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PAH exposure in esophageal tissue and risk of esophageal squamous cell carcinoma in northeastern Iran

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Abstract

Objective—To evaluate the association of polycyclic aromatic hydrocarbon (PAH) exposure in esophageal epithelial tissue and esophageal squamous cell carcinoma (ESCC) case status in an ESCC case-control study in a high-risk population in northeastern Iran.

Design—Immunohistochemical staining of tissue microarrays (TMAs) of non-tumoral esophageal biopsies from ESCC cases and control subjects. Immunohistochemistry was performed using monoclonal antibodies 8E11 and 5D11, raised against benzo[a]pyrene (B[a]P) diol epoxide

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COMPETING INTERESTS

None

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ETHICS COMMITTEE APPROVAL

The Golestan Case-Control study was reviewed and approved by the Institutional Review Boards of the Digestive Disease Research Center (DDRC) of Tehran University of Medical Sciences, and the US National Cancer Institute. Both cases and controls gave written informed consent for the interview and all endoscopic and biopsy procedures.

AUTHORS' CONTRIBUTIONS

All authors contributed to the conception and design of the study. BA-A, FK, SMH, PH, MS, CCA, and SMD participated in data collection and assembly. BA-A, FK, and CCA analyzed the data. All authors contributed to data interpretation and the writing of the manuscript.

(BPDE)-I-modified guanosine and BPDE-I-modified DNA, respectively. Staining intensity was quantified by image analysis, and the average staining in three replicates was calculated.

Setting—Rural region in northeastern Iran.

Participants—Cases were patients with biopsy-proven ESCC. Controls were GI clinic patients with no endoscopic or biopsy evidence of ESCC.

Main outcome measure—Adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) for the association between antibody staining intensity and ESCC case status.

Results—Cultured ESCC cells exposed to B[a]P *in vitro* showed dose-dependent staining with 8E11, but not with 5D11. With 8E11, sufficient epithelial tissue was available in the TMA cores to analyze 91 cases and 103 controls. Compared to the lowest quintile of 8E11 staining in the controls, adjusted ORs (95% CIs) for the 2nd to 5th quintiles were 2.42, 5.77, 11.3, and 26.6 (5.21–135), respectively (*P* for trend < 0.001). With 5D11, 89 cases and 101 controls were analyzed. No association between staining and case status was observed (ORs (95% CIs) for the 2nd to 5th quintiles were 1.26, 0.88, 1.06, and 1.63 (0.63–4.21), *P* for trend = 0.40).

Conclusions—Dramatically higher levels of 8E11 staining were observed in non-tumoral esophageal epithelium from ESCC patients than from control subjects. This finding strengthens the evidence for a causal role for PAHs in esophageal carcinogenesis in northeastern Iran.

Keywords

esophageal squamous cell carcinoma; polycyclic aromatic hydrocarbons; immunohistochemistry; tissue microarray

INTRODUCTION

Esophageal cancer is the sixth most common cause of cancer death worldwide, and esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer. [1;2] Incidence and mortality rates of ESCC show striking variations across different geographic regions, with over 50-fold differences between the lowest and highest risk areas of the world.[1;2] With reported rates over 50/10⁵ person-years, Golestan Province in northeastern Iran is one of the highest-risk areas for ESCC in the world.[3;4]

In most low-risk areas of the world, tobacco smoking and heavy alcohol consumption are the major causes of ESCC.[5;6] In contrast, in high-risk areas such as Golestan Province, Iran and Linxian, China, a much smaller proportion of ESCC cases are attributable to these two factors.[7;8] In Golestan, about 40% of men and 3% of women are current or former smokers,[9] yet ESCC rates are very similar in the two sexes.[3;4] Likewise, in Linxian, the prevalence of smoking is far higher in men than in women,[8] but their ESCC rates are similar. There is also very limited alcohol consumption in these two high-risk areas, especially in Golestan Province.[8;9] Therefore, other etiologic factors must play an important role in these high-risk areas. Other candidate factors include nutritional deficiencies,[10] hot beverages,[11] tooth loss,[12;13] and ethnicity.[3;14]

People may be exposed to some of the carcinogens found in tobacco smoke, such as polycyclic aromatic hydrocarbons (PAHs),[15] in ways other than smoking tobacco. PAHs are produced during incomplete combustion of organic materials, and their major sources, other than tobacco smoke, are food products,[16] atmospheric air,[17] and occupational exposure.[18] The International Agency for Research on Cancer (IARC) has classified benzo[*a*]pyrene (B[a]P), a prototypical PAH, as a Group 1 carcinogen for humans, based on mechanistic studies, and has classified several other individual PAHs as probable or possible carcinogens for humans.[19]

The people of Golestan may be highly exposed to PAHs. Dietary intake of B[a]P has been reported to be higher in Golestan than in lower-risk Fars Province.[20] Measurement of 1-hydroxypyrene glucuronide (1-OHPG), a stable PAH metabolite and signal compound for PAH exposure, in urine samples from 99 Golestan inhabitants showed that 83% of the study participants had high or very high levels of this biomarker.[21] However, evidence for an association between PAH exposure and ESCC in Golestan is only indirect, and to date, no evaluation in any population has examined the association between a direct measure of PAH exposure in individual subjects and ESCC in a case-control or cohort study.

To obtain a more direct evaluation of the role of individual exposure to PAHs in ESCC carcinogenesis, we used immunohistochemistry (IHC) to measure the PAH content of non-tumoral esophageal tissues from ESCC patients and control subjects in a case-control study in Golestan Province.

MATERIALS AND METHODS

Participants

Between December 2003 and June 2007, ESCC cases and control subjects were enrolled in the Golestan Case-Control study in Golestan Province, Iran. This study was a collaboration of the Digestive Disease Research Center (DDRC) of Tehran University of Medical Sciences, the US National Cancer Institute (NCI) and the International Agency for Research on Cancer (IARC). The study was approved by the Institutional Review Boards of DDRC and NCI, and all participants gave written informed consent before enrollment in the study. The methods of this study and selected results have been published previously.[7;11;13;22] In brief, local internists were asked to refer all patients with upper gastrointestinal (GI) tract symptoms suspicious of cancer to Atrak Clinic, a referral clinic in Gonbad, a major city in Golestan Province. After written informed consent, Atrak Clinic patients with biopsy-proven ESCC were enrolled as cases, and other Atrak Clinic patients who had no endoscopic or biopsy evidence of ESCC were enrolled as clinic controls. All subjects underwent an in-person interview which elicited detailed information on known and suspected risk factors for ESCC. In all, 300 ESCC cases and 300 clinic controls were enrolled.

Tissue samples

Each case and control underwent esophagogastroduodenoscopy and biopsy sample collection according to a predefined protocol. The esophagus and stomach were examined before and after staining the esophagus with 3% Lugol's iodine solution. At least seven biopsies were obtained from all upper GI tumors, and additional standard biopsies were taken in all patients from endoscopically normal sites in the gastric antrum, the gastric body, the cardia, and the mid-esophagus. Biopsy specimens were oriented, fixed in 70% ethanol, and embedded in paraffin.

For this study, we constructed tissue microarrays (TMAs) from the endoscopically normal standard mid-esophageal biopsies from 120 ESCC cases and 120 control subjects. We chose to evaluate non-tumoral rather than tumor biopsies from the cases to make the compared tissues from cases and controls as similar as possible in PAH metabolism. The specific case and control biopsies were chosen because their size and orientation made them the most suitable for use in TMA construction. The biopsies from cases included 4 showing squamous dysplasia, 3 showing esophagitis, and 113 showing histologically normal mucosa, and the biopsies from controls included 5 with esophagitis and 115 with normal mucosa. Two TMAs with one millimeter in diameter cores were made, using a MTA-1 manual arrayer (Beecher Instruments, Sun Prairie, WI). Each TMA contained 60 cores from ESCC cases (one core per case) and 60 cores from control subjects (one core per control).

Antibodies to BPDE-I

We performed immunohistochemistry (IHC) with two monoclonal antibodies, 8E11 and 5D11 (Cell Sciences, Canton, MA), to evaluate the PAH content of the esophageal epithelium in the TMA cores. The 8E11 antibody was raised against 7 β ,8 α -dihydroxy-9 α , 10 α -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE-I) modified guanosine conjugated with bovine serum albumin (BPDE-I-G-BSA), and it detects BPDE-I-deoxyguanosine (BPDE-I-dG) adducts, BPDE-I-DNA adducts, BPDE-I-RNA adducts, BPDE-I-protein adducts, BPDE-I tetraols (hydrolysis products of free BPDE-I), and other non-adducted PAHs.[23–26] The 5D11 antibody was raised against BPDE-I modified DNA, and it primarily detects BPDE-I-DNA adducts.[23;24] It is thought that 5D11 recognizes antigenic determinants on both the BPDE-I ring structure and DNA, and is thus fairly specific for BPDE-I-DNA adducts, while 8E11 mainly recognizes determinants present on the BPDE-I ring, and therefore reacts with a broader range of BPDE-modified biological molecules. [23;24]

Immunostaining of cultured cells exposed to benzo[a]pyrene

To evaluate the staining characteristics of the 8E11 and 5D11 antibodies, cultured squamous cell carcinoma cell lines TE1, TE6 and TE13 were exposed for 3 to 48 hrs to concentrations of B[a]P ranging from 0.1 to 20 micromolar in dimethylsulfoxide (DMSO; Merck, Germany). These cells have been previously shown to be capable of metabolizing PAH and generating BPDE-DNA adducts (K. Vahakangas, personal communication). Exposed cells were washed with PBS and recovered by trypsinization, and cell pellets were fixed in 70% ethanol before embedding in paraffin. Sections were prepared and stained with antibodies 8E11 and 5D11 using the same protocol that was used for the TMA sections.

Immunohistochemical analysis

Sections were deparaffinized in xylene, rehydrated in graded ethanol, and then antigen retrieved for 20 minutes with a steamer using a pH 10.0 antigen retrieval buffer (Dako target retrieval solution high pH, Dako, Carpinteria, CA). Endogenous peroxidase block was applied for 5 minutes, and the primary antibody (8E11 or 5D11, 1:100 dilution, Cell Sciences, Canton, MA) was incubated for 1 hour at room temperature, followed by 15 minutes of incubation with the biotinylated secondary antibody, Streptavidin-HRP (Dako, Carpinteria, CA), and the reaction was visualized using 3,3'-diaminobenzidine solution (DAB). All staining reactions were performed in triplicate, using a Dako autostainer (Dako Autostainer Plus Link, Dako, Carpinteria, CA).

Image analysis and slide scoring

The slides were imaged using a ScanScope T2 digital scanner (Aperio, Vista, CA) with a 20X objective, and the images were imported into TMALab software (Aperio, Vista, CA) for image management and analysis. The epithelial portion of each core was outlined manually by a pathologist (BA-A) for image analysis. Because 8E11 reacts with a wide range of adducted products, most of which are found in the cytoplasm, it is expected to produce predominantly cytoplasmic staining. 5D11, however, stains only BPDE-I-DNA adducts, so it is expected to generate primarily or exclusively nuclear staining. Therefore, 8E11 staining was quantified using a pixel count algorithm and 5D11 staining was quantified using a nuclear algorithm (Aperio, Vista, CA). The pixel algorithm assigns a staining intensity score (0–255) to each pixel in the selected area. For statistical analysis, these pixel scores were grouped into four groups, 0 to 3+, with 0 indicating the weakest and 3+ indicating the strongest staining. The nuclear algorithm identifies and assigns a staining intensity score (0 – 255) to each nucleus in the selected area. These scores were also grouped into four groups, 0 to 3+.

Two pathologists (BA-A and SMD) examined the slides independently for staining quality. Both pathologists concluded that the quality of the first 5D11 staining was poor. Therefore, only the second and third 5D11 staining results were analyzed.

Statistical analysis

8E11—The image of the outlined epithelium in each core contained thousands of pixels, and each pixel had a staining intensity score ranging from 0 to 3+. To obtain a summary staining intensity score for each core, we calculated a weighted average for all of the pixel staining intensity scores within the outlined area of that core (weights 0 to 3+). To account for differences in staining intensity between slides, we calculated a standardized Z score for each core based on the mean and standard deviation of the summary staining intensity scores of the control subjects analyzed on that core's TMA slide. That is, we subtracted the mean summary staining intensity score of the control subjects on each slide from the summary staining intensity score of each core on that slide and divided the resulting number by the standard deviation of the summary staining intensity scores of the control subjects on that slide. Therefore, on each slide, the mean Z score for controls was 0 and the standard deviation for controls was 1. Finally, because each TMA slide was stained in triplicate, we calculated the average of the three standardized Z scores for each core, which became that core's final staining intensity score. Cores with fewer than 20,000 pixels were considered to have inadequate tissue and were excluded from the analysis.

5D11—The image of the outlined epithelium in each core contained hundreds of nuclei, and the staining intensity scores given to each nucleus ranged from 0 to 3+. Analytical methods were similar to those for 8E11. In the final step, averages of the two Z scores (from stainings 2 and 3) were calculated. Cores with fewer than 100 nuclei were considered to have inadequate tissue and were excluded from the analysis.

Differences in demographic variables were tested using T-tests (age) and chi-square tests (all other variables). Pearson's correlation coefficients were calculated for the standardized Z scores between each pair of the stainings. T-tests were used to compare the means of the standardized scores between cases and controls in each staining. Logistic regression analysis was done to compare the final staining intensity scores between case and control tissue samples. Quintiles were made based on the final staining intensity scores among control subjects, and the unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) were computed for the 2nd to 5th quintiles compared to the lowest quintile. Regression models were adjusted for age, sex, education (any versus none), ethnicity (Turkmen versus non-Turkmen), tobacco use (ever versus never), and opium use (ever versus never). Because the lag time between PAH exposure and the highest density of PAH-DNA adducts may be relatively short,[27] regression models were also performed adjusting for age, sex, education, ethnicity, current tobacco use (yes versus no) and current opium use (yes versus no). *P*-values for trend were calculated using the logistic regression models by assigning values of 1–5 to quintiles 1–5, respectively. All reported *P*-values are two-sided and those <0.05 were considered statistically significant. All analyses were done using STATA software, version 10.0 (StataCorp, College Station, TX).

Results

Immunostaining of the TE6 squamous cell carcinoma cell line is shown in Figure 1. Similar results were obtained with the TE1 and TE13 cell lines (data not shown). With the 8E11 antibody, unexposed cells did not show any evidence of staining, and a clear, dose-dependent increase in both cytoplasmic and nuclear staining was observed. With the 5D11 antibody, background nuclear staining was observed in cells not exposed to B[a]P. Although

a modest increase in staining was seen with 12.5 μ M B[a]P, there was no clear dose-dependent increase in staining.

Examples of 8E11 staining of the TMA cores are shown in Figure 2. Predominantly cytoplasmic staining is seen in superficial squamous cells throughout the epithelium, with sparing of the basal layer around most vascular papillae. In the TMAs, different cores showed different levels of staining intensity.

Table 1 shows the demographic characteristics, tobacco use, opium use and education of the case and control subjects who were analyzed for esophageal PAH exposure with 8E11 staining. Case subjects were significantly older and less educated than control subjects, but the other variables were not significantly different by case status. The distribution of these characteristics was similar among the total group of cases and controls whose biopsies were used to prepare the TMAs and among the cases and controls who were analyzed with 5D11 staining (data not shown).

Ninety-one cases and one hundred and three control subjects had adequate tissue (> 20,000 pixels) for 8E11 image analysis. The Pearson's correlation coefficients for the standardized Z scores obtained in stainings 1 and 2, 1 and 3, and 2 and 3 were 0.46, 0.24, and 0.47, respectively. Mean 8E11 staining intensity Z scores were significantly higher in cases than in controls in each of the three stainings (Table 2). ORs (95% CIs) for the association between 8E11 staining intensity and ESCC are shown in Table 3. Compared to the lowest quintile (the lowest intensity of 8E11 staining), adjusted ORs (95% CIs) for the 2nd to 5th quintiles were 2.42 (0.39 – 14.8), 5.77 (1.06 – 31.4), 11.3 (2.16 – 59.6), and 26.6 (5.21 – 135), respectively (*P* for trend < 0.001). Regressions adjusting for current tobacco and opium use (rather than ever use of these substances) gave similar results (data not shown).

Eighty-nine cases and one hundred and one control subjects had sufficient tissue (> 100 nuclei) for 5D11 image analysis. The Pearson's correlation coefficient between the standardized Z scores obtained in stainings 2 and 3 was 0.50. The mean 5D11 staining intensity score was slightly higher in cases than in controls in staining 2 and was the same in cases and controls in staining 3 (Table 2). ORs (95% CIs) for the association between 5D11 staining intensity and ESCC are shown in Table 3. Compared to the lowest quintile, adjusted ORs (95% CIs) for the 2nd to 5th quintiles were 1.26 (0.46 – 3.45), 0.88 (0.31 – 2.50), 1.06 (0.35 – 3.20), and 1.63 (0.63 – 4.21), respectively (*P* for trend = 0.40). Regressions adjusting for current tobacco and opium use (rather than ever use of these substances) gave similar results (data not shown).

DISCUSSION

Investigations of the role of PAHs in human cancers can be traced back to 1775, when Percivall Pott found an association between exposure to soot and scrotal cancer in chimney sweeps.[28] More recently, the International Agency for Research on Cancer (IARC) recognized some complex PAH mixtures (e.g. coal tar) and industrial processes (e.g. coke production) as carcinogenic in humans, but individual PAHs were considered only probable or possible carcinogens in humans.[18] It was only in the most recent IARC review that benzo[a]pyrene exposure in occupational settings was categorized as a definite human carcinogen.[19]

An individual's exposure to PAHs can be estimated in several ways, including measuring PAH metabolites in urine, staining tissues with anti-PAH antibodies, and chemical analysis of target or surrogate tissues (eg. esophageal tissue or blood) for adducted or non-adducted PAHs. Urinary metabolites of PAHs, such as 1-hydroxypyrene glucuronide (1-OHPG), reflect total body exposure in the 24–72 hours prior to collection,[29] which may be useful

for estimating habitual exposures but may not capture episodic exposures. Antibodies raised against PAH immunogens can be used to estimate PAH exposure in specific tissues of interest, such as the esophagus, but they may vary in their specificity and are only semiquantitative. Chemical analysis of adducted or non-adducted PAHs is the most specific and quantitative way to measure PAH exposure, and this analysis can be performed in the target tissue for carcinogenesis (eg. the esophagus) or in surrogates (eg. blood). Since DNA adducts can be repaired, they are usually less permanent than protein adducts.[30] Studies in experimentally exposed rats using ^{32}P postlabelling have shown that maximum DNA adduct levels are reached three days after a single dose of BaP, followed by a rapid decay.[27] Because of the absence of active repair, the stability of protein adducts varies over a longer time scale, which depends on the protein's stability and the rate of cell turnover in the mucosa.[30] The strengths and limitations of measuring PAH adducts by immunoassays, ^{32}P -postlabelling, and mass spectrometry have been extensively discussed. [30]

In the current study we tested the association between PAH exposure and ESCC risk in a high-risk population in Golestan Province, Iran. We estimated PAH exposure by immunohistochemical staining of esophageal biopsies. Immunostaining has been previously used successfully to detect PAH-DNA adducts in a pilot study of 5 archival esophageal biopsies in subjects from Linxian, China, another high-incidence area with a pattern of risk factors similar to those identified in Golestan.[31] Recent studies have also used antisera to BPDE-DNA adducts to evaluate PAH adducts in cancers of the cervix [32] and prostate.[33] In our study, we used two monoclonal antibodies, 8E11 and 5D11, to evaluate the PAH-ESCC association. The 8E11 stain showed an appropriate dose-response in the B[a]P-dosed cell lines, and it showed a very strong dose-response association between 8E11 staining and case status, with an adjusted OR (95% CI) for the fifth versus first quintile of staining of 26.6 (5.21–135). The 5D11 staining, on the other hand, did not show the expected dose-response in the dosed cell lines, and did not show a difference in staining between case and control cores on the TMAs. Based on the cell line results, the 5D11 staining patterns observed in the TMAs appear to be non-specific.

Our 8E11 results indicate that PAHs and their metabolites are detectable in epithelial cells of the esophagus (the target of esophageal carcinogenesis), and that the quantity of these compounds in non-tumoral epithelial biopsies is strongly associated with ESCC risk. The large magnitude and clear dose-response pattern of this association argues that this is not a chance finding. The known staining profile of 8E11 suggests that the compound causing this association is most likely a PAH or PAH derivative, however other cross-reactive molecules also cannot be ruled out. There is a need for more specific evaluations to identify the exact compound(s) mediating this association. This association also does not prove that PAHs are causing mutagenesis in these tissues.

As an additional comparison, we examined the 8E11 staining intensity (mean Z score) of current smokers and current non-smokers of tobacco, stratified by case status. Among cases, the mean Z scores were 0.87 in current smokers and 0.61 in non-smokers, a difference of 0.26 ($p=0.21$). Among controls, the mean Z scores were 0.19 in current smokers and -0.02 in non-smokers, a difference of 0.21 ($p=0.41$). Thus in both cases and controls, 8E11 staining intensity was greater in those with this known PAH exposure, consistent with a valid measure of this exposure, although the power was too low to be sure that this was not due to chance. Additionally, in both cases and controls, the difference in staining intensity (Δ mean Z-score) between smokers and non-smokers was only about one-third of the difference in staining intensity between cases and controls (Δ mean Z-score= 0.66 , Table 2), implying that the elevated staining in cases was largely due to other exposures. This is consistent with our previous analysis of urinary 1-OHPG in the same area, which showed that 83% of the

population was highly exposed to PAHs and only 15% of the variance could be explained by known risk factors.[21] Thus there is also a need to look for other PAH sources that are significant exposures in this population.

Strengths of this study include the analysis of the target tissue of interest (the esophageal epithelium) rather than a surrogate, the analysis of non-tumoral epithelial tissue (rather than tumor tissue) from both cases and controls in a well designed case-control study, the use of TMAs containing both case and control tissues to minimize differences in how these tissues were processed or evaluated, conducting and averaging triplicate staining measurements, the use of an automated image analysis system to quantify the staining, and the collection of detailed information on environmental factors which allowed adjustment for possible confounders. On the other hand, this study also has limitations. The case-control design of the study leaves open the possibility of reverse causality, that the development of esophageal cancer caused the patients to be more highly exposed to PAHs or to increase their reactivity to 8E11 through some other mechanism. A possible explanation for such an association in this population would be patients smoking opium to relieve the pain from their cancer. In earlier studies we have shown that opium use is relatively common in Golestan [7;9] and that people in this area respond truthfully and accurately to questions regarding their opium use.[34] Such questions were included in the questionnaires filled out by all cases and controls in this study, and adjustment for the answers to these questions did not change the staining associations. But reverse causality due to another unmeasured factor cannot be ruled out.

In conclusion, we found a very strong dose-response relationship between intensity of esophageal tissue staining with 8E11 antibody and ESCC risk in a case-control study in Golestan Province, Iran. This finding strengthens the evidence for a causal role of PAHs in the etiology of esophageal cancer in this high-risk population. It will be important to replicate this study in other high-risk populations, to perform similar studies in prospective cohorts, and to undertake studies with more specific and quantitative chemical analyses to identify the PAH compounds associated with ESCC risk.

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Abbreviations

1-OHPG	1-hydroxypyrene glucuronide
95% CI	95% confidence interval
B[a]P	benzo[a]pyrene
BPDE	benzo[a]pyrene diol epoxide
DAB	3,3'-diaminobenzidine
DDRC	Digestive Disease Research Center
ESCC	esophageal squamous cell carcinoma
GI	gastrointestinal

IARC	International Agency for Research on Cancer
IHC	immunohistochemistry
NCI	U.S. National Cancer Institute
OR	odds ratio
PAH	polycyclic aromatic hydrocarbon
TMA	tissue microarray

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SUMMARY BOX

What is already known about this subject

- Tobacco smoking and heavy alcohol use are major risk factors for esophageal squamous cell carcinoma (ESCC)
- In some populations with a high incidence of ESCC, tobacco and alcohol use are not prominent risk factors, but people may be exposed to carcinogens found in tobacco smoke, such as polycyclic aromatic hydrocarbons (PAHs), in ways other than smoking tobacco
- In several diverse populations at high risk for ESCC, high levels of urinary PAH metabolites have been found in non-smoking adults, suggesting that PAH exposure from non-tobacco sources may be an important risk factor for this disease

What are the new findings?

- This study measured the staining intensity of anti-PAH antibodies in non-tumoral esophageal biopsies from ESCC cases and controls from a high-risk population in northeastern Iran; it found dramatically higher staining levels in the esophageal epithelium of the cases, even after adjusting for tobacco use.
- This association of high levels of PAH exposure in the target esophageal epithelium and ESCC risk significantly strengthens the evidence for a causal role for PAH exposure, from tobacco or other sources, in esophageal carcinogenesis.

How might the findings of this study impact clinical practice in the foreseeable future?

- If PAH exposure, from any source, is an important causal factor for ESCC, interventions can target this exposure to reduce the risk of this disease, especially in high-risk populations.

Figure 1. Immunostaining of the TE6 squamous cell carcinoma cell line with 8E11 and 5D11 antibodies. With 8E11, unexposed cells show no evidence of staining, and staining increases in a dose-dependent manner with increasing exposure to B[a]P. With 5D11, background nuclear staining is seen in the unexposed cells, and there is no clear dose-dependent increase in staining with increasing B[a]P exposure.

Figure 2. Immunostaining of the TMA cores with the 8E11 antibody. Figures A–C (400x) show a spectrum of increasing staining intensity; the staining is predominantly cytoplasmic and involves the full thickness of the epithelium. Figure D (200x) shows full-thickness staining with sparing of the basal cells around the vascular papillae.

Table 1

Demographic characteristics and habits of ESCC case and control subjects from the Golestan Case-Control Study who were examined for esophageal PAH exposure with 8E11 staining

	Cases (N = 91)*	Controls (N = 103)*	P-value†
<i>Mean Age (s.d.)</i>	66.0 (10.1)	56.4 (13.1)	<0.001
<i>Gender</i>			
Male	43 (47%)	46 (45%)	0.72
Female	48 (53%)	57 (55%)	
<i>Ethnicity</i>			
Turkmen	47 (52%)	47 (46%)	0.44
Non-turkmen	44 (48%)	55 (54%)	
<i>Tobacco use</i>			
Never	54 (59%)	71 (69%)	0.16
Ever	37 (41%)	32 (31%)	
<i>Opium use</i>			
Never	60 (67%)	79 (77%)	0.12
Ever	30 (33%)	24 (23%)	
<i>Education</i>			
No education	83 (91%)	72 (70%)	<0.001
Some education	8 (9%)	31 (30%)	

* Numbers for individual variables may be less than the total because of missing data.

† Differences in distributions were tested using T-tests (age) and chi-square tests (all others).

Table 2

Mean (standard deviation) of the summary staining intensity Z scores in ESCC case and control subjects from the Golestan Case-Control Study

Stain	Mean Z score (s.d.)		
<i>8E11</i>	Cases (N=91)	Controls (N=103)	<i>P</i> -value [†]
First staining	1.03 (1.32)	0.00 (1.00)	< 0.001
Second staining	0.39 (0.94)	0.00 (1.00)	0.006
Third staining	0.58 (1.02)	0.00 (1.00)	<0.001
Total average	0.66 (0.80)	0.00 (0.76)	<0.001
<i>5D11</i>	Cases (N=89)	Controls (N=101)	<i>P</i> -value
First staining [*]	---	---	
Second staining	0.32 (1.21)	0.00 (1.00)	0.05
Third staining	0.00 (1.10)	0.00 (1.00)	0.98
Total average	0.16 (0.99)	0.00 (0.88)	0.23

^{*} The quality of the first 5D11 staining was poor, so this staining was not analyzed.

[†] Differences in the mean Z scores of cases and controls were tested using T-tests.

Table 3

Odds ratios and 95% confidence intervals for the association between staining intensity and esophageal squamous cell carcinoma in the Golestan Case-Control Study

	Case subject Number	Control subject Number	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*
8E11				
First quintile (< -0.65) [†]	2	20	Referent	Referent
Second quintile (-0.65 to -0.29)	6	21	2.85 (0.51 – 15.9)	2.42 (0.39 – 14.8)
Third quintile (-0.29 to 0.05)	14	21	6.66 (1.34 – 33.1)	5.77 (1.06 – 31.4)
Fourth quintile (0.05 to 0.52)	20	21	9.52 (1.96 – 46.1)	11.3 (2.16 – 59.6)
Fifth quintile (> 0.52)	49	20	24.5 (5.23 – 115)	26.6 (5.21 – 135)
5D11				
First quintile (< -0.85)	18	20	Referent	Referent
Second quintile (-0.85 to -0.16)	18	20	1.00 (0.41 – 2.46)	1.26 (0.46 – 3.45)
Third quintile (-0.15 to 0.30)	13	21	0.69 (0.29 – 1.76)	0.88 (0.31 – 2.50)
Fourth quintile (0.31 to 0.75)	11	20	0.61 (0.23 – 1.62)	1.06 (0.35 – 3.20)
Fifth quintile (> 0.75)	29	20	1.61 (0.69 – 3.79)	1.63 (0.63 – 4.21)

* Adjusted for age, sex, education, ethnicity, tobacco use and opium use

[†] Average normalized Z-score from multiple stainings (see methods)