Diagnosis of bladder cancer with urinary cytology, immunocytology and DNA-image-cytometry

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Received 23 September 2000
Accepted 9 December 2000

DNA-image-cytometry and antibodies directed against the Lewis X- and the 486p 3/12 antigen were applied to improve diagnostic accuracy of urinary cytology for the detection of bladder cancer. Cytology, immunocytology and DNA-image-cytometry were performed in spontaneously voided urine samples and barbotage bladder washings from 71 patients. The DNA content was determined using the CM-1 Cytometer according to the recommendation of the ESCAP Consensus Report on Standardization of DNA-image-cytometry (1995). For immunocytological examination we used the monoclonal anti Lewis X antibody P-12 and antibody 486p 3/12. All patients underwent subsequent cystoscopy and for any suspicious lesion biopsy or transurethral resection was done. Histological findings revealed 31 patients with transitional cell carcinomas of different stages and grades of malignancy. 40 patients had various benign diseases of the urinary bladder. Cytology yielded a sensitivity of 68% and a specificity of 100%. DNA aneuploidy was detected in 81% of cancer patients with a specificity of 100%. By combining these two methods the overall sensitivity increased to 87%. Immunocytology with Lewis X and 486p 3/12 antibodies showed reactivity in 84% and 87% in combination with a specificity of 80% and 70%, respectively. By combining urinary cytology, immunocytology and/or DNA-image-cytometry the overall sensitivity increased to 94% with no change in specificity. DNA-image-cytometry should be used to evaluate particularly urothelial cells suspicious for malignancy in urinary specimens. Because of low specificity the monoclonal antibodies against Lewis X- and 486p 3/12 antigens are not helpful in screening for bladder cancer. Nevertheless, their high sensitivity may justify their use in case DNA image cytometry is not available and in the follow up of patients with transitional cell carcinoma.

Keywords: Lewis X antigen, 486p 3/12 antigen, DNA- and flow cytometry, urinary cytology, bladder cancer

1. Introduction

Transitional cell carcinoma of the urinary bladder is a very common disease with approximately 54,000 new cases diagnosed in the United States in 1998 [24]. Still cystoscopic and biopitic evaluation are warranted in the diagnosis and the follow up of patients with bladder carcinoma. Urinary cytology is the most commonly used noninvasive method in early detection of bladder cancer with a sensitivity of 43 to 72% and a specificity of 80 to 100% [18,22,23,30,33,35].

DNA-cytometry is based on the biological principle of detection of DNA aneuploidy which is the quantitative cytometric equivalent of chromosomal aneuploidy. Chromosomal aneuploidy is defined as the presence of numerical and/or structural chromosomal aberrations. Like chromosomal aneuploidy, DNA aneuploidy is a well accepted marker for neoplasia. Aneuploidy rates of 0 to 29% for low grade and of 70 to 90% for high grade urothelial carcinomas are reported in the literature as detected by DNA-image-cytometry or DNA-flow-cytometry [11,19,20,26,29,38]. Recently, with improvements in DNA-image-analysis systems we were able to achieve a sensitivity of 73.6% in 60 cases of grade 1 urothelial carcinomas [31,32].

To further enhance the sensitivity of urinary cytology we used two newly developed antibodies against...
the Lewis X antigen and the 486p 3/12 antigen. P12 is a monoclonal anti-Lewis X antibody directed against the Lewis X determinant. The Lewis X antigen belongs to the Lewis system, occurs in secretions and serum lipoproteins and is bound to the cell membranes of erythrocytes. It is a surface antigen whose immunodeterminant structure is the trisaccharide galactose B 1-4 (fructose 1-3) N-acetylglucosamine. It is expressed by few normal cells such as epithelial cells of the gastrointestinal tract and granulocytes and by a number of tumors originating from tissues which do not normally express the antigen. It is not detected in normal urothelium except occasionally in superficial cells [13,34]. Lewis X is expressed by more than 90% of papillomas and transitional cell carcinomas irrespective of stage, tumor grade, blood group and secretory status [14]. 486p 3/12 is an IgM monoclonal antibody which belongs to the class of CEA comparable antibodies. It is directed against a membrane bound 200 kD-glycoprotein, an oncofetal antigen expressed by fetal bladder mucosa and some superficial bladder cells in epithelium of normal bladder. An identification rate of 90% of bladder cancer patients has been reported regardless of tumor grade [1,22,23].

Despite some improvements current methods are not sufficiently accurate to serve for screening of bladder cancer and as a basis for clinical decision making. The aim of the present study was to improve the sensitivity of urinary cytology in bladder cancer by simultaneously using DNA-image-cytometry and immunocytochemistry with antibodies against Lewis X- and 486p 3/12 antigens.

2. Material and methods

2.1. Patient characteristics

We analyzed Papanicolaou stained smears of urinary sediments from 31 patients with transitional cell carcinoma and 40 patients with benign diseases of the urinary bladder such as prostatic hyperplasia of the prostate, patients with storage symptoms of the bladder or those with no pathological status by conventional cytology and performed immunocytochemical examination of the urine using P12 anti-Lewis X and 486p 3/12 monoclonal antibodies. In all cases spontaneously voided urine samples or bladder washing samples were taken. Additional bladder washings were performed in all cases.

The bladder was washed 5 to 10 times using at least 100 ml normal saline. Concomitant or previous malignancies, chemotherapy or immunotherapy were exclusion criteria. Histological evaluation was performed according to the TNM classification of the International Union against cancer. The grading system was performed in compliance with the World Health Organization criteria. Grading and staging were assessed by histological evaluation of tumor specimen obtained by transurethral resection of bladder tumors. In all bladder tumors or suspicious lesions a bladder mapping including biopsies of all sides of the bladder was performed. In patients without a visible tumor and negative urinary cytology no biopsies were taken.

All 31 carcinomas and all 40 controls were also examined by DNA-image-cytometry and cystoscopy. There were 21 pTa, 4 pT1, 1 pT2, 3 pT3 transitional cell carcinomas and 2 carcinoma in situ of the urinary bladder. These consisted of 11 grade 1, 17 grade 2, and 3 grade 3 urothelial carcinomas. The mean age of the 31 patients with transitional cell carcinoma was 67 years (55–78); the mean age of the 40 controls was 64 years (30–81).

2.2. Conventional urinary cytology and immunocytochemistry

The specimens were divided into two portions: one for conventional cytology and DNA-cytometry and the other for immunocytochemical examination. At first both specimens were centrifuged at 2000 rpm for 10 minutes. For cytological examination, these cytopins were fixed in an equal volume of Saccomaninos fixative and then smears there of stained according to Papanicolaou. Atypical and suspicious cells without clear evidence of malignancy were considered as non malignant cytology.

For immunocytochemical examination one part of the sediment was fixed in an equal volume of 50% ethanol for Lewis X and the other in an equal volume of PBS buffer for 486p 3/12. Cytocentrifugation specimens were prepared and immediately processed after air-drying. For 486p 3/12 we used the alkaline phosphatase immunocytochemical method and for Lewis X the avidin–biotin-complex immunoperoxidase method [22,30]. At least 100 urothelial cells per specimen were required, and superficial umbrella cells, granulocytes, blood cells, transitional cells and squa-
mous cells had to be distinguished. During each staining a negative control was used. A specimen was considered to be immunocytologically positive for monoclonal antibody 486p 3/12 if there were more than 30% reactive cells, including superficial cells. The majority of stone patients exhibited a high rate of positive immunocytochemistry is reported with the monoclonal antibody 486 P 3/12 [22]. Since normal urothelial cells are also positive for this antigen a threshold of 30% is justified. Granulocytes served as an internal positive control. A specimen was considered Lewis X positive if 5% or more of the urothelial cells stained positive. This antigen is occasionally also expressed on some normal superficial urothelial cells. Therefore, granulocytes and superficial umbrella cells were excluded from examination. As these cells are sometimes difficult to distinguish from tumor cells a limit of 5% appears to be justified.

2.3. DNA-image-cytometry

After cytological examination the Papanicolaou stained smears can be used for DNA-image-cytometry. All slides were uncovered in xylene, hydrolyzed, destained and rehydrated with Schiff’s reagent according to Feulgen in a temperature-controlled Shandon Varistain 24 staining machine [5,6,10]. They were examined separately for malignancy by two pathologists [7]. The DNA content of the urothelial cells was determined with a CM-1 Cytometer. This cytomter consists of a light microscope with a 40/0.65 objective, an interference filter with 570 ± 10 nm half-width, an inverted 20× objective as condenser for reduction of stray light with a fixed low numerical aperture of 0.25, a camera adapter with 1.6-fold secondary magnification and a CCD black-and-white camera with 625 lines (Pulnix TM 765, USA). The TV-image-analysis system consists of a 486 IBM compatible PC with frame grabber, software with glare correction, VGA monitor, mouse and laser printer. The TV-image-analysis system and the software were developed and advanced to the current status by us in the past years [24,25]. To determine the normal 2c content 50 granulocytes from each patient were measured and consecutively 250 normal, respectively abnormal urothelial cells on the same slide for ploidy evaluation. Those in which less than 250 epithelial nuclei were counted were excluded. Interpretation of the data was performed using the analytical software of the system, whereby the stemline interpretation according to Böcking et al. [6] assumes DNA aneuploidy if comparison of the G0/G1 fractions of the reference and test cells in the Kolmogoroff–Smirnow test produces a p value < 0.001 [4,6,7,28]. The G0/G1 fraction was determined interactively from the histogram displayed on the monitor [4]. According to the conventional classification DNA aneuploidy is not assumed until a stemline ploidy 1.8c > STP > 2.2c.

In order to test the interobserver reproducibility of DNA-cytometry and immunocytochemistry 10 preparations were examined by a second examiner. The intraobserver reproducibility was tested by having 10 preparations measured twice by the same examiner.

3. Results

The cytological examination revealed a sensitivity of 68% and a specificity of 100% (Table 1). Conventional interpretation of the DNA stemline ploidy (1.8c > STP > 2.2c) revealed DNA aneuploidy in 60%, stemline interpretation according to Böcking et al. [6] in 81%, both with a specificity of 100%. The combination of DNA-image-cytometry and urinary cytology improved the sensitivity to 87%. The immunocytochemical examination for Lewis X showed a sensitivity of 84% and a specificity of 80%. The immunocytochemical examination for 486p 3/12 revealed a sensitivity of 87% and a specificity of 70%. Lewis X, 486p 3/12 antigen and DNA-image-cytometry showed a significantly higher detection rate in low grade tumors as compared to conventional cytology (Table 2). Different combinations of cytology, DNA-image-cytometry, immunocy-

| Grade | n | Cytology | Lewis X | 486p 3/12 | DNA aneuploidy |
|-------|---|---------|---------|---------|----------------|
| 1     | 11| 5 (45%) | 9 (82%) | 8 (73%) | 8 (73%) |
| 2     | 17| 13 (76%)| 14 (82%)| 16 (94%)| 14 (82%) |
| 3     | 3 | 3 (100%)| 3 (100%)| 3 (100%)| 3 (100%) |
Table 3

Improvement of detection of bladder cancer in 71 patients by combination of cytology, DNA-image-cytometry and immunocytology

|                      | Sensitivity (n = 31) | Specificity (n = 40) |
|----------------------|----------------------|----------------------|
| Cytology + DNA-image-cytometry | 27 (87%) | 40 (100%) |
| Cytology + Lewis X | 29 (94%) | 32 (80%) |
| Cytology + DNA-image-cytometry + Lewis X | 29 (94%) | 32 (80%) |
| Cytology + 486p 3/12 | 29 (94%) | 28 (70%) |
| Cytology + Lewis X + 486p 3/12 | 29 (94%) | 28 (70%) |

Fig. 1. DNA histograms displaying typical distribution patterns for grade 1 (A) and grade 3 (B) aneuploid urothelial carcinomas. Aneuploidy was detected by comparison of the reference and test cells. Stemline ploidy of reference cells is 2c.

4. Discussion

Detection of bladder tumors and the follow up of patients after therapy traditionally require invasive procedures such as cystoscopy or biopsies. The only non-invasive method for diagnosis and surveillance in routine clinical use is the cytological examination of voided urine specimens. The high primary incidence of urothelial carcinomas with a share of 6% of all new cancer cases is further aggravated by a local recurrence rate of around 40–80% reported in the literature [9,18,21,24,40]. The search for sensitive noninvasive methods for screening and follow up has a high priority. Urinary cytology alone clearly fails in this respect because of its low sensitivity of 43 to 72%, especially in low grade lesions where it ranges from 0 to 59% [18,22,23,30,33,35]. We recently reported achieving an overall detection rate of 80% and a sensitivity of 73.3% in low grade urothelial tumors by using an improved DNA-image-cytometry system [30,32]. In the present study comprising urothelial carcinomas of different grades, the combination of urinary cytology and DNA-image-cytometry yielded a sensitivity of 87%. Van der Poel et al. [39] and Wiener et al. [41] reported on sensitivities of 59 and 69% respectively using the Quanticyt-system which combines a nuclear roundness factor and the 2c Deviation-Index of DNA-distribution [3] as diagnostic criteria. Quanticyt requires barbotage bladder washings to obtain at least 500 cells for analysis whereas voided urine samples are sufficient for DNA-image-cytometry where only 250–300 cells need to be evaluated [6,39,41]. Nevertheless, even the combination of urinary cytology and DNA-image-cytometry reported here may still not be sufficiently sensitive for screening purposes.

Many authors have evaluated the use of monoclonal antibodies against tumor associated antigens. Recently, Immunocyt – a combination of the antibodies M.344, LDQ10 and 19A211 – was reported to achieve an overall sensitivity of 86% [27]. The P-12 monoclonal antibody against the Lewis X antigen has been used by a number of groups for detection of urothelial tumors and yielded a sensitivity of 75 to 86% being in good correlation with 84% reported in this study [18,30,33,35,37]. Interestingly, Golijanin et al. were able to achieve at least 95% sensitivity by examination of two consecutive voided urine samples [18,33]. Monoclonal antibody 486p 3/12 typically showed a sensitivity of 71 to 91% with 87% in this study [1,21–23,25,40]. On the basis of the heterogeneity of bladder carcinomas with different subpopulations of cells we do not expect...
a 100% positive reaction for tumor cells with any of the available tumor markers [1,16]. Therefore, combining two or more monoclonal antibodies could possibly enhance the overall sensitivity by detecting different antigens from different subsets of cells [1,21,22,40]. To assess this possibility we added a second antibody against the 486p 3/12 antigen to our previously published study with the Lewis X antibody [30]. Nevertheless, in our present study different combinations of urinary cytology, DNA-image-cytometry and the two antibodies Lewis X and 486p 3/12 yielded a sensitivity of 94% which exhibited no improvement over the use of only one of the antibodies in conjunction with standard cytology. One explanation could be that the antibody 486p 3/12 does not recognize an additional antigen but an immunodeterminant structure shared by Lewis X or even the Lewis X antigen itself [27,30].

One of the common drawbacks of the antigens studied so far is that a specificity of around 90% could not be surpassed [8,12,15,17,20,36]. In the present study Lewis X and 486p 3/12 achieved a specificity of 80% and 70%, respectively. For this reason none of the currently known immunological markers are suitable for screening where large numbers of patients would yield too many false positive results. As a consequence the more valuable task for tumor markers lies in the follow up of patients with superficial bladder carcinoma where false positive results can be accepted because of the smaller pool of patients combined with a high rate of tumor recurrence.

5. Conclusion

Since the combination of DNA-image-cytometry and conventional cytology is more sensitive as compared to cytology alone we do recommend its use in case that cytology renders a suspicious result. If DNA-image-cytometry reveals DNA aneuploidy, consecutive cystoscopy and biopsy seems to be justified in the light of its high specificity. The combination of conventional cytology and immunocytoLOGY for Lewis X or 486p 3/12 increased the sensitivity to 94%. The combined use of both antibodies showed no further improvement. Considering their low specificity we believe that both antibodies are not suitable for screening purposes in general. But in case DNA-image-cytometry is not available both antibodies are helpful to increase the sensitivity of urinary cytology and should, thus, encourage cystoscopy in patients with positive immunoreactivity although at a low specificity. Nevertheless, because of their high sensitivity in conjunction with conventional cytology they are both equally useful in the prediction of recurrent lesions in bladder tumor patients.

Acknowledgements

We want to thank Ms Hylia Erdag for help in the cytological evaluation, Ms Heike Raupach for technical support and Dr. Ellen Striepecke for second examination of samples and critical advise.

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