Analysis of lung cancer-related genetic changes in long-term and low-dose polyhexamethylene guanidine phosphate (PHMG-p) treated human pulmonary alveolar epithelial cells

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Abstract

Background: Lung injury elicited by respiratory exposure to humidifier disinfectants (HDs) is known as HD-associated lung injury (HDLI). Current elucidation of the molecular mechanisms related to HDLI is mostly restricted to fibrotic and inflammatory lung diseases. In our previous report, we found that lung tumors were caused by intratracheal instillation of polyhexamethylene guanidine phosphate (PHMG-p) in a rat model. However, the lung cancer-related genetic changes concomitant with the development of these lung tumors have not yet been fully defined. We aimed to discover the effect of long-term exposure of PHMG-p on normal human lung alveolar cells.

Methods: We investigated whether PHMG-p could increase distorted homeostasis of oncogenes and tumor-suppressor genes, with long-term and low-dose treatment, in human pulmonary alveolar epithelial cells (HPAECs). Total RNA sequencing was performed with cells continuously treated with PHMG-p and harvested after 35 days.

Results: After PHMG-p treatment, genes with transcriptional expression changes of more than 2.0-fold or less than 0.5-fold were identified. Within 10 days of exposure, 2 protein-coding and 5 non-coding genes were selected, whereas in the group treated for 27–35 days, 24 protein-coding and 5 non-coding genes were identified. Furthermore, in the long-term treatment group, 11 of the 15 upregulated genes and 9 of the 14 downregulated genes were reported as oncogenes and tumor suppressor genes in lung cancer, respectively. We also found that 10 genes of the selected 24 protein-coding genes were clinically significant in lung adenocarcinoma patients.

Conclusions: Our findings demonstrate that long-term exposure of human pulmonary normal alveolar cells to low-dose PHMG-p caused genetic changes, mainly in lung cancer-associated genes, in a time-dependent manner.

Keywords: Polyhexamethylene guanidine phosphate, Humidifier disinfectant, Human pulmonary alveolar epithelial cells, Total RNA sequencing, Lung cancer related genes

Background

In Korea, humidifier disinfectants (HDs) have become a national concern. Epidemiological investigations and medical and biological research have revealed that polyhexamethylene guanidine phosphate (PHMG-p),...
a main constituent of HD, is highly correlated with inflammatory lung fibrosis. These concomitant lung injuries following respiratory exposure to HDs are known as HD-associated lung injuries (HDLI) [1–3]. Although there are many reports on the association between PHMG-p and clinical manifestations, including lung fibrosis and inflammation, there are few long-term and low-dose studies on its carcinogenic potential. There is convincing evidence that studies are needed to elucidate the relationship between PHMG-p and carcinogenesis. First, in our previous studies, the possibility of tumorigenesis in PHMG-p-instilled rat lung was confirmed using computed tomography (CT) image analysis and an elevated expression of several cancer-related genes [4, 5]. Indeed, in our recent 52-week follow-up study on PHMG-p toxicity, we suggested the possibility of PHMG-p as a lung carcinogen by confirming that PHMG-p causes squamous cell carcinoma in the rat lung [6]. Second, although no specific pattern was observed in the relationship between PHMG and malignant neoplasms, it has been reported that the difference in the effect marginally appears in some infants, within the malignant neoplasms of the digestive tract, respiratory and intrathoracic organs, and leukemia [7]. Third, it is reported that polyhexamethylene biguanide (PHMB), which is structurally similar to PHMG, develops angiosarcoma in the liver during oral exposure [8].

Although the cancer-associated pathways affected by PHMG-p treatment have already been elucidated in a human alveolar A549 cell line, which originates from lung carcinoma, the fact that the research focus was on the epithelial to mesenchymal transition (EMT) and that the in vitro research was conducted in lung adenocarcinoma cells, not by normal cells, might be considered limitations in terms of carcinogenesis research [9, 10]. To overcome these limitations, we introduced human pulmonary alveolar epithelial cells (HPAEpiCs) consisting of type I and type II alveolar cells that occupy more than 99% of the internal surface area of the lung. If the cytotoxicity of PHMG-p itself causes acute cell death, long-term subculture cannot be conducted. Therefore, the cytotoxicity of each concentration was measured over a wide range of concentrations and terms, and a concentration that did not cause superficial damage to cells was set and applied to long-term HPAEpiCs sub-culture.

In this study, we showed that PHMG-p induced changes in the transcriptional expression of oncogenes and tumor suppressor genes in terms of lung carcinogenesis in human pulmonary alveolar normal cells through the analysis of total RNA sequencing data. In addition, we designed this study to secure the reliability of candidate genes by comparing three sets of the PHMG-p short-term treatment group and three sets of the long-term treatment group, either individually or together.

Methods
Reagents.
Polyhexamethylene guanidine phosphate (PHMG-p) was purchased from BOC Sciences (NY, USA) with CAS registry number 89697–78–9. Cell Counting kit-8 (CCK-8) reagent was obtained from Dojindo (Kumamoto, Japan). TRIzol™ reagent (#15,596,026) and DEPC-treated water (AM9906) were purchased from Thermo Fisher Scientific (MA, USA). Chloroform (C2432-25ML), ethyl alcohol (E7023-1L) and 2-Propanol (278,475-250ML) were obtained from Sigma-Aldrich (MO, USA). TaqMan microRNA reverse transcription kit (#4,366,596) and TaqMan™ universal PCR master mix (#4,304,437) were purchased from Applied Biosystems (MA, USA).

Cell culture and treatment.
Human pulmonary alveolar epithelial cells (HPAEpiC, #3200; ScienCell Research Laboratories Inc., CA, USA) were maintained in alveolar epithelial cell medium (EpiCM, #3201; ScienCell) supplemented with epithelial cell growth supplement (EpiCGS, #4152; ScienCell) and 2% (v/v) fetal bovine serum (FBS, #0010; ScienCell), and 1% (v/v) antibiotic solution (P/S, #0503; ScienCell) at 37 °C under saturated humidity in 5% CO2. The PHMG-p stock solution was diluted in the culture medium and used at a final concentration of 1 µg/mL, and sub-cultured and maintained in the set condition in which PHMG-p was present for 35 days.

Cell viability assessment.
HPAEpiCs were incubated in 96-well plates overnight at approximately 80% confluence and treated with PHMG-p (0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 µg/mL) for 24, 48, and 72 h. CCK-8 reagent (Dojindo, Japan) was used to evaluate cytotoxicity under PHMG-p-containing conditions according to the manufacturer’s instructions. The absorbance of the processed solution containing cells was measured at 450 nm using a microplate reader (SpectraMax M2e; Bucher Biotec, Basel, Switzerland).

RNA isolation.
Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions. RNA quality assessment was performed using an Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, Netherlands), and RNA samples were quantified using an ND-2000 spectrophotometer (Thermo Inc. DE, USA).
RT-qPCR analysis.
Isolated RNA was used as a template to synthesize cDNA using the amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, TX, USA). To quantify mRNA levels, real-time quantitative polymerase chain reaction (RT-qPCR) was performed using Power SYBR® Green PCR Master Mix from Applied Biosystems. To assess the downregulated genes in PHMG-p long-term treated samples, GAPDH mRNA was used as the loading control.

Library preparation and sequencing.
Libraries were prepared from total RNA using the NEBNext Ultra™ II Directional RNA-Seq Kit (NEW ENGLAND BioLabs Inc., MA, USA). Ribosomal RNA was eliminated using the RiboCop rRNA depletion kit from LEXOGEN Inc. (Vienna, Austria). RNAs that do not contain rRNA were used for cDNA synthesis and shearing, following the manufacturer’s instructions. Indexing was carried out using Illumina indexes 1–12. The enrichment step was performed by PCR. Subsequently, libraries were verified using the Agilent 2100 Bioanalyzer (DNA High Sensitivity Kit) to assess the average fragment size. Quantification was performed using a library quantification kit by applying a StepOne Real-Time PCR System (Life Technologies Inc., CA, USA). High-throughput sequencing was performed as paired-end 100 sequencing using a NovaSeq 6000 (Illumina Inc., CA, USA).

Data analysis.
Quality control of the raw sequencing data was performed using FastQC [11]. Low-quality reads (< Q20) and adapters were eliminated using FASTX-Trimmer [12] and BBMap [13]. Then, the trimmed reads were mapped to the reference genome using TopHat [14]. Gene expression levels were estimated using fragments per kilobase per million reads (FPKM) by Cufflinks [15]. The FPKM values were normalized based on the quantile normalization method using EdgeR within R [16]. Data mining of mRNA expression profiling and graphic visualization, including Venn diagrams, was carried out using ExDEGA from Ebiogen Inc. (Seoul, Korea). Gene ontology analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources using input and output data files obtained from ExDEGA [17, 18]. A hierarchical clustering (HCL) map was created using MultiExperiment Viewer (MeV).

Kaplan–Meier plot analysis.
To determine the prognostic values of the ISG15, MMP1, TRPA1, KRT19, PLAU, FMO3, COL14A1, FMO2, TIMP3, and SLITRK6 mRNA, transcription levels were measured using a Kaplan–Meier (KM) plotter, an open-source database (www.kmplot.com) that consists of gene expression profiles and survival lifetime data of patients with lung adenocarcinoma. The analysis was performed on a total of 719 lung adenocarcinoma patients. Only the JetSet best probe set was used for analysis. The patients were divided into low (black line) and high (red line) expression levels using the optimal expression cutoff point based on the log-rank test and median split procedure. Other statistical results, including hazard ratios (HRs), 95% confidence intervals, and log-rank P values, were also calculated and presented using this database. Statistical significance was set at P < 0.05.

Statistical analyses.
All data were analyzed using GraphPad Prism v.5.0 (GraphPad Software, CA, USA) and are expressed as the mean ± standard deviation. Statistical significance was set at P < 0.05. The log-rank test was used to evaluate the survival differences.

Results
PHMG-p-induced cytotoxicity in HPAEpiCs.
To determine the long-term exposure concentration that is not superficially reactive, such as cell death by PHMG-p, cell viability assays were carried out in HPAEpiCs (Fig. 1). Cytotoxicity and cell viability assays were performed at 24 h, 48 h, and 72 h. The concentration range was set to under 8 ug/ml through a preliminary experiment. The viability of HPAEpiCs decreased in a time- and dose-dependent manner following PHMG-p exposure. The survival rate was 90% with PHMG-p at a concentration of 5 to 6 µg/mL at 24 h, but the concentration gradually decreased in a time-dependent manner, and with 2 µg/mL at 72 h, the viability was approximately 90%. In contrast, a higher susceptibility to PHMG-p was reported in lung-derivived IMR-90, A549, and BEAS-2B cells [19]. Taken together, HPAEpiCs constituting the internal surface area of the lung have lower susceptibility to PHMG-p treatment than the various lung-originated cells mentioned above, so it is a suitable model for identifying genetic changes that are not biased towards cell death signaling in terms of long-term PHMG-p toxicity. Considering the period for long-term sub-culture intervals with PHMG-p exposure, the maximum treatment...
concentration of 1 ug/ml, presenting cell viability of greater than 90% at 72 h, was selected.

**PHMG-p exposure time-dependently increased number of altered genes.**

To investigate the genetic changes between the six sets comprising the short-term treatment group (within 10 days from 4 days; consists of 3 sets; #1 vs #2, #3 vs #4, and #5 vs #6) and the long-term treatment group (within 35 days from 27 days; consists of 3 sets; #13 vs #14, #15 vs #16, and #17 vs #18), we performed subcultures for 35 days using medium containing PHMG-p at a concentration of 1 µg/mL (Fig. 2). When heatmaps were generated with genes whose transcriptional expression changed more than twofold or less than 0.5-fold in three sets per group, only MT1G and NARR were protein-coding genes in the short-term treatment group (Table 1). Although a total of 17 genes with altered expression were selected in the three sets (1 vs #2, #3 vs #4, and #5 vs #6) constituting the short-term treatment group, genes with altered expression in the set constituting the long-term treatment group had increased to 45 genes in total. We confirmed that there were 24 protein-coding genes (MMP1, IFI6, ISG15, MX1, CDKN1A, HMGA2, KRT19, PLAT, PLAU, IL-33, AK5, NT5E, TRPA1, HBG2, NDUFA4L2, HBG1, MGP, FMO2, FMO3, SLITRK6, COL14A1, TBX4, GPX3, and TIMP3) with consistently increasing and decreasing trends in the three sets. We found that
a significant number of genes were involved in multiple mechanisms related to lung cancer (Fig. 3a and Table 2). Of these genes, GO analysis indicated that in PHMG-p long-term treated HPAEpiCs, genes mainly involved in response to external stimuli and cell proliferation were upregulated. On the other hand, the genes involved in cell cycle arrest and negative regulation of the cell cycle were increased in response to short-term treatment with PHMG-p (Fig. 3b).

From the above results, to ensure higher reliability, we selected genes with a common tendency to increase or decrease in all sets, not in each set in the same group. The numbers of changed genes are shown in a Venn diagram (Fig. 4a), and the number of genes that changed in common within each of the three sets of the short-term and long-term treatment groups are shown graphically (Fig. 4b). When the PHMG-p exposure period was prolonged, the number of overlapping genes between the two sets in each group increased from 45 to 57 genes (1.27-fold), but the number of overlapping genes between the three sets increased from 7 to 29 genes (4.14-fold) (Fig. 4a and b). These results indicated that the 29 overlapping genes in all sets were, with a high probability, altered due to long-term exposure to PHMG-p.

Table 1 Overview of common gene expression level changes in PHMG-p short-term treated HPAEpiCs and their reported expression changes in lung cancer

| Gene symbol | Entrez ID | Description | Biotype | Fold change (FC)* | Expression | Reference(s) |
|-------------|-----------|-------------|---------|------------------|------------|--------------|
| SNORD95     | 619570    | small nucleolar RNA, C/D box 95 | snoRNA  | 6,000            | Up         | [20]         |
| MT1G        | 4495      | metallothionein 1G | protein coding | 6.15 | 3.92         | 2.93        | Up | [21, 22] |
| CDC37L1-AS1 | 101929351 | CDC37L1 antisense RNA 1 (head-to-head) | lncRNA  | 2.06            | 4.35       | 2.43         |                   |
| SNORA5A     | 654319    | small nucleolar RNA, H/ACA box 5A | snoRNA  | 35.0            | 1.06       | 62.7         |                   |
| SNORA7S     | 654321    | small nucleolar RNA, H/ACA box 75 | snoRNA  | 0.0328          | 0.0290     | 0.0533       | Up | [20, 24] |
| SNORA28     | 677811    | small nucleolar RNA, H/ACA box 28 | snoRNA  | 0.0367          | 0.0169     | 0.0105       |                   |
| NARR        | 100861437 | nine-amino acid residue-repeats | protein coding | 0.479 | 0.289        | 0.349       |                   |

(Dn: Down), (*: The FC value is provided as whole number, and the number of significant figures is specified as three.)
PHMG-p responsive genes associated with lung cancer
in PHMG-p-treated HPAEpiCs in a time-dependent manner.

We found that the expression of SNORD95 (small nucleolar RNA, C/D box 95) was significantly elevated in PHMG-p-exposed HPAEpiC samples (Fold change (FC), #2/#1: 6,000, #4/#3: 8,540, #6/#5: 8,720). Conversely, the expression of SNORA75 (small nucleolar RNA, H/ACA box 26 snoRNA), SNORA28 (small nucleolar RNA, H/ACA box 28), and NARR (nine-amino acid residue-repeat gene) was repressed in our results (FC, SNORA75, #2/#1: 0.0328, #4/#3: 0.0290, #6/#5: 0.0533; SNORA28, #2/#1: 0.0367, #4/#3: 0.0169, #6/#5: 0.0105; NARR, #2/#1: 0.479, #4/#3: 0.289, #6/#5: 0.349). MT1G (Metallothionein 1G) was upregulated in all three sets of short-term treatment groups (FC, #2/#1: 6.15, #4/#3: 3.92, #6/#5: 2.93) (Table 1). The expression levels of CDC37L1-AS1 and SNORA5A were increased (FC, CDC37L1-AS1, #2/#1: 2.06, #4/#3: 4.35, #6/#5: 2.43; SNORA5A, #2/#1: 35.0, #4/#3: 2.10, #6/#5: 62.7); however, the transcriptional levels of these genes showed a fluctuating pattern over time (Table 1). In contrast to the results of short-term PHMG-p treatment, in the long-term treatment, the
transcriptionally altered genes consisted of 24 protein-coding genes (IFI6, MX1, MMP1, ISG15, HMGA2, PLAT, KRT19, IL33, TRPA1, AK5, NT5E, PLAU, CDKN1A, TBX4, SLITRK6, TIMP3, COL14A1, FMO3, FMO2, GPX3, NDUFA4L2, HBG1, MGP, and HBG2), two lncRNAs (H19 and LOC103021295), 2 microRNAs (miR-3687–2 and miR-490), and 1 snoRNA (SNORA26), and most of them were identified as protein-coding genes. Of these genes, IFI6 and MX1 tended to increase serially (FC, IFI6, #14/#13: 2.23, #16/#15: 3.81, #18/#17: 7.01; MX1, #14/#13: 2.25, #16/#15: 4.14, #18/#17: 4.41) as the exposure time of PHMG-p increased (Table 2). Upregulated levels of MMP1, HMGA2, TRPA1 and PLAU (MMP1, #14/#13: 4.79, #16/#15: 4.79, #18/#17: 3.76; HMGA2, #14/#13: 2.27, #16/#15: 2.95, #18/#17: 3.20; TRPA1, #14/#13: 2.48, #16/#15: 2.33, #18/#17: 2.50; PLAU, #14/#13: 2.23, #16/#15: 2.93, #18/#17: 2.06) were identified, and the fold changes were increased by FC value. In the case of ISG15, COL14A1 and HBG2 (ISG15, #14/#13: 2.19, #16/#15: 4.72, #18/#17: 3.36; COL14A1, #14/#13: 0.432, #16/#15: 0.334, #18/#17: 0.448; HBG2, #14/#13: 0.160, #16/#15: 0.158, #18/#17: 0.245), they increased by FC value (Table 2). The anti-apoptotic ability of PLAT in non-small cell lung cancer (NSCLC) was increased by 2.87-, 3.25-, and 3.20-fold (FC) in sets #14/#13, #16/#15, and #18/#17, respectively. KRT19, which binds to the COOH-terminal domain of HER2 and activates HER2-Erk signaling, was upregulated in
It was confirmed that the altered fold change of 0.486, and the fold changes were decreased by FC value, #14/#13: 0.351, #16/#15: 0.174, #18/#17: 0.358; #14/#13: 0.291, #16/#15: 0.454, #18/#17: 0.358; #14/#13: 0.457, #16/#15: 0.295, #18/#17: 0.415; #14/#13: 0.463, #16/#15: 0.454, #18/#17: 0.470; #14/#13: 0.291, #16/#15: 0.295, #18/#17: 0.454; and #14/#13: 0.387; #16/#15: 0.351, #18/#17: 0.358). The transcriptional level of Tbx4 was decreased in all sets of the long-term treatment group (FC, #14/#13: 0.409, #16/#15: 0.384, #18/#17: 0.448). We also investigated the levels of Timp3, Fmo2, Hbg1, and nduFA4L2 (Timp3, #14/#13: 0.463, #16/#15: 0.454, #18/#17: 0.470; Fmo2, #14/#13: 0.457, #16/#15: 0.295, #18/#17: 0.415; Hbg1, #14/#13: 0.291, #16/#15: 0.454, #18/#17: 0.358; nduFA4L2, #14/#13: 0.351, #16/#15: 0.174, #18/#17: 0.486), and the fold changes were decreased by FC value (Table 2). It was confirmed that the altered fold change of non-coding RNAs (ncRNAs) is large scale (the absolute value of FC, from 2.01 to 10,900) compared to the degree of change in the protein-coding genes (the absolute value of FC, from 2.01 to 7.01) in the long-term treatment group. The genes whose expression was altered were Snora26, Mir3687-2 and Mir490 (Snora26, #14/#13: 75.6, #16/#15: 39.7, #18/#17: 38.6; Mir3687-2, #14/#13: 2.22, #16/#15: 10,900, #18/#17: 2.01; Mir490, #14/#13: 0.0412, #16/#15: 0.0163, #18/#17: 0.0237), all of which consist of ncRNAs. Mir3687-2 and Mir490 increased in all sets, but in the case of Mir3687-2, the FC value increased significantly (FC=10,900), especially in sets #16 and #15. In the case of Mir490, it was confirmed that it decreased significantly in all sets (Table 2).

**RT-qPCR validation of altered protein coding genes**

To validate the candidate genes selected according to the criteria (more than 2.0-fold or less than 0.5-fold) on total RNA sequencing, RT-qPCR was performed. To confirm the increased transcriptional level in upregulated candidates, the expression of Mxi1, Krt19, Hmga2, Isg15, il33, MMP1, Trpa1, Ifi6, Plau, Cdkn1a, Plat, Ak5, and Nt5e was determined by RT-qPCR. In particular, it was confirmed that Mxi1 significantly increased from 5.4-fold to less than 13.3-fold in the long-term treated sets (Fig. 5). Based on the total RNA sequencing results, Cdkn1a was not selected because it did not meet the criteria in the PHMG-p short-term treatment group (set of 4 days), but in this validation phase, it increased up to 6.7-fold in the short-term group (set of 10 days). As a result, it was confirmed that Cdkn1a increased 3.5-fold in the short-term treatment group and 3.3-fold in the long-term treatment group (Fig. 5). All selected protein-coding genes were found to be increased in the long-term treatment group (within 35 days from 27 days). Unlike other candidate genes, it has been reported that Ak5 expression was reduced due to methylation of the promoter region within the CpG islands in lung adenocarcinoma [45]. The downregulated expression of nduFA4L2, Col14A1, Slitrk6, Timp3, Fmo3, Hbg1, Mgp, Hbg2, Tbx4, Gpx3, and Fmo2 was also confirmed (Fig. 6). However, the basal expression of Col14A1 was very low, and the cycle threshold (Ct) value of RT-qPCR was not measurable; therefore, it was excluded from Fig. 6. In addition, contrary to the downregulated tendency, nduFA4L2 was overexpressed in human NSCLC, reported to occur under hypoxic conditions, one of the characteristics of cancer. Its overexpression is a key factor for maintaining NSCLC growth [69]. The tendency of transcriptional alteration of all candidate genes in RT-qPCR was 100% consistent with the total RNA sequencing results because the candidate genes were selected based on matching in all three sets (#13 vs #14, #15 vs #16, and #17 vs #18).

**Clinical significance of Isg15, Mmp1, Trpa1, Krt19, Fmo3, Col14A1, Fmo2 and Timp3 in patients with lung adenocarcinoma**

To estimate the survival function of Isg15, Mmp1, Trpa1, Krt19, and Plau, we performed KM plotter analysis generated for groups of lung adenocarcinoma patients based on their expression levels. As indicated in Fig. 7a and S1a, lung adenocarcinoma patients with high expression levels of Isg15, Mmp1, Trpa1, Krt19, and Plau (red line) were significantly associated with poor survival rates (log rank P value: 2.3e-07, 0.00034, 0.00037, 0.00057, and 0.023, respectively) as compared to those with low expression (black line). In addition to the results of increased gene expression, downregulated Fmo3, Col14A1, Fmo2, Timp3, and Slitrk6 also showed a poor survival rate (log rank P value: <1.0e-16, 2.2e-10, 2.3e-0.8, 0.00016, and 0.0042, respectively) compared to their high expression (Figs. 7b and S1b Figure). Hazard ratio (HR) scores of the five upregulated genes were 1.86 (1.47 · 2.37), 1.53 (1.21 · 1.94), 1.52 (1.21 · 1.92), 1.51 (1.19 · 1.92), and 1.31 (1.04 · 1.65), respectively. Also, HR scores of five downregulated genes were 0.36 (0.28 to 0.46), 0.45 (0.35 to 0.58), 0.50 (0.39 to 0.64), 0.64 (0.50 to 0.81), and 0.70 (0.55 to 0.90). These data indicate that upregulated transcriptional levels of Isg15, Mmp1, Trpa1, Krt19, and Plau and decreased expression of Fmo3, Col14A1, Fmo2, Timp3, and Slitrk6 are highly associated with unfavorable overall survival of lung adenocarcinoma patients.
Discussion

In this study, we confirmed the distorted homeostasis of oncogenes and tumor suppressor genes that change in a time-dependent manner by PHMG-p in normal human lung alveolar cells. Previous in vitro studies have investigated the effect of PHMG-p exposure on lung cells and (i) focused on fibrotic inflammation, (ii) used immortalized cells or lung cells derived from malignant tumors, and (iii) identified a molecular mechanism that changes with short-term PHMG-p treatment [2, 9]. To overcome these limitations, we (i) focused on carcinogenesis-related genetic changes, (ii) introduced human type I and II normal alveolar cells that make up most of the inner surface of the lungs, and (iii) obtained results through a long-term exposure procedure. In the case of RNA sequencing, instead of repeating the experiment with same conditions, 3 groups were selected from short-term treatment and 3 groups from long-term treatment to increase

Fig. 5 RT-qPCR validation for upregulated protein coding genes in the long-term treatment group. To validate the upregulated candidate genes on total RNA sequencing, the expression of MX1, KRT19, HMGA2, ISG15, IL33, MMP1, TRPA1, IFI6, PLAU, CDKN1A, PLAT, AK5 and NT5E was determined by RT-qPCR. All selected genes were verified to be increased in the long-term treatment group (within 35 days from 27 days). All experiments were performed 3 times with technical replicates.
the reliability of this research. Moreover, each gene in the Venn diagram of Fig. 4 was selected by reflecting the fold change, p-value, and normalized data (log2) values rather than reflecting only a single variable. Therefore, it is likely that the differences in the selected genes between each group will be increase. However, we think that the number of genes that change in common during long-term culture is more reliable because they were commonly selected despite applying 3 variables mentioned above. In addition, we suggested the possibility that PHMG-p can increase lung carcinogenesis by performing GO, heatmap cluster, differentially expressed gene (DEG) analysis including pre-mRNA, lncRNA, and miRNA, and KM plotter analysis with selected genes.

Within 10 days of exposure to PHMG-p, two protein-coding genes and five non-coding genes were altered. In the PHMG-p group treated for 27–35 days, 24 protein-coding and 5 non-coding genes were identified in the PHMG-p group. Interestingly, in our selection criteria for candidate genes, the number of ncRNAs did not change, but the number of protein-coding genes increased by a factor of 12. In addition, it was found that the degree of change in the case of ncRNAs was largely compared to the degree of change in the protein-coding genes (Tables 1 and 2), but it is not clear whether this phenomenon is a transcriptional characteristic of ncRNA itself or a characteristic of reactivity in PHMG-p treatment.

To confirm whether the selected genes are related to lung cancer, we searched all published papers and also listed genes that contradict our hypothesis to eliminate bias. MT1G was upregulated in all three short-term treatment groups. Although the expression of MT1G in breast, thyroid cancer, and hepatocellular carcinoma was downregulated compared to that in non-cancerous tissue, it has been reported that the expression of MT1G in NSCLC is higher than that in non-malignant lung tissues [21, 77–79]. Furthermore, MT1G was enriched in the most aggressive large-cell lung carcinoma, and high expression of MT1G correlated with poor prognostic values in 24 lung large-cell lung carcinomas [22].
However, only one group contended that the expression of MT1G was lower in lung cancer tissues than in peri-cancer tissues [23]. In a different trend from altered genes, CDKN1A mRNA gradually increased in the short-term treatment group and continuously decreased in the long-term treatment group. Inferring from the above results, the possibility exists that expression of CDKN1A was upregulated to inhibit cell growth in the short term, but it is thought that as many oncogenes increase, they lose their ability to regulate homeostasis with respect to growth. IL-33, a member of the IL-1 family that promotes the production of Th2-related cytokines, was increased in our study (Table 2). In addition, reports on the elevated expression levels of IL33 and NT5E have focused on NSCLC [40–42, 46–48]. Indeed, blockage of IL-33 is known to prevent the growth of NSCLC by inhibiting M2 macrophage polarization and reducing the accumulation of Treg cells in the tumor microenvironment [40]. NT5E is a novel target for the treatment of many cancer types, and various NT5E/CD73 inhibitors are currently being tested in clinical trials. In NSCLC, the level of NT5E, a target gene of miR-30a-5p, is increased due to decreased expression of miR-30a-5p. NT5E also contributes to the survival of NSCLC by inhibiting its function by trapping miR-134 [46, 48]. IFL6 and MX1 tended to increase serially as the exposure time of PHMG-p increased. The IFL6 protein plays a central role in resistance to apoptosis in various cancer types [80, 81], and MX1 protein levels are increased in lung adenocarcinoma [25]. Furthermore, it has been reported that MMP1, HMG2, TRPA1, and PLAU are highly expressed in various subtypes of lung cancer [26–28, 31–34, 43, 44, 49–51]. However, ISG15, COL14A1 and HBG2 are mainly decreased in lung adenocarcinoma [30, 64, 70, 71].

TBX4, which is involved in the regulation of embryonic developmental processes, is downregulated in lung cancer [56–58], and it was decreased in all sets of the long-term treatment group. TIMP3 is also known to play a role in inducing apoptosis and suppressing NSCLC growth [59, 60]. The COL14A1 promoter region was confirmed to be hypermethylated, with a probability of 60.4% in 48 NSCLC patient samples [64]. Although the role of FMO2, whose main function is an NADPH-dependent enzyme, in tumorigenesis is still unclear, it has been reported to play a role as a tumor suppressor in lung adenocarcinoma [65, 66]. GPX3, a scavenger of reactive oxygen species, is known to inhibit the growth, invasion, and migration of various lung cancer cells, including h157, h460, h1299, h1650 h1975, and A549 [67, 68]. HBGI1 and HBG2, the gamma globin genes, have been reported to have low expression in NSCLC, including adenocarcinoma and squamous cell carcinoma [70, 71]. MGP expression was
found to be reduced during the symptomatic illness stage in lung cancer [72].

In malignant tumors, there are reports of distorted homeostasis between oncogenes and tumor suppressor genes, including failure to regulate the expression levels of ncRNAs [82, 83]. Most of these ncRNAs cannot directly bind to ribosomes and can be translated into proteins, but they can interact with other coding genes or non-coding genes; by binding to several proteins, they affect the interactions between proteins and serve as sponges for microRNAs [84, 85]. The expression of SNORD95 was upregulated in PHMG-p-exposed samples, and it was also reported that SNORD95 was increased in 11 lung squamous cell carcinoma and 11 lung adenocarcinoma samples compared to their matched normal samples [86]. Conversely, the expression of SNORA75, SNORA28, and NARR was downregulated in our study. However, there are no previous reports that the three genes described above are decreased in lung cancer. In the case of MIR3687-2, the FC value was significantly increased, especially in the #16/#15 set. In the case of MIR490, a significant decrease was observed in all sets. Therefore, there is a need to find the putative target genes of miRNA-3687 and microRNA-490-3p and -5p. FGFR1 (fibroblast growth factor receptor-like 1), NCS1 (neuronal calcium sensor 1), UCN2 (urocortin 2), TMEM167B (transmembrane protein 167B), and CDR1 (cerebellar degeneration-related protein 1) are predicted targets of miR-3687. VDAC1 (voltage-dependent anion channel 1), TMOD3 (tropomodulin 3), COMMD10 (COMM domain containing 10), HNRNPA1 (heterogeneous nuclear ribonucleoprotein A1), PIK4K2B (phosphatidylinositol-5-phosphate 4-kinase, type II, beta) are putative targets of miR-490-3p. ZNF627 (zinc finger protein 627), TTC29 (tetratricopeptide repeat domain 29), BTC (betacellulin), CST8 (cystatin 8), and LILRA3 (leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3) are putative targets of miR-490-5p which were predicted in the microRNA target prediction programs, such as TargetScan and miRDB. Surprisingly, it was recently reported that among the two miRNAs that change in a PHMG-p-dependent manner, miR-3687 is increased in lung squamous cell carcinoma and miR-490 is decreased in lung squamous cell carcinoma and lung adenocarcinoma [52].

Furthermore, since the HPAEpiCs used in this research are known to consist of pulmonary alveolar type I (AT1) and alveolar type II (AT2), we identified AT1 and AT2 specific gene markers. AT1-specific genes, IGFBP2, CAV1, and CAV2, indicated a tendency to increase as the subculture days prolonged. Therefore, we inferred that AT2 cells in HPAEpiCs gradually differentiated into AT1 cells. In addition, considering the normalized data, we confirmed that most HPAEpiCs consisted of AT1 cells, and that AT2 cells were also included (Table S2) [87–92].

Our follow-up studies will be focused on delineating the transcriptional regulation of ncRNAs including microRNA, snoRNA, and lncRNA altered by PHMG-p, discovering their interacting genes. Also, there is a need to investigate the molecular mechanisms in human bronchial and tracheal epithelial cells that are not limited to alveolar epithelial cells but are primarily exposed upon inhalation of respirable particles containing PHMG-p.

Taken together, for the first time, we confirmed and validated the distorted regulation of repetitively altered genes in three experimental sets of PHMG-p long-term treatment groups using normal pulmonary alveolar cells, which constitute the majority of the internal surface of the lung. In addition, most of the altered genes are closely related to lung cancer and the survival rate of patients with lung adenocarcinoma.

**Conclusions.**

Based on our description, we suggest that PHMG-p, as the main gradient of humidifier disinfectants, has a carcinogenic potential in normal human lung alveolar cells in case of long-term exposure.

**Abbreviations**

PHMG-p: Polyhexamethylene guanidine phosphate; HDs: Humidifier disinfectants; HDLI: HD-associated lung injury; HPAEpiCs: Human pulmonary alveolar epithelial cells; CT: Computed tomography; GGO: Ground glass opacity; PHMB: Polyhexamethylene biguanide; EMT: Epithelial-to-mesenchymal transition; LncRNA: Long non-coding RNA; NcRNA: Non-coding RNA.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s40360-022-00559-5.

**Additional file 1. Supplementary Figure 1. Clinical significance of selected genes in patient with lung adenocarcinoma.**

**Additional file 2. Supplementary Table 1. Primers sequence**

**Additional file 3. Supplementary table 2. Evaluation of expression of AT1 and AT2 cell specific genes**

**Acknowledgements**

Not applicable

**Authors’ contributions**

H.L. conceptualized this study, analyzed the sequencing data, searched references to candidate genes, and prepared the original draft of this manuscript. J.L. supervised the study and acquired the funding source. S.H.J., C.K., E.P., and J.L. reviewed the study and edited the manuscript. H.L. performed RT-qPCR. Y.J.N., J.Y.K., M.O.S., J.Y.C., J.K., and Y.B. contributed to searching references to gene candidates involved in lung cancer. All authors have read and approved the final manuscript. All authors contributed to this research. (H.L.: Hong Lee, H.L.: Hyejin Lee.)

**Funding**

This study was supported by a grant from the National Institute of Environment Research (NIER), funded by the Ministry of Environment (MOE) of the Republic of Korea (grant number NIER-2021–04-03–001).
Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate.
Not applicable.

Consent for publication.
Not applicable.

Competing interests
The authors declare no competing financial interests.

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Received: 12 October 2021   Accepted: 21 March 2022
Published online: 30 March 2022

References
1. Kim HY, et al. Protective Effects of Nintedanib against Polyhexamethylene Guanidine Phosphate-Induced Lung Fibrosis in Mice. Molecules. 2018;23:8.
2. Song JA, et al. Polyhexamethyleneguanidine phosphate induces severe lung inflammation, fibrosis, and thymic atrophy. Food Chem Toxicol. 2014;69:267–75.
3. Song MK, DI DI Kim, K Lee. Kathon Induces Fibrotic Inflammation in Lungs: The First Animal Study Revealing a Causal Relationship between Humidifier Disinfectant Exposure and Eosinophil and Th2-Mediated Fibrosis Induction. Molecules. 2020;25(20):4684.
4. Jeong S-H, et al. MTF1 Is Essential for the Expression of MT1B, MT1F, and MT1H in the Human Pulmonary Alveolar Epithelial Cells. Toxins. 2021;9:203.
5. Kim C, et al. Evaluation of polyhexamethylene guanidine-induced lung injuries by chest CT, pathologic examination, and RNA sequencing in a rat model. Sci Rep. 2021;11:6318.
6. Kim C, et al. Evaluation of the long-term effect of polyhexamethylene guanidine phosphate in a rat lung model using conventional chest computed tomography with histopathologic analysis. PLoS ONE. 2021;16:e0256756.
7. Lim JH, et al. Study for improving recognition and judgement standard of health damage of humidifier disinfector (I), in National Institute of Environmental Research. 2018.
8. Kim S, Paek D. Humidifier disinfectant disaster: what is known and what needs to be clarified. Environ Health Toxicol. 2016;31:e2016025.
9. Jeong MH. Akt and Notch pathways mediate polyhexamethylene guanidine phosphate-induced epithelial-mesenchymal transition via ZEB2. Toxicol Appl Pharmacol. 2019;390:114691.
10. Park YJ, et al. Guanidine-based disinfectants, polyhexamethylene guanidine-phosphate (PHMG-P), polyhexamethylene biguanide (PHMB), and oligo(2-(2-ethoxethyl)ethoxyl) guanidinium chloride (PGH) induced epithelial-mesenchymal transition in A549 alveolar epithelial cells. Inhal Toxicol. 2019;31:161–6.
11. Simon A. <Simon, Andrews. FastQC. Babraham Bioinformatics. FastQC. Babraham Institute. last modified January 08 2019. Version 0.11.9 released, 2010.pdf> 2010.
12. Hannon. <Hannon Lab. FASTX toolkit. FASTQ A short-reads pre-processing tools. last modified February 02 2010. Version 0.0.13 released, (2014). pdf> 2014; Available from: http://hannonlab.cshl.edu/fastx_toolkit/.
13. Bushnell B. BBMap. BBMap short read aligner, and other bioinformatic tools. SourceFORGE. last modified August 11 2021. 2014; Available from: https://sourceforge.net/projects/bbmap/.
14. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009;25:1105–11.
15. Adam Roberts, CT, Donaghey Julie, Rinn John L. Improving RNA-Seq expression estimates by correcting for fragment bias. Genome Biology. 2011;12:R22.
16. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing. 2018: Vienna, Austria.
17. Huang da W, B.T. Sherman, R.A. Lempicki. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4:44–57.
18. Huang da, W., B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment pathways: tools path toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37:1–13.
19. Jung HJ, et al. Cytotoxicity and gene expression profiling of polyhexamethylene guanidine hydrochloride in human alveolar A549 cells. Toxicol In Vitro. 2014;28:684–92.
20. Mouriki NE, et al. snoRNAs Offer Novel Insight and Promising Perspectives for Lung Cancer Understanding. 2020; 9:3.
21. Werynska B, et al. Metallothionein 1F and 2A overexpression predicts poor outcome of non-small cell lung cancer patients. Exp Mol Pathol. 2019;104:301–8.
22. da Motta LL, et al. Oxidative stress associates with aggressiveness in lung large-cell carcinoma. Tumour Biol. 2015;36:4681–8.
23. Liang Gui-You, S.-X.L., Gang, Liu Xing-Da, Jian Li, Zhang Deng-Shen. Expression of metallocation and Hif2 pathway genes in lung cancer and cancer-surrounding tissues. World Journal of Surgical Oncology. 2013;11:199.
24. Gao L, et al. Genome-wide small nucleolar RNA expression analysis of lung cancer by next-generation deep sequencing. Int J Cancer. 2015;136:E623–9.
25. Hu Su-Chiung, Wu C, Hsieh Chuen, Wang Chih-Liang, Wu Yi Cheng, Wu Chih-Ching, Liu Chin-Ching, Yu Jau-Song, Chang Yu-Sun, Yu Chi-Jung. Identification and characterization of potential biomarkers by quantitative tissue proteomics of primary lung adenocarcinoma. The American Society for Biochemistry and Molecular Biology. 2016;15:2396–410.
26. Foley C, et al. Matrix metalloprotease 1 deficiency suppresses tumor growth and angiogenesis. Oncogene. 2014;33:2264–72.
27. Sauter W, et al. Matrix metalloproteinase 1 (MMP1) is associated with early-onset lung cancer. Cancer Epidemiol Biomarkers Prev. 2008;17:1127–35.
28. Gabasa M, et al. MMP1 drives tumor progression in large cell carcinoma of the lung through fibroblast senescence. Cancer Lett. 2021;507:1–12.
29. Mustachio Lisa Maria Mustachio, MX’Yun Lu, Rodriguez-Canales Jaime, Mino Barbara, Behrens Carmen, Wistuba Ignacio, Rabasedas Neus Bota, Yu Jun, Lee Jack, Roszik Jason, Zheng Lin, Liu Xi, J Sarah. Freemantle1 and Ethan Dmitrovsky1,2,7, The ISG15-specific protease USP18 regulates stability of PTEN. nctoaret. 2017:8:3–14.
30. Qu T, et al. ISG15 induces ESRP1 to inhibit lung adenocarcinoma progression. Cell Death Dis. 2020;11:511.
31. Gao X, et al. HMGA2 regulates lung cancer proliferation and metastasis. Thorac Cancer. 2017;8:501–10.
32. Mansoori B, et al. HMGA2 as a Critical Regulator in Cancer Development. Genes (Basel). 2021;12:22.
33. Meyer B, et al. HMGA2 overexpression in non-small cell lung cancer. Mol Carcinog. 2007;46:503–11.
34. Sarhadi VK, et al. Increased expression of high mobility group A proteins in lung cancer. J Pathol. 2006;209:206–12.
35. Yan F, et al. Knockdown of PLAT enhances the anticancer effect of gefitinib in non-small cell lung cancer. J Thorac Dis. 2020;12:712–23.
36. Lin CY, et al. ADAM9 promotes lung cancer progression through vascular remodeling by VEGFA, ANGPT2, and PLAT. Sci Rep. 2017;7:15108.
37. Wang W, et al. KRT8 and KRT19, associated with EMT, are hypomethylated and overexpressed in lung adenocarcinoma and link to unfavorable prognosis. Biosci Rep. 2020;40:7.
38. Yuan X, et al. Prognostic significance of KRT19 in Lung Squamous Cancer. J Cancer. 2021;12:1240–8.
39. Ohtsuka T, et al. Interaction of cytokeratin 19 head domain and HER2 in the cytoplasm leads to activation of HER2-Erk pathway. Sci Rep. 2016;6:39537.

40. Kailing Wang1, Shan Shan1 3,*, Zongjun Yang2, Xia Gu, Yuanyuan Wang, Chunchong Wang, Tao Ren. IL-33 blockade suppresses tumor growth of human lung cancer through direct and indirect pathways in a preclinical model. Oncotarget. 2017;8:68571–82.

41. Wang C, et al. IL-33 signaling fuels outgrowth and metastasis of human lung cancer. Biochem Biophys Res Commun. 2016;479:461–8.

42. Zhou X, et al. IL-33 Promotes the Growth of Non-Small-Cell Lung Cancer Cells Through Regulating miR-128-3p/CD1P Signalling Pathway: Cancer Manag Res. 2021;13:279–89.

43. Buch TRH, et al. Role of Chemosensory TRP Channels in Lung Cancer. Pharmaceuticals (Basel). 2018;11:44.

44. Schaefer EA, et al. Stimulation of the chemosensory TRPA1 cation channel by volatile toxic substances promotes cell survival of small cell lung cancer cells. Biochem Pharmacol. 2013;85:426–38.

45. Tessema M, et al. Concomitant promoter methylation of multiple genes in lung adenocarcinomas from current, former and never smokers. Carcinogenesis. 2009;30:1132–8.

46. Zhu J, et al. CD73/NT5E is a target of miR‑30a‑5p and plays an important role in the pathogenesis of non-small cell lung cancer. Mol Cell. 2017;61:34.

47. Yusuke Inoue KY. Nobuya Kurabe, Tomohiro Kihara, Akizawa Kusunaga, Matsuyaki Tanashashi, Hiroshi Ogawa, Naoki Inui, Kazuhiro Funai, Kazuya Shimura, Hiroshi Niwa, Takafumi Suda, Haruhiko Sugimura, Prognostic impact of CD73 and AZA adenosine receptor expression in non-small-cell lung cancer. Oncotarget. 2017;8:8738–51.

48. Lingyun Dong JZ. Yun Gao, Xiaoting Zhou, Weizhen Song, Jianan Huang, Chunzhou Chen, Weizhong Liu. The circular RNA NTSE promotes non-small cell lung cancer cell growth via sponging microRNA-134. Aging (Albany NY). 2020;12:3936–49.

49. Wang JM, et al. Gene expression profiling in patients with chronic obstructive pulmonary disease and lung cancer. Ann R Respir Crit Care Med. 2008;177:402–11.

50. Almasi CE, et al. The liberated domain I of urokinase plasminogen activator receptor—a new tumour marker in small cell lung cancer. APMS. 2013;121:189–96.

51. M Salden, T.A. WS, Peters HA, M. P, MP, Timmermans M, J. P, A. M. van Meerbeeck, J. A. Foekens. The urokinase-type plasminogen activator receptor: a new tumour marker in small cell lung cancer. APMIS. 2013;121:189–96.

52. Kicking T, et al. In-depth proteomic analysis of nonsmall cell lung cancer to discover molecular targets and candidate biomarkers. Mol Cell Proteomics. 2012;11:916–32.

53. Fahrmann JF, et al. Proteomic profiling of lung adenocarcinoma indicates heightened DNA repair, antioxidant mechanisms and identifies LASP1 as a potential negative predictor of survival. Clin Proteomics. 2016;13:31.

54. Fabrizio Bianchi JH. Giuseppe Pelosi, Rosalia Cirincione, Mary Ferguson, Cathy Ractiwe, Pier Paolo Di Fiore, Kevin Gatter, Francesco Pezzella, Ugo Pastoreno, Lung Cancers Detected by Screening with Spiral Computed Tomography Have a Malignant Phenotype when Analyzed by cDNA Microarray. Clin Cancer Res. 2004;10:6023–8.

55. Meng L, et al. Mitochondrial NDUFAL2 protein promotes the vitality of lung cancer cells by repressing oxidative stress. Thorac Cancer. 2019;10:676–85.

56. Kikuchi T, et al. Long Noncoding RNA H19 Facilitates Small Cell Lung Cancer Tumorigenesis Through miR‑140‑3p/FGF9 Axis. Onco Targets Ther. 2017;10:104831–54.

57. Liu Z, et al. The screening of immune-related biomarkers for prognosis of lung adenocarcinoma. Bioengineered. 2021;12:1273–85.

58. An BC, et al. GPx3-mediated redox signaling arrests the cell cycle and acts as a tumor suppressor in lung cancer cell lines. PLoS One. 2018;13:13.

59. An BC, et al. Epigenetic and Glucocorticoid Receptor-Mediated Regulation of Glutathione Peroxidase 3 in Lung Cancer Cells. Mol Cells. 2016;39:636–81.

60. Meng L, et al. Mitochondrial NDUFAL2 protein promotes the vitality of lung cancer cells by repressing oxidative stress. Thorac Cancer. 2019;10:676–85.

61. Chang Jer‑Wei, P.‑I. H, Hsu Han‑Shui, Wen Chiao‑Kai, Chang Yu‑Sun, Chang‑Wei An, Tsai Ying‑Ming, Chong Inn‑Wen, Kuo Po‑Lin. Identification of novel gene expression signature in lung adenocarcinoma by using next-generation sequencing data and bioinformatics analysis. Oncotarget. 2017;8:104831–54.

62. Liu Z, et al. The screening of immune-related biomarkers for prognosis of lung adenocarcinoma. Bioengineered. 2021;12:1273–85.

63. Li X, et al. Long Noncoding RNA H19 Facilitates Small Cell Lung Cancer Tumorigenesis Through miR‑140‑3p/FGF9 Axis. Onco Targets Ther. 2020;13:3525–34.

64. Chang Jer‑Wei, P.‑I. H, Hsu Han‑Shui, Wen Chiao‑Kai, Chang Yu‑Sun, Chang‑Wei An, Tsai Ying‑Ming, Chong Inn‑Wen, Kuo Po‑Lin. Identification of novel gene expression signature in lung adenocarcinoma by using next-generation sequencing data and bioinformatics analysis. Oncotarget. 2017;8:104831–54.

65. Liu Z, et al. The screening of immune-related biomarkers for prognosis of lung adenocarcinoma. Bioengineered. 2021;12:1273–85.

66. An BC, et al. GPx3-mediated redox signaling arrests the cell cycle and acts as a tumor suppressor in lung cancer cell lines. PLoS One. 2018;13:13.

67. An BC, et al. Epigenetic and Glucocorticoid Receptor-Mediated Regulation of Glutathione Peroxidase 3 in Lung Cancer Cells. Mol Cells. 2016;39:636–81.

68. Kikuchi T, et al. Long Noncoding RNA H19 Facilitates Small Cell Lung Cancer Tumorigenesis Through miR‑140‑3p/FGF9 Axis. Onco Targets Ther. 2017;10:104831–54.

69. Meng L, et al. Mitochondrial NDUFAL2 protein promotes the vitality of lung cancer cells by repressing oxidative stress. Thorac Cancer. 2019;10:676–85.

70. Kikuchi T, et al. In-depth proteomic analysis of nonsmall cell lung cancer to discover molecular targets and candidate biomarkers. Mol Cell Proteomics. 2012;11:916–32.
83. Rigoutsos I, et al. N-BLR, a primate-specific non-coding transcript leads to colorectal cancer invasion and migration. Genome Biol. 2017;18:98.
84. Statello L, et al. Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol. 2021;22:96–118.
85. Wu XS, et al. LncRNA-PAGBC acts as a microRNA sponge and promotes gallbladder tumorigenesis. EMBO Rep. 2017;18:1837–53.
86. Jipei Liao LY, Yuping Mei, Maria Guarnera, Jun Shen, Ruiyun Li, Zhenqiu Liu, Feng Jiang. Small nucleolar RNA signatures as biomarkers for non-small-cell lung cancer. Mol Cancer. 2010;9:198.
87. Beers MF, Moodley Y. When Is an Alveolar Type 2 Cell an Alveolar Type 2 Cell? A Conundrum for Lung Stem Cell Biology and Regenerative Medicine. Am J Respir Cell Mol Biol. 2017;57(1):18–27.
88. Fujino N, et al. Isolation of alveolar epithelial type II progenitor cells from adult human lungs. Lab Invest. 2011;91(3):363–78.
89. Kasper M, et al. Loss of caveolin expression in type I pneumocytes as an indicator of subcellular alterations during lung fibrogenesis. Histochem Cell Biol. 1998;109(1):41–8.
90. McElroy MC, Kasper M. The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. Eur Respir J. 2004;24(4):664–73.
91. Newman GR, et al. Caveolin and its cellular and subcellular immunolocalisation in lung alveolar epithelium: implications for alveolar epithelial type I cell function. Cell Tissue Res. 1999;295(1):111–20.
92. Wang Y, et al. Pulmonary alveolar type I cell population consists of two distinct subtypes that differ in cell fate. Proc Natl Acad Sci U S A. 2018;115(10):2407–12.

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