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ORIGINAL ARTICLE

ANALYSIS OF THE MITOCHONDRIAL 4977 bp DELETION IN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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ABSTRACT

Mutations in the mitochondrial (mt) genome that result in mt dysfunction, have long been proposed to play important roles in the pathogenesis of hepatocellular carcinoma (HCC). Among these, the common mtDNA 4977 bp deletion is one of the most frequent mutations observed in various cancers. To understand the relationship between the mtDNA 4977 bp deletion and HCC, we performed mutational screening for the presence of this deletion in 105 HCC patients and 69 unrelated healthy subjects. After nested-polymerase chain reaction (nested-PCR) amplification, we found that there were 10 patients carrying the mtDNA 4977 bp deletion, and this deletion was absent in control subjects. Moreover, HCC patients carrying this deletion showed a marked increase in reactive oxygen species (ROS) level and mtDNA copy number when compared with the healthy controls. Taken together, our data indicated that the mtDNA 4977 bp deletion may play important role in the carcinogenesis of HCC, possibly *via* the alternation of mtDNA copy number and oxidative stress.

Keywords: Hepatocellular carcinoma (HCC); Mitochondrial DNA (mtDNA); 4977 bp deletion; Copy number; reactive oxygen species (ROS).

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. It is a common cancer with 500,000-1,000,000 new cases annually, leading to ~600,000 deaths each year [1]. Clinically, HCC is characterized by its propensity to invade the vas-culature within the liver. For most patients with HCC, the diagnosis is delayed and the prognosis is poor. The median overall survival in patients with advanced HCC is less than 1 year, mainly owing to the absence of effective therapies [2]. However, when the diagnosis is confirmed, 70.0 to 80.0% of HCC patients have lost the opportunity to undergo complete tumor resection [3]. Thus, there is an urgent need to develop novel biomarkers for early diagnosis and detection of HCC.

Mitochondria contain their own genetic material: the mitochondrial DNA (mtDNA), which encodes essential molecular elements required for electron transport by the respiratory chain where oxygen is consumed [4]. Due to the lack of histone protection and a poor DNA repair system, mtDNA has a higher mutation rate than nuclear DNA, somatic and germline mtDNA mutations have been reported for a wide variety of cancers, such as colon cancer, head and neck tumors [5,6]. It has also been shown that oxidative modified DNA is especially prone to mispairing of repetitive elements and is correlated with deletions; in fact, large scale deletions were among the first mtDNA mutations identified to cause human diseases [7]. Up to now, more than 100 deletions have been reported to be associated with various diseases (http://www.mitomap. org/).

In this study, to explore the association between mitochondrial DNA (mtDNA) 4977 bp deletion and HCC, we initiated a comprehensive mutational screening for this deletion in 105 patients with HCC and 69 healthy subjects. Moreover, we evaluated the reactive oxygen species (ROS) level and mtDNA copy number in those subjects carrying these deletions.

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MATERIALS AND METHODS

Clinical Samples and DNA Extraction. Blood and tissues of 105 patients with HCC were collected at Zhuji People's Hospital, Shaoxing, People's Republic of China (PRC). The lesion and the nearby normal tissue from the subjects were confirmed histologically by a pathologist. Moreover, a total of 69 peripheral blood samples from age-matched donors who had a cancer free history and had no other known diseases that could be associated with mt defects were also recruited at the physical examination center in our hospital. Informed consent from all participants in this study was obtained under protocols approved by Zhuji People's Hospital. Tumor tissues on slides were extracted separately under a microscope, and genomic DNA was isolated as previously described [8]. Briefly, total DNA was extracted from formalin-fixed, paraffin-embedded tissues using the E.Z.N.A.® FFPE DNA isolation kit (Omega BioTek Inc, Norcross, GA, USA; http://omegabiotek.com/store/product/e-z-n-a-tissue-dna-kit/), according to the manufacturer's protocol. The isolated DNA was eluted in 100 µL Tris-EDTA buffer included in the E.Z.N.A.® FFPE DNA kit and stored at –20 °C until required. Moreover, the genomic DNA from peripheral blood leukocytes of the patients and the controls were also extracted, using the universal Genomic DNA Extraction kit version 3.0 (Takara Bio Inc, Dalian, Japan). The quality of the isolated DNA was assessed by NanoDrop spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific Inc, Waltham, MA, USA). The optical density values of all samples ranged from 1.8 to 2.0.

Detection of the Mitochondrial DNA 4977 bp Deletion. To screen the 4977 bp deletion in mtDNA, nested-polymerase chain reaction (nested-PCR) analysis was performed; we used the DNA samples extracted from the tissues as templates. In brief, two pairs of nested primers for detection of the 4977 bp deletion were as follows: 1F: 5'-AAC CAC AGT TTC ATG CCC ATC-3'; 1R: 5'-TGT TAG TAA GGG TGG GGA AGC-3'; 2F: 5'-ACC CTA TTG CAC CCC CTC TAC-3'; 2R: 5'-CTT GTC AGG GAG GTA GCG ATG-3'. The PCR condition was as follows: pre denaturation at 94 °C for 5 min.; then 30 cycles at 94 °C for 10 seconds, 58 °C for 45 seconds and 72 °C for 50 seconds; and a final extension at 72 °C for 10 min. The PCR products were then electrophoresed on a 2.0% agarose gel. The presence of the 4977 bp deletion was indicated by the appearance of a 358 bp band that was verified by sequencing analysis [9]. The wild-type mtDNA as the template would not yield any PCR products under such conditions because of the large flanking region (>5 kb).

Analysis of Mitochondrial DNA Copy Number. The DNA extracted from blood samples was used to determine its mtDNA copy number. The mtDNA copy number was measured with a real-time-PCR using an Applied Biosystems 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). One primer pair specific for the mtDNA (*ND1*) and another primer pair specific for the nuclear DNA (*18s*) were designed for relative quantification of the mtDNA copy number. The primer sequences for the mt *ND1* gene were: forward primer (*ND1*-F): 5'-CCC TAA AAC CCG CCA CAT CT-3'; reverse primer (*ND1*-R): 5'-GAG CGA TGG TGA GAG CTA AGG T-3'. The primer pair used for the amplification of the nuclear gene *18s* was as follows: forward primer (*18s*-F): 5'-TAG AGG GAC AAG TGG CGT TC-3'; reverse primer (*18s*-R): 5'-CGC TGA GCC AGT CAG TGT-3'. The 14 µL PCR mixture contained 1X SYBR Green mixture, 215 nM *18s*-R primer, 215 nM *18s*-F primer, and 0.4 ng of genomic DNA for *ND1* and *18s*. The thermal cycling conditions were 95 °C for 10 min., followed by 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 min. for *ND1* and 62 °C for 1 min. for *18s*. Standard curves made by serial dilution of a reference DNA sample was used to determine the ratio of mtDNA copy number to the amount of nuclear DNA that was proportional to the mtDNA copy number in each cell. The efficiency of all quantitative PCR ranged from 99.0 to 110.0%. The R^2 for all standard curves was ≥0.99. Standard deviations (SD) for the cycle of threshold (Ct) duplicates was ≤0.25. All samples were assayed in duplicate on a 96-well plate.

Determining the Reactive Oxygen Species Levels in Plasma. The blood samples were immediately placed into sterile EDTA test tubes and centrifuged at 1500 g for 20 min. at 4 °C to collect plasma. Plasma was stored at –70 °C until assayed. The concentration of ROS was analyzed by enzyme-linked immunosorbent serologic assay (ELISA) using commercial kits (Nova Biomedical, Boston, MA, USA), in accordance with the manufacturer's instructions.

Statistical Analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 17.0 (SPSS Inc, Chicago, IL, USA). The mtDNA relative content and common deletion level were analyzed by the independence-samples *t*-test. A p value of <0.05 was considered statistically significant.

RESULTS

Analysis of the Common Deletion in Mitochondrial DNA. Nested-PCR is a highly sensitive method for detecting large scale deletions, even at very low levels.

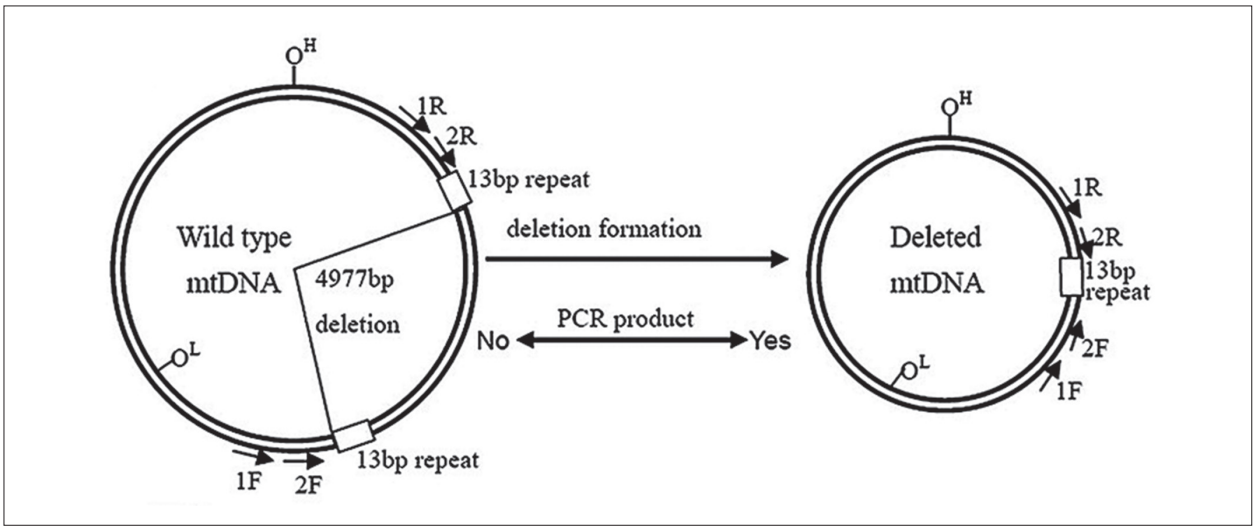

Figure 1. Identification of the mtDNA 4977 bp deletion. M: DL2000 Marker; lane 1: control subjects without the 4977 bp deletion; lanes 2 and 3: patients with the mtDNA 4977 bp deletion.

To investigate clinical implications of the common mt deletion, we first extracted the DNA from HCC patients and the normal tissues, as well as 69 age- and sex-matched healthy subjects. We also summarized some clinical characteristics and other risk factors for HCC in these patients including gender, age, history of alcohol consumption and tumor metastasis (Table 1). Of the 105 HCC patients, 10 (9.52%) showed the 4977 bp deletion, while this deletion was absent in healthy subjects and normal tissues (Figure 1). By molecular level, the 4977 bp deletion occurs between two 13 bp direct repeats at position 13447-13459 and 8470-8482, removing all or part of the genes encoding four complex I subunits, one complex IV subunit, two complex V subunits and five transfer RNA (*tRNA*) genes, which are indispensable for maintaining normal mitochondrial function.

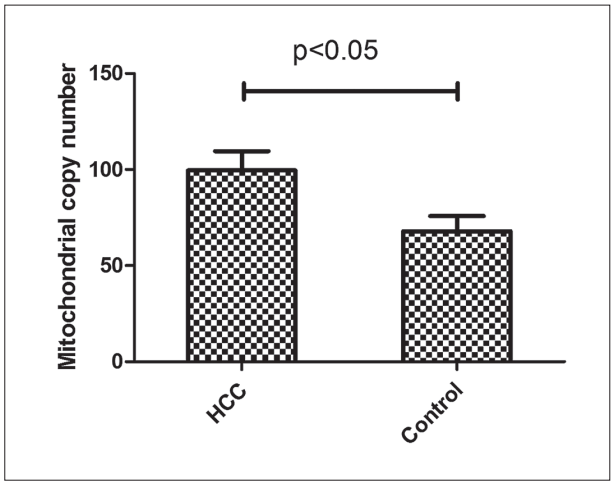

Figure 2. Comparison of mtDNA content between HCC patients with the mtDNA 4977 bp deletion and control subjects.

Table 1. Characterization of patients with hepatocellular carcinoma.

Parameters	Number of HCC Patients	mtDNA 4977 bp Deletion Frequency *n* (%)
Cases	104	10 (9.52)
Males Females	65 40	6 (5.71) 4 (3.81)
Age ≥ 55 Age <55	75 30	7 (6.67) 3 (2.85)
History of alcohol consumption: yes no	 85 20	 10 (9.52) 0 (0.00)
Metastasis: positive negative	 35 70	 4 (3.81) 6 (5.71)

HCC: hepatocellular carcinoma; mtDNA: mitochondrial DNA; *n*: number.

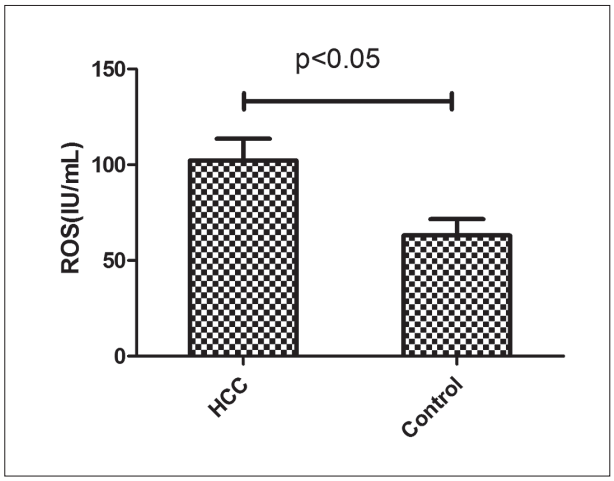

Figure 3. Reactive oxygen species concentrations in HCC patients carrying the mtDNA 4977 bp deletion and control subjects.

Qualification of Mitochondrial DNA Copy Number. Alternations in mtDNA content have been reported in increasing numbers of cancer types [10]. To investigate the mtDNA copy number changes and to determine if there was a correlation between the 4977 bp deletion and the overall mtDNA content in HCC, we further analyzed the mtDNA copy numbers in those individuals carrying the 4977 bp deletion. For this purpose, we used real-time-PCR to determine the mtDNA content. As shown in Figure 2, the mtDNA content was significantly higher in HCC patients carrying the 4977 bp deletion when compared with the healthy subjects (p <0.05), suggesting that the 4977 bp deletion may increase the mtDNA copy number.

Reactive Oxygen Species Analysis. As shown in Figure 3, the concentration of ROS was significantly higher in HCC patients than the controls (p <0.05).

DISCUSSION

In the present study, we investigated the frequency of the mtDNA 4977 bp deletion in patients with HCC. The mtDNA mutations have been implicated in various human diseases including cancer [11], a long-term process that involves multiple steps driven by different genetic and epigenetic alternations. Among these, the 4977 bp deletion is one of the first described and most studied mtDNA mutations, Kao *et al.* [12] initially demonstrated that two 13 bp direct repeats, present at nucleotides (nts) 8470-8482 and 13447-13459 bp position, which are prone to 4977 bp deletion in mtDNA [12].

Both animal studies and cell model analysis showed the mtDNA 4977 bp deletion played an important role during the course of tumorigenesis [13,14]. However, the results of various studies dissecting the role of this mutation in cancer development are conflicting. For example, the study of Ye *et al.* [15] found there was no correlation between the common mtDNA 4977 bp deletion and cancer risk, while Tseng *et al.* [16] demonstrated that the detection frequency of common deletions was higher in adjacent normal tissue than carcinoma tissue in their 60 breast cancer patients from Taiwan.

In this study, we found a positive correlation between mtDNA 4977 bp deletion and HCC, we noticed that this mutation had a high frequency in HCC patients (9.52%), whereas this deletion was absent in healthy subjects and normal tissues, suggesting that the 4977 bp deletion may served as a molecular marker for HCC. Mitochondrial DNA depletion had been associated with infantile neurogenetic disorders that caused unexplained weakness, hypotonia and developmental delays in early childhood [17]. Moreover, alternations in mtDNA content had been reported in many types of cancer, and multiple mechanisms had been proposed including deregulation of mtDNA replication [18,19]. Loss of mtDNA copy number control was associated with aging and was likely to be linked to either nuclear or mtDNA mutations [20]. In the current study, we found that, compared with the healthy subjects, the mtDNA content in HCC patients was much higher possibly due to increased oxidative damage to the replication machinery [21,22].

Because the common mtDNA deletion has been suggested as an indicator of oxidative damage, we further investigated the generation of ROS in blood samples of HCC and healthy controls. The ROS in mitochondria were by-products of the β oxidation pathway for fatty acid metabolism and they were generated via electron leakage from mitochondrial electron transport resulting in the activation of oncogenic pathways [23]. In our study, we showed that the ROS level was higher in HCC patients than those matched control individuals (Figure 3). At lower concentrations, ROS were important signaling molecules involved in cellular proliferation, migration, and apoptosis [24,25]. However, at higher concentrations, these molecules could be useful against pathogens, resulting in increased leukocyte and platelet activation, and increased leukocyte recruitment [26], which finally caused the oxidative stress that was responsible for HCC.

In conclusion, our investigation provided that the mtDNA 4977 bp deletion played a putative role in the pathogenesis of HCC, we also found that tumor with this common deletion, mtDNA content could be increased probably due to a retrograde effect. Furthermore, we proposed that the 4977 bp deletion may be served as a potential biomarker for HCC, possibly via the alternation of mtDNA content and increasing the ROS production.

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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