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Advances in the Diagnosis of Urothelial Neoplasia

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Urothelial neoplasia is a unique cancer in that it consists of a spectrum of tumors with different biologic behaviors. The most common urothelial neoplasm is the low grade superficial papillary carcinoma or papilloma which may recur numerous times but does not result in significant morbidity or mortality. A variant of the superficial papillary carcinoma, which represents approximately 10% of the tumors, is the noninvasive papillary neoplasm which progresses to a less differentiated invasive transitional cell carcinoma (TCC). Considerable effort has been directed at identifying which of the superficial well differentiated papillary tumors will persist, recur, and progress to invasive cancer. Current approaches to identifying such tumors include cytogenetics, molecular biology, and flow cytometric DNA analysis. In the final group of bladder carcinomas, the high grade invasive neoplasms, evidence suggests that these life-threatening tumors arise de novo without identifiable precursors. Unfortunately, 75% to 90% of invasive TCCs are classified in this group, with the remaining minority progressing from preexisting recurrent superficial papillary carcinomas. Obviously the biologic behavior of these aggressive poorly differentiated tumors is life-threatening, and application of traditional diagnostic procedures and new technologies need to be directed at early diagnosis. (Henry Ford Hosp Med J 1989;37:19-23)

Transitional cell carcinoma (TCC) of the urinary bladder is a relatively common neoplasm. The American Cancer Society estimates that 45,400 new cases of TCC will be diagnosed in 1989, accounting for 4.71% of new cancers and approximately 10,000 deaths. Death from TCC is invariably due to metastases which are a direct sequelae of invasion into the bladder wall. Large size and deep invasion correlate with the presence of metastatic disease. Unfortunately, the vast majority (75% to 90%) of invasive TCCs develop de novo without a recognized precursor and only 10% to 25% are preceded by papillary noninvasive TCC. The biologic progression of precursors and noninvasive TCC is complex and not completely understood. Two separate and distinct problems in diagnosing urothelial cancer involve 1) early diagnosis of invasive high grade neoplasms, and 2) identification of predictors of recurrence/progression in patients with noninvasive papillary TCC. Exfoliative urine cytology and cystoscopy-directed biopsy with direct visualization of the affected urothelium remain the standards for diagnosis and classification of TCC.

At initial diagnosis approximately 75% of TCCs are noninvasive tumors (Ta), although some will have microinvasion into the lamina propria (T1). These neoplasms are regarded as superficial tumors and, although symptomatic, are generally not considered life-threatening. Most noninvasive papillary TCCs are well differentiated grade I neoplasms with histologic (and cytologic) appearances similar to hyperplastic urothelium. Unfortunately, approximately two-thirds of these patients will develop recurrent tumors, and 10% to 20% of these recurrences will have a less differentiated histologic grade and/or invade into the lamina propria or muscle layers of the bladder. Parameters associated with recurrence and/or progression include 1) multifocal tumors, 2) coexisting severe dysplasia/carcinoma in situ (CIS), 3) loss of differentiation antigens such as blood group related antigens (BGRA), 4) development of marker chromosomes or abnormal cellular DNA content measured by flow cytometry or image analysis systems, and 5) histologic (cytologic) dedifferentiation to a higher grade. The last three factors are closely related as loss of expression of differentiation antigens is associated with development of marker chromosome abnormalities and deviation from normal (diploid) DNA con-

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tent. These genetic changes usually result in morphologic loss of differentiation. We present the current status of diagnostic procedures and techniques available for classification of TCC, as well as our experience at Henry Ford Hospital in the application of new technologies that are being studied in numerous institutions.

Pathology

The surgical pathology of urothelial neoplasia can be divided into three major groups. The most common is the papillary (noninvasive) urothelial tumor which represents approximately 75% of TCC diagnosed. These neoplasms consist of a proliferation of urothelium which is cytologically similar in appearance to normal or hyperplastic urothelium and are well differentiated/grade I TCC. The propensity to proliferate (but not invade) leads to an exophytic growth above the adjacent mucosa into the bladder lumen, resulting in the characteristic appearance at cystoscopy. The exophytic and orderly growth of differentiated papillary urothelial neoplasms is a central fibrovascular core providing blood supply and mechanical support of the expanding neoplastic urothelium. The well differentiated nature (grade I) of the majority of papillary urothelial neoplasms is reflected by the difficulties in cytologic diagnosis. Exfoliated cells from grade I papillary neoplasms are morphologically similar to cells shed from nonneoplastic inflammatory/reactive or normal urothelium. Urinary tract instrumentation or nephrolithiasis/urolithiasis may also result in exfoliation of cells, resulting in a cytologic picture indistinguishable from grade I TCC. The diagnosis of well differentiated papillary tumors requires cystoscopy with biopsy confirmation. Many patients will have numerous recurrences of papillary urothelial neoplasms, either in the same site (persistence) or at other sites of the urothelium (recurrence). Some evidence indicates that patients with severe dysplasia or CIS in the adjacent, normal-appearing urothelium are more likely to have recurrent neoplasms (1-3). The National Bladder Cancer Collaborative Group concluded that coexisting severe dysplasia/CIS at sites removed from the papillary tumor are at even higher risk for progression to invasive cancer (3,4). A second group of patients likely to develop invasive carcinoma are those who have recurrent papillary neoplasms with a less differentiated histologic grade (5). Although the frequency of progression to invasive cancer in patients with dysplasia/CIS is not accurately defined, it is unquestionably substantial. In one series, 42% of patients with differentiated papillary tumors and coexisting CIS developed invasive cancer over a 42-month period (6).

Invasive TCC represents the stage of disease responsible for tumor-related deaths. Generally invasive TCCs are poorly differentiated, high grade (III) neoplasms (5). Curiously, early focal or microscopic invasion into the submucosa or lamina propria (stage A) is not considered life-threatening because these tumors are not associated with significant frequency of regional or distant metastases and are often grouped with other "superficial" forms of TCC. However, one series found that 30% of stage A (microinvasive) TCC progressed to muscle invasion over an average follow-up period of 39 months (7). This observation supports the ominous nature of any form of tumor invasion. Nevertheless, most invasive urothelial TCCs are not associated with or preceded by papillary TCC (8). In a series of 104 patients with invasive TCC, only 20 had histories of preceding papillary (noninvasive) tumors (8). This infrequent progression of papillary tumors has been observed by others (5), and thus it is generally accepted that the majority of invasive TCCs appear de novo, without a preceding diagnosis of urothelial pathology.

Cytology

Urinary cytology is a valuable diagnostic tool in the detection and follow-up of patients with urothelial neoplasms. Cytology is diagnostic of high grade neoplasias, either in situ, papillary, or invasive. Cytologic evaluation can be made from a variety of specimens including voided urine or urine collected by instrumentation, such as catheterization or cystoscopy and bladder/urethral/ureteral washings or brushings. The diagnostic yield and accuracy depend on the method of specimen collection, cytopreparatory technique, histologic grade of the neoplasm, and the skill of the cytopathologist.

In a hypertonic environment such as urine, exfoliated cells rapidly degenerate. Hence, a 24-hour specimen or a first morning sample is suboptimal for cytologic evaluation. This is also necessary for urine from a catheter collection bag or for voided urine that contains residual urine in patients with obstructive uropathy. A voided urine sample must be submitted fresh to the laboratory and processed immediately. If delay is anticipated, the urine specimen may be fixed in an equal amount of 50% ethyl alcohol and refrigerated. The best method can be chosen with consultation of the cytopathologist. The advantage of the voided specimen is that it can be easily collected and repeated without any invasive techniques.

Instrumentation can dislodge large numbers of benign epithelial cells, both isolated and in tissue fragments. The latter may also be seen in the presence of urolithiasis. In a fluid environment these tissue fragments of benign epithelium can assume a papillary configuration and are extremely difficult to differentiate from epithelial tissue fragments exfoliated from low grade well differentiated urothelial neoplasms. A history of instrumentation or urolithiasis represents situations where false-positive diagnosis of well differentiated (grade I) TCC can occur. Indeed, the presence of benign-appearing tissue fragments of transitional epithelium in a voided urine sample is abnormal and indicative of grade I carcinoma, particularly in a patient with no history of recent instrumentation or lithiasis. It is critical that such a history be reported to the cytopathologists in order to avoid a false-positive diagnosis.

Diagnostic yield and accuracy depend greatly on the histologic grade of the tumor. With adequate sample, almost all high grade TCCs (in situ, invasive, or rarely papillary) can be identified from cytologic evaluation since malignant cells exfoliating from these lesions present with definite, recognizable criteria.

Grade I (noninvasive) TCCs lack most of the morphologic features of malignancy. They spontaneously exfoliate fewer cells because of their intercellular attachments associated with differentiation. In cytologic presentation, these neoplasms show tight cell clusters, rather than single cells, as the tips of the papillary fronds break off easily. The frequency of finding these

papillary tissue fragments depends on the size of the neoplasm and to some extent on the tumor's location. The cytomorphology of these cells resembles normal urothelium so closely that their cytologic detection is reportedly low, ranging from zero (9) to 33% (10). The cells of grade I TCC present subtle cellular and nuclear abnormalities such as mildly increased nuclear size, increased chromatin content with granularity, and irregularity of the nuclear membrane and nucleoli. By considering such features, Murphy et al (11) reported 70% diagnostic accuracy for grade I carcinomas. Grade II TCCs form a spectrum in-between grade I and grade III. The exfoliated cells usually show sufficient cellular atypia to be detected in urinary samples. The reported diagnostic accuracy of cytology in grades II and III neoplasms varies greatly, with reports ranging from 51% (9), to 73% (10), and 94% (11) of patients diagnosed.

Urinary cytology, especially bladder washing, is a reliable diagnostic test in identifying CIS. These intraepithelial carcinomas may be difficult to visualize by cytoscopy and often require random biopsies for diagnosis. The malignant cells from CIS or invasive TCC are generally high grade and readily detectable by cytology. CIS exfoliates large numbers of single cells, allowing their identification in virtually 100% of cases. However, the presence of high grade malignant cells does not indicate the presence or absence of invasion, and the differentiation of CIS and invasive carcinoma requires cystoscopy examination.

Urinary cytology is useful in monitoring patients with a history of TCC, particularly those cases which progress to less differentiated neoplasms. Bladder wash specimens sample a large proportion of the bladder mucosa and have the potential to identify nonvisualized foci of CIS.

Urothelial Surface Antigens

Epithelial cells express differentiation antigens on their membrane surface, including the blood group related ABO(H) and histocompatibility antigens. The expression of these antigens reflects a complicated biochemical pathway beginning with the gene(s) coding for the antigen, gene transcription, and protein synthesis and transport through the cytoplasm to the membrane surface. With some tumors progressing from papillary (noninvasive) to invasive TCC, a loss of BGRA occurs. This loss most likely reflects genetic alterations with either loss of the specific loci coding for BGRA or a defect in the transcription, synthesis, or transport phase.

Bergman and Javadpour (12) and Lange et al (13) demonstrated that ABO(H) antigen deletion correlated with advanced grade and stage of disease. Unfortunately, ABO(H) antigens are extremely sensitive to glycolipid extracting solvents, and the detection of these antigens is optimum only on fresh or frozen cells (14). Application of ABO(H) detection loses sensitivity when applied to formalin-fixed, paraffin-embedded biopsies of urothelial neoplasms (15).

Acquisition or expression of T antigen (Thomsen-Friedenreich) (16) appears to represent another surface antigen with prognostic value. T antigen is related to the M and N group on normal erythrocytes and can be unmasked by removal of terminal sialic acid residues with neuraminidase. The presence of T antigen is abnormal and is expressed primarily in high grade TCC. The greatest value of T antigen detection is in combination with ABO(H) expression in patients with superficial, low grade TCC. Deletion of the ABO(H) group and expression of T antigen predicts progression and tumor invasion with greater accuracy than ABO(H) deletion alone (17). The detection of T antigen also is best when performed on fresh or optimum-fixed cells or on tissue biopsy and is unreliable in formalin-fixed, paraffin-embedded biopsies.

DNA Content and Distribution

Evaluating urothelial neoplasms for DNA content is also helpful in the diagnosis and characterization of bladder tumors. The optimum technique for measurement of DNA in tumor cells is by staining with DNA dyes, such as propidium iodide, and quantitating the DNA content of individual cells using flow cytometric analysis. This new technology requires single cell suspensions which complicates analysis of solid tumors but is easily adapted for examination of urine, especially bladder washes containing large numbers of single cells. The instrument uses an exciting light (laser) which results in excitation of the DNA dye (or fluoresceinated antibody, etc) with the emission of a light of specific wave length. The emitted light for each event (cell) is measured by photosensitive light tubes and analyzed by computer. In this manner, the instrument is capable of analyzing hundreds of cells per second.

DNA analysis by flow cytometry is capable of measuring two major parameters: the total DNA content of the tumor cell population, and the fraction of cells synthesizing DNA (specific-pathogen free [SPF]). The former is calculated relative to expected DNA content of normal (diploid) cells that contain 46 chromosomes and is expressed as the DNA index. For instance, a DNA index of 1 is normal; less than or greater than 1 reflects loss or increase of chromosomal material and is abnormal (aneuploid). The SPF is expressed as a fraction of the tumor cell population which is synthesizing DNA; the greater this percentage, the more cells that are actively synthesizing DNA and presumably actively proliferating.

Flow cytometry is a technologic advance that can quantitatively measure subcellular changes not evident with the light microscope. Using propidium iodide, which differentially stains DNA, cellular changes in DNA content (ploidy) can be quantitatively measured in single cell suspensions (18). In our study of 75 bladder washings comparing flow cytometric DNA analysis of ethanol-fixed cells with cytospin prepared Papanicolaou (standard) cytologic analysis, 45% of the samples examined had normal cytology but contained abnormal DNA (hyperdiploid and tetraploid) (19). Abnormal DNA content is an indicator for the aggressive potential of bladder tumor cells since it is associated with higher histologic grades II and III and a higher frequency of progression to invasion (20,21) (Table 1). Thus, flow cytometry is useful in monitoring patients with known bladder carcinomas and may predict the papillary tumors likely to progress to invasive TCC if sufficient chromosomal abnormalities exist.

Cytogenetics

Cytogenetic markers are established parameters for the diagnosis and management of patients with hematologic disorders.

Table 1
Urothelial Neoplasia: Henry Ford Hospital Experience*

Histologic Grade	Grade I	II	III
Noninvasive (Ta)	4	7	0
Submucosal invasion (T1)	0	2	1
Muscle invasion (T2+)	0	0	12
Diploid range DNA	4	8	2
Aneuploid DNA	0	1	11
Total	4	9	13

*From Crissman JD, Liu BS, Zarbo RJ, et al. Diagnosis of urothelial neoplasia. Cytometry 1988;2(suppl):8.

Table 2
Relation of Blood Group Related Antigen, H-Ras Product (p21), and DNA Studies: Henry Ford Hospital Experience*

Tumor Cells Positive	ABH (%)	T (%)	p21 (%)
Diploid range DNA (14)	50.0	19.8	56.0
Normal cytogenetics (4)	29.4	17.4	67.0
Abnormal cytogenetics (9)	40.0	20.6	49.0
Aneuploid DNA (12)	27.9	14.6	40.3
Cystitis (8)	33.6	13.4	16.6

*From Crissman JD, Liu BS, Zarbo RJ, et al. Diagnosis of urothelial neoplasia. Cytometry 1988;2(suppl):8.

Several characteristic markers have been identified in lymphomas and leukemias, such as the translocation (15,17)—a specific rearrangement for acute promyelocytic leukemia. This chromosome change has not been described in any other form of cancer. Virtually all subgroups of acute nonlymphocytic leukemias have been further classified based on the presence of specific chromosome markers. The application of cytogenetic marker studies in hematopoietic neoplasms has progressed; cooperative cancer study groups are developing protocols for clinical trials stratified by cytogenetic study. Technical limitations have hampered progress in the cytogenetics of solid tumors. Improvements in tissue culture including tumor disaggregation by enzyme digestion, application of enriched media, and synchronization of cells in culture have contributed to advances in cytogenetic studies of solid tumors including TCC.

The value of chromosome analysis as a prognostic factor in patients with TCC was recognized as early as 1971 by Falor and Ward. Marker chromosomes predicted for tumor recurrence in a later follow-up study by the same investigators (22). Trisomy 7 and monosomy 9 have been established as primary markers in bladder cancer because these two changes were observed as sole abnormalities in some tumors (23). Establishing primary and secondary chromosome abnormalities for any tumor type is important because they may be involved in initiation and/or progression of the tumor. We identified several nonrandom changes in TCC: deletion of short arm (p) of chromosome 11, duplication of 3p, deletion of long arm (q) of 6, trisomy 7, and duplication of 1q (24). Trisomy 7 is a good candidate for a primary genetic event, whereas others are most likely secondary changes. In addition, tumors with 11p deletion and 3p duplication were associated with a higher likelihood of tumor progression. We continue to perform cytogenetic analysis on all bladder tumors in an attempt to better define chromosomal abnormalities of prognostic value (Table 2).

Oncogenes as Tumor Markers

Oncogenes are genes that cause or are associated with cancer. They represent altered versions of a group of normal genes (proto-oncogenes) that exist in every cell. When these proto-oncogenes are changed by point mutations or by translocation from their usual position on one chromosome to another or have their transcription-initiation sites altered, they may produce a protein with a different configuration, an excess of their protein product, or may express this product at an inappropriate time of the cell cycle. These changes have been shown to confer transforming properties leading to neoplasia, and therefore these altered genes are referred to as oncogenes (25-27).

DNA from certain naturally occurring human tumors can induce malignant transformation in the NIH-3T3 fibroblast cell line. The first oncogene identified in a human tumor was the *ras* oncogene, which was isolated from a human bladder cancer cell line. Examination of the *ras* gene identified in the bladder cancer cell line indicates that a point mutation at codon 12 is responsible for its transforming capabilities. This mutation has been found in some other human cancers including lung and colon carcinomas. Slamon et al (28) demonstrated cellular oncogene activity in tissues obtained from fresh human tumors. They studied tumors from 54 patients representing 20 different cell types and tested for the presence of 15 different oncogenes. The *ras* oncogene, as well as *myc* and *fos* oncogenes, were expressed in nearly all of the tumors studied. Other oncogenes were expressed infrequently, and some were not detected. Recent studies using restriction fragment length polymorphism (RFLP) of the human C-Ha-ras-1 gene indicated the association of variable tandem repeat sequences in some cancer patients with the possession of a rare Ha-ras allele (29,30). A similar association between rare alleles of C-mos and certain human cancers has also been reported (31,32), providing further evidence for the usefulness of RFLPs at proto-oncogene loci in determining predisposition of certain cancers including urothelial neoplasia.

The *ras* oncogene produces an immunogenic protein (p21) that can be used to generate antibodies which can recognize this oncogene product. The nature of bladder cancer invites application of monoclonal antibody techniques for early diagnosis and monitoring of disease since it is readily sampled. We have identified that p21 expression is a better monitor of neoplastic cell populations than BGRA deletions or expression of T antigen (33) (Table 2). Urothelial carcinomas arise in the superficial lining of the urinary tract, and these neoplastic cells are bathed constantly in urine and exfoliate readily. Cellular metabolites of the cancer may also be presumed to be secreted or shed into the urine. Using antisera generated against the *ras* oncogene product, we have identified *ras* oncoproteins in urine of patients with TCC. Using an immunologic assay (enzyme-linked immunosorbent assay), we have demonstrated the presence of *ras* expression in urine in 70% of patients with bladder cancer. Furthermore, increased level of *ras* expression was associated with

higher tumor grade and increasing stage. We also detected the *ras* oncogene product within the cytoplasm of exfoliated tumor cells in patients with elevated *ras* oncoprotein expression in their urine.

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