

## Evaluation of Relationship between Tissue Levels of Polycyclic Aromatic Hydrocarbon (PAHs) in Gastric Cancer by Immunohistochemistry

### Abstract

Gastric cancer is one of the most common cancers in the world and the north and northwest of Iran. Given the high prevalence of polycyclic aromatic hydrocarbons (PAHs) in the environment as one of the influential factors in cancer, the present study was conducted to evaluate the relationship between tissue levels of polycyclic aromatic hydrocarbon (PAHs) and history of food exposure to environmental contaminants. Immunohistochemistry was performed in patients with gastric cancer. The study population included 30 patients with gastric cancer. Thirty tissue samples were randomly selected among patients with gastric cancer and a questionnaire was used to assess the role of environmental factors and nutritional factors in the incidence of gastric cancer. Patients' tissue blocks were examined by BPDE-5D11 monoclonal antibody to determine the tissue expression of PAH using immunohistochemistry (IHC). Statistical analysis of data was performed using SPSS16 software. The results showed that the tissue level of PAH is associated with factors such as the place of birth of patients (rural or urban), gender of patients (male and female), type of PAH expression (diffuse, focal), and smoking ( $p < 0.05$ ). Also, an investigation of PAH agonists in this study showed that smoking increases the risk of gastric cancer. Based on the results of the present study, it is recommended that contact with PAH sources such as smoked and grilled foods and cigarette smoke be strictly avoided.

**Keywords:** Gastric Cancer, Aromatic Polycyclic Hydrocarbons, Immunohistochemistry, environmental Contaminants, Nutritional Factors

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### Introduction

#### 1-Introduction

Gastric cancer is a malignant tumor that stems from the epithelium of the gastric mucosa. The most common type is gastric adenocarcinoma, which is present in 90% of cases and is approximately 5% of malignant lymphoma tumors (1). This cancer is the fourth most common cancer and the second cause of cancer-induced death in the world (2). The most high-risk areas with an age-standardized rate (ASR) of more than 20 people per 100000 people per year are Japan, China, and Korea (3). In Iran, the northern and north western areas are at higher risk for gastric cancer and Ardabil province has the highest rate of gastric cancer with ASR 49.1 and 25.4 in men and women in Iran, respectively (4). Helicobacter pylori infection is one of the leading causes of gastric cancer, accounting for more than 60% of all infections. Other common causes include deficiencies in antioxidants, salted foods, tobacco, genetic factors, and environmental factors (5). Various environmental and chemical factors are involved in the development of gastric cancer, and PAH is one of them. Multi-ring aromatic hydrocarbons consist of two or more fused aromatic rings that are in the form of different isomers. In pure form, they are colorless to pale white or yellow solids and are used in the painting and production of plastics, pesticides, and road asphalt. These compounds have low solubility in water and are highly lipophilic (6).

PAHs are a large group of environmental carcinogens that are seen everywhere as environmental pollutants, including in water, soil, and air. These materials are obtained from natural

disasters such as forest fires, volcanic activity, and incomplete combustion processes of fossil fuels. The most important foods for receiving PAHs are oils and fats, smoked products (meats, fish, and shellfish), spices, and dried fruits and grains (7, 8). The most important PAHs are naphthalene, anthracene, fluorine, phenanthrene, benzo [a] pyrene, benzo [k] fluoranthene, etc. (9). Some of the very important effects of PAHs in humans are mutagenic and carcinogenic effects of some PAHs, including benzo [a] pyrene. Poly-aromatic hydrocarbons (PAHs) are xenobiotic compounds that can play a role in gastric cancer (10). Aryl hydrocarbon (AhR) receptors mediate the metabolism and toxicity of xenobiotics (11). By binding to the aryl hydrocarbon (AhR) receptor, PAH increases the expression of cytochrome-PuSO-CYP1a1, and CYP1b1 enzymes, and after metabolic changes and conversion to electrophilic reactors, it becomes a carcinogen that can react with DNA and cause cellular macromolecules damages, including DNA. It indicates the PAH's ability to cause cancer (12, 13). Given the increasing prevalence of environmental pollution in Tehran and other metropolitan areas to polycyclic aromatic hydrocarbons (PAHs), the present study was conducted to investigate the relationship between tissue levels of polycyclic aromatic hydrocarbons (PAHs) and the history of food exposure to environmental contaminants in people with gastric cancer by immunohistochemistry method.

#### 2-Materials and Methods

In the present study, the study population included 30 patients with gastric cancer referred to the pathology ward of the Cancer Institute of Imam Khomeini Hospital. Inclusion criteria

of the study included: the patient's survival, the presence of the patient's tissue block and his or her pathology report, and the absence of a history of gastric cancer in his first-degree family. After completing the questionnaire and examining the medical files of the pathology laboratory of Imam Khomeini Cancer Institute during 2014-2015, 30 patients were the candidate to participate in the present study. After confirming their slides, the desired blocks were separated and 5 slides were prepared from each of them. A total of 150 slides were prepared to determine the tissue expression of the PAH marker. The slides were transferred to the Toxicogenomics Research Laboratory of the Pharmaceutical Sciences Research Center of Tehran Azad University for the IHC staining method.

## **2-1-Preparation of samples**

### **2-1-1-Fixation**

Since cell degradation begins immediately after the organism's death, it is necessary to fix the tissue to prevent damage. Fixation typically involves cross-linking between proteins and complete tissue removal. This practice usually takes 24 hours. 10% formalin, equivalent to 4% formaldehyde or paraformaldehyde, is commonly used. There are often two methods for fixation, including the perfusion method and the immersion method. The perfusion method uses an animal capillary network to deliver the fixing compound throughout the tissue. Thus, the fixation operation is uniform and fast. In the immersion method, which is easier than the first method, the tissues of a laboratory animal can be fixed in different cells. The most important disadvantage of this method is the slow penetration of the fixer and the surface areas are better fixed than the deep areas.

### **2-1-2- Dehydration**

To prepare paraffin or plastic from block tissue, it must be dehydrated. This is done by placing the tissue in increasingly concentrated alcoholic solutions.

### **2-1-3- Preparation of paraffin blocks**

After fixation, the samples were divided into 3-5 mm pieces. To make a paraffin block, the tissues must first be transparent. It involves using an interstitial fluid of ethanol and paraffin. Since these two compounds are not mixable, the used compounds include benzene, chloroform, toluene, and xylene. Toluene is the most common cleanser. First, the tissue is immersed in a 50-50 solution of absolute ethanol and toluene for two hours. Finally, it is placed in a water bath (56-58 ° C) which is the melting temperature of paraffin, and then it is placed in pure paraffin for one hour and transferred to a special container containing paraffin for 2-3 hours. After that paraffin penetrated completely into the tissue, it is placed in the relevant container to form a block. After the blocks turned yellow, they are ready for testing.

### **2-1-4- Preparing the cut and placing it on the slide**

For histological experiments, cuts of 3-5 microns are prepared from paraffin sections by a special microtome and placed on a slide to perform the necessary tests on them.

### **2-2- Fixed method for testing**

In this method, xylene organic solvent is used to deparaffinize the samples to remove paraffin on the slide (xylene 1: 20 minutes and xylene 2: 20 minutes).

Dehydration stages

It is performed with different alcohols in this way:

Placing slices on absolute alcohol

Absolute alcohol 1: 5 minutes

Absolute alcohol 2: 5 minutes

Placing slices on 96% alcohol

Alcohol 96% 1: 5 minutes

Alcohol 96% 2: 5 minutes

Placing slices on 70% alcohol

70% alcohol 1: 5 minutes

70% alcohol 2: 5 minutes

Placing slices on 50% alcohol

50% alcohol 1: 5 minutes

50% alcohol 2: 5 minutes

After these stages, the slides should be washed as follows:

### **2-2-1- Ag-retrieval stage:**

It is the supply of antigen on the surface of tissue by citrate buffer with pH = 6-6.2 at the temperature of 900W at for 10-20 minutes (Varies depending on the type of antibody). Since it is possible for the tissues to be removed in the boiling citrate buffer, after a few minutes, we check the slides and continue heating again. We place the citrate buffer slides in the laboratory while still in the citrate buffer to cool. If the optimal time for Ag-retrieval is low, the antigen is not exposed and if the temperature is high, it is over-exposed. After heating in the buffer, the slides should be completely cooled in the solvent and reach room temperature.

### **2-2-2-Quenching step**

The endogenous peroxidase enzyme must be inactivated and as we have exogenous peroxidation in the staining, so we quench the endogenous one. It is performed with H<sub>2</sub>O<sub>2</sub> (0.3%). We place the slides in the vicinity of H<sub>2</sub>O<sub>2</sub> for 10-15 minutes. We place the slides in a wet chamber so that the surface does not dry. Then, we wash it with Tris 1 and 2 buffers with pH = 7.2-7.4. then, we place the slides in each tray for 5 minutes.

### **2-2-3-Blocking**

It is performed through different methods. The best work to do is to use Bovine Serum Albumin (BSA). BSA powder is available. We dissolve three grams of the powder in 100 ccs of trays. This serum covers the entire surface of the slides and helps to add Ag in the next stage. It prevents Ab from sticking to the slide glass surface and unwanted surfaces and the

possibility of non-specific binding between the tissue protein and the primary antibody. It causes the slide background and the tissue not to remain clean at the end of work. After this operation, we wash the slides with Tris 1 and 2 buffers (5 minutes each) and shake them several times.

#### **2-2-4- Incubation with substance: Adding primary Ab**

The primary antibody should be at the optimum concentration. We use Bovine Serum Albumin (BSA) to dilute the antibodies. Staining was performed three times to obtain this optimum BPDE-SD11 concentration. First, a microliter of antibody with BSA at a volume of 200 microliters is prepared, but due to the inappropriateness of the antibody concentration, a concentration of 1 to 100 was obtained, and again, due to inappropriate antibody concentration, a concentration of 1 to 150 (a microliter of antibody with BSA with a volume of 150 microliters) was prepared. This concentration was considered the appropriate concentration, and at this stage, the antibody was incubated in the refrigerator in the vicinity of slides for 24 hours. At this time, the protein, which is the tissue antigen or antibody, forms a complex. Finally, the slides were washed with Tris 1 and 2 (each for 5 minutes).

#### **2-2-5- Detector kit for secondary staining**

At this stage, envision kit was used and the slides were incubated with this substance at room temperature for 30 minutes. Then, it was washed with Tris 1 and 2 buffers.

#### **2-2-6- Adding chromogen**

At this stage, a substance called diaminobenzidine (DAB) was used, which was diluted with chromogen buffer and then incubated at room temperature for 15 minutes. The DAB was prepared by diluting one drop of DAB + chromogen with one drop of DAB + substrate buffer and the compound was kept in a refrigerator after using it. This substance is very toxic and carcinogenic and safety precautions must be observed. During this operation, the slides were washed with distilled water several times with pressure.

#### **2.2.7. Background staining**

This method uses hematoxylin. The slides are placed beside this substance for 10-15 minutes. Hematoxylin is the background dye that stains the nucleus. After this step, the final washing was performed.

#### **2-2-8- Dehydration stage**

At this stage, dehydration must be performed again to preserve the tissues. To do this, the slides were placed in 50%, 70%, 96% alcohol, and then absolute alcohol for 5 minutes each, respectively, and then dipped in xylene.

#### **2-2-9- Mounting**

At this stage, a special mounting adhesive was added and the slide was placed on the slide.

#### **2-2-10-Scoring**

Samples were scored using a light microscope with the presence or absence of color and intensity of colors. The colors were measured based on the intensity of the nuclear color and were divided into 4 classes. The classification was performed by a pathologist qualitatively and proportionally.

1- Negative: Color is observed in less than 5% of cells.

2- +1 (Mild): Color is observed in less than 5-25% of cells

3- +2 (Mild to Moderate): Color is observed in 25-50% of cells.

4- +3 (Moderate): Color is observed in 50-75% of cells.

5- +4 (Moderate to Severe): Color is observed in more than 75% of cells.

6- +5 (Severe): color is observed in almost all cells.

#### **2-3-Use of control**

In each IHC test, both positive and negative controls are required to measure the desired antigen. In the case of positive control, the tissue or cell as well as the user has already been tested and generated a signal. The selected antibody must be closely related to the previous antibody, and in the case of negative control, the use of tissue or cell is the same as the desired tissue without antigen in it is performed with the initial removal while the other steps will be done without change.

#### **2-4- Statistical analysis**

In the present study, SPSS19 software was used for data analysis, and the significance level (P-value) was considered less than 0.05.

### **3- Results**

Among the study population, which included 30 patients with gastric cancer, 86.7% of them had positive PAH expression. Among tumor tissue samples, two cases of score = +1, in seven cases, score = +2, in nine cases, score = +3, in five cases, score = +4, and in three cases, score = +5 were found. In the present study, it was found that in patients, in which PAH expression was positive, all of them had diffuse expression and a significant relationship was reported between PAH expression in gastric tumor tissue and its expression ( $P = 0.044$ ).

3-1- Comparison between demographic factors and PAH expression in gastric cancer tissue

Among the evaluated variables related to demographic factors of patients with gastric cancer, place of birth with  $P=0.034$  and gender with  $p=0.025$  showed a significant relationship with PAH expression in gastric tumor tissue of patients with gastric cancer. In the present study, it was found that 75% of patients with positive PAH expression were born in urban areas and a significant relationship was found between patients' place of birth and PAH expression. Also in the present study, the number of males with gastric cancer was more than females with gastric cancer, but given a smaller number of women,

PAH expression in them shows a higher percentage and there is a significant relationship between gender and PAH expression (Table 1).

Table 1. Investigation of the relationship between demographic factors, risk factors, underlying factors, and PAH expression in gastric cancer tissue

Characteristic	PAH overexpression (+ 4, + 5)	PAH lowexpression (0, + 1, + 2, + 3)	P-value
Age (yrs)	57 (14.67)	62.59 (8.41)	0.201
<b>Gender</b>			
Male	4 (19.04%)	17 (80.96%)	*0.025
Female	4 (44.4%)	5 (55.6%)	
<b>BMI</b>			
Weight (kg)	55.13 (7.56)	59.05 (7.62)	0.222
Height (cm)	167.75 (6.08)	169.00 (9.92)	0.742
BMI			0.42
≤ 24.9 kg/m <sup>2</sup>	8 (100%)	21 (95.45%)	
25-29.9 kg/m <sup>2</sup>	0	1 (4.55%)	
≥ 30 kg/m <sup>2</sup>	0	0	
<b>Place of Birth</b>			
Town	6 (75%)	6 (27.27%)	*0.034
Village	2 (25%)	16 (72.73%)	
<b>Living Place</b>			
Town	8 (100%)	16 (72.73%)	0.155
Village	0	6 (27.27%)	
<b>Physical Activity</b>			
Yes	3 (37.5%)	6 (27.27%)	0.666
No	5 (62.5%)	16 (72.73%)	
<b>Duration of Physical Activity</b>			
None	5 (62.5%)	15 (68.19%)	0.055
1-30 minute	0	6 (27.27%)	
30-60 minute	3 (37.5%)	1 (4.54%)	

2-3- Comparison between the presence of underlying diseases and PAH expression in gastric cancer tissue in patients with gastric cancer

The subjects were examined in terms of the history of underlying diseases such as cardiovascular disease, diabetes, depression, etc. Among them, five subjects with gastric cancer also had underlying diseases, and out of these 5 subjects, one had a positive expression of PAH in their cancer tissue. However, in these investigations, no significant relationship was found between the underlying diseases and the expression of PAH in patients with gastric cancer (p = 0.248).

**3-3- Comparison of nutritional risk factors and PAH expression in gastric cancer tissue**

In the present study, patients were divided into four groups in terms of oil consumption: animal oil, liquid oil, solid oil, butter, or margarine oil. In terms of consumption of mayonnaise sauce, they have also been divided into four classes: no consumption, daily, weekly, and sometimes. There was no significant relationship between nutritional factors and PAH expression in gastric cancer tissue (p> 0.05).

**3-4- Comparison between exposure to PAH agonists and PAH expression in gastric cancer tissue**

Among the studied factors in the relationship between PAH expression and PAH agonists in gastric cancer tissue, a

significant relationship was found between smoking and PAH expression with P = 0.043. In the present study, it was found that 62.5% of patients with PAH expression were smoking and

there was a significant relationship between smoking and PAH expression (Table 2).

Table 2. Investigation of the relationship between exposure to PAH agonists and PAH expression in gastric cancer tissue

Characteristics	PAH overexpression (+ 4, + 5)	PAH low expression (0, + 1, + 2, + 3)	P-value
<b>Regular use of plastic dishes and bottles</b>			
No	7 (87.5%)	19 (86.36%)	0.146
Daily	1 (12.5%)	0	
1-2 in a week	0	3 (13.64%)	
<b>Grilled Meat</b>			
No	2 (25%)	4 (18.18%)	0.958
1-2 in week	3 (37.5%)	9 (40.9%)	
Above 2 in week	1 (12.5%)	2 (9.1%)	
Under 1 in week	2 (25%)	7 (31.82%)	
<b>Recreational Smoking</b>			
No	3 (37.5%)	13 (59.1%)	*0.043
Yes	5 (62.5%)	9 (40.9%)	
<b>Habitual Smoking</b>			
None	3 (37.5%)	13 (59.09%)	0.379
1-10 cigarettes/ day	1 (12.5%)	1 (4.54%)	
10-20 cigarettes/ day	4 (50%)	5 (22.72%)	
> 20 cigarettes/ day	0	3 (13.63%)	
<b>Opium and CNS stimulants (Addiction)</b>			
No	8 (100%)	20 (90.9%)	1.000
Yes	0	2 (9.1%)	
<b>Occupational Exposure to PAHs</b>			
Yes	3 (37.5%)	5 (22.72%)	0.643
No	5 (62.5%)	17 (77.28%)	
<b>Microwave Usage</b>			
Yes	1 (12.5%)	3 (13.63%)	1.000
No	7 (87.5%)	19 (86.37%)	
<b>Microwave Using Model</b>			
None	7 (87.5%)	19 (86.38%)	0.733
Daily	0	1 (4.54%)	
Weekly	0	1 (4.54%)	
Monthly	1 (12.5%)	1 (4.54%)	
<b>Living near PAH-producing factories</b>			
Yes	0	1 (4.54%)	1.000
No	8 (100%)	21 (95.46%)	

#### 4-Discussion

The present study aimed to find the possible relationship between physiological factors, environment, and pollutants and also to evaluate the role of PAH in the incidence of gastric cancer in Iran. In this regard, we examined the differences in lifestyle and diet of 30 patients with gastric cancer and tissue expression of PAHs was determined by BPDE-5D11

monoclonal antibody using the immunohistochemistry method. Thus, some biological habits, living near the factories producing PAHs, cooking at high temperatures, and air pollution were examined. To achieve these goals, questionnaires were designed based on environmental factors affecting the incidence of gastric cancer and completed by patients with gastric cancer who underwent surgery at Imam

Khomeini Hospital during 2014-2016. Investigation of demographic factors in the present study showed that patients' place of birth and gender increases the risk of gastric cancer. It means that all patients, with a positive PAH expression, were born in urban areas. The results of previous studies suggest that level of PAH in the air of urban areas is 10 times that of rural areas and diesel engines are an important source of air pollution in urban areas. The emissions of these engines often include smoke and toxic compounds, and industrial activities are one of the causes of entering PAHs into the air of urban areas (14). Concerning the gender of patients, some studies have reported that males are more prone to gastric cancer than females (15). No clear reason has been found for this difference, but occupational and environmental exposures might be involved in this regard, for example, males smoke more than females (16). Estrogen can also be the reason for this difference. These hormones protect women against gastric cancer during the reproductive years, but their effect decreases after menopause (17, 18).

### 5-Conclusion

Investigation of PAH agonists in the present study showed that smoking increases the risk of gastric cancer. In explaining this result, it can be stated that based on studies conducted on gastric tissue in people with gastric cancer and normal gastric tissue in 1998, high levels of BPDE-I-DNA were reported in tumor tissues compared to normal tissue and smokers showed higher levels of BPDE-I-DNA than non-smokers, which has higher levels of BPDE-I-DNA in tumor tissues than normal tissue, which may be associated with gastric cancer (19). The rate of gastric cancer increases with increasing age. Among the cases studied in the United States between 2005 and 2009, almost 1% occurred in people aged between 20 and 34 years, while 29% occurred in people aged between 75 and 84 years (20). The present study, like other studies, suffers some limitations, the most important of which were lack of easy access to patients and obtaining the required information. Nevertheless, the present study is the first serious study conducted to investigate the tissue expression of PAH in patients with gastric cancer. It also summarizes the possible risk factors and recommends an appropriate lifestyle and diet to prevent gastric cancer. Environmental pollution and hormonal disorders interact with each other, and since these pollutants are always present in the environment and cannot be avoided and Iran is also known as one of the polluted countries in terms of the level of compounds such as aromatic hydrocarbon polycyclic, it is recommended to use a proper diet and avoid fatty foods. Also, it is recommended to avoid contact with PAH sources such as smoked and grilled foods and contact with cigarette smoke.

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### Conflict of interest

None.

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### Ethics Statements

None.

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