Short Communication

Focal Loss of CD44 Variant Protein Expression is Related to Recurrence in Superficial Bladder Carcinoma

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The majority of papillary transitional cell carcinomas of the bladder are localized tumors at initial diagnosis; identification of those developing recurrence and an aggressive behavior is important. CD44 variant proteins have been implicated in tumor progression and metastasis, and a correlation with adverse prognosis has been demonstrated in a variety of human tumors. Here, the usefulness of conventional CD44 protein immunohistochemistry as a prognostic parameter for recurrence of superficial transitional cell carcinomas was assessed in paraffin sections of 241 tumors with long-term follow-up. A highly significant association was found between focal loss of CD44v3 and -v6 immunostaining and short recurrence-free interval in noninvasive (pTa) transitional cell carcinomas ($P = 0.005$), but not in minimally invasive (pT1) carcinomas ($P = 0.78$). Our results indicate the value of conventional CD44 immunohistochemistry as an additional tool for identifying patients at high risk for recurrence of pTa transitional cell carcinomas. They also point to biological differences between noninvasive and minimally invasive transitional cell carcinomas of the bladder. (*Am J Pathol* 1999, 155:1427–1432)

Transitional cell carcinoma (TCC) of the urinary bladder presents a spectrum of tumors ranging from indolent, noninvasive to diffuse, invasive, highly aggressive bladder cancer. The majority of TCCs present as either localized noninvasive (pTa) or minimally invasive (pT1) tumors at time of initial diagnosis. At that time, the identification of TCCs with a high probability of developing recurrence and an aggressive course is important for the management of the patients. The histological grade, tumor stage, and tumor number are considered important prognostic factors in bladder cancer.^{1–3} However, the grading and staging of urinary bladder cancer suffer from low inter-observer reproducibility.^{4,5} There is a need for better prognosticators that are not only independent of grade and stage but are also more reproducible than the currently used histological parameters.

CD44 glycoproteins are promising candidates, because the CD44 variant glycoprotein containing sequences encoded by exon 6 has been shown to confer metastatic potential on rat pancreatic carcinoma cells^{6,7} and to be implicated in progression and metastasis formation in various human malignancies.⁸ The transitional epithelium of urinary bladder and cell lines derived from it, as well as cell lines from TCCs, have been shown to express various CD44 isoforms including CD44v3, -v4, -v5, and -v9.^{9,10} In addition, retention of certain intronic sequences has been reported to occur frequently in TCC but hardly ever in normal transitional epithelium.¹¹ Recent studies have shown that CD44v6 expression is reduced in poorly differentiated, invasive TCCs, as compared to well or moderately differentiated noninvasive tumors.^{12–14} Furthermore, a correlation between CD44 expression and prognosis has been reported.¹³ In long-term follow-up, strong expression of CD44v6 was related to high survival

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probability and independently related to favorable outcome in muscle invasive tumors, whereas standard CD44 was an independent prognostic factor in superficial tumors. Other investigators, however, failed to establish a prognostic value for CD44v6 for tumor recurrence and patient survival.¹⁵

Based on our previously obtained data on a small collection of superficial TCCs,¹⁶ we have evaluated the value of CD44 immunohistochemistry in predicting recurrence in 241 superficial (pTa and pT1) TCCs from patients for whom long-term follow-up data were available. Here we show that focal loss of immunostaining, observed with monoclonal antibodies against CD44v3 and -v6 protein, is a significant prognostic factor that helps to identify patients at high risk for recurrence of noninvasive (pTa) TCCs.

Materials and Methods

Patients and Tissue Samples

Formalin-fixed, paraffin-embedded tissue samples of primary TCCs of the urinary bladder were available from the archives of the Institute of Pathology at the University of Basel, the Cantonal Hospital St. Gallen, and the Triemli Hospital in Zurich, Switzerland. The series consisted of 241 superficial bladder carcinomas (pTa and pT1). Tumor stage and grade were defined according to UICC and WHO classifications.^{17,18} One pathologist (G. S.) reviewed all slides of all tumors. Stage pT1 was defined by the presence of both unequivocal tumor invasion of the suburothelial stroma and tumor-free fragments of the muscular bladder wall. Biopsies containing carcinoma with stroma invasion, but lacking fragments of the muscular bladder wall, were excluded, as were true papillomas (grade 0). Of the 241 cases of superficial TCCs, 167 (69.3%) were pTa and 74 (30.7%) were pT1; 65 (27.4%) were G1, 127 (52.7%) were G2, and 49 (20.3%) were G3.

Clinical histories of patients (194 males, 47 females) with superficial bladder cancer were retrospectively evaluated by reviewing the patients' charts and contacting the attending physicians. The average age of these patients was 65.6 years (range, 20–92 years). Regular follow-up cystoscopies had been performed at least at 3, 9, and 15 months, then annually until the endpoint of this study (recurrence, progression, or last control). Information on the number of tumors present in the bladder was obtained for 172 patients (75 multicentric, 97 unicentric). The medium follow-up period was 54 months (range, 6–167 months). Intravesical treatment had been performed in 60 patients (mitomycin in 29, BCG in 26, epirubicin in 2, fenofurubicin in 2, adriablastin in 1, tigason in 1, and tumosteron in 1 patient). Recurrences were defined as cystoscopically visible tumors. Tumor progression was defined as the presence of muscle invasion (stage pT2 or higher) in a subsequent biopsy. An increase in stage from pTa to pT1 was not defined as progression because not all subsequent biopsies of our patients were available for review. Such a review

is mandatory for studies investigating pTa-to-pT1 progression because of the interobserver variability among different pathologists, ranging from 25 to 40%, for the distinction of pTa and pT1.^{4,5}

Immunohistochemistry

CD44 protein expression was analyzed immunohistochemically, employing protein A-purified mouse monoclonal IgG reactive with CD44 protein isoforms containing human variant exons 3 (clone 3G5) and human variant exons 6 (clone 2F10) purchased from R&D Systems (Abingdon, UK). Immunostaining for CD44 was performed on paraffin sections, applying microwave antigen retrieval as previously described.¹⁹ After quenching of endogenous peroxidase activity (0.6% H₂O₂ in absolute methanol, 15 minutes) and section conditioning²⁰ with 1.5% normal horse serum and 4% fat-free milk in isotonic Tris-HCl buffer, pH 6.5, for 20 minutes, sections were incubated with primary antibodies (1 µg/ml) for 60 minutes. Following buffer rinses, sections were processed by a streptavidin-biotin-peroxidase technique according to the manufacturer's recommendations (Jackson ImmunoResearch Laboratories, West Grove, PA). Peroxidase activity was revealed according to Adams.²¹

Specificity controls included the omission of primary and/or secondary antibodies as negative controls and the use of normal skin as positive controls.

Evaluation of Immunostaining and Statistical Analysis

During the whole immunohistochemical evaluation, the observers were blinded for disease outcome. A first evaluation scheme assessed the CD44-positive tumor cut surface area regardless of the staining intensity. Tumors were grouped into those with ≥50% and those with <50% positive tumor cut surface. Furthermore, focal loss of immunostaining for both CD44v3 and -v6 was evaluated in serial sections. Focal loss of immunostaining was defined as the appearance of variably sized regions of the urothelium lacking immunoreactivity with the monoclonal antibodies throughout its entire thickness. The second scheme evaluated the dominant pattern of immunostaining. Homogenous staining was defined as extending evenly through the entire thickness of the neoplastic urothelium, whereas restricted immunostaining was defined as extending through less than ¾ of the thickness.

Relationships between variables obtained in form of frequency tables were statistically analyzed using the χ^2 test.²² Survival curves regarding tumor recurrence were obtained according to Kaplan-Meier and the significance of their differences was assessed by the log-rank test. Hazard ratios indicating the relative risk of recurrence of one group compared with the other were calculated. A

Cox proportional hazard model was used to determine the parameters, with greatest influence on the risk of recurrence and progression.

Results

CD44v3 and -v6 Expression in Normal Transitional Epithelium and Superficial TCC

Transitional epithelium adjacent to tumors exhibited immunolabeling in the basal cell layer, decreasing in intensity toward the surface, with the most superficial layers of the urothelium including the umbrella cells being unlabeled, as reported by others.^{10,12,13} In the 241 TCCs investigated, two prevailing staining patterns were observed: restricted staining extending through less than $\frac{3}{4}$ of the urothelium thickness and homogenous staining extending evenly through the entire thickness of the neoplastic urothelium (Figure 1). Tumors exhibiting either of these labeling patterns could present variably sized regions with loss of immunostaining (Figure 1). Such a focal loss of immunostaining was strongly associated with advanced tumor stage and high histological grade. A focal loss of immunostaining was found in 89 of 167 pTa (53.3%), 66 of 74 pT1 (89.2%; $P < 0.0001$ for pTa vs. pT1), 28 of 65 G1 (43.1%), 79 of 127 G2 (62.2%), and 48 of 49 G3 tumors (98.0%, $P < 0.0001$, three-group contingency table analysis for grade).

Relationship between CD44 Immunostaining Pattern and Tumor Recurrence and Progression

In our collective, 129 of 241 (53.5%) patients suffered from recurrent disease. To determine the predictive value of CD44 immunohistochemistry for recurrence of superficial TCCs, the initial biopsies of all pTa and pT1 tumor patients, with and without recurrent disease, were analyzed. Among the parameters analyzed, a significant difference was observed only in association with focal loss of immunostaining for both CD44v3 and -v6 protein. The group of pTa/pT1 TCCs exhibiting unlabeled areas for both CD44v3 and -v6, irrespective of their size ($\geq 50\%$ or $< 50\%$ positive tumor cut surface), had a significantly shorter recurrence-free interval ($P = 0.0132$, Figure 2a) and was at significantly higher risk of developing tumor recurrence (hazard ratio 1.26, confidence limits 1.05–1.53). None of the other analyzed staining patterns distinguished tumors with recurrence from those without (data not shown). In addition, several subgroups of the patient collective were analyzed regarding the association between focal loss of immunostaining and recurrence. Although patients with noninvasive pTa tumors showed a significant difference in recurrence-free survival ($P = 0.005$, hazard ratio 1.34, confidence limits 1.09–1.67, Figure 2b), the group of minimally invasive pT1 tumors did not ($P = 0.864$). The association with recurrence was preserved in the subgroup of patients with pTa tumors who had not received chemotherapy

($P = 0.031$). The group of pTa TCCs with chemotherapy was too small for statistical analysis ($n = 33$). Multicentricity ($P = 0.008$), but not grade ($P = 0.750$, Figure 2c) or stage ($P = 0.689$), was linked to an increased risk of tumor recurrences in univariate analysis. A multivariate analysis of our collective, including grade ($P = 0.58$), stage ($P = 0.50$), multicentricity ($P = 0.013$), and focal loss of immunostaining ($P = 0.063$), revealed that only multicentricity ($P = 0.013$) was an independent predictor of recurrences, whereas focal loss of immunostaining ($P = 0.063$) did not reach the level of significance.

Only eleven of our patients (eight pT1, three pTa) suffered from disease progression, thus limiting the potential of this patient set to identify molecular changes associated with progression. The progression rate for pT1 carcinomas was low in this patient set, probably because of our stringent definition of stage pT1. Tumor stage ($P = 0.0003$, Figure 2d) and, to a lesser extent, multicentricity ($P = 0.050$) and focal loss of immunostaining ($P = 0.055$), were related to tumor progression in univariate analysis, but not the histological grade ($P = 0.301$). In multivariate analysis, only stage ($P < 0.0001$) and multicentricity ($P = 0.014$) were linked to subsequent tumor progression, not grade ($P = 0.645$) or focal loss of immunostaining ($P = 0.141$).

Discussion

Recurrence of TCC cannot be predicted unequivocally at time of initial diagnosis using criteria such as histological grade, depth of invasion, and number of tumors. Hence, additional prognosticators for recurrence of TCC would be desirable. In the present study, we have investigated the relationship between the immunohistochemical expression of two CD44 protein variants and various clinicopathological features of superficial TCCs. Our data provide evidence for the value of CD44v3 and -v6 immunohistochemistry in identifying patients with superficial TCC at high risk for tumor recurrence.

We found that focal loss of CD44v3 and -v6 immunostaining in the biopsies proved to be of predictive value for recurrence in superficial TCCs. Although the loss of immunostaining varied in size, variation of this parameter was not of additional significance, because TCCs exhibiting $\geq 50\%$ behaved similarly to those with $< 50\%$ unstained tumor cut surface area. Focal loss of immunostaining occurred in the great majority of invasive and in half of noninvasive tumors. Although related to recurrence in the noninvasive group, CD44 was not related to progression in the two groups. Because focal CD44 loss occurred in most tumors, it is unlikely to be a causal event in progression. It will be interesting to determine how focal loss of CD44 immunostaining is related to other prognostic factors in superficial bladder cancer. It must be emphasized that our study was performed on routinely formaldehyde-fixed and paraffin-embedded tumor tissue using a standard antigen retrieval technique,²³ commercially available reagents, and a standard immunohistochemical procedure. Thus, detection of focal loss of immunostaining for CD44v3 and -v6 is easily practicable in

Figure 1. Immunohistochemical demonstration of CD44v3 and -v6 protein in noninvasive (pTa) transitional cell carcinoma; serial paraffin sections; heavy metal-intensified diaminobenzidine reaction. Tumors exhibit either a homogeneous immunostaining extending through all layers of the urothelium (**a**, H&E; **b**, CD44v3; **c**, CD44v6) or a staining restricted to the basal cell layers (**d**, H&E; **e**, CD44v3; **f**, CD44v6). The focal loss of immunostaining can be limited (arrowhead in **g**, H&E; **h**, CD44v3; **i**, CD44v6) or can involve large areas of the neoplastic urothelium (asterisks in **k**, CD44v6; **j**, H&E). The field outlined in **k** is shown at higher magnification in **l** (H&E) and **m** (CD44v6). Original magnifications, $\times 50$ (**a-f**, **j**, **k**), $\times 60$ (**g-i**), $\times 280$ (**l**, **m**).

surgical pathology laboratories. We observed differences between noninvasive (pTa) and minimally invasive (pT1) tumors. Patients with noninvasive TCCs displaying areas with loss of labeling for both CD44v3 and -v6 showed a significantly shorter recurrence-free survival time, whereas no such correlation could be established for minimally invasive (pT1) tumors. These findings are consistent with other marked genetic differences found be-

tween pTa and pT1 TCCs.^{24,25} From the present results it can be concluded that loss of immunostaining for CD44v3 and -v6 protein is an event occurring early during progression of TCC and represents a useful prognostic factor for recurrence. It could be speculated that CD44v3- and -v6-negative tumor areas might consist of an expanding subpopulation of tumor cells with more aggressive growth properties.

Figure 2. Kaplan-Meier curves of recurrence-free interval. Focal loss of immunostaining for both CD44v3 and -v6 in the group of pTa and pT1 together (**a**), as well as in the pTa group alone (**b**), is correlated with significantly shorter recurrence-free interval of TCC. No correlation was observed between histological grade and recurrence in pTa tumors (**c**). Tumor stage and progression were correlated (**d**).

In agreement with previously published data on CD44 expression,^{12-14,26} an inverse correlation existed between the expression of CD44v3 and -v6 proteins and histological grade, as well as tumor stage. Similar observations were made for carcinomas derived from other stratified epithelia, such as squamous cell carcinomas of the skin,¹⁹ and for breast carcinoma.²⁷ Thus, reduced expression of CD44 variant forms may have prognostic value in certain carcinoma types, whereas in others, including renal cell²⁸ and colorectal carcinomas,²⁹ overexpression seems to be positively correlated with tumor differentiation and to have prognostic value.

From the present findings arises the question whether the altered CD44 protein expression and tumor recurrence in TCCs are causally related. We recognize that immunohistochemistry neither permits direct studies of the complex splice patterns present in carcinomas, including TCC,⁹ nor reveals the composition of these different variants and their functions. One might speculate that the absence of larger CD44 variant molecules at the cell surface may facilitate cell detachment and favor interaction of shorter CD44 molecules with the extracellular matrix to permit migration and invasion, as shown for melanoma cell lines.^{30,31} It is also possible that the changed CD44 expression represents solely an epiphenomenon of dedifferentiation, which occurs in parallel to processes that cause tumor recurrence and invasion in TCCs. Indeed, a simultaneously occurring decrease in the expression of CD44v6, E-cadherin,^{12,15} and $\alpha_6\beta_4$ integrin³² could be demonstrated in invasive TCC.

In conclusion, our current findings provide further evidence of the involvement of CD44 splice variants in the tumorigenesis of TCC. In terms of diagnosis and practicability, they show that detection of focal loss of immunohistochemical staining for CD44v3- and -v6-containing isoforms may provide an additional parameter in identifying patients with TCC at risk for tumor recurrence and that this information can be obtained on formalin-fixed, paraffin-embedded tissues. This immunohistochemical evaluation may be especially useful because the extent of loss of staining does not seem to be important and the identification of one area with unequivocal loss of immunostaining may be sufficient to define an increased risk for recurrence in affected patients.

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