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Overview

The p53/p63/p73 family of proteins – the focus on isoforms and mutants in cancer

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Abstract

p53 tumor suppressor protein is critical for the cell growth control and the maintenance of genomic stability. These activities are due, at least in part, to its ability to form tetramers that bind to specific DNA sequences and activate transcription. Later discovered p53 homologues – p63 and p73 share remarkable structural and functional similarity with p53. All three genes have two promoters and undergo alternative splicing to generate multiple isoforms that might play important roles in carcinogenesis. Two groups of isoforms are generated: transactivating forms (p53/TAp63/TAp73) with tumor suppressor activities as well as a number of amino-terminally truncated transactivation deficient isoforms (called ΔNp53/ΔNp63/ΔNp73). It was recently discovered that p53, like p63 and p73, has a second internal promoter that leads to the synthesis of multiple isoforms whose function is not yet fully clear. Moreover, arising from alternative splicing of exons 6 to 9, new p53 splice variants were identified. In this review we describe different isoforms of p53, p63, p73 and their roles in tumorigenesis. Defining the interactions between p53/p63/p73 would give us new insight into the roles of these proteins in tumor formation. 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. The study of p53 mutational spectra could give us clues about etiology of cancer. Recently, we reported the presence of specific p53 mutations in tumor tissue of patients from Croatia and Bosnia and Herzegovina with endemic nephropathy. These data support the hypothesis that dietary exposure to AA is a major risk factor for endemic (Balkan) nephropathy.

TP53 GENE FAMILY

The first p53 family member, gene *TP53*, was detected 30 years ago as a cellular protein which interacts with the oncogenic T antigen SV40 (*1*, *2*). Tumor suppressor gene *TP53* plays a key role in tumorigenesis (*3*) – it governs cellular responses to a several stress signals (DNA damage, hypoxia, oncogenic stress) by inducing DNA repair and, if DNA is not repairable, transient or permanent cell cycle arrest or apoptosis. p53 function is almost always compromised in tumor cells, usually as a result of somatic mutations (*4*). Mutations of the *TP53* 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. Apart from the loss of tumor suppressor activity, some mutant p53 proteins gain oncogenic potential resulting in uncontrolled growth of tumor cells (*4*).

Two related genes *TP63* and *TP73* were identified in 1997 (*5, 6*). The *TP53* homologue, *TP73* gene is located at position 1p36.2-3, the region which is often deleted in neuroblastoma, colon cancer, melanoma and breast cancer (*5*). A new family member, *TP63* gene is located at the position 3q27-29, the region of the chromosome that is often doubled in the different types of tumors, suggesting a possible oncogenic effect of p63 (*6*).

Structure of p53/p63/p73

The members of p53 family have a very high structural similarity in protein organization (Figure 1A). All three family members contain amino-terminal transactivation domain (TAD), centrally located DNA-binding domain (DBD) and carboxy-terminal oligomerization domain (OD) (*7*). Between DBD and OD there is a small nuclear localization signal – responsible for localization in the nucleus (NLS). In addition, all representatives of the p53 family contain at least one proline rich domain (PRD) with PXXP motif (where P = proline, and X = any other amino acid). p63 and p73 have a carboxy-terminal inhibitory region and an area with a sterile α-motif (SAM) (*8*). At carboxy terminus of p53 protein there is a basic domain (BD).

All *p53* gene family members have two promoters (Figure 1B): P1 located upstream of exon 1 and P2 located within exon 3 (*p63* and *p73*) or exon 4 (*p53*). Using alternative promoters and alternative splicing, different isoforms are encoded. Transcribing from P1 promoter

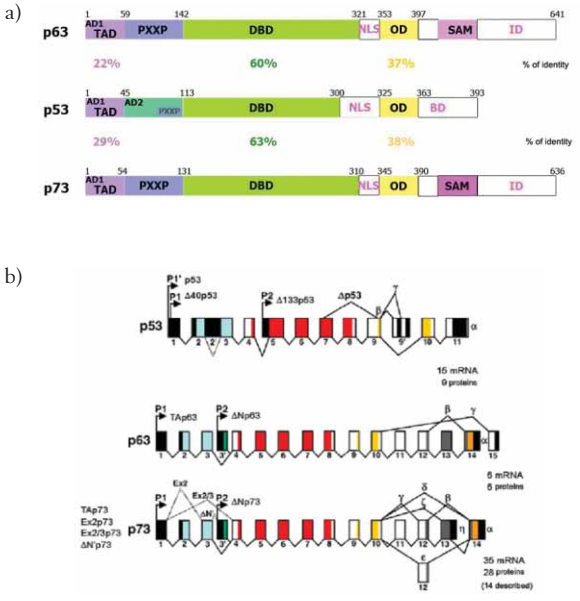

Figure 1. *Human p53/p63/p73. (A) Comparative structure of p53, p63 and p73 proteins. All proteins consist of transactivation domain TAD, DNA binding domain DBD, nuclear localization signal NLS, oligomerization domain OD. Genes* p63 *and* p73 *have sterile α motif SAM and inhibitory domain ID, while p53 has basic domain at the carboxy end. The highest homology is in DBD. (B) Schema of the human* p53, p63 *and* p73 *gene structure. Alternative splicing (α, β, γ) and alternative promoters (P1 and P2) are indicated.*

gives rise to isoforms with transactivation domain – p53, TAp63, TAp73. Combining the alternative splicing of the 5' end and different promoters, additional protein isoforms of p63 and p73 arise. Alternative splicing of *p73* gene transcripts that are transcribed from P1 promoter forms isoforms Ex2p73, Ex3p73 and Ex2/3p73. Isoform Ex2p73 is lacking exon 2, isoform Ex2/3p73 exons 2 and 3, and isoform Ex3p73 exon 3. Using the alternative P2 promoter amino-terminal truncated isoforms without TAD (ΔNp53, ΔNp63, ΔNp73) are produced. ΔNp63 and ΔNp73 have the dominant-negative effect on the activity of p53, p63 and p73, and may directly inactivate their target genes (*7*, *9*, *10*). Most of the alternative splicing, however, occurs at the 3' end of exon 10–13, which creates isoforms that differ in their carboxy-terminal. According to that, in normal cells there are at least six isoforms of protein p73 (α-ξ) (*11*) that differ in their biochemical functions. Protein p63 has three isoforms (α, β and γ) which differ in their carboxy-end as result of alternative splicing (*12*).

Isoforms TAp63/TAp73 and ΔNp63/ΔNp73 act as tumor suppressors and oncogenes, respectively. Therefore, we often talk about the structure of »two genes in one«.

It was established recently that the *p53* gene encodes for a number of different isoforms, with still unclear biological function. Isoform Δ133p53 is transcribed from the alternative promoter P2 within intron 4 (*13*), while the isoform Δ40p53 (p47) is transcribed from the P1, but does not have the same initiation site as the wild type p53, or it is formed by alternative splicing of intron 2 (*14–16*) (Figure 1B). Isoforms Δ133p53 and Δ40p53 are collectively called ΔNp53 because they lack part or entire transactivation domain.

Alternative splicing of intron 9 of *p53* gene, gives rise to p53, p53β and p53γ (last two without oligomerization domain). Up to now, nine isoforms of p53 protein are distinguished: p53, p53β, p53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δ40p53, Δ40p53β and Δ40p53γ (Figure 1B).

However, besides the above listed p53 isoforms, we participated in the identification of completely new p53 splice variants: p53ξ, p53Δ and p53ε, arising from alternative splicing of exon 6 and intron 9, respectively (*17*). The existence of these splice variants was confirmed in 18 of 34 ovarian cancer cell lines (52.9%) and 134 of 245 primary ovarian cancers (54.7%). p53 splice variants were evaluated for their clinical relevance. Their expression differs in primary ovarian cancers, implicating that they possess different functions *in vivo*. The novel splice variant p53δ is associated with impaired response to primary platinum-based chemotherapy and constitutes an adverse prognostic marker for recurrence free and overall survival in ovarian cancers. Although the function of p53β is still unclear, we provide first evidence for an adverse clinicopathological marker correlated with worse recurrence-free survival in patients with in ovarian cancers exhibiting functionally active p53 (*17*).

p53 family members in the regulation of transcription

Many parallels can be found between the functional p53, TAp73 and TAp63 on the one hand, and between ΔNp73 and ΔNp63 on the other side. Proteins with transactivation area (TAp73) can imitate the function of p53 transactivating many p53 target genes, whereas proteins without TAD (ΔNp73) inhibit apoptosis and show a dominant-negative effect toward p53 and TAp73.

Protein p53 is a transcription factor – binds to more than 300 different promoters and thus stimulates the expression of different genes. The greatest homology between the family members is found in DNA-binding domain what suggests that the family members bind to the same DNA sequences, and activate the same promoters. With a large overlap, there is some degree of selectivity in the role of individual family members related to the regulation of transcription of various genes (*12*). TAp73 protein activates transcription of a series of the same target genes as p53 (*14-3-3σ* and *Gadd45*, *mdm2*, *bax*, *PUMA*, *cyclin G*, *IGFBP* and *p21Waf1/Cip1*), although not equally effective.

p53, p63 and p73 in tumorigenesis

p53 gene controls cellular response to stress caused by DNA damage, hypoxia or oncogene activation by stimulating apoptosis or cell cycle arrest and thereby prevents the formation of tumors. Therefore, as mentioned before, the tumor suppressor role of *p53* gene is crucial in the prevention of tumor growth. This role is achieved by changing the expression of different target genes responsible for the development of tumors. In most human tumors *p53* gene is not functional because of numerous mutations, loss of $p14^{ARF}$ or overexpression of Mdm2. The result of *p53* germ line mutations is Li Fraumeni syndrome, which includes the development of a number of tumors at an early age (*18*). Although *p73* and *p63* are rarely mutated in tumors (*19*), the consequences of inherited mutations in *p63* are ectrodactyly, ectodermal dysplasia and facial clefting (EEC) and ankyloblepharon, ectodermal defects and clefting (AEC) syndromes (*20*). All but one missense mutation of *p63* gene in EEC syndrome are located in the DNA-binding domain. To date no syndrome in humans associated with mutations of *p73* has been found (*18*).

Mutated p53 is stable, however, does not bind to DNA and does not induce transcription of certain genes (like *p21* and *mdm2*). More than 80% of all p53 mutations can be found in DNA-binding domain (Figure 2), the most important for p53 tumor suppressor role (*8*). Besides the loss of tumor suppressor activity (cell cycle arrest and apoptosis) some mutant p53 gain oncogenic properties (*4*).

p73 location in the region 1p36 frequently undergoes loss of heterozygosity in some tumors: neuroblastoma (*21*), lung (*22*) and ovarian (*23*) cancer. Despite this and the functional similarity with the *p53* gene, *p73* is not a classic Knudson-type tumor suppressor. Moreover, unlike p53 knock out mouse, which spontaneously develops multiple tumors before six months of age, p63 and p73 knock out mice do not develop tumors, but there are a number of developmental disorders (*24*).

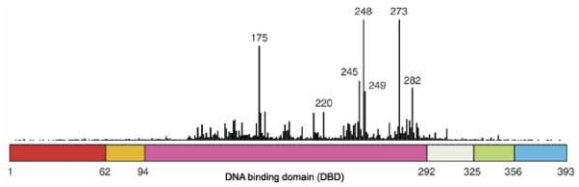

Figure 2. *The most frequent p53 mutations occur in DNA binding domain.*

However, the most common cancer-specific alteration is an overexpression of p73 rather than the loss of function (*7*). There is a higher level of expression of p73 protein in various tumors (neuroblastoma, lung cancer, colon, breast, bladder, liver, ovary) than in the tissues of origin, indicating a worse prognosis for the patient (*12, 25*) Specifically, studies based on measuring the expression of p73 in different stages of development of colon tumors showed that overexpression of p73 is associated with poor outcome of disease (*26*). Namely, both TAp73 and ΔNp73 are overexpressed in many tumors (*25, 27, 28*). In different human tumor cell lines and primary tumors abnormal variations of p73 were observed. Since in different parts of the same tumor may be present different variants of protein p73, p73 heterogenity reflects the biological heterogenity of tumor (*12*). In fact, the changes of the balance between p73 isoform expressions are often hallmark of tumor formation. Dominant-negative ΔNp73 isoforms, rather than TAp73 are relevant components of tumor-associated p73 overexpression, functionally overriding an accompanying increase of TAp73 expression.

We were involved in studies of p73 expression profile in ovarian cancer, where we found that in tumors harboring wild type p53, there is significantly higher expression level of N-terminally truncated isoforms what supports the hypothesis that their expression can alleviate the selection pressure for p53 mutations by the inhibition of the p53 protein function (*17, 25, 28*).

The role of mutant p53 proteins, which mostly lost tumor suppressor activity and acquired inhibitory activity, can be explained by interactions between the oncogenic and tumor suppressor isoforms.

Interactions between p53/p63/p73

p73 and *p63* gene mutations are not common in tumors, but there is an important dominant-negative cross--talk between p53, p63 and p73. This relationship is based on creating heterocomplexes between certain isoforms of p53 and p63/p73 proteins.

Such important inhibitory cross-talk that occurs in tumors between certain p53 and p73 proteins, potentially converts an anti-oncogenic synergism into an oncogenic antagonism. The mixed protein complexes are formed

between some mutant p53 proteins or ΔNp63/ΔNp73 isoforms and wild type p53/TAp63/TAp73. The formation of such mixed heterocomplexes correlates with functional transdominance – loss of p73-mediated transctivation and proapoptotic abilities (*29, 30*). This suggests that »gain-of-function« phenotype of p53 mutant cells might in fact be due to an interference with the suppressor function of p73/p63 (»double hit effect of p53 mutations«). ΔNp73 inhibits p53 and TAp73 activity by direct binding to the proteins of by competing for promoter sites (*28*). Several mutant p53 proteins form heterocomplexes with TAp73 protein. We confirmed the existence of mixed complexes between several p53 isoforms and TAp73 (unpublished data).

Despite the functional inactivity, the hetero-oligomerization mediates the stabilization/accumulation of TAp73 simultaneously with inactivation (*31*). Inhibition of p53 is achieved by competition for binding to DNA, whereas the inhibition of TAp73 by direct protein interactions (*32*). This structure suggests the idea of a disturbed balance between individual proteins in the development of tumors.

Interaction between p53 family proteins is important in the modulation of chemotherapeutic cytotoxicity and the outcome of chemotherapy. Increased expression of certain mutant p53 may lead to the prevention of apoptosis by protein p73 in the chemotherapeutic treatment (*33, 34*). Upon DNA damage p53 cannot induce apoptosis without the presence of either p63 or p73 (*35*). Therefore both p63 and p73 are important for p53-indeced apoptosis and other p53 tumor suppressor activity, they bind to the same promoters and all are important for induction of target genes.

The members of p53 family can regulate each other's expression through several feedback loops. Notably, both p53 and TAp73 can bind to the ΔNp73 promoter and induce its expression, which, in turn, inhibits p53 and TAp73 activity.

Taken together, p53/p63/p73 family members can interact in many ways including the protein interactions among them, regulation of target genes or each other's promoters. Clarifying the interplay between them seems to be exceptionally important aspect of cancer research.

Studying the p53 mutations

Since mutations of the *TP53* gene are the most common changes in human malignancies, their detection is of practical importance. p53 mutant cells are not easily identified. Until recently most studies used immunocytochemical detection of p53 accumulation in tumor samples as marker for *p53* mutations (*36*). Mutant p53 proteins are easily detectable by immunohistochemical (IHC) methods due to their abnormally extended half-life. However, the estimation of p53 status using IHC method is not always accurate enough. Namely, many tumors with *p53* mutation do not accumulate mutant p53 protein and not all tumors with missense mutation are ICH positive. Furthermore, there are some tumors that accumulate functional wild type p53 because of persistent stress signal.

Another method to study p53 missense mutations is single stranded conformational polymorphism (SSCP) analysis which detects DNA sequence changes as a shift in electrophoretic motility (*37*). Methods such as direct gene sequencing and DNA microarray-based sequencing method using AmpliChip p53 GeneChip assay, powered by Affymetrix (improved by Roche Molecular Systems, sequence exons 2–11 of the *TP53* gene) appeared to be more accurate (*38*).

However, the mutations of *p53* are not the only mechanism of inhibiting p53 function (p53 could be inactivated by MDM2 overexpression or deregulation of components of p53 pathway). Functional assay for screening cell lines, blood and tumors which scores for functional p53 has been developed (FASAY assay) (*39*). This assay, based on yeast reporter system, can be used to detect mutations in tumor specimens contaminated with large amounts of normal tissue.

p53 mutations as fingerprints

Various types of carcinogens may cause change-of--function mutations to activate oncogenes or inactivate tumor suppressor genes. The mutational study could give us clues about the mechanisms of carcinogenesis in specific tissues (*40*). The study of *p53* mutational spectra is very informative since *p53* mutations are the most frequent mutations 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) (*41*).

Most mutations are in the DNA binding domain (DBD, Figure 2), responsible for sequence-specific DNA binding and transcriptional activity, as well as for a direct mitochondrial pro-apoptotic activity (*4*).

A significant correlation between p53 mutational spectra and exposure to various carcinogens has been demonstrated. The mutations occur most frequently at CpG dinucleotides (a cytosine followed by guanine) in codons 175, 248, 249, 273 and 282 (Figure 2) which are frequently methylated (*42*) and reflect an endogenous mutagenic mechanism (*43*). Furthermore, G:C → T:A (G → T) transversions, the most frequent substitutions in human cancers, probably are caused by carcinogen--DNA adducts. They are more frequent in lung cancers associated with smoking compared to lung cancers of nonsmokers (*43*). Generally, cigarette smoking has been established as a major risk factor for lung cancer incidence, and p53 mutational hotspots are codons 157, 158, 248, 249 and 273. The G → T transversion on codon 157, is one of the hotspots in lung, breast, and head and neck cancers, but uncommon in other cancer types. Moreover, it was shown that *in vitro* exposure of bronchoepithelial and HeLa cells to tobacco-derived benzo(a)pyrene generates adduct formation at guanine positions in codons 157, 248, and 273 (*44*).

In liver tumors from populations living in endemic areas of Southern Africa and Asia where aflatoxin B1 (mycotoxin consumed in food contaminated with *Aspergillus flavis*) and hepatitis B virus are risk factors for hepatocellular carcinoma, most p53 mutations occur at the third nucleotide position (AGG → AGT) of codon 249 (*45*). 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}) (*46*).

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, producing tandem mutations, characteristic CC → TT double-base transitions *(47)*. Furthermore, the p53 mutational pattern in lung cancer from ^{238}uranium miners associated with ^{222}radon differs from the one in lung cancer caused by smoking alone – 249^{met} mutation appears in lung cancer of never smokers implicating the non-tobacco associated carcinogen (*48*). Moreover, liver angiosarcomas of vinyl chloride-exposed factory workers have higher frequency of p53 A:T → T:A transversions comparing to sporadic angiosarcoma (*49*).

Aristolochic acid as p53 mutagen

Herbal medicines derived from *Aristolochia species* have been used since ancient times to treat disease and are still widely used in traditional and »natural« medicine. However, it was shown that the aristolochic acid (AA), the component of *Aristolochia* plant, is a genotoxic mutagen (*50–53*). The herbal drugs containing *Aristolochia* have been associated with development of a characteristic chronic interstitial nephropathy, called aristolochic acid nephropathy (AAN), previously Chinese herbs nephropathy (*54*). The disease is characterized by proximal tubular damage, renal interstitial fibrosis and slow progression of the disease to the end stage with high prevalence of upper urinary tract urothelial carcinoma, the location that is highly unusual in sporadic urothelial carcinoma. The upper urinary tract cancers are generally associated with exogenous carcinogens (*55*), like aniline dyes, acrylamines and chemicals used in the rubber, leather and petrochemical industries, chronic analgesic abuse and chronic irritation (kidney stone), and aristolochic acid.

AAN cases have been identified in Europe, Asia and the United States. About 100 AAN cases have been identified in Belgium among women undergoing a slimming treatment involving drinking tea with *A. fangchi* (*56, 57*). The pathological and clinical features of endemic (Balkan) nephropathy (EN) closely resemble those associated with aristolochic acid nephropathy (*38, 54*). The EN is present in several rural areas in the valleys of big Danube tributaries in Croatia, Bosnia and Herzegovina, Serbia, Bulgaria and Romania affecting approximately 2–7% of exposed rural farming population (*58, 59*).

Aristolochic acid forms DNA adducts in AAN patients (*38, 60–62*), which are considered to be reliable biomarkers of exposure to it (*38, 62, 63*). DNA adduct 7-(deoxyadenosin-N(6)-yl)aristolactam I (dA-AAI) forms A:T→T:A transversions at codon 61 of *ras* gene in rats and mouse models (wt CAA → CTA) (*64, 65*).

In Belgian AAN patient p53 protein was overexpressed, suggesting that the *p53* gene is mutated in AAN-associated urothelial carcinoma (*55*).

To test direct mutagenic effect of a substance towards human *p53* a sophisticated genetic mutagenesis assay was designed – mouse embryo fibroblasts derived from gene-targeted knock-in mice (Hupki), with substituted endogenous mouse p53 DBD (Ex 4–9) by the human counterpart (*66*). After aristolochic acid I treatment, five of ten established cultures harbored p53 DBD mutations. Of note, four of them were A → T transversions on the non-transcribed strand, a unique hallmark of mutagenesis by AAI (and rare in spontaneous mutations) (*64, 66*). 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) (*66*). 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 (*66*). 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.

Figure 3. Aristolochia clematitis *growing in the wheat field in the Croatian endemic area during harvest time 2007 (kindly provided by dr. Bojan Jelaković).*

The first notion that AA could be the major risk factor for EN came from Ivić more than 30 years ago (*67*). Unfortunately, this hypothesis was forgotten until Hranjec *et al.* (*68*) confirmed that seeds of *Aristolochia clematitis* (Figure 3) were commingled with wheat seeds and contaminated the flour. The environmental toxic substance was ingested through bread by farmers from EN villages. Based on pathological findings, Cosyns et al. (*69*) debate whether AAN could be the clue for EN, caused by the common etiologic agent, aristolochic acid. Finally we confirmed this hypothesis: the presence of specific DNA mutations and AA-DNA adducts in urothelial tumors of Croatian and Bosnian EN patients (*38*). Using AmpliChip p53 microarray (Roche Molecular Systems), exons 2–11 were sequenced. Of 19 base substitutions identified, 17 were at A:T pairs (89%), with the 88% of these (15/17) being A → T (A:T → T:A) transversions (Figure 4A). Of note, p53 mutations in EN patients with urothelial cancers from Croatia and Bosnia are unique and not consistent with IARC p53 database, version R12, November 2007 (*41*). Namely, in the general population of patients with upper urothelial cancers, the A → T transition are rare – only 5% of all p53 mutations (Figure 4B). The latest version – R14, released in November 2009, includes our finding (*38*), rising A → T transversions to 7%.

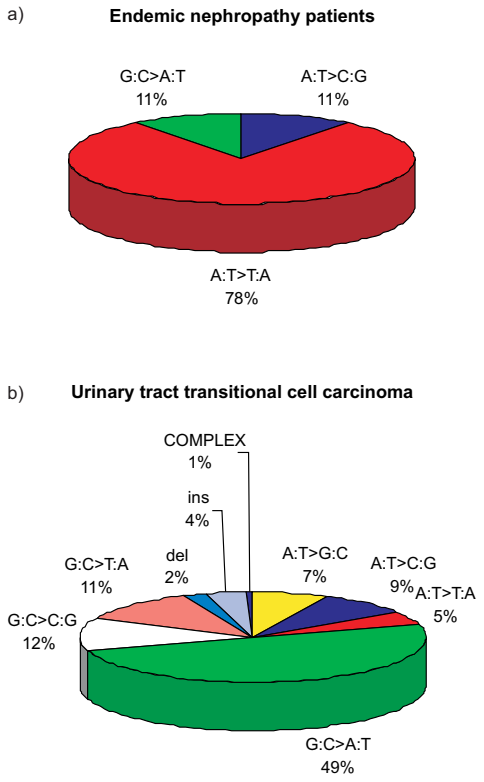

Figure 4. p53 *mutational spectra in upper urinary tract urothelial cancers (UUC). (A) EN patients in Croatia (19 mutations). (B) Urothelial transitional cell carcinoma of kidney, renal pelvis, ureter, bladder and other nonspecified urinary organs (761 mutations). Data from IARC p53 database, R12 released in November 2007 (41); adapted from ref. 38.*

In addition, p53 mutations in our patients 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 → T:A mutations found in EN patients, were also detected in human Hupki cells treated with AAI (*66, 70*). Therefore, the presence of AT → TA transversions serves as a fingerprint for AA EN associated with urothelial cancer cases. These data strongly support the hypothesis that dietary exposure to AA is a major risk factor for endemic (Balkan) nephropathy.

CONCLUSION

The discovery of *TP53* homologous genes, *TP63* and *TP73*, has sparked great expectations in the research of their biological roles. However, today it is clear that, no matter how similar the members of p53 family are, they have their own identity. Recently, it was determined that, like *p63* and *p73*, *p53* gene also has an alternative promoter and together with alternative splicing causes the production of multiple isoforms that lack amino or carboxy end. In short, p53/p63/p73 isoforms have different roles in tumorigenesis, and it is questionable whether they can be regarded as a classic tumor suppressor genes. All these proteins are involved in a complex network of interactions that determine the fate of cells. Therefore, further research on p53 family protein interactions is necessary for the understanding of their individual and collective roles.

On the other side, the study of p53 mutational spectra can give us clues about exogenous and endogenous factors in human carcinogenesis. In such a manner high frequency of A→T transversions in the *p53* gene in tumor tissue of patients from Croatia and Bosnia with endemic nephropathy are strong supplementary evidence of an etiological role of AA in EN-associated urothelial tumors.

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