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Aristolactam-DNA adducts are a biomarker of environmental exposure to aristolochic acid

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Endemic (Balkan) nephropathy is a chronic tubulointerstitial disease frequently accompanied by urothelial cell carcinomas of the upper urinary tract. This disorder has recently been linked to exposure to aristolochic acid, a powerful nephrotoxin and human carcinogen. Following metabolic activation, aristolochic acid reacts with genomic DNA to form aristolactam-DNA adducts that generate a unique *TP53* mutational spectrum in the urothelium. The aristolactam-DNA adducts are concentrated in the renal cortex, thus serving as biomarkers of internal exposure to aristolochic acid. Here, we present molecular epidemiologic evidence relating carcinomas of the upper urinary tract to dietary exposure to aristolochic acid. DNA was extracted from the renal cortex and urothelial tumor tissue of 67 patients that underwent nephroureterectomy for carcinomas of the upper urinary tract and resided in regions of known endemic nephropathy. Ten patients from nonendemic regions with carcinomas of the upper urinary tract served as controls. Aristolactam-DNA adducts were quantified by ^{32}P-postlabeling, the adduct was confirmed by mass spectrometry, and *TP53* mutations in tumor tissues were identified by chip sequencing. Adducts were present in 70% of the endemic cohort and in 94% of patients with specific A:T to T:A mutations in *TP53*. In contrast, neither aristolactam-DNA adducts nor specific mutations were detected in tissues of patients residing in nonendemic regions. Thus, in genetically susceptible individuals, dietary exposure to aristolochic acid is causally related to endemic nephropathy and carcinomas of the upper urinary tract.

Kidney International (2012) **81,** 559–567; doi:10.1038/ki.2011.371; published online 9 November 2011

KEYWORDS: *Aristolochia*; aristolochic acid; DNA adducts; endemic nephropathy; nephrotoxicity; upper urinary tract cancer

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Received 20 July 2011; revised 9 September 2011; accepted 13 September 2011; published online 9 November 2011

Endemic (Balkan) nephropathy (EN) is a chronic, progressive tubulointerstitial disease that affects residents of rural farming villages located along tributaries of the Danube river in Bosnia and Herzegovina, Bulgaria, Croatia, Romania, and Serbia.[1] An unusual feature of EN is its close association with urothelial (transitional cell) carcinomas of the renal pelvis and ureter.[2,3] These upper urinary tract cancers (UUCs), which account for only 5% of all urinary tract cancers worldwide, are present in ~50% of EN cases.[3] Both EN and UUC exhibit a familial but not inherited association, suggesting the importance of environmental factors as well as genetic determinants in this disease.[4–6]

Over the past 50 years, extensive efforts have been made to elucidate the etiology of EN/UUC.[1,5,7] The majority of this research focused on the role of environmental agents, including various heavy metals, mycotoxins, trace elements, and organic chemicals.[8,9] These investigations, however, fail to account fully for the distinctive pathophysiology and epidemiology of this disease.[10]

In 1969, Ivić[11] proposed a role for chronic *Aristolochia* poisoning in the etiology of EN, based on his observation that seeds from these plants, which grew abundantly in local wheat fields, comingled with wheat grain during the harvesting process. Thus, he speculated that human exposure to a toxic component of *Aristolochia* seeds could occur through ingestion of bread prepared with flour derived from contaminated grain. Ivić[11] also demonstrated that, in animal models, *Aristolochia* seeds induced nephropathy and sarcomas of the skin.

We pursued the astute hypothesis of Ivić,[11] stimulated by reports of end-stage renal disease in a cluster of otherwise healthy Belgian women who ingested *Aristolochia fangchi* as part of a weight-loss regimen.[12] This so-called Chinese herbs nephropathy, later renamed aristolochic acid nephropathy (AAN), bears striking similarities to EN in terms of its pathophysiology and association with UUC.[13,14] Importantly, only 5% of the ~1800 Belgian women ingesting *Aristolochia*

herbs over the course of ~1 year developed renal disease or UUC, suggesting a role for genetic susceptibility similar to that reported for EN.[13]

Previously, we identified aristolactam (AL)-DNA adducts in the renal cortex of four patients with EN and in urothelial tumor tissues of three patients with UUC/EN.[15] These adducts were not detected in patients with UUC who were living outside the endemic region. Importantly, the mutational profile of the *TP53* tumor-suppressor gene in tumors of patients with EN/UUC was dominated by A:T to T:A transversions and contained several unique hot spots,[16] a pattern that differed markedly from *TP53* mutational profiles for sporadic cases of UUC reported worldwide.[17] Additionally, mutated adenosine residues were located exclusively on the nontranscribed strand. Thus, this *TP53* mutational 'signature' represents a highly specific bioindicator for the carcinogenicity of aristolochic acid (AA).[15,16]

In this paper, we present molecular and epidemiologic evidence linking both dietary exposure to AA and *TP53* mutational spectra to UUC in residents of the endemic regions of Bosnia, Croatia, and Serbia. Based on these results, we propose the use of these sensitive and specific biomarkers in the diagnosis of AAN and its associated UUC, recently recognized as a global disease.[18,19]

RESULTS

Demographics

Of the 97 cases screened, 77 subjects fulfilled our inclusion/exclusion criteria; these cases were divided into two groups based on residence histories. The majority (67/77) lived in endemic regions for at least 20 years (endemic cases), whereas 10 patients were life-long residents of Zagreb, Belgrade, or other nonendemic sites (nonendemic cases) and, therefore, unlikely to have been exposed to AA-contaminated bread. The key demographic features of these groups are summarized in Table 1. The average age of endemic residents at the time of surgery was 73.4 years whereas subjects from nonendemic regions were slightly younger with a mean age of 66 years ($P = 0.01$). Females outnumbered males by a ratio of 1.5:1 in endemic regions; the comparable ratio in nonendemic regions was 0.67; this difference was not statistically significant ($P = 0.311$). Of the tumors, 70% were localized exclusively in either the renal pelvis or the ureter, with the majority being found in the renal pelvis. In some cases, bladder cancer accompanied tumors of the upper urinary tract. Tumor from one subject was classified as a (urothelial) carcinoma of the duct of Bellini.

Table 1 | Study demographics and tumor sites

N	Endemic cases 67	Nonendemic cases 10
Primary residence		
Bosnia, *N* (%)	29 (43.2%)	0 (0.00%)
Croatia, *N* (%)	18 (26.9%)	5 (50.0%)
Serbia, *N* (%)	20 (29.9%)	5 (50.0%)
Males, *N* (%)	27 (40.3%)	6 (60.0%)
Females, *N* (%)	40 (59.7%)	4 (40.0%)
Age, median	73.0	65.5
Age, mean ± s.d.	73.5 ± 0.8	66.1 ± 4.4
Age, range	57–89	43–85
Tumor site, %		
Renal pelvis	43.3%	60.0%
Ureter	28.4%	10.0%
Renal pelvis and ureter	10.4%	20.0%
Renal pelvis and bladder	4.5%	0.0%
Ureter and bladder	7.5%	10.0%
Renal pelvis, ureter, and bladder	6.0%	0.0%

DNA adducts in renal cortex

Deoxyadenosine (dA)-AL lesions were present in the renal cortex of 47 (70%) of endemic cases, representing 80% of female subjects and 56% of males ($P < 0.055$; Table 2 and Figure 1). dA-AL adduct levels averaged two per 10^8

Table 2 | Aristolactam-DNA adducts in renal cortex from endemic cases

	Percent with adducts
All subjects (*N*=67)	70.1%
Males (*N*=27)	55.6%
Females (*N*=40)	80.0%
Smokers (*N*=25)	64.0%
Nonsmokers (*N*=42)	73.8%

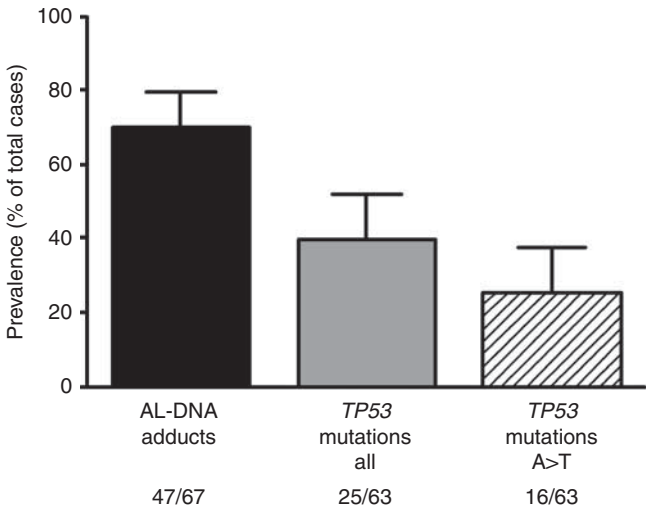

Figure 1 | Prevalence of biomarkers of aristolochic acid (AA) exposure and *TP53* mutations in cases of upper urinary tract cancers (UUCs) from the endemic villages/regions in Bosnia, Croatia, and Serbia. Aristolactam (AL)-DNA adducts, produced during intracellular nitroreduction of AA, were measured in renal cortex by a ^{32}P-postlabeling-polyacrylamide gel electrophoresis (PAGE) assay.[43] Specific mutations in the tumor-suppressor gene *TP53* were identified in UUC samples using p53 AmpliChip technology. A:T→T:A transversions (A>T) are the dominant *TP53* mutations associated with AA exposure in UUC.[16] Tumor DNA was not available for analysis for four cases. Error bars denote 95% confidence intervals for each value. Cortical adducts and tumor *TP53* mutations were not detected in DNA samples obtained from nonendemic cases ($n = 10$; data not shown).