

Mitochondrial DNA sequence analysis of two mouse hepatocarcinoma cell lines

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Abstract

AIM: To study genetic difference of mitochondrial DNA (mtDNA) between two hepatocarcinoma cell lines (Hca-F and Hca-P) with diverse metastatic characteristics and the relationship between mtDNA changes in cancer cells and their oncogenic phenotype.

METHODS: Mitochondrial DNA D-loop, tRNA^{Met+Glu+Ile} and ND3 gene fragments from the hepatocarcinoma cell lines with 1 100, 1 126 and 534 bp in length respectively were analysed by PCR amplification and restriction fragment length polymorphism techniques. The D-loop 3' end sequence of the hepatocarcinoma cell lines was determined by sequencing.

RESULTS: No amplification fragment length polymorphism and restriction fragment length polymorphism were observed in tRNA^{Met+Glu+Ile}, ND3 and D-loop of mitochondrial DNA of the hepatocarcinoma cells. Sequence differences between Hca-F and Hca-P were found in mtDNA D-loop.

CONCLUSION: Deletion mutations of mitochondrial DNA restriction fragment may not play a significant role in carcinogenesis. Genetic difference of mtDNA D-loop between Hca-F and Hca-P, which may reflect the environmental and genetic influences during tumor progression, could be linked to their tumorigenic phenotypes.

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Key words: Hepatocarcinoma; Mitochondrial DNA; Base Sequence

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INTRODUCTION

Mammalian mitochondrial DNA (mtDNA) is a 15-16 kb circular double-stranded DNA. The genome contains genes coding for 13 polypeptides involved in respiration and oxidative phosphorylation, 2 rRNAs and a set of 22 tRNAs that are essential for protein synthesis of mitochondria. In contrast to the nuclear DNA, mtDNA is a naked DNA molecule without introns and is replicated at a much higher rate without an effective

DNA repair mechanism. Therefore, mtDNA is more vulnerable to reactive oxygen species and free radicals that are generated in electron leak pathway of the respiratory chain^[1-3].

The possibility that the mitochondrial genome may be involved in carcinogenesis can be extrapolated back to Warburg, who demonstrated that increased anaerobic glycolysis was a common feature of tumor cells^[4]. One scenario is that the somatic mtDNA mutations in tumor cells play an active role in shifting metabolism away from mitochondrial oxidative phosphorylation and towards enhanced glycolysis^[5,6]. mtDNA mutation is a prominent feature of cancer cells and has been identified in various tumors and tumor cell lines^[7]. However, the direct link between mtDNA mutations of cancer cells and their oncogenic phenotype has not been demonstrated.

Hca-F and Hca-P are two types of cell lines originating from an identical parent cell line, hepatocarcinoma H22, with high and low metastatic abilities, respectively. To inquire into the role of mtDNA mutations in tumorigenic phenotype, the genetic variations of mtDNA from Hca-F and Hca-P cell lines were analyzed by PCR and restriction fragment length polymorphism (RFLP) and DNA sequencing.

MATERIALS AND METHODS

Materials

The restriction endonucleases including *Hae* III, *Bam*HI, *Apa* I, *Nde* II, *Xho* I, *Xba* I, *Alu* I, *Rsa* I, *Stu* I, *Dra* I, *Ava* I, and *Hae* II were purchased from German Boehringer Mannheim and American Promega companies; PCR test kits were also obtained from German Boehringer Mannheim Company. Hca-F and Hca-P hepatocarcinoma cell lines were supplied by Professor Mao-Yin Lin of Dalian Medical University, China.

Amplification of PCR

The primers were synthesized by Shanghai Cell Biological Institute of China (Table 1). Mitochondrial DNA of the tumor cell lines was prepared by the method of nuclei/cytoplasm partition^[8]. PCR amplification was carried out in a final volume of 100 μ L containing 0.5 μ g mtDNA, 0.5 mmol/L of each primer, 2.5 mmol/L MgCl₂, 200 mmol/L of each dNTP, and 2.5 U *Taq* DNA polymerase (TaKaRa Ex TaqTM). PCR (an initial incubation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, at 55 °C for 1 min, and at 72 °C for 1 min; the final step at 72 °C was extended to 10 min) was performed in a Biometra Personal PCR system.

PCR-RFLP analysis of D-loop, tRNA^{Met+Glu+Ile} and ND3 gene fragments

Two U restriction endonucleases and 1.2 μ L 10 \times buffer were added to 5 μ L of every PCR product separately and each dilution was mixed with sterilized water until the total volume was 12 μ L; then incubated at 37 °C overnight. After digested by the restriction endonucleases, each sample was analyzed by 1% agarose gel electrophoresis (the buffer fluid was TBE buffer). Performed at 3 V/cm for 1-2 h, the electrophoresis was observed under ultraviolet and photographs were taken. The standard marker of Huamei Company was adopted as the molecular weight standard to determine the length of the fragments.

Table 1 Primer sequence and length of amplified fragments

| Amplified fragment | Length (bp) | Location | Primer sequence |
|-----------------------------|-------------|--|---|
| D-loop | 1 100 | L strand (15 294-15 320) H strand (98-72) | 5'-TAAACATTACTCTGGTCTTGTAACC-3' 5'-ATTAATAAGGCCAGGACCAAACCT-3' |
| tRNA ^{Met+Glu+Ile} | 1 126 | L strand (3 401-3 419) H strand (4 527-4 508) | 5'-CGGCCCATTCGCGTTATTC-3' 5'-AGGTTGAGTAGAGTGAGGGA-3' |
| ND3 fragment | 534 | L strand (9 364-9 385) H strand (9 897-9 876) | 5'-ACGTCTCCATTTATTGATGAGG-3' 5'-GAGGTTGAAGAAGGTAGATGGC-3' |
| D-loop 3' end fragment | 437 | L strand (15 950-15 968) H strand (91-73) | 5'-AGGCATGAAAGGACAGCAC-3' 5'-ATAAGGCCAGGACCAAACCT-3' |

Table 2 Mitochondrial tRNA^{Met+Glu+Ile}, ND3 and D-loop restriction patterns

| Enzyme | D-loop (1 100 bp) | | tRNA ^{Met+Glu+Ile} (1 126 bp) | | ND3 (534 bp) | |
|----------------|-------------------|------------------|--|----------------|--------------|------------|
| | Site | FL | Site | FL | Site | FL |
| <i>Hae</i> III | 4 | 456 445 122, ... | 4 | 470 290 136... | 1 | 392 142 |
| <i>Apa</i> I | 1 | 655 445 | 1 | 658 469 | 0 | 534 |
| <i>Alu</i> I | 4 | 545 269 102, ... | 3 | 665 318 135... | 1 | 310 224 |
| <i>Bam</i> H I | 0 | 1 100 | 2 | 710 252 164 | 0 | 534 |
| <i>Dra</i> I | 0 | 1 100 | 1 | 634 493 | 1 | 45 183 |
| <i>Stu</i> I | 0 | 1 100 | 2 | 605 297 225 | 1 | 391 143 |
| <i>Nde</i> II | 2 | 84 511 837 | 0 | 1 126 | 0 | 534 |
| <i>Rsa</i> I | 6 | 720 178 125... | 4 | 392 259 228... | 2 | 35 310 378 |
| <i>Xba</i> I | 1 | 680 420 | 0 | 1 126 | 0 | 524 |

FL: fragment length.

DNA sequencing of PCR products

PCR products for the D-loop 3' end fragment of 437 bp in length were sent to United Gene Technology Company, Ltd, Shanghai, China for direct sequencing.

RESULTS

Mitochondrial DNA D-loop, tRNA^{Met+Glu+Ile} and ND3 gene fragments from the hepatocarcinoma cell lines with 1 100, 1 126 and 534 bp in length respectively were analysed by PCR amplification. No amplification fragment length polymorphism and negative amplification were observed. Mitochondrial DNA D-loop, tRNA^{Met+Glu+Ile} and ND3 fragments were cleaved respectively by 12 kinds of restriction endonucleases including *Hae* III, *Bam*H I, *Apa* I, *Nde* II, *Xho* I, *Xba* I, *Alu* I, *Rsa* I, *Stu* I, *Dra* I, *Ava* I and *Hae* II. But no difference was observed in all restriction maps of D-loop, tRNA^{Met+Glu+Ile} and ND3 fragments of mtDNA from hepatocarcinoma cell lines and no variation was found in 41 restriction endonuclease sites (Figure 1, Table 2).

The D-loop 3' end fragment sequence of two hepatocarcinoma cell lines was determined by sequencing. Compared with published mouse mtDNA sequence, we had found 3 mutations: G:C→A:T transition was detected at nucleotide 16 007 in Hca-F and Hca-P cell lines and a T:A→C:G occurred

at nucleotide 16 268 only in Hca-F cell line (Figure 2).

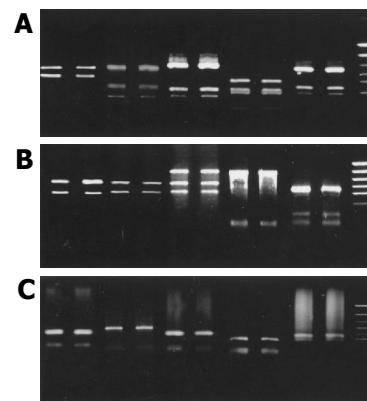


Figure 1 Restriction patterns of amplified fragments. F: Hca-F cells; P: Hca-P cells; M: PCR standard marker. A: Restriction patterns of tRNA^{Met+Glu+Ile} digested by *Apa* I, *Alu* I, *Bam*H I, *Rsa* I and *Stu* I, respectively; B: Restriction patterns of D-loop digested by *Xba* I, *Apa* I, *Rsa* I, *Stu* I and *Alu* I, respectively; C: Restriction patterns of ND3 digested by *Hae* III, *Dra* I, *Stu* I, *Rsa* I and *Alu* I, respectively.

| | | | | | | |
|--------|------------|------------|------------|-------------|------------|--------------------|
| 15 950 | AGGCATGAAA | GGACAGCACA | CAGTCTAGAC | GCACCTACGG | TGAAGAATCA | TTAGTCCGCA A(2) |
| 16 010 | AAACCCAATC | ACCTAAGGCT | AATTATTCAT | GCTTGTTAGA | CATAAATGCT | ACTCAATACC |
| 16 070 | AAATTTTAAC | TCTCCAAACC | CCCCACCCCC | TCCTCTTAAT | GCCAAACCC | AAAAAACACT |
| 16 130 | AAGAACTTGA | AAGACATATA | ATATTAACTA | TCAAACCCTA | TGTCCTGATC | AATTCTAGTA |
| 16 190 | GTTCCCAAAA | TATGACTTAT | ATTTTAGTAC | TTGTA AAAAT | TTTACAAAAT | CAATGTTCCGT |
| 16 250 | GAACCAAAAC | TCTAATCATA | CTCTATTACG | CAATAAACAT | TAACAA 1 | GTTAATGTAG |
| 11 | CTTAATAACA | AAGCAAAGCA | CTGAAAATGC | TTAGATGGAT | AATTGTATCC | CATAAACACA |
| 71 | AAGGTTGGT | CCTGGCCTTA | T | | | |

Figure 2 Variations identified in the D-loop (bases 15 950-91) of mitochondrial DNA from two types of hepatocarcinoma cell lines. Sequence and base number were from the complete mouse mtDNA sequence reported by Bibb *et al.* (Cell 1981; 26: 167-180). The G at nucleotide 16 007 was mutated to A in two hepatocarcinoma cell lines and T at nucleotide 16 268 was mutated to C in Hca-F cell line.

DISCUSSION

Carcinogenesis is a multi-step process involving the accumulation of genetic changes that ends in malignant cell transformation. Much attention has been paid to the genetic events in the nDNA, such as activation of oncogene, inactivation of tumor suppressor gene, and defects of mismatch DNA repair gene. However, several aspects in the process of carcinogenesis are still unclear. Contribution of mtDNA mutations to carcinogenesis was postulated when wide spectra of the mtDNA alterations were reported in different types of cancer: colon, ovarian, lung, pancreatic, liver, thyroid, bladder, prostate, esophageal and gastric cancer^[7,9-12]. Reported sequence changes include point mutations (mostly transitions), multiple deletions and microsatellite instability in coding and noncoding regions. However, of all these mutations and polymorphisms only a few can be linked to a known phenotypic effect. Alonso^[13] suggested different mechanisms, such as clonal expansion, increased oxidative damage and mutator mutations, to explain this high frequency of homoplasmic mtDNA variation in cancer samples. Because most tumors are a clonal expansion of a single cell, it is possible that mtDNA homoplasmic mutations are just the results of clonal expansion of spontaneous somatic mutations, which occur at a very low frequency during previous replication of this precursor cell and later become predominant or homoplasmic by clonal expansion of its cell^[14]. However, the selective advantage of mtDNA changes in the development of tumors cannot be excluded. In principle, these mutations could contribute to neoplastic transformation by changing cellular energy capacities, increasing mitochondrial oxidative stress, and/or modulating apoptosis.

In this study, large-scale deletion mutations of D-loop, tRNA^{Met+Glu+Ile} and ND3 gene fragments in hot-spot regions of mtDNA were analysed by PCR amplification and RFLP techniques. There were no amplification fragment length polymorphism and negative amplification, and all restriction patterns of D-loop, tRNA^{Met+Glu+Ile} and ND3 fragments from Hca-F and Hca-P hepatocarcinoma cell lines were also identical. In contrast to previous studies, we failed to find mtDNA large-scale deletions in tumor cell lines, which differ from the observation that multiple mtDNA deletions were detected in tumors and normal human tissue cells^[15,16]. We propose possible mechanisms to explain the phenomenon. Firstly, the increased proliferation of tumor cell lines promotes the cytoplasmic segregation of deleted mtDNAs, and thus the mtDNA molecules with deletions are passively eliminated in tumor cells. Secondly, the mtDNA deletions accumulated in tumor cells may result in impaired mitochondrial respiration and decrease ATP synthesis. After cytoplasmic segregation of deleted mtDNAs, the cells harboring higher proportion of mtDNA deletions could not survive and lead to dropout from the population. So, we suggest that mtDNA fragment deletion mutations in tumors unlikely play a significant role in carcinogenesis, probably just as age-related increases in DNA damage due to cellular oxidative stress and environmental factors.

Mitochondrial DNA control region (non-coding region, D-loop), containing the origin of replication for H-strand synthesis, mitochondrial transcription promoters, mtTF1 binding site and conserved sequence block, *etc.*, serves as the main site for mitochondrial genomic replication and transcription. Point mutations and genetic instabilities at the D-loop region are potentially involved in the maintenance of structure and function or even the expression of other mitochondrial genes, and probably also involved in the progressive stage of the disease^[17,18]. In the present study, we have found sequence difference in mtDNA D-loop region between two types of hepatocarcinoma cell lines with diverse metastatic abilities. G:C→A:T transition was detected at nucleotide 16 007 in Hca-F and Hca-P cell lines and

T:A→C:G occurred at nucleotide 16 268 only in Hca-F cell line. Little is known about the presence of genetic alterations, especially of point mutations, localized at crucial sites or adjacent place in the mtDNA control region, and their contribution to carcinogenesis. Although it is unlikely that mutations in the mtDNA control region are immediately deleterious or tumorigenic, the frequency of mtDNA mutations may reflect underlying genetic and environmental influences during tumor progression^[19]. It is also possible that sequence variants in the mtDNA control region could influence disease-associated mutations in the coding regions^[20,21]. There is considerable evidence that the mutation rate of both mitochondrial coding region and non-coding region loci is increased as a result of tumorigenicity^[22-25]. It is likely, therefore, that the difference of mtDNA D-loop region observed in different hepatocarcinoma cell lines is a reflection of this increased mutation rate and can be linked to their tumorigenic phenotype. More extensive biochemical and molecular studies will be necessary for determining the pathological effect of these mtDNA genetic alterations.

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