Lung cancer from radon or $^{239}$plutonium exposure has been linked to $\alpha$-particles that damage DNA through large deletions and point mutations. We investigated the involvement of an epigenetic mechanism, gene inactivation by promoter hypermethylation in adenocarcinomas from plutonium-exposed workers at MAYAK, the first Russian nuclear enterprise established to manufacture weapons plutonium. Adenocarcinomas were collected retrospectively from 71 workers and 69 non-worker controls. Lung adenocarcinomas were examined from workers and non-worker controls for methylation of the CDKN2A (p16), $6\text{-methylguanine-DNA methyltransferase}$ (MGMT), death associated protein kinase (DAP-K), and Ras effector homolog 1 genes (RASSF1A). The prevalence for methylation of the MGMT or DAP-K genes did not differ between workers and controls, while a higher prevalence for methylation of the RASSF1A gene was seen in tumors from controls. In marked contrast, the prevalence for methylation of p16, a key regulator of the cell cycle, was increased significantly ($P = 0.03$) in tumors from workers compared with non-worker controls. Stratification of plutonium exposure into tertiles also revealed a striking dose response for methylation of the p16 gene ($P = 0.008$). Workers in the plutonium plant where exposure to internal radiation was highest had a 3.5 times (C.I. 1.5, 8.5; $P = 0.001$) greater risk for p16 methylation in their tumors than controls. This increased probability for methylation approximated the 4-fold increase in relative risk for adenocarcinoma in this group of workers exposed to plutonium. In addition, a trend ($P = 0.08$) was seen for an increase in the number of genes methylated ($\geq 2$ genes) with plutonium dose. Here we demonstrate that exposure to plutonium may elevate the risk for adenocarcinoma through specifically targeting the p16 gene for inactivation by promoter methylation.

**Introduction**

The molecular mechanisms leading to lung cancer from high linear energy transfer (LET) radiation have largely been linked to $\alpha$-particles that cause DNA damage primarily through large deletions and, to a lesser extent, point mutations (1,2). Epigenetic inactivation of genes by CpG island promoter methylation has emerged as a major mechanism in cancer initiation and progression for silencing genes responsible for all aspects of normal cellular function (3). Inactivation of the CDKN2A (p16) gene by methylation is not only common in human and rodent lung tumors associated with tobacco, but also in rat lung tumors induced by $^{239}$plutonium (Pu) (4,5). Epidemiology studies indicate a significantly higher risk for adenocarcinoma in MAYAK workers than in controls and a strong correlation between this tumor type and Pu exposure (6). Lung cancers in these workers were frequently found in the lower and middle lung lobes, and at the periphery, thus reflecting the deposition and retention patterns of inhaled Pu (6). We hypothesized that the risk for adenocarcinoma following radiation exposure increased in part through an increased prevalence for inactivation of genes by promoter hypermethylation. This hypothesis was tested by determining the prevalence for methylation of four genes that play a critical role in the development of adenocarcinoma through their effects on cell cycle control (p16), DNA repair [$6\text{-methylguanine-DNA methyltransferase}$ (MGMT)], apoptosis [death associated protein kinase (DAP-K)] and ras signaling [Ras effector homolog 1 (RASSF1A)] (5,7–9). In addition to the critical role these four genes play in maintaining normal cell function, they are all commonly inactivated in adenocarcinoma at prevalences ranging from 20 to 45% and their loss of function has been associated with unfavorable prognosis that includes increasing pathological stage and reduced survival (7,8,10–13).

**Materials and methods**

**Study population**

This study was approved by Institutional Review Boards in Ozyorsk, Russia and Albuquerque, New Mexico. Lung adenocarcinomas were obtained from MAYAK employees and non-worker controls. The samples used were acquired retrospectively as paraffin-embedded tissue blocks through either the Radiobiology Tissue Repository at the Scientific Center of RF-Biophysics for the MAYAK nuclear facility employees from 1948 until 1972, or through the Ozyorsk or Chelyabinsk city hospital for non-worker controls. Controls were obtained from these hospitals to minimize geographical differences to workers. None of the controls were affiliated with the MAYAK enterprise, thus they had no direct exposure to radiation. The study population comprised 140 persons diagnosed with adenocarcinoma between 1966 and 1991. Non-smokers defined as never having smoked >100 cigarettes in their lifetime were identified retrospectively through review of their medical records. A physician examined MAYAK nuclear facility employees each year and smoking status was recorded as part of the medical record. DETAILED smocking information including pack years and duration was collected for the majority of workers. The medical records provided information on gender, age of diagnosis and age of death and smoking status (yes, no, detailed smoking history not available) for the non-worker controls. Letters were also sent to 70% (relatives not located for other 30%) of family members of non-worker controls to verify

**Abbreviations:** DAP-K, death associated protein kinase; LET, linear energy transfer; MGMT, $6\text{-methylguanine-DNA methyltransferase}$; Pu, $^{239}$plutonium; p16, CDKN2A; RASSF1A, Ras effector homolog 1.

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whether the person smoked. The response rate to the letter was virtually 100% and corroborated the medical record with respect to smoking. Non-malignant lung diseases that included chronic bronchitis, emphysema, acute pneumonia, chronic pneumonia and tuberculosis were all noted for MAYAK workers as part of the medical record. Finally, employment records indicated the plant within the MAYAK facility where each person worked.

**Radiation dosimetry**

External γ-radiation doses for MAYAK workers were recorded through individual film badges, which were worn at all times during work shifts. Exposure assessment has been described in detail (14). The amount of incorporated Pu was estimated by a urine bioassay based on the radiochemical method of double phosphate precipitation of Pu (14,15). Three to five consecutive daily urine samples from each worker were analyzed with mean values calculated. The error was ≤30%, and the detection limit for body burden of Pu was estimated to be 0.26 kBq.

**Histological diagnosis**

All cases were reviewed histologically in concert with review of the pathology reports at the Southern Ural Biophysics Institute. All tumors were classified broadly as adenocarcinoma. A second review at the Lovelace Respiratory Research Institute confirmed the tumor histology.

**Microdissection and nucleic acid isolation**

DNA was obtained by microdissection. Sequential sections from tumors were cut, deparaffinized and stained with toluidine blue to facilitate dissection. A 25-gauge needle attached to a tuberculin syringe was used to remove the lesions under a dissecting microscope. Most adenocarcinomas were contaminating extensively with normal tissue, and the goal for microdissection was to enrich for >50% tumor tissue. DNA was isolated by overnight digestion of the microdissected tissue with pronase (1%) followed by phenol-chloroform extraction and ethanol precipitation.

**Methylation-specific PCR (MSP)**

The methylation status of the p16, MGMT, DAP-K and RASSF1A promoters was determined using a nested, two-stage MSP (16). DNA was first subjected to bisulfite modification, and a multiplex PCR was performed to amplify a 280-, 289-, 209- and 260-bp fragment of the p16, MGMT, DAP-K and RASSF1A genes, respectively, including a portion of the CpG-rich promoter region. The primers used recognize the bisulfite-modified template, but do not discriminate between methylated and unmethylated alleles. Following amplification by stage-I primers, two different stage-II amplifications were performed. In one, stage-II amplification, the primers used a recognized sequence in which CpG sites are unmethylated. These primers allowed us to verify the ability to amplify the target region because stage-I products are often not detectable with DNA amplification from formalin-fixed tissue. The other set of primers used was specific for detecting methylated alleles and was localized to regions in and around the transcription start site of the genes, an area that correlates with loss of gene expression (5). Primer sequences and PCR conditions have been described (16,17). Normal human lung tissue collected from autopsy of never-smokers and cell-lines positive for methylation served as a positive control, respectively. The products were visualized on 2% agarose gels. All samples that gave a product with the stage-II methylation-specific primers were confirmed by a second PCR that also used methylation-specific restriction enzyme digestion after amplification. This approach verifies that CpGs within the amplification product are also methylated (16,17). There were a few samples for which methylation status of a gene could not be confirmed, i.e. neither unmethylated nor methylated alleles were detected.

**Statistical analysis**

A descriptive analysis examined the distribution of internal and external radiation doses, as well as smoking (ever versus never) and promoter methylation status for each gene (p16, MGMT, DAP-K, RASSF1A) in workers and controls. Pu doses were categorized into exposure tertiles. The tertiles were defined to assess multiplicity for methylation and the influence of Pu dose. Associations between methylation multiplicity, groups and dose categories were assessed univariately using chi-square tests and for linear trend using Mantel-Haenzel chi-square tests. All statistical tests for differences in distribution and linear trends were conducted with a two-sided significance level of 0.05. All analyses used SAS software (version 8e; SAS Institute, Cary, NC).

**Results**

**Demographics and exposures**

Adenocarcinomas from 71 MAYAK workers (88.7% male) and 69 non-worker controls (100% male) were evaluated for methylation (Table I). Age at diagnosis did not differ between workers and controls. Smokers comprised ~90% of each group. Median smoking duration and amount were 35 years and 33 pack years, respectively, for workers. Smoking history was limited to ever/never smoking for controls. The median level for exposure to Pu absorbed doses and γ-radiation among workers was 52 and 133 cGray, respectively. Workers, due in part to differences in exposure based on worker location within the MAYAK nuclear facility, were exposed to a large range of both internal and external radiation. For example, persons who worked at the Pu plant received the highest exposure to Pu, while those working at the reactor plant had minimal Pu exposure (Table II).

**Radiation exposure targets the p16 gene for inactivation**

Methylation of the p16 gene was increased significantly in male workers compared with male controls with and without adjustment for smoking status (yes or no) and tumor stage (Table III). The prevalence for methylation of the MGMT and DAP-K genes did not differ between male workers and controls. In marked contrast, adenocarcinomas from male controls had a significantly higher prevalence for methylation of the RASSF1A gene than male workers, which could indicate that other genes in this signal transduction pathway were alternatively affected (Table III). Precedence for gene targeting within a pathway has been seen with differing histologies of lung cancer where mutations in the retinoblastoma gene are common in small cell lung cancer, while p16 methylation is

<table>
<thead>
<tr>
<th>Variable</th>
<th>MAYAK worker n = 71</th>
<th>Non-worker control n = 69</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years)*</td>
<td>59 (31, 75)</td>
<td>60 (38, 76)</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>88.7</td>
<td>100</td>
</tr>
<tr>
<td>Female</td>
<td>11.3</td>
<td>0</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>87.3</td>
<td>89.9</td>
</tr>
<tr>
<td>No</td>
<td>12.7</td>
<td>10.1</td>
</tr>
<tr>
<td>Smoking, duration (years)*</td>
<td>35 (6, 60)</td>
<td>NA*</td>
</tr>
<tr>
<td>Smoking, pack years*</td>
<td>33 (2, 67)</td>
<td>NA*</td>
</tr>
<tr>
<td>Radiation exposure (cGy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha</td>
<td>52 (1, 914)</td>
<td>0</td>
</tr>
<tr>
<td>External gamma</td>
<td>133 (2, 1000)</td>
<td>0</td>
</tr>
</tbody>
</table>

*aMedian (minimum, maximum).

*bNot available.
Methylation of the MGMT, RASSF1A or DAP-K genes was not associated with employment at any workplace (not shown). While a trend towards a protective effect was observed for methylation of RASSF1A (OR = 0.6), the effect did not differ across workplace or reach statistical significance (not shown). Together these findings suggest that exposure to Pu not \( \gamma \)-radiation mediated the increased prevalence for methylation of the p16 gene in adenocarcinomas from workers.

**Radiation dose modulates the prevalence for promoter methylation**

Tertiles of Pu exposure were defined to assess the effect of dose on the prevalence of p16 methylation among workers. The prevalence of p16 gene methylation increased significantly as a function of increasing Pu dose \((P = 0.008\) for linear trend; Figure 1). Smoking (duration or dose) among workers in the three exposure groups did not differ.

The inactivation of multiple genes by methylation, i.e. a methylation phenotype, could be an important factor in the increased risk for adenocarcinoma from Pu exposure. This hypothesis was addressed by examining the effect of Pu dose on the multiplicity for promoter methylation. Because an inverse relationship was seen for RASSF1A and radiation exposure, it was not included in this analysis. The percentages of adenocarcinomas methylated for zero, one or more than/equal to two genes were determined for workers and controls across the same exposure tertiles used to assess the dose–response relationship for the p16 gene. The number of genes methylated showed a trend for increase \((P = 0.08\) for linear trend) with Pu dose (Table IV). In general, the percentage of adenocarcinomas without methylated genes decreased with increasing radiation dose, while the number of tumors with more than/equal to two methylation changes increased across exposure groups (Table IV). The multiplicity for methylation was also associated with working at the Pu plant \((OR = 2.2; C.I. 1.0, 4.6, P = 0.04; Table II)\).

**Discussion**

Our results demonstrate that the elevated risk for radiogenic adenocarcinoma of the lung may, in part, be mediated by silencing tumor suppressor genes through promoter methylation.
The effect of targeting p16 for inactivation on cancer risk is likely substantial because inactivation of this gene is an early event in lung cancer development and plays a critical role in immortalization by allowing cells to escape the mortality checkpoint ‘M0’ (5,21,22). The fact that the 3.5-fold increase in risk for methylation of this gene for workers at the Pu plant approximated the previously reported (6) 4-fold increased risk for adenocarcinoma in this group of workers establishes a link between Pu dose, p16 methylation and cancer risk. As also seen in a limited number of female workers, six of eight who never smoked, it is apparent that Pu exposure alone can lead to inactivation of the p16 gene.

The strong association between methylation and α-rather than γ-radiation probably stems from the fact that α-particles deposit large amounts of energy (high LET) in small volumes along their paths, while γ-rays deposit relatively sparse amounts of energy (low LET). Estimation of DNA damage using p53 expression as a dosimeter indicates that to produce equivalent damage would require an absorbed dose of a low-LET emitter that is 10-fold higher than for α particles (23). Even accounting for differences in effective dose, in vitro exposure of peripheral blood lymphocytes to α-particles resulted in more complex chromosome aberrations that were transmitted through cell division than seen with low LET X-rays (24).

This persistent DNA damage could be the common link as to how Pu exposure targets the p16 gene for inactivation and increases overall the inactivation of genes by promoter hypermethylation in adenocarcinomas from the MAYAK workers. A recent study (25) detected and quantified stable intrachromosomal rearrangements within chromosome 5 in lymphocytes of healthy former workers from the MAYAK facility. The yield of these aberrations was highly correlated with Pu dose to the bone marrow. The extensive DNA damage suggests that radiation exposure to the lung epithelial cells from workers led to the accumulation of massive amounts of chromosomal damage that likely overwhelmed DNA repair pathways. DNA-dependent protein kinase is a serine-threonine kinase activated by double-strand breaks in DNA and may play a major role in non-homologous recombination and transcriptional control (26). Our group demonstrated recently that reduced DNA protein kinase activity, which functionally diminished capacity to repair DNA damage by the radiomimetic bleomycin, was associated with an increased risk for lung cancer (27). The disruption of replication timing because of chromosomal instability or loss of normal transcriptional control has been proposed as a mechanism through which normally unmethylated regions of DNA become aberrantly methylated (28). Although exposure to Pu is not common, radon is a ubiquitous environmental carcinogen estimated to cause 10 000 cases of lung cancer annually in the US (29). Our studies have highlighted a new mechanism for how radiation exposure could impact lung cancer risk by affecting not only a specific tumor suppressor gene, but also globally by epigenetic silencing of multiple key regulatory genes.

Acknowledgements

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**Table IV.** Effect of Pu absorbed lung dose on multiplicity for aberrant methylation in adenocarcinomas from male MAYAK workers compared with controls

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Dose (cGy)</th>
<th>Sample size</th>
<th>Number of genes methylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero</td>
<td>One</td>
<td>≥Two</td>
</tr>
<tr>
<td>Pu&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>66</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>1–15</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>16–137</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>&gt;137</td>
<td>16</td>
<td>19</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not available for 13 male workers.

<sup>b</sup>Sample sizes are slightly lower than the number of workers and controls reported in Table I because not all samples could be assayed as methylated or unmethylated for each gene.

<sup>c</sup>P = 0.08 compared with non-worker controls.

References

dosimetry system for the workers at the MAYAK production association. Health Phys., 83, 72–76.

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