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## EXPRESSION OF MARKERS FOR TRANSITIONAL CELL CARCINOMA IN NORMAL BLADDER MUCOSA OF PATIENTS WITH BLADDER CANCER

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### ABSTRACT

**Purpose:** Because we have found that random mucosal biopsies have no additional prognostic value to conventional histopathology, we studied biopsies of histologically normal bladder mucosa with several molecular markers believed to be associated with the development of transitional cell carcinoma.

**Materials and Methods:** Six groups of patients with an increasing stage of bladder tumor were selected: (1) benign disease (for example, benign prostatic hyperplasia,  $n = 8$ ); (2-4) low ( $n = 10$ ), intermediate ( $n = 9$ ) and high risk ( $n = 7$ ) superficial tumors; (5) progressive superficial tumors resistant to optimal conservative therapy ( $n = 6$ ); (6) invasive or disseminated tumors at presentation ( $n = 5$ ). We studied the expression of cytokeratin (used as an epithelial marker), fibronectin, E-cadherin (HECD-1), I-CAM, human leukocyte antigen (HLA)-I, HLA-II and epidermal growth factor receptor (EGF-R) in cold-cup biopsies of normal mucosa.

**Results:** Fibronectin, HECD-1, I-CAM and HLA-II expression showed no significant changes in the different groups. There was a significant increase in the expression of HLA-I ( $p = .003$ ) and EGF-R ( $p = .0001$ ) with a higher stage of the tumor.

**Conclusion:** An increasing EGF-R expression in normal looking mucosa of patients with increasing stages of bladder tumors could be a prognostic factor or might indicate that this increase in expression is not tumor specific but is seen in the whole bladder.

**KEY WORDS:** bladder; carcinoma, transitional cell; biopsy; tumor markers, biological

Of the patients presenting with transitional cell carcinoma (TCC) of the urinary bladder, approximately 65% have superficial tumors ( $\leq pT1$ ). The remaining patients present with invasive ( $\geq pT2$ ) or metastatic (N+, M+) disease.<sup>1</sup> Superficial bladder cancer is characterized by inconsistent biological behavior. The risk of tumor recurrence and progression can vary. Of all superficial bladder cancer patients a small group will only have 1 episode of superficial tumor. After transurethral resection (TUR) they will be without recurrences with or without adjuvant treatment ("low risk tumors"). Another small group of patients will present with "high risk" tumors from the start. These patients are at risk for progression to invasive disease, despite maximal adjuvant treatment. The majority of patients, however, will have regularly recurrent bladder tumors without obvious signs of progression ("intermediate risk"). To differentiate between these risk groups prognostic factors are used, such as tumor grade, stage, size and multiplicity, recurrence rate, and possibly the result of random mucosal biopsies. As we have shown, these selected mucosal biopsies are of no additional value to conventional histopathology in a multivariate analysis.<sup>2</sup> However, the high recurrence rate and multiplicity in superficial bladder cancer suggest that the entire mucosa is primed to become malignant (field defect theory). Therefore, we looked at molecular changes in mucosal biopsies, using several markers associated with development of TCC. We analyzed normal-appearing mucosa in 6 groups of patients with an increasing stage and grade of bladder tumors. The expression of these markers in normal mucosa is believed to be a potential indicator of a mucosal field defect, and might

be an additional prognostic factor in the work-up of patients with bladder cancer.

### MATERIALS AND METHODS

In all, 6 groups of patients were identified. For each group 10 patients were selected (table 1). In every patient urethroscopy was done, and cold-cup mucosal biopsies were taken from normal-appearing mucosa. At least 2 biopsies were taken from the left and right lateral bladder wall. In case of a tumor on one of the lateral walls, the biopsy was taken 2 cm. away from the tumor. Biopsies were stored in liquid nitrogen. Serial frozen sections were obtained from the biopsy material. One section from each series for the immunohistochemical analysis was stained with hematoxylin and eosin to determine the histopathological diagnosis. In a pilot study we tested the feasibility of this technique, and the results showed that biopsies stored in this manner were adequate for these investigations. There were no artifacts due to storage in liquid nitrogen and there was little mechanical damage.

We studied the expression of cytokeratin (CK18-2), fibronectin, E-cadherin (HECD-1), intercellular adhesion molecule (I-CAM), human leukocyte antigen (HLA) class I, HLA class II and the epidermal growth factor receptor (EGF-R). The antigen G250, which is renal cell carcinoma specific with only minor expression on the major bile ducts, was used as a negative control. The monoclonal antibodies (MAbs) used for this testing are listed in table 2. Expression of the MAbs was tested by means of direct immunohistochemical staining for MAbs for HLA class I and II<sup>3</sup> and indirect methods for the other MAbs. The indirect immunoperoxidase technique used in this study was performed at room temperature. The frozen sections were fixed with acetone for 10 minutes and air dried. Preincubation was done with 10% normal rabbit serum in

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TABLE 1. Risk groups of patients studied: tumor stage, grade and primary versus recurrent at time of biopsy indicated

Group	Risk	N	Description with pathology at the time of biopsy
1	No risk	8	Patients with benign disease, e.g. prostatic hyperplasia
2	Low risk superficial	10	Patients with primary solitary pTa grade I (7) or IIa (3) tumors, remaining >1 year disease free after TUR without adjuvant treatment
3	Intermediate risk superficial	9	Patients with pTa (5) or pT1 (4) tumors, grade II (7) or III (2), recurrent (5) or primary (4), remaining >1 year disease free with adjuvant intravesical instillations
4	High risk superficial	7	Patients with pTa (4) or pT1 (3), grade II (6) or III (1), primary (2) or recurrent (5) tumors, with a superficial recurrence within 1 year in spite of additional therapy
5	Increasing risk	6	Patients with pT1 (3) or in situ (3) tumors, grade II (1) or III (5), primary (2) or recurrent (4), having progression to muscle invasion or metastasis in spite of additional therapy
6	High risk	5	Invasive (2×pT2, 1×pT3 and 1×pT4) or disseminated disease (1×M+) at primary presentation

phosphate-buffered saline (PBS) for 30 minutes and subsequently with primary antibody for 1 hour. The primary antibody concentration was typically determined by using serial dilutions on a positive control. The optimal concentration was chosen as the lowest with which a clear positive signal was obtained at a minimal background. The optimal dilution is also indicated in table 2. After rinsing with PBS samples were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts A/S, Glostrup, Denmark) diluted 1:100 in PBS containing 1% bovine serum albumin (BSA) for 30 minutes, and rinsed again with PBS before detection of the peroxidase activity with 3,3'-diaminobenzidine 0.6 mg./ml. (Sigma Chemical Co., St. Louis, Missouri) in 0.65% imidazol/PBS containing 25  $\mu$ l. of 30% H<sub>2</sub>O<sub>2</sub>. Following brief rinsing with water, the sections were incubated with 0.5% CuSO<sub>4</sub> (in 0.9% NaCl) for 5 minutes to intensify the reactions. After rinsing with water, the sections were counterstained with hematoxylin, dehydrated and mounted. Control experiments were done with 1% BSA in PBS instead of primary antibody.

Staining was scored semiquantitatively by 3 different investigators. For each antigen a total of 10 high-power fields was scored. In each high-power field the percentage of clearly positive staining cells was estimated, after determination of the optimal primary antibody concentration. No variations in intensity of staining were considered.

For statistical analysis of the 6 different groups of patients the Kendall test against rank-correlation was used.

## RESULTS

First, conventional histology of the biopsies was studied. Only patients in whom this histology was normal, that is, without mucosal denudation, dysplasia or tumor, were evaluated. The remaining number of patients in each group was 8 in group 1 (benign disease), 10 in group 2 (low risk), 9 in group 3 (intermediate risk), 7 in group 4 (high risk), 6 in group 5 (progressive superficial tumors in spite of optimal conservative therapy) and 5 in group 6 (invasive or disseminated tumors at presentation).

The epithelial structures in the biopsy specimens were analyzed with the various antibodies. The results of the antibody expression, together with the results of the statistical analysis are listed in table 3. G250 expression, used as a control for nonspecific staining, was negative in all cases.

The epithelial phenotype was confirmed by using cytokeratin 18 (CK18-2), an epithelial-specific intermediate filament protein that is known to be expressed in all cell layers of the mucosa. CK18-2 was chosen because the epitope recognized by this antibody is, unlike the one recognized by RGE53, not partially masked in normal urothelium. Although cytokeratin 18 expression was not used as a tumor marker, aberrant expression patterns have to be kept in mind in relation to stage and grade of TCC.<sup>4</sup> In our study no significant changes

in CK18-2 expression were noted ( $p = .04$ ) although expression decreased in the higher groups.

Evidence for gross changes in the extracellular matrix composition could result in changes in fibronectin (FN) staining, which is a component of the extracellular matrix. Disruption of the FN pattern and the subsequent decreased expression are associated with neoplastic changes *in vitro*.<sup>5</sup> We found no significant variation in either the pattern or intensity.

A recently described progression marker is I-CAM class 1, even though its role has not been studied extensively in bladder cancer. There were no changes found in the expression of this protein in the various groups ( $p = .07$ ).

A protein that marks epithelial integrity is E-cadherin. E-cadherin expression is restricted to epithelial tissues<sup>6,7</sup> and is homogeneously expressed in the urothelium at cell-cell contacts.<sup>8</sup> All biopsies in this study expressed E-cadherin homogeneously; hence no indication for microinvasive subpopulations was evident in any of the groups ( $p = .08$ ).

Loss or decrease in the expression of histocompatibility antigens was reported for TCC and may be instrumental in escape from immune surveillance. Surprisingly, we found a significant increase in the expression of HLA I in the various groups ( $p = .003$ ). Changes in expression of HLA II showed no significant differences between groups ( $p = .31$ ).

A well-described progression marker is EGF-R, which is normally expressed in the basal layers of the urothelium. Expression of EGF-R in both superficial and deeper layers of the mucosa, the so called malignant distribution, is associated with TCC.<sup>9</sup> We found this malignant distribution of EGF-R in all patient groups and also in the mucosa of patients without bladder cancer. Statistical analysis showed a significant correlation between an increased EGF-R expression and a higher stage of the primary bladder tumor ( $p = .0001$ ).

## DISCUSSION

Because superficial bladder cancer is characterized by an unpredictable clinical course, prognostic factors are of particular interest. The high recurrence rate and multiplicity led some investigators to formulate a "field defect theory", presuming that carcinogenic factors in urine can affect the whole mucosa. Therefore, selected mucosal biopsies were believed to carry prognostic value. However, the value of histologic examination of mucosal biopsies of normal-appearing mucosa is contradictory and probably very limited.<sup>2</sup> We investigated such biopsies with several markers, commonly used to study the development of TCC, in 6 groups of patients with tumors of increasing clinically malignant behavior. Of some markers, like cytokeratin 18 and fibronectin, little is known about the relation of the expression of these markers to TCC behavior. The relation of I-CAM expression and cancer has only been described recently. In kidney cancer an increased I-

TABLE 2. Monoclonal antibodies and their concentration used for testing of the mucosal biopsies

Antigen	Antibody*	Dilution	Reference
HLA I	W.36.2	1:200	3
HLA II	B8.11.2	1:200	3
E-Cadherin	HECD-1	10 µg./ml.	8
Cytokeratin	CK18-2	1:2	4
Fibronectin	MF1B	1:10	(Euro-Diagnostica, Apeldoorn, The Netherlands)
I-CAM	P3.58BA-14:25		(Dr. J. Johnson, Inst. für Immunologie, Univ. München, Germany)
EGF-R.	RPN 513	1:100	33

\* Staining was done with rabbit-anti-mouse peroxidase, except fibronectin, which was stained with donkey-anti-goat peroxidase.

CAM expression is found compared with normal kidney tissue.<sup>10</sup> Such expression is also found in bladder cancer cell lines, and a relation was found between I-CAM class I expression and susceptibility for killer cells.<sup>11</sup> Moreover, I-CAM expression was stimulated with interferon-gamma (IFN-γ), indicating a possible mode of action of BCG, which increases urinary IFN-γ. However, the relation between I-CAM expression and the prognosis of bladder cancer is unclear. Loss of E-cadherin expression or inhibition of E-cadherin by addition of antibodies is associated with invasive phenotype of several tumors.<sup>12-17</sup> We found low E-cadherin expression to be associated with invasive bladder cancer.<sup>8</sup> Reduced expression of HLA-I has been described in various tumors<sup>18</sup> and has been associated with the ability of tumor cells to escape immunologic surveillance and with malignant potential (invasiveness, metastases, dedifferentiation) of the tumor, Cordon-Cardo et al., for example, found primary nonmetastatic lesions to be HLA-I positive in 8 of 11 patients, in contrast to 33 of 44 metastatic lesions, which were negative.<sup>19</sup> Normal bladder mucosa expresses HLA-I antigens.<sup>20</sup> In bladder cancer, there is a reduced expression of HLA-I antigens with the change from dysplasia to tumor.<sup>21</sup> Levin et al. found a significant correlation of HLA I positive tumors and survival (p < 0.05) and suggested that HLA-I expression is a prognostic indicator.<sup>18</sup> However, the association between HLA antigens and TCC is not always clear, and may not be clinically relevant.<sup>22</sup> Only scant data on the relation between HLA-II expression and bladder cancer have been published and although HLA-II expression was found in bladder cancer and correlated with BCG therapy<sup>23,24</sup> and was induced by cytokines,<sup>25</sup> no correlation has been found between HLA-II expression and tumor aggressiveness.<sup>24,26</sup>

A consistent value of the epidermal growth factor receptor (EGF-R) in TCC has been recently reported.<sup>9,27</sup> Normally, EGF-R is found only on the basal layer of epithelial cells. In bladder tumors the urinary concentration of EGF can be decreased, but EGF-R expression in tumors is higher than in normal urothelium. Messing found increased expression of EGF-R in superficial and deeper layers of the mucosa ("malignant distribution") in low and high grade TCC, as well as

in normal-appearing mucosa of patients with TCC elsewhere in the bladder.<sup>9</sup> This might favor interaction of premalignant and malignant mucosa with urinary EGF. Neal et al. studied 101 patients with newly diagnosed bladder cancer, with a follow-up of 30 months.<sup>27</sup> They found an association between strong positive immunohistochemical staining of the EGF-R (48 tumors) and high stage bladder cancer (p < 0.001). In pTa and pT1 lesions (52 patients) EGF-R positivity was associated with multiplicity, short time to recurrence and high recurrence rate. In a multivariate analysis EGF-R positivity was predictive of bladder cancer related death in all 101 patients (relative risk 3.42, p < 0.001). In 52 patients with superficial TCC, EGF-R positivity was predictive for time to recurrence (relative risk 2.28, p < 0.03) and time to progression (relative risk 22.12, p < 0.001). For human bladder cancer cell lines transplanted into nude mice, a higher EGF-R expression was also found in the invasive cell lines than in the noninvasive lines.<sup>28</sup>

If the theory of a "field defect" is correct, premalignant abnormalities could be present in normal mucosa of bladder cancer patients.

Sidransky et al. examined 13 tumors from cystectomy specimens from 4 women and found that, in each patient, all the tumors shared 2 chromosomal abnormalities (inactivation of the same X chromosome and loss of the same allele on chromosome 9q).<sup>29</sup> Loss of chromosome 17p and 18q was not the same in each patient, but these losses are late events in tumor progression. The authors concluded that these tumors arise from a single transformed cell after variable subsequent genetic alterations. This finding could indicate that, over time, (pre)malignant cells remain present in the bladder and can be detected. Similar findings about clonality of bladder tumors were published by Miyao et al., who found concordance in loss of heterozygosity of chromosome 9 and 17p and p53 mutations between primary tumors and lymph node metastases in 14 patients.<sup>30</sup> Flow cytometric measurement of DNA ploidy of untreated bladder tumors and grossly normal distant bladder mucosa revealed comparable degrees of aneuploidy, also indicating the malignant potential of the normal mucosa.<sup>31</sup> Rao et al. investigated biochemical markers in tumors, adjacent bladder mucosa and random distant bladder mucosa in 30 patients with superficial bladder tumors.<sup>32</sup> Although there was a decreasing degree of changes in most of these markers from the tumor to the distant mucosa, variable degrees of marker changes in morphologically normal mucosa were present. G-actin, for example, was altered in 58% of distant biopsy specimens in 30 patients compared with 0 in 6 normals. Abnormal EGF-R expression was found in ± 40% of the distant mucosal biopsies.

These data suggest that chromosomal and biochemical abnormalities can be present in normal-appearing mucosa. Therefore, investigation of normal-appearing mucosa remains an interesting approach, with possible prognostic consequences. The difference in our approach is that we com-

TABLE 3. Staining of antigens in mucosal biopsies: percentage (with range) of positive cells in high-power field.

Group	HLA I	HLA II	HECD-1	CK18-2	FIBRO	I-CAM	EGFR
1	60 (WK-100)	25 (0-100)	100	100	WK	30 (10-80)	70 (30-90)
2	20 (10-30)	7 (5-10)	100	100	WK	40 (20-70)	70 (30-80)
3	35 (5-80)	7 (5-30)	100 (90-100)	100	WK	35 (0-50)	75 (60-90)
4	30 (WK-50)	<50 (0-5)	100 (90-100)	100 (90-100)	0	10 (0-30)	85 (70-90)
5	90 (70-100)	25 (5-90)	95 (80-100)	90 (80-100)	WK	25 (5-60)	90 (87-90)
6	100	15 (5-50)	100	100	0	30 (10-60)	90 (75-95)
	p = .003	p = .31	p = 0.08	p = 0.04		p = 0.07	p = .0001

\* WK = weak staining.

In case antigen expression was seen, staining scored as positive (yes versus no). Differences in staining intensity were not considered. Statistic analysis according to Kendall test against rank-correlation (not possible for fibronectin).

pared different groups of patients with an increasing stage of primary tumor. Looking at the markers we used, one might expect a rise in EGF-R expression and a fall in HLA class I and E-cadherin expression with increasing malignant potential of the mucosa. The relation between HLA class II, cyto-keratin, fibronectin and I-CAM expression and TCC behavior is inconsistent. The fact that most markers show no changes in our study indicates that normal mucosa in patients with bladder tumor reacts similarly to normal mucosa, and this does not support the "field defect" theory. The increasing HLA-I expression is the opposite of what was expected and is difficult to understand. Like others, we have found an abnormal distribution of EGF-R expression (not only in basal cells) in normal-appearing mucosa,<sup>9</sup> even in patients without histological evidence of bladder carcinoma. However, the increasing expression of EGF-R might indicate a malignant potential of the normal mucosa and might be of prognostic value. On the other hand, since this increased EGF-R expression especially is found in patients with invasive lesions, it might also indicate that increased EGF-R expression, which is believed to be associated with TCC progression, is a general phenotypic change in the urothelium.

In conclusion, normal mucosa, adjacent to bladder tumors, harbors potential prognostic information. It is unclear whether or how this is related to the progression of transitional cell carcinoma. Immunohistochemical examination of normal-appearing mucosa in bladder cancer patients with the markers presented here was not conclusive. Our results indicate that the expression of EGF-R in normal mucosa might be of value, but that unambiguous interpretation is not possible.

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