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Original Scientific Paper

URINARY BLADDER CANCER RECURRENCE AND EXPRESSION OF LYNCH AND HER MARKERS: SEARCHING FOR IMMUNOHISTOCHEMICAL PATTERNS AMONG 113 TUMORS FROM 33 PATIENTS

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SUMMARY – The aim was to identify immunohistochemical (IHC) markers able to predict recurrence of urinary bladder tumors. The method of multivariate adaptive regression splines (MARS) was applied to IHC data of 33 patients with urinary bladder cancer that relapsed one to six times (24 male and nine female, age 57-87 years). The MARS analysis was used to predict the total number of recurrences and the Ki-67 value by nine IHC markers (epidermal growth factor receptor (EGFR), HER2, HER3, E-cadherin, Ki-67, MLH1, MSH2, MSH6 and PMS2). Data were divided as initial tumors, first and subsequent recurrences, and tumors that relapsed within nine months of previous surgery or later. The IHC markers were semiquantitatively classified into four groups, as follows: 0 means no positive cells; 1, 10% of positive cells; 2, 11%-30% of positive cells; and 3, 31%-100% of positive cells. In predicting the overall number of recurrences, as a surrogate marker of tumor biology, the R^2 value for all tumors was 0.423, for initial tumors 0.686, for first recurrence 0.700, and for subsequent recurrences only 0.233. The key predictors for initial tumors were HER2 and MSH2, while for the first recurrence it was EGFR. For quick recurrences (within nine months), the R^2 was 0.474 with EGFR and HER3 as predictors, while for slow recurrences R^2 was 0.640 due to EGFR and PMS2. In predicting the Ki-67 value of that tumor, the R^2 value for all tumors was 0.300, for initial tumors 0.262, for first recurrence 0.360, and for subsequent recurrences only 0.533. The key predictors for first recurrences were EGFR and MSH6, and for subsequent recurrences HER2, EGFR and all Lynch markers. The R^2 was 0.266 for quick recurrences and 0.370 for slow recurrences. The finding of E-cadherin was not found relevant by any of these MARS models. In conclusion, the MARS results associated multiple IHC markers with the number of recurrences and with Ki-67 values. It is important that differences in predictive markers were found between initial tumors and first recurrences, and between quick and slow recurrences, thus suggesting that tumor biology is different among these subgroups regarding the total number of recurrences and Ki-67 values.

Key words: *Urinary bladder carcinoma; Immunohistochemistry; HER2; EGFR; Lynch markers; Ki-67; Tumor biology*

Introduction

Both aggressiveness and a relapse tendency of transitional cell urinary bladder cancer remain not well understood[1,2]. Despite a high incidence of this common cancer, we still lack predictive factors that

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might help recognize patients at an increased risk of recurrence.

Based on a recent review[3], that individuals with Lynch syndrome are also prone to urinary tract malignancies, this paper presents our attempt to evaluate the predictive power of selected immunohistochemical (IHC) markers in recurrent urinary bladder tumors. Besides testing for Ki-67 value and presence of E-cadherin, Lynch and HER markers were semiquantitatively measured. The idea was to detect immunophenotypic patterns that might predict whether the occurrence of each subsequent transitional cell urinary bladder carcinoma is related to the progression of a previously diagnosed primary tumor or it is an independent event.

Material and Methods

This retrospective study was conducted at departments of the Osijek University Hospital Center. The study included archival tissue specimens of patients more than 18 years of age with at least two histopathologic findings of transitional cell urinary bladder carcinoma (primary tumor and recurrence). The exclusion criterion was the presence of another, non-urinary cancer.

In hospital archives, 33 patients aged 57- 87 years were identified. They had a total of 113 transitional cell urinary bladder cancers. The total number of cancers *per* patient ranged from two (one relapse) to six (five relapses). The following data were collected under a code: gender, patient age at the time of first surgical procedure, and histochemical characteristics of all extirpated tumors with dates of procedures. The personal identity information was protected by coding.

The already archived paraffin cubes of removed tumors were used for supplementary IHC staining to define carcinoma phenotype of each extracted tumor. The positivity of the findings of an individual IHC indicator was evaluated quantitatively or semiquantitatively by two independent pathologists.

The material for histopathologic analysis was collected by a routine and standard histopathologic method of tumor fixation in 10% buffered formalin, fitting the tumor into paraffin blocks, cutting the preparation into 3-5 micrometers, and staining with hemalaun-eosin dye. The preparations were examined by light microscope. All tumors were submitted to IHC analysis using additional incisions of tumor tissue. They were processed in an automated Ventana BenchMark ULTRA IHC staining system using a basic AEC result visualization system. Ventana basic AEC system (Basic AEC detection kit) is an indirect biotin streptavidin system consisting of mouse IgG, mouse IgM, and rabbit polyclonal primary antibodies. The interpretation of staining was always associated with the evaluation of appropriate positive controls. The following primary antibodies were used in IHC analysis (manufacturer/name/clone/catalog number):

- CONFIRM anti-EGFR (3C6) cat. no. 05278341001
- PATHWAY anti- HER2 / neu (45B) cat. no. 05995570001
- DAKO anti-HER3 (DAKH3-IC) cat. no. M7927
- VENTANA anti-E-cadherin (36) cat. no. 05905290001
- CONFIRM anti-Ki-67 (30-9) cat. no. 05278384001
- VENTANA anti-MLH1 M1 cat. no. 06472966001
- VENTANA anti-MSH2 (G219-1129), Ventana cat. no. 05269270001

Treatment of deparaffinized tissue sections up to 5-micron thick was performed during an automated procedure in a Ventana BenchMark Ultra. Optimal results were obtained using pH 9 buffer solutions. Owing to good adhesion of tissue incisions to the slides, silanized slides of Daco (Silanized Slides Code S3003) were used. The preparations were coated with a solution of Neo-Mount (Merck mounting medium for microscopy cat. no. 109106) and a glass slide to achieve durability of the preparations themselves. IHC staining results were expressed by the semiquantitative method as follows:

negative reaction (-): no staining in tumor cells;
poorly positive reaction (+): less than 10% of tumor cells positive;
moderately positive reaction (++): 10%-50% of tumor cells positive; and
strongly positive reaction (+++): more than 50% of tumor cells positive.

Moderately positive (++) and very positive (+++) reactions were considered positive, while negative (0) and weakly positive (+) reactions were considered negative.

Ki-67 was expressed by actual percentage (determined at three sites on tumor tissue *per* 500 adjacent cells in the area of so-called 'hot spot' or the site of the most pronounced staining).

Data were analyzed by non-parametric statistical methods of testing differences and relatedness. The multivariate adaptive regression splines (MARS) method[4] was used to evaluate the predictive value of individual indicators in explaining the biologic characteristics of recurrent bladder tumors. In all statistical tests, the level of significance was set at $p<0.05$. STATISTICA version 10 (StatSoft, Inc., 2011; www.statsoft.com) software package was used on statistical processing.

The plan of the study and research methods, total number, selection, inclusion criteria, information and consent of the respondents were in accordance with ethical and scientific standards. Predictable risks and dangers in relation to the presumed scientific benefit were designed with the least possible exposure to health risks and risks of researchers, research associates and general population in accordance with inaugurated basic principles and human rights in biomedical research in the field of medicine and health, including standards of use and the process of human biologic material in scientific and professional biomedical research. The study was approved by the Ethics Committee of the Osijek University Hospital Center (no. 25-1:831-6/2015) and Ethics Committee of the Faculty of Medicine, Josip Juraj Strossmayer University in Osijek (no. 2158-61-07-18-123).

Results

Table 1 shows age distribution of our 33 patients (24 male and nine female) aged 57-87 years and number of recurrent tumors (one to six recurrences). Table 2 shows expression data of E-cadherin, 4 Lynch and three HER markers. In both tables, there were no significant differences.

Before applying the MARS model, data were stratified by two criteria, as follows: initial tumors (33 tumors) *versus* first (33 tumors) and subsequent recurrences (47 tumors); 80 relapsing tumors divided in two groups: 55 tumors that quickly relapsed (within nine months from the previous surgery) and 25 tumors that relapsed slowly (more than nine months). The finding of E-cadherin was not found relevant by any of the MARS models calculated.

Table 3 shows results of the MARS models in predicting the overall number of recurrences, used here as a surrogate marker of tumor biology. The R^2 value was 0.423 for all tumors, 0.686 for initial tumors, 0.700 for first recurrence, and only 0.233 for subsequent recurrences. The key predictors for initial tumors were HER2 and MSH2, and EGFR for first recurrence. For quick recurrences (within nine months), the R^2 was 0.474 with EGFR and HER3 as predictors, while for slow recurrences R^2 was 0.640 due to EGFR and PMS2.

These results suggest that the biology of the first recurrence in our patients was predictable in 70% by HER2 and MSH2, in contrast to only 23% predictability in subsequent recurrences, suggesting that these subgroups contained tumors of different biology. A tempting possibility that would require a larger number of patients is that first recurrences are real recurrences of the initial tumors, whereas some of subsequent recurrences might come from independent neoplastic foci that have developed in precancerous bladder mucosa. Possibly pointing in the same direction are differences between quick and

Table 1. Age distribution of patients according to total number of bladder cancer recurrences

Number of patients according to total number of bladder cancer recurrences	Patient age at initial diagnosis (years)			Total
	<61	61-74	>74	
One	1	7	5	13
Two	2	1	0	3
Three	2	6	3	11
Four	1	2	0	3
Five	0	0	2	2
Six	1	0	0	1
Total patients	7	16	10	33

Table 2. Distribution of immunohistochemical markers tested: E-cadherin, HER family (EGFR, HER2, HER3) and Lynch family (MLH1, MSH2, MSH6, PMS2)

Bladder cancers according to marker expression	Immunohistochemical cancer marker			
	EGFR	HER2	HER3	E-cadherin
0 (absent)	23	36	56	2
1+	29	40	37	111
2+	28	19	13	0
3+	33	18	7	0
Total bladder cancers	113	113	113	113
Marker expression	MLH1	MSH2	MSH6	PMS2
0 (absent)	4	7	9	4
1+	1	4	6	6
2+	18	23	41	36
3+	90	79	57	67
Total bladder cancers	113	113	113	113

Immunohistochemical markers were semiquantitatively classified into four groups: 0, no positive cells; 1, 10% of positive cells; 2, 11%-30% of positive cells; and 3, 31%-100% of positive cells.

Table 3. Results of the MARS model in predicting the overall number of recurrences as a surrogate marker of cancer biology

Predictor importance in the MARS model for the overall number of recurrences		All bladder cancers included	Cancers in order of appearance			Recurrence type	
			Initial cancer	First recurrence	Subsequent recurrence	Quick (≤9 months)	Slow (>9 months)
Semiquantitative expression of IHC markers (absent to 3+)	EGFR	2	1	2	1	2	2
	HER2	1	2	0	0	0	0
	HER3	0	0	0	0	2	0
	MLH1	0	1	0	0	0	0
	MSH2	2	2	1	0	0	0
	MSH6	0	1	0	1	0	1
	PMS2	3	0	1	1	2	3
	E-CAD	0	0	0	0	0	0
R^2 (index of determination)		0.423	0.686	0.700	0.233	0.474	0.640

Table shows index of determination (R^2) achieved by the model and importance of the immunohistochemical (IHC) markers used in the model

slow recurrences; again, tumor biology was predictable in quick recurrences by EGFR and HER3, whereas EGFR and PMS2 were important in slow recurrences.

Table 4 shows results of the MARS models in predicting the Ki-67 value of that tumor, here used as a marker of the tumor mitotic activity. The R^2 value was 0.300 for all tumors, 0.262 for initial tumors, 0.360 for first recurrence, and 0.533 for subsequent recurrences. The key predictors for first recurrences were EGFR and MSH6, and for subsequent recurrences HER2, EGFR and all Lynch markers. The R^2 was 0.266 for quick recurrences and 0.370 for slow recurrences. These results suggest that Ki-67 values were less predictable by the tested markers than the overall number of recurrences, although here again subsequent recurrences showed different patterns from first recurrences, thus supporting differences in their biology.

Table 4. Results of the MARS model in predicting recurrent tumor Ki-67 value as a surrogate marker of its aggressiveness and biology

Predictor importance in the MARS model for bladder cancer Ki-67 value		All bladder cancers included	Cancers in order of appearance			Recurrence type	
			Initial cancer	First recurrence	Subsequent recurrence	Quick (≤9 months)	Slow (>9 months)
Semiquantitative expression of IHC markers (absent to 3+)	EGFR	1	0	2	1	1	0
	HER2	0	2	0	3	0	0
	HER3	0	0	0	0	0	0
	MLH1	1	0	0	2	0	1
	MSH2	2	1	0	1	0	0
	MSH6	4	1	2	1	1	2
	PMS2	0	0	0	1	1	1
	E-CAD	0	0	0	0	0	0
R^2 (index of determination)		0.300	0.262	0.360	0.533	0.266	0.370

Table shows index of determination (R^2) achieved by the model and the importance of immunohistochemical (IHC) markers used in the model

It can be concluded that MARS models associated multiple IHC markers with the number of recurrences and with Ki-67 values. It is important that differences in predictive markers were found between the initial tumors and first recurrences, and between quick and slow recurrences, thus suggesting that tumor biology is different among these subgroups regarding the total number of recurrences and Ki-67 values.

Discussion

In the study by Kurth *et al.*, 576 patients were analyzed and it was shown that the tumor recurred in 54% of patients, and in 17% the recurrence was visible already at the first cystoscopy after six weeks. The median time to first relapses observed from six weeks after the first surgery to five years of follow-up was 1.8 years. The analysis showed that the factors associated with the first recurrence were the degree, size and number of tumors[5].

The study presented here was based on the question whether the occurrence of each subsequent relapse is related to the initial tumor removed or is an independent event. Therefore, an effort was made to identify the phenotypic features of the initial and recurrent urinary bladder tumors in four subgroups of patients associated with an increased risk of disease recurrence and to analyze differences in the phenotype of early and late relapses. Our results obtained from the analysis of IHC profiles of urinary bladder tumors showed that there was a significant difference in the predictive value of individual IHC indicators of primary tumors, first relapses, subsequent relapses, or of relatively rapid relapses compared to later relapses. The markers applied were selected based on the previous studies describing their role in tumor recurrence and progression.

The Ki-67 value

Ki-67 is a known predictive factor for cancer cell growth. Its expression is present in various tumors and is associated with poorer prognoses. The role of Ki-67 as a prognostic factor for urinary bladder cancer is not completely clear. In a meta-analysis of 52 tumor tissue samples, Tian *et al.* showed that increased Ki-67 expression was associated with increased recurrence rates for Caucasians only[6]. Makboul *et al.* demonstrated association of Ki-67 with progression but not with recurrence ability of urinary bladder cancer[7]. A study by Acikalin *et al.* in 68 patients with invasive carcinoma who underwent transurethral resection, Ki-67 expression was not statistically significantly associated with recurrence, progression and mortality[8]. In several large studies with a total of 5147 urinary bladder cancer patients, Ki-67 expression was found to be significantly associated with a shorter period to disease recurrence, progression, and shorter overall and cancer-specific survival[9].

E-cadherin

E-cadherin is the most important intercellular molecule that stabilizes epithelial cells; it is known that alteration of its expression can lead to a disrupted relationship between cells and development of cancer. Several multicenter studies involving 2089 patients with urinary bladder cancer showed that decreased expression of E-cadherin significantly predicted poorer overall survival[10]. In contrast, some studies showed that there was no association between alteration of E-cadherin expression and prognosis[11]. During the course of this study, the results obtained showed that of all the IHC indicators tested, only E-cadherin undoubtedly had no significant role in the relapse process, which was in accordance with the well-known study by Liang *et al.*, which described association of the loss of E-cadherin expression with invasion and metastasis but not relapse[12]. In the study by Dalesio *et al.*, it was observed that in noninvasive bladder cancer muscle, prognostic factors could play a more important role in development of the disease after transurethral resection than the choice of adjuvant chemotherapy or immunotherapy. Of particular importance was the finding that patients with less than one relapse a year had a prognosis similar to those with a primary tumor only, whereas patients with multiple relapses had poor prognosis. These results indicated that patients with noninvasive carcinoma form a heterogeneous group and that more aggressive therapy could be considered in a subset of more frequent recurrences[13].

Markers of the Lynch family

Changes in Lynch gene expression led to the emergence of microsatellite cell instability. Thibodeau *et al.* report that mutations of MSH2 and MLH1 proteins were found in 19 out of 29 cases of hereditary non-polyposis colorectal cancer, meaning that they were significant predictors of relapse[14]. A study conducted on 95 families showed that carriers of the MLH1, MSH2 and MSH6 mutants and their first degree relatives had an increased tendency to recur and grow urinary bladder cancer. Two patients had the MLH1, 15 MSH2 and 4 MSH6 mutation. Carriers of MSH2 mutations showed the highest risk as compared with the total population where the cumulative risk was 7% for men and 5.8% for women[15]. Garcia-Tello *et al.* found that inactivation of the *PMS2* and *MLH1* genes was an independent marker of good prognosis and, together with the histologic grade, could define the prognosis of the disease[16].

Markers of the HER family

Given the predictive value of HER2, there are studies with very conflicting results. Several studies have argued that HER2 overexpression is a significant predictor of relapse, i.e., disease-free survival (DFS) and progression-free survival (PFS)[17]. Other studies have shown very little or no prognostic value of HER2 expression in the development of urinary bladder cancer[18-21]. In the study by Cormio *et al.*, 67 patients were monitored for high-grade invasive urinary bladder carcinoma, half of whom underwent transurethral bladder resection only, and half had Bacillus-Calmette-Guerin (BCG) treatment installed. The median follow-up of these patients was 75.7 months. DFS and PFS were 35.8% and 73.0%, respectively. Kaplan-Meier survival analysis showed that the traditional prognostic factors such as gender, tumor size, and relapse rate did not predict DFS and PFS. BCG treatment proved to be a significant predictor of DFS but not PFS. HER2 overexpression was shown to be a significant predictor of DFS and PFS[22]. These differences in data could be explained by differences in patient populations and HER2 analysis techniques, but additional data from prospective studies using standardized HER2 expression analysis techniques are certainly needed.

In addition to the known facts proven in numerous studies on the association between HER2 overexpression and amplification, Sanguedolce *et al.* have shown that mismatch repair (MMR) mutations of the *MLH1* and *MSH2* genes could increase the negative predictive value of HER2 expression[23].

In order to determine the prognostic role of the HER family of genes, the factors predicting first recurrence were shown to be multiple tumors, HER2 and HER3, while tumors showing expression of EGFR, HER2 and HER3, or HER2 alone had a significantly higher risk of developing second recurrence. The factors associated with shorter survival were tumor stage, HER2, EGFR-HER2, and HER2-HER3[24].

Lindgren *et al.* analyzed the expression profile and mutations for molecular categorization of a group of 75 urinary bladder cancers. They confirmed the major role of HER3 in the development of noninvasive low-grade urinary bladder cancer[25].

The least data are available on HER3 and HER4 expression. Memon *et al*. in a study of 88 patients at a median follow-up of 23 months found that increased HER3 and HER4 expression was associated with better prognosis. Joint expression of both factors showed better survival than single expression[26].

Conclusions

Our results showed that the total number of relapses and the speed of the relapse process were important biologic potential that depended on the individual patient. By analyzing the total number of tumor recurrences in an individual patient, based on IHC indicators, our results showed that tumors that had only one recurrence were biologically different from tumors that had more than one recurrence, and most of the patients were aged 61 to 74 years (49%) at the time of the first tumor removal. The MARS model best predicted Ki-67 for second and subsequent relapses, with 53% of Ki-67 values explained by the MARS model, and EGFR, HER2, and Lynch markers were included while MLH1 was found to be most important.

Recurrent tumors represented relapses of the underlying disease, and the features of early relapses in altering the IHC profile during the recurrence process could have contained information relevant for predicting the further course of the disease.

Due to the complex mechanisms of urinary bladder carcinogenesis and a limited number of patients, it was difficult to define clinically relevant predictors of relapse, but our results support the notion that such predictors exist in the cancer phenotype of this tumor. A larger study might define relevant predictors and also the existence of other differences between different patient groups. This would support the claim that urinary bladder cancer is a heterogeneous disease with several tumor types.

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Sažetak

RECIDIV KARCINOMA MOKRAĆNOG MJEHURA I EKSPRESIJA BILJEGA LYNCH I HER: ISTRAŽIVANJE IMUNOHISTOKEMIJSKIH OBRAZACA U 113 TUMORA KOD 33 BOLESNIKA

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Cilj rada bio je identificirati imunohistokemijske (IHC) biljege koji mogu predvidjeti recidiv karcinoma mokraćnog mjehura. Metoda multivarijatnih adaptivnih regresijskih splinova (MARS) primijenjena je u IHC podatcima 33 bolesnika s karcinomom mokraćnog mjehura koji se povratio jedan do šest puta (24 muškarca i 9 žena, dob 57-87). Analiza MARS primijenjena je za predviđanje ukupnog broja recidiva i vrijednosti Ki-67 prema devet IHC biljega: receptor epidermalnog faktora rasta (EGFR), HER2, HER3, E-kadherin, Ki-67, MLH1, MSH2, MSH6 i PMS2. Podatci su podijeljeni na početne tumore, prve i naknadne recidive i tumore koji su se vratili unutar devet mjeseci od prethodne operacije ili kasnije. IHC biljezi semikvantitativno su klasificirani u četiri skupine kako slijedi: 0 znači da nema pozitivnih stanica; 1, 10% pozitivnih stanica; 2, 11%-30% pozitivnih stanica; i 3, 31%-100% pozitivnih stanica. U predviđanju ukupnog broja recidiva kao surogat biljega biologije tumora je vrijednost R^2 za sve tumore bila 0,423, za početne tumore 0,686, za prvi recidiv 0,700, a za sljedeće recidive samo 0,233. Ključni prediktori za početne tumore bili su HER2 i MSH2, dok je za prvi recidiv bio EGFR. Za brze recidive (u roku od devet mjeseci) R^2 je bio 0,474 s EGFR i HER3 kao prediktorima, dok je za spore recidive R^2 bio 0,640 zbog EGFR i PMS2. U predviđanju Ki-67 vrijednosti tog tumora vrijednost R^2 za sve tumore bila je 0,300, za početne tumore 0,262, za prvi recidiv 0,360, a za sljedeće samo 0,533. Ključni prediktori za prve recidive bili su EGFR i MSH6, a za sljedeće HER2, EGFR i svi biljezi Lynch. R^2 je bio 0,266 za brze recidive i 0,370 za spore recidive. Nijedan od ovih modela MARS nije smatrao relevantnim otkriće E-kadherina. Zaključno, rezultati MARS-a povezuju više IHC biljega s brojem recidiva i s vrijednostima Ki-67. Važno je da su pronađene razlike u prediktivnim biljezima između početnih tumora i prvih recidiva te između brzih i sporih recidiva, što ukazuje na to da je biologija tumora različita među ovim podskupinama u pogledu ukupnog broja recidiva i vrijednosti Ki-67.

Ključne riječi: *Karcinom mokraćnog mjehura; Imunohistokemija; HER2; EGFR; Biljezi Lynch; Ki-67; Tumorska biologija*