNA deletions is paralleled by declined OXPHOS in the heart and brain. Accumulation of the so-called 'common deletion', which is 4,977 bp, is proportional to chronological age (Cortopassi and Arnheim, 1990). This deletion covers the gene loci encoding ND4, ND4L, ND3, COIII, ATPase 6 & 8, and several tRNAs, which are essential components of OXPHOS. Though the level of common deletion may not be sufficient for pathology (1%) (Soong *et al.*, 1992), many other mutations accumulate as well with the 4,977 bp deletion (Ozawa, 1997).

In the present work, we tried to relate the common deletion in mitochondrial DNA to hepatoma in Korean patients in an attempt to identify the cause of cancer.

Materials and Methods

Materials Biopsied tissues were obtained from the patient and used immediately or kept in a liquid nitrogen tank until the experiment. Normal as well as tumor parts from the same patient were kept separate.

Preparation of mitochondrial DNA Tissues (300 mg) were homogenized in an isolation buffer (0.25 M sucrose, 2 mM hepes, pH 7.4) and mitochondria were obtained (Chung *et al.*,1998). Mitochondria were lysed in a lysis buffer (120 mM NaCl, 10 mM Tris-Cl, I mM EDTA, 1.2% SDS, pH 8.0) for 2 hours at 37°C. DNA was extracted using a conventional phenol extraction procedure (Kim *et al.*, 1997).

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Table 1. Primer and probe sequences of D-loop and COXIII.

Primer for PCR	D-loop	Sense(16017-16037)	5′-TTTCATGGGGAAGCAGATTTG-3′
		Antisense(170-190)	5′-GTTCGCCTGTAATATTGAACG-3′
	COXIII	Sense(7901-7920)	5′-TGAACCTACGAGTACACCGA-3′
		Antisense(13650-13631)	5′-GGGGAAGCGAGGTTGACCTG -3′
Probes for *in situ* hybridization	D-loop	5′-ATTGATTTCACGGAG-GATGGTGGTCAAGGG-3′	
	COXIII	5′-CTCAGGTGATTGA-TACTCCTGATGCGAGTA-3′	

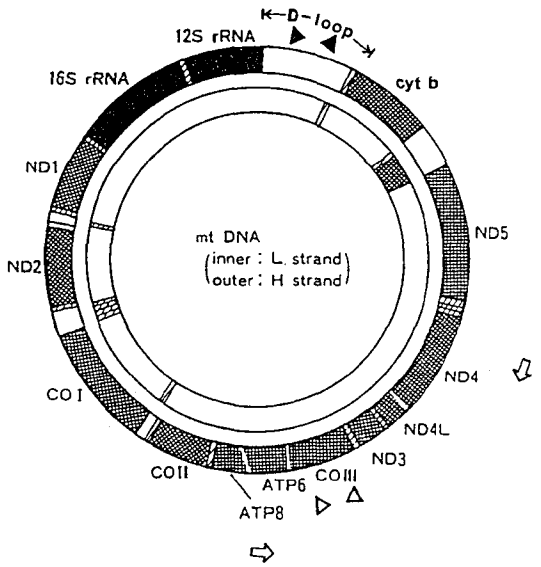

Fig. 1. Human mitochondrial genome map (Ozawa, 1997). The sizes and regions of a probe are shown by symbols outside the circle. The primers for the common deletion are shown as an open arrow. The probes for D-loop and COXIII are symbolized as a solid and open triangle, respectively.

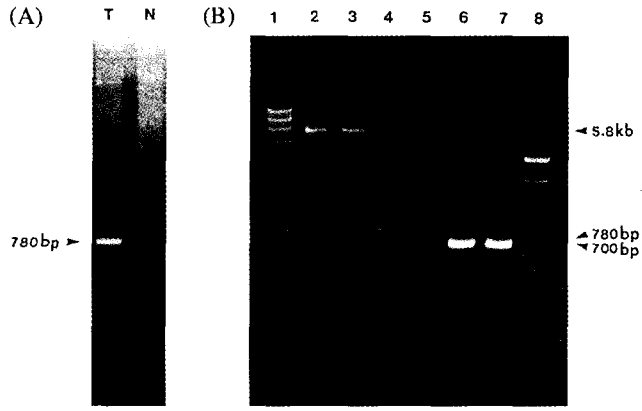

Fig. 2. PCR analysis of common deletion in liver mitochondria from a hepatoma patient on a 1.2% agarose gel. A. DNA was prepared from biopsied tissue using primers for common deletion as in Materials and Methods. Lanes T and N are the PCR products from tumor and normal tissues, respectively. The arrow indicates the band of 780 bp produced as a result of common deletion. B. DNA was obtained from microdissected cells from a slide specimen and PCR was carried out as in A. Lanes 1 and 8 are λ Hind III and 100 bp ladder DNA for molecular weight markers. Lanes 2 and 3 are intact band (5.8 kb) from normal and tumor tissue. Lanes 4 and 5 are common deletion (780 bp) from normal and tumor tissue. Lanes 6 and 7 are D-loop (700 bp) from normal and tumor tissue.

Polymerase chain reaction To detect deletion in biopsied tissues, identical primers (Table 1) and conditions were used as in the previous report (Chung *et al.*, 1998). One of the primers of D-loop has the sequence, 5'-TTTCATGGGGAAGCAGATTTG-3', at position from 16017 to 16037. The sequence of the reverse primer for D-loop is 5'-GTTCGCCTGTAATATTGAACG-3' at position from 170 to 190. To detect common deletion, a forward primer of sequence 5'-TGAACCTACGAGTACACCGA-3' was chosen at 7901-7920 and a reverse primer 5-GGGGAAGCGAGGTTGACC TG-3' at 13631-13650 (Fig. 1). PCR was carried out at 94°C for 1 min, 58°C for 1 min, and 72°C for 40 sec for 30 cycles. For a microdissected tissue sample, approximately 50-200 normal and tumor cells were obtained separately with a needle from the same slide. These cells were lysed in 100 mM Tris-Cl, pH 8.0, 0.1% Tween-20, proteinase K (1 mg/ml), and incubated at 52°C for 48 hours in a shaking water bath. Immediately thereafter, lysis was stopped and denatured at 100°C and stabilized for 30 min at room temperature. PCR was performed under identical conditions for the entire biopsied tissue.

***In situ* Hybridization** A slide of paraffin embedded tissues was washed using the conventional xylene method. After washing with PBS, RNase was added at 20 μg/ml and incubated with pepsin (0.75 mg/ml) for 2-4 min at 55°C. Then, hybridization experiments were carried out according to the protocol by Research Genetics (England). A biotin labeled probe of D-loop, 5'-ATTGATTTCACGGAGGATGGTGGTCAAGGG-3', at 16393 -16422 was prepared by Gibco-BRL (Hong-Kong). A Biotin labeled probe of COXIII, 5'-CTCAGGTGATTGATACTCCTGAT GCGAGTA-3', at 9616-9645 was prepared by Oligos (USA) (Fig. 1).

Results and Discussion

PCR for common deletion In order to find the relationship of mitochondrial DNA mutation to tumorigenesis, common deletion was compared in hepatoma. There was one prominent band of 780 bp indicating a common deletion in

biopsied tissues from a hepatoma patient (Fig. 2A). There were additional 2-3 bands between 200 bp and 1,000 bp. Results from the primer shift experiment confirmed these deletion bands (data not shown). Cells were collected from the slide specimen by needle-scratch so that neither normal nor tumor cells were mixed. Samples from 13 patients were tested for common deletion. A 780 bp band corresponding to common deletion was detected predominantly in tumor tissues. Significantly, 4 patients showed a higher degree of common deletion in tumor compared to normal cells, where the deletion was barely detected (Fig. 2B). These patients also showed a considerable difference with the COXIII probe *in situ* hybridization experiment. In several cases tested, an additional band of the approximate size of 400 bp was observed, which were also found in biopsied tissues. In biopsied tissues, however, a 780 bp band corresponding to common deletion was found irrespective of tumor existence. Based on band density, common deletion occurred higher again in the tumor compared to the normal tissue. But more often, the difference was not found as in other results (Tallini et *al.*, 1994; Cui *et al.*, 1991). One reason could be a low amount of mtDNA recovered from the tumor compared to normal tissue due to the toughness of tumor tissue. Though the PCR technique is highly sensitive, the products obtained are dependent on many factors, such as template amount, purity and incubation condition. Alternatively, the presence of different mtDNA mutations, other than common deletion or stage-specific appearance of the mutations during tumorigenesis, may be possible.

***In situ* Hybridization** Biotin-labeled probes on 5'-end for D-loop and COXIII were prepared. For D-loop labeling, one biotin was attached while two were used for COXIII due to the weak signal. Serial specimens were used for each of the probes to facilitate comparison. Most specimens exhibited a similar degree of signal for the D-loop between the normal and tumor regions (Fig. 3A). However, the COXIII signal varied depending on tissue regions. Out of 34 patients, 14 patients (41%) showed a weak signal (Fig. 3B) and 1 patient (3%) a stronger signal in the tumor region compared to the normal region. There was no significant difference in 19 patients (56%).

The *in situ* hybridization, however, can reveal the difference *per se* compared to the PCR method. D-loop was used as a control for the *in situ* hybridization experiment because of its consistent existence in mitochondrial genome. A COXIII probe was used to detect deletion as it is located inside the common deletion. The signal was stronger with D-loop due to higher copies compared to the COXIII probe. When tumor regions were compared, the COXIII often showed a difference, while the D-loop did not. This difference was confirmed by microdissected PCR. Although the number of patients investigated was not high, the deletion occurred in a significant proportion (41%) compared to stomach cancer (20%) (Chung *et al.*, 1998). A 100% correlation of tumor

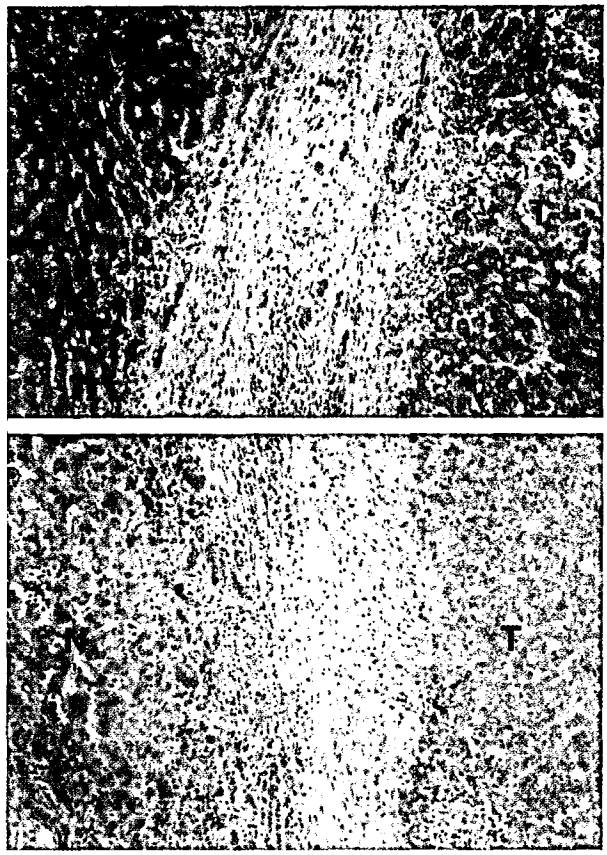

Fig. 3. *In situ* hybridization with COXIII probe. Normal (N) and tumor (T) tissues were hybridized with a D-loop probe (top) and COXIII probe (bottom) (C100). Hematoxylin stained nuclei as either violet (top) or blue (bottom). In top picture, though pink color of the probe in tumor was not distributed as much as in the normal tissue due to diminished cytoplasm, there are distinct pink regions outside the nuclei (violet color) in both the N (normal) and T (tumor) regions. However, in the bottom picture, the pink probe was barely seen in the tumor region (T) compared to the pink spots in the normal tissue (N). Nuclei were seen as a blue color in the bottom picture instead of a violet color.

occurrence with a common deletion of mtDNA is not expected because of various causes of cancer. As mutation in mtDNA is not expected to cause tumorigenesis, 41% in hepatoma seems quite high. A similar deletion of mtDNA was found in liver steatosis caused by alcohol (Fromety *et al.*, 1995) and also in liver cirrhosis (Yamamoto *et al.*, 1992; Muller-Hocker *et al.*, 1997). Even the D-loop deletion was reported in gastric adenocarcinomas (Burgart *et al.*, 1995).

One of the causes of tumorigenesis is the multiple repeats that are present in mtDNA that are associated with deletion (and insertion). A number of mtDNA deletions have been identified that are associated with Chronic External Ophthalmoplegia Plus (CPEO) and Kearns-Sayer Syndrome (KSS). The deleted region is frequently flanked by direct repeats. Maternally inherited deletions and tissue-specific

depletion of mtDNAs have been reported with reduced levels to 2% to 3% in some infants and to 15% in association with multiple deletions in an adult (Wallace *et al.*, 1991).

If deletion occurs, insertion should accompany it. A deleted gene fragment may be released to behave like an oncogenic virus and enter the nuclear genome (Gellissen and Michaelis, 1987; Werbin, 1992). Furthermore, recent studies revealed numerous kinds of 'repeat' sequences within mitochondrial genome (Wallace *et al.*, 1991; Hou, and Wei, 1996), which can participate actively in deletion and insertion. Some of the mitochndrial genes originally encoded on mtDNA transferred to nuclear genome (Hadler *et al.*, 1983) with higher frequency in rat hepatoma compared to the normal (Corral *et al.*, 1989). In the HeLa cell, a similar incorporation was found in the c-*myc* gene (Shay and Werbin, 1992). This insertion can be facilitated by repeat sequences flanking the deletion (Hou and Wei, 1996). With extensive and thorough studies using various primers, a higher proportion of altered mitochondrial genome, including deletion and insertion, can be identified during the progressive stages of tumorigenesis.

The other possibility in causing tumorigenesis is the generation of reactive oxygen species by mitochondria. The lack of essential components of electron transport chain and protein synthesizing machinery may deteriorate mitochondria. These partly damaged mitochondria might generate harmful reactive oxygen species (ROS). For instance, if COXIII is deleted, then the electron transport and proton motive force will not operate properly, so that harmful free radicals may be produced in higher amounts. The radicals initiate the SOS signal for nucleus to switch to the escape pathway. Affected cells choose to either "die" or "survive". Recent studies have shown that H_2O_2 has a profound effect on the mitochondrial permeability transition (MPT) (Hirokawa *et al.*, 1998) and mitogen-activated protein kinase (MAPK) (Guyton *et al.*, 1996). Furthermore, the commitment of cytochrome c in apoptosis demonstrated that mitochondria are involved in the core of the cellular event (Liu *et al.*, 1996). Simultaneously, the capacity to repair may be weakened due to the loss of energy. If it occurs slowly, a cell may have time to change itself for survival and to choose an escape pathway (Gellissen and Michaelis, 1987). During this long process of tumorigenesis, mitochondria may have been altered (Shay and Werbin, 1987; Cavalli. and Liang, 1998) and leave no clue at all by selectively removing damaged mitochondria (Yamamoto *et al.*, 1992). Currently many reports, both pro and con, have piled up. In fact, many failed to detect the mutation in mtDNA that is associated with a tumor (Cui *et al.*, 1991; Bianchi *et al.*, 1995). We suggest that a careful examination of stage-specific tumor cells should find the clues before they disappear. In conclusion, our results suggest mtDNA mutation is a contributory factor in tumorigenesis in hepatoma.

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