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BIOMARKERS FOR DETECTION, TREATMENT DECISION AND PROGNOSIS OF THE URINARY BLADDER CANCER

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Summary. *Currently, there is no perfect test for the detection of bladder cancer. Even the gold standard cystoscopy is increasingly being demonstrated to miss both Tis and papillary bladder cancer. The oldest urine-based biomarker, cytology, has high specificity but has low sensitivity and significant variability in performance. Stage and grade of transitional cell carcinoma are currently the most useful tools for taking therapeutic decisions and evaluating the prognosis of bladder cancer patients. During the last two decades, the better understanding of the molecular mechanisms involved in carcinogenesis and tumor progression has provided a large number of molecular markers of bladder cancer, with a potential diagnostic and prognostic value. Markers that distinguish among bladder cancer, normal urothelium, and benign urothelial conditions are potentially diagnostic, prognostic, and therapeutic targets. Currently, there are many research bladder tumor markers, but only a few are commercially available. The ideal urinary bladder tumor test is still unavailable, but the eventual "gold standard" will consist of multiple assays that analyze nucleic acids and proteins for detection. In addition, these tests would also reveal to the clinician both prognostic information and therapeutic targets for personalized medical treatment.*

Key words: *Urinary bladder cancer, biomarkers, diagnostic, prognostic*

Background

Urinary bladder cancer (UBC) is the fourth most common malignancy in males and the ninth most common malignancy in females in the USA. An average of 260 000 new cases of urinary bladder cancer are diagnosed worldwide every year, with an estimated 63 210 new cases and 13 180 deaths in 2005 in the USA alone (1).

The current treatment for UBC is based on the pathological staging of the tumor. The

traditional TNM classification or the WHO classification system for UC (2) relies on pattern recognition and nomenclature for reporting biopsies, the interpretation of which can be highly subjective. While current histopathological criteria can provide important morphological information about tumors in patient populations, they are unable to specify the risk for progression or response to treatment for an individual patient with UBC. Esrig et al. (3) showed the wide difference in recurrence and survival rates between patients of the same pathological stage with differences in their tumor p53 status. In a cohort of 243 patients with UBC treated by radical cystectomy, the recurrence rates for stage pT1, pT2a and pT2b tumors with negative p53 nuclear reactivity were 7%, 12% and 11%, respectively, in contrast to 62%, 56% and 80%, respectively, for tumors that had p53 immunoreactivity. That study indicated the need to incorporate objective staging methods using molecular markers specific to UBC to complement the morphological approach. Recently, the combined effects of p53, p21 and pRb expression in the progression of UC were published (4). The patients were classified into four groups: group I (no alteration in any marker, 47), group II (any one marker altered, 51), group III (any two markers altered, 42) and group IV (all three markers altered, 24). The 5-year recurrence rates in these groups were 23%, 31%, 60% and 93%, respectively, and the 5-year survival rates were 68%, 56%, 28% and 8%, respectively. These findings point strongly towards the use of multiple markers to better stage tumors, and to better determine the prognosis and predict the therapeutic response of individual patients to specific treatment.

Various histopathological and clinical parameters are known to have prognostic significance in bladder cancer. These parameters include tumor stage, histological grade, multicentricity, tumor growth pattern (solid vs. papillary), carcinoma in situ of the adjacent non-tumor-forming urothelium, and tumor cell proliferation. However, the prognostic impact of all these parameters is not considered reliable enough to assure optimal treatment decisions in individual patients and better prognosticators are urgently needed. Such new prognostic parameters may be derived from our rapidly increasing knowledge on the molecular alterations in these tumors.

Ontogeneses

Ontogeneses are normal molecular genes with implications in cell proliferation. Their products are kinases, growth factors, and their receptors. They can contribute to malignant phenotype either by the over expression of their product or by expression of altered proteins.Oncogeneses that are known to play a role in bladder cancer include erbB-2 (5), Epidermal Growth Factor Receptor (*EGFR*) (6), *c-myc* (7), *Cyclin D1* (8), and *h-RAS* (9).

Tumor suppressor genes

Tumor suppressor genes encode proteins with a protective role against malignant phenotypes. Their inactivation, due to chromosomal alterations, can lead to initiation and progression of carcinogenesis. Recently, with the help of new investigational techniques such as microsatellite analysis and fluorescent in situ hybridization (FISH), chromosomal alterations have been identified, mainly deletions of chromosome 9, 13, 17 in patients with bladder cancer. Tumor suppressor genes that have been found inactivated in bladder carcinomas include *p53* (10), *Rb* (11) and *MTS1* (12). There were reports suggesting that alterations of the p53 gene (10, 13), *erbB-2* amplification (14), *Rb*-inactivation (15) or *EGFR* over expression (16) may herald poor prognosis in bladder cancer patients.

Cell-cycle regulatory proteins

It is well known that cell cycle is a strictly controlled process regulated by protein complexes composed of cyclins and cyclin-dependent kinases (cdks) and also by several tumorsuppressor gene protein products acting at the Go/G1 checkpoint of the cell-cycle (17). Some of these protein products are p53, pRb, p16, and p14. Their role is the regulation of normal cell growth and consecutively normal cell death (apoptosis). Inactivation of one or more tumor-suppressor genes and/or loss of cell cycle control lead to inadequate phosphorylation of key proteins, which represents the first step of carcinogenesis. The inactivation of a gene occurs by different mechanisms such as mutation, deletion or methylation. It is of particular interest that in most cases the inactivation of a gene needs alterations of both alleles with the exception of p53 whereas alteration of only one allele is sufficient for altered phenotype.

Cell adhesion molecules

It is well known that cells interact with neighbouring cells and the extracellular environment. These interactions are mediated through adhesion molecules. The main representatives of the adhesion molecule family are cadherins, integrins, members of immunoglobulin superfamily, and selectins. The role of these transmembrane glycoproteins is to mediate the intercellular matrix adhesion cell-cell adhesion molecules-(CAMs). The adhesion molecules are closely involved in the control of several cellular processes such as differentiation, proliferation, invasion and colonization of distant organs (18). The reduced cell-matrix adhesion allows neoplastic cells to escape the control of differentiation and the loss of intercellular adhesion allows malignant cells to escape from the site of origin and to make new colonizations at distant organs (19). Cadherins are the most important adhesion molecules.

Telomerase

Telomeres are structures with short repetitive sequences at the ends of a chromosome, and once detached from the chromosome, they cannot reattach. Chromosomes lose 50–200 nucleotides from their telomeric structure in every division until they acquire a standard length and lead cells to apoptosis. The telomeric sequence can be reattached via an enzyme named telomerase. Although cells from normal tissue show almost no activity of this enzyme, cancer cells show high activity leading to the consequence of maintaining the telomere length and furthering cell immortality (20). Detailed research has been done for detection of telomerase activity in urine samples of patients with bladder cancer.

Genomic studies in bladder cancer

The tissue microarray (TMA) technology has the potential to significantly accelerate studies seeking for associations between molecular changes and tumor phenotype or clinical endpoints. In this technology tissue cylinders are punched from hundreds of different primary tumor blocks and subsequently brought into one empty "recipient" paraffin block. Sections from such array blocks can then be used for simultaneous in situ analysis of hundreds of primary tumors on DNA, RNA, and protein level. Multiple previous studies have demonstrated that reliable and representative results can be obtained on tissue microarrays despite the small size of the tissue samples analyzed per tumor. Most importantly, similar prevalence of amplification/protein expression and associations with clinical endpoints were always found in TMA studies as previously reported in large section analyses.

In bladder cancer the frequency of cyclin E protein expression increases from stage pTa (22.2%) to pT1 (45.5%; $p < 0.0001$) but then decreases for stage pT2-4 (29.4%; $p < 0.0001$ for pT1 versus pT2-4) (21). Low cyclin E expression is associated with poor overall survival in all patients ($p < 0.0001$), but have no prognostic impact independent of stage. It was concluded that cyclin E over expression is characteristic to a subset of bladder carcinomas, especially at the stage of early invasion.

Both amplifications and deletions of *RAF1* and *FGFR1* are significantly associated with high tumor grade ($p < 0.0001$), advanced stage ($p < 0.0001$), and poor survival ($p < 0.05$) if tumors of all of the stages where ana-

lyzed together (22). *RAF1* amplifications are associated with subsequent tumor progression in pT1 carcinomas ($p < 0.05$).

Amplifications of *HER-2* and the adjacent *TOP2A* are significantly associated with advanced tumor stage (*HER-2* $p < 0.0001$, *TOP2A* $p = 0.0218$), high grade ($p < 0.0001$ for both) and protein overexpression ($p < 0.0001$ for both) (23). Amplification frequency is highest for *TRIO*, compared *TAS2R, ADCY2, DNAH5, CTNND2, TRIO, ANKH* and *MYO10,* all located 5p15.31-5p15.1 (24). TRIO encodes a protein with a putative role in cell-cycle regulation. It's amplification is strongly associated with invasive tumor phenotype, high tumor grade, and rapid tumor cell proliferation (*Ki67 LI*) ($p < 0.0001$ each), but not poor prognosis.

Decreased *p63* immunoreactivity and *p53* overexpression are significantly associated with advanced tumor stages and poor prognosis (25).

DNA methylation

Loss of tumour suppressor gene expression through methylational silencing appears an important malignant characteristic and has been demonstrated in more than one molecular cancer pathway (26,27). Whilst the cause of altered DNA methylation is unknown, tumors with frequent hypermethylation appear to have specific pathological and clinical phenotypes, when compared to tumors with no or low levels of detectable methylation (28). For example, pathologically poorly differentiated tumors have higher levels of methylation than welldifferentiated tumors.

The methylation index (number of loci affected) sequentially increases as the lesion progresses from normal to dysplastic urothelium and finally to invasive carcinoma. This may indicate sequential tumour suppresser gene inactivation and suggest a mechanism for molecular disease progression.

Several reports have shown the presence of promoter methylation to be a poor prognostic marker (29).

Biomarkers

The ideal cancer biomarker should be both 100% sensitive and 100% specific. This means it would always be positive whenever cancer is present and never miss any cancers (sensitivity). It would also always be negative when cancer is not present and never generate false-positive results (specificity). The results based on the biomarker would have to be reproducible so that they can be readily compared between patients and within the same patient. Finally, the test based on the biomarker should be fast, easy to perform, and inexpensive. Unfortunately there is no biomarker with all these characteristics at this time.

Current urinary markers investigate entities at different levels of the cancer cell evolution and can be grouped into broad categories, including tumor-associated antigens, blood group antigens, growth factors, cell cycle/apoptosis, and extracellular matrix proteins.

The original *bladder tumor antigen (BTA)* test was based on the premise that invasive bladder cancer will degrade the basement membrane extracellular matrix, and these antigens will then be released into the urine for detection. The original BTA test has been replaced by quantitative and qualitative tests. The qualitative point-of-care test BTA stat (Polymedco, Redmond, WA) and the quantitative BTA TRAK (Polymedco) assays detect human complement factor H–related protein. The overall sensitivity of these tests ranges from 50% to 80%, whereas the specificity is between 50% and 75%. These tests have a lower specificity than cytology and can be falsely positive in patients with inflammation, infection, or hematuria (30).

The detection of *nuclear matrix protein 22*, a part of the mitotic apparatus released from the urothelial nuclei on cellular apoptosis, is the basis for the NMP-22 BladderChek test (Matritech, Newton, MA). There is a substantially higher level of NMP-22 in the urine of patients with bladder cancer. However, because this protein is released from dead and dying urothelial cells, many benign conditions of the urinary tract, such as stones, infection, inflammation, hematuria, and cystoscopy, can cause a false-positive reading. The sensitivities and specificities can vary substantially and range from 68.5% to 88.5% for sensitivity and from 65.2% to 91.3% for specificity (31). Recently, a multi-institutional trial had been completed with this NMP-22 qualitative point-of-care test, and the results showed that overall, the NMP-22 was more sensitive than cytology but less specific. Of 1331 patients who had the index test, 223 had positive test results and 1108 had negative results. Of the 223 positive-testing patients, 44 had true-positive results, and among the 1108 negative-testing patients, 35 bladder cancers were found. The NMP-22 BladderChek test sensitivities were 50% and 90% for noninvasive and invasive cancer, respectively, with an overall sensitivity of 55.7%. In contrast, cytology performed poorly with comparable sensitivities of 16.7% and 22.2% in noninvasive and invasive bladder cancers, respectively, with an overall sensitivity of 15.8%. Overall specificity was still higher for cytology at 99.2% compared with NMP-22 at 85.7%. The sensitivity of cystoscopy in this study was 88.6%; when combined with NMP-22, however, this increased to 93.7% (32).

The requirements for a biological molecule to be characterized as a molecular marker

- High sensitivity and high specificity
- Increased capability of diagnosing well-differentiated tumors
- Increased capability of diagnosis of tumors in primary stage, mainly *in situ*
- To be independent of subjective factors like methodology and examiner experience
- To provide results at a low cost
- To be a simple and easy method
- To be a method with reproducible results

Other assays

Telomerase is a protein/RNA complex involved in extension of telomeres during cell cycle DNA replication. Cancer cells have a higher level of telomerase than normal cells, and detection of telomerase RNA levels has been used to diagnose cancer. This test has shown very high specificity but poor sensitivity and reproducibility (33).

Hyaluronic acid is a nonsulphated glycosaminoglycan in the basement membrane of tissue; its degradation enzyme is hyaluronidase. These 2 proteins are important in distinguishing cancer cells from normal cells. The sensitivity and specificity for this test is between 80% and 85%, respectively (34).

Cytokeratins are another promising class of proteins, and the specific cytokeratins 18, 19, and 20 are highly expressed in bladder cancer. However, all 3 are also induced with infections. The test for cytokeratin 8 and 18 is the UBC II enzyme-linked immunosorbent assay (IDL Biotech, Borläbger, Sweden).

Conclusion

The rational approach to discovery, design, and implementation of UBC biomarkers is ongoing. New molecular biology technology is being used to examine DNA (single nucleotide polymorphisms, mutation, amplification, and deletions), RNA expression, and proteomics. Markers that distinguish among UBC, normal urothelium, and benign urothelial conditions are of potential diagnostic, prognostic, and therapeutic value. The eventual urinary test may consist of a multiple assay that detects nucleic acids as well as proteins while also being able to prognosticate patient outcome and give clinicians personalized cancer therapeutic targets.

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BIOMARKERI ZA OTKRIVANJE, LEČENJE I PROGNOZU KARCINOMA MOKRAĆNE BEŠIKE

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Kratak sadržaj: *Ne postoji savršen test za utvrđivanje karcinoma mokraćne bešike. Pokazano je da čak i cistoskopija, zlatni standard za dijagnostiku, ponekad ne prepozna tumor in situ i papilarni karcinom mokraćne bešike.Najstariji biomarker urina, citologija, ima visoku specifičnost a malu senzitivnost sa značajnim varijabilitetom u izvođenju. Stadijum i gradus karcinoma prelaznog epitela imaju najveći prognostički i terapijski značaj kod procene karcinoma bešike. Tokom poslednje dve decenije, bolje razumevanje molekulskih mehanizama u karcinogenezi i progresiji malignog procesa dalo je veliki broj molekulskih markera za moguću dijagnozu i prognozu karcinoma mokraćne bešike. Markeri koji prave razliku između karcinoma mokraćne bešike, normlnog urotelijuma i benignih oboljenja imaju dijagnosticki , prognosticki i terapijski znacaj. Trenutno se istražuje vise markera za karcinom mokraćne bešike ali je samo nekoliko dostupno na tržistu. Jos uvek ne postoji idealan test za otkrivanje karcinoma mokraćne bešike ali ce eventualni "zlatni standard" biti sačinjen od brojnih testova koji analiziraju proteine i nukleinske kiseline. Dodatno će ovi testovi moći da otkriju kliničke, prognostičke i terapijske ciljeve za lečenje, prilagođene svakom pacijentu.*

Ključne reči: *Karcinom mokraćne bešike, biomarkeri, dijagnostika, prognoza*