Molecular analysis of *p53* and K-*ras* in lung carcinomas of coal miners

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Abstract. Thirty-three cases of non-small cell lung cancers (NSCLC) from the archives of National Coal Workers' Autopsy Study were studied for mutational alterations in *p53* and K-*ras* using PCR-SSCP, DNA sequencing and PCR-oligonucleotide probe hybridization techniques. Mutations of the *p53* were observed in 4 smokers (19%) and one in a never smoker (8%). Two polymorphisms in smokers were detected at codon 213, a common site for sequence variation. Among the smokers the *p53* mutations were in the heavy smokers. In never smokers there was only a single *p53* mutation and two K-*ras* mutations. In never smokers the frequency of K-*ras* mutations was similar (17%) in smokers, but one never smoker had two K-*ras* mutations. Mutations of *p53* were more frequent in adenocarcinomas (27%) and they were AT→GC transitions. Four of 11 (36%) adenocarcinomas were found to have K-*ras* codon 12 mutations, and one adenocarcinoma had two K-*ras* mutations. There were two large cell undifferentiated carcinomas with *p53* mutation and one with a K-*ras* mutation. Two of the 16 squamous cell carcinomas were positive for *p53* mutation, while no K-*ras* mutations were found in this group. The results of these preliminary studies indicate a moderately different mutational spectrum of *p53* and K-*ras* in coal miners independent of cigarette smoking. The mutational spectrum observed in this study of coal miners with heavy cigarette smoking history suggest a protective effect of coal mine dust in preventing abnormal mutations induced by chemical carcinogens in cigarette smoke or reactive oxygen species. These limited preliminary studies provide insight into the possibility of accurately measuring changes in etiologic markers to unravel the uncertainties of cancer in coal miners.

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Abbreviations: CWP, coal workers' pneumoconiosis; NCWAS, National Coal Workers' Autopsy Study; DNA, deoxyribonucleic acid; TMAC, tetramethyl ammonium chloride; EDTA, ethylene diamine tetraacetate; PCR, polymerase chain reaction; PCR-SSCP, polymerase chain reaction-single strand confirmation polymorphism

Key words: lung cancer, coal workers' pneumoconiosis, *p53* and K-*ras* genes, cigarette smoking, mutational spectrum, etiologic markers

Introduction

Lung cancer is the most common malignancy in the United States and is the leading cause of cancer related death in both men and women (1). Lung cancer is ranked second only to bladder cancer in the proportion of cases thought to be due to occupational exposures (2). The contribution of occupational and environmental factors to the development of lung cancer is well recognized (3). Inhalation of certain non-toxic dusts has been implicated in lung carcinogenesis in animals and humans through the transportation and co-deposition of known carcinogens to the respiratory mucosa (4,5). Polycyclic aromatic hydrocarbons are commonly encountered carcinogens that when activated have been shown to induce G→T transversions in experimental studies (6). Incontrovertible positive evidence exists for the carcinogenicity of cigarette smoke, bis(chloromethyl) ether, certain dyes, asbestos, high concentrations of radon decay products, or any combination of these agents (3,6,7).

Epidemiologic and pathologic studies indicate that lung carcinoma occurs less frequently in coal miners than in general populations with comparable cigarette smoke exposure (8,9). However, there are a few reports in the literature that show an increased risk for lung cancer in coal miners (10-12). This discrepancy may be attributed to inadequate control of contributing factors such as cigarette smoking, radon, and urban air pollutants. Because the overwhelming risk factor for lung cancer is cigarette smoke and human data based on *p53* mutations associated with tobacco smoking is available, we attempted to evaluate the effect of coal mine dust exposure in the etiology of lung cancer using two molecular markers, *p53* and K-*ras*.

A crucial target gene involved in DNA damage repair, cell cycle, apoptosis and inhibition of the development or progression of cancer is *p53* (13-18). Activation of K-*ras* is also a frequent phenomenon in a significant proportion of lung cancers and is particularly associated with smoking. Until recently mutations in *p53* (~60%) and K-*ras* (~30%) were the most frequently observed genetic alterations in human lung cancers and approximately 30% of *p53* mutations in smokers are G:C→T:A transversions (17-19). Transversions of G:C→T:A

Table I. Demographic characteristics of coal miners, with type of CWP and lung cancer cell type.

Age	Mining tenure	Smoking history	Pneumoconiosis	Cancer type
73	37	SM 30 pack-years	Macules	Adenocarcinoma, PD
73	66	SM 23 pack-years	No CWP	Adenocarcinoma, MD
79	45	SM 15 pack-years	Macules, nodules, silicosis	Adenocarcinoma, MD
59	25	SM 25 pack-years	Macules	Adenocarcinoma, WD
56	21	SM 10 pack-years	Macules, nodules, silicosis	Adenocarcinoma, WD
77	33	SM 32 pack-years	Macules	Large cell carcinoma, UD
68	20	SM 50 pack-years	Macules, nodules	Large cell carcinoma, UD
53	8	SM 28 pack-years	Macules	Large cell carcinoma, UD
58	14	SM 45 pack-years	Macules	Large cell carcinoma, UD
71	30	SM 75 pack-years	Macules, silicosis	Large cell carcinoma, UD
68	48	SM 48 pack-years	No CWP	Large cell carcinoma, UD
58	27	SM 60 pack-years	Macules, nodules	Squamous cell, MD
71	15	SM 60 pack-years	Macules, nodules	Squamous cell, PD
73	20	SM 40 pack-years	Nodules	Squamous cell, PD
78	47	SM 5 pack-years	Macules, nodules	Squamous cell, WD
54	10	SM cigars	Macules, silicosis	Squamous cell, WD
59	4	SM 25 pack-years	Macules	Squamous cell, MD
79	15	SM 50 pack-years	Macules	Squamous cell, MD
61	20	SM 45 pack-years	Macules	Squamous cell, WD
69	26	SM 15 pack-years	Macules	Squamous cell, WD
64	18	NS	Macules, nodules	Squamous cell, WD
65	23	NS	Macules	Adenocarcinoma, MD
55	2	NS	No CWP	Adenocarcinoma, WD
62	19	NS	Macules	Adenocarcinoma, WD
75	46	NS	No CWP	Adenocarcinoma, WD, large cell carcinoma
85	30	NS	No CWP	Adenocarcinoma, WD
82	70	NS	Macules	Adenocarcinoma, MD
64	32	NS	Macules	Squamous cell, PD
82	10	NS	Macules	Squamous cell, PD
61	40	NS	Macules	Squamous cell, WD
58	24	NS	Macules	Squamous cell, WD
62	15	NS	Macules	Squamous cell, MD
65	2	NS	Macules	Squamous cell, WD

SM, smoker; NS, non-smoker; CWP, Coal workers' pneumoconiosis; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; UD, undifferentiated.

the samples were resuspended in 9 µl of loading buffer (5:1 deionized formamide: 25 mM EDTA/Blue dextran). From this 2 µl samples were electrophoresed through 6% Long Ranger gel (FMC) at 2X A Run Module in ABI Prism 377 automated DNA sequencer (Applied Biosystems, CA). The sequence data was collected by ABI Prism 377 Collection Software and analyzed by Sequencing Analysis and Sequence Navigator Software (Applied Biosystems, CA).

K-ras mutation analysis (PCR amplification). The extract containing genomic DNA (10 µl) was subjected to PCR in a total volume of 50 µl of reaction mixture. The condition of the reaction mixture for PCR was the same as above except for the omission of ^{32}P-dATP and substitution of different primers. The primer set consisted of 5' ATGACTGAATATAAACTT GT-3' (forward) and 5'-CTATTGTTGGATCATATT-3' (reverse). PCR was carried out in a DNA thermal cycler (Cetus-Perkin Elmer) for 35 cycles. Each cycle of amplification consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C and 2 min of polymerization at 72°C. After the last cycle, polymerization was continued for an additional 7 min at 72°C.

Slot-blot analysis. Slot-blot analysis of PCR amplified samples were carried out using ^{32}P-labeled oligonucleotide probe panel following standard procedure. Amplified DNA was denatured

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79	15	SM 50 pack-years	Macules	Squamous cell, MD
61	20	SM 45 pack-years	Macules	Squamous cell, WD
69	26	SM 15 pack-years	Macules	Squamous cell, WD
64	18	NS	Macules, nodules	Squamous cell, WD
65	23	NS	Macules	Adenocarcinoma, MD
55	2	NS	No CWP	Adenocarcinoma, WD
62	19	NS	Macules	Adenocarcinoma, WD
75	46	NS	No CWP	Adenocarcinoma, WD, large cell carcinoma
85	30	NS	No CWP	Adenocarcinoma, WD
82	70	NS	Macules	Adenocarcinoma, MD
64	32	NS	Macules	Squamous cell, PD
82	10	NS	Macules	Squamous cell, PD
61	40	NS	Macules	Squamous cell, WD
58	24	NS	Macules	Squamous cell, WD
62	15	NS	Macules	Squamous cell, MD
65	2	NS	Macules	Squamous cell, WD

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Table II. A, Frequency of *p53* and K-*ras* mutations in NSCLC cases exposed to coal mine dust and cigarette smoke.

Age	Mining (year)	Smoking	Cancer type	Codon	*p53* mutation DNA sequence amino acid	K-*ras* codon 12 mutation sDNA sequence amino acids
73	37	Yes	ADC	-	-	GGT →CGT Gly →Arg
66	17	Yes	ADC	-	-	GGT →GCT Gly →Ala
79	45	Yes	ADC	213	CGA→CGG Arg-Arg (Polymorphism)	- -
59	25	Yes	ADC	-	-	- -
56	21	Yes	ADC	245	GGC →GGT Gly→Gly	- -
77	33	Yes	LCC	220	TAT →TCT Tyr →Cys	- -
68	20	Yes	LCC	-	-	- -
53	8	Yes	LCC	213	CGA →CGG Arg →Arg (Polymorphism)	- -
58	14	Yes	LCC	-	-	GGT →CGT Gly →Arg
71	30	Yes	LCC	-	-	- -
68	48	Yes	LCC	-	-	- -
65	2	Yes	SCC	-	-	- -
58	27	Yes	SCC	280	AGA→ACA Arg →Thr	- -
71	15	Yes	SCC	245	GGC→AGC Gly→Ser	- -
73	20	Yes	SCC	-	-	- -
78	47	Yes	SCC	-	-	- -
59	4	Yes	SCC	-	-	- -
79	15	Yes	SCC	-	-	- -
61	20	Yes	SCC	-	-	- -
69	26	Yes	SCC	-	-	- -
64	18	Yes	SCC	-	-	- -

B, Frequency of *p53* and K-*ras* mutations in never smoker NSCLC cases exposed to coal mine dust.

Age	Mining (year)	Smoking	Cancer type	Codon	*p53* mutation DNA sequence amino acid	K-*ras* codon 12 mutation sDNA sequence amino acids
65	23	No	ADC	-	-	GGT→CGT Gly→Arg
55	2	No	ADC	-	-	- -
62	19	No	ADC	-	-	- -
75	46	No	ADC	-	-	- -
85	30	No	ADC	274	GTT→TTT Val→Phe	GGT→CGT Gly→Arg GGT→GAT Gly→Asp
82	70	No	ADC	-	-	- -
64	32	No	SCC	-	-	- -
82	10	No	SCC	-	-	- -
61	40	No	SCC	-	-	- -
58	24	No	SCC	-	-	- -
62	15	No	SCC	-	-	- -
54	10	No	SCC	-	-	- -

PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated.

In this study, two polymorphisms at codon 213 were detected in tumor tissues, but it was not found in non-tumor tissues. Recently, Smida *et al* (44) reported similar observation. Using laser-assisted micro-dissection, they found polymorphism at codon 213 in micro-dissected tumor sample, but it was not detected in normal tissue samples obtained using the same procedure. Furthermore, a recent report indicated that the median relapse-free survival of patients with breast

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