Decreased mtDNA Copy Number of Gastric Cancer: a New Tumor Marker?

Fan Li[1]
Xiaosong Wang[2]
Chengbo Han[3]
Jie Lin[1]

[1] Department for High Ranking Officials, First Affiliated Hospital, China Medical University, Shenyang 110001, China.
[2] Pathology Department, Second Affiliated Hospital of China Medical University, Shenyang 110004, China.
[3] The Fourth Laboratory of Cancer Institute, First Affiliated Hospital of China Medical University, Shenyang 110001, China.

Correspondence to: Chengbo Han
E-mail: hancb@126.com

This work was supported by the research grants from the National Natural Science Foundation of China (No. 30371607)

OBJECTIVE To explore the relationship between mtDNA (mitochondrial DNA) and gastric cancer by comparing the difference of mtDNA copy number in gastric cancers and paracancerous tissues.

METHODS The HV1 (hypervariable region) and HV2 of the mitochondrial D-loop region from 20 cases of gastric cancer and 20 paracancerous tissues were amplified by PCR with β-actin serving as a quantitative standard marker. The products were separated by polyacrylamide gel electrophoresis (PAGE) and silver stained in order to compare the difference in mtDNA copy number between gastric cancers and paracancerous tissues. The mtDNA copy number was determined for gastric cancers having various pathological characteristics and the results compared with previous immunohistochemical staining of the tumors.

RESULTS There was a significantly quantitative difference in HV1, HV2 (standardized with β-actin) between gastric cancers and paracancerous tissues ($P<0.01$). The mtDNA copy number was associated with important enzymes in the nucleus such as AKP, cAMP-PDE and cGMP-PDE ($P<0.05$), but not associated with tumor histological type and invasive depth ($P>0.05$).

CONCLUSION The occurrence of gastric cancer was closely associated with decreased mtDNA copy number, which may be a new tumor marker.

KEYWORDS: gastric carcinoma, mitochondrial DNA, quantitative analysis, marker.

In China, gastric carcinoma is the most common malignancy of the gastrointestinal tract, and the second most frequent cause of cancer-related death in the world. Genetic alterations associated with gastric cancer have been described, such as a loss of the genes encoding E-cadherin, P53 or the transforming growth factor-β (TGF-β) receptor. Overexpression of erbB-2 or c-met also have been related to gastric cancer, but these genetic changes have only been found to be associated with a limited number of cases and the underlying mechanism of gastric carcinogenesis is still poorly understood. In recent years, mitochondrial DNA (mtDNA) has been considered to be associated with tumor-genesis. [1] mtDNA is a 16,569-bp double-stranded, closed-circular molecule, which encodes proteins participating in the synthesis of ATP by oxidative phosphorylation. [2] Compared with nuclear DNA, mtDNA is more susceptible to damage

Received May 11, 2004; accepted July 29, 2004.

by mutagens and therefore has a high mutation rate because of a lack of protection by histones, a low level of mismatch repair (MMR) activity, a high level of free radicals and reactive oxygen species (ROS) generated from oxygen in the organelles.

MATERIALS AND METHODS

Materials

A total of a 40 frozen tissues including 20 cases of gastric cancers and 20 adjacent normal gastric mucous tissues, were taken from the resected specimens of 20 patients with gastric cancer in the First Affiliated Hospital of China Medical University, Shenyang. The gastric cancers were classified according to WHO's histological classification as follows: 3 cases of papillary adenocarcinomas, 8 well-differentiated adenocarcinomas and moderately-differentiated adenocarcinomas, 3 poorly-differentiated adenocarcinomas, 2 mucinous adenocarcinomas and 2 signet-ring cell carcinomas. Using Lauren's classification there were 11 diffusive type and 9 intestinal type.

The depths of tumor invasion were as follows: 5 invasion of the muscularis propria, 6 of the subserosa and 8 serosa-exposed. All gastric cancers were subjected previously to enzyme- and immunohistochemistry to analyze the functional differentiation of gastric cancerous cells[3](see Table 1).

Table 1. Results of mtDNA quantitative analysis

Group	*n*	HV1/β-actin	*P*
Tissue type			
Normal	20	2.51 ± 0.29	<0.01
Cancer	20	0.99 ± 0.16	
Lauren's			
Diffusive	9	0.98 ± 0.19	>0.05
Intestinal	11	1.02 ± 0.25	
Invasive depth			
mp	5	0.98 ± 0.17	
ss	6	1.09 ± 0.13	>0.05
se	8	0.95 ± 0.11	
AKP			
-	9	0.83 ± 0.10	<0.05
+~+++	11	0.98 ± 0.13	
LAP			
-	14	0.90 ± 0.13	>0.05
+~+++	6	0.91 ± 0.18	
cAMP-PDE			
-	11	0.78 ± 0.10	<0.05
+~+++	9	0.97 ± 0.18	
cGMP-PDE			
-	14	0.80 ± 0.17	<0.05
+~+++	6	1.09 ± 0.20	

AKP: alkaline phosphatase LAP: L-aminopeptidase
cAMP-PDE: cyclic AMP phosphodiesterase
cGMP-PDE: cyclic GMP phosphodiesterase
mp: muscularis propria se: subserosa se: serosa-exposed

nDNA and mtDNA preparation

Gastric epithelial tissue (30 mg) was homogenized for 30 s, and then digested in 1 ml of 10 mmol/L Tris-HCl, 0.1 mol/L EDTA (pH 7.4) containing 0.1 g/L proteinase K and 5 g/L sodium dodecyl sulfate (SDS). DNA was extracted twice with an equal volume of phenol/ chloroform / isoamyl alcohol (25:24:1), then once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 1/10 volume of 3 mol/L sodium acetate (pH7.4) and 2 volumes of ethanol, and then rinsed with 70% ethanol. The precipitated DNA was recovered in 50 μl of 10 mmol/L Tris-HCl, 0.1 mmol/L EDTA (pH 8.0). The total DNA was quantified using spectrophotometry.

PCR amplification of the D-loop

Two amplified regions in the D-loop spanning HV1 (hypervariable region) and HV2 (this could avoid an occasional error and selective error resulting from a single pair of primers)were selected to predict mtDNA copy number. PCR primers were designed in conserved regions (12S rRNA and $tRNA^{pro}$) in order to avoid artificially negative results due to variations in the primer-combined region of the mitochondrial template strand. Nuclear β -actin genes were amplified simultaneously as an internal control (quantitative standard) to standardize HV1 and HV2 (i.e. mtDNA copy number). PCR primers are seen in Table 2. PCR amplification was conducted in a final volume of 50 μl containing 50 ng total DNA, 0.5 μmol/L of each primer,

2.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, and 2.5 U *Taq* DNA polymerase (TaKaRa Ex Taq™). PCR (an initial incubation at 94 ℃ for 5 min, followed by 30 cycles at 94℃ for 45 s, 60 ℃ for 45 s, and 72 ℃ for 1 min; the final step at 72 ℃ was extended to 3 min) was performed in a Biometra Personal PCR system. Concentration of the template DNA and primers added to the PCR reaction solution were kept rigidly consistent.

Table 2. Primers used for PCR amplification

Name	Primer	Sequence	Length
HV_1	F15974	5'-ACTCCACCATTAGCACCCAAA-3'	591bp
	R16564	5'-TGATGTCTTATTTAAGGGGAACGT-3'	
HV_2	F4	5'-CACAGGTCTATCACCCTATTAACCA-3'	625bp
	R628	5'-GCCCGTCTAAACATTTTCAGTG625-3'	
β-actin	F	5'- AAGGGACTTCCTGTAACAATGCA-3'	140bp
	R	5'-CTGGAACGGTGAAGGTGACA-3'	

HV: hypervariable region of D-loop region

PAGE and silver staining

Polyacrylamide gel electrophoresis (PAGE) was performed on 3% concentrated gel and 8% separated gel. The mixture (5 μl) including 3 μl PCR product and 2 μl sampling buffer solution were added into the hole in the concentrated gel. Electrophoresis was conducted at 8 mA for 20 min, then 15 mA for 30 min. Silver staining procedure: 100 ml/L ethanol fixation for 5min; 11.3 g/L nitric acid for 5 min; washed twice with distilled water for 2 min; dyed twice with 12 mmol/L silver nitrate for 10 min; washed twice with distilled water for 2 min; developed with 30 g/L sodium carbonate/0.5 ml/L formaldehyde solution for several minutes; stopped in 100 ml/L glacial acetic acid for 5 min; 30 ml/L glycerol for 5min; sealed and preserved. Scanning was conducted with a ChemiImager and analyzed with Image J software to quantify HV1, HV2 and β -actin. Values for HV1/β -actin and HV2 /β -actin were then calculated.

Statistical analysis

The statistical differences between different groups were analyzed with a t-test or One-Way ANOVA. A value of $P<0.05$ was considered significant, and $P<0.01$ highly significant. Quantitative data were expressed as the mean ± standard deviation (SD). All data were analyzed by SPSS 10.0 statistical software.

RESULTS

Differences between mtDNA copy number in gastric cancers and normal tissues.

PAGE results showed that the nuclear gene β -actin could be consistently amplified in all gastric cancers and adjacent tissues. HV1 and HV2 could also be amplified in normal tissues, while PAGE showed weak bands or even negative bands in gastric cancers (Fig. 1,2). After standardization with β -actin, there was a highly significant difference in HV1 and HV2 copy number between gastric cancers and normal tissues, $P<0.01$ (Table 1), i.e., the mtDNA copy number in gastric cancer was lower than in normal gastric tissues. HV1 and HV2 were very consistent, coefficient $r=0.91$, $P<0.01$, which showed the artificially negative probability owing to single HV1 or single HV2 was very low.

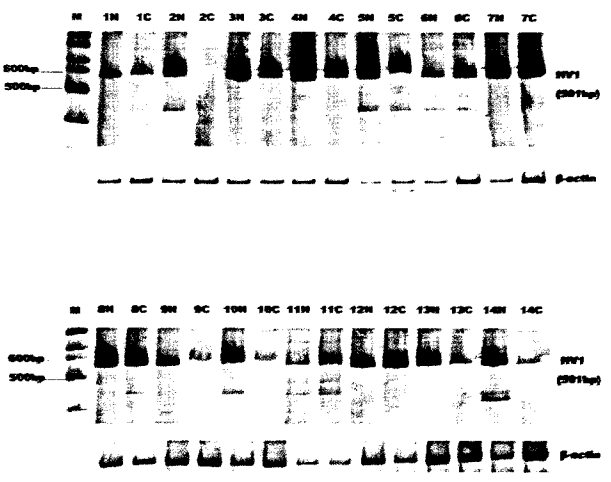

Fig.1. Results of polyacrylamide gel electrophoresis and silver staining of HV1 region amplified by PCR.

M: 100bp Ladder Marker N: normal tissues C: cancerous tissues

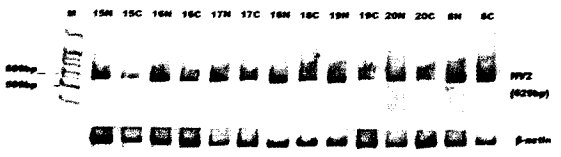

Fig.2. Results of polyacrylamide gel electrophoresis and silver staining of HV2 region amplified by PCR.

Relationships among the mtDNA copy number and pathological characteristics of gastric carcinomas

There was no statistically significant relationship between the mtDNA copy number and the WHO's classification, which may be a result of too few samples or dispersive histological types. In addition there were no statistically significant relationships based on Lauren's classification (P=0.086), or on the depth of invasion as well.

Relationships among the mtDNA copy number and AKP, LAP, cAMP-PDE and cGMP-PDE

After analysis of the gastric cancers using enzymatic and immunohistochemical methods, the results were positive for the following numbers of the tumors: 11 AKP (alkaline phosphatase) +~+++; 6 LAP (Leu-aminopeptidase) +~+++; 9 cAMP-PDE (cyclic AMP phosphodiesterase) +~+++; and 6 cGMP-PDE (cyclic GMP phosphodiesterase) +~+++. The results showed statistically significant correlations between mtDNA copy number and expression of AKP, cAMP-PDE and cGMP -PDE (P<0.05) but not with LAP (P>0.05). Negative expression of AKP, cAMP-PDE and cGMP -PDE might suggest a decreased mtDNA copy number.

DISCUSSION

The present research indicates that the biological features of tumors are not merely directed by nuclear genetic material, but also influenced by extranuclear mtDNA. mtDNA lacks protective histones, and is directly exposed to the oxygen free radicals generated by oxidative phosphorylation in the absence of glutathione to facilitate their disposal. Therefore, mtDNA is susceptible to attack by endogenous damaging factors and exogenous carcinogens. [1] In addition, the high ratio of lipid to DNA in mitochondria preferentially enhances access of lipidophilic carcinogens through the mitochondrial membrane onto mtDNA. Heretofore, mitochondrial mismatch repair (MMR) systems have been found only in yeast strains, where MSH1 is involved in mitochondrial genome repair, and MSH2 in nuclear DNA repair. No MSH1 homologue has been found in mammalian cells and therefore it is widely believed that mitochondria lack MMR a system. [4] mtDNA is a major target of carcinogens, so the degrees of injury and mutation rate of mtDNA are significantly higher than that of nuclear DNA (around 10 times).[5]

The non-coding D-loop region and hypervariable region (HV) are the linking regions of mtDNA with the mitochondrial inner membrane, and consequently are more susceptible to damage from ROS because its triple-strand structure is exposed to lipid peroxides and is highly sensitive to oxidative stress.[6] Chomyn et al.[7] suggested that mtDNA was not necessary for cell apoptosis, but could influence its velocity, because decreased mtDNA or increased damage of mtDNA could result in increased ROS.

Uncontrolled proliferation and various levels of de-differentiation are the properties of cell carcinomatous change. cPDEs are the key enzymes that degrade cAMP and cGMP, nucleotides which play important roles in cell proliferation and differentiation. The alterations of cPDE activities can directly affect the level of cAMP and cGMP, and thus influence cell proliferation and differentiation.[8,9] Our research group previously found that the cAMP level was correlated with patholobiological behavior of gastric carcinomas. Recently, studies have shown that a specific cPDE inhibitor induced cell apoptosis of chronic lymphocytic leukemic cells (CLL), in a dose-dependent manner.[10,11] Moon et al.[11] proposed that the possible mechanism of cell apoptosis induced by rolipram and forskolin, a prototypic PDE4 inhibitor and an adenylate cyclase

activator respectively, might involve the following sequence of events. Activation of protein phosphatase 2A (PP2A), causing dephosphorylation of Bad, a proapoptotic Bcl-2 family member resulting in stimulation of. Bad translocation into a voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and thereby induce mitochondrial depolarization, release of cytochrome c (cyt-c) into the cytosol, and further activate caspase-9 and caspase-3 and thus promote apoptosis. Thus it can be seen that a series of molecular changes induced by cPDE might influence mitochondrial membrane permeability (MMP) and even cell apoptosis. [12] This certainly will affect mitochondrial genetic stabilities. Various apoptosis stimulators lead directly or indirectly to increased ROS. ROS can not only directly damage mtDNA, especially the D-loop region causing diminished replicative efficiency, but also open the VDAC channel and release an apoptotic activator such as cyt-c, AIF and Smac/Diable. Moreover, some types of gastric cancers not only secrete mucus, but also express small intestinal absorptive cell marker enzymes (ACME) such as AKP and LAP that are important marker enzymes for functional differentiation of gastric carcinoma and provide the means for hydrolysis of paracancerous stroma and infiltration into adjacent normal tissues. Gastric cancers with positive expression of AKP or LAP, which lack mucus secretion belong to an absorptive function differentiation type (AFDT) of tumors that include well-differentiated papillary adenocarcinoma and tubular adenocarcinoma. In contrast, gastric cancers with negative expression of AKP and LAP and simultaneous mucus secretion belong to a mucus-secreting function differentiation type (MSFDT) of tumors that includes mucinous adenocarcinoma and undifferentiated carcinoma.[13]

Our study showed that the mtDNA copy number was larger in gastric cancers with positive AKP expression compared to gastric cancers with negative AKP expression ($P<0.05$), which suggests that mtDNA in poorly-differentiated gastric cancers was susceptible to damage, resulting in a lower mtDNA copy number. However, this hypothesis is not fully supported as no correlations were found in the copy number among different levels of histological differentiation. Maybe, the decreased copy number of mtDNA is a very early event for most tumors. The statistically significant correlation between mtDNA copy number and AKP, cAMP-PDE and cGMP-PDE expression further indicates that changes of nuclear genetic materials or signaling molecules can, to some extent, influence the mitochondrial genome, thereby providing an indication that further studies are needed to explore the relationship between mtDNA and carcinogenesis.

We now know there is a significant difference in the mtDNA copy number between gastric cancerous tissues and normal tissues, and hope that the mtDNA copy number will serve as a new tumor molecular marker. However our studies are still in a pilot-study phase, and further research is needed to establish mtDNA as a tumor marker and understand the mechanisms involved.

ACKNOWLEDGEMENTS

The authors thank Prof. Yujie Zhao , Jiaming Ma, Biocenter of China Medical University for generous assistance.

REFERENCES

1 Han CB, Li F, Xin Y. Advances in research for relationship between mitochondrial DNA and solid Tumor. Chin J Cancer. 2002; 21: 809–813.

2 Taanman JW. The mitochondrial genome: transcription, translation and replication. Biochim Biophys Acta. 1999; 1410: 103–123.

3 Xin Y , Zhao FK , Wu DY, et al. Comparative study on the functional and morphological differentiations of 104 human stomach cancer using enzyme –and mucin –histochemical methods. J Chin Med Univ. 1993; 22: 259–262.

4 Habano W, Nakamura S, Sugai T. Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: evidence for mismatch repair systems in mitochondrial genome. Oncogene. 1998; 17: 1931–1937.

5 Carew JS, Huang P. Mitochondrial defects in cancer. Mol Cancer. 2002; 1: 1–12 .

6 Bianchi NO, Bianchi MS, Richard SM. Mitochondrial genome instability in human cancers. Mutat Res. 2001; 488: 9–23.

7 Chomyn A, Attardi G. mtDNA mutations in aging and apoptosis. Biochem Biophys Res Commun. 2003; 304: 519–529.

8 Ogawa R, Streiff MB, Bugayenko A, et al. Inhibition of PDE4 phosphodiesterase activity induces growth suppression, apoptosis, glucocorticoid sensitivity, p53, and p21WAF1/CIP1 proteins in human acute lymphoblastic leukemia cells. Blood. 2002; 99: 3390–3397.

9 Sarfati M, Mateo V, Baudet S, et al. Sildenafil and vardenafil, type 5 and 6 phosphodiesterase inhibitors, induce caspase–dependent apoptosis of B –chronic lymphocytic leukemia cells. Blood. 2003; 101: 265–269.

10 Frame MJ, Tate R, Adams DR, et al. Interaction of caspase–3 with the cyclic GMP binding cyclic GMP specific phosphodiesterase (PDE5a1). Eur J Biochem. 2003; 270: 962–970.

11 Moon EY, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood. 2003; 101: 4122– 4130.

12 Gale DD, Landells LJ, Spina D, et al. Purdue Frederick. Pharmacokinetic and pharmacodynamic profile following oral administration of the phosphodiesterase (PDE) 4 inhibitor V11294A in healthy volunteers. Br J Clin Pharmacol. 2002; 54: 478–484.

13 Xin Y, Li XL, Wang YP, et al. Relationship between the phenotypes of cell –function differentiation and pathobiological behavior of gastric carcinomas. World J Gastroenterol. 2001; 7: 53–59.