Indoor radon exposure increases tumor mutation burden in never-smoker patients with lung adenocarcinoma

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ARTICLE INFO
Keywords:
Radon
Non-small-cell lung cancer
Mutation

ABSTRACT

Objectives: Radon, a natural radiation, is the leading environmental cause of lung cancer in never-smokers. However, the radon exposure impact on the mutational landscape and tumor mutation burden (TMB) of lung cancer in never-smokers has not been explored. The aim of this study was to investigate the mutational landscape of lung adenocarcinoma in never-smokers who were exposed to various degrees of residential radon.

Materials and methods: To investigate the effect of indoor radon exposure, we estimated the cumulative exposure to indoor radon in each house of patients with lung cancer with a never-smoking history. Patients with at least 2 year-duration of residence before the diagnosis of lung adenocarcinoma were included. Patients were subgrouped based on the median radon exposure level (48 Bq/m³): radon-high vs. radon-low and targeted sequencing of tumor and matched blood were performed in all patients.

Results: Among 41 patients with lung adenocarcinoma, the TMB was significantly higher in the radon-high group than it was in the radon-low group (mean 4.94 vs. 2.6 mutations/Mb, P = 0.01). The recurrence rates between radon-high and radon-low group did not differ significantly. Mutational signatures of radon-high tumors showed features associated with inactivity of the base excision repair and DNA replication machineries. The analysis of tumor evolutionary trajectories also suggested a series of mutagenesis induced by radon exposure. In addition, radon-high tumors revealed a significant protein-protein interaction of genes involved in DNA damage and repair (P < 0.001).

Conclusions: Indoor radon exposure increased the TMB in never-smoker patients with lung adenocarcinoma and their mutational signature was associated with defective DNA mismatch repair.

1. Introduction

The incidence of lung cancer in never-smokers has been rising, and approximately 25% of lung cancer cases worldwide are not attributable to tobacco use. Profound differences in the clinical and molecular characteristics of non-small-cell lung cancer (NSCLC) in never-smokers versus smokers, suggest that they are different entities [1]. Multiple recurrent oncogenic drivers such as epidermal growth factor receptor (EGFR) mutation, anaplastic lymphoma kinase (ALK), and ROS1 fusion are known culprits of lung cancer in never-smokers [2–4]. These...
oncogene-driven tumors are extremely sensitive to tyrosine kinase inhibitors (TKIs) and multiple TKIs have been developed or are under development in clinical trials. However, research studies have overlooked the environmental causes of lung cancers in never-smokers. Recently, radon (Rn), natural and noble gas, has been recognized as an important cause of lung cancer [5,6]. Indoor radon exposure or residential radon is the second most common cause of lung cancer, and many previous studies have confirmed the relationship between radon exposure and lung cancer [7–9]. Radon emissions may cause genetic alterations such as increased frequency of DNA-repair gene alterations, or lead to altered cytokine productions [5,10]. It has also been reported that radon exposure may have a cumulative effect and never-smoker patients with lung cancer diagnosed at a younger age may have been exposed to higher level of radon [11]. In fact, a significant linear risk of developing lung cancer due to radon exposure has been reported, with a 16% increase in risk per 100 Bq/m³ increase in indoor radon concentration [12]. In addition, radon exposure is associated with all histological types of lung cancer in never-smokers [13].

However, the series of mutagenesis caused by radon is not exactly known. It is also unclear which genes may be altered in patients who have been exposed to high level of residential radon, and whether radon increases number of mutation in lung cancer. We believed that the effect of radon should be assessed in patients without history of smoking, which is the foremost important source of mutagenesis in lung cancer.

In this study, we aimed to investigate the mutational landscape of lung adenocarcinoma in never-smokers who were exposed to various degrees of residential radon. We also investigated the association between tumor mutation burden (TMB) and radon exposure.

2. Materials and methods

2.1. Patients and data collection

A total of 439 patients were included in the epidemiology study of radon, and completed the questionnaire. For this study, the inclusion criteria of patients were as follows: 1) diagnosed with lung adenocarcinoma, 2) never-smokers, 3) available tissue for next-generation sequencing, and 4) patients lived in the same house where the radon was measured for at least 2 years before the onset of lung cancer. Among 439 patients, 54 patients agreed on next-generation sequencing analysis, but 13 patients were excluded from the final analysis due to the following reasons: smoking history, lack of radon measurement, no matched blood sample or filtered due to low quality of DNA (Supplementary Fig. 1). Tumor tissues (somatic) and matched normal DNA (germline) were collected from prospectively recruited patients with the approval of the Institutional Review Board (IRB) of two institutions (Yonsei University and Ajou University) in Korea. Clinicopathological data were collected from patient charts in accordance with the IRB-approved protocol. Clinical, demographic and lifestyle information including age, sex, tobacco use, job history, clinical stage, treatment history, and survival data were collected (as of June, 2018). Histological analysis was performed by a board-certified pathologist (H.S.S) according to AJCC TNM 8th edition, and they reselected the relationship between radon and lung cancer levels.

2.2. Measurement of indoor radon levels

Between October 28, 2015 and May 30, 2016, indoor radon levels were measured at two sites in each house of subjects in the study population. Alpha-track detectors (Raduet Model RSV-8, Radosys Ltd., Budapest, Hungary) were used as a passive radon measuring device. The average concentration of radon in the indoor air was calculated from two points within the home. The measurement points were selected from the living room and a bedroom, spaces where residents of a house primarily spend most of their time. The measuring devices were positioned away from household electrical appliances, windows, and sealed drawers. The measurement duration was 3 months in each house. After estimating indoor radon levels, we divided the participants into radon-high and radon-low groups based on the median radon level (48 Bq/ m³).

2.3. Targeted sequencing of tumors

Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) samples using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) for the targeted sequencing of 244 cancer-related genes (SI Table). The genomic regions of the 244 genes were captured using the customized SureSelectXT Target Enrichment library generation kit (Agilent, Santa Clara, CA, USA), and sequenced using the Illumina HiSeq 2500 platform with an average depth of coverage 1000× magnification. The median value of estimated tumor purity was 39% among 20 samples with available copy number profiles using THetA algorithm [14].

2.4. Variant calling and functional annotation

Base quality trimming for short reads from the targeted sequencing was performed using Sickle [15] with the default parameters. Filtered reads were mapped to the human reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner (BWA) [16]. All reads mapped with < 20 mapping quality were discarded. The aligned reads were further processed using Genome Analysis Toolkit v3.5, including MarkDuplicates, local realignment, and base quality score recalibration [17]. The germline variants were called using the GATK HaplotypeCaller. Poor-quality variants were filtered using GATK VariantFilter and discarded using the following criteria: reads depth (DP) < 20, quality by depth (QD) < 2.0, Fisher strand (FS) > 60.0, root mean square mapping quality (MQ) < 20.0, MQRankSum < -12.5, ReadPosRankSum < -8.0.

The somatic variants and insertions/deletions were called using MuTect [18] and Scalpel [19], respectively with the default parameters. In the somatic mutation calling, FoxoG sequencing artifacts were removed using IGSB oxoG tools (https://github.com/migbro/IGSB_oxoG_tools) to discard skewed read-orientation variants with the 0.625 FoxoG parameter. The FoxoG parameter was the cutoff point that was 3 standard deviations away from the mean of all FoxoG ratios in samples with the hyper FoxoG artifact. Additionally, DKFZBiasFilter (https://github.com/eilslabs/DKFZBiasFilter) was used to reduce FoxoG and FFPE sequencing artifacts. The variants were annotated using ANNOVAR [20] including the consequences, predicted impacts and reported allele frequencies in the population. In particular, non-rare germline variants (minor allele frequency > 0.05) were discarded to obtain pathogenic variants using the Genome Aggregation Database (gnomAD) [21].

To visualize the overall landscape of mutations, Oncoprint was drawn using maftools [22]. In addition, a Lollipop plot was drawn for frequently mutated genes to determine the recurrence of genomic loci with variants. Mutational signatures were measured using deconstructSigs [23]. The inference of cancer progression model was built using the translation oncology (TRONCO) software [24] with bootstrap (P < 0.05) and Cancer Progression Inference (CAPRI) methods. Protein-protein interactions (PPI) and their functions were analyzed using STRING with the default parameters [25]. In addition, functional enrichment analysis was performed using ToppGene Suite [26].

2.5. Statistical methods

All statistical analyses were performed using the R, Python (Scipy,
Seaborn packages) and SPSS ver. 23.0 (IBM Corp., Armonk, NY, USA) software. To test the group-specific enrichment of germline variants, a one-sided Fisher’s exact test was applied to each called variant, followed by the \( P \)-value cutoff of 0.05. The TMB was measured based on the number of somatic missense mutations per megabase in the range of the targeted capture region. The number of missense mutations per megabase between radon-high and radon-low groups were compared using a one-sided Student’s \( t \)-test and Welch’s \( t \)-test. In addition, Levene’s test was performed to assess the equality of variances for the assumption of the \( t \)-test. The recurrence-free survival (RFS) and overall survival (OS) were estimated using the Cox regression. The function “coxph” in R was used to fit the Cox regression model to the data.

### 3. Results

#### 3.1. Patients

A total of 41 patients were included and the median duration of residence in the house where the measurements were taken was 13.5 years (range, 3–50). The clinical data of all patients are summarized in Table 1. All patients were never-smokers with adenocarcinoma. Most patients had early stage lung cancer at their initial diagnosis, and 28 (68%) patients with stage I–II disease. Twenty-seven (66%) patients had *EGFR* activating mutation, one (2%) had *ALK* fusion, and the remaining thirteen (32%) had *EGFR/ALK/ROS1* wild type. Furthermore, 41 patients received surgery for lung cancer, and among them fourteen (34%) experienced recurrence after surgery. The most common recurrence site was the lung (57%), followed by the brain (28%), bone (21%) and liver (14%). Comparison between radon-high vs. radon-low group revealed no significant difference in clinical parameters. For the whole cohort, the median RFS was 46 months (95% confidence interval [CI], 27–62 months) and OS was not reached at the time of data cutoff. We also investigated the prevalence of occupational and environmental exposure to carcinogens since they are associated with lung cancer occurrence [27]. Occupational risk factors such as metal workers,

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Radon-high group (N = 24)</th>
<th>Radon-low group (N = 17)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex F</td>
<td>22 (92%)</td>
<td>16 (94%)</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>2 (8%)</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>Age (years), median (range)</td>
<td>70 (43-79)</td>
<td>65 (54-83)</td>
<td></td>
</tr>
<tr>
<td>Stage at initial diagnosis I-II</td>
<td>15 (63%)</td>
<td>13 (76%)</td>
<td>0.4986</td>
</tr>
<tr>
<td>III-IV</td>
<td>9 (37%)</td>
<td>4 (23%)</td>
<td>1</td>
</tr>
<tr>
<td>Molecular subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR mutation</td>
<td>16 (67%)</td>
<td>11 (65%)</td>
<td>1</td>
</tr>
<tr>
<td>ALK fusion</td>
<td>1 (4%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ROS1 fusion</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>EGFR/ALK/ROS1 wild type</td>
<td>7 (29%)</td>
<td>6 (35%)</td>
<td></td>
</tr>
<tr>
<td>Surgical resection</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Yes</td>
<td>24 (100%)</td>
<td>17 (100%)</td>
<td>0.7417</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Recurrence after lung surgery Yes</td>
<td>9 (38%)</td>
<td>5 (29%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>15 (62%)</td>
<td>12 (71%)</td>
<td></td>
</tr>
<tr>
<td>Recur site lung</td>
<td>4 (44%)</td>
<td>4 (24%)</td>
<td></td>
</tr>
<tr>
<td>bone</td>
<td>2 (22%)</td>
<td>1 (6%)</td>
<td></td>
</tr>
<tr>
<td>brain</td>
<td>2 (22%)</td>
<td>2 (12%)</td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>2 (22%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>lymph node</td>
<td>1 (11%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Number of hours spent at home ≤ 12 h</td>
<td>14 (58%)</td>
<td>9 (53%)</td>
<td>0.7598</td>
</tr>
<tr>
<td>&gt; 12 h</td>
<td>10 (42%)</td>
<td>8 (47%)</td>
<td></td>
</tr>
<tr>
<td>Area of residence Urban</td>
<td>15 (62%)</td>
<td>10 (58%)</td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>9 (38%)</td>
<td>7 (42%)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Somatic mutational spectra of never-smoker patients with lung cancer detected using targeted sequencing. Samples with ≥ 5% incidence of genetic alterations are shown, stratified by level of radon exposure (high vs. low). Upper bar graph shows number of mutations per sample.
mining, asbestos production and construction workers were not found in our study population. The area of residence, either urban or rural, and number of hours spent at home were also investigated. There were no significant differences between radon-high vs. radon-low groups.

3.2. Overview of somatic mutations

A total of 175 non-silent somatic single nucleotide variants (SNVs) and 45 indels were identified from the targeted sequencing of 41 patients, which corresponded to a rate of 3.8 SNVs per 1Mb (Fig. 1). **EGFR** (73%) was the most commonly mutated gene, followed by tumor protein p53 (TP53, 27%); phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (PIK3CA, 12%); chromodomeline helicase DNA-binding protein 7 (CHD7I, 10%); FAT atypical cadherin 1 (FAT1, 10%); and switch/sucrose non-fermentable (SWI/SNF)-related, matrix-associated, actin dependent regulator of chromatin, subfamily a, member 2 (SMARCA2, 10%). We found no differences in radon concentrations between patients with and without **EGFR** mutations.

3.3. Comparison of radon-high vs. radon-low groups Radon concentrations

To investigate the effect of radon exposure on lung cancer, we categorized patients into two groups based on the median exposure value: radon-high (> 48 Bq/m3) vs. radon-low (≤ 48 Bq/m3) group. We then compared the TMB per megabase between both groups. As shown in Fig. 2A, the radon-high group showed a significantly higher TMB than radon-low group (one-sided Welch’s t-test, P = 0.01). The mean TMB/megabase was 4.94 Mb in the radon-high vs. 2.62 Mb in the radon-low group. On multivariate analysis, TMB was an important variable in discriminating radon-high vs. radon-low group, after age (**Supplementary Fig. 2**). A comparison of the TMB/megabase between early I–II vs. late (III-IV) stage lung cancer did not show any statistical difference (**Supplementary Fig. 3**). We also examined whether high TMB/megabase was associated with poor prognosis (**Supplementary Fig. 4**). After adjusting for stage, the recurrence rates between radon-high and radon-low groups did not differ statistically. Next, we investigated whether different mutation types were present by examining mutational signatures of tumors. Comparison of mutation signature in both groups showed that both were enriched with a C > T change (Fig. 2B). Closers observation of the mutational signatures revealed that the radon-high and radon-low groups exhibited different proportions of each signature. Signatures U2, 2, and 6 were identified in the radon-high group, whereas signatures U1, 1 A, 20, and 19 were identified in the radon-low group (Fig. 2C). Signature 2 is associated with activity of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family, which converts cytidine to uracil, coupled to the activity of the base excision repair and DNA replication machineries [16]. Signature 6 contributes very large numbers of substitutions and small indels, and this pattern is strongly associated with the inactivation of DNA mismatch repair genes [28].

To investigate the mechanisms of the hypermutability of radon-high tumors, we examined the evolutionary trajectories of clonal expansion of these tumors. Fig. 3A depicts the tumor evolutionary trajectories through combinations of mutually exclusive and concurrent mutations observed in radon-high tumors. We noted an enrichment of genes (ToppFun, P < 0.01) involved in DNA damage and repair, marked in red (ATR, ATRX, BRCA1 associated RING domain 1 (BARD1), RAD50, SMARCA4, histone deacetylase 2 (HDAC2), BRCA2, E1 A binding protein p300 (EP300), CDK2A, RB transcriptional coressor 1 (RB1), and inhibitor of nuclear factor kappa B kinase subunit epsilon (IKKBE)). **EGFR**-mutated clones in the radon-high group were expanded to **TP53**-mutated clones, following subclonal expansion of DNA repair and damage-associated mutations. In contrast to radon-high tumors, radon-low tumors did not exhibit abundant mutated clones associated with DNA repair and damage, although **EGFR** and **TP53** mutations were observed (**Supplementary Fig. 5**). We also investigated PPI in radon-high tumors, which revealed significant PPI between genes involved in DNA damage and repair (PPI enrichment, P < 0.001, Fig. 3B). Altogether, we concluded that the hypermutability processes of radon-high tumors were not just random events such as sequencing error, but were a series of mutagenesis induced by radon exposure.

We also investigated the germline mutational landscape of all patients as illustrated in Fig. 4A. Among the germline variants, **FAT1** mutation (73%) was the most commonly identified, followed by lysine methyltransferase 2C (KMT2C, 68%), **FAT4** (68%), and reelin (RELN, 46%), cystic fibrosis transmembrane conductance regulator (CFTR, 46%) mutations in increasing order of frequency. Interestingly, **NOTCH2** mutations were exclusively identified in radon-high tumors of 8 of 20 patients (40%, one-sided Fisher’s exact test, P = 0.001). **NOTCH2** I681 N, R1260H, and I1689 F mutations were identified as shown in the lollipop plot in Fig. 4B. **NOTCH2** R1260H mutations and I1689 F mutations were recurrently identified, although their significance remains unclear.

4. Discussion

Residential radon is a well-known important risk factor for lung cancer [29], both in smokers and never-smokers. At the beginning of our study, we assumed that the role of radon may be greater in lung cancer in never-smokers than in smokers, and we discovered that residential radon exposure was associated with higher TMB in our never-smoker population. Patients in the radon-high group showed a significantly higher number of nonsynonymous mutations than those in the radon-low group did. We also established the mutational signature of radon, which resembled cancers with defective DNA mismatch repair.

Radon is a radioactive gas, which is generated naturally by the collapse of uranium in soils and rocks [30]. Radon can decay into active progenies containing electric charge and can be inhaled into the bronchus when combined with natural aerosols. In lung epithelial cells, radon progenies can produce alpha-particles, resulting in DNA damage through double-strand breaks and chromosomal alterations [30,31]. Additionally, reactive oxygen species (ROS) from radon exposure may increase oxidative stress, which provoke chronic inflammatory processes [32]. During inflammation, enhanced ROS production may induce recurring DNA damage, and inhibition of apoptosis. Therefore, it is conceivable that exposure to residential radon may predispose individuals to lung cancer with high TMB.

TMB was observed in patients in the radon-group at 4.94/Mb, which was considerably higher that of the **EGFR**-mutant population reported previously [33]. Offin et al. recently reported a TMB of 3.77 mutations/megabase in **EGFR**-mutant NSCLC. Considering that **EGFR**-mutant population constitutes most of patient population (74%), we presumed that exposure to high levels of radon resulted in elevated the TMB. We also found that **TP53** mutation was more common in those with high-radon exposure than in those with low exposure, and that the high-radon group showed an unfavorable RFS. **TP53** mutation is a well-known indicator of poor prognosis in NSCLC [34], if the mutations are disruptive. The association between radon exposure and **TP53** mutation is still unclear, but similarly a previous epidemiological study also reported that residential exposure to radon seems to contribute to **TP53** gene mutation [35].

TMB is an emerging predictive biomarker for responses to immune checkpoint blockers (ICBs) in patients with NSCLC. The phase III trial evaluating combinations of the programmed cell death protein 1 (PD-1) inhibitor nivolumab, the CTLA4 inhibitor ipilimumab, and chemotherapy as first-line treatments in newly diagnosed advanced NSCLC, revealed that the two drugs were more effective than chemotherapy in patients with a high TMB, suggesting that TMB could be used as a predictive biomarker in patients who receive upfront immunotherapy [36]. It may be tempting to consider that the findings of this study, which revealed increased TMB in patients exposed to high-
Fig. 2. A. Median tumor mutation burden (TMB) of radon-high group was 4.94 vs. 2.62 mutations/Mb in radon-low group ($P = 0.01$).

B. Comparison of mutation signatures between radon-high vs. radon-low groups. There were six classes of base substitution – C > A, C > G, C > T, T > A, T > C, and T > G, and we incorporated information on the bases located immediately 5’ to 3’ to each mutated base to obtain 96 possible mutations.

C. Proportions of selected mutational signatures in radon-high and radon-low groups.
Fig. 3. A. Tumor evolutionary trajectories through combinations of mutually exclusive and concurrent mutations observed in radon-high tumors. Genes in red circles represent genes that are involved in DNA damage and repair (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

B. Protein-protein interaction (PPI) in radon-high tumors, showing a significant PPI of genes involved in DNA damage and repair.
radon levels, might indicate that ICBs could be more effective in these patients. However, the effective immunogenicity of mutations needs to be assessed [37], and the absolute cutoff of high TMB remains to be established.

**NOTCH2** germline mutations were exclusively identified in radon-high tumors, present in 8 of 20 patients (40%). Notch signaling pathways play a key role in the development of cancer, as they target stem cell populations, organ morphogenesis and homeostasis [38]. Crosstalk between the Notch and p53 pathways have been reported, in which Notch signaling regulates p53 activity, which in turn, also regulates Notch, in positive or negative feedback loops. Although **NOTCH** somatic mutations have been reported in various cancers, the role of germline **NOTCH2** mutations remains unclear.

Regarding the cut-off dose of radon exposure, we adopted the median value (48 Bq/m³) in our cohort in defining radon-high vs. radon-low groups. Different studies have adopted different cut-offs of defining radon-high group. Choi et al. divided subjects into two groups with the cutoff value of 100 Bq/m³ (5), and Elio et al. used the reference value of 200 Bq/m³ in defining lung cancer risk [39]. However, as there is no absolute safe level of radon exposure, and low radon concentration may also cause adverse effects, future large-scaled studies are necessary to more accurately define radon-related lung cancer risks.

A few limitations, mainly associated with the small study sample size, should be considered when interpreting the results of this study. The small sample size makes it difficult to generalize the results and

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**Fig. 4.**

A. Germline mutational spectra in never-smoker patients with lung cancer detected using targeted sequencing.

B. A lollipop diagram of **NOTCH2** germline mutations and their occurring loci.
likely contributed to the large standard errors, leading to the wide CIs and high P-values. Additionally, it would be difficult to conduct a prospective validation study based on our results in patients. In vivo studies may enhance our understanding of the effects of radon on lung cancer development and the mutational landscape. Another limitation is the inclusion criteria of at least 2 years of residence where the radon was measured. Previous literature suggested that it takes at least 5 years to observe a change of lung cancer risk due to radon exposure [40]. Even though the inclusion criteria was a minimum of 2 years in our study, the median duration of residence was 13 years (range 3–38 years) and we adopted a more accurate measurement method of indoor radon by assessing the residence, the environment around residence, and lifestyles of the residents [41].

In conclusion, we discovered that cumulative radon exposure may contribute to increased TMB in never-smoker patients with lung cancer, and the mutational signature was associated with defective DNA mismatch repair. Our findings further increase the awareness of indoor radon exposure as an important risk of lung cancer detected in never-smokers.

Acknowledgement

The biospecimens and data used for this study were provided by the Biobank of Ajou University Hospital, a member of Korea Biobank Network.

Funding

This study was supported by the Korean Ministry of Environment as part of the “Environmental Health Action Program” (grant number 2015001350002) and supported by a grant from the National research foundation (NRF), Republic of Korea (NRF-2016R1C1B1013299, NRF-2017M3A9E9072669, NRF-2017R1D1A1B03029874).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.04.002.

References