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Aryl hydrocarbon receptor expression is associated with a family history of upper gastrointestinal cancer in a high risk population exposed to aromatic hydrocarbons

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Abstract

Background—Polycyclic aromatic hydrocarbon (PAH) exposure is a risk factor for esophageal squamous cell carcinoma (ESCC), and PAHs are ligands of the aryl hydrocarbon receptor (AhR). This study measured the expression of AhR and related genes in frozen esophageal cell samples from patients exposed to different levels of indoor air pollution, who did or did not have high-grade squamous dysplasia (HGD), and who did or did not have a family history (FH) of upper gastrointestinal cancer (UGI Ca).

Methods—147 samples were evaluated, including 23 (16%) from patients with HGD and 48 (33%) from patients without DYS who heated their homes with coal, without a chimney (a “high” indoor air pollution group), and 27 (18%) from patients with HGD and 49 (33%) from patients without DYS who did not heat their homes at all (a “low” indoor air pollution group). Nearly half (64 (44%)) had a FH of UGI Ca. RNA was extracted and Quantitative-PCR analysis was performed.

Results—AhR gene expression was detectable in 85 (58%) of the samples, and was more than 9-fold higher in those with a FH of UGI Ca (median expression (IQR) -1964 (-18000, -610) versus -18000 (-18000, -1036) Wilcoxon $P = 0.02$). Heating status, dysplasia category, age, gender, and smoking were not associated with AhR expression (linear regression, all P-values ≥ 0.1).

Conclusion—AhR expression was higher in patients with a FH of UGI Ca. Such individuals may be more susceptible to the deleterious effects of PAH exposure, including PAH-induced cancer.

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Keywords

Gastrointestinal tract cancer; Esophagus; Aryl hydrocarbon receptor; family history of cancer; gene expression; polycyclic aromatic hydrocarbons

Introduction

Esophageal cancer is the sixth leading cause of cancer death worldwide and is the leading cause of cancer death in Linxian, China, where mortality rates from this disease are around 100/100,000 person-years for both sexes (1). These are some of the highest mortality rates for any single cancer found anywhere. In high risk populations throughout the world, esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal cancer. The primary prevention of ESCC within high risk groups continues to be limited by our inability to identify specific risk factors and etiologic agents.

Having a positive family history of cancer is known to be associated with an increased risk for several cancers (2-4). In Linxian, China, a recent prospective study with 15 years of follow-up and over 3,400 incident upper gastrointestinal cancers, including over 1,900 esophageal squamous cell carcinomas, 1000 gastric cardia cancers and 300 gastric noncardia cancers found a positive family history of esophageal or gastric cardia cancer to be significantly associated with increased risk of cancer at all of these sites (5). A separate case-control study of esophageal squamous dysplasia, the precursor lesion of ESCC, conducted in the same region, confirmed an association between a family history of esophageal or gastric cancer and risk of dysplasia (6). Similar associations have also been reported in other high-risk regions of China (7-9). These include not only associations between a family history of upper gastrointestinal cancer and esophageal cancer risk, but also associations between family history of these cancers and chromosomal aberrations (frequency of allelic loss) (10,11) or gene-environment interactions (7).

High grade (moderate or severe) esophageal squamous dysplasia is also known to be associated with an increased risk of ESCC. In a 13-year follow-up study of a cohort of 682 endoscoped patients in Linxian, those who began with moderate dysplasia were about 10 times as likely and those with severe dysplasia were about 30 times as likely to develop ESCC as those who began with normal esophageal mucosa (12).

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), are likely to play an etiologic role in ESCC. In low risk areas, exposure to PAHs comes primarily from tobacco smoke, but exposure from non-tobacco sources may be more important in high risk areas. Evidence that supports the role of PAHs in the high risk areas of China includes a high prevalence anthracotic peri-esophageal lymph nodes in squamous cell carcinoma resections (13), high levels of B[a]P in uncooked and cooked staple food samples (14), and high concentrations of urine 1-hydroxypyrene glucuronide (1-OHPG), a PAH metabolite and biomarker of recent exposure (15). This exposure may come from indoor air pollution caused by burning soft coal in unventilated rooms, and such coal burning was recently shown to be associated with a two-fold increased risk for esophageal squamous dysplasia (6), the histological precursor lesion of ESCC. In other high risk areas for ESCC, such as Southern Brazil and Northeastern Iran, high levels of urine 1-OHPG have also been detected (16,17).

PAHs are ligands for the aryl hydrocarbon receptor (AhR). Upon binding, the AhR is translocated to the nucleus and binds the Aryl hydrocarbon receptor nuclear translocator (Arnt), resulting in the increased expression of the Cytochrome p450 metabolism genes CYP1a1 and CYP1b1 (18-22), among others. This interrelationship represents part of the AhR/Dioxin

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Response Element (DRE) paradigm (19,23). Studies find higher tissue expression of AhR in response to PAH exposure (22,24-26), and tissue specific metabolic activation or accumulation of PAHs increases DNA adduct formation and mutagenesis (27-29). Variability in gene induction along this pathway may be related both to ligand exposure and to cancer risk (21, 26,23,30); for example, tobacco exposure can induce AhR and can increase lung cancer risk (31), and tobacco smoke can induce CYP1b1 in the gastrointestinal tract (32).

The current cross-sectional study measured gene expression of AhR, Aryl hydrocarbon receptor repressor (AhRR), CYP1a1 and CYP1b1 in esophageal cell samples from adults living in the high ESCC risk region of Linxian, China. These participants and their samples were a subset of a recent cross-sectional esophageal screening study to detect squamous dysplasia or ESCC (33). All of these subjects completed a structured questionnaire that included information about home heating and family history of upper gastrointestional cancer, and they all underwent esophageal cytology examination followed by chromendoscopy with Lugol's iodine staining and biopsy. Gene expression was compared between individuals who heated their homes without a chimney (a “high” indoor air pollution group) and those who did not heat their homes at all (a “low” indoor air pollution group), to assess the effect of this exposure on the AhR pathway. Expression of these genes was also compared between individuals with biopsy-proven high-grade esophageal squamous dysplasia and those with no histological evidence of dysplasia, to evaluate the association between these genes and the precursor lesions of ESCC, and between those with and without a family history of upper gastrointestinal tract cancer.

Materials and Methods

Study subjects and procedures

The subjects in this study were a subset of the subjects who participated in the Cytology Sampling Study 2 (CSS2), a cross-sectional esophageal balloon cytology screening study in Linxian which has been previously described in detail (6,33). The CSS2 study included 40-65 year old volunteers who were apparently healthy and had no contraindications for balloon cytology or upper GI endoscopy.

All subjects in the CSS2 completed a structured questionnaire based on information previously found or suspected to be associated with ESCC in this population, including family history of upper gastrointestinal tract cancer and living conditions such as the use of home heating, with or without a chimney. Each subject then underwent an esophageal balloon cytology examination followed by chromoendoscopy with Lugol's iodine staining and biopsy. The cytologic sampler was either a mechanical balloon (Cytomesh Esophageal Cytology Device, Wilson-Cook Medical, Inc., Winston-Salem, NC) or a traditional Chinese inflatable balloon (CICAMS, Beijing, China). All subjects gave written informed consent, and this study was approved by the Institutional Review Boards of the Cancer Institute of the Chinese Academy of Medical Sciences (CICAMS) and the U.S. National Cancer Institute.

After esophageal sampling, the balloon was placed in 40 ml of saline in a 50 ml centrifuge tube, cut from its catheter, shaken, and transferred on ice to a central laboratory at the CICAMS field station in Yaocun Commune, Linxian.

At the central laboratory, each sample was vortexed for 30 seconds to remove adherent cells from the balloon, the balloon was removed from the tube, and the remaining cell suspension was centrifuged at 1500 rpm for 5 minutes. The excess supernatant was discarded and the cell pellet re-suspended in supernatant to approximately 1.0 ml final volume. Half of this concentrated cell solution was transferred to a 1.25 ml eppendorf tube (Sarstedt Inc. Newton,

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NC), and snap-frozen in liquid nitrogen. The frozen samples were transported from the field laboratory on dry ice and were stored at -80°C until RNA extraction.

The subjects underwent endoscopy at the CICAMS field station within two weeks after their cytology examinations. Endoscopy with Lugol's iodine staining, with targeted biopsies of all unstained lesions and standard biopsies of normal-appearing mucosal sites, was performed as previously described (33). The endoscopic biopsy slides were read separately by two pathologists (N.L, S.M.D), using criteria previously described (34). Discrepant results were adjudicated by joint review. Each subject's esophageal disease status was categorized by his or her worst squamous endoscopic biopsy diagnosis. For this analysis, subjects with biopsy-proven moderate or severe squamous dysplasia were classified as having high-grade dysplasia (HGD), and subjects with biopsies showing only normal mucosa or esophagitis were classified as having no dysplasia.

Sample selection

For the current study, the CSS2 subjects were selected in two ways, by their questionnaire responses related to indoor air pollution and by their histologic evidence of high grade squamous dysplasia.

From the 720 CSS2 subjects with frozen esophageal cytology samples, 572 (79%) had frozen esophageal cytology samples available in our repository. Of these subjects, 344 (60%) could be stratified into either a “high” indoor air pollution group (N=94 (16%)) that heated their homes without a chimney or a “low” indoor air pollution group (N=250 (44%)) that did not heat their homes at all. The remaining 228 (40%) with available biologic samples used other methods to heat their homes, and they were not included in this analysis.

Out of the 344 subjects in the two indoor air pollution groups, 51 (15%) had HGD and 234 (68%) had no evidence of dysplasia. The remaining 59 (43%) samples had other histologic diagnoses, and thus were not included in this analysis.

All 51 subjects with HGD were included, with 23 (45%) representing the “high” indoor air pollution group and 28 (55%) the “low” indoor air pollution group. Similarly, all 49 (7%) subjects without dysplasia who were in the “high” indoor pollution group were selected for inclusion. A random subset of equal size (49 patients) and gender proportions (51% male) was selected from the 185 available subjects without dysplasia who were in the “low” indoor air pollution group.

Thus, 149 participants were selected based on sample availability, biopsy diagnosis and questionnaire data including 23 with high-grade dysplasia and 49 without dysplasia who heated their homes without a chimney (the “high” indoor air pollution group), and 28 with high-grade dysplasia and 49 without dysplasia who did not heat their homes (the “low” indoor air pollution group).

Approximately half (64 (44%)) of these subjects had a family history of upper gastrointestinal cancer (Table 1).

RNA Extraction

A 50 uL aliquot of each frozen esophageal cell sample was thawed by the addition of 100 uL of Arcturus Picopure extraction buffer under RNAse-free conditions (Kit: Cat # KIT0204 Picopure Isolation Kit, Arcturus Mountain view, CA). RNA isolation included on-column DNAse treatment of all samples. The RNA was then eluted into 30 uL of the provided Arcturus elution solution. Quantitation and purity check was performed on 2 uL of each sample by spectrophotometer (NanoDrop). A260/280 Quality analysis was performed on 1 uL of each

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sample by Agilent Bioanalyzer (picochip) and reported as an RNA integrity number, or RIN, on a Scale of 1-10, with 10 representing maximum quality. Samples were run in duplicate.

The mean (SD) RNA yield per sample was 300.1 (581.1) ng, and the mean (SD) RNA concentration per sample was 15.0 (29.0) ng/ul. Post-extraction RNA quality for all the samples, as determined by the RIN index, had a mean (SD) of 2.9 (1.5). Both the RNA concentration and quality were deemed suitable for qt-rtPCR expression analysis, since they were similar to the values obtained from a set of successfully amplified pilot samples (data not shown).

Qt-rtPCR

First Strand cDNA synthesis—The Super Script™ III First Strand Synthesis system for Reverse Transcriptase PCR (Invitrogen Corporation, Carlsbad, CA, USA) was used to synthesize cDNA from total RNA in a 96 well microtiter plate format. A minimum of 1ng of total RNA served as the template for first strand cDNA synthesis and RNase OUT™ (Invitrogen Corporation, Carlsbad, CA, USA) was included in the reaction to prevent degradation of the target RNA by contaminating ribonucleases. Incubations were carried out in a thermocycler at the following conditions: 50°C for 50 minutes, followed by 85°C for 5 minutes, followed by the addition of RNase H, with a continued incubation for 20 minutes at 37°C.

Analysis of Gene Expression by Quantitative-PCR—Quantitative-PCR (Q-PCR) analysis was performed using Taq Man probes (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions in 10μL final volumes in 384-well microtiter plates. Thermocycling conditions, using an Applied Biosystems ABI-7900 SDS, were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 40 cycles of 15 seconds, and 60° C for 1 minute. The primers for the genes of interest, AhR, AhRR, Cyp1a1 and Cyp1b1, and an 18s endogenous control gene were purchased using the Applied Biosystems Assay-on-Demand program; the sequences of these genes/probes are proprietary in nature (Applied Biosystems, Foster City, CA, USA). Raw data from the Q-PCR run was exported into a comparative Ct analysis workbook. In our criterion for selecting real target amplification, the C_T-value (signal above cycle threshold) had to be $\geq 33 C_T$'s and had to be detected 3 C_T's earlier than the negative controls. Quantification of mRNA was performed using a ΔΔCT Log2 transformation approach. The mRNA expression of each target gene was normalized to the expression of the 18s control gene, and then it was compared to the Universal Human Reference RNA sample (Stratagene Los Angeles CA) to yield a relative mRNA expression value (relative to the reference sample). This ΔΔCT sample value (ΔΔCT= ΔCT-Sample − ΔCT-Reference) was then modified as follows: If 2(-ΔΔCT)-1>0, then the result = 2(-ΔΔCT)-1; otherwise, the result =-1/2(-ΔΔCT). This modification changed the range for down-regulation from 0-1 to -∞-0 and the range for up-regulation from 1-∞ to 0-∞.

Analysis

For this analysis, all subjects were classified as having high or low exposure to indoor air pollution and as having HGD or no dysplasia, as described above. Smokers were defined as those who had ever smoked regularly for ≥ 6 months. A family history of cancer was considered positive if a subject reported any upper gastrointestinal cancer in a first degree relative.

Two outliers, one with AhR expression over 22-fold higher and one with Cyp1a1 expression 120-fold higher than the next highest values on the log2 scale were dropped from the analysis. AhR samples with no detectable expression were imputed to be one half the lowest measure, and the results were not sensitive to other imputations such as having these samples be equal to the lowest measure.

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Statistical analyses of gene expression and other variables were performed using Wilcoxon rank-sum tests, Pearson's chi-square tests, and linear regressions, and a two-sided $p \leq 0.05$ was considered statistically significant.

Results

Table 1 shows the distribution of selected demographic and lifestyle factors in all subjects, in those with high and low exposure to indoor air pollution, and in those with and without high-grade esophageal squamous dysplasia. The analytical cohort consisted of 147 subjects with an average age of 54 years, with nearly equal representation of men and women across the heating and dysplasia categories. Slightly more of the subjects with high indoor pollution and high grade dysplasia were smokers, and significantly more of those with high grade dysplasia had a family history of cancer (Pearson's chi-square test p=0.03)

The expression of each transcript was evaluated in duplicate in neighboring wells. The coefficient of variation of these duplicates was less than 2.4% for all genes. Expression of the 18s housekeeping gene was measurable in all 147 samples. AhR gene expression was measurable in 85 (58%) of the 147 samples, but AhRR, CYP1a1 and CYP1b1 gene expression were measurable in only 25 (17%), 13 (9%), and 14 (10%) of the samples, respectively. The low expression of AhRR, CYP1a1 and CYP1b1 prevented detailed comparisons, but expression of Cyp1a1 tended to be more common among those with a family history of cancer (12.5% versus 6%, Table 2).

Subjects with a family history of upper gastrointestinal tract cancer had a relative AhR expression over nine-fold higher than those with a negative family history of these cancers (Wilcoxon rank-sum test p=0.02, Table 3). This association was also positive when tested by univariate linear regression using a family history of upper gastrointestinal tract cancer as a dichotomous variable to predict AhR expression (p=0.02). Because more of the subjects with a positive family history had high grade dysplasia than those with a negative family history (56% versus 44%, respectively, Pearson's Chi-square test p=0.03) and those with a positive family history of upper gastrointestinal tract cancer tended to have higher RNA concentrations than those with a negative family history (median (IQR) 8.7 (1.9, 19) ng/μl versus 3.4 (1.0,12) ng/μl, respectively, p=0.05), the association between family history and AhR expression was tested and persisted after adjusting for dysplasia status and sample RNA concentration (p=0.02). It was also unaltered by the inclusion of variables for indoor air pollution category or smoking status.

Exposure to high levels of indoor air pollution, the presence of high grade squamous dysplasia, and other covariates of potential interest, including age, gender, and smoking, were not associated with AhR expression in linear regression models ($p \geq 0.14$).

Discussion

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of carbon, and several are classified as human carcinogens by IARC and the US National Toxicology Program (35,36). Urine 1-hydroxypyrene glucuronide reflects recent PAH exposure, and using this marker we have shown that several populations at high risk for ESCC in China, Iran, and Brazil are highly exposed to PAHs (15-17). Consequently, studies on the biological effects of this exposure in high-risk populations and its potential relationship with precursor lesions and invasive cancers are needed to further elucidate the association between PAH exposure and ESCC.

The observation that a positive family history of cancer is associated with increased cancer risk is well accepted, and it is particularly evident in gastrointestinal, geniturinary, and

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gynecologic cancers (2-4). This predisposition may be attributable to an inherited genetic susceptibility that, in some instances, is modified by the environment, i.e., a gene-environment interaction (4). One such example is the association between tobacco use and specific Cytochrome P450-related enzyme genotypes that predispose the esophagus of smokers to an even higher risk for DNA damage or cancer (37,38). A recent 15-year prospective study in Linxian has shown a significant association between a positive family history of UGI cancer and an increased risk of both esophageal and gastric cancer (5), and a separate case-control study showed an association between family history of these cancers and risk of esophageal squamous dysplasia (6).

The current study evaluated the expression of genes coding for proteins that are related to the carcinogenic effect of PAH exposure, including aryl hydrocarbon receptor (AhR), CYP1a1, CYP1b1, and aryl hydrocarbon receptor repressor (AhRR), which acts as an AhR antagonist (18). The expression of these genes was evaluated in epithelial cell samples from the esophagus, the target organ of interest in the Linxian population, which has known environmental PAH exposure and high rates of ESCC. Our data showed that median AhR expression was significantly higher in individuals with a positive family history of UGI cancer, but was not associated with apparently higher levels of PAH exposure or the presence of high grade esophageal squamous dysplasia, the precursor lesion of ESCC. Expression levels of AhRR, CYP1a1 and CYP1b1 were all low. These results suggest that individuals in this population with a family history of UGI cancer may be more susceptible to the detrimental effects of PAH exposure, including PAH-induced cancer.

Median AhR expression was not higher among individuals who heated their homes without a chimney or among tobacco smokers, groups that appeared to be exposed to more PAHs. However, our assessments of these exposures were relatively simply and a more accurate measurement of environmental PAH exposure at the time of sample collection may be needed to test this association more completely. Median AhR expression was also not higher among subjects with high grade squamous dysplasia, possibly because the esophageal balloons sample the entire esophageal epithelium and may not accurately detect increased expression that is found only in focal mucosal lesions. In contrast, differences in AhR expression related to familial predisposition would be expected to be present in all cells, so it should be more easily seen in balloon samples.

The current study was limited by the number of samples with expression below our limit of detection. This was most significant for AhRR and the Cytochrome p450 genes. The lack of detectable expression for these genes of interest in the setting of detectable levels of a housekeeping gene is indicative of minimal to no expression (39), and is consistent with previous reports that these genes are variably expressed in the esophagus (40,41). The interpretation that this is a real finding is supported by the fact that the efficiency of our short qRT-PCR products should be relatively “independent” of RNA quality (39), and any potential effect of suboptimal RNA quality would have been minimized after normalization of the target gene expression results to the 18s endogenous control and the Universal Human Reference sample. Finally, the positive association which we found between AhR expression and family history of cancer persisted with a variety of statistical approaches.

Advantageous aspects of our cell sampling method also deserve mention. This minimally invasive approach samples the entire mucosal epithelium of the target organ of interest and theoretically provides an integrated assessment of its exposure and biological potential. Sampling the target organ of interest is important because induction of gene expression is frequently cell and tissue-type specific (42,43). Our cell sampling method also avoids problems that may occur when sampling is limited to neoplastic tissue, such as the inhibition of CYP enzyme expression by inflammatory cytokines associated with such lesional tissue (44) and

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the fact that PAHs may induce xenobiotic metabolizing enzymes more potently in normal squamous cells than in premalignant or tumor cells (45). The cell sampling method used in our study avoids these potential pitfalls and may be suitable for inclusion in other epidemiological studies, especially those looking for variation in constitutive traits.

In summary, we measured gene expression of AhR and related genes in esophageal epithelial cell samples from subjects in a Chinese population at high risk for ESCC, and we compared expression levels in subjects who differed by apparent PAH exposure, by the presence or absence of esophageal squamous dysplasia, the precursor lesion of ESCC, and by family history of upper gastrointestinal tract cancer. We found significantly higher AhR expression in subjects with a family history of upper gastrointestinal tract cancer but no difference in this expression between subjects with different levels of indoor air pollution or the presence or absence of esophageal squamous dysplasia. This is the first report of an association between AhR expression and family history of cancer in humans, and it supports the idea of a familial predisposition to the deleterious effects of PAH exposure in this high-risk population.

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Table 1

Demographic and Lifestyle Factors

	Total	Indoor Air Pollution		Worst Histologic Diagnosis	
		High	Low	With Dysplasia	Without Dysplasia
Samples	147	71	76	50	97
Mean Age (SD)	54 (5.0)	54 (5.1)	55 (4.9)	55 (4.7)	54 (5.1)
Male (%)	74 (50)	38 (54)	36 (47)	25 (50)	49 (50)
Smoking* yes (%)	48 (33)	24 (34)	24 (32)	19 (38)	29 (30)
Family History†,‡ yes (%)	64 (44)	35 (49)	29 (38)	28 (56)	36 (37)

*Smoking: High versus low Indoor Air Pollution, Pearson's Chi-square test p=0.77; With versus without Dysplasia, p=0.32.

†Family History of Cancer: High versus low Indoor Air Pollution p=0.17; With versus without Dysplasia **p=0.03**,

‡Upper gastrointestinal tract cancer in one or more first-degree relatives (father, mother, siblings, or children).

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Table 2

Any versus no gene expression, by family history of upper gastrointestinal tract (UGI) cancer, in the esophageal cell samples

Gene		With a Family History of UGI Cancer N (column %)	Without a Family History of UGI Cancer N (column %)
AhR	No Expression	20 (31%)	42 (51%)
	Expression	44 (69%)	41 (49%)
AhRR	No Expression	52 (81%)	70 (84%)
	Expression	12 (19%)	13 (16%)
Cyp1a1	No Expression	56 (88%)	78 (94%)
	Expression	8 (12%)	5 (6%)
Cyp1b1	No Expression	57 (89%)	76 (92%)
	Expression	7 (11%)	7 (8%)

Pearson's Chi-square test AhR p=0.02; p>0.2 for all other genes

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Table 3

AhR relative gene expression, by family history of upper gastrointestinal tract (UGI) cancer, dysplasia, and indoor air pollution exposure status

Category	Number of Subjects	Fold Change in AhR Gene Expression [Median relative gene expression (IQR)]*
Without a Family History of UGI Cancer (reference)	83	1.0 [-18000 (-18000, -1036)]
With a Family History of UGI Cancer	64	9.2 [-1964 (-18000, -610)]
Without Esophageal Dysplasia (reference)	97	1.0 [-2434 (-18000, -711)]
With High Grade Esophageal Dysplasia	50	0.1 [-18000 (-18000, -847)]
Indoor Air Pollution With Low Exposure (reference)	76	1.0 [-3714 (-18000, -1010)]
With High Exposure	71	1.2 [-3015 (-18000, -623)]
Non Smoker (reference)	99	1.0 [-4904 (-18000, -623)]
Smoker	48	2.0 [-2386 (-18000, -1026)]
Male (reference)	74	1.0 [-3906 (-18000, -1351)]
Female	73	1.4 [-2668 (-18000, -533)]

* Log2 ΔΔCT sample value

Wilcoxon rank-sum and linear regression tests: Family History of Cancer **p=0.02**, **p=0.02**; Esophageal Dysplasia p=0.3, p=0.1; Indoor air pollution p=0.6, p=0.6; Smoking status p=0.4, p=0.4; Gender **p=0.04**., p=0.2.