**Polycyclic aromatic hydrocarbon (PAH)-containing soils from coal gangue
stacking areas contribute to epithelial to mesenchymal transition (EMT)
modulation on cancer cell metastasis**

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Abstract: The total accumulative stockpiles of gangue in China comprise 4.5 billion metric tons, and approximately 659 million tons of additional gangue are generated per year. Considering the stacking characteristics are highly heterogeneous, the potential cancer risks from the presence of polycyclic aromatic hydrocarbons (PAHs) remain elusive. This study aimed to determine whether PAH-containing soil around coal gangue stacking areas poses a potential cancer risk and contributes to cancer cell metastasis. The results indicate that eighteen PAHs, primarily originated from coal gangue, exhibited distance variations from the coal gangues to the downstream villages, and the abandoned colliery posed increased potential carcinogenic risks for humans as a result of long-term stacking of coal gangue. Furthermore, soil samples stimulated HepG2 cell migration and invasion in a PAH-dependent manner, and the action was involved in PPAR γ -mediated epithelial to mesenchymal transition (EMT) modulation. These findings highlight the potential cancer risk of PAH-containing soil samples around coal gangue stacking areas, and identify important biomarkers underlying the risk and targets preventing the outcomes in polluted areas.

Keyword: coal gangue stacking; polycyclic aromatic hydrocarbons (PAHs)-containing soil; cancer risk; cancer cell metastasis; epithelial to mesenchymal transition (EMT).

1. Introduction

Gangue is a by-product with a low combustion value that is discharged from coal excavation and washing (Wang et al., 2013; Zhang et al., 2015). Gangue production accounts for approximately 15% of raw coal production, depending on the geological and mining characteristics (Liu and Liu, 2010). Over the previous 20 years, most electricity in mainland China has been produced from fossil fuels, predominantly from coal, which has resulted in a substantial amount of coal gangue accumulation. The total accumulative stockpiles of gangue in China comprised 4.5 billion metric tons in 2011, and approximately 659 million tons of additional gangue is generated per year (National Development and Reform Commission, China, 2012). To alleviate the potential environmental problems that result from gangue stockpiles, the government of China has recently established several legislative approaches for the utilization and management of coal gangue. To date, the comprehensive utilization level only accounts for 15% of the total gangue production; the remaining waste rocks produced by excavating and washing are stacked around mines in cone types and valley dump types, which are typically adjacent to residential areas and are defined as coal gangue hills. Numerous studies have reported the pollution characteristics of coal gangue, including naturally occurring harmful heavy metals, trace elements, polycyclic aromatic hydrocarbons (PAHs) and other organic pollutants, which may discharge into the surrounding environment, endanger the ecological system and have potential risks to human health (Fan et al., 2013). The main organic hazardous components in coal gangues are PAHs. As persistent organic pollutants, PAHs have been classified as carcinogens by the International Agency for Research on Cancer (IARC). Therefore, it is critical to investigate the potential cancer risk

1 associated with coal gangue stacking areas.

2 The environmental impact of the mining industry is substantial, and exposure to known
3 carcinogens is likely for individuals who reside near coal mines. Many studies have identified
4 an increased cancer incidence and mortality risk for multiple cancer types among coal miners,
5 including bladder, gastric, lung, laryngeal, hypopharyngeal, and liver cancers (Mueller et al.,
6 2015). Since the merger and reorganization of coal mines was initiated in 2010,
7 approximately 2,000 coal enterprises have closed, and the number of discarded gangue
8 dumps has significantly increased. To date, approximately 3,000 hills of coal gangue exist in
9 China (Wang et al., 2008). Currently, the existing disposal dumps comprise many small-scale
10 hills of coal gangue with different geological characteristics and different abandonment times.
11 New problems have emerged for the different abandonment times of coal gangue hills
12 without further disposal. During the substantial coal gangue stacking process, coal dust may
13 gradually diffuse into the surface soil and release native PAHs, which may permeate the deep
14 soil and pollute groundwater through long-term self-ignition, weathering and leaching in
15 different manners, as well as affect food chain transfer and bioaccumulation (Ribeiro et al.,
16 2012; Sun et al., 2009; Wang et al., 2009). Specifically, extremely high levels of PAHs have
17 been identified in the soil and groundwater around abandoned coal gangue dumps following
18 primitive operations (Sun et al., 2009; Wang et al., 2009). These findings indicate that
19 exposure to PAHs from coal gangue hills may be related to the increased cancer incidence
20 around mining areas. However, to the best of our knowledge, no experimental study has been
21 conducted to determine whether soil leaching around coal gangues associated with cancer
22 risk and progression based on PAH pollution characteristics, especially with regards to

evidence from exposure markers and molecular targets.

In this study, we analyzed the distribution behavior of PAHs in the soil around coal gangue hills in Shanxi. The sites included a long-abandoned colliery, a newly abandoned mine and a functioning coal factory. We estimated the cancer risk and determined the potential capacity and mechanism by which PAHs in the soil around coal gangue dumps promote cancer cell metastasis.

2. Materials and Methods

2.1. Site identification and sample collection.

The soil and gangue samples were collected from coal gangue hills located in Qinyuan (QY, 36°34'12.24"N, 12° 8'55.67"E), Gujiao (GJ, 37°59'18.38"N, 112°4'37.80"E) and Yangquan (YQ, 37°52'14.33"N, 113°27'47.90"E) of Shanxi province (Fig. A.1); these sites represented a long-abandoned colliery, a newly abandoned mine and a functioning coal factory, respectively. The sampling was initiated from the gangue-stacked area adjacent to the village. We collected 33 soil samples, at each sampling site, a top soil sample (0-30 cm from the surface) was collected, and each soil sample consisted of three-five subsamples. Following the removal of gravel, all samples were placed in prewashed aluminum jars and immediately stored at -20 °C until further analysis. The GC-MSD (6890 N, HP; 5973, HP) was used to determine the concentration of 18 PAHs followed a modified procedure by Giger and Schaffner (1978).

The soil samples were freeze-dried, and the coal gangue samples were crushed. Following filtration with a 100 mesh sieve, a 100 g sample was added to a sealed beaker with

100 mL double-distilled water. The beakers were shaken at room temperature for 3 d and equilibrated overnight. The mixture was centrifuged at 3,000 rpm for 10 min, and the supernatant was transferred into a sealed glass bottle and maintained in darkness. Leachate was filtered using sterilizing filters to prevent contamination of microbiological in the cell culture.

2.2. Population survey and cancer risk assessment.

The basic population data regarding age, gender, body weight and height were collected in the form of a questionnaire survey from the exposed population in this study. For the risk assessment calculations, we ranked the 18 target PAHs according to their cancer potency relative to BaP (Hu et al., 2007). The incremental life time cancer risk (ILCR) represents the sum of the risks of the three different pathways. The detailed calculation is provided in the supplementary text A.1. Informed written consent was obtained from all participants, and the present study had the appropriate ethical approval from their respective institutions.

2.3. Cell culture and treatment.

The hepatocellular carcinoma HepG2 cell line was purchased from the Cell Bank of Type Culture Collection (CAS, China). The cells were cultured in a monolayer with RPMI 1640 medium (Gibco, USA), which was supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin (Gibco, USA) and 100 U/mL streptomycin (Gibco, USA). The cells were maintained in an incubator with 5% CO₂ and 95% humidity at 37 °C. Prior to treatment, the cells were randomly inoculated into control and treatment dishes. The treatment groups were added various concentrations (Vleachate: Vmedium=1:1, 1:5, 1:25, 1:125) of 100 µL leachate samples from different sites into 35mm dishes for 24 h.

2.4. Cell migration and invasion assays.

Following 24 h of treatment, the cells were harvested, and approximately 8×10^4 cells were seeded in the upper chamber of a 24-well Matrigel-coated (1.25 mg/mL; BD Biosciences) transwell insert plate with serum-free RPMI 1640 (Yue et al., 2015). The lower chambers were filled with RPMI 1640 containing 10% FBS. Following incubation for 24 h, the cells were fixed with 4% paraformaldehyde and stained with crystal violet. The migrated cells were counted under light microscopy (Olympus, Japan) in random fields at a magnification of 400 $\times$.

2.5. Real-time quantitative reverse transcription-PCR.

After a-24 h incubation, HepG2 cells were harvested and total RNA was extracted by TRIzol Reagent (Invitrogen, USA) and then synthesized to first-strand complementary DNA (cDNA) using a reverse transcription kit (TaKaRa, China). E-cadherin and Fibronectin mRNA expressions were determined by real-time PCR. The detailed steps listed in the supporting text A.2 and table A.1.

2.6. Western blot assays.

After being incubated for 24 h, cells were washed twice with PBS and proteins were extracted in ice-cold lysis buffer. The protein concentration was determined according to the Bradford method (1976).. 40 μ g total proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transferred to a PVDF membrane, and blocked with 3% bovine albumin. Then, incubated the blocked membranes overnight at 4 $^{\circ}$ C with monoclonal antibodies for β -actin (1:1000, Cell Signaling, USA) and polyclonal antibodies for human PPAR γ (1:200, Bioss, China) respectively. Then the membranes were

incubated with fluorescently labeled secondary antibody (1:5000) (IRDye 800 CW goat anti-rabbit IgG, LI-COR), and finally detected by LI-COR Odyssey Infrared Fluorescent System.

2.7. Molecular docking.

LigandFit from Discovery Studio 2.5 (Accelrys) was performed to simulate the potential molecular docking mode. Reference protein coordinates for docking were obtained from the X-ray structure of FL, pHE, and PPAR γ (PDB ID: 2PRG). The docking position was determined using a shape-based mode, which was based on the crystal structure of PPAR γ . DREIDING force field and flexible fit were selected, and the conformations were generated via Monte Carlo simulations. Ten ligand conformations were saved after they were minimized with the default rigid body minimization parameters. We subsequently assessed the docking results with a set of scoring functions in Discovery Studio 2.5, including Dockscore, LigScore1, LigScore2, PLP1, PLP2, JAIN and PMF.

2.8. Chromatin immunoprecipitation (CHIP) assay.

HepG2 cells were seeded in 100-mm dishes and treated with extract from the soil sample closest to the village in YQ.. The CHIP assay was conducted according to the instructions of the Chromatin Immunoprecipitation Assay Kit (Millipore, USA). Briefly, following 24 h of treatment, the cells were fixed with formaldehyde and washed twice with PBS that contained protease inhibitors. The cells were subsequently harvested by scraping with PBS that contained protease inhibitors, the pelleted cells were resuspended in SDS lysis buffer and sonicated to obtain a DNA lysate. The samples were centrifuged, and the supernatants were diluted with Chip dilution buffer. The samples were subsequently

pre-cleared to reduce the nonspecific background and then incubated with primary antibody (PPAR γ) overnight. The antibody/histone complex was collected via the addition of Salmon Sperm DNA/Protein A Agarose Slurry, washed with different wash buffers, and eluted from the antibody; the cross-linking of protein and DNA was reversed with NaCl. Finally, DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and a PCR reaction and DNA agarose electrophoresis were conducted to obtain the final results.

2.9. Statistical analyses.

The data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), and the results are presented as the mean $\pm$ standard deviation of three independent experiments. Significant differences were identified via one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test and were established at $p < 0.05$.

3. Results and Discussion

3.1 Determination of PAH concentration and source.

PAHs are carcinogens that represent the main organic hazardous components in coal gangues; PAHs discharge into the surrounding environment, endanger the ecological system and also have potential risks to human health (Fan et al., 2013). Here, we first analyzed the levels of 18 PAHs in coal gangue and soil samples collected around coal gangue hills in Shanxi with different dump-time characteristic and the contents are listed in Fig.1A and Table A.2. The total concentrations of the 18 PAHs in the coal gangue samples were 8,210.04 $\mu\text{g/kg}$ for QY, 11,146.42 $\mu\text{g/kg}$ for GJ and 3,498.15 $\mu\text{g/kg}$ for YQ. Interestingly, the total concentrations of the 18 PAHs in the different soil sampling sites around the coal gangue hills

had ranges of 317.44-4,133.58, 241.56-591.29, and 380.33-1,222.34 $\mu\text{g/kg}$ for QY, GJ and YQ, respectively. The total PAH concentrations in the coal gangue samples from QY, GJ and YQ were lower than those obtained from a northern city of China, i.e., the Jiulong coal mine (levels generally above 16,220 $\mu\text{g/kg}$) (Sun et al., 2009), and were similar to those measured from the coal gangue in the neighboring city Gequan (4,290 $\mu\text{g/kg}$) (Fan et al., 2013). The total concentrations of the 18 PAHs in the different soil sampling sites around coal gangue hills clearly exceeded the natural PAH concentrations, 1-10 $\mu\text{g/kg}$ in soil, which are primarily derived from plant synthesis and natural fires (Edward, 1983). Furthermore, the PAH concentrations in the QY and YQ soil samples in this study were increased compared with other areas of northern China, such as the Tiefert coal mine in Liaoning province (arithmetic mean of 1,118.3 $\mu\text{g/kg}$) (Liu et al., 2012), the Anhui coal mine district (mean value of 840 $\mu\text{g/kg}$) (Liu et al., 2012), and Xiangfen county in Shanxi province (mean value of 722.35 $\mu\text{g/kg}$) (Pan et al., 2015); however, the concentrations were not increased compared with the soil samples in GJ. Considering the coal gangue dump status, the highest PAH levels in the GJ gangue sample and the relatively lower PAH contents of the soil samples may be because the Luyukou coal mine, which represents the biggest mine in the GJ gangue coal gangue dump, was recently abandoned; thus, the PAHs have not permeated the soil through weathering and leaching. In contrast, the highest PAH concentrations in the soil samples in QY were attributed to the Qinxin coal mine, which was the largest mine in the QY coal gangue dump and had been abandoned many years earlier; thus, PAHs were released from the coal gangue and had accumulated in the soil.

To determine the potential transferring behavior of PAHs in soils around the coal gangue,

we subsequently analyzed the relationship between the total PAH concentration and the distance from the different coal gangue dumps. For QY, the contents of the PAHs increased with an increase in the sampling distance from the gangue hill and reached 317.44 $\mu\text{g/kg}$ for 100 m, 930.86 $\mu\text{g/kg}$ for 700 m and 4,133.58 $\mu\text{g/kg}$ for 1,000 m. In contrast, the PAH levels exhibited the opposite trend in YQ and were 1,222.34 $\mu\text{g/kg}$ for 100 m, 636.06 $\mu\text{g/kg}$ for 650 m and 459.35 $\mu\text{g/kg}$ for 1,000 m. There were no obvious changes in the total PAH concentrations at the different distances for GJ. These findings imply that the PAHs in the soils downstream of gangue hill predominately originate from gangue stacking, and the distance-dependent alteration of PAHs may be attributed to the different stacking times of coal gangue hills in different districts. To confirm the implication, a coefficient of divergence (CD) analysis (Kong et al., 2010) was subsequently conducted to analyze the similarities between the soil samples closest to the coal gangue hills and the soil samples closest to the villages for PAH compositions (Fig. 1B). The CD values of QY, YQ and GJ were 0.289, 0.108 and 0.213, respectively; therefore, the PAH compositions were similar between the soil samples closest to the coal gangue hills and the soil samples closest to the villages. Interestingly, the CD value of QY was higher than that of YQ or GJ. This finding may be attributed to the altered pollution characteristics of the PAHs by long-term stacking of coal gangue. The temperate continental climate in the QY, which is cool and dry, may lead to poor volatilization of PAHs. PAHs may also be subjected to photo-oxidation and biodegradation by microorganisms, which likely further led to the high level of PAHs enriched in the soils around the coal gangue hill of QY (Wilcke, 2000). Moreover, Qin River, which is a branch of the Fen River, flows through this area; thus, the river flow may transport PAHs to other areas

as a result of distillation effects. Furthermore, the sample of QY coal gangue exhibited a relatively low concentration of total PAHs.

To address the coal gangue-resourced PAHs, we further determined the ring classes of the PAHs in the soil samples closest to the village. As shown in Fig. 1C, 3, 4 and 5 ring PAHs were dominant in the soil samples collected from the three sites closest to the villages, which provides further evidence for the coal gangue source of PAH-containing soils in the sampled areas. Previous studies have demonstrated that PAHs in soil around coal mines were related to the native PAHs in raw coal (Xue et al., 2007; Yang et al., 2008; Zhao et al., 2014). Other studies have also reported that coal gangue is the major source of PAHs in mining areas. For example, benzo(a)pyrene pollution in maize around mine zones originates from coal gangue piles (Li et al., 2008), and the most abundant PAHs around the mining area are located in the soil near the gangue dump (Yang et al., 2007). PAHs are apt to transfer, and because of their photochemical degradation properties, the determination of their precise source from the environmental media is difficult and complex. Low-weight PAHs are subject to transport and degradation, whereas high-weight PAHs are prone to rapid deposition, which may occur close to the emission source (Wang et al., 2010; Yang et al., 2010). Thus, both agricultural soils and lake bank soils showed increased percentages of low-molecular-weight PAHs compared with the mining zone soils.

3.2 Estimation of cancer risk for PAHs in soils nearest village.

Epidemiological and experimental studies have indicated that PAH exposure is associated with cancer development (Ba et al., 2015; Chen et al., 2002; Mordukhovich et al., 2016). Here, the equivalent concentration of BaP (BaP_{eq}) of the soil samples from QY, YQ

and GJ were 340.956, 82.317 and 23.169 $\mu\text{g/kg}$, respectively (Table 1). The BaP_{eq} values varied with the characteristics of coal gangue stacking, and the values in QY were 4-8 times higher than those in GJ. We compared our values with the values obtained from other locations in China and abroad; both values exceeded the agricultural soil acceptance criteria of New Zealand (27 $\mu\text{g/kg}$) (Cavanagh, 2006), which did not occur for GJ. The extremely high level of BaP in QY was higher than the coal gangue dump district of other northern cities in China, such as the Tiefa coal mine district (90.1 $\mu\text{g/kg}$) in Liaoning (Liu et al., 2012), the coal production area surrounding Xinzhou (34 $\mu\text{g/kg}$) in Shanxi (Zhao et al., 2014), and the coke production base in Shanxi (44.6 $\mu\text{g/kg}$) (Duan et al., 2015). Furthermore, BaP exposure to agricultural soil may create a potential risk via enrichment in the food chain. Considering that PAH exposure is associated with cancer risk, the PAH-bound soil exposure in the area may play an important role in increased cancer morbidity and mortality.

To provide additional evidence for the quantitative cancer risk (Hu et al., 2007), we calculated ILCR. An ILCR greater than 10^{-4} indicates a high potential health risk; an ILCR between 10^{-6} and 10^{-4} denotes a potential risk; and a smaller value indicates an acceptable range (Liao and Chiang, 2006). As shown in Table 2, the ILCR values for QY, YQ and GJ were 2.78×10^{-6} , 6.72×10^{-7} and 1.89×10^{-7} , respectively. Only the ILCR value of QY was significantly higher than the safety level, which indicated a potential risk for elderly individuals because of exposure to soil PAHs. The ILCR value in YQ was slightly lower than 10^{-6} and also indicated a potential cancer risk for elderly individuals. In contrast, the value in GJ, which was lower than the safety level, suggested a lower cancer risk. These findings were consistent with the epidemiological conditions of the local residents. Specifically, the highest

cancer risk of QY is attributed to PAHs in the soil, and the relatively high risk level of YQ may occur because there is a maximum content of PAHs due to the short distance between the village and the gangue hill.

3.3 Identification of HepG2 cell migration caused by PAH-containing soil samples.

The high mortality of cancer is attributed to early metastasis, and local tumor cell invasion and distant metastasis promote the malignant progression of tumors (Ebos and Kerbel, 2011). To confirm the cancer risk associated with the environmental behavior of PAH-containing soils using experimental evidence, we determined whether the soil samples collected from the three coal gangues promoted the migration and invasion of human tumor cells using HepG2 cells as a model. As presented in Fig. 2A, the migration ability of HepG2 cells exposed to soil samples varied as a function of the distance-dependent PAH levels. In detail, the migration ability of HepG2 cells was increased by treatment with soil samples collected in QY with an increased distance from the gangue dumps because of the increased PAH concentrations. In contrast, the opposite trends were identified following YQ soil sample treatment, and the invasion of HepG2 cells decreased with an increasing distance from the gangue dump, which indicated a similar trend with the variation of the PAH level. However, no distance-dependent change in the cell numbers recovered from the lower chamber was identified following exposure to the soil sample from GJ because there was no significant change in the total PAH concentrations in the different distance sampling sites. Importantly, the soil samples in the closest villages from the coal gangue stimulated a concentration-dependent increase in HepG2 cell migration. As shown in Fig. 2B, the migration rates appeared to rapidly increase following exposure to soil samples at 1:125, 1:25,

1 1:5 and 1:1 ($V_{\text{leachate}}: V_{\text{medium}}$) and reached levels of 1.29-, 1.38-, 2.26- and 2.55-fold,
2 respectively, greater than the control for QY, 1.33-, 1.53-, 2.24- and 2.47- fold, respectively,
3 greater than the control for YQ, and 1.47-, 2.07-, 1.89-, and 2.38-fold, respectively, greater
4 than the control for GJ. The significant differences occurred after 1:5 and 1:1 ($V_{\text{leachate}}:$
5 V_{medium}) sample exposure. The quantitative data demonstrated that the soil samples around
6 the coal gangue areas stimulated HepG2 cell migration, and posed the potential cancer risk.

7 To quantitatively confirm that PAHs in soil samples are the major contributor to the
8 migration and invasion process, we conducted a correlation analysis between the PAH levels
9 in the sampling sites closest to the villages and HepG2 cell invasion. As shown in Table 3,
10 among the 18 PAHs, FL and pHE were the main components related to cancer cell invasion.
11 To confirm the correlative analysis, we exposed HepG2 cells to FL and pHE at their actual
12 environmental concentrations in the soil samples. The two chemicals stimulated cell invasion,
13 and the migration rates were increased to 1.43- and 2.09-fold of the control (Fig. A.2). These
14 findings, coupled with the previously described distance- and concentration-dependent
15 HepG2 cell invasion, specify the role of PAHs in soil samples regarding cancer risk and
16 cancer cell metastases.

17 **3.4 Mechanism for HepG2 cell migration induced by PAHs in soil samples.**

18 The epithelial to mesenchymal transition (EMT) is a crucial event in cancer progression
19 whereby epithelial cells lose their cell polarity and cell-cell adhesion and gain migratory and
20 invasive properties to become mesenchymal cells. EMT has increasingly been recognized to
21 occur during the progression of various carcinomas (Ding et al., 2010; Firrincieli et al., 2010;
22 van Zijl et al., 2009). The hallmarks of EMT are the down-regulation of E-cadherin in

epithelial cells and the increased expression of fibronectin in mesenchymal cells to reinforce the destabilization of adherens junctions (Benzoubir et al., 2015). To clarify the potential mechanism for HepG2 cell migration induced by PAHs in soil samples, we first determined the transcriptional alteration of E-cadherin and fibronectin based on the samples from QY and YQ. As shown in Fig. 3A, all soil samples closest to the villages showed a concentration-dependent decrease in the E-cadherin mRNA level, and the values reached 0.81-, 0.78-, 0.78- and 0.58-fold, respectively, of the control for QY, 0.78-, 0.73-, 0.64- and 0.75-fold, respectively, of the control for GJ, and 0.92-, 0.72-, 0.88-, and 0.41-fold, respectively, of the control for YQ. Correspondingly, the samples showed increased fibronectin mRNA expression in a concentration-dependent manner and reached 1.40-, 1.44-, 1.32-, and 1.03-fold, respectively, of the control for QY, 1.03-, 1.07-, 1.13-, and 1.24-fold, respectively, of the control for GJ, and 1.07-, 1.28, 1.38-, and 1.19-fold, respectively, of the control for YQ. Similarly, the protein level of E-cadherin and fibronectin showed the same trend in Fig. 3B. The lack of adhesion marker expression (down-regulation of E-cadherin) and the acquisition of the mesenchymal phenotype (up-regulation of fibronectin) result in an increased motility of liver cancer cells and eventually lead to tumor metastasis (Shih and Yang, 2011). Importantly, the changes in E-cadherin and fibronectin expression were consistent with cell invasion. Therefore, loss of desmosomes and adherens junctions likely contributed to the ability of PAH-containing soil samples obtained in proximity to villages around coal gangue hills to induce tumor cell metastasis and increase the cancer risk.

Traditionally, some of the PAHs were thought to bind to arylhydrocarbon receptors (AhRs) to form a ligand-AhR complex, which activates the downstream cascade response

1 and is responsible for biotoxicity. However, recent studies based on a luciferase reporter gene
 2 assay system have further indicated that PAHs significantly activate PPARs, increase PPAR- γ
 3 target gene expression, and ultimately stimulate tumor metastasis (Lin et al., 2014). PPARs
 4 are ligand-activated transcription factors; the PPAR family mainly includes three subtypes:
 5 PPAR α , PPAR β/δ and PPAR γ . PPARs play key roles in the regulation of inflammatory gene
 6 transcription, lipid metabolism, cell differentiation and tumor cell metastasis (Panigrahy et al.,
 7 2005; Scirpo et al., 2015; Shen et al., 2012). Therefore, we hypothesized that PAH-containing
 8 soil samples activate PPAR γ via chemical docking and subsequently cause EMT-mediated
 9 cell invasion through PPAR γ binding. To test this hypothesis, we first confirmed that PAHs in
 10 the soil samples firmly bound to the active pockets of PPAR γ and activated PPAR γ
 11 expression. Considering that FL and pHE were the main components related to cancer cell
 12 invasion, thus, the molecular docking of these two types of PAHs and PPAR γ was evaluated
 13 using the Ligand Fit program (He et al., 2015; Zhang et al., 2014). As shown in Fig. 4, both
 14 FL and pHE firmly bound to the active pockets of PPAR γ (Pocket A for pHE: ASN312,
 15 VAL315, THR316, LYS319, LYS474, ASP475, GLN628 and SER630; pocket B for FL:
 16 ASN312, VAL315, THR316, LYS319, LEU401, GLU471, LYS474, ASP475, GLN628 and
 17 SER630). Moreover, FL (distance: 5.26211 Å: FL:A-LYS474:NZ; 5.1311 Å:
 18 FL:C-GLN628:N; 6.7649 Å: FL:C-GLN628:N) and pHE (distance: 5.18161 Å:
 19 pHE:C-GLN628:N; 6.39185 Å: pHE:C-GLN628:N; 5.09571 Å: pHE:A-LYS474:NZ; 4.6667
 20 Å: pHE:C-GLN628:N) formed an additional Pi-Cation bonding interaction with GLN628 and
 21 LYS474 of PPAR γ . Correspondingly, we identified increased PPAR- γ expression in response
 22 to QY and YQ soil sample exposure. As presented in Fig. 5A, the PPAR γ expression was

significantly increased in a concentration-dependent manner and reached the maximum at the highest concentration ($V_{\text{leachate}}: V_{\text{medium}}=1:1$). Both increases were significantly attenuated by PPAR γ inhibitor (GW9662, 5 μ M) pretreatment.

Next, it was important to clarify whether tumor cell metastasis induced by EMT-related gene modulation occurred via PAH-activated PPAR γ transcription. The loss of E-cadherin, a molecular hallmark of EMT, reinforces the destabilization of adherens junctions between cancer cells (Lamouille et al., 2014). To this end, we searched binding sites (BSs) of PPAR γ in the promoter region of the E-cadherin gene through TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and identified at least two PPAR γ BSs. These BSs were confirmed by CHIP analysis (Fig. 5B) and validated by qPCR analysis. Importantly, the increased cell invasion, the attenuated E-cadherin expression and the increased Fibronectin expression following PAH-containing soil sample exposure were significantly reversed by the PPAR γ antagonist GW9662 (Fig. 5C). These findings suggest that the EMT process is involved in PAH-containing soil sample-induced cancer cell metastasis, primarily being mediated through an inhibitory effect of PPAR γ on E-cadherin transcription. To our knowledge, PAHs, which are PPAR ligands, have also been demonstrated to negatively regulate E-cadherin expression, thereby obstructing transcription in a process referred to as ‘transrepression’. The PPAR γ crystal structure indicates that the acceptor site for the small ubiquitin-related modifier is buried in the ligand-binding pocket; thus, the binding of ligand to PPAR γ may expose this site to PIAS1-mediated sumoylation (Bailey and Ghosh, 2005). It has been reported that sumoylated PPAR γ interacts with the nuclear receptor corepressor (NCoR-HDAC3) complex and thereby blocks the subsequent recruitment of the

ubiquitination machinery responsible for clearing the promoter of the repressive complex; this step is required before gene expression may be initiated (Bailey and Ghosh, 2005). This model therefore may explain how E-cadherin expression may be inhibited by transrepression through PPAR γ ligand PAHs. Combined with the previously discussed findings, the PAH-containing soil samples activated PPAR γ expression, down-regulated the E-cadherin level by inhibiting the transcription of PPAR γ , and ultimately caused tumor cell metastasis.

4. Conclusion

These findings demonstrate that PAH exposure in soil media around coal gangue hills potentially poses cancer risk and stimulates cancer development, and PPAR γ -mediated EMT modulation on cell metastasis might be important biomarkers underlying the risk and targets preventing the outcomes in polluted areas.

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Tables

Table 1. The BaP_{eq} concentrations of PAH-containing soil samples nearest to the villages

PAH species	BaP _{eq} ($\mu\text{g/kg}$)		
	Qinyuan	Gujiao	Yangquan
Naphthalene (Na)	0.298	0.021	0.232
Acenaphthylene (AcL);	0.011	0.004	0.006
Acenaphthene (Ac);	0.033	0.003	0.009
Fluorene (FL);	0.206	0.009	0.076
Benzo(g,hi)perylene (BghiP);	2.336	0.137	0.862
Indeno(1,2,3-cd)pyrene (IP);	13.735	0.913	3.199
Dibenzo(a,h)anthracene	87.637	7.095	31.394
Benzo(b)fluoranthene (BbFa)	47.667	4.229	17.551
Coronene (CoR)	0.123	0.012	0.079
Phenanthrene (pHE);	0.984	0.067	0.267
Anthracene (An);	0.334	0.055	0.166
Fluoranthene (Fa);	14.072	1.164	3.346
Benzo(a)anthracene (BaA);	17.355	1.062	1.959
Chrysene (ChR);	11.087	1.043	3.100
Pyrene (Py);	0.241	0.023	0.072
Benzo(a)pyrene (BaP);	136.000	6.520	17.960
Benzo(e)pyrene (BeP);	2.536	0.212	0.953
Benzo(k)fluoranthene (BkFa);	6.301	0.600	1.086
ΣPAHs	340.956	23.169	82.317

1 Table 2. Incremental life time cancer risk of the PAH-containing soil samples nearest to the villages

ILCR	Qinyuan	Gujiao	Yangquan
	2.78×10^{-6}	1.89×10^{-7}	6.72×10^{-7}

2

3

1 Table 3. Correlations between PAH concentrations in soil samples nearest to the villages with capacity
2 of HepG2 cell invasion

PAHs	Gujiiao	Qinyuan	Yangquan
Na	0.728	0.879	0.936
AcL	-	-	0.404
Ac	0.710	0.839	0.928
FL	0.873	0.917	0.922
BgHiP	0.783	0.828	0.920
Ip	0.810	0.907	-0.260
DbAhA	0.741	0.657	0.891
BbFa	0.737	0.876	0.645
CoR	0.523	0.729	0.950
pHE	0.913	0.886	0.984
An	0.728	0.834	0.937
Fa	0.943	0.785	-0.098
BaA	0.951	0.808	-0.241
ChR	0.657	0.867	0.951
Py	0.991	0.561	0.693
BaP	0.813	-0.226	-0.329
BeP	0.801	0.903	0.816
BkFa	-	0.682	-0.353

3

4

Figure Captions

Fig. 1. Analysis of PAH pollution characteristics in gangue and soil samples. (A) The PAH contents of gangue and soil samples. (B) The similarities in PAH compositions between the gangue sampling site and soil collecting site closest to the corresponding village based on CD analysis. (C) The benzene ring structure of PAHs in soil samples obtained closest to the village.

Fig. 2. Effects of PAH-containing soil samples on HepG2 cell invasion. (A) The distance-dependent effects of the soil samples from the coal gangue to the closest villages on HepG2 cell invasion. (B) The concentration-dependent effects of the soil samples collected from the closest villages on HepG2 cell invasion. HepG2 cells were incubated with soil samples collected from QY, GJ and YQ at different distances or concentrations for 24 h; the invasive ability was subsequently determined using a cell invasion assay (400×). (*) $p < 0.05$, (**) $p < 0.01$ vs the control group.

Fig. 3. Effects of PAH-containing soil samples on E-cadherin and fibronectin expression in HepG2 cells. HepG2 cells were treated with soil samples obtained closest to villages at various concentrations ($V_{\text{leachate}}: V_{\text{medium}} = 1:125, 1:25, 1:5$ and $1:1$) for 24 h. (A) The mRNA levels of E-cadherin and fibronectin were subsequently determined. (B) The protein levels of E-cadherin and fibronectin were subsequently determined. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ vs the control group.

Fig. 4. Proposed binding modes between PAHs (FL and pHE) and PPAR γ .

Fig. 5. PPAR γ -involved modulation of E-cadherin expression in response to PAH-containing soil sample exposure. (A) Effects of PAH-containing soil samples on PPAR γ expression.

1 HepG2 cells were treated with soil samples obtained closest to villages collected from QY,
2 GJ and YQ at various concentrations ($V_{\text{leachate}}: V_{\text{medium}} = 1:125, 1:25, 1:5$ and $1:1$) for 24 h; the
3 protein expression of PPAR γ was subsequently determined. (B) Binding of the PPAR γ
4 subunit in the promoter of the E-cadherin gene following QY soil sample exposure in HepG2
5 cells. HepG2 cells were treated with a QY soil sample obtained closest to the village at a
6 concentration of $1:5$ ($V_{\text{leachate}}: V_{\text{medium}}$) for 24 h; the binding between PPAR γ and E-cadherin
7 was subsequently detected using CHIP analysis of the promoter binding activity of PPAR γ . A
8 1,200 bp DNA ladder was used for the marker. (C) PPAR γ disruption restored the decreased
9 E-cadherin expression and increased cell invasion in response to QY soil sample treatment.
10 HepG2 cells were treated with the QY soil sample obtained closest to the village at a
11 concentration of $1:5$ ($V_{\text{leachate}}: V_{\text{medium}}$) for 24 h in the absence or presence of pre-treatment
12 with the PPAR γ antagonist GW9662 (5 μM); the E-cadherin mRNA level, cell invasion and
13 protein levels of E-cadherin and Fibronectin were subsequently assessed. (*) $p < 0.05$, (**) $p <$
14 0.01 , and (***) $p < 0.001$ vs the control group ($n=3$).

Figure 1

A

B

C

Figure 2

A

B

Figure 3

Figure 4

FL-PPAR γ

pHE-PPAR γ

Figure 5