RESTRICTION ENDONUCLEASE ANALYSIS OF MITOCHONDRIAL DNA FROM HUMAN LUNG ADENOCARCINOMA CELL LINE SPC-A-1

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ABSTRACT

Objective: To understand the role of mitochondrial DNA (mtDNA) in carcinogenesis. Methods: single-step method was used to isolate the mtDNA from human lung adenocarcinoma cell line SPC-A-1. The mtDNA was analyzed by restriction fragment length polymorphism (RFLP) with 11 kinds of restriction endonuclease, which were Pvu II, Xho I, Pst I, EcoR I, BstE II, Hind III, Hpa I, Bcl I, EcoR V, Sca I and Xba I. Restriction map of mtDNA from SPC-A-1 cell was obtained by the single and double-digestion method. Results: It was found that no variation at 32 restriction-sites could be detected in the coding region of mtDNA from SPC-A-1 cell line. But a new site was found at nucleotide 16276 (EcoR V) within the noncoding region. Conclusion: These results indicate that the primary structure of gene coding region of mtDNA isolated from SPC-A-1 cell is highly stable. While the major variation of nucleotide is probably located in the noncoding region.

Key words: Lung carcinoma, Mitochondrial DNA, Restriction fragment length polymorphism, Mutation

Mitochondria contain their own genetic systems for replication, transcription and translation.[1] Human mitochondrial DNA (mtDNA) is a 16 569-base pair (bp) double-stranded, closed circular molecule, which codes for both a small (12S) and a large (16S) ribosomal RNA gene, 22 transfer RNAs and 13 protein-coding genes.[2] All of the mitochondrial genes encode subunits of the oxidative phosphorylation (OXPHOS) enzymes that are responsible for the energy-generating pathway. There are several copies of the mitochondrial genome in each mitochondria as well as hundreds to thousands of mtDNA copies per cell. MtDNA, which lacks histones and has a low replication fidelity, is thought to be highly susceptible to damage by mutagens.[3] However, to date, very few structural characteristics of mtDNA alterations in carcinoma cells have been reported. Therefore, we analyzed mtDNA polymorphism in the human lung adenocarcinoma cell line SPC-A-1 by using restriction mapping with 11 restriction enzymes. The aim of the present study was to determine whether mtDNA mutation could be associated with carcinogenesis.

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MATERIALS AND METHODS

Enzymes

Eleven kinds of restriction endonuclease, including Pvu II, Xho I, Pst I, EcoR I, BstE II, Hind III, Hpa I, Bcl I, EcoR V, Sca I, and Xba I, were purchased from Boehringer Mannheim Biochemica.

Cell Line

The human lung adenocarcinoma cell line SPC-A-1 was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences.

Extraction and Restriction of mtDNA

One-step Method, according to Hu Yide et al. extracted the mtDNA from fresh culture cells.[4] It was digested 6 hours at 37℃ with the indicated restriction endonucleases (1-2U/μ g of mtDNA). Buffers and other conditions were specified by the vendor for each enzyme. The digestion was terminated by addition of 0.2 vol of gel loading buffer (6 × buffer = 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water).

Electrophoresis and Restriction Map Construction

Restriction fragments were separated by electrophoresis on 1 to 2% agarose gels and stained with ethidium bromide. The size of fragments was

determined by using λ DNA/Hind III and λ DNA/Hind III + EcoR I as size markers. Restriction patterns were viewed and photographed under UV illumination. The sizes of the mtDNA fragments were then determined according to the published mtDNA sequence. Maps for restriction sites in humans of unknown sequence can be derived by comparing their fragment patterns with that predicted from the Cambridge sequence. [5]

RESULTS

MtDNA Molecular Length

Fragments of mtDNA from SPC-A-1 cell were obtained by the single and double-digestion methods. The total molecular length was 16.5 kilobase (kb).

Single-digestion Pattern

The mtDNA sample from SPC-A-1 cell was mapped for restriction site polymorphism with 10 restriction enzymes: Pvu II, Xho I, Pst I, EcoR I, BstE II, Hind III, Hpa I, Bcl I, Sca I and Xba I. Single-digestion pattern was completely consistent with the Cambridge sequence pattern.

While the 6.877 kb fragment disappeared, two new fragments (3.474kb and 3.403 kb) appeared when it was digested with EcoR V (Table 1).

Double-Digestion Pattern

Same as in the above, the 6.877kb fragment disappeared and two new fragments (3.474 kb and 3.403 kb) appeared when it was digested with EcoR V combined with Hpa I. The 6.666 kb fragment disappeared and two new fragments (6.137 kb and 0.529 kb) appeared when it was digested with EcoR V combined with Pvu II.

Double-digestion pattern of the other enzymes was completely consistent with the Cambridge sequence pattern (Table 2).

Cleavage Site Polymorphism

Table 3 shows the locations of the 33 cleavage sites mapped in this study. They are widely distributed among coding region and noncoding region of the mtDNA molecule. It was found that no variation at 32 restriction-site could be detected in the coding region of mtDNA from SPC-A-1 cell line. However a new site was found at nucleotide 16276 (EcoR V) within the noncoding region. The maps of mtDNA from SPC-A-1 cell line were constructed as Figure 1.

Table 1. mtDNA restricted fragments sizes and patterns from SPC-A-1 cell line with individual enzymes

Enzyme	Recognition sequence	Fragments size (kb)					Patterns compare with Cambridge sequence
		A	B	C	D	E	
Pvu II	CAG↓CTG	16.569					No change
Xho I	C↓TCGAG	16.569					No change
BstE II	G↓GTNACC	14.771	1.798				No change
Pst I	CTGCA↓G	14.459	2.110				No. change
EcoR I	G↓AATTC	8.050	7.366	1.153			No change
Hind III	A↓AGCTT	10.202	5.477	0.890			No. change
Hpa I	GTT↓AAC	9.854	4.323	2.392			No change
Bcl I	T↓GATCA	8.306	3.999	3.333	0.934		No change
Sca I	AGT↓ACT	13.122	1.506	1.159	0.575*	0.012*	No change
Xba I	T↓CTAGA	7.506	4.488	1.970	1.760	0.845	No change
EcoR V	GAT↓ATC	6.137	3.555	3.474	3.403		Different

*: fragments invisible on the agarose gels.

DISCUSSION

The human mtDNA genome contains 16.569 bp and plays a limited but essential role in the biogenesis of the organelles that contain these. Since 1988, mtDNA variation resulting from base substitutions, insertions, or deletions have been postulated to play an important role in a broad spectrum of human diseases.[1,2] Whereas only about 7% of the nuclear DNA (nDNA) is ever expressed at any particular differentiated stage, the expression of the whole mtDNA is essential for the normal functioning of the cells. Genetic mutation, whether spontaneous or induced by chemical mutagens, is a random process and occurs in the mtDNA as well as nDNA, but the

Table 2. mtDNA restricted fragments sizes and patterns from SPC-A-1 cell with double enzymes

Enzyme	Fragments size (kb)								Patterns compare with Cambridge Sequence
	A	B	C	D	E	F	G	H	
Hind III+Xho I	8.817	5.477	1.385*	0.890*					No change
Hind III+EcoR I	8.050	5.477	1.153	0.929					No change
Hind III+Bcl I	7.657	3.089	2.545	1.454	0.934	0.649	0.241*		No change
Hind III+Pvu II	6.651	5.477	3.551	0.890					No change
Xho I+EcoR I	7.366	5.735	2.315	1.153*					No change
Xho I+Pvu II	12.303	4.266							No change
Xho I+Hpa I	7.307	4.323	2.547	2.392					No change
Bcl I+Pvu II	7.300	3.999	3.330	1.006*	0.934*				No change
Bcl I+BstE II	8.306	3.330	1.798	1.162	1.039	0.934			No change
Hpa I+Pvu II	6.813	4.323	3.041	2.392					No change
Pst I+EcoR I	8.050	3.616	2.110	1.640	1.153				No change
Xba I+EcoR I	5.122	2.384	2.167	1.970	1.760	1.168	1.153	0.845	No change
Hpa I+EcoR V	3.474#	3.403#	3.280	2.512	2.392	1.043	0.465*		Different
Pvu II+EcoR V	6.137@	3.555	3.403	2.945	0.529@				Different

*: Indicate the fragments invisible on the agarose gels; "#" two new bands of 6.877kb fragment restricted by EcoR V; "@" two new bands of 6.666kb fragment restricted by EcoR V.

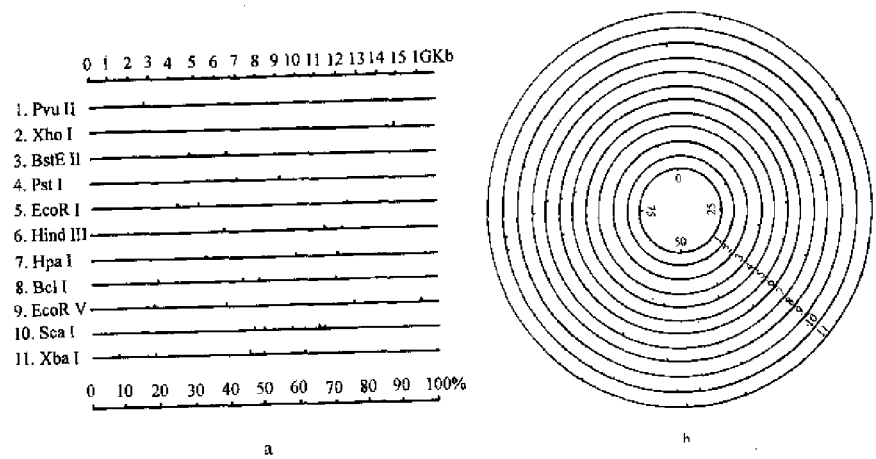

Fig 1. Restriction maps of mtDNA from SPC-A-1 cell line (a. linear form, b. circular form)

Table 3. Polymorphic sites for the 11 enzymes of mtDNA from SPC-A-1 cell line

Enzyme	Restricted sites				
	A	B	C	D	E
Pvu II	2652				
Xho I	14955				
BstE II	4820	6618			
Pst I	6914	9024			
EcoR I	4121	5274	12640		
Hind III	6203	11680	12570		
Hpa I	5693	10016	12408		
Bcl I	3658	7657	8591	11921	
EcoR V	3181	6736	12873	16276*	
Sca I	8013	8588	9747	11448	11460
Xba I	1193	2953	7441	8286	10256

*: One new restricted point of EcoR V was found in mtDNA from SPC-A-1 cell line.

mitochondrial mutation rate is about 10 times higher than that of nDNA.[6] MtDNA is thought to be more susceptible to damage by mutagens than nDNA for the following reasons: (a) mtDNA polymerase γ replicates the DNA with poor fidelity, (b) mtDNA is a naked molecule to which chemical carcinogens can easily bind, while nDNA is protected by histones and (c) a high concentration of reactive oxygen species in mitochondria can induce mtDNA damage.[7]

Alterations in the energy metabolism of cancer cells have been reported for many years. Few previous studies have demonstrated structural changes in mtDNA in human cancer, including large-scale deletions (>50bp) in gastric and renal cell carcinomas.[8] Our main goal was to detect mtDNA alterations and specific mitochondrial genomic structural differences (RFLPs) implicated in the malignant transformation processes of lung cancer. To this end, we have studied the complete mtDNA by means of very sensitive techniques. The use of 11 restriction endonucleases allowed us to identify 33 restriction sites. It was found that no variation at 32 restriction-sites could be detected in the coding region of mtDNA from SPC-A-1 cell line. But a new site was found at nucleotide 16276 (EcoR V) within the noncoding region. These results indicate that the primary structure of gene coding region of mtDNA isolated from SPC-A-1 cell is highly stable, while the major variation of nucleotide is probably located in the noncoding region.

Welter, et al. reported that no major structural changes were observed between the mtDNA of five human colon cancers and that of adjacent healthy tissues of the same patients when the restriction fragment patterns obtained by 10 restriction endonucleases were analyzed. They suggested that a strong selective mechanism exists conserving the primary structure of mtDNA in tumorigenesis.[10] Monnat, et al., who analyzed mtDNA of different individuals suggested that a mechanism or mechanisms exist that limits the development of nucleotide sequences divergence in mtDNA.[11] How such a mechanism might work is not clear. Two families of nuclear genes that will be particularly important to analyze in this manner are cellular protooncogenes and those genes whose products play essential roles in cell growth and division.[9]

In addition, the noncoding region contains the main regulatory elements of the mitochondrial genome: the two promoters (HSP and LSP) and the origin of replication of the H strand (OH). Therefore, we suggested that the mutation in the starting area of H strand replication might be associated with carcinogenesis or susceptibility to carcinoma. This hypothesis might be tested in the future by studying more types of the malignant cells and endonucleases or by a total sequencing of the mtDNA noncoding region.

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