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Polycyclic Aromatic Hydrocarbons Regulate the Occurrence and Development of Nasopharyngeal Carcinoma by Regulating Aryl Hydrocarbon Receptor

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Nasopharyngeal carcinoma (NPC) has hidden onset, low rate of early diagnosis, and most of them have metastases at the time of diagnosis. The specific pathogenesis of NPC is still unclear. Polycyclic aromatic hydrocarbons (PAHs) are a large group of contaminants produced by the incomplete combustion of organic matter and widespread in the air. Many of these compounds are mutagenic and carcinogenic. PAHs plays an important role in mutagenic and carcinogenic, while its role in NPC still needs further elucidation. In this study, CNE-2 cells were stimulated by PAHs, then the expression of aryl hydrocarbon receptor (AhR) and CYP1A2 were respectively examined using Real-Time fluorescence quantitative PCR (qRT-PCR) and Western Blot. CNE-2 cells proliferation, migration, invasion and apoptosis were examined by CCK-8, Wound-Healing Assay, Transwell, Flow Cytometry, respectively. We found that AhR expression was increased while the level of apoptosis was inhibited by PAHs. While the ability of cell invasion was weakened, proliferation and migration were not significantly different. After treated by PAHs and ITE, the effect of PAHs on promoting AhR expression was significantly inhibited and apoptosis was up-regulated. The present study found that, PAHs inhibit apoptosis of NPC cells and promote the expression of AhR. Besides, PAHs participates in NPC occurrence and development by regulating AhR expression. Collectively, these findings may provide a possible strategy for the clinical treatment of NPC.

Keywords: apoptosis; aryl hydrocarbon receptor; invasion; nasopharyngeal carcinoma; polycyclic aromatic hydrocarbons
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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy of the head and neck which is characterized by distinct geographical distribution and has a high incidence in Southeast Asia and North Africa (Tang et al. 2021). There is not any recognizable symptom in the early stage of NPC. Thus, most patients have metastasized by the time of diagnosis (Chen et al. 2019). Prior research has shown that many factors can lead to NPC. So far, there is no apparent pathogenesis. Therefore, further study on the molecular mechanism of the occurrence and development of NPC, tumor-related signaling pathway, effective inhibition of cell proliferation or promotion of tumor cell apoptosis is of great clinical significance and scientific value for the prevention and treatment of NPC.

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Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds that contain two or more benzene rings but no heteroatoms which are found almost everywhere (air, water, food, soil, etc.). PAHs in the air mainly from the incomplete combustion of fuel, garbage or other organic matter such as tobacco and plant material (Kim et al. 2013). Many of these compounds are genotoxic, mutagenic, teratogenic, and carcinogenic, and thus pose a significant danger to human health and the well-being of humans (Mallah et al. 2022). Exposure to PAHs increases the development of many cancers, such as Lung cancer, breast cancer, bladder cancer, throat cancer, skin cancer, colon cancer, etc. (Wagner et al. 2015; Cheng et al. 2021; Pedersen et al. 2021; Holme et al. 2023; Jubber et al. 2023; Rahman et al. 2023). As the nasopharynx is in close contact with PAHs in the air, it is speculated that PAHs can affect the occurrence and development of nasopharyngeal carcinoma, but there is no relevant study on PAHs in NPC.

The aryl hydrocarbon receptor (AhR) is defined as a receptor that binds and mediate toxic effects induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and structurally related halogenated aromatics. It is activated by a variety of endogenous and exogenous ligands (Kolluri et al. 2017). Related studies have shown that the expression level of AhR in the nucleus of many invasive cancers is increased. After heterodimerization with the AhR nuclear translocator (ARNT), AHR can induce the transcription of several cytochrome P450 (CYP) enzymes, which play an important role in the metabolism and biological activity of PAHs (Murray et al. 2014). PAHs can regulate the expression of P450 (CYP) enzyme in cells by forming a complex with AhR to modulate its molecular events (Shi et al. 2017). However, it is still uncertain whether PAHs affects the development of NPC by regulating AhR.

To date, the role of PAHs in NPC is poorly understood, and it is also unknown whether PAHs can affect the expression of AhR. In this study, after CNE-2 cells were stimulated by PAHs, the effects of PAHs on proliferation, apoptosis, migration, invasion and AhR expression of NPC cells were observed. In addition, CNE-2 cells were treated with AhR exogenous ligand and PAHs simultaneously to analyze the probable action pathway, in order to further clarify the pathogenesis and metastasis mechanism of the disease, so as to establish a more effective therapeutic strategy.

Materials and Methods

PAHs mixture preparation

The airborne fine particulate matter with an aerodynamic diameter equal to or smaller than 2.5 μm (PM 2.5) was collected every day for four months (January, April, July and October) in 2018 at Fudan University (31.3°N, 121.5°E) in Shanghai, China (Hong et al. 2021). PAHs in PM 2.5 were measured using a gas chromatography-mass spectrometer (GC-MS; Agilent, USA) (Hong et al. 2016). The 16 Environmental Protection Agency (EPA) priority PAHs analyzed in PM 2.5 samples were as follows: acenaphthylene, acenaphthene, anthracene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[123-cd]pyrene, naphthalene, phenanthrene and pyrene. According to our results of PAHs analysis as shown in Table 1, 16 pure PAHs were used to compound the PAHs mixture for cells exposure in this study.

Cell culture

The human CEN2 were cultured in RPMI-1640 (Hyclone) medium containing with 10% fetal bovine serum (FBS) (Capricorn), supplemented with 100 μg/ml streptomycin and 100 UI/ml penicillin (Gibco). A humidified 5% CO_2 incubator at 37°C was used for all cells to be cultured. The researcher generated the cells once two days. When the cell density was about 75%-80%, the medium was abandoned and stimulated with PAHs diluted at the ratio of 1:2,000 for 48 h. In another group, CNE-2 cells were simultaneously treated with 1:2,000 PAHs and 10 μM ITE.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from CNE-2 cells using TriPure Isolation Reagent (CA, USA), following the manufacturer's instructions. RevertAid First Strand cDNA Synthesis Kit (USA) was used for total RNA. SYBR Green Mix (CA, USA) was used for real-time quantitative amplification. All real-time quantitative PCR (RT-qPCR) reactions were performed using the GoTaq® RT-qPCR Master Mix System (Promega). The primer sequences for AhR amplification were: forward 5′-CTTCCAAGCGGCATAGAGAC-3′ and reverse 5′-AGTTATCCTGGCCTCCGTTT-3′. Primer sequences CYP1A2 amplification were: forward 5′-CAATCAGGTGGTGGTGTCAG-3 and reverse 5′-GCTCCTGGACTGTTTTCTGC-3′. The relative expression calculated by the $2-\Delta\Delta$CT method.

Western blot assay

CNE-2 cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% TritonX-100, 0.1%-1% deoxycholate, 0.1% SDS). BCA Protein Assay Kit (USA) was used for protein concentration determination. The protein samples were subjected to 12% SDS-PAGE and transferred onto PVDF membranes. The blots were blocked at room temperature for 1 h and washed with TBS (50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.6). They were then incubated overnight with primary antibody at 4°C. The horseradish peroxidase labeled secondary antibody (Goat anti Rabbit IgG, 1:2,000 dilution) was diluted with 5% BSA and then placed in PVDF membrane and reacted at room temperature for 1 h. The blot was visualized and the image captured using ChemiDocTMMP detection system (Bio-Rad Laboratories, Inc).

CCK-8 of cell proliferation

CNE-2 cells were pre-cultured in 96-well plate in 5%

Table 1. The concentrations 16 EPA priority PAHs in PM2.5 and PAHs mixture.

PAH	Concentrations of PAH in PM2.5 (ng/mg)	PAHs composition profile (%)
Acenaphthylene (Acy)	24.01 ± 1.11	1.46
Acenaphthene (Ace)	18.72 ± 0.67	1.14
Anthracene (Ant)	6.3 ± 0.44	0.38
Benz[a]anthracene (BaA)	65.35 ± 3.34	3.97
Benzo[b]fluoranthene (BbF)	262.12 ± 8.11	15.93
Benzo[k]fluoranthene (BkF)	97.14 ± 1.55	5.90
Benzo[a]pyrene (BaP)	87.35 ± 4.56	5.31
Benzo[g,h,i]perylene (BghiP)	76.22 ± 2.45	4.63
Chrysene (Chr)	145.16 ± 4.66	8.82
Dibenz[a,h]anthracene (DBA)	16.48 ± 1.99	1.00
Fluoranthene (Flu)	254.65 ± 17.89	15.48
Fluorene (Fl)	8.98 ± 0.76	0.55
Indeno[123- cd]pyrene (IND)	116.74 ± 13.17	7.10
Naphthalene (NaP)	6.87 ± 0.74	0.42
Phenanthrene (Phe)	251.61 ± 20.18	15.29
Pyrene (Pyr)	207.37 ± 9.13	12.61
$\sum_{16}$ PAHs	1645.07 ± 90.75	100

EPA, environmental protection agency; PAHs, polycyclic aromatic hydrocarbons; PM2.5, airborne fine particulate matter with an aerodynamic diameter equal to or smaller than 2.5 μm.
16 pure PAHs were used to compound the PAHs mixture for cells exposure.

CO_2 at 37°C, and then these cells were treated with different PAHs and PAHsITE for 48 h. Afterward, the solution of 10 μl CCK-8 (Biosharp, China) was supplemented to each well and these cells were incubated for 2 h at 37°C under the humid condition of 95% air and 5% CO_2. Finally, the absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Flow cytometry of cell apoptosis

Cell apoptosis was assessed by using flow cytometry assay (BD, FranklinLakes, NJ). After treatment, the cells were prepared for flow cytometry with 5 μl Annexin V-FITC for 15 min at 4°C in a dark. The cells were washed by 200 μL 1 Binding buffer and centrifugation at 1,200 RPM /min for 5 min. Each tube was suspended by 190 μL 1 Binding buffer. 10 μL PI was added to each tube and the cells were analyzed by flow cytometry. Flow cytometry analysis was performed by a FACS can (Beckman Coulter, Fullerton, CA, USA).

Flow cytometry of cell cycle

Cell cycle distribution was determined with a cell cycle assay kit (Keygen, China). Briefly, cells were digested by 0.25% trypsin and collected. And then the cells were washed twice with PBS and fixed in 75% cold ethanol at least 4 h. After fixation, PI containing RNase1 were applied to stain the DNA for 30 min at room temperature in the dark. The samples were analyzed for DNA content profile by flow cytometry. The percentage of cells in G0/G1, S, and G2/M phases was obtained by ModFit LT 4.0.

Wound-Healing of cell migration

The cells were seeded into six-well plates. About 7×10^5 cells were added to each well. A pipette tip was used to scratch a straight line in the cell layer to create a wound. Then, the cells were washed with PBS and treated with RPMI 1640 medium without FBS. The wound images were observed under light microscope 48 h later.

Transwell of cell invasion

Cell invasion was detected by using Transwell assay with a pore size of 8 μm (Corning, USA). 50 μL basement membrane Matrigel (BD Bioscience) was used for coating the upper surface of the transwell chamber, followed cells were suspended in the serum free medium and adjusted cell density to 1×10^5. Then, total of 200 μl cells suspension and drugs were added to the upper compartment of transwell culture chamber. The lower compartment was filled with 500 μl of 20% serum medium. After incubation 48 h at 37°C, the non-traversed cells in the upper compartment were wiped by using a wet cotton swab. After washing with PBS, the chamber was fixed in 4% polymethyl alcohol for 20 min. Then these cells were stained with 1% crystal violet (Sigma, USA) for 30 min. Then, the stained cells were evaluated under the microscope (Olympus, Tokyo, Japan). Image analysis software image-Pro Plus 6.0 (Media Cybernetics, USA) was used to analyze the images.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software. All experiments were performed at least in tripli-

cate, and the data are presented as mean standard deviation. The results of the present study are presented as the mean ± standard deviation (SD). Statistical analyses were performed by using Graphpad 8.0 statistical software (San Diego, California, USA). The significant difference between two groups was analyzed by student's *t*-test. A *p*-value of < 0.05 was considered to indicate a statistically significant result.

Results

PAHs composition profile

The PM 2.5 samples was collected every day for four months (January, April, July and October) in 2018 at Fudan University (31.3°N, 121.5°E) in Shanghai, China. The 16 Environmental Protection Agency (EPA) priority PAHs were analyzed in PM 2.5 samples. As shown in Table 1, the concentration of 16 US EPA priority PAHs was 1645.07 ± 90.75 ng/mg in PM 2.5, and BbF (15.93%), Flu (15.48%), Phe (15.29%) and Pyr (12.61%) were the major compounds. According to our results, 16 pure PAHs were used to compound the PAHs mixture for cells exposure in this study.

The expression of AhR and CYP1A2 in CNE-2 cells

In order to explore the relationship between PAHs and AhR, CYP1A2, we used qRT-PCR to detect the expression levels of AhR and CYP1A2 after PAHs treatment. The expression level of AhR in CNE-2 cells was significantly increased after PAHs treatment ($p < 0.001$, Fig. 1A), while the expression level of CYP1A2 was decreased ($p < 0.05$, Fig. 1A). According to the result of western blotting, the PAHs treatment could not elevate the transcription level of AhR ($p > 0.05$, Fig. 1B), but decrease the transcription level of CYP1A2 ($p < 0.001$, Fig. 1B). These results indicated that PAHs could promote the expression of AhR at gene, and inhibit the expression of CYP1A2 both at gene and protein levels.

In order to further explore the effect of PAHs on the expression of AhR in CNE-2 cells, we stimulated CNE-2 cells with PAHs and ITE at the same time. The result showed that compared with PAHs stimulation alone, AhR overexpression was inhibited by ITE ($p < 0.001$, Fig. 1A), and the expression level of CYP1A2 significantly increased ($p < 0.001$, Fig. 1A). According to the result of western blotting, AhR protein expression was inhibited by ITE ($p < 0.001$, Fig. 1B), and the protein expression level of CYP1A2 significantly increased by ITE ($p < 0.001$, Fig. 1B).

PAHs inhibited the apoptosis of CNE-2 cells, but had no effect on the proliferation

CNE-2 cells were stimulated with PAHs or stimulated with PAHs and ITE at the same time, and then was measured by CCK-8 assay. The results showed that cell proliferation was no significant differences among the three groups ($p > 0.05$, Fig. 2A). While the apoptosis rate of CNE-2 cells decreased significantly after treated with PAHs ($p < 0.05$, Fig. 2B). In the subsequent experiments, CNE-2 cells were stimulated with PAHs and ITE at the same time, the apoptosis rate of CNE-2 cells increased ($p < 0.001$, Fig.

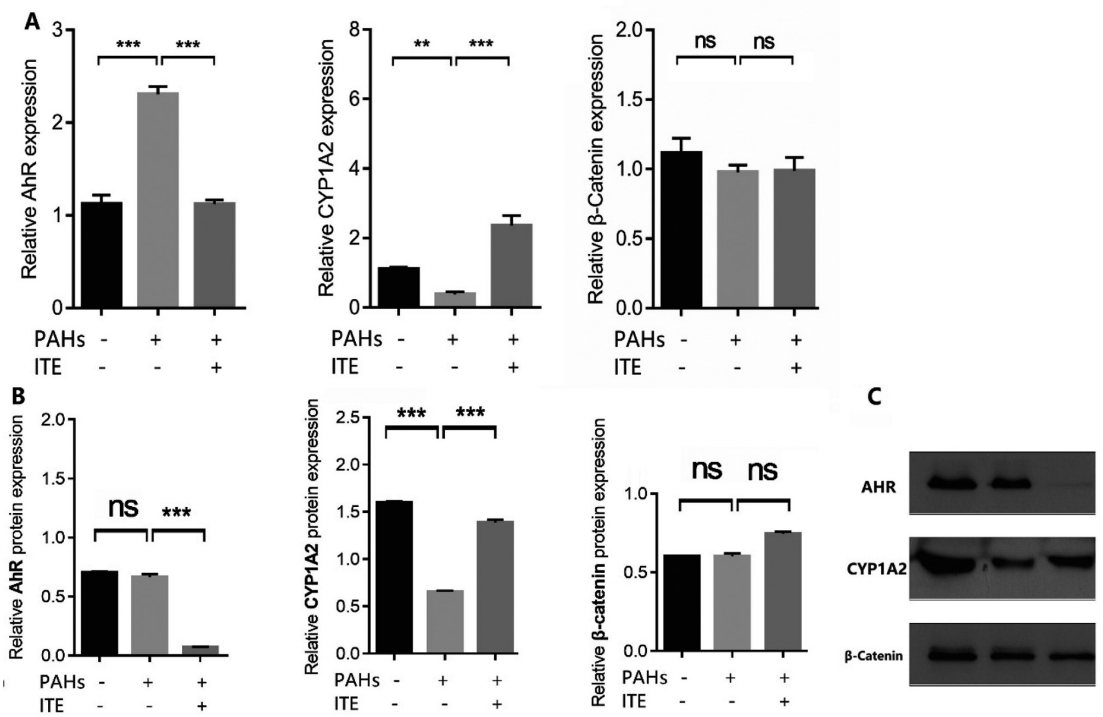

Fig. 1. Effect of PAHs on AhR and CYP expression in CNE-2 cells.
(A) Relative mRNA expressions of AhR and CYP1A2 in CNE-2 cells. (B) Relative protein expressions of AhR and CYP1A2 in CNE-2 cells. (C) Immunoblot images of western blot assay. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

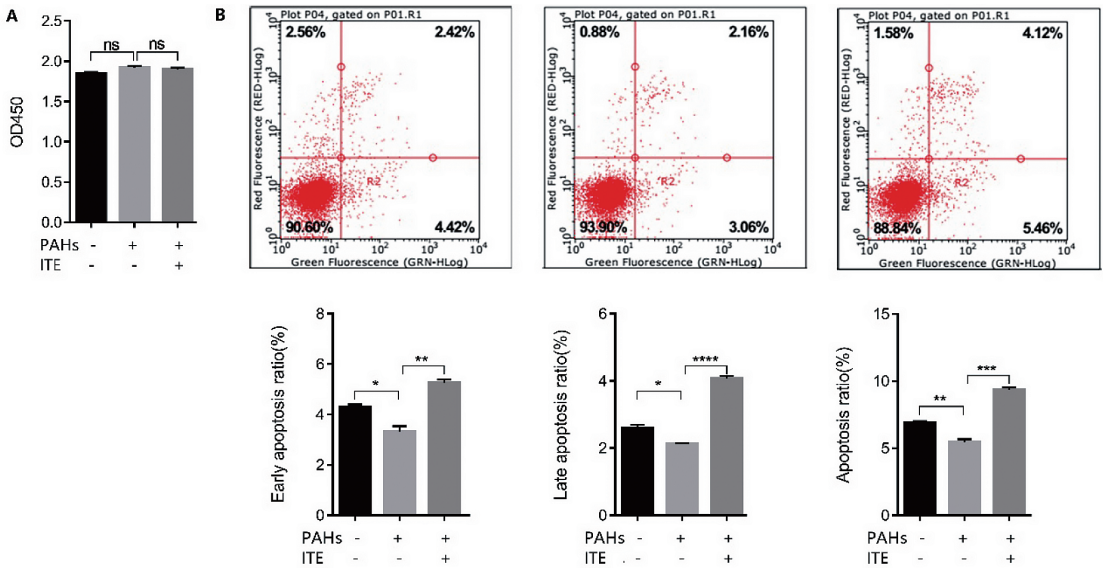

Fig. 2. Effects of PAHs on cell proliferation and apoptosis.
(A) Cell proliferation was measured using CCK-8 assay, then DO_{450} was measured. (B) The effect of PAHs on cell apoptosis were analyzed by flow cytometry. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

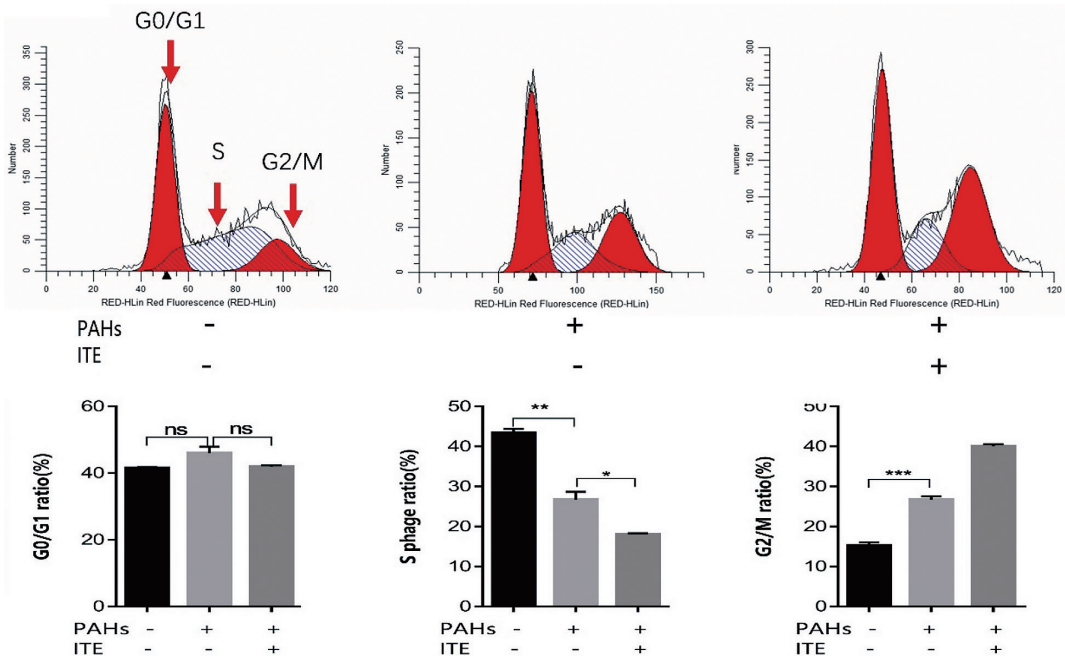

Fig. 3. Effects of PAHs on cell cycle distribution.
The cell cycle distribution was measured used flow cytometry assay. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

2B). These results indicated PAHs inhibited the apoptosis of CNE-2 cells, but had no effect on the proliferation.

PAHs change the cell cycle distribution

The cell cycle detection showed that PAHs increased the proportion of G2/M phase cells ($p < 0.001$, Fig. 3), and decreased the proportion of S phase ($p < 0.05$, Fig. 3). ITE could not change the cell cycle distribution.

PAHs inhibited the invasion of CNE-2 cells, but had no effect on cell migration

Cell invasion and migration of CNE-2 cells were measured by Transwell assay and Wound-Healing assay respectively in the study. The result showed that cell invasion was remarkably suppressed by PAHs compared with control ($p < 0.001$, Fig. 4A). we stimulated CNE-2 cells with PAHs and ITE at the same time. The result showed that compared with PAHs stimulation alone, and the invasion ability increased ($p < 0.05$, Fig. 4A). Nevertheless, PAHs and ITE

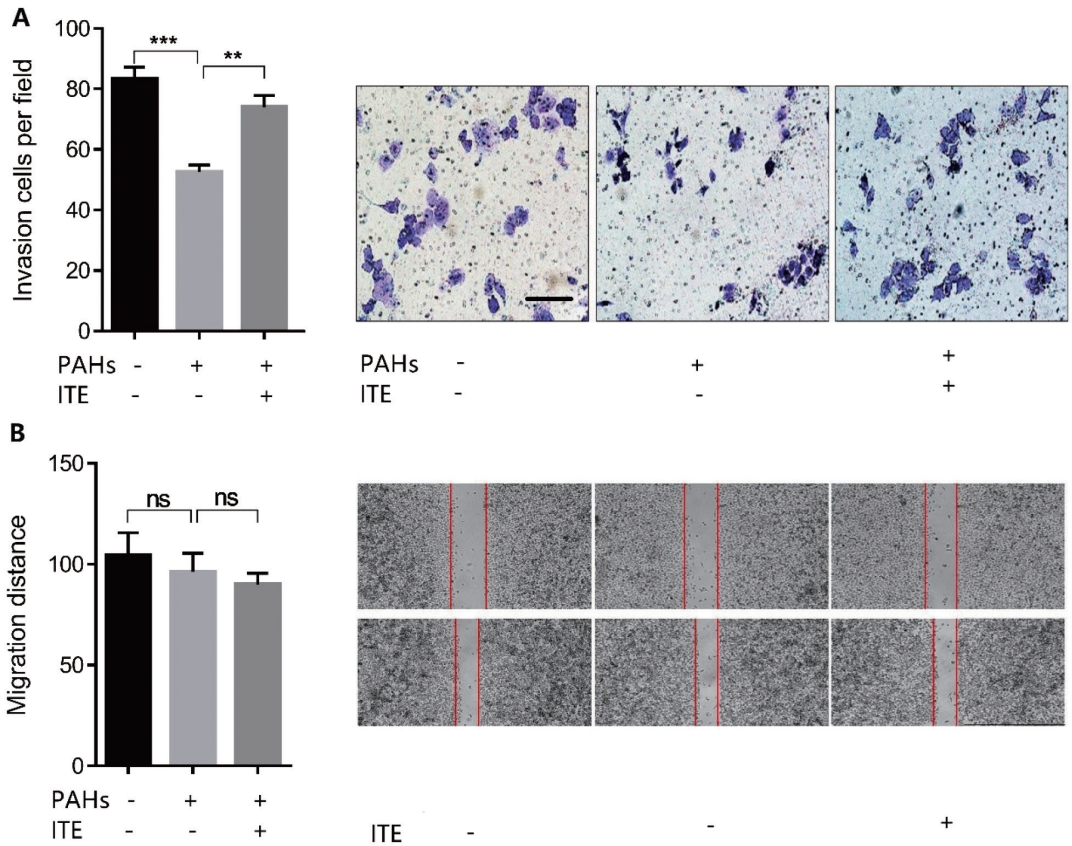

Fig. 4. Effects of PAHs on cell invasion and migration.
(A) PAHs remarkably inhibited cell invasion on CNE-2 cells. (B) PAHs had no significant effect on CNE-2 cell migration. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

had no significant effect on CNE-2 cell migration ($p > 0.05$, Fig. 4B). Taken together, these data indicated that PAHs could inhibit cell invasion in CNE-2 cells but has no effect on cell migration.

Discussion

In this study, we studied the effect of PAHs on nasopharyngeal carcinoma CEN-2 cells. A better understanding of the mechanisms of actual PAHs lesions is necessary. However, because of variations in actual PAHs in the air, studying actual PAHs-induced damage to human health is difficult and unrepeatable. The concentrations 16 EPA priority PAHs in PM 2.5 were measured, and used compound the PAHs mixture for our cells exposure. We found that PAHs promotes the expression of AhR, inhibits CYP1A2, inhibits apoptosis, inhibits cell invasion, and has no significant effect on cell proliferation and migration. In order to determine whether PAHs plays a role by affecting AhR expression. We added exogenous non-toxic AhR ligand to participate in the regulation of AhR receptor, and we found that the expression of AhR in CNE-2 cells was decreased, while the expression of CYP1A2 was increased, and cell apoptosis was increased. Therefore, we speculated that PAHs might affect the apoptosis and invasion of nasopharyngeal carcinoma cells by regulating AhR expression, which provides a new idea for the clinical treatment of NPC.

AhR ligand can regulate tumor development by mediating the transcription of AhR, so the expression of AhR may play a role in the occurrence and development of tumors. PAHs, as a high affinity ligand of AhR, can promote the transcription of AhR. Previous studies have found that the expression level of AhR is related to the occurrence and development of breast cancer, prostate cancer, lung cancer and hepatocellular carcinoma (Zhu et al. 2021; Sweeney et al. 2022; Yu et al. 2022; Wang et al. 2023). Similar with these studies, our results revealed that PAHs can promote the expression of AhR in NPC cells.

The CYP1 family, a member of the CYP superfamily, mainly includes CYP1A1, CYP1A2 and CYP1B1 (Nelson et al. 2004). The combination of PAHs and AhR mediates the transcriptional activation of CYP1 family, which is involved in the bioactivation and drug metabolism of procarcinogens to carcinogenic derivatives (Go et al. 2015). Continued exposure to environmental carcinogens is thought to increase the expression of CYP1A1 in extrahepatic tissues via AhR (Androutsopoulos et al. 2009). Interestingly, recent studies have shown that CYP1A2 has a tumour-suppressing function in patients with lung adenocarcinoma and hepatocellular carcinoma (Gastelum et al.

2020; Zhang et al. 2021). In the experiment, we found that the expression level of CYP1A2 mRNA decreased after the addition of PAHs, which suggested that the activity and expression level of CYP1A2 might be down-regulated in nasopharyngeal carcinoma. When we added AhR antagonist, the expression level of CYP1A2 mRNA was up-regulated. This could further prove that PAHs inhibits the expression of CYP1A2 in nasopharyngeal carcinoma.

AhR is involved in regulating apoptosis and cell cycle through a variety of mechanisms (Yin et al. 2016). Previous studies have shown that PAHs inhibit apoptosis in human bronchial epithelial cells by activating AhR (Ferecatu et al. 2010). In our study, we found that PAHs inhibited the apoptosis of CNE-2 cells. When ITE was added, it was found that the inhibition of apoptosis by PAHs was reduced, and the expression of AhR was also decreased. It can be seen that PAHs can regulate the apoptosis of NPC cells by regulating the expression of AhR. However, whether the apoptosis of CNE2 is related to the expression of CYP1A1 still needs further study. B[a]P was found to promote cell proliferation through AhR in lung cancer (Jimma et al. 2019). However, in our study, PAHs had no significant effect on the proliferation CNE-2 cells. Whether this is related to the concentration of PAHs remains unclear and further studies are needed. PAHs can affect the apoptosis and invasion of NPC cells, and affect AhR expression of NPC cells. But it is still unclear whether the apoptosis and invasion of NPC cells are related to AhR expression, and further studies are needed.

The invasion and metastasis of cancer cells are important manifestations of cancer progression. Our study found that PAHs had no significant effect on the migration of CNE-2 cells, but could inhibit cell invasion through AhR. Interestingly, in a study of tongue squamous cell carcinoma, B[a]P significantly increased the cell invasion and migration ability (Huang et al. 2020). In another oral squamous cell carcinoma study, AhR ligand increased oropharyngeal squamous cell migration, and AhR inhibitors inhibited oropharyngeal cell migration (Stanford et al. 2016). We speculated that it was related to the composition and content of PAHs.

In conclusion, PAHs can affect the apoptosis and invasion of NPC cells by regulating AhR expression, but has no significant effect on the proliferation and migration of nasopharyngeal carcinoma cells. This study can provide a new direction for the study on the occurrence and development of nasopharyngeal carcinoma, and further studies are needed to reveal the effect of PAHs on nasopharyngeal carcinoma and its potential mechanism.

Conflict of Interest

The authors declare no conflict of interest.

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