Journal of Cellular Biochemistry, Supplement 16I:85–92 (1992)

Strategies of Chemoprevention Based on Antigenic and Molecular Markers of Early and Premalignant Lesions of the Bladder

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Abstract Using monoclonal antibodies, we have identified a series of tumor-associated antigens selectively expressed on tumor subtypes with distinct clinical behaviours. The mucinous antigen M344 and the gp200 surface antigen 19A211 are preferentially expressed on papillary superficial tumors and carcinoma *in situ* lesions of the bladder. The combination of these two antigenic markers in immunocytology and flow cytometry studies of exfoliated cells has improved the sensitivity of detection for bladder tumors. Moreover, the detection of M344- and 19A211-positive exfoliated cells from previously treated but currently tumor-free patients appears to be predictive of tumor recurrence on follow-up. These results, as well as results of bladder mapping studies in tumor patients, suggest that these antigenic changes occur in a premalignant stage and may provide tools to monitor the efficacy of chemopreventive measures. Other markers, such as the surface antigen T138 and the soluble molecules autocrine motility factor (AMF) and tumor collagenase stimulating factor (TCSF), are produced by primary or recurrent tumors with a higher metastatic potential. They may be useful in identifying high risk patients for distant failure. The highly restricted antigen 19A211 is also expressed on cervix condylomas and carcinoma. This observation led us to investigate a possible viral etiology of some bladder cancers. Using PCR techniques, we detected the presence of human papillomavirus (HPV) 16 DNA sequences in a significant proportion of bladder tumors. HPV positivity was inversely correlated with the presence of p53 mutations in exons 5–9 of the same tumors as measured by PCR-SSCP technique. This combination of markers may provide a basis for chemoprevention strategies targeted to distinct etiological events.

Key words: HPV, p53 mutations, tumor antigens, tumor etiology, tumor monitoring

Cancer is a complex disease process resulting from a variable contribution of exogenous factors and genetic susceptibility. Cancer control may be achieved by primary prevention targeted at modifying dietary and smoking habits or lifestyle factors and eliminating environmental carcinogens [1,2]. However, there is an important need for secondary prevention to reduce the risk of cancer development in those individuals genetically predisposed, or those already exposed, to cancer initiators. The newer approach of chemoprevention uses nontoxic agents to inhibit or reverse the process of carcinogenesis [3,4]. The efficacy of chemopreventive measures will depend on the proper understanding of tumor biology in a given system, as well as on reliable means to identify high risk populations. Thus the development of chemoprevention is intimately related to developments in tumor biology and particularly to the availability of biomarkers [5]. The identification of genetic and phenotypic alterations occurring in the multistep process of carcinogenesis in a given tumor system provides marker lesions to better identify risk categories. In addition, such biomarkers may be used to assess the effect of chemopreventive measures and serve as intermediate endpoints that reflect the effect of treatment on cancer development [6].

One fundamental concept of carcinogenesis in aerodigestive and genitourinary cancers is the concept of "field cancerization" [7]. This concept is documented by the frequent association of

This work was supported by grants from the NCI-USA (CA47526-06) and NCI Canada (3515). Y.F. is a recipient of a scientist award from the Medical Research Council of Canada.

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tumors with premalignant lesions in the field at risk and by the synchronous or metachronous development of multiple primary tumors. One may thus presume that precancerous changes preceding the occurrence of a first tumor may be analogous to those observed in patients treated for a primary cancer who are at risk of recurrence. It may then be useful to consider chemoprevention as primary or secondary depending on whether it applies to the former or the latter group of risk patients.

BLADDER CANCER AS A MODEL FOR CHEMOPREVENTION

Bladder cancer is in many ways a unique model for the development of strategies of chemoprevention. First, there are known exogenous risk factors such as cigarette smoking and industrial exposure that may help define appropriate chemoprevention approaches [8,9]. Second, most bladder cancers initially present as superficial disease effectively treated endoscopically but with high risk of recurrence. This identifies a high risk population to test secondary chemoprevention with relatively short term endpoints. Third, the ease of access to the bladder or to exfoliated or shed material provides a means of noninvasive monitoring of early and premalignant lesions, assuming tests of suitable sensitivity and specificity are available [10].

Cumulative evidence from clinical and biological studies, however, suggests that two disease processes exist. Most frequently, superficial bladder tumors will be papillary and highly recurrent with a low risk of progression to invasive cancer. These tumor cells are more frequently diploid and not effectively monitored by urinary cytology or DNA flow cytometry. By contrast, carcinoma *in situ* is a high grade aneuploid lesion frequently leading to invasive cancer. It is the typical type of lesion observed in patients exposed to industrial carcinogens. It is thus possible to hypothesize, as in Figure 1, that individuals may be predisposed genetically to the influence of distinct carcinogens leading to papillary tumors or carcinoma *in situ* and invasive cancers. Strategies of chemoprevention must take into account these two potentially different disease pathways. Moreover, the risk of progression to invasive cancer must be carefully assessed and monitored in patients with

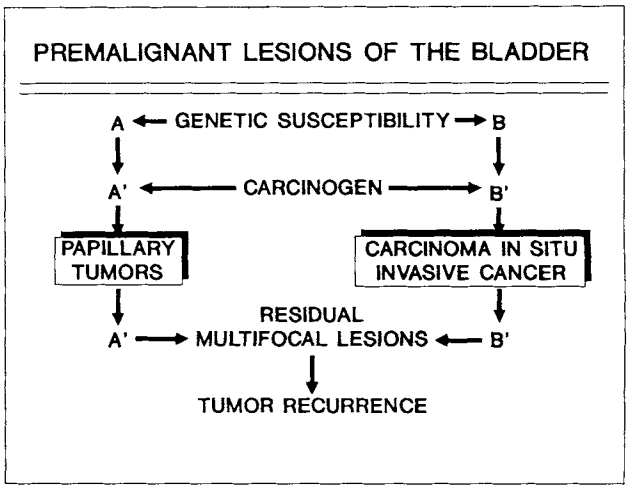

Fig. 1. Schematic representation of hypothetical pathways of carcinogenesis in bladder cancer. An assumption is made that precancerous lesions leading to primary tumor or tumor recurrence after treatment are similar.

high grade carcinoma *in situ*. Recent advances in tumor marker research have led to the identification of promising biomarkers in the stratification and monitoring of bladder cancer [11]. Several of these markers have reached the stage of clinical trials and may become intermediate endpoints for studies in chemoprevention in the near future.

BIOMARKERS IN BLADDER CANCER

Tumor immunology, molecular biology and genetic studies have opened numerous avenues for developing markers defining genetic susceptibility as well as early or premalignant lesions of the bladder. These advances are based on a better understanding of the genetic and molecular mechanisms of tumor initiation and progression, as well as on technological advances that allow evaluation of a variety of markers on minimal amounts of clinical material. As a result of these technological advances, it is now possible, for example, to study cytogenetic anomalies using *in situ* hybridization with fluorescent DNA probes [12,13] or using restriction fragment length polymorphism (RFLP) [14,15] to identify chromosomal deletions in bladder cancer. Loss of material on the long arm of chromosome 9 has been consistently observed in a majority of superficial bladder cancer and frequently in invasive bladder cancer and may

be an early genetic event in bladder transitional cell carcinogenesis.

The recent discovery of tumor suppressor genes also provides significant new perspectives in the biology of cancer cells. Most extensively studied and investigated in bladder cancer are the p53 and the retinoblastoma suppressor gene products, as reported in this symposium. The biochemical function of p53 and the retinoblastoma (Rb) gene product has been partly elucidated [16–18]. The function of these proteins may be altered by genetic deletions or, in the case of p53, by point mutations that may be induced by carcinogens. Identification of such mutations in exfoliated cells may allow the identification of patients at risk of developing bladder cancer in a cohort of exposed individuals. The functions of p53 and Rb may also be altered by binding viral oncoproteins such as those from human papillomavirus (HPV) [19–22]. Using polymerase chain reaction, we recently identified HPV 16 sequences in 34% of Ta, T1 and 36% of T2 human bladder tumors [23]. Preliminary results also suggest that HPV-positive tumors rarely contain mutations in the p53 gene. If confirmed by more extensive studies, these results may determine an anomaly that could also be measured in exfoliated cells of urine and help identify a distinct population at risk of bladder cancer.

Another approach in the identification of potential biomarkers has been the use of monoclonal antibodies to study phenotypic changes in tumor cells. These immunological reagents provide investigative tools for the identification of new markers as well as optimal reagents for tests on exfoliated cells and soluble shed or biopsy material. Research conducted in our laboratory using monoclonal antibodies has allowed us to identify a series of tumor-associated antigens selectively expressed on tumor subtypes with distinct clinical behaviour [11]. Two of these antigens are part of a new family of cancer mucins preferentially expressed in papillary superficial tumors and carcinoma *in situ* lesions of the bladder.

Another group of markers, of which T138 antigen is a prototype, identifies tumors with a more aggressive behaviour and a high metastatic potential [24]. These monoclonal antibodies may be valuable in the design of strategies for chemoprevention in bladder cancer.

BLADDER CANCER MUCINOUS ANTIGENS

Cells from low grade papillary tumors are phenotypically very similar to normal urothelial cells. This results in a low level of tumor detection by urinary cytology or flow cytometry as well as a paucity of antigens identified in early monoclonal antibody studies. However, using strategies aimed at inducing passive tolerance to dominant antigens of normal urothelium, we were able to obtain a series of monoclonal antibodies defining two new antigenic systems of papillary superficial bladder tumors and carcinoma *in situ* [25,26]. These antigens of early bladder cancer have been extensively studied immunohistochemically, in flow cytometric studies of exfoliated cells, and biochemically to determine the nature of the target molecules and epitopes recognized by the monoclonal antibodies. First identified was the M344 antigen, a high molecular weight mucinous molecule (Fig. 2) expressed selectively in 70% of papillary Ta, T1 bladder tumors. The antigen is rarely expressed in invasive cancers but frequently in associated carcinoma *in situ*. No normal tissue was found to express the antigen, including normal bladder urothelium from a large number of individuals, which suggests a tumor-specific antigen. The antigen was expressed in rare mucinous adenocarcinoma of breast and prostate, distinguishing it from other known cancer mucins [27]. The second antigen was identified by monoclonal antibody 19A211 [26] and found to react with a sialyl epitope of a protein complex with a dominant 200 kD moiety (Fig. 2) in superficial bladder tumors. This antigen is also expressed by urothelial umbrella cells in 25% of normal individuals, but not by other normal cells in immunohistochemistry studies. These characteristics and the results of competition assays clearly demonstrate that the 19A211 antigen is distinct from the Lewis X blood group antigen to which several monoclonal antibodies to bladder cancer were found to be reactive [26]. The 19A211 antigen is also expressed on 70% of Ta, T1 bladder tumors and transitional cell carcinoma *in situ* (TIS) lesions, but in contrast to the M344 antigen, it is reactive with a significant proportion of invasive bladder cancers. The antigen is also expressed in a few adenocarcinomas of the breast and colon, but most interestingly, is

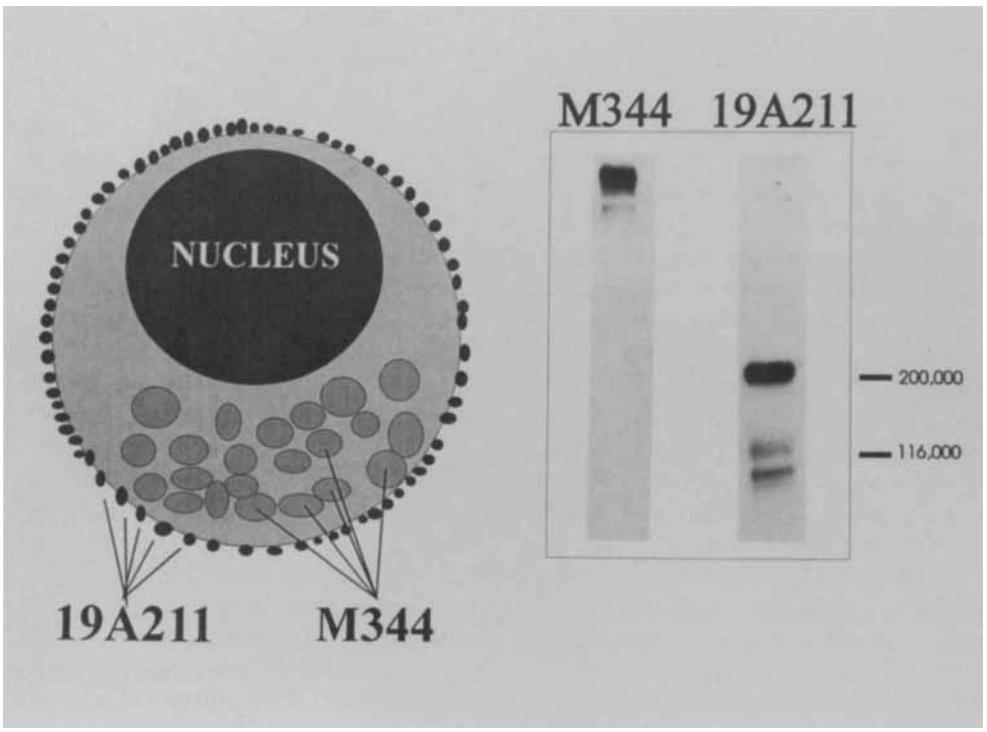

Fig. 2. Bladder cancer mucinous antigens. M344 is a very high molecular weight cytosolic antigen while the 19A211 200 kD tumor-associated antigen is a surface molecule. Both are highly glycosylated.

expressed on a majority of uterine cervix cancers and condylomas. This finding is intriguing in view of the results of our recent study demonstrating HPV DNA in subsets of bladder tumors [23].

These two novel antigenic systems have some common features suggesting that they both belong to a family of highly polymorphic cancer mucinous antigens. The expression of both antigens is influenced by the spatial configuration of tumor cells [28]. Both molecules are highly glycosylated, with the 200 kD moiety of 19A211 antigen, for example, having 60% sugar in its composition. Capture experiments and competition assays have shown, however, that these two antigens are different from the known mucinous antigens of breast, pancreas, colon and prostate [29,30]. Ultrastructural studies by immunoelectron microscopy also indicated that M344 and 19A211 antigens may be part of distinct families. M344 localizes to large electron lucid cytoplasmic vacuoles, while the tumor-specific 200 kD molecule of 19A211 is strongly expressed as a membrane antigen on the same tumor cells (Fig. 2) [31]. Results of ongoing biochemical and molecular biology studies should reveal information on the nature of these tumor antigens.

Of particular relevance to the present discussion is the high rate of positivity of either one or both of these antigens in Ta, T1 and TIS lesions of the bladder, or in exfoliated cells obtained by bladder irrigation. In a prospective study of 260 irrigations from 140 patients, we analysed reactivity to M344 and 19A211 antibodies in combination with ploidy determination by dual-parameter flow cytometry [32]. The antibodies were positive in 95% of Ta, T1 tumors with positive urinary cytology and in 80% of those with negative cytology. In contrast, the frequency of aneuploidy was detected in 50% of the Ta, T1 tumors with positive urinary cytology and 30% of tumors with negative cytology. Even more importantly, in 77 irrigations from patients with previous tumors but negative control cystoscopy, the antibody test

was positive 30% of the time. The recurrence rate within 6 months was 75% in patients with a positive antibody test compared to only 20% in those with a negative antibody reaction. Aneuploidy or positive cytology was observed in 10% of these irrigations and the recurrence rate at 6 months was nearly 60% in those cases. The two bladder mucinous antigens can also be easily detected on exfoliated cells found in voided urine. The M344 antigen was studied in voided urine in collaboration with Hemstreet *et al.*, [33] at Oklahoma University, in patients with bladder tumors and in control patients and was found to be both sensitive and specific.

Moreover, studies of biopsies of apparently normal mucosa in patients with bladder tumors indicate that M344 antigen is an early marker of urothelial transformation as suggested by the results from irrigations in control patients. Thus M344 and 19A211 antigens must be considered high on the list of candidate biomarkers of early and premalignant lesions of the bladder in association with ploidy analysis by flow cytometry or image analysis. The sensitivity and specificity of these tests will be investigated in a trial by the NIH bladder tumor marker network.

TUMOR ANTIGENS OF INVASIVE BLADDER CANCERS

Other tumor markers are identified on more aggressive bladder cancers and are associated with higher metastatic potential. The loss of expression of the Rb gene product is one that has been recently investigated in two separate studies and found to be associated with poor prognosis in invasive cancers [34,35]. It is not known, however, whether these changes may be observed in early lesions which lead to invasive cancers.

On the other hand, alterations in p53 nucleoprotein expression, presumably due to gene deletion or point mutations, have been observed in early tumors and TIS which appear to be at higher risk of cancer progression [36]. Whether such alterations may be observed in premalignant states remains to be investigated. More studies are required before p53 and Rb can be considered as useful biomarkers in chemoprevention studies.

T138 antigen, a surface glycoprotein of 25 kD initially defined by monoclonal antibodies to bladder cancer [37,38], is also a marker expressed on cancers with a significantly higher risk of progression to metastasis and death. This was demonstrated in a prospective study of 68 patients in whom bladder irrigations were analysed for T138 and DNA by flow cytometry [39]. In this study, death was observed in 35% of patients with diploid, T138-positive tumors compared to 0% in those with diploid, T138-negative tumors, and in 65% with aneuploid T138-positive tumors. Moreover, 4 of 5 patients with positive Ta, T1 tumors later progressed to invasive and metastatic cancer. Recent analysis of antigen expression in a cohort of 350 newly diagnosed primary Ta, T1 bladder tumors showed T138 expressed in 15% of cases, suggesting that this molecular alteration may be observed early in the pathway of aggressive bladder cancer. The low affinity monoclonal antibody available to the T138 antigen is not suitable for assays on fixed cells. Work is currently in progress in our laboratory to produce better reagents to this most interesting molecule.

Other potential biomarkers of more aggressive cancers may be molecules such as the autocrine motility factor (AMF), collagenase, and the tumor collagenase stimulating factor (TCSF) [40] which have been associated with invasive bladder cancers and can be measured as free molecules in urine. Such urine assays would be optimal tests for noninvasive monitoring of the status of carcinogenesis. However, little is known at this time about the sensitivity and specificity of these tests, or about their status of expression in early cancers and premalignant lesions of the bladder. With more focused studies it is likely that some of these promising markers will be of value in chemoprevention trials.

BIOMARKERS AND STRATEGIES OF CHEMOPREVENTION IN BLADDER CANCER

Bladder cancer offers unique opportunities for investigating the efficacy of chemopreventive agents in reversing the process of carcinogenesis. As illustrated in Figure 1, there is a significant proportion of patients at risk for tumor recurrence, presumably due to the persistence

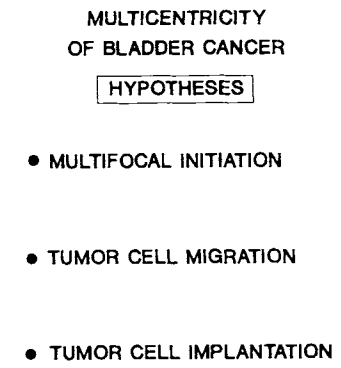

Fig. 3. Multicentricity of bladder cancer. Possible hypotheses to explain multicentric synchronous and metachronous bladder tumor recurrence.

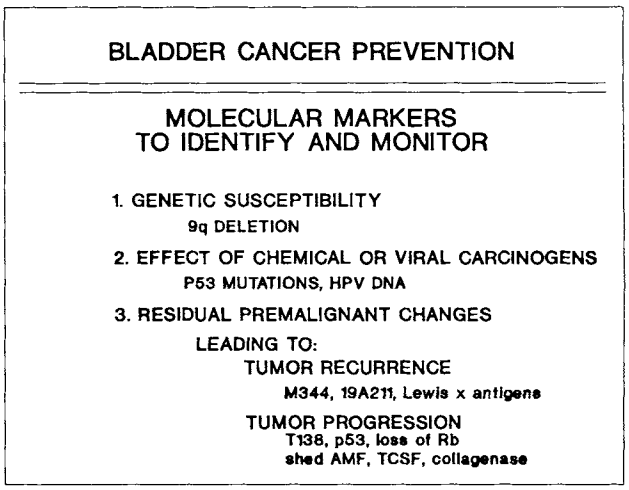

Fig. 4. Bladder cancer prevention. Hypothetical classification of some promising tumor markers in bladder cancer according to potential steps of carcinogenesis.

of multifocal premalignant lesions. It was recently suggested, based on very limited observations, that multifocal bladder cancer was monoclonal in origin [41]. According to this hypothesis, tumor recurrence would be due to persistent tumor cells that have migrated or have implanted away from the primary site (Fig. 3). Although this event is likely in a subset of cases mostly associated with high grade invasive cancers, the prolonged delay between the primary and the recurrent tumor suggests that multifocal initiation is involved in a significant proportion of cases. An agent effective in preventing recurrence under those circumstances would probably be also effective in primary chemoprevention to prevent the initial tumor event.

Another interesting feature of bladder cancer as a model for chemoprevention research is the emergence of several phenotypic biomarkers that may help identify distinct disease pathways and, perhaps, steps in the progression of malignancy. Some of the promising markers discussed herein are summarized in Figure 4 and are classified according to their potential value. Although this working hypothesis is still highly speculative, sufficient data are available on some of these markers, such as M344, 19A211 and Lewis X antigens, to justify their investigation in clinical trials. More information will be required, however, before any of these biomarkers can be used as intermediate endpoints in chemopreventive studies.

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