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RESEARCH

Divergent Squamous Differentiation in Upper Urothelial Carcinoma—Comparative Clinicopathological and Molecular Study

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Abstract Upper urothelial carcinoma (UUC) has a plasticity to demonstrate divergent differentiation with squamous metaplastic elements. There was no previous study exploring profiling of molecular markers in metaplastic squamous upper urothelial carcinoma (SUUC) and conventional upper urothelial carcinoma (CUUC). The aims of this study was to compare expression of the phenotypic characteristics of tumors and molecular markers (p53, p16, cyclin D1, E-cadherin, HER-2, Ki-67, Bcl-2, Bax) in SUUC and CUUC. SUUC was detected in 20% of 44 patients. There was significant difference between SUUC and CUUC in the pathological stage, grade, growth and presence of lymphovasular invasion ($p<0.05$; 0.05; 0.05; 0.01 respectively). The mean Ki-67 and p53 labeling index was significantly higher in SUUC than in CUUC ($p<0.05$; 0.05). There was no significant difference in the expression of p16, cyclin D1, E-cadherin, HER-2, Bcl-2 and Bax between SUUC and CUUC. Univariant model showed that SUUC was significantly associated with lymphovascular invasion ($p=0.007$), Ki-67 activity ($p=0.016$) and growth ($p=0.026$). Exploration of UUC with squamous divergent differentiation showed changes in phenotypic characteristics and Ki-67, as well as similar molecular profile with CUUC.

Keywords Divergent differentiation · Squamous metaplasia · Molecular markers · Proliferative activity · Upper urothelial carcinoma

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Introduction

Conventional urothelial carcinoma accounts for most carcinomas of the urinary tract lining. However, neoplastic urothelium has the capacity to demonstrate enormous plasticity. In addition, urothelial carcinoma has a propensity to demonstrate divergent differentiation with glandular, squamous, small cell neuroendocrine, lymphoepithelioma-like, sarcomatoid or other elements [1, 2].

The origin of this unusual lesion is controversial. Some investigators believe that it represents the collision of two separate malignant tumors occurring independently and synchronously in the same location. Other suggest that they have a monoclonal origin with subsequent differentiation into its components [3, 4].

Squamous differentiation in upper urothelial carcinoma (SUUC) is result of metaplastic change. SUUC has

prognostic and therapeutic implications, including a poor response to radiotherapy [5–7].

Less than 10% of all tumors in the upper collecting system are squamous cell carcinomas, and adenocarcinomas are extremely rare. Squamous cell carcinoma, accounts for only 7% of renal pelvic tumors; this type of cancer is commonly associated with inflammatory processes. These tumors tend to be deeply invasive and thus are associated with a poorer prognosis [6].

Molecular genetic evidence has emerged recently supporting a close relationship between urothelial carcinoma and various divergent elements [1].

No previous study explored the profiling of molecular markers in metaplastic SUUC and conventional upper urothelial carcinoma (CUUC) with respect to tumor suppressor proteins: p53, p16 and E-cadherin, oncogenes: HER-2 and cyclin D1, proliferative protein Ki-67 and apoptotic markers—Bcl-2 and Bax. The aim of this study was to compare expression of these molecular markers and clinicopathological characteristics in SUUC and CUUC.

Materials and Methods

Patient's Population

We studied 44 consecutive patients with upper urothelial carcinoma (UUC) who had undergone nephro-ureterectomy with removal of bladder cuff. Extended lymphadenectomy was not routinely performed. All cases of UUC were diagnosed at the Institute of Pathology, Faculty of Medicine, Nis. Tumor specimens were obtained from 37 pelvis and 7 ureteral tumors. The histological sections were processed from tissue fixed in 10% formalin by standard techniques, and stained with haematoxylin and eosin (H&E). H&E-stained slides were used to assess histological grade (low and high grade) [8], pathologic stage (pT) [9], growth of tumor (papillary/solid), and lymphovascular invasion (LVI). Authors compare low stage non-muscle invasive tumor (pTa-pT1) and high stage muscle invasive (pT2-pT4) tumor [10].

According to the WHO criteria for the diagnosis of histological variants of urothelial cancer squamous differentiation was defined as the presence of intercellular bridges or keratinization. The usual criteria for squamous metaplastic change in UUC including abundant eosinophilic cytoplasm, large oval nuclei with an open chromatin pattern and prominent nucleoli [8].

Immunohistochemistry and Scoring

Tumors were analyzed using the mouse monoclonal antibody against p53 (Pab 1801, IgG1/Newcastle), p16 (Clone 6H12, IgG2b/Newcastle), E-cadherin (Takara Biomedical, Kyoto, Japan), cyclin D1 (P2D11F11, IgG2a/Newcastle), HER-2 (Code A 0485/Dako), Ki-67 [(MIB-1 (8), isotope IgG1, kappa/Dako)], Bcl-2 (Clone 124, M 0887/Dako), and Bax (Code A 3533/Dako), at dilution 1:50, 1:40, 1:1500, ready to use, 1:300, 1:100, 1:50, 1:1000 respectively, and a standard avidin-biotin immunoperoxidase complexes detection system, according to the manufacturer's protocol (Dako LSAB2R system-HRP).

Before quantifying the immunohistochemical results, the technique quality was assessed and those areas with greater positivity were selected, avoiding peripheral area measurement, necrosis or artifact.

Slides were reviewed independently by three investigators. Interobserver discrepancies were resolved using a double headed microscope. Only nuclear expression was recorded for p53, p16, cyclin D1, and Ki-67. The number of distinctly positive tumor cell nuclei was counted under high power (×400) using a 10×10 eyepiece grid. In total, 1,000 tumor cells were assessed. The number of positive nuclei was expressed as a percentage of all tumor cell nuclei counted. On this way we defined p53, cyclin D1 and Ki-67 index.

The staining protocol for p16 nuclear protein includes heterogeneous, homogeneous and negative findings. Tumor was considered to have a normal heterogeneous p16 pattern if it had relatively weak nuclear staining with considerable differences in nuclear intensity, including many negative cells. Strong 16 staining considered if the majority of the malignant cells had intensive p16 nuclear expression and p16 negative tumor cells were rare. Tumor was termed p16 negative if no malignant cells had positive staining. Tumor without or with over expression of p16 was categorized as altered [11, 12].

E-CD expression was scored according to established criteria [13, 14]. In every case, it was clarified whether the membrane or the cytoplasm was dyed [13, 14].

For testing HER-2 (C-erbB2) status we used HercepTest scoring system devised by DAKO. HER-2 cell membrane specific immunoreactivity were scored by estimating the percentage of positive tumor cell as follows: score 0, no immunoreactive cells; score +1, positivity in <5% cancer cells; score +2, positivity in 5%–50% cancer cells; and score +3, positivity in >50% of cancer cells. The specimens were considered HER-2 positive when the score was ≥2+.

Immunohistochemical reaction of Bcl-2 and Bax was scored as follows: negative if ≤10% of cells were stained, and positive if ≥10% were stained. Cytoplasmatic staining intensity was scored using a scale of 0 to 3 (0, no staining; 1, weak; 2, moderate; and 3, intense). Both markers were placed in one of the two categories, altered or normal. Bcl-2 and Bax immunoreactivity was considered altered when samples demonstrated positivity in >10% of tumor cells with an intensity of 2 or 3.

Statistical Analysis

For purposes of analysis, pathological tumor stage (low vs. high), grade (low vs. high), growth pattern (papillary vs. solid), LVI (yes vs. no), and clinical parameters [gender (M vs. F), localization (pelvis vs. ureter), side (left vs. right)] were evaluated as dichotomized variables. The Fisher's exact test were used to evaluate the association of morphological parameters (stage, grade, growth, lympho-vascular invasion), and immunohistochemical expression (p16, HER-2, E-cadherin, Bcl-2, Bax) with metaplastic squamous characteristic of UUC. Other molecular markers (p53, Cyclin D1, Ki-67) are expressed as means ± standard deviation, and statistical significance between these groups was estimated according to the Student's *t*-test for unpaired samples. Influence of molecular markers and morphological parameters to squamous differentiation in UUC was estimated by logistic regression analysis.

The result was considered statistically significant if $p<0.05$. All analyses were performed with the SPSS statistical package (SPSS version 10.0 for Windows).

Results

Clinical and Pathological Features

There were 29 males (66%) and 15 females (34%), with relation M:F = 2:1. The median age was 63.5 years (range, 22–87 years). Tumor localization was more frequent on the left side [29 (66%) vs. 15 (34%)]. Among the 44 patients with UUC, 35 (80%) had solitary tumor, while 9 (20%) patients had multifocal tumors at the time of presentation. Squamous metaplastic change in UUC was present in 9 (20%) patients. There was not difference between SUUC and CUUC in age (64.3:63 years), gender (males/females=5/4 versus 24/11), location (pelvis/ureter=7/2 versus 30/5) and side (left/right=8/1 versus 21/14). SUUC presented as high stage tumors (9/0 versus 20/15, $\chi^2=5.85$, $p<0.05$), with solid growth (solid/papillary=8/1 versus 14/21, $\chi^2=6.84$, $p<0.01$), lymphovasular invasion (yes/no=6/3 versus 6/29, $\chi^2=8.85$, $p<0.005$), and high grade (high/low=6/3 versus 11/24, $\chi^2=3.75$, $p<0.05$) in comparison to CUUC.

Evaluation of Immunohistochemical Staining

Expression of molecular markers in relation to squamous metaplastic change in UUC is summered in Table 1. SUUC have higher the mean Ki-67 and p53 labeling index than CUUC [(17.49 versus 8.69, $p<0.05$) and (17.99 versus 4.02, $p<0.05$)]. There was not any difference in the mean cyclin D1 index between squamous metaplastic and nonmetaplastic UUC, as well as in expression of p16, HER-2 and apoptotic markers—Bcl-2 and Bax between investigated groups of UUC.

Univariant model of analysis showed influence of phenotypic characteristics and expression of molecular markers, p53, p16, cyclin D1, E-cadherin, HER-2, Ki-67, Bcl-2, and Bax, in divergent differentiation of UUC. SUUC was significantly connected with lymphovascular invasion (Wald 7.34, $p=0.007$), proliferative Ki-67 activity (Wald 5.80, $p=0.016$) and growth (Wald 4.96, $p=0.026$). Other

Table 1 Expression of molecular markers in relation to squamous metaplastic change in UUC

Molecular markers	Conventional UUC *N*=35	Squamous UUC *N*=9	p
Ki-67 index	8.69±7.93[a]	17.49±9.94	<0.05
Cyclin -D1 index	11.80±11.67	12.36±12.17	N.S.
p53 index	4.02±5.77	17.99±31.66	<0.05
p16			
Normal	16	3	N.S.
Altered	19	6	
HER-2 score			
Normal	14	5	N.S.
High	21	4	
E-cadherin			
Membranous	31	6	N.S.
Cytoplasmatic	4	3	
Bcl-2			
Normal	31	9	N.S.
Altered	4	0	
Bax			
Normal	18	3	N.S.
Altered	17	6	

[a] Values are means±SD

parameters (molecular and morphological) in univariant analysis were not associated with divergent differentiation in UUC. Multivariate logistic regression model, that included parameters marked as significant by univariant analysis, has shown statistical significance (chi-square=14.647 p=0.005), but reveled no significant influence on these variables.

Discussion

Clinical significance of squamous differentiation in urothelial tumors remains unsettled with evidence suggesting that it might be an indicator of poor response after radical surgery, radiation or systemic chemotherapy [5, 15]. Most reports to date have concentrated on bladder urothelial tumors showing squamous differentiation, but evidence concerning renal pelvis tumors is lacking [16]. Our results showed that 20% of UUC was with squamous differentiation and is usually found in high grade tumors, with deeply invasive behavior, with solid growth and lymphovascular invasion. Similar results were obtained in earlier studies by Lopez-Beltran et al. [17] who also found a significant association between squamous differentiation and tumor grade and stage. The same authors did not found association between squamous differentiation and survival status in both renal pelvis and bladder cancer suggesting that its effect on survival is probably related to the association with higher grade and stage [18].

The use of immunohistochemistry in assessing squamous differentiation in UUC is helpful in detecting some change at the molecular level. No previous study explored the profiling of molecular markers in SUUC. Current study is the first which describe important change in p53 and Ki-67 index with squamous differentiation of UUC. The mean expression rate of p53 and Ki-67 was higher in SUUC than in CUUC. This finding can be connected with phenotypic characteristics of that tumor. It is well known that the majority of aggressive and invasive UUC have alterations in the tumor suppressor genes products p53. P53 plays a vital role in the regulation of cell cycle. The defective p53 in human cancer leads to the loss of p53-dependent apoptosis, proliferative advantage, genomic instability and DNA repair and angiogenic control loss [4, 19, 20]. Also proliferative marker Ki-67 has a prognostic value in urothelial neoplasms of the urinary bladder [21], pelvis and ureter [22], being associated with tumor grade, stage, recurrence and prognosis of urothelial carcinoma [19, 20, 23, 24], as well as with LVI, and metastases to lymph nodes [19, 24]. Its high expression is related to a poor survival [19, 22, 25, 26]. Also, the combination of p27 and Ki-67 might identify a subgroup of patients that should benefit from a more intensive course of therapy [22].

The present study did not found aberrations in other G1/S regulatory proteins, such as cyclin D1 or p16; oncogene HER-2, apoptotic markers—Bcl-2 and Bax, as well as expression of E-cadherin between SUUC and CUUC. By univariate analysis, which included investigated molecular markers and morphological parameters, we detected significant influence of lymphovascular invasion and growth, as well as Ki-67 activity with squamous differentiation in UUC. These finding suggest that SUUC and CUUC have similar molecular profile supporting by immunohistochemistry. The sample size limited our ability to detect other differences in the expression of these molecular markers.

Molecular genetic evidence has emerged recently supporting a close relationship between urothelial carcinoma and various divergent elements [1]. Investigation of histogenesis of sarcomatoid urothelial carcinoma of the urinary bladder support a monoclonal cell origin and suggest that clonal divergence may occur during tumor progression and differentiation [3, 4].

Some authors suggested in urothelial carcinoma with divergent differentiation the use of more sophisticated cDNA expression arrays as well as the use of proteomic and functional genomic tools. For any of the target-based therapies to be successful, accurate diagnosis that pinpoints the molecular defects in individual patients will be crucial for achieving an optimal response [27].

In conclusion, morphological and molecular study of squamous differentiation in UUC showed change in phenotypic characteristics and Ki-67 activity, as well as similar molecular profile with CUUC.

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Conflict of interest Authors declare none.

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