Aberrant Promoter Methylation of $p16^{INK4a}$ and O^6 -Methylguanine-DNA Methyltransferase Genes in Workers at a Chinese Uranium Mine

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Abstract: Aberrant Promoter Methylation of $p16^{INK4a}$ and O^6 -Methylguanine-DNA Methyltransferase Genes in Workers at a Chinese Uranium Mine: Shibiao Su, *et al.* Department of Toxicology, School of Radiation Medicine and Public Health, Soochow University, China—To find the possible association of gene methylation of $p16^{INK4a}$ and O^6 -Methylguanine-DNA Methyltransferase (O^6 -MGMT) with occupational exposure to radon, 91 male miners from a uranium mine in China were divided into 4 groups according to the cumulative doses of radon exposure from 2 to 425 WLM (working-level months), and aberrant promoter methylation of $p16^{INK4a}$ and O^6 -MGMT genes in sputum samples was determined by a specific PCR assay. The results revealed that the methylated rates of $p16^{INK4a}$ gene ($z=2.844$, $P=0.005$) and O^6 -MGMT gene ($z=3.034$, $P=0.002$), and the total methylated rate of these two genes ($z=3.859$, $P=0.0001$) increased significantly with the cumulative doses of radon among the miners. This methylation could be applied as a potential marker for the detection of early DNA damage induced by occupational radon exposure. (*J Occup Health* 2006; 48: 261–266)

Key words: Radon exposure, Methylation, $p16^{INK4a}$ gene, O^6 -MGMT gene

Human exposure to radon (^{222}Rn) is ubiquitous, occurring as a result of the seepage of this inert gas from uranium-containing rocks and soil into enclosed areas such as underground mines and homes. The carcinogenicity of radon has been convincingly documented through epidemiological studies of

underground miners, all showing a substantial increased risk of lung cancer¹⁾. In a pooled analysis of 11 miners studies, Lubin *et al.*²⁾ reported RRs for lung cancer at 10 WLM as ranging from 1.2 to 6.1. Extrapolating these data using the linear non-threshold theory of radiation carcinogenesis to predict risk from residential exposure to ^{222}Rn , the authors concluded that in the USA, exposure to radon progeny may account for 10% of all lung cancer deaths and 30% of lung cancer deaths in non-smokers, while an estimate from the National Academy of Sciences BEIR VI committee suggested 21,800 lung cancer cases annually result from radon exposure with uncertain bounds from 3,000 to 33,000, making this the second leading cause of lung cancer in the United States¹⁾. The damage done to epithelial cells of the lung occurs when radiation interacts either directly with DNA in the cell nucleus or indirectly through the affect of free radicals.

Aberrant DNA (cytosine) methylation within the so-called CpG islands is among the earliest and most common alteration in human malignancies leading to abrogation of tumor suppression in a broad spectrum of genes^{3,4)}. Aberrant promoter methylation of the $p16^{INK4a}$ tumor suppressor gene, which plays a key role in cell cycle regulation by inhibiting cyclin-dependent kinases (CDK) 4 and 6, is an early and very frequent event in squamous cell carcinoma (SCC) of the lung. O^6 -MGMT is a DNA repair enzyme that protects cells from the carcinogenic effect of alkylating agents by removing adducts from the position of guanine. O^6 -MGMT is usually inactivated by aberrant promoter methylation in non-small cell lung carcinoma (NSCLC)⁵⁾. Thus, $p16^{INK4a}$ and O^6 -MGMT genes are strong candidates as biomarkers for the early detection of lung cancer.

The present study was aimed at exploring the association of aberrant promoter methylation of $p16^{INK4a}$ and O^6 -MGMT genes with cumulative dose of radon exposure to predict the risk of lung cancer among occupational workers.

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Materials and Methods

Subjects

The subjects were 91 male underground miners, ground miners and office staff at a Chinese uranium mine aged from 42 to 55 yr old. They were divided into 4 groups according to cumulative dose of radon exposure in terms of working level month (WLM) (>120 WLM, 60–120 WLM, 30–60 WLM, 2–30 WLM) and the level of adverse effect induced by radon that was reported in our previous studies^{6, 7)}. One WLM is the cumulative exposure equivalent to one working level (WL) for a working month (170 h). A WL is any combination of short-lived radon progeny in one liter of air that will result in the emission of 1.3×10^5 MeV of potential alpha energy. A home exposure of 4 pCi/L for 70 yr would approximately equal a cumulative exposure of 54 WLM. All subjects consented to participate in the study and volunteered to offer their sputum samples.

Questionnaire

A questionnaire was conducted to collect the following information. (1) Personal status: gender, age, status of marriage and education background. (2) History of occupation: work site, type of work, length of service and work hour per day. (3) Habitation conditions: house type, construction materials and ventilation. (4) Smoking history: never smoked, former smoker, current smoker and median packs/year. (5) Family history of lung cancer. (6) Shielding mask use: never, sometimes or often. All questionnaires were answered and completed by the subjects.

Environmental monitoring

The radon concentration at 29 work sites was monitored by the method of solid nuclear traces for 90 d from September to November.

Calculation of average radon dose

The concentration of radon exposure was calculated by the following formula (1) and converted to the annual exposure dose (WLM) of radon progeny at the work place according to formula (2). The cumulative dose (WLM) of radon progeny was estimated by formula (3)⁸⁾.

$$(1) C (\text{Bq/m}^3) = (N - Nb) \times 1000 / (K \cdot t)$$

C— the average radon concentration of exposure hours (Bq/m^3)

N— the trace density of sample detector (trace/cm^2)

Nb—the trace density of background detector (trace/cm^2)

K— scale factor of detector [$(\text{trace/cm}^2)/(\text{kBq/m}^3 \cdot \text{h})$]

t— exposure time (h)

$$(2) \text{Annual exposure dose for service (WLM)} = C (\text{Bq/m}^3) \times 1.26 \times 10^{-3} (\text{WLM/Bq/m}^3)$$

$$(3) \text{Cumulative exposure dose (WLM)} = \text{Annual exposure dose (WLM)} \times \text{yrs (length of service or habitation)}$$

Sputum sampling

Sputum was induced by a nebulized normal saline solution⁹⁾. The induced sputum was collected and stored in Saccamanno's solution until DNA extraction.

DNA extraction

DNA was extracted from sputum samples by digestion with proteinase K solution followed by the standard phenol-chloroform extraction and ethanol precipitation. The extracted DNA was stored at -20°C until use.

Methylation Specific PCR (MSP)

Methylation of the *p16^{INK4a}* and *O⁶-MGMT* gene promoters in sputum samples was determined by a nested, two-stage MSP assay¹⁰⁾. Genomic DNA isolated from the sputum was modified by the bisulphite reaction using the CpGenome DNA Modification Kit (CHEMICOM., Purchase, US) as reported⁶⁾, which converts only unmethylated cytosines to uracil. PCR primers specific to both methylated and unmethylated template were used. The primers for the methylated (*M_F* and *M_R*) and unmethylated (*U_F* and *U_R*) alleles of *p16^{INK4a}* and *O⁶-MGMT* are described in Table 1^{10, 11)}. MSP of *O⁶-MGMT* was carried out on a final volume of 50 μl containing 250 ng of bisulphate modified DNA, 250 μM dNTP (Applied Biosystems, Foster City, CA), 1 μM of each primer (Sangon, Shanghai, China), 1.5 mM MgCl_2 , 5 μl DMSO, 10 $\times$ PCR buffer with $(\text{NH}_4)_2\text{SO}_4$, and 2 units of Taq DNA Polymerase (Applied Biosystems, Foster City, CA), by an MJ PTC-200 thermocycler (M. J. Research, Inc., Cambridge, MA) with the following cycling parameters: 94°C for 5 min; 35 cycles of 94°C for 1 min, specific annealing temperature (Table 1) for 1 min, then 72°C for 1 min; and a final extension step at 72°C for 10 min. Products were visualized on 3% agarose gels. MSP of *p16^{INK4a}* was performed on a final 25- μl aliquot containing 50 ng of bisulphate modified DNA, 250 μM dNTP (Applied Biosystems, Foster City, CA), 150 ng of each primer (Sangon, Shanghai, China), 2 mM MgCl_2 , 10 $\times$ PCR buffer with $(\text{NH}_4)_2\text{SO}_4$, and 2 units of Taq DNA Polymerase (Applied Biosystems, Foster City, CA), in an MJ PTC-200 thermocycler (M. J. Research, Inc., Cambridge, MA) with the following cycling parameters: 94°C for 5 min; 35 cycles of 94°C for 45 s, specific annealing temperature (Table 1) for 45 s, then 72°C for 45 s; and a final extension step at 72°C for 7 min. Products were visualized on 2% agarose gels. The two-stage MSP assay requires only nanogram quantities of DNA and can detect methylated alleles in the presence of unmethylated alleles at a sensitivity of 1 in >50,000 copies⁹⁾.

Table 1. Primers for MSP

Primer	Sequence (5'–3')	Annealing temperature	Product size
<i>p16</i> ^{INK4a} M _F ^a	TTATTAGAGGGTGGGGCGGATCGC	65°C	150 bp
<i>p16</i> ^{INK4a} M _R	GACCCCGAACCGCGACCGTAA		
<i>p16</i> ^{INK4a} U _F	TTATTAGAGGGTGGGGTGGATTGT	60°C	151 bp
<i>p16</i> ^{INK4a} U _R	CAACCCCAAACCACAACCATAA		
<i>O</i> ⁶ - <i>MGMT</i> M _F	TTTCGACGTTTCGTAGGTTTTCGC	56°C	81 bp
<i>O</i> ⁶ - <i>MGMT</i> M _R	GCACTCTCCGAAAACGAAACG		
<i>O</i> ⁶ - <i>MGMT</i> U _F	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	59°C	93 bp
<i>O</i> ⁶ - <i>MGMT</i> U _R	AACTCCACACTCTTCCAAAAACAAAACA		

^aM_F, forward methylated primer; M_R, reverse methylated primer; U_F, forward unmethylated primers; U_R, reverse unmethylated primers.

Table 2. Quantification of radon exposure in workers of different groups

Group	Radon concentration at worksites (Bq/m ³)	Annual exposure dose (WLM)	Length of service (yr)	Cumulative dose (WLM, mean ± SD)
1	95–767	0.09–0.73	14–33	12 ± 6
2	1,090–1,764	1.03–1.67	13–33	45 ± 15
3	2,240–2,837	2.13–2.69	14–35	78 ± 10
4	8,325–13,507	7.90–12.81	13–34	294 ± 132

Statistical analysis

Statistical analyses were performed using the SAS software package (version 8.1; SAS Institute, Cary, NC USA). One way analysis of variance (ANOVA) and χ^2 -test were applied to explore differences in variables such as age, length of service, work hour and smoking status. The Cochran-Armitage trend test was used to correlate the methylation rate of *p16*^{INK4a} gene, *O*⁶-*MGMT* gene, and the total methylation rate of these two genes with the radon exposure dose.

Results

The concentrations of radon measured at different work sites are shown in Table 2, and ranged from 95 to 13,507 Bq/m³. The annual exposure dose of the workers increased from group 1 to group 4, leading to a corresponding increase of the cumulative dose (WLM). Although the cumulative dose in group 4 was significantly higher than the other 3 groups, the length of service of the workers among the different groups was almost the same.

No statistically significant differences could be found in variables such as mean age, length of service, daily work hours and smoking status among the four groups workers (Table 3). All the subjects claimed no family history of lung cancer, and they never used radon-proof masks while on duty.

As shown in Table 4, the methylated rates of the *p16*^{INK4a} gene, *O*⁶-*MGMT* gene, and total methylated rate of these two genes increased significantly along with the increasing cumulative doses of radon exposure from group 1 to group 4. The methylated rate of *O*⁶-*MGMT* gene was higher than that of *p16*^{INK4a} gene in groups 2, 3 and 4, but with no statistical significance. In group 4 in which the exposure dose surpassed 120 WLM, the methylated rate of the *p16*^{INK4a} gene, *O*⁶-*MGMT* gene and the total methylated rate of the two genes were as high as 20%, 28% and 40%, respectively.

Discussion

Upon inhalation, radon progeny in a conjugated state is mainly deposited in the lung, whereas most of its unconjugated forms are deposited in the nasal pharynx and trachea bronchus. Because of the good liposolubility, radon and its progeny easily reach the lung and are absorbed into the blood. The lung and blood cells thus become the main target of irradiation from radon exposure^[12].

Increasing evidence suggests that radon and its decay products are carcinogenic in the lungs of both experimental animals and humans^[13, 14]. Lung cancer is now the leading cause of cancer-related death in the United States, as well as in China, and is projected to reach epidemic levels in the world during the 21st

Table 3. Demographics of the subjects in the four groups

Variables	Group (number)				<i>p</i> -value
	1 (17)	2 (25)	3 (24)	4 (25)	
Age (yr) (mean $\pm$ SD)	43.8 $\pm$ 5.2	45.1 $\pm$ 6.4	44.5 $\pm$ 5.4	43.5 $\pm$ 3.5	>0.05
Occupational history length of service (yr) (mean $\pm$ SD)	21.9 $\pm$ 7.4	23.2 $\pm$ 5.8	22.1 $\pm$ 4.4	22.0 $\pm$ 4.3	>0.05
work hour (hr/d)	6	6	6	6	
Smoking status					>0.05
never(%)	18	16	13	16	
former(%)	29	28	29	32	
current(%)	53	56	58	52	
median pack/year	15	16	15	16	
Radon-proof mask	No	No	No	No	
Family history of lung cancer	No	No	No	No	
Habitation condition					
house type	Uniform	Uniform	Uniform	Uniform	
construction materials	Uniform	Uniform	Uniform	Uniform	
ventilation	Yes	Yes	Yes	Yes	

Table 4. Measurement of *p16*^{INK4a} and *O*⁶-*MGMT* methylation in subjects of 4 groups

Group	n	Range of cumulative dose (WLM)	<i>p16</i> ^{INK4a} methylation		<i>O</i> ⁶ - <i>MGMT</i> methylation		Total methylation of <i>p16</i> ^{INK4a} and <i>O</i> ⁶ - <i>MGMT</i>	
			+	%	+	%	+	%
1	17	2–	0	0.00	0	0.00	0	0.00
2	25	30–	0	0.00	1	4.00	1	4.00
3	24	60–	1	4.17	2	8.33	2	8.33
4	25	120–	5	20.00	7	28.00	10	40.00
z-value				2.844		3.034		3.859
<i>p</i> -value				0.005		0.002		0.0001

century¹⁵). Mortality from this disease could be reduced greatly through the development of molecular markers that identify individuals at the earliest stages of lung cancer in which curative resection is feasible. Candidate biomarkers should have high sensitivity and specificity and appear early enough in the course of disease for medical intervention to improve prognosis. Finally, the markers must be present in a biological fluid that can be obtained non-invasively, making its collection feasible for population-based screening.

To assess the potential of a molecular-based marker approach for the early detection of human lung cancer induced by radon, a rather specific mutational spectrum was observed, for example, in the *p53* gene from lung tumours of uranium miners with occupational exposure to radon¹⁶). In a study on 20 non-smoking residents living in houses with different airborne radon concentrations, it

was concluded that radon concentrations >200 Bq/m³ were associated with an increasing mutant frequency in the *HPRT* locus in circulating lymphocytes¹⁷). Another study reported that the frequency of aberrant methylation of the *p16*^{INK4a} (18%) and *O*⁶-*MGMT* genes (36%) occurred in 22 uranium workers with exposures ranging from 3 to 577 WLM, among whom 75% were exposed to over 100 WLM. However, the association of methylation of DNA with radon progeny was not revealed because of the small sample size¹⁸).

Previous studies have revealed an increased lung cancer risk for underground workers in mines, with all probability related to radon progeny exposure. The calculated population etiologic fraction was about 45% for underground mining and about 80% for smoking¹⁹). The effects of smoking and exposure to alpha radiation from radon daughters were nearly additive, and exposure

to radon daughters at home accounts for an appreciable number of cases of lung cancer in the general population²⁰). It was obvious that smoking status and habitation condition are confounding factors in a study on the adverse effects induced by radon. That is the reason why variables including age, occupational history, smoking status and habitation condition should be considered and restricted in investigations like this present study.

In our previous study on the correlation of adverse effects of radon and its progeny on target tissues in rats, we reported that the distance of DNA migration in bronchi alveolar lavage fluid and peripheral blood mononuclear cells increased in a dose-dependent manner when male Wistar rats were exposed to from 2 to 117 WLM of radon inhalation⁶). Another previous study stressed the importance of *p16^{INK4a}* as an early marker of lung cancer diagnosis, but did not mention the *O⁶-MGMT* gene¹⁰). In the present study, a similar trend was revealed for both *p16^{INK4a}* and *O⁶-MGMT* methylation in uranium miners with cumulative doses from 2 to 425 WLM. These findings suggest that long-term exposure to radon may induce DNA damage to the tracheal epithelial cells even if at the concentrations lower than the current occupational limit (3,700 Bq/m³). There is overwhelming evidence that altered DNA methylation patterns are important sign of DNA damage leading to carcinogenesis. The result that the methylated rate of *O⁶-MGMT* was greater and occurred earlier than that of *p16^{INK4a}* in each group (Table 4) might imply a higher sensitivity of the *O⁶-MGMT* gene to radon α irradiation¹⁸).

The results of this study suggest that the methylation status of the *p16^{INK4a}* and *O⁶-MGMT* genes could be applied as a potential marker to the detection of early DNA damage induced by occupational radon exposure.

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