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• GASTRIC CANCER •

Quantitative analysis of tumor mitochondrial RNA using microarray

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Abstract

AIM: To design a novel method to rapidly detect the quantitative alteration of mtRNA in patients with tumors.

METHODS: Oligo 6.22 and Primer Premier 5.0 bio-soft were used to design 15 pairs of primers of mtRNA cDNA probes in light of the functional and structural property of mtDNA, and then RT-PCR amplification was used to produce 15 probes of mtRNA from one normal gastric mucosal tissue. Total RNA extracted from 9 gastric cancers and corresponding normal gastric mucosal tissues was reverse transcribed into cDNA labeled with fluorescein. The spotted mtDNA microarrays were made and hybridized. Finally, the microarrays were scanned with a GeneTAC™ laser scanner to get the hybridized results. Northern blot was used to confirm the microarray results.

RESULTS: The hybridized spots were distinct with clear and consistent backgrounds. After data was standardized according to the housekeeping genes, the results showed that the expression levels of some mitochondrial genes in gastric carcinoma were different from those in the corresponding non-cancerous regions.

CONCLUSION: The mtDNA expression microarray can rapidly, massively and exactly detect the quantity of mtRNA in tissues and cells. In addition, the whole expressive information of mtRNA from a tumor patient on just one slide can be obtained using this method, providing an effective method to investigate the relationship between mtDNA expression and tumorigenesis.

Key words: Mitochondrial RNA; Gastric cancer; Microarray technique

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INTRODUCTION

Tumor mitochondria often differ very significantly from their normal counterparts. Alterations in organelles accompanying neoplastic transformation of cells are reflected in mitochondrial morphology, enzymatic composition and cellular content[1,2]. Mitochondrial DNA (mtDNA) is a 16 569 bp double-stranded, closed circular molecule, which codes for a small (12S) ribosomal RNA gene and a large (16S) ribosomal RNA gene, 22 transfer RNAs and 13 protein-coding genes[3]. The mtDNA-encoded polypeptides are enzyme complex subunits of the oxidative phosphorylation (OXPHOS) system responsible for the synthesis of ATP[4]. In addition, mtDNA lacks introns, and is susceptible to reactive oxygen species (ROS)[5-7], and involved in carcinogenesis because of its high susceptibility to mutations and limited repair mechanisms in comparison to nuclear DNA[8-10]. Quantitative alteration of transcripts (mtRNA) of mtDNA may be a general characteristic of cancer cells[2]. Increased transcripts of mtDNA may lead to decreased apoptosis of tumor cells and subsequent carcinogenesis[11]. Microarray technique can monitor the expression of many genes in parallel, thereby speeding up the identification of differentially expressed genes and the construction of differential expression profiles. Microarray analysis has become an increasingly popular tool to study the functions of genes, especially those genes involved in tumor formation and growth[12-15].

MATERIALS AND METHODS

Materials

One normal gastric mucosal tissue, 6 gastric cancer tissues and corresponding normal gastric epithelial mucosal tissues adjacent to cancerous lesions were obtained from the First Affiliated Hospital Hospital of China Medical University, which were all diagnosed pathologically by hematoxylin and eosin staining.

Methods

Primer designation Both the primer design software Oligo 6.22, Primer Premier 5.0 and Mitomap database (human mitochondrial genome database of Emory) were used to analyze the whole mitochondrial genome, and 15 pairs of primers were screened out to amplify the probes of mtRNA (Table 1). BLAST analysis was used to exclude the influence of nuclear pseudogenes.

RNA isolation Total RNA was extracted from normal gastric tissues, gastric cancerous and para-cancerous mucosal tissues. A total of 50-100 mg frozen tissue was pulverized in a mortar containing liquid nitrogen. The powder was dissolved in TRIzol reagent, and then chloroform was added to precipitate the protein. RNA was isolated by precipitation with isopropanol. RNA pellet was washed in 750 mL/L ethanol, air-dried and dissolved in water treated with diethylpyrocarbonate (DEPC). RNA was stored at -80 ℃ until use.

Probe preparation RNA extracted from normal gastric samples was amplified via reverse transcription PCR (RT-PCR) to produce the probes of mtRNA. Reverse transcription procedure referred to the manual of TaKaRa AMV RT-PCR kit. PCR reaction was carried out in a final volume of 50 μL in a Biometra personal PCR system, with an initial incubation at 94 ℃ for

Table 1 Primer pairs used for PCR-amplified probes of mtRNA

Functional domain	Primer	Sequence	Length (mer)	Tm (℃)	GC%	Product size (bp)
12SrRNA	F1097	GCCCTAAACCTCAACAGT	18	54.1	50.0	264
	R1360	CATTTCTTGCCACCTCAT	18	44.5	44.4	
16SrRNA	F2618	TAGGGACCTGTATGAATGG	19	48.2	47.4	485s
	R3102	ATAGAAACCGACCTGGAT	18	48.0	44.4	
ND1	F3927	GTCTCAGGCTCAACATC	17	42.1	52.9	273
	R4199	TAGGGTGAGTGGTAGGAA	18	51.1	50.0	
ND2	F5022	CCCACATAGGATGAATAA	18	40.2	38.9	466
	R5487	GCGATGAGGATGGATAGAG	19	46.2	52.6	
COI	F6043	TCTAGGTAACGACCACATCTACAAC	25	62.4	44.0	614
	R6656	CGAAGCCTGGTAGGATAA	18	51.9	50.0	
COII	F7841	TAACAGACGAGGTCAACG	18	50.5	50.0	351
	R8191	TTGCTCCACAGATTTCAG	18	43.1	44.4	
ATPase8	F8366	TGCCCCAACTAAATACTA	18	49.4	38.9	191
	R8556	CAATGAATGAAGCGAACA	18	41.6	38.9	
ATPase6	F9000	CGCCTAACCGCTAACATTACTG	22	64.2	50.0	148
	R9147	AGGCGACAGCGATTTCTA	18	53.8	50.0	
COIII	F9321	CCATAACGCTCCTCATAC	18	47.8	50.0	203
	R9523	TAGGCTGGAGTGGTAAAA	18	51.8	44.4	
ND3	F10200	GCGTCCCTTTCTCCATAA	18	52.4	50.0	203
	R10402	TTCGGTTCAGTCTAATCCTT	20	51.8	40.0	
ND4	F11581	ATCTGCCTACGACAAACA	18	48.3	44.4	444
	R12024	GTGGTGGGTGAGTGAGCCC	19	61.3	68.4	
ND4L	F10573	AATAATACTATCGCTGTTCA	19	45.4	30.0	189
	R10761	CATTGGAGTAGGTTTAGG	18	46.2	44.4	
ND5	F13028	CTGACTCCCCTCAGCCATAGA	21	57.2	57.1	276
	R13303	TGTGGTTGGTTGATGCCG	18	53.3	55.6	
ND6	F14322	GTTTACCACAACCACCAC	18	52.1	50.0	291
	R14612	TCTAAGCCTTCTCCTATTT	19	48.8	36.8	
Cyt-b	F15002	GCGCCTCAATATTCTTTATCTGC	23	58.7	43.5	305
	R15306	GAAGGGCAAGATGAAGTGAAA	21	53.4	42.9	

ND: NADH dehydrogenase; Cyt-b: Cytochrome b; ATPase: adenosine triphosphatase; CO: cytochrome c oxidase.

4 min, followed by 30 cycles, each consisting of denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 2 min. Finally a further extension was performed at 72 ℃ for 4 min. Meanwhile, two housekeeping genes β-actin (X16940) and glyceraldehyde phosphate dehydrogenase (GAPDH, M33197) and two hepatitis C virus (HCV) genes were amplified as the positive internal control and negative control, respectively. Polyacrylamide gel electrophoresis (PAGE) confirmed the integrity of the probes and control genes. Probes were purified according to the manual of QIAquick PCR purification kit and quantified with UV absorbance at 260 nm, finally re-suspended in 0.1×carbonate buffer (pH 9.0) or in an aqueous solution of 150 mmol/L sodium phosphate (pH 8.5) plus 0.1 g/L SDS[16].

RT-PCR of cDNA labeled with fluorescence One microgram of RNA extracted from gastric cancerous and para-cancerous tissues was reverse-transcribed into cDNA in 20 μL reaction volume with a hexamer primer (TaKaRa AMV RT-PCR Kit) and was labeled with fluorescein isothiacyanate dUTP (FITC-dUTP). To remove RNA complementary to cDNA, 1 μL of *E.coli* RNAase H was added and incubated at 37 ℃ for 20 min to digest the residue RNA. The resulting cDNA was resuspended in 20 μL deionized water, and stored at -20 ℃.

Construction of microarray

Amino-slides Coverslides were soaked for over 24 h in the mixture of dichromic acid and stronger sulfuric acid, rinsed with tap water, and then plunged into 1 mol/L NaOH for 1 h. The slides were washed with an ultrasonic washing device for 3×3 min, and dipped in acetone for 3 min, in 50 mL/L arm element KH-550 (with acetone) for 6 min, in acetone for 5×3 min again, and then baked for 1 h at 104 ℃.

Spotting and hybridization Fifteen pairs of mitochondrial DNA probes together with positive control housekeeping genes and negative control HCV gene were spotted onto amino-modified slides by a touching needle-dipping device (Micro Grid II device, England). To sufficiently analyze the results and preclude the interference of occasional errors, we spotted 9 spots per sample. The 9 spots were placed in a wet chamber with a humidity of 95% at 37 ℃ for 2 h, baked at 80 ℃ for 1 h, dipped in blocking solution (100 mL/L iodized skellysolve butane and 900 mL/L anhydrous alcohol) for 1.5 h.

Eight μL of RT-PCR products and 2 μL of hybridization buffer containing 300 mL/L DMSO (dimathyl sulfoxide) and 700 mL/L 20×SSC were mixed. The amino-modified slides with probes and cDNA mixture above were denatured respectively at 95 ℃ for 5 min, dipped quickly into ice-cold water for 3 min. The mixture was added onto the slides, and then the silicon-slide was placed on the top of the array to make them fully hybridized, the slides were placed in a well-sealed hybridization chamber, and incubated in a 55 ℃ oven for 12-14 h.

Slide washing The slides were washed in 0.5×SSC/0.1 g/L SDS solution at 42 ℃ for 5 min and in 2×SSC at 37 ℃ for 3 min with gentle agitation, stored in a lightproof slide box.

Detection Chips were scanned with a scanning array system at 10 μm resolution (GeneTAC™ laser scanner, USA). The obtained images were analyzed using ImaGene3.0 software (BioDiscovery, Los Angeles, USA).

Northern blot In order to evaluate the reliability of the microarray method, the RNA extracted from gastric cancerous and non-cancerous tissues was subjected to Northern blot analysis. Probes of NADH dehydrogenase 4 (ND4), cytochrome C oxidase I (COI) were labeled with a random prime DNA labeling kit (Boehringer-Mannheim). Equal amounts of RNA determined by quantitation of optical densities at 260 nm and further normalized using the housekeeping genes, were loaded onto agarose gels containing 2.2 mol/L formaldehyde, and transferred to nylon membranes. The membranes were dried and prehybridized at 42 ℃ for 3 h, and then hybridized with labeled ND4 and COI at 42 ℃ for 18 h.

RESULTS

Hybridization and data analysis

The hybridized spots were distinct, with a clear border and no black cavity, the background was consistent and clear for image analysis (Figure 1). In All arrays, the housekeeping genes showed positive signals, whereas HCV genes showed negative signals. The intensity of each spot represented the quantity of FITC-dUTP, hybridized to each spot. In order to enhance the confidence of the results, the overall intensities were normalized with a correction coefficient obtained using the ratios of housekeeping genes (Figure 2). After data were standardized, the results showed that the expression levels of some mitochondrial genes in gastric carcinoma were higher than those in the para-cancerous tissues. Since the samples were limited, further detailed analyses would be reported with a large size of samples.

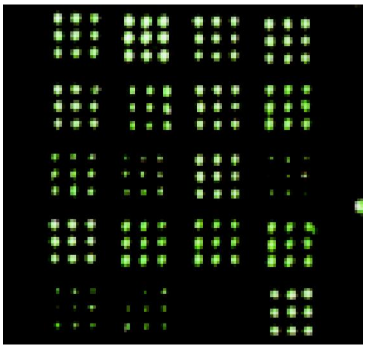

Figure 1 Scanning picture of hybridization. In the microarray, the signals of housekeeping genes were positive and those of HCV genes were negative. The 15 probes of mtRNA showed different hybridizing signals in the same gastric tissues, and some probes showed different hybridizing signals in cancerous tissue and normal gastric mucosal tissue.

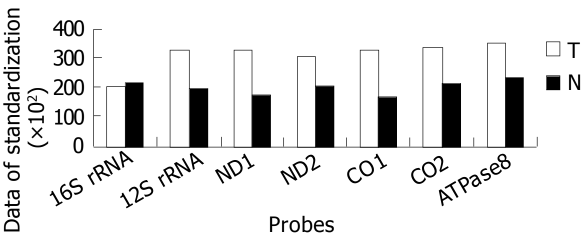

Figure 2 Quantitative analysis of mtRNA in different gastric tissues. After standardization of the two groups of data with housekeeping genes, the quantity of mtRNA in different gastric tissues using microarray was different. Moreover, the expression in different genes of mtDNA was different. T: gastric cancerous tissue; N: normal gastric mucosal tissue adjacent to cancer.

Confirmation of designed microarray by Northern blotting

Northern blot showed that transcripts of some parts of mtDNA in cancerous tissues were higher than those in non-cancerous tissues, and increased differently in different parts of mtRNA. ND4 and COI were used to serve as two representatives of the whole probes of mtRNA (Figures 3-5). The Northern blot results displaying a high concordance with the microarray further indicated that the established microarray method was reliable.

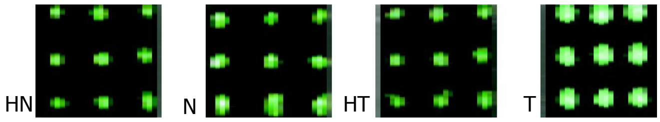

Figure 3 Reliability of mtRNA expression microarray method. HN: hybridization of housekeeping gene β-action in normal gastric mucous tissue. N: hybridization of ND4 or CO I in normal gastric mucosal tissue. HT: hybridization of housekeeping gene β-action in cancerous tissue; T: hybridization of ND4 or CO I in cancerous tissue.

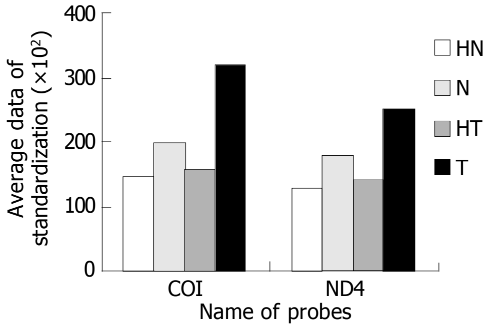

Figure 4 Data after standardization.

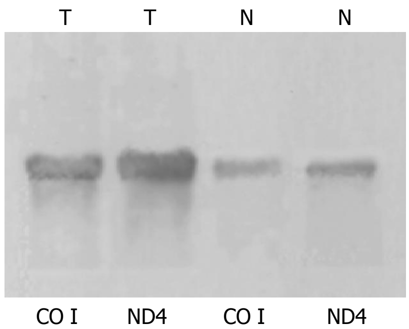

Figure 5 Northern blot of mtRNA extracted from gastric carcinoma and normal gastric mucosal tissues adjacent to the carcinoma. T: gastric cancerous tissue; N: normal gastric mucosal tissue.

DISCUSSION

Tumor cells, in general, contain fewer mitochondria than corresponding normal tissues[2,17]. The diminished content of mitochondria in tumor cells thus might reflect a reduced expression of mitochondrial or nuclear genes for mitochondrial proteins in response to neoplastic transformation[1,18]. However, paradoxically enhanced expression of mitochondrial genes in cancer has been reported[1,11,19]. In solid tumors, an elevated expression of mtDNA-encoded subunits of the mitochondrial electron respiratory chain might reflect mitochondrial adaptation to perturbations in cellular energy requirements[11]. Increased mtRNA levels might possibly suppress tumor cell apoptosis, and subsequently lead to the overgrowth of tumor cells[11]. The precise relationship between mitochondrial mass, level of mitochondrial mRNA, and mtDNA copy number has to be examined. Whether the levels of tumor cell mtDNA increase or decrease, specific alterations of mtDNA gene transcripts might be a tumor marker. Some differentially expressed gene profiles might accompany a specific or a type of carcinoma[20-22]. Hence, we can use the novel method to screen the sense parts of mtRNA as a tumor marker or even as a different tumor marker.

Some traditional methods can be applied to this work such as RT-PCR, Northern blot and real-time PCR, but they are time-consuming and rather expensive. A single microarray could provide information on the expression of tens of thousands of genes. The success of fully exploiting these powerful approaches depends on several criteria[23-27]: accurate selection, amplification and location of probe molecules, accurate reference sequence information, identification of unique probes, accurate distinction among multiple products of a single gene, accurate reconstruction of expressed sample nucleotide sequences, precision map scanning, and reproducible and accurate transformation of image files to numerical data. Expression analysis using glass slide microarrays is typically performed by the competitive hybridization of two targets (typically known as test and reference), each labeled with a specific fluorescent dye like FITC, Cy3 and Cy5. There are also a number of reasons why data must be normalized, including unequal quantities of starting RNA, differences in labeling or detection efficiencies between the fluorescent dyes used, and systematic biases in the measured expression levels[28-32]. Though we also could use two different fluoresceins to label gastric cancerous and normal tissues respectively, yet considering the above difficulties of microarray data normalization and map transformation, we only labeled one fluorescent FITC to gastric cancer and normal gastric tissues, and used the housekeeping gene to normalize the hybridization signals of mtRNA probes. In this study, in the course of constructing the microarray, We paid more attention to the good and consistent background of the microarrayt[33,34]. The processing procedure of slides is directly related to the data analysis. Moreover, since single point data can not draw exact data on account of accidental errors, spotting several spots is essential. Otherwise the results might be artificially positive or negative. In conclusion, mitochondrial microarray is a reliable and repeatable method to detect the loss or changes of mtDNA expression levels, but the precise mechanisms by which the two genomes interact and integrate with each other are poorly understood.

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