

Sequence polymorphisms of the mitochondrial displacement loop and outcome of non-small cell lung cancer

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Abstract. Accumulation of single-nucleotide polymorphisms (SNPs) in the displacement loop (D-loop) of mitochondrial DNA (mtDNA) may be associated with disease outcome. Our team investigated the prediction power of D-loop SNPs in non-small cell lung cancer (NSCLC) outcome. In an overall multivariate analysis, allele 16390 was identified as an independent predictor for NSCLC outcome. The length of survival of patients with allele 16390A was significantly shorter than that of patients with allele 16390G (relative risk, 0.323; 95% CI, 0.109-0.951; $p=0.040$). The analysis of genetic polymorphisms in the mitochondrial D-loop can help identify NSCLC patient subgroups at a high risk for a poor disease outcome.

Introduction

Lung cancer is the most frequent cancer and one of the leading causes of cancer-related deaths worldwide, accounting for 30% of all cancer-related deaths. An epidemiological study estimated that the number of deaths due to lung cancer in 2010 was 1.5 million, rendering lung cancer a major public health challenge (1,2). The annual mortality rate of lung cancer in China is high with approximately 400 thousand deaths (3). Non-small cell lung cancer (NSCLC) accounts for 89% of all lung cancers and approximately one third of NSCLC patients are diagnosed at a locally advanced stage (4,5). Despite aggressive treatment, the prognosis of NSCLC patients is still poor with a 5-year survival rate of approximately 10% and a median survival time of 16-18 months (6,7). Many clinical factors, such as tumor stage, metastasis, gender and weight loss, are predictors of prognosis of NSCLC patients (8), but there are few studies concerning the relationship between oxidative markers and NSCLC prognosis (9).

Lung cancer carcinogenesis is associated with increased oxidative stress which results in DNA damage (10,11). The human mitochondrial genome is a 16-kb closed-circular duplex molecule that contains 37 genes, including two ribosomal RNAs and a complete set of 22 tRNAs (12). Mitochondrial DNA (mtDNA) is believed to be more susceptible to DNA damage and acquires mutations at a higher rate than nuclear DNA because of high levels of reactive oxygen species (ROS), lack of protective histones and the limited capacity for DNA repair in mitochondria (13-15). Somatic mtDNA mutations and polymorphisms are associated with a wide variety of degenerative diseases and cancers (16,17), and can be homoplasmic by clonal expansion (18,19), or heteroplasmic in tumor tissues (20,21). In many cancers, somatic mutations and polymorphisms are located in an mtDNA non-coding region called the displacement loop (D-loop) (22,23), which contains 1122 bp (nucleotides 16024-16569 and 1-576; www.mitomap.org). This region is important for the regulation of both replication and expression of the mitochondrial genome as it contains the leading-strand origin of replication and the main promoter for transcription (24).

Sequence changes have been examined extensively in the D-loop in cancers, but few single-nucleotide polymorphisms (SNPs) have been selected for predicting cancer risk and outcome; their predictive values are still unclear (25-29). In this study, we assessed the prediction power of these SNPs on the outcome of NSCLC patients.

Materials and methods

Tissue specimens and DNA extraction. Blood samples were collected at The Fourth Hospital of Hebei University from NSCLC patients who received treatment at the Department of Respiratory Medicine between 2001 and 2009. The genomic DNA was immediately extracted using the Wizard Genomic DNA extraction kit (Promega, Madison, WI, USA) and stored at -20°C. All procedures were supervised and approved by the Human Tissue Research Committee of our hospital, and an informed consent was obtained from all participants.

PCR amplification and sequence analysis. The forward primer, 5'-CCCCATGCTTACAAGCAAGT-3' (nucleotide 16190-16209) and reverse, 5'-GCTTTGAGGAGGTAAGC TAC-3' (nucleotide 602-583) were used for amplification of a

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Table I. Univariate analysis of clinical characteristics associated with overall survival in the NSCLC patients.

Characteristics	No. of cases	2-year survival rate (%)	p-value
Gender			0.382
Male	48	77.1	
Female	28	67.9	
Age (years)			0.715
≤45	7	28.6	
>45	69	26.1	
TNM classification			0.520
III	20	25.0	
IV	56	26.8	
Smoking			0.253
Yes	36	19.4	
No	40	32.5	
Treatment ^a			0.000
Yes	67	29.9	
No	9	0	
Histology			0.669
SQ	25	28.0	
AC	51	25.5	

NSCLC, non-small cell lung cancer. ^aTreatment includes chemotherapy, radiotherapy, and chemotherapy plus radiotherapy. SQ, squamous cell carcinoma; AC, adenocarcinoma.

982-bp product from the mtDNA D-loop region. PCR was performed according to the protocol included in the PCR Master Mix kit (Promega) and purified prior to sequencing. Cycle sequencing was carried out with the Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and the products were then separated on the ABI PRISM Genetic Analyzer 3100 (Applied Biosystems). Polymorphisms were confirmed by repeated analyses from both strands.

Statistical analysis. Survival curves were calculated using the Kaplan-Meier method, and compared with the log-rank test. Multivariate survival analysis was performed using a Cox proportional hazards model. All of the statistical analysis was carried out with the SPSS 13.0 software package (SPSS Co., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

A total of 80 patients were enrolled in this study and a follow-up review was conducted every 3 months for 2 years. One patient was lost to follow-up at the first year and 3 patients were lost at the second year. The remaining 76 patients shared the same performance status (ECOG score, 0). Of these, 20 patients were at stage III, 56 at stage IV and 56 died during follow-up. The data collected during the 2-year follow-up were

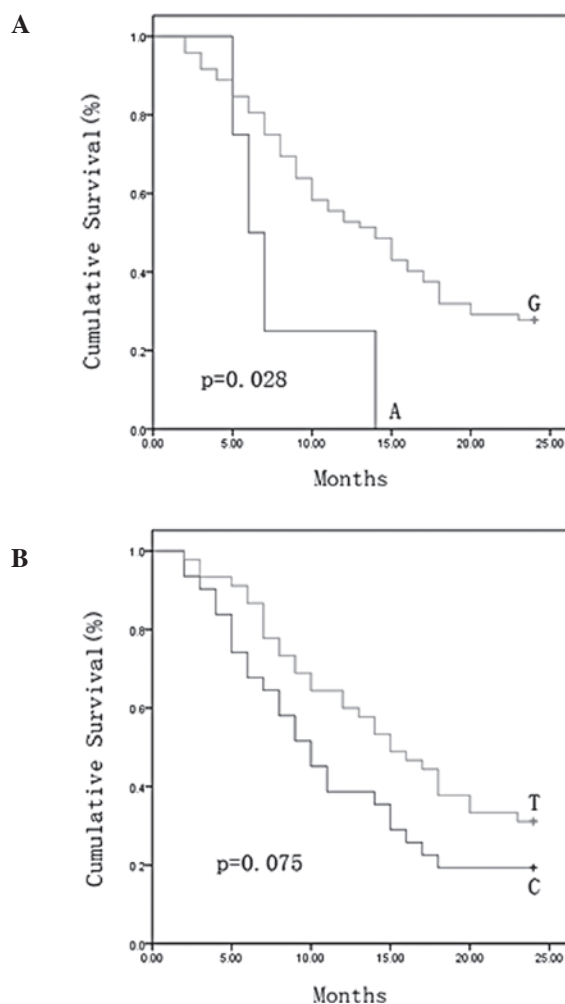


Figure 1. Survival curves according to the nucleotide at position (A) 16390 and (B) 16519 in the displacement loop (D-loop) of NSCLC patients.

analyzed for clinical characteristics using the Kaplan-Meier method and were compared by the log-rank test. Gender, age, TNM classification, smoking and histology were not statistically significant predictors of the length of overall survival, however treatment was correlated with survival in these patients (Table I).

We sequenced the D-loop region in all of the 76 NSCLC patients and 121 SNPs were identified. The relationship between survival and the 121 SNPs was examined. The NSCLC patients were divided into two groups on the basis of their genotype at each SNP site, and overall survival curve was plotted using the Kaplan-Meier method for all NSCLC patients at these sites. A dramatic difference in survival rate was found for nucleotide 16390 ($p=0.028$) and another nucleotide of 16519 was also identified with borderline level of difference ($p=0.075$). The minor allele 16390A and 16519C were associated with a shorter length of survival (Fig. 1A and B). We performed multivariate analysis for these predictors including these two SNPs and treatment with the Cox proportional hazards model. As shown in Table II, the 16390 alleles and treatment were identified as independent predictors for NSCLC outcome. The length of survival for patients with the minor allele 16390A genotype was significantly less than that for patients with the common allele 16390G (relative risk, 0.323;

Table II. Multivariate analysis of prognostic factors associated with overall survival in NSCLC patients with Cox proportional hazards model.

Factors	Relative risk	95% CI	p-value
Treatment	0.143	0.065-0.315	0.000
Nucleotide			
16390	0.323	0.109-0.951	0.040
16519	0.628	0.361-1.093	0.100

NSCLC, non-small cell lung cancer.

95% CI, 0.109-0.951; $p=0.040$) at the 16390 site. These data demonstrated the strong prediction power of nucleotide 16390 on outcome for NSCLC patients.

Discussion

We previously identified cancer risk and outcome-associated SNPs of the D-loop in several types of cancer (25,26,30). In this study, selected SNPs in the mtDNA D-loop were examined for their ability to predict cancer outcome in NSCLC patients. Two SNPs, 16390 (G/A) and 16519 (T/C), were identified by the log-rank test for their association with overall survival. Multivariate survival analysis identified 16390 (G/A) as independent prediction markers for NSCLC outcome.

The mtDNA D-loop is important for regulation of mitochondrial genome replication and expression. SNPs in this region may affect mtDNA replication so as to alter the electron transport chain, which is responsible for the high ROS release and nuclear genome damage as well as cancer initiation and promotion (31-33). Overexpression of protein binding to the D-loop region has been proven to increase ROS and affect tumor progression (34).

In conclusion, SNPs in the mtDNA D-loop were found to be independent prognostic markers for NSCLC outcome. The analysis of genetic polymorphisms in the D-loop may help to identify patient subgroups at a high risk for poor disease outcome, thereby helping to refine therapeutic decisions in NSCLC.

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