RESEARCH ARTICLE

Mitochondrial Genome Microsatellite Instability and Copy Number Alteration in Lung Carcinomas

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Abstract

<u>Objective</u>: Mitochondrial DNA (mtDNA) is considered a hotspot of mutations in various tumors. However, the relationship between microsatellite instability (MSI) and mtDNA copy number alterations in lung cancer has yet to be fully clarifieds. In the current study, we investigated the copy number and MSI of mitochondrial genome in lung carcinomas, as well as their significance for cancer development. <u>Methods</u>: The copy number and MSI of mtDNA in 37 matched lung carcinoma/adjacent histological normal lung tissue samples were examined by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) assays for sequence variation, followed by sequence analysis and fluorogenic 5'-nuclease real-time PCR. Student's t test and linear regression analyses were employed to analyze the association between mtDNA copy number alterations and mitochondrial MSI (mtMSI). <u>Results</u>: The mean copy number of mtDNA in lung carcinoma tissue samples was significantly lower than that of the adjacent histologically normal lung tissue samples (p < 0.001). mtMSI was detected in 32.4% (12/37) of lung carcinoma samples. The average copy number of mtDNA in lung carcinoma samples containing mtMSI was significantly lower than that in the other lung carcinoma samples (P < 0.05). <u>Conclusions</u>: Results suggest that mtMSI may be an early and important event in the progression of lung carcinogenesis, particularly in association with variation in mtDNA copy number.

Keywords: MtDNA - lung cancer - MSI - copy number - mutation

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Introduction

Mitochondria have long been suspected to be involved in carcinogenesis. Alterations in the oxidative phosphorylation of tumorigenic cells may lead to tumor formation or visible clinical symptoms, and can increase the malignant potential of the tumor (Augenlicht and Heerdt, 2001). Studies on the relationships between mitochondria and certain kinds of cancers can be traced back to 70 years ago. These studies have mainly concentrated on the form and energy metabolism of mitochondria in tumor cells. The involvement of the mitochondrion in cancer cell metabolism, function, and therapeutic potential is well documented and recognized. These intracellular organelles have been described by cytologists in as early as the mid-19th century. However, the role of the mitochondria in oxidative energy metabolism was established in detail only in the mid-20th century. One of the major fields in cancer research in relation to mitochondrial function was established in the first quarter of the 20th century by Otto Warburg (1956) and several other investigators. Since then, the "Warburg effect" has been used to refer to the significance of cellular energy metabolism in the pathophysiology of cancer cells.

central role in cellular metabolism. The primary metabolic function of mitochondria is oxidative phosphorylation, an energy-generating process that couples the oxidation of respiratory substrates to the synthesis of ATP (Modica-Napolitano and Singh, 2004). Mitochondrial DNA (mtDNA) results in a high mutation rate because of the lack of introns and protective histones, inefficient DNA repair systems, and continuous exposure to the mutagenic effects of oxygen radicals generated by oxidative phosphorylation (Lightowlers et al., 1997). Scientific knowledge about mitochondria, which play a central role in apoptosis (Tan et al., 2013) and cancer-inducing biological processes, has continued to expand in recent years. Thus, mtDNA mutations are fast becoming hotspots of cancer research. Mitochondria are involved in apoptosis and tumorigenesis, and as such, researchers have begun to examine the potential roles of mtDNA alterations in the development and maintenance of cancers (Purdue et al., 2012). The mtDNA mutations are seen in various forms of cancer and tumor cell lines (Guerra et al., 2012; Guo et al., 2013). Deletions, point mutations, insertions, duplications, and microsatellite instability (MSI) have been detected throughout the genome, and certain mtDNA mutations are associated with specific cancers.

Mitochondria are dynamic organelles that play a

Mitochondrial MSI (mtMSI) and changes in

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Target	Primer and probe name	Sequence(5'-3')	Length of PCR product(bp)
β-globin	Probe	CTCCTGAGGAGAAGTCTGCT	110
	Forward primer	ACACAACTGTGTTCACTAGC	
	Reverse primer	CAACTTCATCCACGTTCACC	
HV1	Probe	CTCCCCATGCTTACAAGCAAGTACAGCAAT	128
	Forward primer	TTGCACGGTACCATAAATACTTGAC	
	Reverse primer	GAGTTGCAGTTGATGTGTGATAGTTG	

Table 1. Primer and TagMan Probe Sequence and Length of PCR Pro

Table 2. Primer Sequences	Used in mtDNA	Amplification
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Repeated sequence	Starting position(nt)	Mt region(nt)	Primer sequence(5'-3')
(PolyC)n	303	D-loop(1) 267-423	TCC ACA CAG ACA TCA TAA CA AAA GTG CAT ACC GCC AAA AG
(CA)n	514	D-loop(2) 453-637	CCT CCC ACT CCC ATA CTA CTA A GTG ATG TGA GCC CGT CTA AAC A
(PolyC)n	16184	D-loop(3) 16112-16379	CAC ACT GAA TAT TGC ACG CAA GGG ACC CCT ATC TGA GG

mtDNA copy numbers in various cancers (e.g., ovarian, endometrial, esophagial and colorectal cancers) have also been analyzed in previous works (Sun and Fu, 2006; Lin et al., 2012a, b; Wang and Shen, 2012). According to these studies, mitochondrial and nuclear MSI showed no significant associations, suggesting that different systems are responsible for mitochondrial and nuclear genetic instability in tumor cells. Are there variations in the mtDNA copy numbers in primary lung cancer? Is there a relationship between mtMSI and changes in mtDNA copy numbers? These questions aroused our interest. The content and MSI of mtDNA in 37 matched lung carcinoma/adjacent histologically normal lung tissue samples from patients were analyzed by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) assay, followed by sequencing and fluorogenic 5'-nuclease real-time PCR technique. Student's t test and linear regression analysis were employed to analyze the association between mtDNA copy number alterations and mtMSI.

Materials and Methods

Case materials

Surgical samples from 37 patients with primary lung cancer and 37 adjacent histologically normal lung tissue samples were obtained from the Xingiao Hospital of the Third Military Medical University from 2003 to 2004. This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the Third Military Medical University. Written informed consent was obtained from all participants. None of the patients recruited for the study had a known history of industrial or occupational exposure to asbestos or organic solvents. All the tissues were kept in liquid nitrogen immediately after surgical resection, in accordance with standard protocol. Smoking habits were divided into three categories: (1) current smokers, consisting of 15 subjects who had smoked more than one cigarette or part thereof daily for more than 1 year; (2) ex-smokers, consisting of 9 subjects who had been previously reported as smokers and had quit smoking for more than 1 year; and (3) 13 nonsmokers, consisting of subjects who had never

smoked. Median age was 55 years old (range: 30-75). The surgical lung cancer specimens included squamous cell carcinoma (n = 23) and adenocarcinoma (n = 14). The institutional regional review board (IRB) approved the research proposal.

Extracting the histological genomic DNA

Phenol, chloroform, and isoamyl alcohol were used to extract genomic DNA samples (including the mtDNA). The purified DNA was eluted in 50 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). An ultraviolet spectrophotometer was used to measure the absorbance of the samples at 260 nm (A 260 nm), and the absorbance values were used to calculate DNA concentration. The extracts were stored at -20 °C.

PCR primers and TaqMan probe

All the primers were synthesized and marked by Shanghai Gene Core BioTechnologies Co., Ltd. The fluorescent detecting materials used in this experiment were FAM (6-Carboxy-fluorescein) and TAMRA (6-Carboxy-tetramethy- rhodamine). In particular, FAM was used as R reporter (marked with TaqMan probe at the 5'end), and TAMRA was used as Q quencher (marked with TaqMan probe at the 3'end). The primer pair was designed in order to flank the region where the TaqMan probe and the desired gene were located. The sequences of the primers for the amplification of mtDNA HV1 (Hipervariable loop-1, HV1), β -globin nuclear gene and TaqMan probe are given in Table 1. The primer sequences for mtMSI and microsatellite loci analyses are shown in Table 2 (Bendall et al., 1995; Maximo et al., 2001).

Preparation of the RT-PCR standard

A 25 μ L PCR was prepared containing the following components: 2 U Taq enzyme (Takara Bio Inc. Otsu, Shiga, Japan), 2.5 mmol/L MgCl₂, 200 μ mol/L dNTP, 0.5 μ mol/L of forward and reverse primers, and about 100 ng of DNA template. PCR was performed using a thermal cycler with the following cycling profile: 35 cycles of denaturation at 94°C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 40 s. Final extension was done at 72 °C for 10 min.

After the purification of PCR samples using the TaKaRa purification kit, the 128 bp HV1 and 110 bp β -globin amplification products were cloned into the PMD18-T carrier cloning vector (Takara Bio Inc., Otsu, Shiga, Japan). Segments containing the facility for screening the carrier were also inserted, and the resulting plasmid constructs were transformed into JM109 cells. Plasmid extraction was then performed using the Qiagen plasmid extraction kit (QIAGEN Inc., Shanghai China). The partly extracted plasmids were evaluated by PCR and sequencing, with the sequencing reaction conducted by the Shanghai GeneCore BioTechnologies Co., Ltd. An ultraviolet spectrophotometer was used to detect the OD260 value. Plasmid concentration was calculated using the following formula: plasmid concentration $(ng/\mu l) =$ $OD260 \times 50 \ \mu g/ml \times dilution \ (ml) \times 1000 \ / \ the \ original$ solution volume (µl). Copy number was calculated using the following equation: copy number (Copies/ μ l) = plasmid concentration $(ng/\mu l) \times 6.02 \times 1014/660 \times bases$ (insert segments and the carrier). Dilution gradients of HV1 and β -globin plasmids were prepared (>10 fold with sterile deionized water) in order to obtain a quantitative PCR standard curve with a template concentration ranging from 103 copy/ μ l to 107 copy/ μ l.

RT-PCR

The prepared 25 μ L RT-PCR reaction contained the following components: ABI TaqMan 1x PCR Master mix, 3.5 mmol/L MgCl₂, 200 μ mol/L dNTP, 0.5 μ mol/L of forward and reverse primers, 50 nmol/L fluorescent probes, and 2.0 μ l of template. Three sets of no template control (NTC) were used for each of the detections. Three parallel experiments were performed for each of the samples.

The RT-PCR cycling profile used was as follows: 50 °C for 2 min, 95 °C for 10 min; 50 cycles of 95 °C for 15 s, 60 °C for 30 s. The TaqMan kits, optical PCR Core PCR, and 7700 type PCR used in this study came from PE Company (PerkinElmer Inc., Massachusetts USA).

Different concentration gradients of the standard and test samples were used to conduct quantitative PCR amplification and to generate the standard curve. Separate fluorescence real-time PCR were conducted to measure the respective quantities of HV1 in the mitochondria and the nuclear β -globin gene in the test sample. The HV1 in mtDNA and the single copy nuclear β -globin were used as markers of copy numbers in the mitochondria and nuclear DNA, respectively. The relative copy numbers of mtDNA/ diploid nuclear genome were calculated to be equivalent to the value of 2 × nuclear HV1/ β -globin (Miller et al., 2003).

PCR- SSCP analysis

PCR was used to amplify the mtDNA microsatellite sequences (Table 2). Three control sequences from microsatellite reference materials of Maximo and Bendall, by Shanghai GeneCore BioTechnologies Co., Ltd., were used. The primer sequences are given in the Table 2. PCR reactions were carried out using the TaKaRa PCR kit (Takara Bio Inc., Otsu, Shiga, Japan) in a 50 mL reaction volume with 200 ng DNA template, 2 U TaKaRa Taq DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan), 2.5 mmol/L MgCl₂, 250 mmol/L of each dNTP, and 0.5 mmol/L of each primer. After denaturation at 94 °C for 30 s, the reaction mixture was cycled 30 times at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min, and finally extended at 72 °C for 10 min.

About 5 μ L was taken from the amplification product, to which an equal volume of buffer was added; the mixture was then denatured by incubation at 90 °C for 5 mins followed by rapid freezing in crushed ice. The PCR products were analyzed by 7.5% agarose gel electrophoresis at 50 V (using 1 × TAE tank buffer) for 3 hours. The electrophoresed gels were observed under ultraviolet light and were photographed for documentation. All experiments were repeated at least twice to rule out any artifacts.

Sequencing

The PCR-SSCP products from both lung cancer and normal lung tissue samples were subjected to non-denaturing polyacrylamide gel electrophoresis, after which the generated bands were recovered and reamplified by PCR using the original primers. The resulting amplicons were then purified and used for plasmid cloning sequence analysis. All the samples have undergone DNA sequencing. Plasmid cloning and sequencing were conducted by Shanghai GeneCore BioTechnologies Co., Ltd.

Statistical analysis

The statistical analysis of experimental values was done using student's t test and linear regression analysis, using the SPSS 15.0 software for Windows. A statistically significant difference was accepted at a p value of < 0.05, similar to other medical studies.

Results

Cloning plasmid appraisal

The screening plasmid was extracted in order to generate a standard curve for the template, which was determined through PCR and sequencing methods. Realtime PCR results showed strong fluorescent signals, indicating that the HV1 and PCR fragments of the β -globin gene were successfully inserted into the PMD18-T carrier plasmid.

Preparation of the standard curve

The standard curve (Figure 1) was generated using different concentrations of fluorescent signals to set



Figure 1. Standard Curves of Real-time PCR for Mitochondrial DNA HV1 (A) and Nuclearβ-globin Gene (B)



MtDNA copy number in non-tumour tissue

Figure 2. MtDNA Copy Number in Lung Carcinoma and Adjacent Normal Samples



Figure 3. Sequence Electropherogram of a Part of the mtDNAD-loop Showing a Comparison of Normal DNA00.0 nd D16184). Statistics showed that three microsatellite (above) and Tumor DNA (below). mtMSI in the C-stretch, 13 cytosine at nt16,184-nt16,193 in the normal DNA, but only 11 in the tumor DNA (primary tumor)

the threshold number (threshold cycle, CT). The values obtained from the gradient concentrations of the standard template and CT value showed a linear correlation.50. Correlation Quantitative PCR analyses of the standard curves for HV1 and the β -globin genes showed related coefficient values were 0.998 and 0.999, respectively, as well as slopes of 25.0^{cases} 998, 3.159, and 3.400.

Changes in the copy number of lung mtDNA

We aimed to understand the mitochondrial DNA copy number changes in lung cancer tissue. Thus, we performed two separate real-time quantitative PCR analyses of lung cancer tissue, fluorescence, and corresponding lung cancer samples of the mtDNA to precisely quantify their copy numbers (copy number/cell). Linear regression analysis showed that the number of copies of lung tissue mtDNA and the corresponding normal lung tissue mtDNA copy numbers have a direct correlation (r = 0.589, p < 0.01) (Figure 2). The average copy number of mtDNA in lung cancer tissue/cell was 395±125, and the corresponding average copy number for normal lung tissue was $733 \pm$ 196, wherein the former was significantly lower than the latter (p < 0.001).

According to gender, age, smoking habit, and pathological type, we took 37 cases of samples from each group. The results showed that the copy numbers of mtDNA in lung cancer patients did not change with gender, age, smoking, and cancer pathological types (p > p)0.05). Results are shown in Table 3.

mtMSI testing results

Through single conformation polymorphism analysis and sequencing, 12 out of 37 (32.4%) cases of lung cancer samples in this study were found to contain mtMSI. A total

Table 3. MtDNA Copy Number Per Nucleus in **Relation to Clinicopathological Variables and mtMSI** in Lung Cancer

]	No. of Cases	MtDNA copy number	P value
Sex			0.501
Females	9	419±82	
Males	28	396±123	
Age			0.306
≥55	19	429±129	
<55	18	380±91	
Histological type			0.948
Squamous cell cancer	23	388±111	
Adenocarcinoma	14	427±119	
Smoking status			0.685
Smoker	24	393±114	
Nonsmoker	13	419±116	
mtMSI			0.017
Positive	12	334±40	
Negative	25	434±124	

of 11 patients had instability in only one region (29.7%), while 1 patient (2.7%) had instability in two regions (D310



Mitochendrial genomes are short cire ilar molecules that, with the exception of viguses, represent the most economiesally packed forms of DNA in the whole biosphere The mit chondrial genome in human cells is extremely small (16,569) compared with the nuclear DNA, although every mitochondrion contains between Eto 20 copies of mEDNA. The copy number of mitochondrial geomes per cell ranges from several hundreds for more than 10,000 depending on the cell type (typizally around 1000 mitochondria per cell). The identification of increased or reduced mtDNA content has been reported in cancer cells; this area, along with the regulation mechanism of mtDNA copy number in cells, has been extensively reviewed in recent years (Clay Montier et al., 2009).

Studies in recent years have focused on basic mitochondrial genetics because of its high incidence and broad distribution. mtDNA copy numbers for different types of cells are not identical. Moreover, in cell differentiation, hormone therapy and training process, mtDNA copy number can also change significantly. Southern cross and organizational comparison using PCR detection are two of the most commonly used methods



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to determine the mtDNA copy number. However, these methods are qualitative or half quantitative in nature. Matsuyama et al. (2005) used hybrid technology to compare the mtDNA contents of various organs in mice. Their results showed that the mtDNA contents of the tested organs (15 month-old mice), from low to high, are as follows: bone marrow, spleen, lung, liver, brain, kidney, skeletal and heart. In the current study, we made two separate real-time quantitative fluorescent PCR to measure the mtDNA copy number in adjacent histologically normal lung tissue samples (copy number/cell). In this method, the amount of nuclear DNA was chosen as the internal reference for mtDNA copy numbers. The β -globin gene is known to be a single copy nuclear gene, making it a good diploid nuclear genome content marker for this kind of experiment. Through this method, we found that the average copy number/cell of mtDNA in normal lung tissue samples is 733±196, which is relatively lower than the lung tissue mtDNA copy number average data obtained by fluorescent real-time quantitative PCR. Given that there is almost no quantitative research done on mtDNA copy number in lung tissue cells, comparisons with other works cannot be made. The said value, however, is slightly higher than the 409±148 of peripheral monocytes, but lower than the 1811±546 of skeletal muscle and 6970±920 of myocardial cells (copy number/cells). Meanwhile, the mtDNA copy number in different types of cells may depend on its energy metabolism and aerobic ATP production needs. Thus, our results are consistent with the fact that the energy metabolism and aerobic ATP production of lung cells is lower than that of the skeletal and myocardial cells.

Many solid tumors have reduced mtDNA copy numbers that are often associated with reduced protein levels during oxidative phosphorylation (Simonnet et al., 2002; Isidoro et al., 2004), and correlated with clinico-pathologic parameters and tumor aggressiveness in various cancer types (Yamada et al., 2006; Xing et al., 2008). It is possible that a deficit in the mitochondrial biogenesis of cancer cells can lead to a decrease in mtDNA copy number. In human lung carcinoma A549 cells, the mitochondrial stress induced by the decreased mtDNA copy number induces phenotypic changes, tumor progression, and invasive behavior (Amuthan et al., 2002). These findings concerning mtDNA alterations in cancer cells, as well as their correlations to malignant phenotypes of cancers, provide important evidence to support the hypothesis proposed by Warburg, which states that there are impairments in the mitochondrial oxidative phosphorylation (OXPHOS) and respiratory functions in cancer cells. These findings also strongly indicate that a decreased mtDNA copy number, coupled with reduced contents of OXPHOS complexes, contribute to the abnormal bioenergetics of many cancer cells, which is characterized by an increase in glycolytic activity accompanied by impaired respiration. In the current study, linear regression analysis showed that the copy numbers of lung cancer tissue mtDNA and the corresponding normal lung tissue mtDNA copy numbers have a direct correlation, with the average copy number of mtDNA in lung cancer tissue being significantly lower than that of the

corresponding normal lung tissue. However, correlations were not observed between the change in copy number, on the one hand, and gender, age, smoking habit, or other pathologic cancer types on the other hand.

The mechanism as to how cancer cells obtain a decreased mtDNA copy number still remains unclear. Considering that the D-loop region is a major control site for mtDNA replication and transcription, and that the np 303-309 poly C tract is most sensitive to damage and inefficient repair compared with other regions of mtDNA (Mambo et al., 2003), the high frequency of somatic mutations in mtDNA D-loop may thus lead to a decrease in mtDNA copy number in many cancer types. Except for the D-loop zone, there are few non-coding bases between neighboring genes. Thus, the segment 1,120 bp between position 16,024 and position 575 of the D-loop is often called the controlled zone, which is responsible for regulating the replication and transcription of the whole mtDNA molecule (Wang et al., 2007). The D-loop region of mtDNA, where the heavy chain replication origin and the binding place of several transcription factors are located, is highly polymorphic and contains hotspots for genetic instability in various tumor types. The mtDNA mutation rate is 10- to 100-fold higher than that of the nuclear DNA, and this can be attributed to the high concentration of reactive oxygen species in the mitochondrial inner membrane, fewer repair mechanisms, and the absence of mtDNA-coating proteins, such as histones, in the nucleus. In the past few years, mtDNA mutations have been demonstrated in various types of human cancer (Penta et al., 2001; Modica-Napolitano and Singh, 2004; Guerra et al., 2011; Guo et al., 2013). These mtDNA mutations detected in cancer cells included somatic point mutations, deletions, insertions, depletions and mtMSI. Most of the somatic mutations found in mtDNA of cancer cells are homoplasmic, and the d D-loop region is a hotspot for mutations in the mtDNA of cancer cells (Peng et al., 2011).

mtMSI refers to the change in the length of short base-repetitive sequences in the mtDNA (Bianchi et al., 2001). Some researchers have analyzed mtMSI in various cancers, including breast, ovarian, endometrium, and colorectal cancers (Jeong et al., 2010). Although studies about the mitochondrial mutation in the aforementioned cancer types have identified the role of mitochondrial genetic instability, reports on mtMSI in lung cancer are rare. MSI is regarded as one of the phenotypes of defective DNA mismatch repair system and, consequently, as a marker of high risk for cancer. The present study showed that most of the mtMSI in cancer were commonly detected in the D-loop region. For this particular purpose, we chose three microsatellite loci at the D-loop of mtDNA, namely, D303, D514 and D16184, to be analyzed for MSI in lung cancer. Out of the 37 cases of lung cancer samples, 12 were detected to contain mtMSI (32.4%). This value is lower than the reported 66% in colon cancer and 42.5% in breast cancer (Wang et al., 2006), whereas it is higher than the 21.2% in liver cancer, 16% in gastric cancer, and 2.6% in kidney cancer. This suggests that the D-loop is a hotspot for alterations in lung tumor. Previous studies have also suggested that MSI at the non-coding D-loop

Ji-Gang Dai

region could alter the rate of mtDNA replication, thereby disrupting mitochondrion-induced apoptosis (Yin et al., 2004; Mambo et al., 2005). In addition, MSI at the mtDNA D-loop is correlated with less differentiated hepatocellular carcinoma (HCC), late-stage progression and poor prognosis of non-small cell lung cancer (Matsuyama et al., 2003), as well as poor prognosis in colorectal cancer (Lièvre et al., 2005) and in breast cancer (Tseng et al., 2006).

Cancer cells with mtMSI and reduced mtDNA copy number are also associated with tumor aggressiveness in renal cell carcinoma, with less differentiation in gastric carcinoma, and with tumor size and cirrhosis of HCC. These results suggest that mitochondrial genome instability and reduced mtDNA copy number may play an important role in the initiation and progression of human cancers. In a study on HCC, Lee et al. (2004) found that the copy numbers of mtDNA have significant correlations with point mutations near the replication origin of the H-strand of mtDNA. In the current study, we also observed that the average copy number of mtDNA in 12 lung carcinoma samples containing mtMSI was significantly lower than the other 25 lung carcinoma samples. The decrease in mtDNA copy number was highly associated with the occurrence of point mutations near the replication origin of the H-strand of mtDNA. Therefore, it is highly possible that the D-loop mutations affect the binding activity of mitochondrial transcription factor A (TFAM), thereby resulting in reduced replication and transcription, as well as a reduction in mtDNA copy number in cancer cells. Therefore, these results suggest that mtMSI may be an early and important event in the progression of lung carcinogenesis, especially in the changes in the mtDNA copy number.

In summary, we have been unable to demonstrate the significant correlations between any alteration in the mtDNA copy number and the mtMSI primarily due to the limited sample size in this study. Nevertheless, the relatively high frequency of mtMSI regions support the hypothesis that changes in the D-loop mononucleotide repeat may be used as a new tool for cancer detection (Shidara et al., 2005). In this regard, the role of mtMSI in pre-malignant lung lesions, which can serve as a surrogate marker of the risk of lung cancer development, requires further investigation.

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