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p53 mutations as fingerprints for aristolochic acid - an environmental carcinogen in endemic (Balkan) nephropathy

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Running title: Aristolochic acid as a human carcinogen

Abstract

The activation of protooncogenes and inactivation of tumor suppressor genes are considered to be the main molecular events in the multistep process of carcinogenesis. Mutations of the TP53 tumor suppressor gene have been found in nearly all tumor types and are estimated to contribute to more than 50% of all cancers. Most mutations lead to the synthesis of highly stable, inactive proteins that accumulate in the nucleus of cancer cells. Among the 393 codons of the human p53 gene, 222 are targets of 698 different types of mutations. Alterations of codons 175, 248, 273 and 282 correspond to 19 % of all mutations and are considered general hot spot mutations. Dietary exposure to aristolochic acid (AA), an established nephrotoxin and human carcinogen found in all *Aristolochia* species was shown to be the causative agent of aristolochic acid nephropathy (previously called Chinese herbs nephropathy). This syndrome is characterized by proximal tubular damage, renal interstitial fibrosis, slow progression to the end stage renal disease and a high prevalence of upper urinary tract urothelial carcinoma (otherwise a highly unusual location). AA preferentially binds to purines in DNA and is associated with a high frequency of A → T transversions in the p53 gene. Rats treated with AA develop A:T → T:A mutations in codon 61. The pathological and clinical features of endemic (Balkan) nephropathy closely resemble those associated with aristolochic acid nephropathy except for the slower progression to end stage renal disease and longer cumulative period before the appearance of urothelial cancer. Recently, we reported the presence of AA-DNA adducts in renal cortex and A → T p53 mutations in tumor tissue of patients from Croatia and Bosnia with endemic nephropathy. These data support the hypothesis that dietary exposure to AA is a major risk factor for endemic (Balkan) nephropathy.

Key words: environmental carcinogen, aristolochic acid, p53 mutations, DNA adducts, endemic nephropathy, aristolochic acid nephropathy

Introduction

A principal objective of cancer research is to understand the etiology of environmentally induced malignancies, with the long-term objective of goal of reducing the incidence of human cancer. The most common cancer-related genetic changes in humans are mutations within the p53 gene. TP53 is a tumor suppressor gene that governs cellular responses to a broad spectrum of stress (DNA damage, hypoxia, oncogenic stress) by inducing transient or permanent cell cycle arrest or apoptosis and plays a key role in the prevention of cancer development.

Both exogenous exposure to carcinogens and endogenous biological processes are known to cause mutations in DNA. Chemical carcinogens induce mutations by forming covalent adducts with the nucleotides in DNA, increasing the probability of enzymatic errors during DNA replication (1). Studies have shown that some carcinogenic agents produce a specific mutational pattern, a "DNA fingerprint" (2), because specific types and locations of DNA adducts are linked to a specific mutational spectrum in a DNA sequence (3). The mutational spectra of cancer-related genes differ depending on cancer type, but provide a molecular link between cancer and etiological agent, and give clues to the nature of carcinogens responsible for genetic alterations (4).

Specifically, the study of mutational spectra of p53 and other important cancer-related genes can give us clues about carcinogen-DNA interactions, functions of gene products and mechanisms of carcinogenesis in specific tissues (3). The p53 gene is a good choice for mutational spectrum analysis for several reasons. First, p53 mutations are the single most frequent in human tumors and are directly involved in cancer formation (more than 20, 000 occurrences of human p53 mutations have been registered to date in the International Tumor Registry IARC p53 database

(http://www-p53.iarc.fr) (5). Second, the p53 gene is relatively small (11 exons coding for 393 amino acids), permitting the mutational study of the entire coding region (6,7). p53 is highly conserved throughout vertebrates, allowing data comparison in animals. Third, most mutations fall into the DNA binding domain (DBD), responsible for sequence-specific DNA binding and transcriptional activity, as well as for a direct mitochondrial pro-apoptotic activity.

Many studies have demonstrated a significant correlation between p53 mutational spectra and exposure to various types of carcinogens. Mutational hotspots at CpG dinucleotides in codons 175, 248, 249, 273 and 282 reflect an endogenous mutagenic mechanism (8). On the other hand, G:C → T:A (G → T) transversions, the most frequent substitutions in human cancers, are likely to be caused by carcinogen-DNA adducts, and are more frequent in lung cancers of smokers compared to lung cancers of nonsmokers (8). Cigarette smoking has been established as a major risk factor for the incidence of lung cancer, and p53 mutational hotspots are codons 157, 158, 248, 249 and 273. Codon 157, G → T transversions, one of the hotspots in lung, breast, and head and neck cancers, is uncommon in other types of cancer, and is associated with smoking in lung cancer patients. Moreover, the proof for causality became solid when it was shown that *in vitro* exposure of bronchoepithelial and HeLa cells to tobacco-derived benzo(a)pyrene generates strong and selective adduct formation at guanine positions in codons 157, 248, and 273 (9). Similar benzo(a)pyrene data was later shown e.g. for hotspots of liver cell carcinoma.

In liver tumors from populations living in endemic areas where aflatoxin B1 and hepatitis B virus are risk factors for hepatocellular carcinoma, most p53 mutations occur at the third nucleotide position (AGG to AGT) of codon 249^{ser} (10,11,12). The 249^{ser} p53 mutant is more effective in inhibiting wild-type (wt) p53 transcriptional

activity in human liver cells than other p53 mutants (143^{ala}, 175^{his}, 248^{trp} and 282^{his}) (13).

Another association between p53 mutational spectra and carcinogen exposure was found in skin carcinoma caused by UV irradiation – p53 mutations are located at dipyrimidine sites, generating CC → TT double-base transitions (14). Furthermore, the p53 mutational pattern in radon-associated lung cancer from uranium miners differs from the one in lung cancer caused by smoking alone (15). Moreover, liver angiosarcomas of vinyl chloride-exposed factory workers have higher frequency of p53 A:T → T:A transversions comparing to sporadic angiosarcoma (16).

In summary, these differences in mutational frequency and spectra among human cancer types may be traced to exogenous and endogenous factors to human carcinogenesis (17).

Aristolochic acid

Aristolochic acid (AA) is an intrinsic component of *Aristolochia sp*. (*Aristolochia clematitis, A. fanghi*, etc.). The structurally related aristolochic acid I (AA-I) and aristolochic acid II (AA-II) are nitrophenanthrene carboxylic acids. Herbal medicines derived from *Aristolochia* have been used since ancient times to treat disease (18). However, in the 1980s AA was shown to be a strong carcinogen in rats and, in short-term tests, to be a genotoxic mutagen (19,20,21,22). The US Food and Drug Administration (FDA) has issued warnings regarding the medicinal use of *Aristolochia* herbals. Nevertheless, *Aristolochia* is still widely used in traditional medicines and so called "natural" remedies.

AA is metabolized and activated in a living organism through two major metabolic pathways: (*i*) demethylation of AA I by cytochrome P450 generates a non-

nephrotoxic product, that is biotransformed to glucuronide or sulphate conjugates (23); (*ii*) reactions catalyzed by cellular nitroreductases (such as NADP(H), quinonoxidoreductase (NQO1), xanthine oxidase (XO), CYP1A1 and CYP1A2) generate reactive intermediate cyclic nitrenium ion (24,25). This electrophilic intermediate subsequently reacts with proteins and DNA, the latter leading to gene mutation and induction of tumors (26,27). The activation of AA takes place mainly in the liver and kidneys, targeting the kidney (cytotoxicity) and forestomach (cancer) in rodents (28).

The herbal drugs containing *Aristolochia* have been associated with development of a characteristic chronic interstitial nephropathy associated with proximal tubular damage and severe interstitial fibrosis starting from cortex with paucy cellular infiltratates sparing glomeruli called aristolochic acid nephropathy (AAN). This tubulointerstitial kidney disease is associated with urothelial carcinoma of the renal pelvis and upper ureter (29,30). This location is highly unusual as sporadic urothelial carcinoma occurs mostly in the lower urinary tract, typically the bladder. Urothelial cancers in the Western world at large occur in the bladder, ureter and renal pelvis at a ratio of 50:3:1. Thus, upper urinary tract cancers are uncommon and typically associated with particular exogenous carcinogens. (31). Atypia of urothelial cells throughout the renal tubules, pelvis and ureter are commonly reported in AAN (29). About one hundred AAN cases have been identified so far in Belgium among women undergoing a slimming treatment involving ingestion of *A. fangchi* that was inadvertently put into the slimming pills for several months (29,32,33). Additional AAN cases have been identified in Europe, Asia and the US. Subsequently, approximately 40%-46% of AAN patients developed urothelial carcinoma in the renal pelvis and ureter within 2-6 years either as non-invasive urothelial cancer (papillary

carcinoma) or as invasive flat urothelial carcinoma (30,34,35,36). Known risk factors for upper urothelial cancer are occupational exposure to aniline dyes, acrylamines and chemicals used in the rubber, leather and petrochemical industries, chronic analgesic abuse and chronic irritation (kidney stone). Cigarette smoking is a major risk factor for urothelial and squamous cell carcinoma at all sites. Patients with upper urothelial cancers in general have a 30-50% chance of developing subsequent bladder cancer (37). Very recently, occurrence of bladder cancer 15 years after kidney transplantation in AAN patients was reported (38). Bilateral nephrectomy was performed several years before bladder cancers were diagnosed, preventing the possibility that upper urothelial cancers could have been detected (38).

AA as a mutagen

AA forms covalent DNA adducts in rodents (40,41,42) as well as in AAN patients (36,43,44,45). Covalently bound AA-DNA adducts, present for the life time in rodents and for many years in humans, now are considered to be a reliable biomarker of exposure to this environmental carcinogen (25,27,44,45,46).

The enzymatic activation of AA leads to formation of the ultimate carcinogen aristolactam-nitrium ion (cyclic N-acylnitrenium ion) (Figure 1), which binds to DNA, preferentially to exocyclic amino groups of purine nucleotides (deoxyadenosine and deoxyguanosine), forming 7-(deoxyadenosin-N(6)-yl)aristolactam I (dA-AAI, compound 2, Figure 1) and 7-(deoxyguanosin-N(2)-yl) aristolactam I (dG-AAI, compound 3, Figure 1), 7-(deoxyadenosin-N(6)-yl) aristolactam II (dA-AAII) and 7-(deoxyguanosin-N(2)-yl) aristolactam II (dG-AAII). The activation of AA is an unusual case of intramolecular acylation, producing the ultimate carcinogen (27,39).

The dominant and most persistent DNA adduct, dA-AAI, is a mutagenic lesion leading to AT → TA transversions *in vitro*. DNA binding studies confirmed that AAI and AAII bind to the adenines of mouse *ras* codon 61, forming adducts associated with distinct activation of H-*ras* by a specific A → T transversion at codon 61 (wt CAA → CTA). This mutation occurs selectively at the first adenine of codon 61 in *all* AA-induced squamous cell carcinomas of the forestomach and ear duct examined in rat (47,48). Similar mutations, but at lower frequency, were demonstrated at c-Ki-*ras* codon 61 in 1 of 7 ear duct tumors (CAA → CAT) and in 1 of 8 tumors of the small intestine (CAA → CTA), as well as at c-N-*ras* codon 61 (CAA → CTA) in a pancreatic metastasis (47). Of note, neither have other *ras* codons nor *ras* mutations in urothelial cancers been studied to date.

Urothelial carcinoma as well as urothelial atypia have been associated with overexpression of p53 protein in urothelial atypia and neoplastic cells from 10/10 and 4/4 Belgian AAN patients, respectively, suggesting that the p53 gene is mutated in AAN-associated urothelial carcinoma (29,34). Hollstein et al. (49) designed a powerful and elegant genetic mutagenesis assay that allows proofing the direct mutagenic effect of a test substance towards human p53 in primary tissue culture cells. Mouse embryo fibroblasts derived from gene-targeted knock-in mice (Hupki), that had substituted their endogenous mouse p53 DBD (Ex 4-9) by the human counterpart, were exposed to AAI (100 μM for 48 h) and then subjected to 9-13 passages. Five of the 10 established cultures harbored p53 DBD mutations that produced aberrant nuclear overexpression of the mutant protein. Of note, all were transversions, including 4 A → T transversions on the non-transcribed strand, a rather unique hallmark of mutagenesis by AAI (and rare in spontaneous mutations) (47,49). Moreover, the characteristic d-adenosine and d-guanosine DNA adducts of AAI were

detected in the DNA of the outgrowing fibroblast lines (50). Remarkably, urothelial carcinoma cells from an AAN-patient in UK also harbored an A → T transversion (AAG → TAG) on the non-transcribed strand at codon 139 of exon 5 in the p53 gene, leading to a stop (Lys → Stop) (50). Moreover, the mutated base adenine has the same neighboring bases in codon 139 of the p53 gene as in codon 61 (CAA) of the H-*ras* gene, suggesting a sequence specific mechanism during mutagenesis (50). Another mutation (G → A) was found in codon 245 (hotspot for p53 mutations) in the p53 gene in DNA from the breast and liver tumors of the same AAN patient (50). Since G → A transitions are not typical of AA, it is not likely that the p53 mutation in the breast and liver tumors was induced by AA and probably only the urothelial tumor was causally related to AA exposure. Importantly, this study provides a direct etiologic link between a defined exposure to a chemical carcinogen and human cancer and clear additional support for the carcinogenicity of AA.

Recently, the same Hupki system was used for studies of the AA mutation signature in the human p53 gene. Six immortalized cultures from 18 primary cultures exposed to AAI (50 μM for 48 h) again harbored p53 mutations in the human DNA binding domain. The most frequently observed mutation was A → T transversion (51). One of the mutations was identical to the A → T transversion in codon 139, originally seen in the urothelial cancer of an AAN patient with documented AAI exposure (50). In contrast, among the seven p53 mutations identified thus far in >60 Hupki cell lines that immortalized *spontaneously* (i.e. no carcinogen treatment), none were A:T → T:A transversions. In addition, no A → T substitutions were identified among the previously reported set of 18 mutations in Hupki cell lines derived from benzo(*a*)pyrene treatment, in which transversions at G → C base pairs predominated (51). Finally, using AAI and AAII-exposed DNA from the human breast cancer cell

line, the AA-DNA binding spectrum within the p53 gene was mapped preferentially to purines in Exons 5-8 of p53 (52).

The relationship between AA-induced DNA adducts and mutations in rat liver and kidney (the tissues that activate AA) was recently reported by Mei et al. (28). Strong linear dose-responses for AA-induced DNA adducts were found in treated rats.. Kidneys had at least two-fold higher levels of DNA adducts and mutation frequencies than livers, with no significant difference between the mutation spectra in AA-treated livers and kidneys (mostly A:T → T:A transversions). However, there was a significant difference between the mutation spectra in both kidney and liver of AA-treated and control rats (mostly G:C → A:T transitions). These results link AA exposure that eventually results in kidney tumors in rats, to a significant increase in AA-induced DNA adduct formation with a characteristic mutation in kidney tissue. Although the same treatment does not produce tumors in rat liver, it does induce DNA adducts and mutations in this tissue, albeit at lower levels than in the kidney (28). Moreover, the mutation frequency in kidneys of AA-treated rats was shown to correlate with tumor incidence in the kidney (53).

Aristolochic acid nephropathy and endemic (Balkan) nephropathy

Endemic nephropathy (EN) is characterized by chronic tubulointerstitial nephritis with slow progression to terminal renal failure. EN is present in several rural areas in the valleys of big Danube tributaries in Bosnia and Herzegovina, Bulgaria, Croatia, Romania and Serbia affecting approximately 2-7% of exposed rural farming population (54,55). EN has several epidemiological characteristics: (*i*) it is present only in certain villages with completely unaffected villages located in close proximity; (*ii*) household (not inherited) pattern of disease was observed; (*iii*) it

affects only adult population; (*iv*) there is strong association (~30-50%) with upper urothelial cancers (UUC). The specific mortality associated with UUC in this region is 50 times higher than elsewhere in Europe (56). Epidemiologic findings and striking geographical correlation of two very rare diseases pointed from the beginning to a common environmental etiological agent. EN was first described more than 50 years ago and many toxic agents have been investigated (57). In the last two decades ochratoxin A (OTA) was a major focus of interest. This hypothesis was supported by the detection of so-called "OTA-associated" DNA adducts (deoxyguanosin adducts) in urothelial tumors of Bulgarian patients (58). However, presence of OTA in these adducts could not be confirmed (59,60). Although there are reports on higher concentrations of OTA in food, blood and urine of inhabitants in EN regions compared to other regions, there is no evidence that OTA is a risk factor for EN (61, 62). According to the European branch of the *International Life Sciences Institutes*, there is no convincing evidence from human epidemiology to confirm the association between OTA exposure and the prevalence of EN and/or upper urothelial cancers (63). *The Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority* also concluded that epidemiological data are incomplete and do not justify the classification of OTA as a human renal carcinogen (64). OTA induces renal adenomas in rodents and no urothelial cancers. This is one of the strongest arguments against OTA as a major risk factor for EN-associated urothelial malignancy. Pathological findings in EN differ also in some very important elements from findings in animal models of OTA nephrotoxicity but are almost identical with findings in aristolochic acid nephropathy (30).

Vanherweghem and his group (33) suggested that the hypothesis that AA was an etiologic agent in EN should be evaluated. Based on pathological findings, Cosyns et

al. (30) debate whether AAN could be the clue for EN, caused by the common etiologic agent, AA. More than 30 years ago Ivić (65) made remarkable observations that implicated AA as a major risk factor for EN. Unfortunately, during the next three decades, this hypothesis was put aside, and we confirmed (66) for the first time experimentally that seeds of *Aristolochia clematitis* were commingled with wheat seeds and contaminated the flour used by farmers from EN villages (Figure 2). Unlike in AAN, where the exogenous carcinogen was present in slimming pills, in EN, according to our data, this environmental toxic substance was ingested through bread (66,67). Finally, this hypothesis was confirmed by *prima facie* evidence of the presence of AA-DNA adducts in DNA extracted from the renal cortex and from upper urinary tract cancer tissue of the patients with EN whom we tested (44). In addition, we have reported on the p53 mutations in urothelial tumors of Croatian and Bosnian EN patients (44). Using AmpliChip p53 microarray, Exons 2-11 were sequenced and 19 base substitutions were identified. The mutations at A:T pairs accounted for 89% (17/19) of all mutations, with the 78% of these (15/17) being A:T $\rightarrow$ T:A transversions (Figure 3A). Of note**,** p53 mutations in EN patients with urothelial cancers from Croatia and Bosnia are unique and not consistent with IARC p53 database R12, November 2007 (6). Namely, in other parts of the world (in the general population of patients with upper urothelial cancers) the A $\rightarrow$ T transition account for only 4% of all p53 mutations (Figure 3B). In addition, p53 mutations in the patients whom we tested appear to cluster between amino acid residues 270 and 290 and at four sites mutations occurred twice (179-2, 274-3, 280-3, and 291-1). The 209-1 and 280-3, both A:T $\rightarrow$ T:A mutations found in EN patients, were also detected in human Hupki cells treated with AAI (49,51).

Conclusion

Epidemiological, pathological, clinical and biochemical studies confirm that AA is a major risk factor for EN. The presence of covalently bound AA-DNA adducts is a strong biomarker of prior exposure to this environmental carcinogen. Clinical and pathological features of EN and AAN, coupled with characteristic p53 mutational spectra in the upper urinary tract malignancies found in this population (the presence of AT → TA transversion) are strong supplementary arguments in favor of an etiological role of AA in EN-associated urothelial tumors and allow us to suggest that EN, CHN and AAN are the same disease and an already acknowledged worldwide problem (69)

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Abbreviations used in this paper: aristolochic acid (AA), endemic nephropathy (EN), DNA binding domain (DBD), aristolochic acid - associated nephropathy (AAN), transitional cell carcinoma (TCC), ocratoxin A (OTA), International Agency for Research on Cancer (IACR).

Figure legend:

Figure 1. Formation of AA-derived DNA adducts (AAI (R=OCH_3) and AAII (R=H).

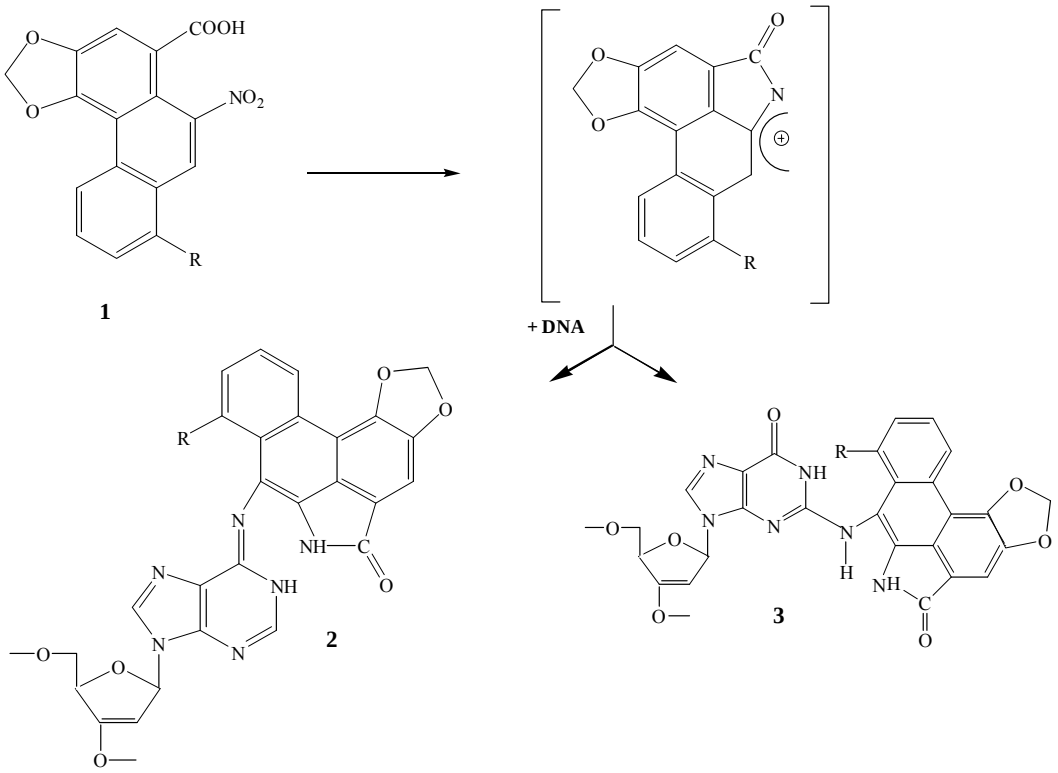
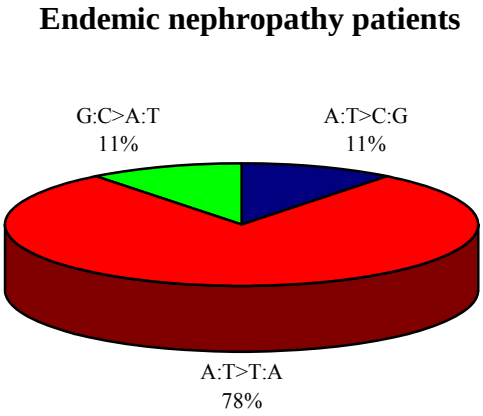


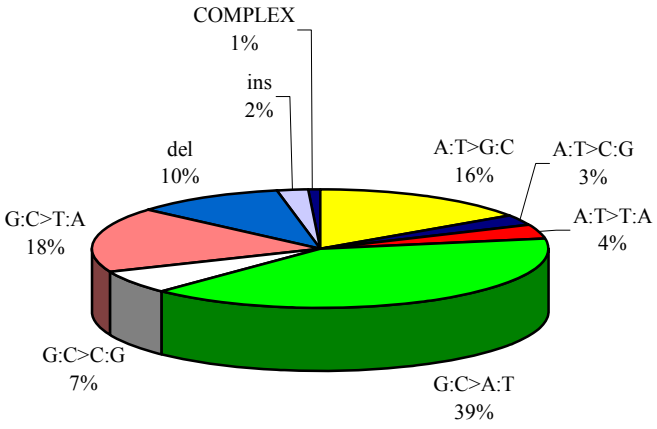
Figure 2. *Aristolochia clematitis* growing in the middle of wheat field in the Croatian endemic village of Kaniža during harvest time 2008.

Figure 3. p53 mutational spectra in upper urinary tract urothelial cancers (UUC). (A) UUC of EN patients in Croatia (19 mutations). (B) Urothelial cancers of kidney, renal pelvis, ureter and urethra, excluding bladder (230 mutations). Data from IARC p53 database, R12 released in November 2007 (6); adapted from ref. 44.

A


Endemic nephropathy patients
G:C>A:T
11%
A:T>C:G
11%
A:T>T:A
78%

B


Urinary tract transitional cell carcinoma
COMPLEX
1%
ins
2%
del
10%
A:T>G:C
16%
A:T>C:G
3%
G:C>T:A
18%
A:T>T:A
4%
G:C>C:G
7%
G:C>A:T
39%