

# Quantitative detection of *common deletion* of mitochondrial DNA in hepatocellular carcinoma and hepatocellular nodular hyperplasia

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## Abstract

**AIM:** To study the deletion of mitochondrial DNA in hepatocellular carcinoma and hepatocellular nodular hyperplasia and its significance in the development of cancer.

**METHODS:** Deleted mtDNA (CD-mtDNA) and wild type mtDNA (WT-mtDNA) were quantitatively analyzed by using real-time PCR in 27 hepatocellular carcinomas (HCC) and corresponding noncancerous liver tissues and 27 hepatocellular nodular hyperplasias (HNH).

**RESULTS:** A novel CD (4 981 bp) was detected in 85% (23/27) and 83% (22/27) of HCC and HNH tumor tissues, respectively, which were significantly higher than that in paired noncancerous liver tissues (57%, 15/27) ( $P < 0.05$ ). The CD/WT-mtDNA ratio in HCC tumors was 0.00092 (median, interquartile range, 0.0001202-0.00105), which was significantly higher than that in paired noncancerous liver tissues (median, 0.000, quartile range, 0-0) ( $P = 0.002$ , Mann-Whitney Test), and was 25 of times of that in HNH tissues (median, 0.0000374, quartile range, 0-0.0004225) ( $P = 0.002$ , Mann-Whitney test).

**CONCLUSION:** CD-mtDNA mutation plays an important role in the development and progression of HCC.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common malignancies worldwide, and has been ranked the 2nd cancer killer in China. Hepatitis B and C viruses (HBV and HCV) and dietary aflatoxin intake remain the major causative factors of HCC<sup>[1]</sup>. Previous

studies also revealed that frequent genetic aberrations were involved in hepatocarcinogenesis<sup>[2,3]</sup>. However, the molecular mechanisms of hepatocarcinogenesis remain unclear. Recently, morphological features of the tumor, both gross and histological, have been found to be significantly associated with tumor recurrence and patient survival.

Nuclear gene alterations are correlated to invasion, metastasis, recurrence of HCC, which are regarded as biomarkers for the malignant phenotype of HCC, and related to the prognosis and therapeutic outcomes. These biomarkers include p53 gene mutation<sup>[4]</sup>, VEGF overexpression<sup>[5,6]</sup>, apoptosis related genes, cell adhesion and extracellular matrix related genes such as E-cadherin,  $\beta$ -catenins, CD44s, MMPs and their inhibitor TIMPs<sup>[7-13]</sup>.

Human mitochondrial DNA (mtDNA) is located in cytoplasm, is becoming the study hotspot for its alteration in correlation with its tumorigenesis. Mitochondria are involved in apoptosis<sup>[14]</sup>, and probably also tumorigenesis<sup>[15]</sup>, which has led researchers to examination the potential roles of mtDNA alterations in the development and maintenance of cancers.

The most abundant change in mtDNA is called common deletion (4 977 bp, CD). CD-containing mitochondrial DNA (CD-mtDNA) was first observed in patients with mitochondrial myopathies, and was also found to accumulate in patients with heteroplasmic mtDNA mutations and in normal individuals during aging, particularly in postmitotic tissues such as muscle and brain<sup>[16]</sup>. The CD-mtDNA mutation has been detected in several types of human tumors including thyroid Hürthle cell tumor<sup>[17]</sup>, gastric cancer<sup>[18]</sup>, and hepatocellular carcinoma<sup>[19]</sup>. However, knowledge about the common deletion of mtDNA in HCC in China is poor. This is the first report of a high incidence (70%) of a novel CD-mtDNA (4 981 bp) in tumor tissues of HCC and hepatocellular nodular hyperplasia (HNH) from southern China, and the first analysis correlating CD-mtDNA level to clinicopathological parameters and age.

## MATERIALS AND METHODS

### *Clinical data and histopathologic analysis of tumor samples*

Patients with histologically proved HCC and HNH at the Cancer Center, Sun Yat-Sen University (Guangzhou, China) were recruited with informed consent from January 1999 to January 2003. Samples consisted of 27 surgically resected HCC and 27 HNH specimens. In HCC specimens, the tumor tissue and paired adjacent noncancerous liver tissue were obtained independently for mtDNA analysis. In serum, hepatitis B viral surface antigen and HCV antibody titer were detected by enzyme-linked immunosorbent assay and radio-immunoassay. Grading of differentiation was performed according to the method of Edmondson and Steiner. Tumors were classified into well differentiated group (grades 1 and 2) and poorly differentiated group (grades 3 and 4). The tumor size was classified into small (tumor mass  $< 3$  cm in greatest diameter) and large (tumor mass size  $> 3$  cm in greatest diameter). There were 24 males and 3 females aged from 40 to 79 years, with an average age of 58 years.

### *DNA extraction*

Total (nuclear and mitochondria) DNA was extracted from

**Table 1** TaqMan primers and probes for detection of WT-mtDNA and CD-mtDNA

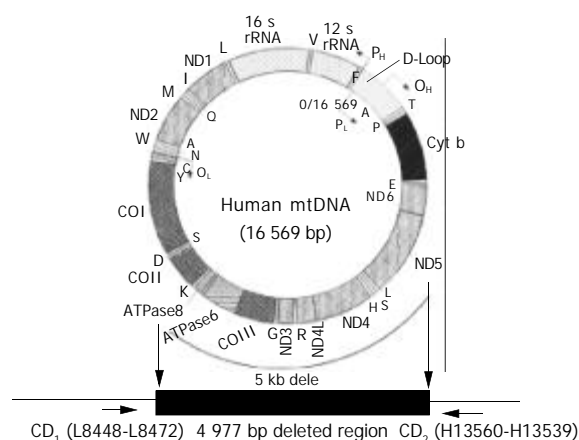
Target	Amplicon	Oligonucleotide sequence (5' -3')
Wild Type mtDNA	101 bp	WT1 forward primer (7 878-7 897): 5'-AATCAATTGGCGACCAATGG-3' WT2 reverse primer (7 979-7 958): 5'-CGCCTGGTTCTAGGAATAATGG-3' WT probe (7 899-7 917): 5' FAM-ACTGAACCTACGAGTACAC-MGB-3'
Common Deletion mtDNA	132 bp	CD1 forward primer (8 448-8 472): 5'-TATTAACACAAACTACCACCTACC-3' CD2 reverse primer (13 560-13 539): 5'-GGCTCAGGCGTTTGTGTATGAT-3' CD probe: (13 456-13 471): 5' FAM- ACC ATTGGC AGC CTA G -MGB 3'

paraffin-embedded tissues of HNH, HCC and adjacent noncancerous liver tissues using the QIAamp DNeasy Tissue Kit (Qiagen, Hilden, Germany). Prior to DNA extraction, a microdissection technique was used in certain cases when tumor tissues and non-tumor tissues were mixed in one sample to enrich tumor cells<sup>[20]</sup>. Five 10- $\mu$ m thick sections were cut and placed into a 1.5 mL Eppendorff tube. The sections were deparaffinized twice with xylene and alcohol.

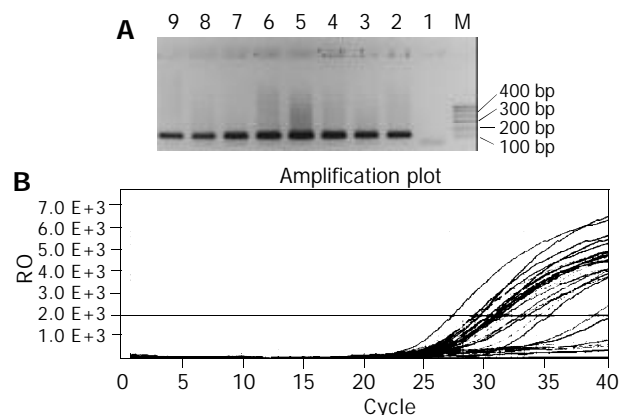
### Conventional PCR detection of CD-mtDNA

In this investigation, we developed a real-time PCR protocol that reliably quantified mtDNA through amplification of different regions of the mitochondrial genomes: one just outside the CD region in cytochrome c oxidase II (MTCO2) coding region (IS), and one overlapping the CD itself (Figure 1). PCR primers for detection of CD-mtDNA were designed according to MITOMAP Human mtDNA Cambridge Sequence data (www.mitomap.org). Real-time PCR primers and fluorogenic probes for regions of WT-mtDNA (forward primer, L7878-7897; reverse primer, H7979-7958; probe, L7899-7917) and CD-mtDNA (forward primer, L8448-8472; reverse primer, H13560-13539; probe, L13456-13471) were designed with the Primer Express software (Table 1).

Conventional PCR was performed in 20  $\mu$ L volume consisting of 2  $\mu$ L 10 $\times$ PCR buffer, 25  $\mu$ mol/L of each dNTP, 2.5 U Taq polymerase, 15 pmol/L of each primer and 50 ng of DNA template. The reaction was performed in a PE2700 thermocycler (Applied Biosystem Inc., USA). PCR reaction included at 95  $^{\circ}$ C for 10 min, 40 cycles at 94  $^{\circ}$ C for 30 s, at 60  $^{\circ}$ C for 30 s and at 72  $^{\circ}$ C for 30 s. The PCR products were separated on 20 g/L agarose gels at 80 V for 60 min, visualized by ethidium bromide staining and UV light, and photographed. The results of amplification of CD-mtDNA by conventional PCR in HCC and HNH tissues are shown in Figure 2A.



**Figure 1** Human mitochondrial DNA showing the 4 997-bp deletion. The genes disrupted by the 4 997 bp deletion between nucleotide positions 8 469 and 13 447 encode four polypeptides for complex I (ND3, ND4, ND4L and ND5), one for complex IV (CO III) and two for complex V (ATP8 and ATP6), and five tRNA genes for the amino acids G, R, H, S and L. CD represent the PCR primers position that flank the common deletion region.



**Figure 2** Detection results of CD-mtDNA in HCC. A: Conventional PCR results: Lane M, marker, Lane 1, negative control, Lane 2, positive control, Lane 3-4, nasopharyngitis samples, and Lane 5-8, NPC samples; B: Real-time PCR result shows the amplification plot of fluorescence intensity against the PCR cycle. Each plot corresponds to a HCC sample. The X axis denotes the cycle number of a quantitative PCR reaction. The Y axis denotes the Rn, which is the fluorescence intensity over the background. The correlation coefficient is 0.994.

### TaqMan-PCR

The principles of real-time PCR and methods for absolutely quantification of target DNA were described<sup>[18]</sup>. The quantitative TaqMan-PCR method could provide real-time measurement of target input.

Triplicate amplification reactions were performed in a 96-well microplate. Total WT-mtDNA and CD-mtDNA reactions (25  $\mu$ L) each containing 100 ng DNA, 1 $\times$ TaqMan Universal PCR Master Mix, 300 mmol/L of each dNTP and 300 mmol/L of each IS or CD primer were performed. The reactions were completed by adding 100 nmol/L of the specific WT or CD probe. PCR and fluorescence analysis were performed using the ABI GeneAmp 7900HT sequence detection system (Applied Biosystems Inc., USA). Amplification conditions included at 50  $^{\circ}$ C for 2 min (for optimal AmpErase UNG activity), at 95  $^{\circ}$ C for 10 min (for deactivation of AmpErase UNG and activation of AmpliTaq Gold), then 40 cycles at 95  $^{\circ}$ C 15 s and at 60  $^{\circ}$ C for 1 min (for probe/primer hybridization and DNA synthesis).

Analysis of the reactions was carried out in an ABI PRISM 7900HT sequence detector equipped with the sequence detection software version 2.0 (PE Applied Biosystems, Foster City, USA). Absolute DNA quantification was performed using the standard curve method. Reactions were carried out with different concentrations ( $10^{10}$ ,  $10^9$ ,  $10^8$ ,  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$  copies/mL) of two standard plasmids in parallel with test reactions. The standard plasmids, one carrying sequences flanking the common deletion and one carrying a unique mtDNA sequence independent of the CD, allowed the generation of two standard curves showing the number of copies of total WT-mtDNA or CD-mtDNA versus the measured CT. The CT values of samples were then converted to the number of DNA copies by comparing the sample CT to that of a known concentration of plasmid DNA.

The amount of mutation corresponded to the concentration ratio of CD-mtDNA to WT-mtDNA within each sample. If DNA was not detected within 40 cycles (CT=40), it was considered absent from a particular sample. The amplification plot CD-mtDNA detected by TaqMan PCR in HCC tissues is presented in Figure 2B.

The CD-mtDNA PCR products were sequenced using the ABI PRISM BigDye termination cycle sequencing ready reaction kit on an ABI PRISM377 sequencer (Applied Biosystem Inc., USA). Blast sequencing analysis confirmed that PCR products of the CD-mtDNA were homologous to the Cambridge version of the mtDNA sequence.

### Statistical analysis

The levels of CD/MT-mtDNA ratio in different groups were compared using the Mann-Whitney rank-sum test. The chi-square test and Fisher's exact test were used to assess the difference in different groups. A *P* value less than 0.05 was considered statistically significant.

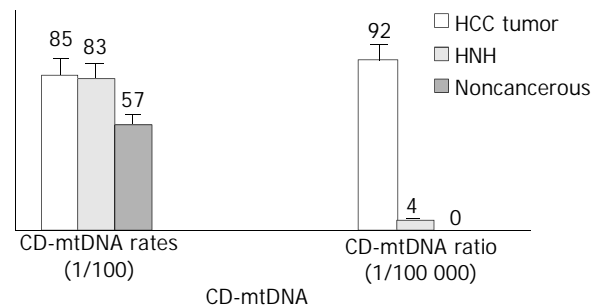
## RESULTS

The 132 bp PCR fragment amplified from CD-mtDNA was cloned and sequenced to confirm the deletion junction created by the CD, which was characterized by the presence of one of the two 13 bp repeats that normally flanked wild-type mtDNA. Sequence comparison (Human mtDNA Cambridge Sequence data, www.mitomap.org) revealed that the common deletion region in our HCC samples was a 4 981 bp fragment extending from position 8 470 bp to 13 450 bp (Figure 3). This was a novel mtDNA deletion belonging to the 4 977 bp deletion subtype that was first reported in liver diseases.

### Incidence of CD-mtDNA by conventional PCR detection

CD-mtDNA was detected by conventional PCR in 70% (19/27), 63% (17/27) and 44% (12/27) in HCC tumors, HNH tissues and HCC adjacent liver tissues, respectively. The detected rate of CD-mtDNA in HCC and HNH tumors by conventional PCR was significantly higher than that in adjacent liver tissues ( $P < 0.05$ , chi-square test). There was no significant difference in CD-mtDNA detected rate between HCC and HNH ( $P > 0.05$ , chi-square test).

By quantitative TaqMan-PCR, the detection rate of CD-mtDNA in HCC tumors, HNH tissues and adjacent liver tissues was 70% (19/27), 63% (17/27) and 44% (12/27), respectively. When these results were combined with those of conventional PCR, CD-mtDNA was detected in 85% (23/27) of HCC, 83% (22/27) of HNH, and 57% (15/27) of HCC paired adjacent liver tissues. The detected CD-mtDNA rate in HCC and HNH tumors was significantly higher than that in paired noncancerous liver tissues ( $P < 0.05$ , chi-square test). (Figure 4).



**Figure 4** Correlations of CD-mtDNA with HCC and HNH. The CD-mtDNA rate in HCC and HNH lesions was significantly higher than that in paired noncancerous tissues; the CD/WT-mtDNA ratio in HCC was significant higher than that in paired noncancerous liver tissues ( $P = 0.02$ ), and was about 25 times that in HNH lesions ( $P = 0.02$ ).

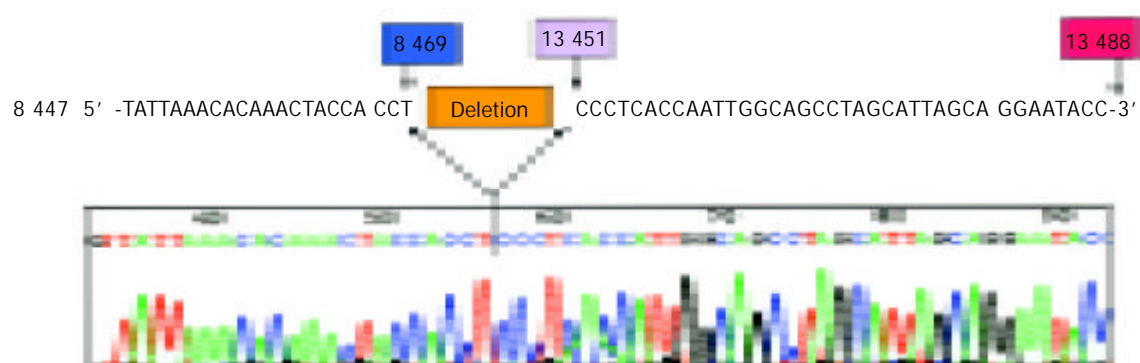
### CD/WT-mtDNA ratio in HCC and HNH lesions

We evaluated the relative level of CD-mtDNA by calculating the ratio of CD-mtDNA to WT-mtDNA in each sample. In this study, the CD/WT-mtDNA ratio was 0.00092 (median, interquartile range, 0.0001202-0.00105) in HCC tumor, 0.000 (median, quartile range, 0-0) in paired noncancerous liver tissues, and 0.0000374 (median, quartile range, 0-0.0004225) in HNH tissues. The CD/WT-mtDNA ratio in HCC tumor was significantly higher than that in paired noncancerous liver tissues ( $P = 0.002$ , Mann-Whitney test), and was 25 times of that in HNH tissues ( $P = 0.002$ , Mann-Whitney test, Figure 4). No correlation of the detected rate and the ratio of CD-mtDNA with ageing, staging, tumor size, HBV infection and differentiation of patients with HCC were found.

## DISCUSSION

It has been found that human mitochondrial DNA (mtDNA) has a double-stranded circular molecule of 16 569 bp that encodes 37 genes: 2 rRNAs, 22 tRNAs and 13 polypeptides<sup>[21]</sup>. The mtDNA was present in high copy levels ( $10^3$ - $10^4$  copies per cell) in virtually all cells, and the vast majority of an individual's copies were identical at birth<sup>[22]</sup>. It has been generally accepted that high mutation rates of mtDNA are caused by a lack of protective histones, inefficient DNA repair systems, and continuous exposure to mutagenic effects of oxygen radicals generated by oxidative phosphorylation<sup>[23,24]</sup>. The deletion was thought to be the product of an intragenomic recombination event between two 13 bp direct repeats (positions 8 470-8 482 and 13 447-13 459) after a single-strand break caused by ultraviolet A (UVA) or ROS<sup>[25]</sup>.

Recently, somatic mutations in mitochondrial DNA (mtDNA)



**Figure 3** Sequencing result of the amplified CD-mtDNA from HCC. Comparison with the MITOMAP Human mtDNA Cambridge Sequence data (www.mitomap.org), the deletion region (red bar region) is a 4981 bp deletion from position 8 470 to 13 450 of the mtDNA.

have been detected in various cancers including HCC. Mutations in the D-loop were frequent events and could be used as a molecular tool to determine clonality of HCC<sup>[26]</sup>. There are controversial reports of CD-mtDNA mutation in HCC. CD-mtDNA was reported to be detected in HCC tumors and noncancerous liver tissues, whereas lower level even no CD-mtDNA was detected in HCC tumors<sup>[27,28]</sup>. In this investigation, we detected a novel CD-mtDNA mutation (4 981 bp) in 85% HCC, which was higher than that in gastric cancer (50%)<sup>[18]</sup>, but less than that in thyroid Hürthle cell tumors (100%)<sup>[17]</sup>. Moreover, we found that both the detected CD-mtDNA rate and CD/WT-mtDNA ratio in HCC were higher than those in paired noncancerous liver tissues of individuals with HCC. This result was different from previous studies. The different risk factors and different genetic background in HCC tumorigenesis between Chinese and Japanese might explain the disparity of the results. Further studies are required to determine if CD-mtDNA mutation is correlated with malignant transformation.

However, the biological impact of mtDNA deletion on HCC tumors is not entirely clear. Defects in mitochondrial function have long been suspected to contribute to the development and progression of cancer. These mutations could contribute to neoplastic transformation by changing cellular energy capacities, increasing mitochondrial oxidative stress, and/or modulating apoptosis<sup>[25]</sup>. Diaz *et al.*<sup>[29]</sup> reported that mtDNA with large deletions, but not with pathogenic point mutations, repopulated organelles significantly faster than wild-type genomes in the same cell. Under proliferating conditions, cells harboring relatively high levels of deleted mtDNAs showed a slight reduction in the mutated fraction. This was consistent with the observation that patients with mitochondrial diseases had relatively low percentages of mutated mtDNA in proliferating peripheral blood cells and fibroblasts<sup>[30]</sup>. This situation paralleled the accumulation of large-scale mtDNA deletions in postmitotic tissues, where selection based on cellular growth or survival did not take place, and abnormal organelle proliferation would lead to an increase in mtDNA replication rates<sup>[31,32]</sup>. Amuthan<sup>[33]</sup> showed that damage to mtDNA and the mitochondrial membrane might change nuclear gene expression, leading to overexpression of genes including cathepsin L, transforming growth factor (TGF $\beta$ ), and mouse melanoma antigen (MMA), which are well known markers for tumor progression. Singh<sup>[34]</sup> showed that mtDNA played an important role in cellular sensitivity to cancer therapeutic agents. Since each cell contains many mitochondria with multiple copies of mtDNAs, it is possible that wild-type and mutant mtDNA can co-exist in a state called heteroplasmy. Thus, the biological impact of a given mutation may vary, depending on the proportion of mutant mtDNAs carried by individuals.

It has been shown that mtDNA deletions accumulate with age in many tissues. However, in tumors, the CD has been associated with external factors such as radiation and cigarette smoking<sup>[35-37]</sup>. In this investigation, we found that although there was no significant difference in detected CD-mtDNA rate between HCC and paired adjacent liver tissues as well as HNH, the CD-mtDNA ratio in HCC was significantly higher than that in HNH (25-fold) and paired noncancerous liver tissues. These results suggest that CD-mtDNA mutation may be accumulated during the hepatocyte transformation. No correlation of CD-mtDNA with ageing, staging and HBV infection of the individuals with HCC and HNH was found. In this investigation, the high detected rate and high ratio of CD-mtDNA in HCC and HNH suggested that this rapid and quantitative assay of CD-mtDNA and WT-mtDNA copy number was potentially useful in a variety of molecular and evolutionary fields of HCC.

In conclusion, this is the first quantitative study of frequent occurrence of CD-mtDNA mutations in patients with HCC. This study provides further evidence that CD-mtDNA mutation

might play an important role in the development and progression of HCC. Studies evaluating the CD-mtDNA mutations as a biomarker may be potentially useful for early diagnosis of HCC.

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