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# Aristolochic acid and the etiology of endemic (Balkan) nephropathy

by

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**Abbreviations:** EN, endemic (Balkan) nephropathy; AAN, aristolochic acid nephropathy; AA, aristolochic acid (mixture of AA-I and AA-II); AL, aristolactam; dA-AL-I, 7-(deoxyadenosin-*N*<sup>6</sup>-yl) aristolactam-I; dG-AL, 7-(deoxyguanosin-*N*<sup>2</sup>-yl) aristolactam-I; dA-AL-II, 7-(deoxyadenosin-*N*<sup>6</sup>-yl) aristolactam-II; dNs, nucleotides/nucleosides; QIT/MS, 2-D linear quadrupole ion trap mass spectrometer; LC-ESI/MS/MS<sup>n</sup>, liquid chromatography-electrospray ionization/multi-stage mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

## ABSTRACT

Endemic (Balkan) nephropathy (EN), a devastating renal disease affecting men and women living in rural areas of Bosnia, Bulgaria, Croatia, Romania and Serbia, is characterized by its insidious onset, invariable progression to chronic renal failure and a strong association with transitional cell (urothelial) carcinomas of the upper urinary tract. Significant epidemiologic features of EN include its focal occurrence in certain villages and a familial, but not inherited, pattern of disease. Our experiments test the hypothesis that chronic dietary poisoning by aristolochic acid is responsible for EN and its associated transitional cell (urothelial) cancer. Using  $^{32}\text{P}$ -postlabeling/PAGE and authentic standards, we identified dA-AL- and dG-AL-DNA adducts in the renal cortex of patients with EN but not in patients with other chronic renal diseases. In addition, urothelial cancer tissues were obtained from residents of endemic villages with upper urinary tract malignancies. The AmpliChip p53 microarray was then used to sequence exons 2-11 of the p53 gene where we identified 19 base substitutions. Mutations at A:T pairs accounted for 89% of all p53 mutations, with 78% of these being A:T→T:A transversions. Our experimental results, namely, (a) DNA adducts derived from aristolochic acid are present in renal tissues of patients with documented EN; (b) these adducts can be detected in transitional cell cancers; and (c) A:T→T:A transversions dominate the p53 mutational spectrum in the upper urinary tract malignancies found in this population, lead to the conclusion that dietary exposure to aristolochic acid is a significant risk factor for EN and its attendant transitional cell cancer.

Endemic (Balkan) nephropathy (EN), a chronic tubulointerstitial disease found in Bosnia, Bulgaria, Croatian, Romania and Serbia occurs exclusively in farming villages situated in valleys of tributaries of the Danube River (1,2). This striking geographic distribution has remained constant since the disease was first described in the late 1950s (3-5). Significant epidemiologic features of EN include its focal occurrence in certain villages, a familial, but not inherited, pattern of disease, initial manifestation after residence in an endemic village for 15 years or more (6) and strong association with upper urinary tract transitional cell (urothelial) cancer (7).

Despite extensive research conducted over the past 50 years, the etiology of EN remains obscure (8). The potential involvement of environmental toxins, including mycotoxins, phytotoxins, heavy metals, viruses and trace element deficiencies, has been widely explored (9-11), with ochratoxin A being a primary focus of EN research in recent years (12, 13). Our investigations into this subject were prompted by the striking clinical and histopathologic similarities between EN and aristolochic acid nephropathy (AAN) (14,15). This insightful observation generated the hypothesis guiding our studies, namely, that aristolochic acid (AA) is a risk factor for both EN and the transitional cell cancer that frequently accompanies it.

We initiated our research by conducting a pilot epidemiologic study in the endemic region of Croatia where bread, the dietary staple of the region, is prepared traditionally from flour made from locally grown wheat (16). We confirmed earlier observations that *Aristolochia clematitis*, a plant native to the endemic region, often grows in cultivated fields (17-19) where its seeds co-mingle with wheat grain during the annual harvest (16,18). We estimate that bread prepared from flour ground from grain contaminated by a few seeds of *A. clematitis* would provide dietary exposure to aristolochic acid (AA) equivalent to that documented for patients with AAN (16). Thus, residents of the endemic region who ingested home-baked bread prepared from contaminated grain may be exposed, over a period of years, to toxic amounts of AA (16,18).

Following metabolic activation, AA reacts with DNA to form covalent dA- and dG-aristolactam (AL) adducts (20, 21). The dA-AL adduct persists for an extended period of time (22), facilitating its detection in target tissues. Thus, building on epidemiologic, environmental, and agricultural evidence (6,16-19), we examined renal tissues of EN patients for these biomarkers of exposure to AA. Additionally, AL-DNA adducts are known to be mutagenic (23-28), leading us to determine the p53 mutational spectrum of transitional cell cancers in patients with EN.

In this paper, we report the detection of dA-AL and dG-AL adducts in the renal cortex of patients with EN and in transitional cell cancers of endemic village residents. The p53 mutational spectra of these

cancers are dominated by A:T→T:A transversions, resembling the mutational “signature” observed in cultured cells and rodents treated with AA (23-28). These novel findings provide support for our guiding hypothesis that dietary exposure to AA is a risk factor for EN and that AA-derived DNA adducts initiate the transitional cell cancers associated with this disease.

## RESULTS

**Identification and quantitation of aristolactam-DNA adducts in human tissues --** Prophylactic bilateral nephrectomy was performed, with informed consent, on an American woman who developed end-stage renal failure following usage of an herbal remedy containing *Aristolochia*. AA-derived DNA adducts were detected in the renal cortex, medulla and pelvis of this patient (1.1 - 3.4 adducts/ $10^7$  dNs for dA-AL and 0.02 – 0.1 adducts/ $10^7$  dNs for dG-AL) three years after stopping *Aristolochia* treatment (**Fig. 1**). Oligonucleotides containing a single dA<sub>3'</sub>P-AL-I or dG<sub>3'</sub>P-AL-I adduct were digested in parallel as controls for the <sup>32</sup>P-postlabeling/PAGE assay (29). The chemical identity of dA-AL-I- and dA-AL-II-DNA adducts in the renal cortex of this patient was established by mass spectrometry (**Fig 2**) with the full product ion spectra for the dA-AL-I and dA-AL-II adducts being identical to those of synthetic standards (supporting information Fig 7A). The signal corresponding to dG-AL was below the level of detection (<1 adduct/ $10^8$  dNs). We estimate that the level of dA-AL-I adducts was much greater (~70 fold) than that of dA-AL-II, consistent with reports using <sup>32</sup>P-postlabeling analysis (22).

**AL-DNA adducts in renal tissues of patients with EN --** Criteria established by the World Health Organization (30) were used to establish the clinical diagnosis of EN. In most cases, the clinical diagnosis was confirmed by the unique renal histopathology; in some, the pattern of interstitial fibrosis characteristic of EN was obscured by changes associated with end-stage renal disease. Formalin-fixed renal tissues embedded in paraffin blocks were obtained from four patients who met the diagnostic criteria. Histopathologic examination of these tissues, performed independently by three renal pathologists, revealed the typical EN pattern of dense interstitial fibrosis with minimal inflammation and relative sparing of glomeruli (31). AL-DNA adducts were detected in all EN patient samples by the <sup>32</sup>P-postlabeling/ PAGE assay, with levels ranging from 0.8 –5.9 adducts/ $10^7$  dNs for dA-AL and 0.2 – 6.2 adducts/ $10^7$  dNs for dG-AL (**Fig 3A**). dA-AL and dG-AL adducts were not detected in the renal cortex of five patients with upper urinary tract transitional cell cancers who resided in a non-endemic region of Croatia (**Fig 3B**) or in five patients with common forms of chronic renal disease (data not shown). Using 10 µg DNA, the limit of detection in the <sup>32</sup>P-postlabelling/PAGE assay is  $3 \times 10^9$  adducts/dNs (29).

**AL-DNA adducts in upper urinary tract transitional cell cancers --** Urothelial and renal cortical tissues were obtained from three long-term residents of endemic villages who had upper urinary tract

malignancies. Following unilateral nephroureterectomy, tumor tissue was frozen in liquid nitrogen for DNA adduct analysis. All tissues were fixed in buffered formalin, stained with H&E and Mallory's Trichrome and subjected to histopathologic examination. DNA adduct levels, determined following <sup>32</sup>P-postlabelling PAGE, were 0.7-1.6 adducts/10<sup>8</sup> dNs for the dA-AL adduct and 0.3-0.5 adducts/10<sup>8</sup> dNs for the dG-AL adduct (**Fig 4**).

**p53 mutations in transitional cell cancers** – The seven women and four men in this study had resided in endemic villages for a minimum of 15 years. With two exceptions, all were born prior to 1934. Histopathologic examination revealed all tumors to be transitional cell carcinomas. Ten of the 11 cancers analyzed were localized in the renal pelvis and/or ureter. The one patient whose bladder cancer was analyzed (#9) also had cancer of the renal pelvis. In two patients, insufficient renal tissue was available for histopathologic analysis; eight of the nine remaining patients exhibited changes in their renal cortex that were diagnostic or highly suggestive of EN (31). p53 mutational analysis was performed on DNA isolated from fresh tumor tissues frozen in liquid nitrogen or, for five of the patients, fixed in formalin and embedded in paraffin. Only those tumors in which >10% of the tumor cells stained positive with a highly specific p53 monoclonal antibody (DO1, Santa Cruz Biotechnologies) were used for mutational analysis. Tumor tissue was enriched in p53 by manually excluding cells that failed to stain with anti-p53. The AmpliChip p53 microarray, powered by Affymetrix, was used to sequence exons 2-11 of the p53 gene. Full sequence data was obtained on nine patients but was limited to exons 5-8 for two others. Results of these mutational analyses are summarized in **Table 1**.

Nineteen base substitution mutations were identified (**Table 1**). In three patients, changes also were detected in the second base of codon 72 and, in one patient, in the third base of codon 36. These well-known polymorphisms (32) were excluded from this analysis. Tissues from four patients displayed a single base substitution, six patients had two mutations and one patient had three. Importantly, mutations at A:T pairs account for 89% (17/19) of all mutations, with the majority of these (15/17) being A:T→T:A transversions, representing 78% of all base substitutions detected in the p53 gene. All but one patient had at least one A:T→T:A mutation. A→C (T→G in Table I) and G→A transitions were each observed in two patients. Mutations appear to cluster between amino acid residues 270 and 290 (**Fig 6**), a region that includes the H2 helix of the DNA binding domain of p53. Mutations occurred twice at four sites (179-2, 274-3, 280-3 and 291-1), three of which were A:T→T:A transversions, representing putative hot spots for AA. The 209-1 and 280-3 mutations also were observed in human p53 knock-in mouse embryonic fibroblasts (Hupki cells) treated with aristolochic acid (**Table 1**).

## DISCUSSION

The principal results of these studies include: (a) identification of DNA adducts derived from AA in the renal cortex and medulla of patients with documented EN; (b) detection of these adducts in transitional cell cancers of long-term residents of endemic villages in Croatia; and (c) demonstration that A:T→T:A transversions, a mutational “signature” for exposure to AA, dominate the p53 mutational spectrum in these malignancies. Taken together, these data suggest that AA is the environmental agent responsible for EN and its attendant transitional cell cancer.

Identification of DNA adducts formed by aristolochic acids in renal tissues represents *prima facie* evidence of exposure to established nephrotoxins/carcinogens. Toward that end, we used a highly sensitive <sup>32</sup>P-postlabeling/PAGE method (29) to quantify such AL-DNA adducts, and validated this approach by performing this analysis on renal tissue from a patient with documented exposure to AA. Remarkably, dA-AL adducts were detected in renal tissue three years after the patient terminated her exposure. In this individual, mass spectrometry (LC-ESI/MS/MS<sup>3</sup>) also was used to confirm the chemical identities of the dA-AL-I and dA-AL-II adducts. To our knowledge, this is the first study to employ MS<sup>3</sup> product ion spectra to identify environmental carcinogen DNA adducts in humans. With this procedure, dA-AL adducts can be detected at <0.2 fmol on column. With the incorporation of stable, isotopically labeled adducts as internal standards, we expect to quantify dAL-AL adducts at levels approaching several adducts per 10<sup>9</sup> dNs, when 100 µg DNA is used for analysis. This level of sensitivity approaches the detection limit of the <sup>32</sup>P-postlabeling methods while simultaneously providing quantitative measurements and spectral data to confirm details of adduct structure.

The <sup>32</sup>P-postlabeling/PAGE method was applied to the analysis of AL-DNA adducts in the target tissues of patients with clinically and histopathologically confirmed EN. In these individuals, dA-AL- and dG-AL-DNA adducts were identified by demonstrating co-migration of putative adducts with authentic standards. In this analysis, dA-AL-I and dA-AL-II adducts are not fully resolved; thus, the presence of AA-II, an intrinsic component of *Aristolochic clematitis*, remains to be established.

AL-DNA adducts were detected in the renal cortex of patients with EN but not that of patients with other forms of chronic renal disease. In addition, dA-AL and dG-AL adducts were detected, albeit at significantly lower levels, in transitional cell cancers of residents of EN villages. The presence of these adducts in target tissues is consistent with their postulated role in the initiation of urothelial cancer. In a separate study (33), Arlt et al used <sup>32</sup>P-postlabeling methods to detect dA-AL-I, dA-AL-II dG-AL-I and putative OTA-DNA adducts in kidney tissues of two patients from an endemic region of Croatia. One patient was diagnosed with upper urothelial tract malignancy, while the other reportedly



suffered from ureteral stenosis. The lack of clinical, epidemiologic and histopathologic data in this study prevents classification of these patients as suffering from EN.

AL-DNA adducts serve as biomarkers of exposure and give rise to a specific pattern of mutations in the p53 gene. The significance of these mutational spectra is best appreciated by comparing the data in Table 1 with that reported for sporadic transitional cell carcinomas in the October, 2006 edition (R11) of the largest international p53 mutational data base (<http://www.p53.iarc.fr>) (32). In that database, A:T→T:A mutations occur infrequently in transitional cell carcinomas of the renal pelvis (0%), ureter (5.0 %), and bladder (4.8%), and at somewhat higher frequency (14.2%) in other (unspecified) urinary tract organs (**Fig 5B**). In contrast, the frequency of A:T→T:A mutations in transitional cell carcinomas in patients with EN or suspected EN is 78% (**Fig 5A**). Nine of the 19 mutations observed in our study (seven sites) are unique among the 696 transitional cell carcinomas listed in the IARC database.

The spectrum of p53 mutations observed in transitional cell cancers of patients with EN is dominated by A:T→T:A transversions. (**Fig 5A**). This result is consistent with the mutational spectra induced by AA-I (or by a mixture of AA-I and AA-II) in (a) the H-*ras* gene of rats (23,24), (b) transgenic rodent models (25,26), (c) the p53 gene in a urothelial cancer from a patient with AAN (34), (d) site-specific mutagenesis studies in which a single dA-AL adduct is transfected into NER-deficient human cells (Yang and Moriya, unpublished), and (e) nine of 11 immortalized mouse cell lines (Hupki cells) carrying the human p53 gene (27,28). Indeed, the predominance of A:T→T:A transversions in the p53 mutational spectra may properly be regarded as a “signature” mutation (35) for human exposure to aristolochic acid.

Confidence in our conclusion regarding AA and the etiology of EN rests, in part, on the biomarker data reported here and, in part, on studies of AAN in human populations, in particular, a cluster of cases affecting women in Belgium (36). The clinical and histopathologic features of EN and AAN are remarkably similar (14). Hallmarks of both diseases include proximal renal tubular dysfunction manifested by low molecular weight proteinuria (37,38) and dense interstitial fibrosis, decreasing in intensity from the outer to the inner cortex while sparing the glomeruli, an infrequent finding in other forms of renal disease (15). Also, in both disorders, urothelial cell atypia and/or malignant transformation develop with similar frequencies (40-50%) (7, 39). Interestingly, based on the prevalence of EN in endemic villages (6) and on the mean cumulative exposure to AA in the cluster of women with AAN (40), only about 5% of individuals exposed to AA develop overt manifestations of disease, suggesting that susceptibility to the toxin is markedly influenced by genetic factors.

Exposure to aristolochic acid potentially could occur either through ingestion of wheat-based products or through the use of herbal remedies prepared from *Aristolochia* plants. The latter possibility was excluded by the results of a questionnaire administered in 2005 to 1081 residents of the endemic region as part of an ongoing epidemiologic investigation of EN (unpublished data). An important clue that AA poisoning might be involved in the etiology of EN first appeared in reports of renal failure in horses fed hay contaminated with *A. clematitis* (17,19). The prevalence of *A. clematitis* in cultivated crop fields and meadows was specifically noted in these early accounts. As far back as 1967, Ivic reported that flour prepared from *A. clematitis* seeds was nephrotoxic and carcinogenic to rats and rabbits and noted the similarity to the histopathologic changes observed in horses (41). Based on his observations that seeds of *A. clematitis* comingled with grain during the harvesting of wheat in an endemic region of Serbia, Ivic suggested that such seeds, ingested in the form of home-baked bread, might harbor the etiologic agent of EN (18). Recently, similar observations were made in our epidemiologic study in the endemic region of Croatia (16).

During the past 50 years, various hypotheses have been proposed to explain the etiology of EN and its associated urothelial cancer (9-11). Considerable attention has been devoted to the hypothesis that a mycotoxin, ochratoxin A (OTA), is the environmental toxin responsible for EN (12,13,42). However, solid epidemiologic evidence supporting an association between OTA exposure and the prevalence of EN or upper urothelial cancer is lacking (43). In fact, a search of the world literature failed to uncover any clearly documented human case of OTA-induced nephrotoxicity. Furthermore, studies using highly sensitive methods of DNA adduct detection, including accelerator mass spectrometry (44), have shown that OTA does not bind to DNA in reactions catalyzed by human and rat enzymes (45). Additionally, although OTA is strongly carcinogenic in male rats, covalent OTA-DNA adducts have not been detected in this species (46). These investigations cast considerable doubt on claims for genotoxicity as a mechanism for the toxic actions of OTA (42).

In conclusion, our data strongly support the hypothesis that chronic dietary poisoning with AA is an etiologic factor in the development of EN and its associated transitional cell cancer.

## **METHODS**

**DNA adduct analysis** -- Methods used for the <sup>32</sup>P-postlabeling/ PAGE analysis of aristolochic acid-derived DNA adducts were described previously (29). The mass spectroscopic methods used in this study have been reported (47); experimental details are provided in the legends to Fig 2 and Fig 7 (supporting information).

**P53 mutational analysis** – Transitional cell cancer tissues obtained from patients residing in endemic villages were analyzed in compliance with Institutional Review Boards of Stony Brook University and the School of Medicine, University of Zagreb. DNA was extracted from frozen and paraffin-embedded tissues using a Qiagen kit. Exons 2-11 were amplified and the fragmented DNA amplicons labeled with biotin at their 3' termini. The mixture was hybridized to oligonucleotides on the AmpliChip p53 microarray and then washed and stained with phycoerythrin, a streptavidin-conjugated fluorescent dye.

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**Table 1: Human p53 mutations observed in tumors of EN patients\***

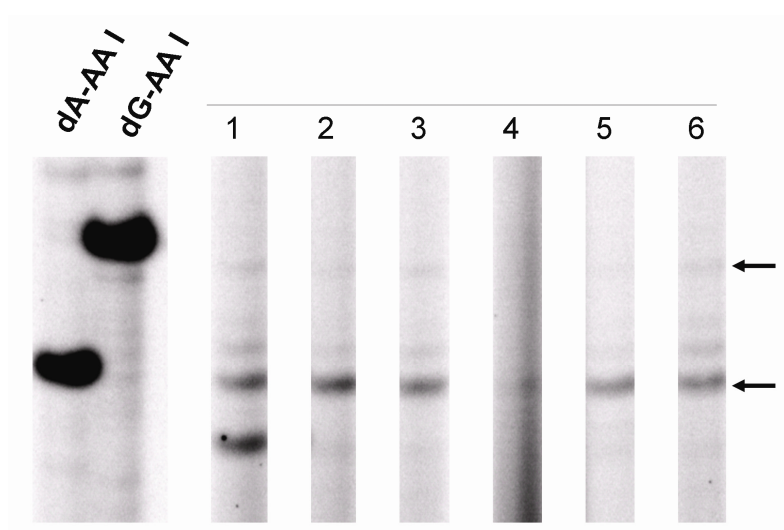
Patient #	Codon/position	Type of mutation	Amino acid change
<b>1</b>	274-3	GTT → GT <b>G</b>	V → V
<b>2*</b>	251-1	<b>A</b> TC → <b>T</b> TC	I → F
<b>3</b>	179-2	<b>C</b> AT → <b>C</b> TT	H → L
<b>4*</b>	241-1	<b>T</b> CC → <b>A</b> CC	S → T
<b>5</b>	158-2	<b>C</b> GC → <b>C</b> AC	R → H
	280-3	<b>A</b> GA → <b>A</b> GT	R → S
<b>6</b>	274-3	GTT → GT <b>G</b>	V → V
	291-1	<b>A</b> AG → <b>T</b> AG	K → Stop
<b>7</b>	162-1	<b>A</b> TC → <b>T</b> TC	I → F
	319-1	<b>A</b> AG → <b>T</b> AG	K → Stop
<b>8</b>	179-2	<b>C</b> AT → <b>C</b> TT	H → L
	282-1	<b>C</b> GG → <b>T</b> GG	R → W
<b>9</b>	131-1	<b>A</b> AC → <b>T</b> AC	N → Y
	291-1	<b>A</b> AG → <b>T</b> AG	K → Stop
<b>10</b>	280-1	<b>A</b> GA → <b>T</b> GA	R → Stop
	286-3	<b>G</b> AA → <b>G</b> AT	E → D
<b>11</b>	144-2	<b>C</b> AG → <b>C</b> TG	Q → L
	209-1	<b>A</b> GA → <b>T</b> GA	R → Stop
	280-3	<b>A</b> GA → <b>A</b> GT	R → S
Mutations in human p53 gene in a mouse cell model (28,29)	130-2	<b>C</b> TC → <b>C</b> AC	L → H
	139-1	<b>A</b> AG → <b>T</b> AG	K → Stop
	158-1	<b>C</b> GC → <b>G</b> GC	R → G
	176-3	<b>T</b> GC → <b>T</b> G <b>G</b>	C → W
	193-2	<b>C</b> AT → <b>C</b> TT	H → L
	203-3	<b>G</b> T <b>G</b> → <b>G</b> T <b>C</b>	V → V
	209-1	<b>A</b> GA → <b>T</b> GA	R → Stop
	280-3	<b>A</b> GA → <b>A</b> GT	R → S
	281-2	<b>G</b> AC → <b>G</b> T <b>C</b>	D → V
	286-2	<b>G</b> AA → <b>G</b> T <b>A</b>	G → V
	313-1	<b>A</b> GC → <b>T</b> G <b>C</b>	S → C

\*Colors designating patient and codon/position correspond to those in Fig 5.

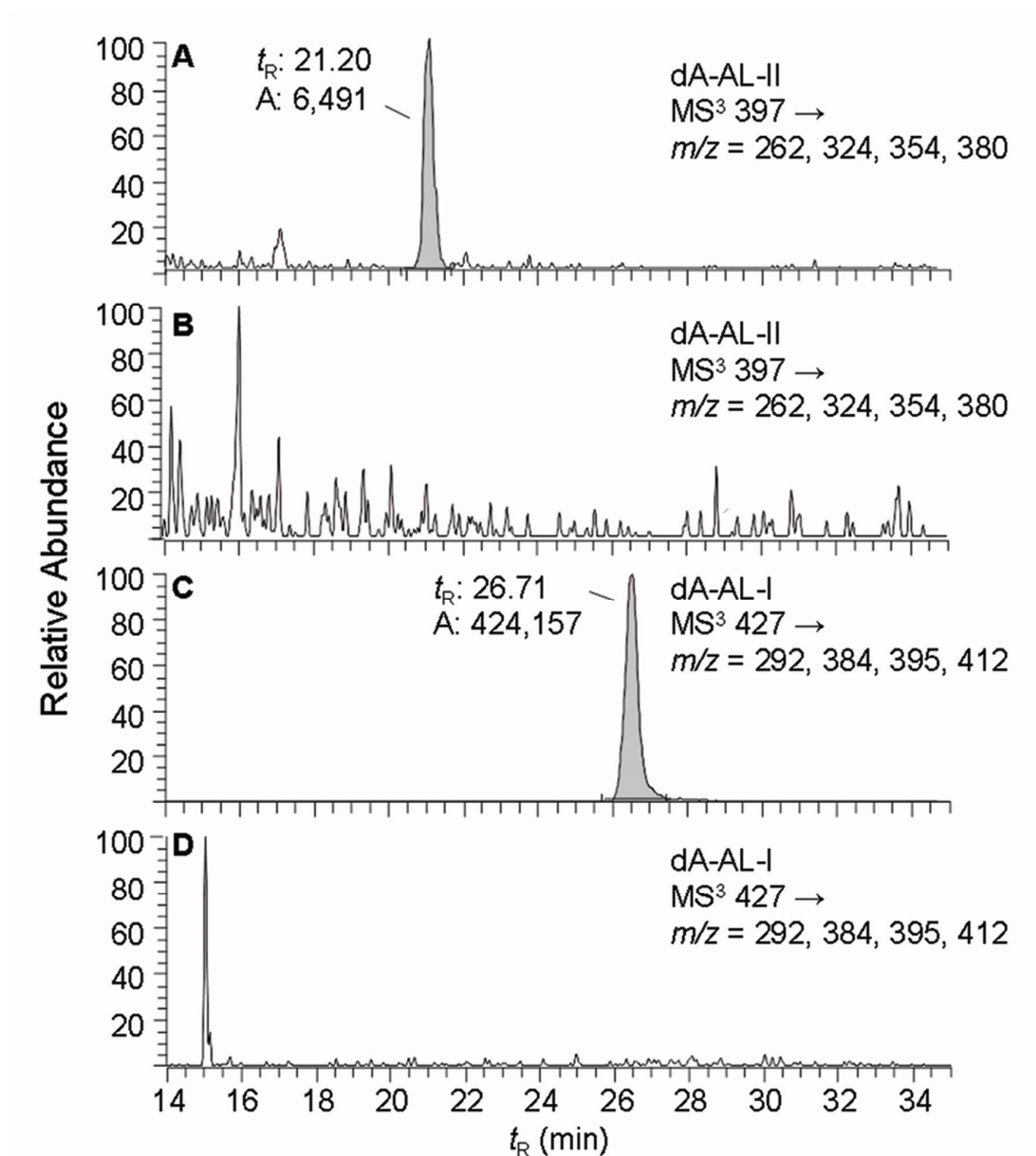
\*Only exons 5-8 were sequenced in these patients.

## FIGURE LEGENDS

**Fig 1. Detection of AL-DNA adducts in renal tissues of a patient with AAN.** DNA (20 ug) was extracted from the renal cortex, medulla and pelvis of an American woman who had developed end-stage renal failure after treatment with an herbal remedy containing *Aristolochia*. The level of AL-DNA adducts in these tissues was determined by quantitative  $^{32}\text{P}$ -postlabeling/PAGE analysis (29). Lanes 1-3 and 4-6 were excised from the right and left kidney respectively. Lanes 1 and 4 are from the renal cortex; lanes 2 and 5, from the renal cortex and medulla; and lanes 3 and 6, from the renal pelvis. Oligonucleotides containing dA-AL-I and dG-AL-I (1 adduct/ $10^6$  dNs), digested in parallel, were used as standards.

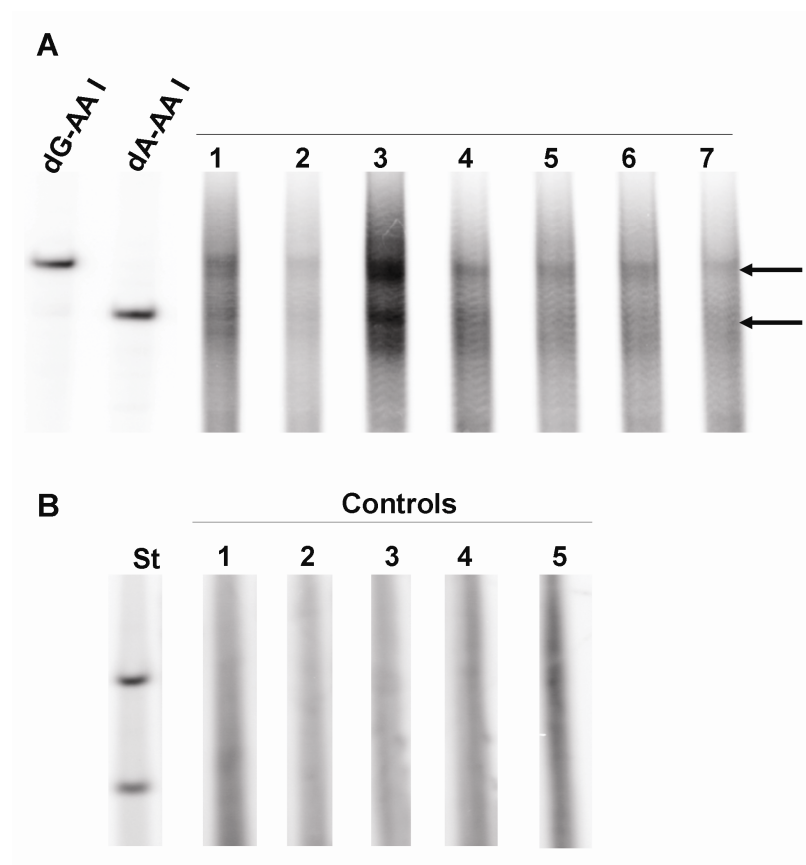


**Fig 2. LC-ESI/MS/MS<sup>3</sup> analysis of dA-AL adducts in renal tissue.** A&C: DNA from renal tissue of the patient described in Fig 1. B,&D: DNA from renal tissue of a subject not exposed to Aristolochia. DNA (80 µg) was subjected to enzymatic hydrolysis, followed by solid phase extraction enrichment of AL-DNA adducts (47). Twenty µg DNA was injected on column. Mass chromatograms (A & B) were monitored for dA-AL-II:  $m/z$  513  $\rightarrow$  397  $\rightarrow$  150 - 500 Da; C&D for dA-AL-I:  $m/z$  543:  $\rightarrow$  427  $\rightarrow$  150 - 500 Da, with the QIT/MS operating in MS/MS<sup>3</sup> mode.

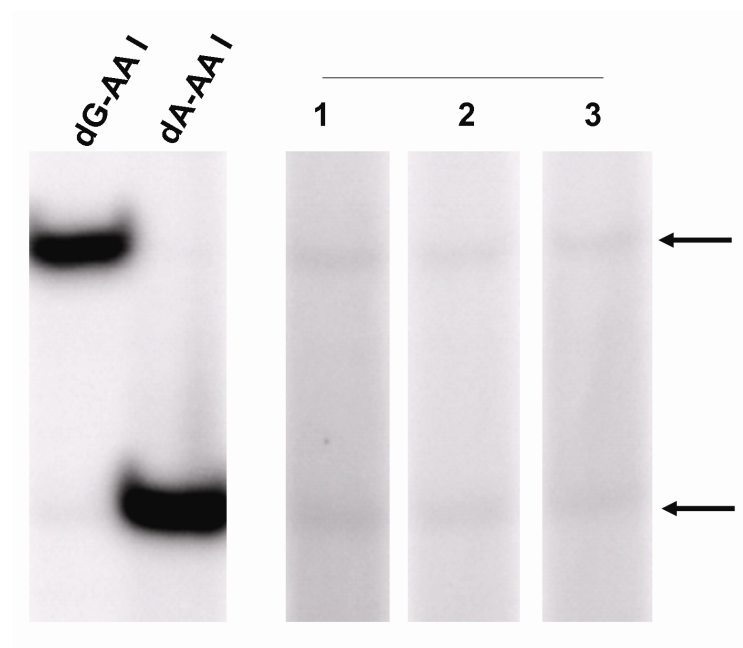




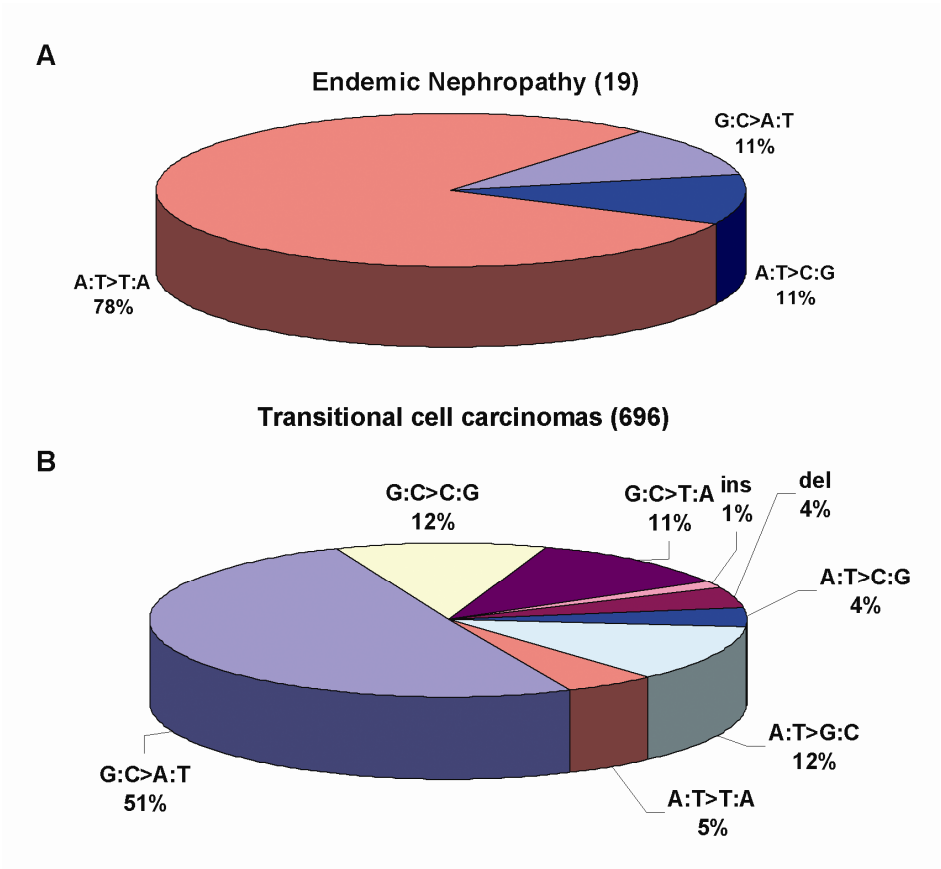
**Fig 3. Detection of AL-DNA adducts in the renal cortex of patients with EN.** DNA (20 ug) was extracted from 7 formalin-fixed, paraffin- embedded renal cortical tissues obtained from four Croatian patients who met the diagnostic criteria for EN, and used for quantitative analysis of AL-DNA adducts by <sup>32</sup>P-postlabeling/PAGE techniques. Lanes 1&2, patient 1; lane 3, patient 2; lane 4&7, patient 3, lane 5&6, patient 4.



**Fig 4. Identification of AL-DNA adducts in urothelial cancer tissues of patients residing in endemic villages.** DNA (10 ug), extracted from upper urinary tract cancer tissues of patients residing in endemic villages of Croatia, was used for quantitative analysis of AL-DNA adducts.



**Fig 5. p53 mutational spectra in transitional cell carcinomas. A)** Transitional cell carcinomas from patients with EN (this study, data from Table 1) **B)** Transitional cell carcinomas in kidney, renal pelvis, ureter, bladder and non-specified urinary organs (data from the IARC p53 database (32)).



**Fig 6. Position of p53 base substitution mutations in patients with EN.** Arrows above and below the bar indicate mutations observed at G:C and A:T pairs, respectively. Arrows in colors other than black represent mutations in the same patient. Single mutations are represented by a black arrow. Numbers corresponding to amino acid residues are shown below the bar.

