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PROGNOSTIC VALUE OF P53, C-ERBB2 AND TUNEL DATA IN UPPER UROTHELIAL CARCINOMA ASSOCIATED WITH BALKAN NEPHROPATHY

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Abstract – A characteristic tumor suppressor protein 53 (p53) mutational profile of genotoxic action of aristolochic acid was identified in the upper urothelial carcinoma (UUTT) associated with Balkan nephropathy (BEN). In the present study, we examined the prognostic value of tissue-based molecular markers in overall-survival (OS) risk after surgical treatment of UUTT, adjusted for gender, age and urological characteristics in 32 patients with BEN. Immunohistochemical examination of p53, the proliferation cell nuclear antigen (PCNA), the human epidermal growth factor receptor 2 (c-ErbB2; also known as HER-2/neu) proto-oncogene and the *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for apoptosis detection were used to examine serial tumor sections. The median OS-time was 60 months for UUTT operation; the mortality rate (18.7%) was related to (new) disease (re)occurrence or invasion in 12-216 months. High-grade (p=0.029), TUNEL>0.36%+ cells (p=0.010), and c-ErbB2+ cells (p=0.014) can define the risk of tumor invasion. Patients with Balkan nephropathy that develop UUTT at a stage greater than pT1 (with apoptosis TUNEL+ cells >0.36% and p53+ cells greater than 10%) were at high risk of poor-OS after the tumor surgery (h(x)=6.35; p=0.045).

The obtained data present evidence for p53, cErbB2 and apoptosis deregulation, as a result of environmental toxin action. This is the first report of molecular biomarker linkage with OS for BEN-associated UUTT.

Key words: upper urothelial carcinoma; Balkan nephropathy; apoptosis; p53; cErbB2; overall survival

INTRODUCTION

Upper urinary tract tumors (UUTT) associated with Balkan endemic nephropathy (BEN) appear with an incidence that is 30 times higher than in the general population (Petronic, 2000) in several villages along the tributaries of large rivers in Serbia, Bosnia, Croatia, Romania and Bulgaria. These tumors occur considerably more frequently as synchronous or successive bilateral tumors, while an additional bladder carcinoma may also develop (Petronic et al., 1991). A third of the patients suffer from both BEN and UUTT. Overt renal insufficiency is common. However, according to literature data, the UUTT observed in patients from BEN regions is not an aggressive tumor. Not one author has reported on the

lower survival of patients with UUTT from BEN regions, although these patients experience increased risk burdens. These patients had equal or even greater survival than patients that develop UUTT outside the endemic areas (Jankovic et al., 1998; 2013). Petronic and Savin (2001) have suggested slow growth of the UUTT in BEN patients that were on hemodialysis. A new or recurring urothelial carcinoma has been evidenced in 20% of patients after 5-12 years, indirectly implying considerable patient survival in BEN regions (Petronic et al., 1995, 1997).

UUTT presents molecular deregulation of the cell cycle in a biologically aggressive neoplasm. Classical tumor grading by light microscopy characteristics is less efficient than by molecular markers. There is a strong evidence for aristolochic acid genotoxic action and UUTT development in BEN patients presenting a characteristic p53 mutational profile (Grollman et al., 2007; Jelacic et al., 2012). The aim of the present study was to evaluate the prognostic potential of p53, PCNA, c-ErbB2, and TUNEL data in patients undergoing surgical treatment of BEN-associated UUTT. We found that neither classical tumor marker grades for cell atypia and stage of local invasion, or a single molecular marker such as p53 PCNA, cErbB2 or TUNEL data cannot predict patient survival. The presence of c-ErbB2+ cells and increased apoptosis could define tumor invasiveness. A positive [stage>1; TUNEL+ cells >0.36%; p53+cells >10%] triple marker points to poor OS, which is a verification of the mutual genotoxic and cytotoxic action of putative agent (aristolochic acid) in BEN regions.

MATERIALS AND METHODS

UUTT developed in members of affected families by BEN and/or UUTT in endemic villages in Serbia, with no other risk factors of urothelial cancer, such as heavy smoking or frequent use of analgesics or NSAIDs, were recorded. Histological diagnosis of UUTT by light microscopy was used to confirm upper urothelial carcinoma grade (1-3) and pT stage (1-4) for local spreading (Mostofi et al., 1973; Sobin et al., 2002), with simultaneous examination for characteristic tubulointerstitial pictures of BEN (nephrectomy or kidney biopsy). The recurring or new urinary tract tumors and renal function were regularly monitored after the operation. Tissue-based molecular biomarkers of UUTT were analyzed without knowledge of the clinical information or pathological findings; serial 4 micrometer-thick sections of tumor specimens were fixed in 3-4% formalin immediately after surgical procedure.

The TUNEL assay was performed using the *in situ* cell death detection POD kit (Boehringer Manheim, Germany) essentially as described previously. Slides were rinsed in distilled water, lightly counterstained with Mayer's hematoxylin and mounted under Dako Glicer gel mounting medium.

Mouse anti-human 1/100 MoAb PC-10 (anti-PCNA, DAKO), rabbit polyclonal anti-human c-cErbB2 antibodies (1/100) (HER-2 Code A 0485/ Dao) and mouse anti-human monoclonal antibody Pan p53 (Clone D07, Dako), and the avidin-biotin peroxidase method (LSAB-2 kit; DAKO) were used. A minimum of 1 000 cells was counted on each tumor section at 400 x magnification. More cells were counted in areas of positive cell clusters to provide a sample representative of staining across the entirety of a tumor for the final calculation of the percentage of positive tumor cells for each biomarker.

Statistical analysis was performed using SPSS 18.0. Each biomarker score was classified as positive with regard to the number of positive cells, as follows: PCNA+ cells >9.7%, cErb-B2+ cells, apoptosis index >0.36% TUNEL+ cells and p53+ cells greater than 10%. These cut-off values for tumor grade and stage greater than pT1 were evaluated by ROC curves in the analysis of a total of 80 cases of UUTT with endemic Balkan nephropathy, as we previously described, Savin and Petronic (2002). The apoptosis index of 0.4% TUNEL+cells placed between threshold values for grade 3 and pT3 progression was included in the analysis. OS after a tumor operation was expressed as the mean and median time. The Cox regression model was applied to calculate risk factors for OS that included the biomarkers PCNA, p53, c-ErbB2 and TUNEL+, together with classical

pathological tumor grade and stage, urological characteristics, family history of BEN and UUTT, renal insufficiency, hemodialysis treatment, all adjusted to age and gender. Kaplan Meier analysis defined groups of patients with greater survival by investigated parameters (Log rank test; $p<0.05$). The biomarker affirmation for predictor of tumor grade or pT-stage progression was approved in a discriminate upon the multinomial regression analysis.

RESULTS

The investigation included 32 patients (20 males and 12 females) aged 59.5±7.5 years with UUTT associated with BEN. The patients were inhabitants, mostly from birth, of established endemic villages in Serbia (median time of admission = 57 years). Several family members were commonly affected by one or both diseases, accounting for 60% of the family burden for BEN, and one third for urinary tract tumor (Table 1). UUTT was found either in the renal pelvis of 22 patients, or in the upper part of the ureter, as multiple tumors in 13 cases. Bilateral simultaneous tumors were diagnosed in 3 patients, while 4 suffered from bilateral successive UUTT; urinary bladder carcinoma was observed in 6 patients (18.7%). The patients underwent surgical treatment: 23 underwent total nephroureterectomy, and 9 patients underwent conservative kidney-ureteral operations at the Urology Clinic of Belgrade from 1977-1997. The patient OS median time of 60 months was calculated for the observation period of 78+46 months after surgery. Six out of 32 patients (18.7%) succumbed to disease reoccurrence or invasiveness during the follow-up period (1, 12, 14, 48, 132, 135 and 216 months). The medical records confirmed severe sepsis and distant metastases in one patient who died 12 months after the operation. After 5 years of follow-up, 10 patients with normal renal function censored the subsequent medical survey. Not one of the general characteristics or the listed urological parameters of tumor presentation and growth (Table 1) directly affected the patient OS rate, except for a worse outcome for bilateral, and especially for successive bilateral tumors ($F=11.55$; $p=0.0031$; Fig. 1). Patient OS did not significantly differ with the surgical option, whether a conservative approach or radical total nephroureterectomy were performed. Patients with a lethal outcome were mainly treated by the radical option. The majority of non-invasive tumors (60%) were grade 2; the median stage/grade ratio was 0.83. There was a direct relation between tumor grade of cell atypia and stage of local spreading ($r=0.48$; $p=0.005$). The OS rate decreased with the grade of progression and was 100%, 89.5% and 63.6% for grades 1, 2 and 3, respectively. However, the grade and stage may not account for the risk of poor outcome after the surgical treatment, as revealed by OS Cox regression analysis.

The analysis of tumor specimens obtained at the operation verified that one third of the patients (12/32; 37.5%) developed p53+UUTT (>10% of p53+cells); 51.6% of UUTT contained c-ErbB2+ cells; the distribution of PCNA+ >9.7% of tumor cells (16/32; 50%) was linked with a high proliferation index and increased apoptosis, calculated as TUNEL+ >0.3% cells (16/32; 50%). These biomarkers may define the cut-off for grade 2 and stages >1, reported in our previous investigation of 80 UUTT from BEN regions (Savin and Petronic, 2002). Molecular marker c-ErbB2 overexpression was more frequently present in advanced grade 3 than intermediate grade 2 (0.90+-0.48 vs. 0.33+-0.48; $F_{cErbB2}=11.33$; $p=0.002$), together with local invasion pT stage 2.7+-1.3 vs. 1.6+-1.0 ($F_{stage}=8.42$; $p=0.001$). Multinomial regression analysis revealed that c-ErbB2+ was a factor of grade progression ($p=0.007$); c-ErbB2+ ($p=0.014$); apoptosis index (TUNEL>0.36% positive cells; $p=0.010$) and the high grade of cell atypia ($p=0.029$) were biomarkers of tumor local invasion by pT, stage>1. However, none of the single tissue-based biomarkers, PCNA+, c-ErbB2+, apoptosis index (TUNEL+) or p53+, can significantly predict OS after the surgery. To overcome the limitations of a single marker, the patients were subjected to selection for two positive biomarkers for OS that allows for the calculation of their mutual contribution to cancerogenesis and OS. We found that the combinations of c-ErbB2+, p53+ status or a high apoptosis index could reveal OS risk after tumor operation. PCNA+ did not provide any information about patient survival.

Table 1. Urological characteristics of UUTT and family history for BEN/UUTT

Clinical characteristics			No	%
UUTT presentation	abdominal pain	no / yes	22 / 10	68.7 / 31.3
	hematuria	no / yes	4 / 28	12.5 / 87.5
	pyuria	no / yes	24 / 8	75 / 25
	localization	pyelum ureter	22 10	68.75 31.25
	growth	solitary multiply	19 13	59.37 40.63
	*bilateral	unilateral simultaneous *successive	25 3 4	78.13 9.37 12.5
light microscopy	grade 1-3	2 / 19 / 11	6.3 / 59.4 / 34.4	
	pT-stage 1-4	17 / 3/ 6 / 6	53.1 / 9.4 / 18.8/ 18.8	
bladder tumor	at time of UUTT diagnosis	no tumor before concomitant after	23 6 2 1	71.9 18.7 6.3 3.1
renal insufficiency	at presentation	none mild overt ESRD - HD dependent	16 6 5 5	50.0 18.8 15.6 15.6
	the kidney with UUTT	functional declined function non-functional	16 4 12	50.0 12.5 37.5
	BEN at UUTT operation	no / yes	0 / 32	
	HD at presentation	no / yes	27 / 5	84.4 / 15.6
	HD after the surgery	no / yes	19 / 13	59.3 / 40.7
family history	BEN	no / yes	13 /19	40.7 / 59.3
	UUTT	no / yes	20 / 12	62.5 / 37.5
	co-morbidity (excluding BEN)	No KVS DT- ulcer operation severe infection (sepsis) other	13 7 5 4 (1) 2	40.6 21.9 15.6 15.6 6.3
treatment and outcome	surgery of UUTT	radical conservative	25 7	78.1 21.9
	patient outcome	live death (tumor /other)	26 6 (6/1)	81.3 18.7
	follow-up time (month)		78+-46	Medin 60

* significant for patient OS,
Abbr. BEN – Balkan nephropathy, UUTT – upper urinary tract carcinoma, OS – overall survival, HD – hemodialysis

In double-positive markers containing c-ErbB2+, the mutated c-ErbB2 increases the prognostic probability of p53+ (F=12.85; p=0.0050) or TUNEL+ (F=8.90; p=0.0307) for the risk of late and delayed OS for a new or recurring tumor according to the Kaplan Meier stratification (Figs. 2 and 3). The latter points to the importance of the association of apoptosis increase and tumor invasion for patient

Table 2. Predictors of poor OS risk for UUTT associated with BEN: bilateral successive UUTT and triple positive [p53+, TUNEL>0.36% +cells in stage over 1]

Multivariate Cox-regression OS analysis	Exp (B)	95% CI	p
Bilateral successive UUTT	4.15	1.10-15.61	0.035
s>1; TUNEL>0.36%; p53+	7.07	1.03-48.32	0.046

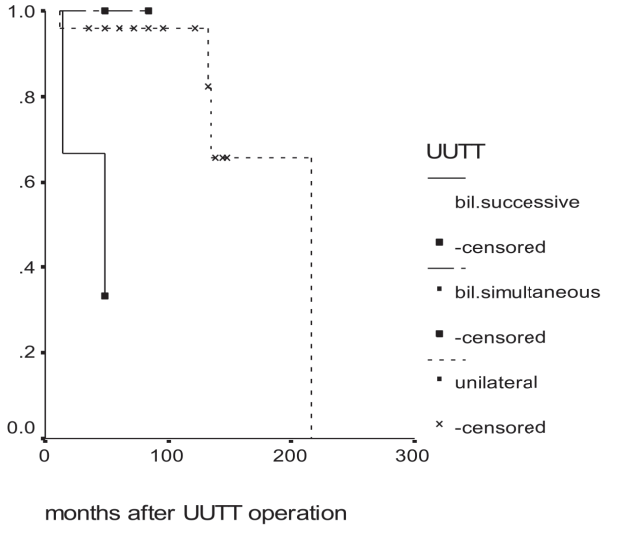

Fig. 1. Kaplan Meier analysis of upper urinary tract epithelial carcinoma by tumor bilateral appearance (log rank F=11.55; p=0.0031).

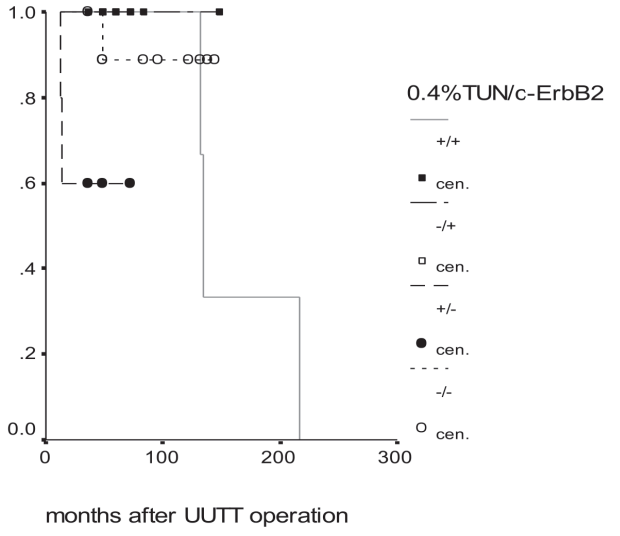

Fig. 2. Kaplan Meier analysis of upper urinary tract epithelial carcinoma by double marker TUNEL+ (>0.4%+ cells) and c-ErbB2+ (Log Rank F=8.90; p=0.0307).

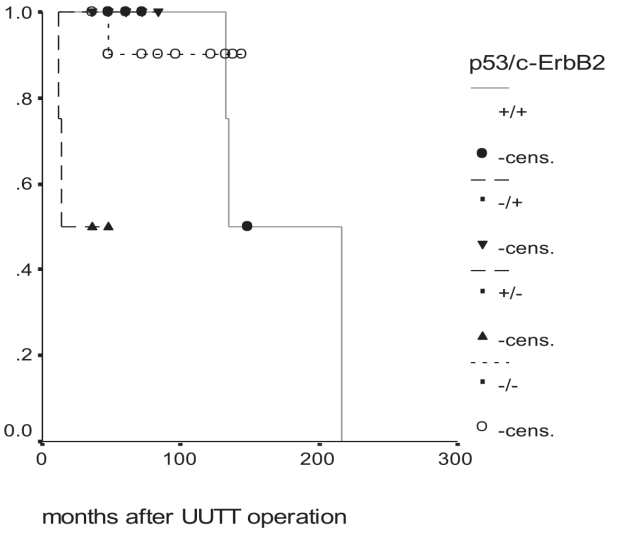

Fig. 3. Kaplan Meier analysis of upper urinary tract epithelial carcinoma by double marker p53+ (>10%+ cells) and c-ErbB2+ cells >0 (log rank F=12.85;p=0.0050).

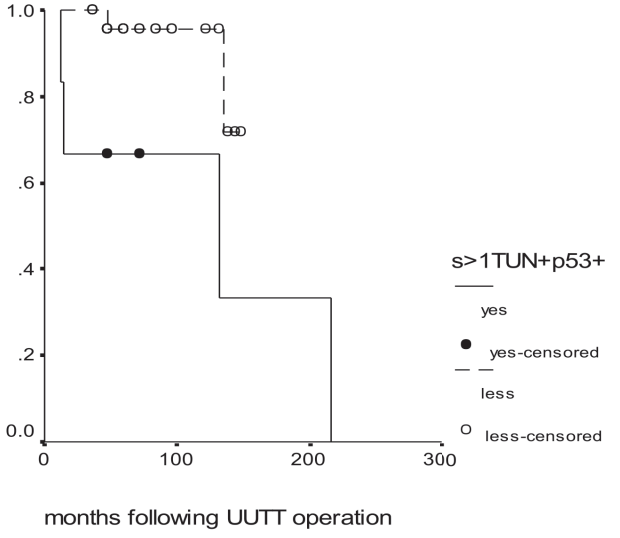

Fig. 4. Kaplan Meier analysis of upper urinary tract epithelial carcinoma by triple marker >pT1/TUNEL+/p53+ (log rank F=5.23; p=0.0222).

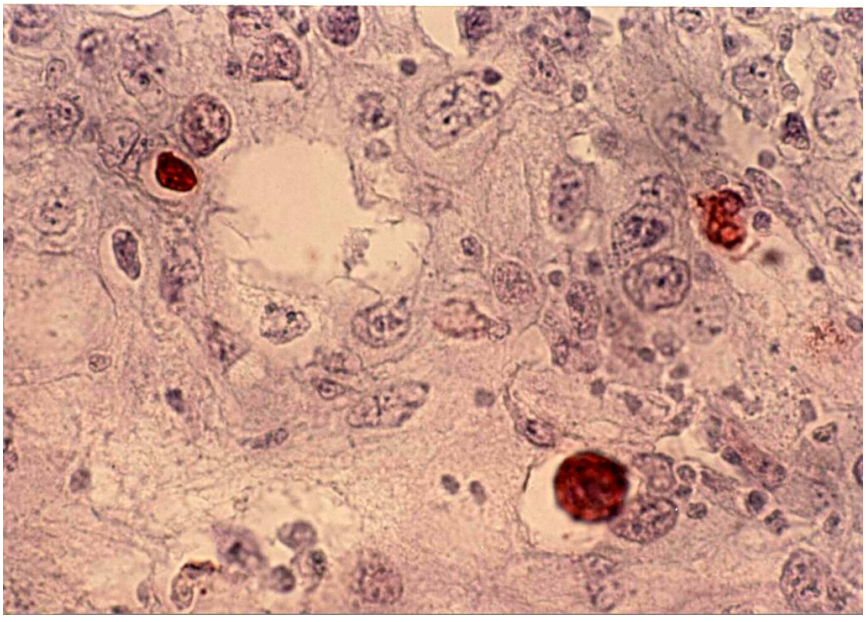

Fig. 5. TUNEL+ cells (>0.4%) in UUTT of grade 3 and stage pT3 developed in a patient with Balkan nephropathy.

survival, although the stage of local invasion in combination with single molecular marker, either p53 or the apoptosis index, may not predict the risk of patient OS. Finally, cross-relations of the last three markers, stage>1, high apoptosis index and p53+ overexpression may define more efficiently the risk of poor OS. An apoptosis index >0.4% and p53+ tumor status in pT>1 are linked with poor survival in two survival tests (Cox regression analysis; (p=0.045) and log rank test (p=0.0222) (Figs. 4, 5)). The same risk of 6.35 times (95%CI=1.04-38.65) of poor OS was calculated by decreasing the apoptosis index from [>0.40%] to [>0.36%]; the last was the putative cut-off for pT1 into pT2 stage of local expansion that suggests the possibility of an early worse prognosis for BEN-related tumors that are restricted to urothelial mucosa [stage>1, apoptosis index>0.36, p53+]. A triple-positive marker was associated with the risk of poor outcome, particularly in patients with bilateral successive UUTT, by multivariate Cox regression analysis (Table 2).

DISCUSSION

Balkan endemic nephropathy frequently appears in several villages of endemic areas in Serbia, with the same incidence as 50 years ago when Petkovic discovered UUTT associated with BEN (Petkovic, 1975; Jankovic et al., 2013). Recurring and/or new urothelial tumor appearance is common and a large portion of patients undergo hemodialysis for several years due to the slow progression of BEN after radical surgical treatment of UUTT. Our follow-up time was prolonged in order to examine the usefulness of tumor tissue-based molecular biomarkers in OS prediction. Six out of 32 patients died (mortality rate of 18.7%) due to recurring disease or invasion from 12-216 months. The great OS median time of 60 months after the operation illustrated the slow progression of BEN and confirmed a long induction time for new or recurring urothelial tumor and the slow growth of multiple tumors (Petronic, 2000). A bias for an improved outcome after conservative vs radical kidney operation was not observed; however, all mortal outcomes were reported after radical surgical treatment; sepsis after surgery was not common (Savin, 2013). In fact, tumor bilateral growth was the only urologic factor pointing to poor OS, probably because of several additional unfavorable conditions, including the need for regular hemodialysis.

Krasteva et al. (2006) screened 90 Bulgarian BEN patients for p53 gene mutations, establishing mutations in the patients. The investigations of Grollman et al. (2007) and Jelakovic et al. (2012) revealed that aristolochic acid is genotoxic, inducing a characteristic p53 mutational profile of UUTT associated with BEN in rural households in Croatia. It has been suggested that exposure to aristolochic acids could result from the consumption of wheat contaminated with seeds of *Aristolochia clematitis* (Hranjec et al., 2005). These investigations provided additional support for the toxic etiology of UUTT in BEN regions, and that BEN-associated tumors possess specific biological features. Analysis of UUTT associated with chronic aristolochic acid intoxication in Taiwan has ascertained a dose-dependent relationship between cumulative aristolochic acid exposure and end-stage renal disease or UUTT development (Wu and Wang, 2013), and increased risk of new urothelial malignancies (Lemy et al., 2008).

Our study suggests that the most reliable predictor for OS was the combination of two positive biomarkers, the apoptosis index >0.36% and p53>10% cells that provided for 6.35-fold higher risk for poor OS after surgery (pT stage greater than 1). This is an evidence for p53 overexpression/mutation and apoptosis deregulation in tumor invasiveness under environmental toxin action. This is the first report on the linkage of tissue-based molecular biomarker to mortality rate for BEN-associated urothelial carcinoma, suggesting that mutagenic and cytotoxic effects play a role in the development of UUTT associated with BEN. We have previously discovered early deregulation of apoptosis in BEN-related UUTT that may have even two times lower apoptosis cut-off value for progression of cell atypia than for tumors diagnosed outside of the endemic regions (0.37 vs 0.61% of TUNEL+ cells). A delayed increase in apoptosis is apparent in high grade UUTT associated with BEN (Petronic and Savin, 2001; Savin and Petronic, 2002). Jankovic Velickovic at al. (2011) demonstrated decreased pro-apoptotic activity in tumors arising in endemic regions. The authors discovered a connection between Bax overexpression with the stage of tumor invasion. Our results indicate that an apoptosis index greater than 0.36% of TUNEL+ cells could define tumor invasiveness besides c-ErbB2+ or increased grade of cell atypia. The prognostic value of tumor apoptosis was not restricted to BEN-related tumors. Jeong et al. (2009) displayed that T stage and early deregulation of apoptosis were associated with survival in UUTT of unspecified etiology. They applied the TdT-mediated DUTP nick-end labeling method to obtain the apoptotic index.

Tumor-based molecular investigation of UUTT from the South Morava River basin in Serbia revealed p53 protein overexpression (>10% cells) in 60% cases, as a specific marker for BEN-related UUTT (Jankovic Velickovic et al., 2009). p53+ overexpression was displayed by only 37.5% tumors. Results of meta-analysis suggest that p53+ might be a prognostic factor of OS for patients with UUTT. The analysis included 514 UUTT patients from the general population (Ku et al., 2013). We found that neither p53+ nor the apoptosis index can predict OS in UUTT associated with BEN, however a double marker [p53+, c-ErbB2+] or [TUNEL+, c-ErbB2+] pointed to a worse OS in the Kaplan Meier analysis. The c-ErbB2 mutation is regarded as an important risk marker of tumor progression. We concluded that c-ErbB2+ enhanced the instability of tumors with suppressor protein p53 deregulation, the same as cErbB2+ acts together with deregulated apoptosis (over 0.40% of TUNEL+ cells). The finding points to a specific mutation of c-ErbB2, similarly as documented for p53. Chae et al. (2012) performed a molecular epidemiologic study of UUTT cases in Taiwan where *Aristolochia* herbal remedies have been used extensively for many years. They found several specific mutational signatures dominated by otherwise rare A:T to T:A transversions, mainly in *p53* (identical to that observed in UUTT associated with BEN), and *HRAS* and *FGFR3* gene. Deoxyadenosine (dA)-AL adducts were detected in the majority (10/13, 76.9%) of patients carrying A→T transversions. Prominent *p53* mutational hotspots include the adenine bases of $^{5'}$AG (acceptor) splice sites located almost exclusively on the non-transcribed strand. A:T to T:A mutations were also detected at activating positions in the *FGFR3* and *HRAS* oncogenes. The EGFR family is a

major class of receptor tyrosine kinase (RTK) proto-oncogenes, including EGFR, ErbB2 (Her2-neu), EGFR3 and EGFR4, whose homo- and heterodimers are activated by EGF (Lee et al., 1989). The amplification of c-ErbB2 and overexpression of its product in urothelial carcinoma has been associated with tumor grade, stage and patient outcome (Tsai et al., 2005). These authors, using multivariate analyses, suggested that tumor stage and c-ErbB2 expression were independent predictors of disease progression and OS. Investigations included 94 cases with UUTT in Taiwan that consumed traditional remedies with aristolochic acid. We presume that c-ErbB2/EGFR3 heterodimers are dysfunctional, especially in cErbB2+ (overexpression) UUTT from BEN regions, and that this probably results in deregulation of intracellular signaling. Additionally, the c-ErbB2 gene may be specifically mutated (Petronic and Savin, 2002), the same as p53 and EGFR3 genes. These patients were prone to recurring or new disease, also a characteristic of UUTT associated with BEN.

Our investigation supports the concept that specific gene deregulation is responsible for specific features of BEN-related tumors. Molecular prognostic tumor mapping of risks to patient OS needs to include several markers, including p53, c-ErbB2 and apoptosis deregulation (Savin et al., 2001).

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