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## SHORT COMMUNICATION

# High urine 1-hydroxypyrene glucuronide concentrations in Linxian, China, an area of high risk for squamous oesophageal cancer

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Most squamous cell carcinomas of the oesophagus in low-risk populations are attributable to alcohol and tobacco consumption, but the aetiologic agents in many high-risk populations have yet to be definitively identified. Linxian, China has some of the highest oesophageal cancer rates in the world. Recent studies suggest that an association exists between high-level exposure to carcinogenic polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (B[*a*]P), and the development of oesophageal cancer. The inhabitants of this high-risk region extensively use coal and wood for cooking and heating in unvented stoves, and thus may be exposed to PAHs produced during the incomplete combustion of these fuel sources. High levels of B[*a*]P were recently detected in staple food samples from Linxian and histopathologic changes that may be associated with PAH exposure have also been identified in oesophagectomy specimens from the region. In an effort to determine whether this high-risk population is exposed to high levels of PAHs, voided urines from non-smokers ($n = 22$) without occupational exposure were collected and analysed using immunoaffinity chromatography and synchronous fluorescence spectroscopy for 1-hydroxypyrene glucuronide, a PAH metabolite and index biomarker for mixed PAH exposure. The median urine 1-hydroxypyrene glucuronide concentration (2.06 pmol $ml^{-1}$) was equivalent to concentrations detected in current smokers. To the authors' knowledge, this represents the first report of elevated urine 1-hydroxpyrene glucuronide concentrations in Linxian, and the first biologic confirmation that the inhabitants of this rural, non-industrial, high oesophageal cancer risk region are exposed to carcinogenic PAHs.

*Keywords*: 1-hydroxypyrene-glucuronide, squamous oesophageal cancer, polycyclic aromatic hydrocarbons, China, biomarker.

*Abbreviations*: 1-OHPG, 1-hydroxypyrene-glucuronide; 1-OHP, 1-hydroxypyrene; SFS, synchronous fluorescence spectroscopy; PAH, polycyclic aromatic hydrocarbons; B[*a*]P, benzo[*a*]pyrene.

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## Introduction

Linxian, a rural county in Henan Province, north central China, has some of the highest rates of oesophageal squamous cell carcinoma in the world, with average annual age-adjusted incidence rates of 241/100 000 for males and 149/100 000 for females (Zhang *et al*. 1990). In comparison, the national incidence rates for China are 27/100 000 for males and 14/100 000 for females (Li *et al*. 1982). Although most squamous cell carcinomas in low-risk populations are attributable to alcohol and tobacco consumption, the aetiologic agents in high-risk populations remain less clear. In addition to high-level exposure to nitrosamines and deficiencies of trace elements and nutrients, studies suggest that an association exists between high-level exposure to carcinogenic polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (B[*a*]P), and the development of oesophageal cancer (Mahboubi *et al*. 1977, Li *et al*. 1982, Hogstedt *et al*. 1982, Lu *et al*. 1991, Taylor *et al*. 1994, Nadon *et al*. 1995, Ward *et al*. 1997, Mark *et al*. 2000, Yang 2000). These include an animal study conducted by the National Center for Toxicological Research that identified a dose–response relationship between B[*a*]P food levels and the incidence of squamous cell carcinoma of the oesophagus in mice (Culp *et al*. 1998). The inhabitants of Linxian extensively use coal and wood for cooking and heating in unvented stoves, and thus may be exposed to carcinogenic PAHs produced during the incomplete combustion of these fuels (Finkelman *et al*. 1999, Phillips 1999, Siwinska *et al*. 1999). This hypothesis is supported by the recent finding of high levels of B[*a*]P in staple food samples from Linxian (Roth *et al*. 1998a) and the finding of histopathologic changes suggestive of PAH exposure, including anthracotic lymph nodes and arteriosclerotic changes in oesophagectomy specimens from this region (Roth *et al*. 1998b).

1-Hydroxypyrene glucuronide (1-OHPG) is a stable PAH metabolite (Spierto *et al*. 1997, Bouchard and Viau 1999, Strickland, unpublished data) excreted in the urine and it is an index biomarker that reflects recent (within the past 24 h) exposure to mixed PAHs (Zhao *et al*. 1990, Jongeneelen *et al*. 1994, Buckley *et al*. 1995, Kang *et al*. 1995a, b, Merlo *et al*. 1998). 1-OHPG represents the unhydrolysed form of 1-hydroxypyrene (1-OHP) and, therefore, is comparable with urine 1-OHP concentration after enzymatic treatment, which is the metabolite measured in many previous studies. (Strickland *et al*. 1994). Jongeneelen *et al*. (1987) suggest that 10–25% of urinary 1-hydroxypyrene may be unconjugated. However, in a similar study performed in our laboratory, unconjugated 1-OHP was not detected in immunoaffinity-purified or unpurified urine samples prior to enzymatic hydrolysis, indicating that most of the hydroxylated pyrene excreted in the urine is conjugated and detectable as 1-OHPG (Strickland *et al*. 1994). Similarly, Singh *et al*. (1995) showed that 1-OHPG accounted for 81–100% of total pyrene metabolites excreted in urine from five coal gasification workers.

Exposure to PAHs due to domestic heating and cooking with coal-burning stoves is associated with significantly increased levels of 1-OHPG, and ingestion of food contaminated with PAHs is among the major sources of interindividual variability in 1-OHPG excretion (van Rooij *et al*. 1994, Siwanska *et al*. 1999). To determine if the inhabitants of Linxian are exposed to high levels of PAHs, urine concentrations of 1-OHPG were measured in samples collected from non-smokers with no known occupational exposure. The study provides the first biologic confirmation that the inhabitants of Linxian are exposed to elevated levels of environmental PAHs.

## Materials and methods

### *Description of the study population*

The authors conducted a study in the high-risk rural region of Linxian, China, as part of their ongoing attempt to understand its high rates of oesophageal cancer and to follow-up previous studies suggesting that PAHs may play a role in this process. The study was approved by the institutional review boards at both the National Cancer Institute, Bethesda, MD, and the Cancer Institute, Chinese Academy of Medical Sciences, Beijing. Participants were selected from eligible volunteers in this region with no history of occupational PAH exposure or smoking, and included a male and female from each of 11 households. Informed consent was obtained from each participant. In January 1997, each subject was given a sterile container and up to 100 ml of first-voided morning urine was collected and placed in a light-protective bag. Trained study personnel administered a brief questionnaire to each participant, including basic demographic variables, current smoking status, time spent cooking during the 24 h prior to specimen collection, and the type of cooking fuel used. The urine samples were then shipped frozen to the USA for analysis.

### *Assay for 1-hydroxypyrene- glucuronide*

Urinary 1-OHPG and creatinine concentrations are stable over time (Spierto *et al.* 1997, Bouchard and Viau 1999, Strickland, unpublished data). All samples were analysed in the same laboratory (P. T. S.) at the same time over 2 weeks in February 1998. Samples were assayed in batches of 10, one batch per day. Urine 1-OHPG was measured using immunoaffinity chromatography and synchronous fluorescence spectroscopy (S. F. S.) as described (Strickland *et al.* 1994). Urine samples (2 ml) were treated with 0.1 N HCl (90°C, 60 min), neutralized and loaded onto Sep-Pak C18 cartridges (Waters). After washing with 30% methanol, the relatively non-polar metabolites were eluted with 4 ml 80% methanol and the volume of eluate was reduced to 0.5 ml by evaporation. The concentrated samples were diluted to 4 ml with 15 mM phosphate-buffered saline (PBS) and loaded onto immunoaffinity columns prepared with 0.8 ml cyanogen bromide activated sepharose 4B (Sigma) coupled with monoclonal antibody 8E11 that recognizes several PAH–DNA adducts and metabolites (Santella *et al.* 1986). It has been shown that 1-0HPG binds to these columns (Strickland *et al.* 1994). After washing the columns three times with 4 ml 15 mM PBS, compounds bound to the monoclonal antibody 8E11 were eluted with 2 ml 40% methanol in three fractions. Eluate fractions were analysed by synchronous fluorescence spectroscopy with a Perkin-Elmer LS50 fluorescence spectrometer. The excitation–emission monochromators were driven synchronously with a wavelength difference of 34 nm. Under these conditions 1-OHPG produces a characteristic fluorescence emission maximum at 381 nm (347 nm excitation) and was identified in all 22 subjects. As shown previously, samples assayed by SFS give comparable quantitative results when further purified by HPLC (Strickland *et al.* 1994, Lee *et al.* 1999). Fluorescence intensity was used to quantify 1-OHPG; the limit of detection was 0.03 pmol $ml^{-1}$. The interbatch coefficient of variation of the assay was 8–10% during the period of sample analysis. The urinary 1-OHPG concentrations are presented both as uncorrected concentrations and normalized to urine creatinine.

### *Statistical methods*

Urinary 1-OHPG concentrations are presented by group as medians and interquartile ranges (25–75%). Group differences were tested using the Wilcoxon rank-sum test. Multiple linear regression was performed using log-transformed, creatinine-corrected, urinary 1-OHPG concentration to evaluate further the influence of age, gender and cooking time on changes in 1-OHPG. Two-sided statistical tests are used throughout; $p < 0.05$ was considered as significant.

## Results and discussion

The study included 11 male and 11 female non-smoking Linxian participants, with a median age of 42 years living in households that predominantly used coal as a fuel source for cooking and heating. All participants had no known occupational exposure to PAHs.

The median uncorrected 1-OHPG concentration for the participants was 2.06 pmol $ml^{-1}$ (table 1) with male participants having significantly higher urine 1-OHPG concentrations than the females (2.91 versus 1.55 pmol $ml^{-1}$, Wilcoxon rank-sum $p < 0.02$). However, this difference was not significant after creatinine correction (males versus females, 0.45 versus 0.31 μmol $mol^{-1}$ Cr, $p = 0.22$).

Table 1. Urinary 1-OHPG concentrations, uncorrected (pmol ml$^{-1}$) and with creatinine correction (μmol mol$^{-1}$), in adult residents of Linxian, China.

| | | |
|---|---|---|
| Overall | | $n = 22$ |
| | uncorrected (pmol ml$^{-1}$) | 2.06[a] (1.24–2.97) |
| | creatinine corrected (μmol mol$^{-1}$) | 0.38 (0.30–0.51) |
| Male[b] | | $n = 11$ |
| | uncorrected | 2.01 (1.97–3.44) |
| | creatinine corrected | 0.45 (0.33–0.51) |
| Female[b] | | $n = 11$ |
| | uncorrected | 1.55 (0.74–2.13) |
| | creatinine corrected | 0.31 (0.20–0.51) |

[a] Median (interquartile range).
[b] Linxian males versus females, uncorrected $p < 0.02$, corrected $p = 0.22$.



Women spent significantly more time cooking than men in the 24 h prior to urine collection (140 versus 30 min, $p = 0.0001$), but multiple linear regression, adjusting for age and gender, failed to find a statistically significant association between time spent cooking and creatinine corrected 1-OHPG concentrations ($p > 0.22$ for all comparisons). To our knowledge, the present study represents the first report of urine 1-OHPG concentrations from Linxian, a high-risk region for squamous cell carcinoma of the oesophagus. These results are consistent with our previous finding of higher B[*a*]P concentrations in uncooked than in cooked wheat and similar B[*a*]P concentrations in both uncooked and cooked corn in Linxian (Roth *et al.* 1998a). Furthermore, these findings suggest that sources of exposure other than cooking, such as ingestion of food contaminated by deposited particulate matter, may be significantly associated with urine 1-OHPG concentration, and these other potential exposures need further investigation (Kang *et al.* 1995b).

The urine 1-OHPG levels of the Linxian inhabitants were similar to those found in current smokers from South Korea and The Netherlands (Jongeneelen 1990, Kang *et al.* 1995a). Although the Linxian 1-OHPG levels were less than the urine concentrations found in US individuals who consumed a diet rich in PAHs secondary to ingestion of well-done charbroiled beef (range 10–83 pmol ml$^{-1}$) (Kang *et al.* 1995b) and were also less than the levels found in several studies of occupationally exposed workers in South Korea, the USA, The Netherlands and Poland (Jongeneelen *et al.* 1990, Overbo *et al.* 1994, 1995, Kang *et al.* 1995a, b), they clearly reflect a significant PAH exposure. To help put the Linxian results in context, we concurrently reanalysed a set of 63 urine samples previously collected from non-smokers with no occupational exposure living in a low-risk population in Korea (Kang *et al.* 1995a, 1997, Strickland and Kang 1999). The Korean samples were stored similarly to the Linxian samples, exposed to similar freeze–thaw cycles, and analysed in the same laboratory, using the same precise, validated assay for 1-OHPG measurement. The results from the primary analysis performed by this laboratory a few months after their initial collection in 1992 and 1996 were comparable with the results of the more recent, reanalysis, thus confirming the stability of this metabolite under these storage conditions (data not shown). The current analysis of the Korean samples showed a median uncorrected 1-OHPG level of 0.33 pmol ml$^{-1}$ and a median creatinine corrected 1-OHPG level of

$0.037\,\mu mol\,ml^{-1}$. Thus, the median Linxian 1-OHPG concentration was over six times higher than the median concentration found in the subjects from this low-risk population, which had a 1-OHPG concentration similar to baseline levels found in several non-smoking populations with no known occupational or medicinal exposure to PAHs (Jongeneelen *et al.* 1990, Overbo *et al.* 1994, 1995, Kang *et al.* 1995b). The difference in 1-OHPG concentration between the Linxian and Korean samples is the equivalent of smoking almost 20 additional cigarettes a day (van Rooij *et al.* 1994).

In conclusion, the present results support the possibility that the inhabitants of Linxian are exposed to elevated levels of carcinogenic PAHs, which may be aetiologically related to their cancer. Additional studies that include more extensive environmental and biological sampling are needed to characterize better this exposure and to determine whether it is aetiologically associated with Linxian's high oesophageal cancer rates.



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