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ND4 mutation in transitional cell carcinoma: Does mitochondrial mutation occur before tumorigenesis? ☆

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Abstract

To investigate how mitochondrial mutation occurs in cancers, we analyzed *ND4* mutation in 53 transitional cell carcinomas (TCCs) of the upper urinary tract and the normal counterpart (perirenal soft tissue). Three methods, i.e., DNA sequencing, restriction fragment length polymorphism (RFLP), and denaturing high-performance liquid chromatography (DHPLC), were employed because of their different sensitive of detecting mutation. The results of sequencing and RFLP showed that *ND4* mutations were only found in 24.5% (13/53) of tumor. However, 11 of these mutations could also be identified in the normal tissue by DHPLC, indicating that most mitochondrial mutations identified in tumors preexist as minor components, which are too low in quantity to be detected by less sensitive methods such as DNA sequencing. The result suggests that mtDNA mutation occurs before tumorigenesis and become apparent in cancer cells.
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1. Introduction

The association between mitochondria and tumor was first mentioned in 1983 by Wilkie et al. who pointed out the mitochondrial abnormalities in various human cancers (Wilkie et al., 1983). Later studies by other investigators further showed that tumor cells had an impaired respiratory system (Shay and Werbin, 1987) and changes in mitochondrial gene expression (Sharp et al., 1992). The direct evidence of the relationship between mitochondria and cancer comes from the recent observations that mitochondrial DNA (mtDNA) mutations are frequently found in human cancers. For example, mtDNA mutations were identified in 80% of pancreatic cancers (Jones et al., 2001), 74% of breast cancers (Tan et al., 2002), 70% of colorectal tumors (Polyak et al., 1998), 60% of ovarian cancers (Liu et al., 2001), 64% of cases of bladder cancers (Fliss et al., 2000), 46% of head and neck cancers (Fliss et al., 2000), 43% of lung cancers (Fliss et al., 2000), and 23% of papillary thyroid carcinomas (Yeh et al., 2000).

Because carcinogenesis is resulted from the aberrant interplay of oncogenes and suppressor genes, it remains to be answered why and how mtDNA is mutated in tumor. In this regard, two schools of hypothesis have been proposed to explain how homoplasmic mtDNA mutant occurs in tumor cells. The classical selection theory postulates that cells harboring mutated mtDNA has proliferative advantage and, therefore, becomes the dominant population. This theory, however, cannot explain why synonymous

mutations without proliferative advantage can become homoplasmic in tumor cells. The genetic drift theory postulates that intracellular clonal expansion can randomly occur without any intrinsic proliferative advantage. However, this theory cannot explain why the homoplasmic mutated mtDNA can arise from homoplasmic wild-type mtDNA without *de novo* mutagenesis.

mtDNA is a double-stranded circular DNA of 16,569 bp in length (Wallace, 1999). Although mutations can occur in any nucleotide of mtDNA, some cancers have their preferential site of mtDNA mutation. Recently, Fliss et al. reported that transitional cell carcinoma (TCC) of the urinary bladder was specifically associated with mitochondrial mutations in D-loop and NADH dehydrogenase subunit 4 gene (*ND4*) (Fliss et al., 2000). The association between *ND4* mutation and TCC provides a focus for the study of the mechanism of mtDNA mutation in cancers.

By studying *ND4* mutation in TCC, the aim of this study was to understand the mechanism by which mtDNA mutation occurs in the tumor development. For this purpose, we analyzed TCC of the upper urinary tract (UUT) instead of the bladder tumor because advanced bladder TCCs are usually treated before cystectomy and, therefore, may harbor therapy-induced mutations, whereas TCCs of the UUT are usually diagnosed following ureteronephrectomy without the interference of the prior therapeutic effects. In addition, we used three different techniques, i.e., DNA sequencing, restriction fragment length polymorphism (RFLP), and denaturing high-performance liquid chromatography analysis (DHPLC), to analyze same sample. This is because we believe the controversy between the above mentioned theories is caused, at least in part, by the insensitive detection of mutation by DNA sequencing.

2. Materials and methods

2.1. Patients

We analyzed 52 primary and 1 recurrent TCCs of the renal pelvis or ureter from 23 male and 29 female patients, mean age 68.8 years (range 53.6–86.7), enrolled during the period from 1996 to 2002. This study was approved by the Institutional Review Board of Mackay Memorial Hospital.

2.2. DNA extraction, polymerase chain reaction (PCR) and DNA sequencing

To isolate DNA from formalin-fixed, paraffin-embedded tumor, representative blocks were cut at 8 μm using a clean disposable microtome blade for each sample. Excess tissue was trimmed before sectioning to ensure representative sampling. The first and the last section from each ribbon were examined under light microscope after routine hematoxylin and eosin staining. Because the interface between the TCC and the adjacent normal renal tissue was generously trimmed, both the first and the last sections

indeed showed that the selected specimens contained nearly uniform cell populations. For control, perirenal soft tissue without evidence of tumor involvement was used for each paired tumor. DNA was extracted from both tumors and normal tissues according to the previously described method (Tzen et al., 2003).

ND4 gene was amplified by three pairs of oligonucleotide primers: primer 10733A 5′-CTA CGT ACA TAA CCT AAA CC-3′ (nts 10,733–10,752) and primer 11189B 5′-AGG AAG TAT GTG CCT GCG TT-3′ (nts 11,189–11,208) were used for producing a 476 bp fragment; primer 11163A 5′-GAT GAG GCA ACC AGC CAG AA-3′ (nts 11,163–11,182) and primer 11633B 5′-ACT ACG AGG GCT ATG TGG CT-3′ (nts 11,633–11,652) for a 490 bp fragment; and primer 11625A 5′-CTT CAA TCA GCC ACA TAG CC-3′ (nts 11,625–11,644) and primer 12105B 5′-GGT AAT GAT GTC GGG GTT GA-3′ (nts 12,105–12,124) for a 500-bp fragment. The PCR and DNA sequencing were carried out as previously described (Tzen et al., 2003).

2.3. RFLP and DHPLC

Restriction enzymes RsaI (Promega, Madison, Wisconsin, USA), XcmI and MaeII (New England Biolabs, Beverly, Massachusetts, USA), MnlI, MwoI, BspMI and HphI (GeneMark, Tainan, Taiwan) were commercially available. Enzyme digestion was carried out overnight in reaction conditions according to manufacturer's instructions. The products were fractionated in 2% agarose gel for RFLP analysis.

For heteroduplex formation, PCR products were denatured at 95 °C for 10 min, slowly cooled to 65 °C at a rate of 1 °C/min, incubated at 65 °C for 5 min, and then cooled to 4 °C at a rate of 1 °C every 5 s. A helix DHPLC system (Varian, Inc., Palo Alto, California, USA) was used for DHPLC analysis according to previously described method (Conley et al., 2003). Resolution temperatures of heteroduplexes were calculated by the DHPLC Melt Program (http://insertion.stanford.edu/melt.html). Data were analyzed by using Star Chromatography Workstation Software Version (Varian).

3. Results

3.1. Analysis of ND4 mutation in 52 patients with transitional cell carcinoma

A total of 53 paired normal and tumor tissues from 52 patients were analyzed at the *ND4* by PCR amplification and DNA sequencing. The results of sequence chromatogram showed that 11 TCCs (from 10 patients) had DNA alterations at 13 nucleotide positions in *ND4* (Table 1). Among these 13 mutations, 8 were synonymous mutations and 5 (cases #19, #24, #25, #33, and #49) were nonsynonymous mutations, including one frameshift and four missense mutations (Table 1).