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Heteroplasmic mutation of mitochondrial DNA D-loop and 4977-bp deletion in human cancer cells during mitochondrial DNA depletion

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Abstract

Somatic mutations in mitochondrial DNA (mtDNA) have been demonstrated in various human cancers. Many cancers have high frequently of mtDNA with homoplasmic point mutations, and carry less frequently of mtDNA with large-scale deletions as compared with corresponding non-cancerous tissue. Moreover, most cancers harbor a decreased copy number of mtDNA than their corresponding non-cancerous tissue. However, it is unclear whether the process of decreasing in mtDNA content would be involved in an increase in the heteroplasmic level of somatic mtDNA point mutation, and/or involved in a decrease in the proportion of mtDNA with large-scale deletion in cancer cells. In this study, we provided evidence that the heteroplasmic levels of variations in cytidine number in np 303–309 poly C tract of mtDNA in three colon cancer cells were not changed during an ethidium bromide-induced mtDNA depleting process. In the mtDNA depleting process, the proportions of mtDNA with 4977-bp deletion in cybrid cells were not significantly altered. These results suggest that the decreasing process of mtDNA copy number per se may neither contribute to the shift of homoplasmic/heteroplasmic state of point mutation in mtDNA nor to the decrease in proportion of mtDNA with large-scale deletions in cancer cells. Mitochondrial genome instability and reduced mtDNA copy number may independently occur in human cancer.

Keywords: Cancer; Mitochondrial DNA; Point mutation; Deletion; Copy number

1. Introduction

Human mitochondrial DNA (mtDNA) is present in thousands of copies per cell, and encodes essential genes for energy production. Because lack of protective histone proteins, limited DNA repair systems, and generating reactive oxygen species (ROS) as a toxic by-product in mitochondria, mtDNA is more susceptible to oxidative damage and has a higher mutation rate compared with nuclear DNA. Mitochondrial dysfunction resulted from the accumulation of somatic mutations in the mtDNAs was proposed to play a central role in a wide range of age-related disorders and various forms of cancer (Wallace, 2005).

In the past few years, mtDNA mutations have been demonstrated in various types of human cancer (Polyak et al., 1998; Fliss et al., 2000; Penta et al., 2001; Modica-Napolitano and Singh, 2004). These mtDNA mutations detected in cancer cells included somatic point mutation, deletion, insertion, and depletion. Most of the somatic mutations

Abbreviations: DMEM, Dulbecco's modified eagle medium; EtBr, ethidium bromide; HCC, hepatocellular carcinoma; mtDNA, mitochondrial DNA; np, nucleotide position; OXPHOS, oxidative phosphorylation; PCR, polymerase chain reaction; ROS, reactive oxygen species.

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found in mtDNAs of cancer cells are homoplasmic, and D-loop region is a hot spot for mutation in mtDNAs of cancer cells (Sanchez-Cespedes et al., 2001). On the other hand, occurrence and accumulation of mtDNA with large-scale deletions are less frequently in cancer as compared with non-cancerous tissue (Pang et al., 1994; Lee et al., 2001; Yang et al., 2004; Yin et al., 2004; Dani et al., 2004; Wu et al., 2005; Tseng et al., 2006). Moreover, it has been demonstrated that many cancers have a reduced copy number of mtDNA as compared with corresponding non-cancerous tissue (Lee et al., 2004, 2005; Yin et al., 2004; Wu et al., 2005; Tseng et al., 2006).

In addition, mtDNA D-loop mutations were recently shown to be correlated with less differentiated hepatocellular carcinoma (HCC) (Tamori et al., 2004), with late-stage progression and poor prognosis in non-small cell lung cancer (Matsuyama et al., 2003), and with poor prognosis in colorectal cancer (Lievre et al., 2005) as well as in breast cancer (Tseng et al., 2006). Cancer cells with reduced copy number of mtDNA were also found to be associated with tumor aggressiveness in renal cell carcinoma (Simonnet et al., 2002), with less differentiation in gastric carcinoma (Wu et al., 2005), and with tumor size and cirrhosis of HCC (Yamada et al., 2006). These results suggest that mitochondrial genome instability and reduced copy number of mtDNA may play an important role in initiation and progression of human cancers. These findings of mtDNA mutations in cancer cells and of their correlations to malignant phenotypes of cancers provide important evidences to support Warburg's hypothesis that there is impairment in mitochondrial oxidative phosphorylation (OXPHOS) and respiratory function in cancer cells (Warburg, 1930, 1956).

In this study, we were interested in studying whether the process of decreasing in copy number of mtDNA could contribute to an increase in level of point mutation and/or a decrease in proportion of mtDNA with large-scale deletions in cancer cells. To address this issue, we treated cancer cells with low concentrations of ethidium bromide (EtBr) to decrease the copy number of mtDNA, and evaluated the alterations in the heteroplasmic levels of mtDNA D-loop mutations as well as the proportions of the mtDNA with 4977-bp deletion in human cancer cells. The relationships among the decrease in the copy number of mtDNA, the mtDNA D-loop mutations, and the proportions of the mtDNA with 4977-bp deletion in human cancer cells were further discussed.

2. Materials and methods

2.1. Cell culture and experimental treatments

Human cancer cell lines, including hepatoma cells (HepG2, Hep3B, and HA22T/VGH), gastric carcinoma cells (SC-M1 and NUGC-3), colon cancer cells (HT-29, Colo205, HCT-15, SW480, and SW620), prostate cancer cells (PC3 and DU145), and breast cancer cells (MCF-7), were cultured at 37 °C in 5% humidified CO_2/95% air in Dulbecco's modified eagle medium (DMEM) or RPMI (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin G and 50 mg/ml streptomycin sulfate. A series of cybrid clones harboring different proportion of mtDNA with 4977-bp deletion (1-3-16, 14-6, 51-10, and 51-12) were a gift from Professor Yau-Huei Wei. The cybrid cells were generated by fusing enucleated skin fibroblasts from a chronic progressive external ophthalmoplegia patient with mtDNA-less (ρ^0) human osteosarcoma cells. Cybrids were grown at 37 °C in 5% humidified CO_2/95% air in DMEM supplemented with 10% FBS, 100 mg/ml pyruvate, 50 mg/ml uridine, 50 U/ml penicillin G and 50 mg/ml streptomycin sulfate as previously described (Wei et al., 2001). To decrease the copy number of mtDNA, cancer cells and cybrids were treated with 50 ng/ml of EtBr for 7 days according to the standard EtBr method (King and Attardi, 1996), and then total DNA was extracted and the copy number of mtDNA was quantified to determine the extent of mtDNA depletion.

2.2. DNA extraction

Total cellular DNA of the cancer cells was extracted by the QIAamp DNA Mini kit (QIAGEN) according to the instructions of the manufacturer. The final DNA pellet was dissolved in double distilled water and frozen at −30 °C until use.

2.3. Analysis of mtDNA D-loop mutation and its heteroplasmic level

The somatic mutation in the D-loop region of mtDNA was analyzed by direct sequencing of the products of polymerase chain reaction (PCR) as described previously (Lee et al., 2005; Wu et al., 2005). The primer pairs L16190 (np 16190–16209, 5′-CCCCATGCTTACAAGCAAGT-3′) and H602 (np 602–583, 5′-GCTTTGAGGAGGTAAG CTAC-3′) were used for the amplification of a 982-bp DNA fragment from the D-loop region of mtDNA. The primer L76 (np 76–100, 5′-CACGCGATAGCATTG CGAGACGCTG-3′) was used for sequencing to determine the heteroplasmic states of the mutations in np 303–309 poly C tract. PCR was performed in an ABI GeneAmp PCR System 9700 DNA thermal cycler. The reactions were carried out for 30 cycles in a 50 μl reaction mixture containing 100 ng DNA, 200 μM of each dNTP, 20 pmol of each primer, 2.5 U of *PfuUltra* high-fidelity DNA polymerase (Stratagene), and 1 × *PfuUltra* HF reaction buffer. The PCR cycles consisted of 15-s denaturation at 94 °C, 15-s annealing at 58 °C and 90-s primer extension at 72 °C. All the PCR products thus obtained were subjected to nucleotide sequencing on an ABI PRISM® 3100 Genetic Analyzer according to the instructions of the manufacturer.

2.4. Quantification of proportion of mtDNA 4977-bp deletion

The proportion of the mtDNA with 4977 bp deletion in cybrids was quantified by a competitive PCR methods, using