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STATE-OF-THE-ART IN THE MANAGEMENT OF CANCER

BLADDER CANCER — PART II
SCREENING, DIAGNOSIS, STAGING AND FOLLOW-UP

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STATE-OF-THE-ART IN THE MANAGEMENT OF CANCER

BLADDER CANCER — PART II
SCREENING, DIAGNOSIS, STAGING, AND FOLLOW-UP

Bladder cancer is primarily a disease of older males and occurs almost twice as often in whites as in blacks of either gender. Approximately 80% of newly diagnosed cases of bladder cancer in both men and women occur in those >60 years-of-age. Global epidemiology statistics by gender and putative etiology for bladder cancer were presented in FO, pp 1225-1229. The hallmark at presentation is blood in the urine with approximately 80% of bladder cancers causing microscopic or grossly visible hematuria. Other symptoms include irritable or obstructive voiding.

Various testing methodologies are being developed and evaluated to screen asymptomatic populations at high risk for bladder cancer, diagnose disease based on symp-

tomatology, stage disease to choose optimal management options, and provide follow-up to ensure early detection of recurring disease. These approaches that provide a continuum of care, also represent an attractive worldwide product opportunity. Although the incidence (new cases) of bladder cancer is low, the prevalence (survivors with cured or active bladder cancer) is high. Today, in the USA, there are over 500,000 bladder cancer survivors requiring lifelong management, with over 300,000 being routinely monitored for recurrence. It is recommended that after treatment, most patients with superficial (noninvasive) bladder tumors should undergo standard follow-up, currently involving cystoscopy and urine cytology, every 3 months in the first year, every 6 months in the second year, and once-a-year thereafter. The large prevalence of bladder cancer and the recommended frequency of follow-up, has made it an attractive target for the development of methods to detect recurrences early for effective intervention. Increased detection rates of recurrence may result in fewer cancers missed and improved outcomes.

SCREENING

Because bladder cancer mostly occurs in older males, and is often associated with certain professions and/or smoking, it may be logistically possible to eventually screen populations at risk using recently introduced approaches, or those in development. However, screening asymptomatic populations for bladder cancer is probably impractical using today's tools. The gold standard approach, cytology followed by cystoscopy, does not lend itself to screening large asymptomatic populations. Cystoscopy is invasive and expensive while urine cytology lacks sufficient sensitivity. However, newer diagnostic approaches, that are based on detecting certain markers in urine, show promise as screening tests. Most tests currently on the market or in development to detect/diagnose bladder cancer in symptomatic patients (see Exhibit 1), are also being evaluated as screening options.

Also, at this point numerous markers associated with bladder cancer have been identified (see FO, pp 1242-1244), creating an opportunity for dedicated chip arrays for the screening of this disease. Many companies are developing chip arrays for numerous applications, including the diagnosis and monitoring of bladder cancer. In July 2000, Ciphergen Biosystems (Freemont, CA) opened its first Biomarker Discovery Center dedicated to proteomics research and protein biomarker discovery, including differential protein expression, protein characterization, assay development and validation, and protein interaction studies using the company's ProteinChip systems and arrays. BDC's initial projects include collaborations in the field of bladder cancer with M. D. Anderson Cancer Center (Houston, TX).

Using the company's surface-enhanced laser desorption ionization (SELDI) technology, researchers at Eastern Virginia Medical School (Norfolk, VA) identified a potentially novel biomarker of a relative molecular mass of 3,400 that was observed in 100% (5/5) of the urine samples of pathologically documented transitional cell carcinoma (TCC) of the bladder, but only in 16.7% (2/12) of cases with benign conditions, and 18.2% (2/11) of the normal samples. Also, 2 of 7 samples from patients with a past history of TCC but with a negative diagnosis at the time of collection, displayed this protein. sThe sensitivity and specificity in diagnosing TCC with this biomarker was 100% and 80%, respectively (Vlahou A, etal, AACR-NCI-EORTC99, Abs. 688).

Further research using urine samples from 14 patients with pathologically documented TCC at the time of specimen collection, 12 patients with benign conditions of the urogenital tract, and 12 normal volunteers, identified two potentially novel TCC biomarkers, each detecting TCC with approximately 50% sensitivity and 85% specificity. However, when considered in combination, sensitivity increased to 72% with no significant change in specificity. A large scale study is currently under way to evaluate the clinical utility of these TCC-associated proteins and to identify additional markers that could lead to the development of highly sensitive diagnostic tests for this disease (Vlahou A, etal, and AACR00, Abs. 5416:852-3).

One of the problems associated with screening of nonsymptomatic populations at risk arises from the fact that bladder cancer is a rapidly growing malignancy, implying that screening would need to be performed at frequent intervals. To determine at what interval screening should be repeated to detect bladder cancer before it becomes muscle invasive, 856 men who had 14 negative daily home tests for hematuria with a chemical reagent strip 9 months previously, were asked to perform repeat tests. During the second 14-day screening period, a positive test for hematuria was obtained in 50 (5.8%) of these men. When 38 were further evaluated, 15 (39.5%) had significant urological pathologic conditions, including 8 malignancies. Bladder cancer was diagnosed in 7 (0.82%) men, with no tumor invading the muscularis propria. The finding of 7 bladder cancers in 856 men with negative hematuria tests 9 months previously, indicates that bladder cancer has a brief preclinical duration, and that testing must be repeated at least annually for screening to detect this disease consistently before invasion occurs (Messing EM, etal, J Urol, Jul 1995;154(1):57-61).

However, although home screening to detect hematuria with a chemical reagent strip results in the identification of high-grade cancers before they become muscle invading, and significantly reduces bladder cancer mortality (Messing EM, etal, Urology, Mar 1995;45(3):387-96; discussion 396-7), it does not identify low-grade tumors in the asymptomatic general population more effectively than the rate reported in symptomatic populations. This finding is based on a study that compared outcomes of 1,575 men with those of all newly diagnosed bladder cancer in men ≥50 years-of-age reported to the Wisconsin cancer registry in 1988, as well as an additional control group that declined to take part in screening. The proportion of low-

Exhibit 1
***In Vitro* Tests for the Screening, Diagnosis, and Monitoring of Bladder Cancer**

Products ❑ Developer ❑ Marketer/Licensor	Description of Diagnostic Approaches	Comments
	Cytology	
Routine cytology	Urine cytology of voided-urine samples performed by visual inspection in the laboratory	Cytology's ability to detect low-grade bladder tumors is limited; also, results require interpretation by a pathologist and are, therefore, subjective
Automated Cellular Imaging System (ACIS) ❑ ChromaVision Medical Systems	A laboratory-based cell-imaging system for cell-based disease assessment that combines proprietary, color-based imaging technology with automated microscopy	ACIS was approved by the FDA in July 1999; as of September 2000, 11 tests, including one for p53, were introduced aimed at providing early disease detection, more effective staging, and better targeted therapy in a variety of cancers, including bladder cancer; an ACIS-based test to perform DNA ploidy analysis was introduced in June 2000, and an ACIS-based test for EGFr in October 2000
ImmunoCyt ❑ DiagnoCure	ImmunoCyt is a fluorescent immunohistochemical assay using 3 MAbs, two against a mucin glycoprotein antigen, and one against CEA	ImmunoCyt was approved in the USA in March 2000 for monitoring for recurrences in conjunction with cytology; it is being investigated in screening asymptomatic populations at risk for bladder cancer
Quanticyt ❑ Urological Research Laboratory at University Hospital Nijmegen	Quanticyt is an automated, objective, reproducible, quantitative karyometric test providing cytologic findings based on two nuclear features, the 2c-deviation index (2cDI) of cell nuclei, and the mean nuclear shape; the system consists of an image processor that analyzes regular light microscopic images of bladder-wash samples; samples are scored as low-, intermediate-, or high-risk; a DNA histogram of the studied cell population is also included	Quanticyt was produced as software for the Windows-95 operating system several years ago; currently, the system is in use in several hospitals in the Netherlands but is not marketed outside the country; the largest database, 15,000 samples from 6,000 patients, is available at the Department of Urology, University Hospital Nijmegen (Prof. J. A. Schalken, j.schalken@uro.azn.nl)
Biomoda	Cytology based on photoreactive compound, (5,10,15,20-tetrakis [4-carboxyphenyl porphine] (TCPP) that is selectively taken up by cancer cells; when cells treated with TCPP are viewed under a microscope and exposed to ultraviolet light, those which have taken up TCPP fluoresce in the visible spectrum, and can be seen through a microscope	Currently, TCPP testing is being evaluated in lung cancer diagnosis and screening but may also be applicable to bladder cancer
	Cytokeratin-based Tests	
Cyfra 21-1 ❑ Fujirebio, Roche Diagnostics, etc.	Cyfra 21-1 detects soluble CK19 fragments in serum using two specific MAbs, Ks 19.1 and BM 19.21, and is available in IRMA and ELISA formats	Cyfra 21-1 tests are sold in certain European countries and Japan but have not been commercialized in the USA
Elecsys 2010 ❑ Roche Diagnostics	Cyfra 21-1 test based on electrochemiluminescent (ECL) methodology	Elecsys 2010 is a laboratory system with a multiple tumor-marker detection capability
IDeaL TPAcyk IRMA ❑ IDL Biotech	MAb-based IRMA for Cyfra 21-1	
IDeaL UBC tests ❑ IDL Biotech	IDeaL UBC tests detect CK8 and 18; there are several formats including an ELISA, an IRMA, and a rapid test	The ELISA and IRMA tests are 2-hour quantitative urine tests, while the UBC Rapid test is a 10-minute semiquantitative test based on a immunochromatographic method that qualitatively detects the presence of fragments of CK8 and CK18 in urine
Prolifigen TPA IRMA and TPA-M IRMA ❑ Sangtec Medical	Polyclonal and MAb-based IRMA to measure tissue polypeptide antigen (TPA), a serological tumor marker of CK8, 18 and 19	TPA may be used in the follow-up of non squamous epithelium-derived neoplasms

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TPS IRMA ❑ Beki Diagnostics	IRMA-based test for TPA	
Nuclear Matrix Protein (NMP) Tests		
BLCA-4 ❑ Eichrom Technologies/University of Pittsburgh	An ELISA-based quantitative assay and a qualitative one-step rapid test are under development to measure/detect NMP BLCA-4	Under investigation for the diagnosis and monitoring of bladder cancer
NMP-22 ❑ Matritech/ Massachusetts Institute of Technology (MIT)	NMP-22 is measured by an ELISA test using two MAbs, performed on stabilized voided urine	NMP-22 was approved in the USA in July 1996 for monitoring recurrence of TCC of the urinary tract and, in January 2000, for testing previously undiagnosed individuals who have symptoms, or are at risk for bladder cancer, in conjunction with the current standard of care
Other Tests		
AccuDx (formerly AuraTek FDP) ❑ Intracel/Mentor	Fibrin/fibrinogen degradation products (FDP) are detected by a simple, rapid immunoassay based on a MAb that detects fibrin D; the test is performed at the point-of-care and results are available in <7 minutes	AccuDx, an approved test, was removed from the market in 1999, and is being resigned to improve its performance; it is expected to be reintroduced in the USA in 2001
Ames Hemastix ❑ Bayer	Hemoglobin dipstick	Hemoglobin dipstick is a simple test to detect microscopic hematuria
BTA Stat ❑ Bion Diagnostic Sciences (Polymedco)	The bladder tumor antigen (BTA) Stat test is a rapid, single step immunoassay, based on two MAbs that detect the presence of human compliment Factor H related proteins (hCFHrp) in urine; it is performed at the point-of-care and results are available in <10 minutes	BTA Stat has been cleared by the FDA for the qualitative detection of hCFHrp in bladder cancer patients; the test is indicated for use as an aid in the management of bladder cancer in conjunction with cystoscopy; may exhibit low sensitivity for CIS
BTA Trak ❑ Bion Diagnostic Sciences (Polymedco)	BTA Trak is a quantitative ELISA immunoassay based on the same premise as BTA Stat	BTA Trak was approved by the FDA in April 1998 for the quantitative detection of hCFHrp in bladder cancer patients; may exhibit low sensitivity for CIS
Cancer Research Campaign Institute and the University of Cambridge	A two-site time-resolved immunofluorometric assay for detection of urothelial cancers, based on ectopic expression of the DNA replication protein Mcm5	This test does not give false positives in cases of cystitis
HA-HAase test	The HA-HAase test consists of two separate urine tests, one that detects the presence of HA, and the other the enzyme HAase which promotes the growth of new blood vessels	
Lexon	Western blot test to detect an unspecified antigen in urine	The company is developing an ELISA version of this test
SELDI mass spectrometry and ProteinChip technology ❑ Ciphergen Biosystems	Using laser capture microdissection (LCM), cells are extracted from solid tumors and proteins associated with specific forms of cancer, are identified by the proprietary surface-enhanced laser desorption ionization (SELDI)-based ProteinChip technology	This system is used to identify proteins that may represent diagnostic markers for various cancers and to monitor cancer progression, as well as potential targets for therapeutic drugs
TRAP and hTert assays ❑ Geron/Roche Diagnostics	TRAP and hTert assays are PCR-based tests that detect telomerase in urine	
UroCor/Ambion	Nonisotopic RNase cleavage assay (NIRCA)-based urine test that detects p53 mutations	A urine-based test to detect mutations in p53 has been available in the USA since 1998; a second generation test is under development
UroVysion ❑ Vysis	Multicolor fluorescence *in situ* hybridization (FISH) assay that detects genetic instability and quantifies chromosomes 3, 7, and 17, and the 9p21 region in urine or bladder washings	UroVysion was filed for approval in the USA in September 2000 for diagnosis of bladder cancer recurrence; a follow-on clinical trial to substantiate the product for early detection of bladder cancer is also planned

grade (Grade I/II) superficial (Stages Ta/T1) versus high-grade (Grade III) or invasive (≥Stage T2) cancers in cases detected by hematuria screening (screened cases), and those reported to the tumor registry (unscreened cases) were not significantly different (52.4% versus 47.7% in 21 screened and 56.8% versus 43.3% in 511 unscreened cases). Among the high-grade or invasive cases, however, the proportion of late-stage (≥T2) tumors was significantly lower in the screened compared to unscreened cases. Within a 3- to 9-year follow-up, no one in the screened group had died of bladder cancer, while 16.4% of unscreened cases had died within 2 years of diagnosis. Also, 23 (1.2%) of the 1,940 men who were solicited but chose not to participate in screening were diagnosed with bladder cancer within 18 months after what would have been their last home screening date, compared with 1.3% of participants diagnosed with bladder cancer detected by screening (Messing EM, etal, Urology, Mar 1995;45(3):387-96; discussion 396-7).

A recent promising development involves sponsorship and participation in bladder screening programs by certain industries with working environments that may expose employees to substances associated with an increased risk for bladder cancer. Various trials are ongoing to evaluate marker-based tests to screen asymptomatic populations at high risk for bladder cancer. A large screening study, initiated in November 1999, sponsored by Phillips Petroleum (Bartlesville, OK), is expected to enroll 1,500 to 2,000 patients in 3 years. It appears that the study's initial screening method consists of hematuria detection. As part of this study, Seth Paul Lerner, MD, at Baylor College of Medicine (Houston, TX), is also investigating the potential role of BTA Stat in the screening of populations at high risk for bladder cancer. Also, in January 2000, DiagnoCure (Sainte-Foy, Quebec, Canada) signed a 5-year agreement with Alcoa (Pittsburgh, PA) to supply its ImmunoCyt test as part of a clinical investigation to evaluate its role in the screening of workers at risk for bladder cancer.

However, despite promising results from various studies, no approach is currently deemed effective in the screening of asymptomatic populations for the early detection of bladder cancer.

DIAGNOSIS AND FOLLOW-UP

Several diagnostic procedures, of varying complexity, sensitivity, specificity and cost, are being employed to evaluate patients presenting with the two most common symptoms of bladder cancer, i.e., hematuria and irritative voiding symptoms. These tests span the gamut from simple *in vitro* urine evaluations to invasive procedures, and include clinical history, physical examination, voided-urine cytology, intravenous pyelography (IVP) or retrograde pyelography, and cystoscopy in combination with adequate tissue harvest by transurethral resection (TUR). Combined, urine cytology with confirmatory cystoscopy are at present the gold standard in bladder cancer diagnosis, usually in combination with IVP. Bladder-wash cytology, bimanual examination to detect palpable masses, systematic biopsies and/or bladder mapping, sonography, computed tomography (CT), bone scans, magnetic resonance imaging (MRI), positron emission tomography (PET) scanning and other, more involved procedures, are not considered routine examinations, and are only used in special situations. PET scanning has been shown to be very helpful and even more accurate than CT or MRI in this setting, but it is not in common use yet.

Because superficial bladder cancer is characterized by a low risk of progression but a high incidence of recurrence, in addition to techniques used to accurately diagnose bladder cancer, it is necessary to employ detection approaches that may identify early recurrences. The prevalence of recurrence after initial treatment, and the natural history of bladder cancer, mandates long-term follow-up.

In Vitro Testing Approaches

In vitro tests, particularly when performed in voided-urine samples, represent the ideal approach to the management of bladder cancer from screening asymptomatic populations to monitoring treatment outcomes. Other sample sources include bladder washings, biopsy tissue and serum. Tests are based on a variety of approaches from simple dipstick tests for hematuria to complex PCR-based technologies that may allow the sensitive detection of cancer-related genetic mutations in exfoliated tumor material, potentially being so accurate as to obviate the use of invasive techniques.

Several new tests to diagnose/monitor bladder cancer have been approved for clinical use, and numerous others are under evaluation (see Exhibit 1). Some of these tests, such as nuclear matrix protein (NMP)-22 assay, bladder tumor antigen (BTA) Trak, BLCA-4, telomerase activity, etc., are objective and quantitative, while others such as BTA Stat and fibrin/fibrinogen degradation products (FDP) are qualitative. All of these tests that exhibit a higher sensitivity than cytology, aim to detect clinically occult bladder cancer and thus increase the interval of cystoscopic evaluation. However, some have a lower specificity than cytology. If a marker has high specificity, any test-directed biopsies are frequently positive; if a test has high sensitivity, there is a low risk that deferring cystoscopy will cause a cancer to be missed. Currently, no marker or combination of markers has 100% sensitivity and specificity (Grossman HB, Semin Urol Oncol, Feb 1998;16(1):17-22).

So far, approved tests have achieved only a marginal role in clinical practice. In order to fulfill their goal, i.e. to replace urine cytology and limit or even eliminate the need for cystoscopy, these tests must exhibit higher and more consistent levels of sensitivity and specificity. The potential opportunity in diagnostic and follow-up use of bladder cancer tests in the USA probably involves about 2.25 million to 3.25 million first-time tests, and about 750,000 follow-up tests annually. Prices at the manufacturer's level for the new rapid tests in the USA range from $10.00 to $18.00 per procedure.

The performance of urine cytology, BTA Stat, NPM-22, FDP, telomerase, chemiluminescent hemoglobin and hemoglobin dipstick, were prospectively evaluated at Mayo Graduate School of Medicine (Rochester, MN), in terms of sensitivity and specificity in the detection of bladder cancer (Ramakumar S, etal, J Urol, Feb 1999;161(2):388-94). Single voided-urine specimens were obtained from 57 patients with bladder cancer, and from 139 without evidence of bladder malignancy on cystoscopy, or a negative biopsy of indeterminate lesions. A cytology report, available for 125 patients, was interpreted independently. BTA Stat, NPM-22 and FDP were analyzed according to manufacturer specifications while the telomerase assay was performed on cells collected from urine by centrifugation in preparation for polymerase chain reaction (PCR)-based amplification using the telomeric repeat amplification protocol (TRAP) assay. The chemiluminescent screening assay for hemoglobin in urine was based on the pseudoperoxidase activity of hemoglobin on hydrogen peroxide, and subsequent oxidation of 7-dimethylaminonaphthalene-1,2-dicarbonic acid hydrazide to generate chemiluminescence emission. Hemoglobin dipstick was interpreted as positive if the hemoglobin content in the urine was trace or greater. Results were as follows:

Assay	Overall Sensitivity (%)	Overall Specificity (%)
Cytology	44	95
BTA Stat	74	73
NMP-22	53	60
FDP	52	91
Telomerase (TRAP)	70	99
Chemiluminescent hemoglobin	67	63
Hemoglobin dipstick	47	84

Telomerase exhibited the strongest association with bladder cancer among all tests with a 69% overall concordance. Telomerase was also positive in 10/11 (91%) cases of carcinoma *in situ* (CIS). Urinary telomerase had the highest combination of sensitivity and specificity for bladder cancer screening in these patients, and was the strongest predictor with superior accuracy in patients with Grade I noninvasive tumors (Ta), and extremely useful in patients with CIS. Telomerase appears to be promising, and outperformed all other evaluated tests in the prediction of bladder cancer.

In most direct comparisons, the NPM-22 and BTA Stat tests were found to be better than voided-urine cytology in detecting low-grade bladder tumors but not high-grade tumors. Urine cytology consistently had fewer false-positive results than did either NPM-22 or BTA Stat alone. In a prospective study, undertaken to evaluate the diagnostic efficacy of BTA Stat and NMP-22, compared with voided-urine cytology, in the detection of primary and recurrent bladder cancer, 147 patients (85 with no bladder cancer history and 62 previously diagnosed with superficial bladder cancer) provided a single voided-urine sample prior to cystoscopy. A group of 21 healthy age-matched volunteers served as controls. Bladder cancer was confirmed histologically in 99 patients, 62 with primary tumors and 37 with recurrent ones. The overall sensitivity and specificity were 71.7% and 56.5% for BTA Stat, 62.6% and 73.9% for NPM-22, and 38.4%, and 94.2% for voided-urine cytology. BTA Stat was significantly more sensitive than cytology in detecting bladder cancer in all stage and grade subgroups, except Grade III. However, NPM-22 was significantly more sensitive than urine cytology only in Stage Ta, Grade I and II patients. The sensitivity of BTA Stat was higher but not significantly different than NMP-22. Results of this study indicate that both BTA Stat and NMP-22 are superior to cytology in the detection of bladder cancer with BTA Stat being more sensitive than NMP-22, and NMP-22 more specific than BTA Stat. Ruling out diseases with potential interference can increase the overall specificity of both tests. Also, false-positive results of either test in patients followed-up for bladder cancer seem to correspond to future recurrences (Giannopoulos A, etal, Urology, Jun 2000;55(6):871-5).

However, one of the limitation of urinary tumor marker tests is their low specificity and positive predictive value, which clinically result in a high false-positive rate. When investigators at Cleveland Clinic analyzed the false-positive data of NMP-22 and BTA Stat, they concluded that awareness and exclusion of conditions that produce false-positive results can increase the specificity and enhance the clinical usefulness of NMP-22 and BTA Stat tests. Similarly, treating an atypical cytology as positive can enhance the sensitivity and usefulness of urinary cytology. In this study, single voided-urine samples from 278 symptomatic patients (microscopic hematuria=112, gross hematuria=77, and chronic symptoms of urinary frequency or dysuria=89) were divided into 3 aliquots, and each was evaluated by NMP-22, BTA Stat and cytology. All patients subsequently underwent office cystoscopy and bladder biopsy, if indicated.

Of 34 (12%) cases of histologically confirmed bladder cancer, NMP-22 detected 28 (82.4%), BTA Stat 23 (67.7%), and cytology only 10 (29.4%). When atypical cytologies were considered positive, cytology detected 19 (55.9%) cases. Elevated NMP-22 values were positive in 28 cases and false-positive in 44 for a specificity of 82%, and a positive predictive value of 38.9%. Similarly, BTA test was positive in 23 cases and false-positive in 43, for a specificity of 82.4%, and a positive predictive value of 34.9%. When atypical cytologies were considered positive, the specificity and positive predictive value of cytology were 93% and 55.9%, respectively. Greater than 80% of the false-positive results were clinically categorized as benign inflammatory or infectious conditions, renal or bladder calculi, recent history of a foreign body in the urinary tract, bowel interposition segment, another genitourinary cancer, or an instrumented urinary sample. History of ureteral stents or

any bowel interposition segment had a 100% false-positive rate. Exclusion of all 6 clinical categories improved the specificity and positive predictive value of NMP-22 to 95.6% and 87.5%, and of BTA Stat to 91.5% and 69.7%, respectively (Sharma S, etal, J Urol, Jul 1999;162(1):53-7).

Urine cytology/cytopathology microscopically identifies the presence of abnormal, malignant cells, which are shed into the urine of patients with bladder cancer. Although most cytology is performed on voided-urine samples, bladder-wash cytology provides superior results. Voided-urine cytology is still the most commonly used noninvasive test to diagnose and monitor bladder cancer, with between 3 million and 4 million urine cytologies being performed in the USA every year. In the USA, routine voided-urine cytology is reimbursed at a $50.00 rate.

Urine cytology is noninvasive, and quite accurate in detecting high-grade bladder cancer and CIS, exhibiting high specificity with few false-positive results. However, its ability to identify only bladder tumors is limited, resulting in many false-negatives, especially in superficial and low-grade tumors, and its results require interpretation by a pathologist, are not available immediately, and are subjective.

Although much effort is being invested in identifying markers of early bladder cancer that can be detected or measured with simple, reproducible, objective tests, considerable effort is also being directed towards improving cytology. Some approaches involve improving the quality of the sample while others concentrate on optimizing cell image analysis.

ImmunoCyt, developed by DiagnoCure, is an immunocytochemical test that uses a cocktail of three MAbs, two of which, M344 and LDQ10, bind to mucin glycoprotein antigens, and MAb 19A211 against CEA. In TCC of the bladder, these three antigens are present in exfoliated urothelial cells of patients previously diagnosed with bladder cancer. In ImmunoCyt, the antibodies are labeled with fluorescent markers, and cells expressing the three tumor antigens are detected by fluorescence microscopy.

In a prospective study performed at the University of Vienna in Austria, voided-urine specimens from 264 consecutive patients, including 114 with symptoms suggestive of bladder cancer, and 150 who were being followed after complete transurethral resection of superficial TCC, were evaluated by standard cytology and ImmunoCyt. In all cases cystoscopy was subsequently performed, and any suspicious lesions were evaluated by biopsy. Histologically proven TCC was diagnosed in 79 patients. The overall sensitivity of combined ImmunoCyt and cytology was 89.9% (Grade I=84%, Grade II=88% and Grade III=96.5%). A total of 34 patients (43%) were positive on ImmunoCyt only, 3 (3.8%) on cytology only; in 34 (43%) cases both evaluations were positive, and in 8 cases (10.1%) both tests were negative. Overall, the sensitivity of ImmunoCyt alone was 86.1% (Grade I=84%, Grade II=84% and Grade III=89.6%), and its specificity was 79.4%, compared to a sensitivity of 46.8% for cytology alone (Grade I=4%, Grade II=52% and Grade III=79.3%), and a specificity of 98.2%. In this study ImmunoCyt proved to be a highly sensitive test for detecting TCC of all grades and stages. Therefore, combined with conventional cytology, it may replace cystoscopy in select patients, especially in follow-up protocols for low-grade TCC (Mian C, etal, J Urology, May 1999;161 (5):1486-9). Based on these results, however, it appears that invasive disease is less readily detected using this test.

In the USA, ImmunoCyt obtained 510(k) approval (#K994356) from the FDA in March 2000 for early detection of bladder cancer recurrences, was launched in July 2000, and is being marketed by Dako (Carpinteria, CA). ImmunoCyt is also marketed in Canada by Faulding (Montreal, Ontario, Canada) and is distributed in Europe and North Africa by various other companies.

Quanticyt, a karyometric system developed at the Urological Research Laboratory at University Hospital (Nijmegen, The Netherlands), is a quantitative automated bladder-wash test providing cytologic findings based on two nuclear features, the 2c-deviation index (2cDI) of cell nuclei, and the mean nuclear shape. The test is an objective reproducible quantitative alternative for routine cytology. Samples are scored as low, intermediate, or high risk. The system that consists of an image processor that analyzes regular light microscopic images of bladder-wash samples, is small and easy to use in daily practice, and also includes a DNA histogram of the studied cell population. Because data is stored in a database system, Quanticyt scores from earlier findings can easily be compared with current readings to detect any deviations that may signal the need for a change in patient management.

In a random set of 100 bladder-wash samples from a population of 1,614 bladder cancer cases, both Quanticyt and routine bladder-wash cytology produced similar results in detecting cytoscopically determined malignant lesions. When cytologic and image analysis results were compared for prediction of a cystoscopic lesion, histologic abnormalities, and tumor recurrence in the same 100 Papanicolaou-stained slides read and interpreted by four experienced pathologists, both the image analysis system and the cytologic examination detected all of the high-grade lesions, but image analysis was superior to routine cytology for the prediction of tumor recurrence after normal findings at cystoscopic examination (van der Poel HG, etal, Mod Pathol, Oct 1997;10(10):976-82). Also, adding consecutive quantitative cytology to urine cytopathologic evaluation improved the detection rate of high-grade lesions. Combining quantitative cytology and visual cytology

provided a more accurate prediction of tumor stage (van der Poel HG, etal, Urology 2000 Oct 1;56(4):584-8).

Various other approaches based on image analysis are being pursued to enhance the performance of urine cytology. One such approach uses quick-staining urinary cytology and bladder-wash image analysis to enhance the diagnosis of recurrent bladder cancer, and identify patients in need of more intensive follow-up. This technique also seems to overcome the diagnostic limits of cystoscopy and cytology that are attributable to the effects of most therapeutic regimens on the bladder epithelium. In an attempt to improve results of cytology, voided-urine and bladder-wash specimens were gathered in 223 follow-up sessions of 124 patients with a history of bladder cancer. Hemacolor (Merck KGaA; Darmstadt, Germany)-stained cytospin preparations of voided-urine specimens were evaluated by visual cytology, and Feulgen-Schiff-stained cytospin preparations of bladder washings by image analysis. A special software was used to classify the DNA histogram by a risk factor for bladder cancer. Follow-up of patients revealed 83 tumor recurrences. Depending on tumor grade, the sensitivity of quick-staining cytology was 13.6%, 46.2%, and 86.4%, for Grade I, II and III TCC, respectively. By combining the results of both methods sensitivity increased to 31.8%, 66.7%, and 90.9%, respectively. In the case of 24 of 140 image analyses that denoted high risk for bladder cancer without simultaneously visible tumor, correct evidence of high risk was found in 92.2% of cases (Wiener HG, etal, Cancer, 25 Oct 1999;87(5):263-9).

Neural network-based digitized cell imaging, using bladder-wash specimens, may also evolve into a promising cytology technique. Investigators at University Medical Centre (Nijmegen, The Netherlands) evaluated, in a pilot study, whether it is possible to apply neural network-based diagnostics on bladder washings to detect bladder cancer. Slides of 85 bladder-wash samples chosen at random from cases in which cystoscopy, histology, and follow-up data concerning tumor recurrence were available, were scanned by the neural network-based digitized cell image system. This system provided 128 digitized cell images. Visual cytology, reported as negative, low-grade tumor, and high-grade tumor, was performed by an experienced cytopathologist. Finally, an automatic Quanticyt analysis was performed on the same specimen, classifying findings as low, intermediate, and high risk. The sensitivity for diagnosing a histologically confirmed tumor was 92% for the neural network, 50% for conventional cytology, and 69% for Quanticyt. For the prediction of a positive cystoscopy, the highest area under the curve (AUC) was found for neural network imaging, at 0.71, compared with 0.58 for cytology. Quanticyt analysis had the highest AUC value (0.62) for predicting tumor recurrence after a negative cystoscopy, with a 0.50 value for neural network imaging (Vriesema JL, etal, Diagn Cytopathol, Sep 2000;23(3):171-9).

However, in another study image-analysis cytometry using catheterized urine as the specimen, provided a limited but not significant advantage over conventional urine cytology in the detection of primary superficial TCC, but seemed to be indicated for the prediction of tumor recurrence and/or progression. Among 23 primary superficial TCC, 11 (48%) were detected by urinary cytology, 12 (52%) by image-analysis cytometry, and 13 (57%) by combined cytology and cytometry. Of 42 recurrent superficial TCC, 29 (69%) were detected by urinary cytology, 19 (45%) by cytometry, and 29 (69%) by combined cytology and cytometry (Desgrippes A, etal, BJU Int, Mar 2000;85(4):434-6).

Bladder tumor antigen (BTA) test was originally a latex agglutination test designed to detect a basement membrane protein antigen that is released into the urine as tumor invades the underlying bladder wall. Initially, the BTA test was reported to be better than urine cytology at identifying patients with bladder cancer, and more accurate in monitoring patients with a history of bladder cancer for recurrent disease. However, subsequent studies did not support these results, especially because tests were falsely positive in patients with other genitourinary diseases (i.e., cystitis). In view of its poor sensitivity and positive predictive value, the BTA test appeared to have a limited role in the initial management of hematuria (Chong TW and Cheng C, Singapore Med J, Sep 1999;40(9):578-80).

Subsequently, the BTA test was replaced by 2 MAb-based tests, BTA Stat, a qualitative test (positive or negative), and BTA Trak, a quantitative assay, that detect a human complement factor H-related protein (hCFHrp) that is similar in composition structure and function to human complement factor H (hCFH) whose action protects tumor cells from the body's natural immune system (Corey MJ, etal, J Biol Chem, 28 Apr 2000;275:12917-25). Produced by several bladder cancer cell lines, and by human bladder cancers, hCFHrp is not associated with other epithelial cell lines (Kinders R, etal, Clin Cancer Res, Oct 1998;4:2511-20). The two MAbs used in these kits were obtained by immunizing mice with partially purified protein preparations derived from the urine of bladder cancer patients. Both these immunoassays have been approved by the FDA.

The rationale for the qualitative detection of hCFHrp in the follow-up of bladder cancer patients was investigated in a study conducted at Freie Universitat (Berlin, Germany). Urine samples from 354 individuals (healthy volunteers=76, patients with benign urologic disorders=111, and patients with histologically proven bladder cancer=167) were tested prior to therapy for the presence of hCFHrp. Overall sensitivity of this qualitative test was 62.9%. Sensitivity of low-grade/low-stage tumors was <50%, comparable to the published sensitivity range of

cytology. In late-stage, high-grade tumors sensitivity was 100% and 77.3%, respectively. Superficial tumors with high risk of progression (T1, Grade III) were detected significantly better (88.9% sensitivity) than low-risk superficial tumors (Ta, Grade I-III, and T1, Grade I-II; 48.2%). The overall specificity (healthy individuals and patients with benign urologic disease) was 93.0%. The sensitivity of hCFHrp was comparable to cytology and its specificity was high (Heicappell R, etal, Urol Int 2000;65(4):181-184).

Both the BTA Stat and Trak tests accurately identify about two thirds of patients with bladder cancer. Various studies found BTA Stat to be highly sensitive for the diagnosis of primary bladder cancer, especially for tumors >2 cm, or high-grade and invasive tumors. However, both tests are limited by false-positive results in patients with recent genitourinary trauma (including cystoscopy), stone disease, urinary tract inflammation, and other genitourinary tumors. In addition, administration of intravesical BCG within the preceding 2 years may affect results.

The BTA Stat test has been cleared by the FDA for the qualitative detection of bladder cancer recurrence in patients diagnosed with bladder tumors. BTA Stat is similar in concept to the urine dipstick test, relying on a color change reaction to indicate the result. It consists of a test strip where five drops of voided urine are deposited and can be read in five minutes by the appearance of a colored line in the test window, while a colored line appears in a "check" window to indicate the test is working properly. The test is indicated for use as an aid in the management of bladder cancer patients in conjunction with cystoscopy. It is cleared in the USA for use by clinical and physician laboratories, and can also be used by the bladder cancer patient at home when prescribed by a physician.

The original BTA test was marketed by Bard Diagnostic Sciences, a subsidiary of C. R. Bard. In the USA, this test has been supplanted by the BTA Stat test and the BTA Trak assay, developed by Bion Diagnostic Sciences (BDS; Redmond, WA), formerly Bard Diagnostic Sciences, and now a subsidiary of Polymedco (Cortlandt Manor, NY) that markets these two tests in the USA and abroad, and a latex agglutination version of BTA in Japan.

In a prospective multicenter study, and the largest of its kind to date, conducted at Tampere University Hospital, in Finland, voided-urine samples of 151 patients with newly diagnosed bladder cancer were obtained before TUR. The samples were divided for cytology and BTA Stat testing. The overall sensitivity of BTA Stat and cytology for detecting primary bladder cancer was 81.5% and 30.3%, respectively. Sensitivity of each test increased as tumor size, number, histologic grade and stage increased, and sensitivity of BTA Stat was superior to that of cytology in all tumor categories (Raitanen MP, etal, J Urol, Jun 2000;163(6):1689-92).

Specificity of the BTA Stat test has been reported as 72%-95% (Sarosdy MF, etal, Urology, Sep 1997;50(3):349-53). In a study, conducted at Seinajoki Central Hospital (Tampere, Finland), the specificity of the test was evaluated in 100 healthy volunteers. Voided-urine samples were collected and tested using BTA Stat. In the case of a positive result, IVP, cystoscopy, ultrasound and retesting were performed and BTA Stat was repeated three months later. No bladder cancer was found in 2 subjects who tested positive by BTA Stat. Specificity of BTA Stat in the studied healthy population was 98% (Raitanen MP and Tammela TL, Scand J Urol Nephrol, Aug 1999;33(4):234-6).

BTA Stat, however, does not represent an alternative to cystoscopy, because it has a low sensitivity in Grade I and in Ta and T1 tumors. BTA Stat and cytology were performed on a sample of recently voided urine, obtained prior to cystoscopy, from 122 asymptomatic patients on follow-up for superficial bladder carcinoma. Cystoscopy and TUR were subsequently performed in those patients suspected of having recurrent bladder carcinoma. Of the 122 patients, 51 had bladder cancer and 71 were tumor free. BTA Stat was superior to cytology in the follow-up of patients with bladder cancer (Gutierrez Banos JL, etal, Arch Esp Urol, Oct 1999;52(8):856-61). Results are presented in Exhibit 2.

Based on a multicenter study conducted at the Hadassah Medical Center in Israel, led by Dr. Dov Pode, it was concluded that BTA Stat can be used as a screening test for bladder cancer in symptomatic patients. Voided-urine samples were collected from 250 patients recruited from three medical centers and tested by cytology and BTA Stat; of these patients, 162 were monitored following resection of bladder tumors, and 88 were evaluated for the first time for hematuria or irritative voiding symptoms. All patients underwent cystoscopy and biopsies were obtained when a bladder tumor was seen or if CIS was suspected. Cystoscopy found no tumors in 122 patients, primary TCC was found in 71, and recurrent tumors in 57. BTA Stat sensitivity was 90.1% in the symptomatic (primary cancer) group, and 73.7% in the surveillance group for an overall sensitivity of 82.8%. All patients with CIS, high-grade tumors, muscle-invasive cancer, and tumors larger than 2 cm were diagnosed by BTA Stat. Therefore, BTA Stat can be used as a screening test for bladder cancer in patients with hematuria or irritative voiding symptoms, and for surveillance of those who have not been treated with intravesical BCG (Pode D, etal, J Urol, Feb 1999;161(2):443-6).

BTA Stat was compared with voided-urine cytology on the same specimens from 100 patients with no history of bladder cancer who had signs and symptoms of dysuria, incontinence, and gross hematuria and microhematuria. Patients were followed for up to 12 months with repeated urine cytologic testing, cystoscopy, and bladder biopsy, when clinically indicated. BTA found 19 positive, and 81 negative cases whereas cytology diagnosed 3 cases as unequivocally positive for TCC, 93 cases as negative, and 4 cases in which unqualified atypical urothelial cells were noted. TCC was confirmed by cystoscopy and bladder biopsy in all 3 cases diagnosed by cytology, and in 3 of 19

Exhibit 2
Comparison of BTA Stat and Cytology in Bladder Cancer Monitoring

Test	Sensitivity (%)							Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
	Overall	G 1	G2	G3	Ta	T1	T2-4 and Tis			
BTA Stat	60.78	23.00	71.40	92.80	46.60	42.00	100.00	77.50	77.50	75.60
Cytology	45.00	15.30	37.50	85.70	26.60	40.00	80.00	85.16	85.16	70.5
Cystoscopy	98.00							87.70	87.70	98.46

cases that tested positive by the BTA Stat, for a sensitivity of both tests of 100% and an 84% false-positive rate for BTA Stat and no false-positive cases for cytology during the 12-month follow-up period. These results indicate that although the sensitivity of BTA Stat is comparable to that of cytology, its relatively high false-positive rate limits the role of BTA Stat as an adjunct to urine cytology for bladder cancer screening (Nasuti JF, etal, Diagn Cytopathol, Jul 1999;21(1):27-9).

The BTA Trak assay is a quantitative sandwich enzyme-linked immunsorbent assay (ELISA), that can only be performed by a laboratory. Use of BTA Trak allows tracking any rise or fall of hCFHrp. Thus, the BTA Stat and Trak tests provide a continuum of care. In a retrospective study, BTA Trak was positive in 156 of 216 samples from patients diagnosed with bladder cancer, for an overall sensitivity of 72%. Mean values increased with progressing disease grade and stage. In the comparison between BTA Trak and cytology, the overall sensitivities were 68% and 25%, respectively. For Stages Ta and T1 and for all tumor grades, BTA Trak sensitivity was significantly greater than that of cytology. The specificity of the TRAK assay ranged from 75% in samples from patients with genitourinary disease, to 97% in healthy volunteers (Ellis WJ, etal, Urology, Dec 1997;50(6):882-7).

However, although BTA Trak is a sensitive test for detection of bladder cancer and for identification of patients at high risk, because of its high rate of false-positives in patients with benign urologic diseases, the test should not be used in unselected populations. In a study using BTA Trak to examine urine samples of 298 individuals (healthy volunteers=76, patients with benign urologic disorders=118, and patients with histologically proven bladder cancer=104), obtained prior to therapy, for the presence of hCFHrp, in comparison to healthy volunteers, patients with TCC had significantly higher urinary levels of hCFHrp (117.60 versus 2.05 U/ml). Levels of hCFHrp were positively correlated with tumor grade and stage; patients with invasive TCC had significantly higher levels of hCFHrp than patients with superficial TCC. Marker levels in superficial bladder cancer at high risk of tumor progression (T1, Grade III) were significantly higher as compared to low- and intermediate-grade superficial cancers. Elevated levels of hCFHrp were also found in patients with benign urologic disorders (median=72.65 U/ml). Using a cutoff of 17.1 U/ml, BTA Trak had a sensitivity of 72.1% and, because of a high rate of false-positive determinations in patients with benign urologic disorders, an overall specificity of 50.5% (Heicappell R, etal, Eur Urol, Jan 1999;35(1):81-7).

Nuclear matrix proteins comprise the structural component of the nucleus that play a central role in the regulation of a number of nuclear processes. The nuclear matrix, first described in 1974, is the nonchromatin structure that supports nuclear shape, organizes DNA, and has a part in DNA replication and transcription and in the processing of RNA.

NPM-22 test is a quantitative enzyme immunoassay (EIA), developed by Matritech (Newton, MA), that detects and measures, in stabilized voided-urine, the amount of NPM-22, nuclear mitotic apparatus (NuMA), found in human epithelial cells to detect TCC of the urinary tract. NMP-22, found in the nuclear matrix of all cell types, is involved in proper distribution of chromatin to daughter cells during cell division. NMP-22 is thought to be released from the nuclei of tumor cells after they die and can be detected in the urine. It has been shown that the majority of patients with bladder cancer release large quantities of this NMP into their urine, and although NPM-22 proteins are also found in all bladder cells, elevated levels (>25-fold) are correlated with the presence of bladder cancer.

The NPM-22 test was approved by the FDA in July 1996 for monitoring recurrence of TCC of the urinary tract. In July 1999 Matritech submitted to the FDA results from clinical trials involving 1,147 subjects at 33 clinical sites in the USA, that demonstrated that the sensitivity of NPM-22 in detecting the later stage, more life-threatening forms of bladder cancer in previously undiagnosed patients who presented with a risk profile that included smoking and/or hematuria, was 85%. Overall, the sensitivity of the test was 70% for all forms of bladder cancer combined, and was more than twice as sensitive as urine cytology in identifying early-stage, low-grade bladder cancer. In addition, based on infor-

mation gleaned from the trials, NPM-22, when used in conjunction with urine cytology, identified 100% of the late-stage, invasive bladder cancers, whereas 45% of these serious conditions would have been missed using urine cytology alone. Based on these findings, in December 1999, an FDA advisory panel unanimously recommended approval of the NPM-22 test for the expanded claim of testing previously undiagnosed individuals who have symptoms, or are at risk for bladder cancer. This recommendation was based on two conditions, namely an increase in the NMP-22 "cutoff" value and the inclusion of language on the label suggesting the test is to be used in conjunction with the current standard of care. After these conditions were met, the FDA approved marketing of NMP-22 in this setting in January 2000.

Regarding the cutoff value, if NPM-22 value is low (≤10 U/ml) 10 or more days after surgery, there is a high probability that the follow-up cystoscopic examination will not indicate a recurrence of disease and, therefore, the urologist may decide to postpone this exam in order to reduce the cost, anxiety and risk to the patient. Similarly, an NPM-22 value >10 U/ml indicates a higher probability that the follow-up cystoscopic examination will indicate a recurrence of disease.

Matritech licensed the research on which the NMP-22 test is based from the Massachusetts Institute of Technology (MIT; Cambridge, MA). In March 1999 Matritech was issued patent #5,882,876 by the U.S. Patent and Trademark Office for using a NMP in the NPM-22 test for bladder cancer which provides additional protection for use of this methodology until 2015.

The NMP-22 test is currently approved in the USA, Europe and Japan for the management of patients with TCC of the urinary tract, and in Japan and the USA for urothelial cancer screening. In the USA the test is billed at $50 at the patient level. Revenues of NPM-22 test are estimated at about $500,000 in 2000.

The test was launched in Europe in April 1995 and is currently marketed there by various distributors. After an agreement with Hybritech (San Diego, CA) to comarket the test in the USA and Europe ended in November 1995, Matritech, in March 1998, entered into a coexclusive distribution agreement for the NPM-22 test kit in the USA with Fisher Diagnostics, a Fisher Scientific (Hampton, NH) company. Pursuant to this agreement, Matritech retained its right to distribute NPM-22 in the USA through its own sales force, but granted Fisher an otherwise exclusive right to distribute NPM-22 to hospitals and commercial laboratories within the USA. Matritech has retained worldwide manufacturing rights.

Matritech has entered into various distribution agreements abroad. Paladin Labs (Toronto, Canada) is the exclusive distributor of NPM-22 in Canada. In May 1998, Konica (Tokyo, Japan), Matritech's Japanese marketing partner for NPM-22 since 1994, announced that the Japanese Ministry of Health and Welfare approved NPM-22 for monitoring as well as testing high risk, previously undiagnosed bladder cancer patients. In August 1999, based on clinical data compiled at the Beijing Tumor Institute, the State Drug Administration (SDA) in the People's Republic of China approved NPM-22 for the detection and management of bladder cancer. NPM-22 is being distributed in China by General Biologicals (Hsinchu, Taiwan) which also launched the test in September 1999 in Taiwan for use as part of an annual screening program for the early identification of individuals with bladder cancer. The company has also been shipping kits to hospitals in Hong Kong and Singapore. In June 2000, Matritech acquired ADL (Freiburg, Germany), a European distributor of NMP-22 and other diagnostic products.

Extensive clinical trials have been conducted with the NPM-22 test both to detect recurrences in populations previously diagnosed with TCC, and/or as a screening tool in symptomatic populations at risk for bladder cancer. In a study conducted at Massachusetts General Hospital, Boston Medical Center, in Boston, and at M. D. Anderson Cancer Center, a total of 231 patients with a history of TCC provided, before cystoscopic examination, 288 voided-urine samples (53 patients were re-evaluated at least once) that were assayed using the NMP-22 test. Selected patients underwent biopsy with appropriate additional therapy. Cytology was performed in 200 cases. Negative cystoscopy was an indication of absence of tumor while presence of tumor was determined by positive biopsy. In positive biopsies, NMP-22 values were correlated with tumor stage and grade and, in patients with complete data, they were compared to cytology results. There were 208 negative cystoscopies (158 with cytology) and 66 positive cystoscopies with biopsy (42 with cytology), and 14 cases were eliminated from statistical analysis because of incomplete data. Sensitivity of NMP-22 was 67% compared to 31% or 40%, depending on the definition of positive cytology, and specificity of NMP-22 was 80%. NMP-22 results were superior to those obtained with urine cytology for detection of TCC of the bladder with sensitivity being as much as twice that of cytology when a reference value of 6.4 U/ml was used. Also, NMP-22 analysis was less costly than cytology, and was operator independent (Stampfer DS, etal, J Urol, Feb 1998;159(2):394-8; comments pp. 399-400; erratum in J Urol, May 1998;159(5):1650). The overall sensitivity of NPM-22 for high-risk tumors was 90%.

In another study, performed at Cleveland Clinic Foundation, voided-urine samples from 330 patients were evaluated with NMP-22 and cytopathology prior to a cystoscopic examination. There were 114 (34.5%) patients with microscopic hematuria, 66 (20.4%) with gross hematuria and the others presented with atypical

cytology, or unexplained voiding symptoms refractory to medical therapy. There were 18 patients with biopsy-confirmed bladder cancer, and 312 with benign conditions of the bladder. Urinary NMP-22 values in the bladder cancer group were significantly higher than those in the benign condition group with a median NMP-22 reference value for the malignant bladder tumors of 31.6 U/ml, and 4.3 U/ml for benign conditions. Sensitivity of NMP-22 was 100% with a specificity of 85% at a reference value of 10.0 U/ml, while cytology had a sensitivity of 33%, and a specificity of 100%. Given a negative predictive value of 100% for NMP-22, a cost savings of $28,302 to $111,072 was achievable, depending on reimbursement rates, if this test was used alone as the indication for cystoscopy (Zippe C, etal, J Urol, Jan 1999;161(1):62-5 and Zippe C, etal, Anticancer Res, Jul-Aug 1999;19(4A):2621-3).

In a multicenter trial, conducted in Japan, the clinical utility of NMP-22 was evaluated as a screening test for patients with microscopic hematuria. Urine samples, collected before cystoscopy, were divided into two portions and tested with NMP-22 and voided-urine cytology. Of the 309 patients with microscopic hematuria, 22 (7.1%) cases of urothelial cancer, and one case of prostate cancer, were detected. Among the remaining cases, 128 (41.4%) were benign disease, and there was no evidence of disease (NED) in the remaining 158 (51.1%). Median NMP-22 values for urothelial cancer, other diseases and NED, were 35.5 U/ml, 6.7 U/ml and 6.0 U/ml, respectively. Sensitivity of the NMP-22 test for urothelial cancer was 90.9% (20/22), whereas the sensitivity of voided-urine cytology was 54.5% (12/22) (Miyanaga N, etal, Int J Urol, Apr 1999;6(4):173-7).

BLCA-4 is a bladder cancer-specific NMP detected at high levels in the urine of almost all patients with bladder cancer tested but is present in very low levels in the urine of healthy people. These findings indicate that an assay using this marker may be a promising urine-based test for bladder cancer, and that it may be significantly better than the NPM-22 test. Led by Robert Getzenberg, PhD, investigators at the University of Pittsburgh Cancer Institute identified six NMP (BLCA 1-6) that are unique to bladder cancer, and three others that are found only in normal bladder tissue. BLCA-4, one of these 6 NMP, can be used to differentiate individuals with bladder cancer from those that do not have the disease (Nguyen T-ST, etal, AACR99, Abs. 4800:727). These investigators recently obtained a large portion of the cDNA sequence for BLCA-4 by isolating two fragments of 200 bp and 700 bp, and are completing construction of the cDNA encoding this protein.

An ELISA assay successfully detected BLCA-4 in the urine showing that levels of this marker are significantly higher in patients with bladder cancer. In one study, BLCA-4 protein was detected in 9/12 (75%) of tumor samples, 12/12 (100%) of the morphologically normal tissue from the same bladders, and 0/11 (0%) in bladders from organ donors. Even when studies were controlled for age, the association between BLCA-4 and disease states was highly significant. These data also support the presence of a field effect in the bladder, suggesting that even cells that appear to be morphologically normal, have undergone alterations. The average value for BLCA-4 in the urine of healthy controls was 3.5 ± 3.7 and 36.39 ± 21.8 in those with bladder cancer. Using a cutoff value of 13 optical density units, all individuals with bladder cancer could be separated from those without disease. BLCA-4, therefore, appears to be the first marker that can distinguish, absolutely, patients with bladder cancer from those without the disease.

Urinary BLCA-4 determination may prove useful for screening and monitoring bladder cancer in the general population, and in groups at high risk for the disease, such as those with spinal cord injury. Patients with spinal cord injury have approximately a 400 times greater risk for bladder cancer compared to other individuals. In addition, because cystitis is very prevalent in this population, it is important to determine if BLCA-4 would be elevated in cases of cystitis, particularly because most of the currently available urine based markers for bladder cancer have been shown to be falsely positive in this setting.

A BLCA-4 ELISA test was evaluated on urine samples from 54 patients with bladder cancer, 202 with spinal cord injury, and 51 healthy volunteers, to determine the BLCA-4 level in these 3 groups, and assess its utility as a screening test in those with spinal cord injury. The association of BLCA-4 level was matched with tumor grade and stage, urine cytology and bladder cancer history in the healthy controls. Similarly, parameters associated with BLCA-4 in spinal cord injured patients, such as spinal cord injury duration, catheterization, history of urinary tract infection, smoking and urine culture, were compared. BLCA-4 level was less than the cutoff point of 13 optical density units/mg protein for the BLCA-4 assay in all 51 normal controls, while in 53 of the 55 urine samples (96.4%) of patients with bladder cancer, and in 38 of the 202 (19%) of spinal cord injured patients, urinary BLCA-4 was greater than the cutoff. Therefore, elevated urinary BLCA-4 levels may accurately identify bladder cancer, and distinguish patients with malignancies from healthy individuals. There is no correlation of urinary BLCA-4 with a history of urinary tract infection, smoking, catheterization or cystitis, considered independently (Konety BR, etal, J Urol, Sep 2000;164(3 Pt 1):634-9, Comment in: J Urol 2000 Sep;164(3 Pt 1):690-1).

Among the 55 patients with histologically proven bladder cancer, the sensitivity of BLCA-4 was found to be 96.4% and its specificity was 100%. Among 93 patients with cystitis, 26 (28%) were positive for BLCA-4

in the urine, and 67 (72%) were negative. However, urine cytology in 42 of these patients demonstrated the presence of infection in 29 (69%) but was negative for malignant cells in all samples. These results indicate that the presence of cystitis does not necessarily correlate with positive BLCA-4 values (Konety BR, etal, AACR00, Abs. 2862:433).

In February 2000, Eichrom Technologies (Darien, IL) licensed the BLCA-4 test from the University of Pittsburgh Cancer Institute (UPCI). Eichrom plans to develop an ELISA-based quantitative assay and a qualitative one-step rapid test based on BLCA-4. Plans to enter into a multisite clinical trial in 2000 to validate the use of BLCA-4 in the diagnosis and monitoring of bladder cancer are also underway. This trial will be coordinated by Badrinath Konety, PhD, in the Department of Urology.

Fibrin/fibrinogen degradation products (FDP) are protein fragments generated by the action of the fibrinolytic system on fibrin and fibrinogen. These plasma proteins leak from blood vessels of tumors into the surrounding tissue. Clotting factors rapidly convert the fibrinogen in the plasma into an extravascular fibrin clot, which is degraded by plasmin, and activated by urokinase. FDP products are either absent or present at exceedingly low levels in the urine of healthy individuals, but are present not only in bladder cancer but also in various nonspecific inflammatory conditions of the urinary tract.

PerImmune, now a part of Intracel (Rockville, MD), developed an FDP test that uses a MAb to detect fibrin D, which was marketed in Europe as AuraTek FDP and, in the USA, as AccuDx. However, problems with the absorbent paper used in the test prompted the company to withdraw it from the market in 1999. A redesigned version under the brand name AccuDx is expected to be re-introduced in 2001, to be marketed by Mentor (Santa Barbara, CA), Intracel's commercial partner. Originally, the FDP test was an ELISA but subsequently was redesigned in a rapid test format that can be performed in the office with results available in about 7 minutes. Interestingly, the rapid test was first developed to detect high levels of FDP products in patients with glomerulonephritis, to monitor the effectiveness of antibiotic treatment. When it was also shown that the presence of FDP is far more prevalent in urine from patients with bladder cancer, interest shifted in developing the test for this indication. Intracel is also targeting the bladder cancer screening market with this test, and also plans to introduce a home-testing version.

Although the role of FDP testing in bladder cancer has not been categorically established, several features of the test make it a potential adjunct in the diagnosis and monitoring of bladder cancer. For instance, the fact that urinary FDP levels tend to be higher in patients with tumors of a higher grade and stage, results in the test's improved sensitivity in detecting more aggressive tumors. Although, the overall accuracy of the current MAb (fibrin D-dimer) immunoassay that ranges from 75% to 80%, suggests that the urine FDP test should not be used alone for the surveillance of superficial bladder cancer, when FDP assays are combined with urine cytology, the sensitivity for detecting tumors is improved significantly. Also, prospective data is needed to determine whether using these tests in combination can safely permit a reduced frequency of endoscopic surveillance (Tsihlias J and Grossman HB, Urol Clin North Am, Feb 2000;27(1):39-46). Because of its relatively high specificity, Intracel plans to position the test as a tool in selecting patient populations in whom the presence of cancer is of the highest probability, thus prompting urologists to perform very comprehensive cystoscopic evaluations to find the tumor site.

When Intracel's rapid immunoassay was compared to urinary cytology and hemoglobin dipstick for the detection of bladder cancer in 192 patients with a history of bladder cancer, it was significantly more sensitive (68%) than conventional urinary cytology (34%), or hemoglobin dipstick (41%), particularly in the detection of low-stage, low-grade disease. In subjects with invasive disease (Stage T2-T4), the sensitivity of the FDP test was 100% and its specificity was 96% for healthy subjects, 86% for patients with urologic disease other than bladder cancer, and 80% for patients under surveillance for bladder cancer but with a negative cystoscopic finding at the time of assay. In this evaluation, the FDP test proved superior to conventional urine cytology and hemoglobin dipstick as an aid in the detection of bladder cancer (Schmetter BS, etal, J Urol, Sep 1997;158(3 Pt 1):801-5).

Telomerase is an enzyme composed of a catalytic protein component and an RNA template which synthesizes DNA at the ends of chromosomes (telomeres) and confers replicative immortality to cells. Telomeres shorten during each round of cell replication, suggesting that they dictate the number of times a cell can divide before it reaches senescence. By expressing telomerase, an enzyme that, in effect, rebuilds the chromosomal tips, cancer cells bypass senescence, becoming, immortalized. Telomerase activity is detected in >90% of various cancers, including urothelial cancers. Of the three subunits comprising the telomerase complex, human telomerase reverse transcriptase (hTERT) is the rate-limiting determinant of the enzymatic activity of telomerase.

Telomerase-related testing may evolve into an accurate diagnostic methodology for a variety of malignancies, including bladder cancer. A leader in the development of telomerase-based tests in oncology is Geron (Menlo Park, CA) that is developing PCR-based telomerase tests in collaboration with Roche Diagnostics (Mannheim, Germany). Among the various assays that can be used to detect telomerase activity in human tissue or cells in culture, in current use and/or in development, are a test for hTERT, a second generation assay, and the original TRAP assay, both developed by Geron. The very high detection rate, 1 in 1,000,000 cells, compared to 1 in 10,000 cells for urine

cytology, may be an important characteristic of reverse transcriptase-polymerase chain reaction (RT-PCR)-based tests such as TRAP.

However, the role of telomerase detection in bladder cancer diagnosis and monitoring is unclear. Reports from various investigators paint a picture of uncertainty regarding the value of testing for telomerase in urine using various targets. When the RNA component (hTR) and the catalytic subunit (hTERT) of human telomerase were detected in urine, using labeled primer and an automated sequencer, as well as a new PCR ELISA platform, and telomerase activity was detected with a TRAP assay, hTR was the most reliable telomerase component in urine. Increased levels of hTR were found in 85% (specificity 83%) of urine samples from patients with bladder cancer whereas hTERT was weakly expressed in urine samples, detectable in 30% of those from patients with bladder cancer, and telomerase activity was detectable in only 5% (Müller M, etal, Journal of Clinical Ligand Assay 1999;22:354–357). Detection of hTR by RT-PCR may represent a promising new urine test for bladder cancer and may be practicable for routine clinical use.

In a study to evaluate the TRAP assay for detection of telomerase as a potential new method for bladder cancer detection and compare it to cytology, voided-urine and bladder-wash samples were obtained from 63 patients with a history of bladder cancer. Cytological evaluation was performed only on voided urine, while the TRAP assay was performed on both samples. The overall sensitivity of the TRAP assay was 35% in voided urine, and 50% in bladder wash, whereas the overall clinical sensitivity of voided-urine cytology was 71%. The sensitivity of voided-urine cytology for papillary and noninvasive tumors (Ta) was 50%, compared to 92% for T1 and 62% for T2+ tumors, and 100% for high-grade flat CIS. The sensitivity of the TRAP assay using voided urine was 46% for Ta, 50% for T1, 18% for T2+, and 20% for CIS. In voided-urine samples, the sensitivity of the TRAP assay in Ta disease was similar to that of cytology. There was a strong association between the total number of exfoliated malignant cells and the sensitivity of the TRAP assay. The sensitivity of the TRAP assay in bladder washes was 44% for Ta, 67% for T1, 46% for T2+, and 43% for CIS (Dalbagni G, etal, Clin Cancer Res, Sep 1997;3:1593-1598).

In order to evaluate the usefulness of detecting hTERT mRNA in urine samples for bladder cancer screening, investigators examined the expression of hTERT mRNA in spontaneously voided-urine specimens from 33 patients with bladder cancer, and 26 individuals without bladder lesions. RT-PCR analysis revealed that approximately 80% of urinary sediments from patients with bladder cancer expressed hTERT mRNA, regardless of clinical stage or pathologic grade, compared to only 4% of sediments from patients without urothelial lesions. Interestingly, hTERT mRNA expression was observed even in some urine samples from bladder cancer patients with negative urine cytology. These findings suggest that the expression of hTERT in urine sample may be a useful diagnostic marker for bladder cancer (Ito H, etal, Clin Cancer Res, Nov 1998;4(11):2807-10).

When voided urine from patients with bladder cancer and from control patients with either hematuria, or with no urologic conditions, were examined for telomerase activity, the overall sensitivity of this assay in detecting bladder cancer was 85% (88/104); it was 79% (23/29) in Grade I, 84% (32/38) in Grade II, and 87.5% (28/32) in Grade III tumors, and 100% (5/5) of CIS tested positive. This compared favorably with urinary cytology which had an overall sensitivity of 51% and sensitivity of 13%, 44%, 82%, and 100% for Grades I, II and III tumors, and CIS, respectively. The specificity of telomerase in patients with benign causes of hematuria was 66%, and in healthy volunteers it was 100%. Assessment of NMP-22 suggested comparable sensitivity and specificity but BTA was less sensitive for low-grade disease and less specific, because it was influenced by inflammation and instrumentation. Thus, telomerase may detect low-grade tumors in voided urine, and may be advantageous in patient follow-up for recurrent disease (Droller MJ, etal, Keio J Med, Sep 1998;47(3):135-41).

Telomerase activity may also be a potential marker in preneoplastic bladder lesions, and a prognostic marker of bladder tumor relapse or progression. Tissue telomerase activity was assayed by two techniques, TRAP-PCR and a telomerase PCR-ELISA, to assess its utility in the diagnosis of bladder cancer. Malignant and inflammatory bladder lesions, and their adjacent normal tissues, were assessed for telomerase activity in a group of 18 patients, 14 with urothelial carcinoma, and 4 with a nonspecific inflammatory lesion of the bladder. Among the 14 tumor samples analyzed, 11 were telomerase-positive, and 2 of the 3 telomerase-negative tumor samples expressed a detectable 'telomerase inhibitor'. In the apparently normal tissues next to bladder tumors, 4 of the 14 specimens were telomerase-positive. Interestingly, these lesions were always next to high-grade muscle-invasive bladder tumors (T2, Grade III). Of the 4 nonspecific inflammatory lesions, 2 (one from cystitis glandularis and one from severe dysplasia), known to be preneoplastic lesions, were also telomerase-positive. These results strongly suggest that the reactivation of telomerase may be an early event in bladder carcinogenesis, preceding morphologic changes related to malignant transformation (Lancelin F, etal, BJU Int, Mar 2000;85(4):526-31).

p53 mutations and other genetic markers, detected and characterized in urine, bladder-wash, or tissue specimens of patients with bladder cancer, may lead to the clinical applicability of molecular methods of disease monitoring. However, it is necessary to improve sensitivity in detecting mutations in a sample containing a mixture of normal and malignant cells. A urine-based test, developed by UroCor (Oklahoma City, OK), under a license from Ambion (Austin, TX), that uses the latter's nonisotopic

RNase cleavage assay (NIRCA) for the high-throughput detection of mutations, has been on the market since 1998. This test is used by urologists to identify patients with a mutated p53 tumor suppressor gene, which indicates their bladder cancer may be more aggressive, and more likely to recur. Because it is urine-based it obviates the need for invasive biopsy to obtain a tissue sample. In July 2000, UroCor obtained a Phase II SBIR grant (No. 2 R44 CA76823-02) of $693,828 from the National Cancer Institute (NCI) for a 2-year, multicenter trial to produce a second generation of its p53 mutation assay for bladder cancer prognosis with enhanced technical performance. This award follows an earlier Phase I NCI grant, which was completed in June 1999.

A pilot study investigated the feasibility of detecting mutations in exons 5-8 of the p53 gene using single-stranded conformational polymorphism (SSCP) analysis in 31 bladder-wash specimens from 27 patients with bladder cancer. In one case bladder washings were available from the same patient on two separate occasions with one washing demonstrating a mutation and the other not. In two other cases a mutation was demonstrated in the bladder tumor but not in the corresponding washing. An abnormal additional SSCP band was detected in 5 samples from five different patients suggesting the presence of a p53 mutation. In all 5 cases the same abnormal SSCP pattern was demonstrated in samples of the corresponding bladder tumor (Phillips HA, etal, Br J Cancer, Jan 2000; 82(1):136-41).

However, according to a study of p53 oncoprotein immunohistochemistry in the follow-up of bladder carcinoma, this marker was not found to be useful in the characterization of carcinoma of the urinary bladder. This study, performed on tissue specimens between November 1992 and November 1993 and during the follow-up period until July 1998, analyzed the utility of semiquantitative determination of p53 in TCC of the bladder by an immunohistochemical technique using NCL-p53-DO7, supplied by Novocastra Laboratories (Newcastle upon Tyne, UK). This prospective clinical cohort study was conducted on 81 patients, comprising two groups, 20 healthy patients and 61 patients with bladder carcinoma (Moreno Sierra J, etal, Arch Esp Urol, Oct 1999;52(8):840-8).

Testing for other genetic markers, in addition to p53, is also being evaluated in the diagnosis and monitoring of bladder cancer. In a study to evaluate the relative sensitivity of molecular markers compared to urine cytology as screening tools for bladder cancer, data was analyzed from 25 patients with bladder cancer (CIS=20 and invasive=5). Using a bank of specific markers, genetic changes were identifiable in cells extracted from urine of 18/25 (72%) bladder cancer patients which compared favorably to cytology which was positive in only 3/25 (12%). Of the 18 patients with genetic changes, 10 demonstrated changes in chromosome 9 (in the region of p16), and 7 in chromosome 14 [in the area of the Machado-Joseph disease (MJD) gene]; in 2 patients there were changes in both areas. It is reasonable to assume that these 2 areas are important loci for malignant transformation. Changes in the region of p53 (chromosome 17) were observed among patients with invasive tumors (2/5) exclusively. After removal of tumor, and 3 monthly evaluations using both techniques, 3 patients developed positive genetic markers identical to those detected in the primary tumor (none by cytology) and all 3 were subsequently found to have recurrent tumor at cystoscopy. This preliminary data indicates that testing cells extracted from urine with specific genetic markers, is much more sensitive than urine cytology. Further evaluation is necessary to optimize the battery of genetic markers and to elucidate the specificity, sensitivity and cost-effectiveness of this approach (Halle D, etal, AACR00, Abs. 4562:717).

As of May 2000, the Feist-Weiller Cancer Center, in association with the Department of Pathology at Louisiana State University Health Sciences Center (LSUHSC; Shreveport, LA), has been using a panel of ACIS tissue tests including HER2/neu, ER, PR, Ki-67 and p53, to quantify tumor marker protein expression for the clinical assessment of breast cancer to assist physicians to accurately characterize a patient's tumor. The Center also plans to use ACIS to further research studies in the areas of malignant lymphoma, and bladder cancer.

In September 2000, Vysis (Downers Grove, IL) filed for approval of its UroVysion multicolor fluorescence *in situ* hybridization (FISH) assay for determination of chromosome-specific abnormalities in cells obtained from urine or bladder washings. UroVysion is designed for interphase cell analysis to detect and quantify chromosomes 3, 7, 17 and the 9p21 region. UroVysion consists of three alpha-satellite repeat sequence probes, CEP 3 SpectrumRed, CEP 7 SpectrumGreen and CEP 17 SpectrumAqua, that hybridize to the centromere regions of chromosomes 3, 7 and 17, respectively, and a unique sequence probe, LSI 9p21 SpectrumGold, that hybridizes to the 9p21 region of chromosome 9. Direct fluorophore labeling of the DNA probes results in hybridization with bright clear probe signals, and little or no background noise, providing for easy interpretation.

In June 1999, Vysis entered into a two-year CRADA with the National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH; Bethesda, MD), and the Institute of Pathology at the University of Basel, in Switzerland, to investigate, under PI Olli-P Kallioniemi, MD, PhD, a combination of the Vysis' GenoSensor and FISH Systems and NHGRI's tissue microarrays, to define amplifications of specific genes in various cancers, and then to determine the potential clinical significance of these amplifications. As part of the agreement, Vysis has an option to negotiate a license with NIH and/or the University of Basel for inventions that arise from the CRADA research. NHGRI's tumor tissue microarrays permit simultaneous screening of up to 1,000 different tumor specimens with one array, while Vysis' GenoSensor array enables simultaneous analysis of hun-

dreds of gene amplifications. The project will examine more than 130 tumor types with emphasis on cancers of the breast, bladder, prostate, and brain. In February 2000, Vysis obtained an exclusive royalty-bearing license agreement from Yale University (New Haven, CT) covering Yale's European patent EP 0 444 115 B1, "Chromosomal *in situ* Suppression Hybridization," and other related USA and foreign patents and pending patent applications, that complement Vysis' existing intellectual property portfolio consisting of patent rights exclusively licensed from the University of California and the Vysis direct-label probe patents covering its core FISH technology platform.

DNA replication proteins are also being investigated as diagnostic cancer markers. One of these proteins, Mcm5, belonging to the minichromosome maintenance (MCM) family of proteins, persists in all cell-cycle phases except quiescence. It is believed that expression of Mcm5 is a consequence of maturation-arrested cancer cells failing to exit the proliferative cycle. When affinity-purified polyclonal antibodies against Mcm5 were applied to histologic sections of normal bladder urothelium (n=7), and noninvasive (n=27) and invasive (n=41) TCC of the bladder, Mcm5 immunostaining was restricted to cells in the basal proliferative compartment in normal urothelium while, in noninvasive and invasive TCC, Mcm5 was expressed in basal, intermediate, and superficial epithelial cells. Similar patterns of MCM expression were found in normal and cancerous epithelia in many other organ systems, indicating that presence of Mcm5 protein in superficial exfoliated cells, including urothelial cells, may be predictive of cancer. Also, the percentage of immunostained cells correlated with the pathologic grade of TCC, averaging 78% for Grade III, 70% for Grade II, and 45% Grade I tumors. In related work it was also shown that anti-Mcm5 antibodies can improve the Papanicolaou smear test for cervical dysplasia and neoplasia (Stoeber K, etal, Lancet, 30 Oct 1999; 345 (9189):1521-5).

Dr. Ron Laskey and colleagues at the Cancer Research Campaign Institute and the University of Cambridge, in the UK, have developed a two-site time-resolved immunofluorometric assay for detection of urothelial cancer, based on ectopic expression of Mcm5. Urine samples from 36 patients with hematuria or suspected urinary cancer, and from those previously diagnosed with TCC, undergoing follow-up cystoscopy, were obtained in a blinded fashion at a urology clinic, Addenbrookes NHS Trust (Cambridge, UK). Soluble cellular proteins, isolated from 50-80 ml urine samples, were directly applied to the immunoassay. When results of the immunoassay were compared with cytoscopy and pathologic diagnosis, Mcm5 protein was detected in 8 patients, 7 cases with TCC of the bladder and one with Grade III TCC of the ureter, indicating the potential of this noninvasive approach to detect urothelial malignancy of the bladder, ureters, or renal pelvis. TCC of the bladder, diagnosed on pathologic assessment of biopsy material, included Grade I, Ta (n=1), Grade I, T1 (n=2), Grade II, Ta (n=2), Grade II, T1 (n=1), and Grade III, T2 (n=1) disease. In contrast, Mcm5 protein was detected in only 1 of the remaining 28 cases without cytoscopic evidence of malignancy, in a patient with a chronic hypotonic bladder associated with bacterial infection and mucosal ulceration. Ulceration would be expected to give a false-positive result because the proliferating basal compartment is exposed to urine. Importantly, the immunoassay did not produce false-positive results in 5/28 patients without a malignancy who were clinically diagnosed with inflammatory cystitis. Biopsy samples from patients with cystitis show that intraepithelial/mucosal acute and chronic inflammatory cells, associated with cystitis, are negative for Mcm5 expression; Mcm5 protein remains restricted to the normal basal proliferative compartment (Stoeber K, etal, *ibid*).

Cytokeratin (CK) fragments may also be valuable urine or serum markers for TCC of the bladder. There are 20 identified cytokeratins, with several being considered as targets for both diagnostic and prognostic tests in bladder cancer.

One test, Cyfra 21-1 that detects soluble CK19 fragments in urine or serum using two specific MAbs, Ks 19.1 and BM 19.21, is available as an immunoradiometric assay (IRMA), and an ELISA in Europe, marketed by Roche Diagnostics, among others, and in Japan by Fujirebio (Tokyo, Japan), but has not been approved in the USA. A newer approach involves an electrochemiluminescent (ECL) immunoassay for Cyfra 21-1, as part of Roche Diagnostic's Elecsys 2010 immunoassay system. In urine samples voided by patients with bladder cancer and controls, at a threshold value of 5.7 µg/l, sensitivity was 81.0% and specificity was 97.2% (Sanchez-Carbayo M, etal, Clin Chem Nov 1999;45(11):1944-53).

Investigators evaluated Cyfra 21-1 as a diagnostic modality in bladder TCC in the serum and urine of 182 patients, including 66 with bladder TCC (group 1), 66 with another urological pathology (group 2) and 50 free of urothelial disease (group 3). Mean urinary Cyfra 21-1 was 154.39±49.00, 22.6±8.9 and 2.40±0.14 ng/ml in groups 1, 2, and 3, respectively. Optimal sensitivity was 96.9%, and specificity 67.2% at a threshold value of 4 ng/ml, implying that urinary Cyfra 21-1 is a useful marker for diagnosing TCC, and provides good sensitivity in low-grade disease (Pariente JL, etal, J Urol 2000 Apr;163(4):1116-9).

Another cytokeratin, CK20, expressed in urothelial carcinoma cells but not in normal urothelial cells, is a sensitive marker of urothelial differentiation, and may be useful in the identification of urothelial papillomas. In one study, aimed to examine whether CK20 expression could serve as a noninvasive test to detect and monitor malignant urothelial cells in urine, investigators used RT-PCR methods to determine the expression of CK20 in cells separated from the urine of 87 individuals, divided into 2 groups comprising 14 healthy volunteers without any known history of TCC, and 73 patients with hematuria suspected for TCC of the bladder. For control purposes,

CK20 expression was examined in cells of bladder carcinoma tumors of 5 patients, in the blood of either patients with bladder carcinoma (n = 5) or healthy controls (n = 5), and in three different cell lines. The CK-20 amplification band (370 bp) was obtained with mRNA extracted from TCC cells of either bladder tumor, or HT-29 line (a CK20 colon carcinoma line). Sensitivity was 91% and specificity 67%. Among 7 false-positive cases, 3 showed atypia, 3 hyperplasia, and 1 metaplasia, and 2 underwent previously successful TCC tumor removal, suggesting that the CK20 test also detected premalignant lesions. No false-positive cases were found in the healthy control group. No other preparation, including blood of patients with TCC, showed the CK20 amplification band (Klein A, etal, Cancer, 15 Jan 1998;82(2):349-54).

However, when a nested RT-PCR assay which amplifies CK20 transcripts was used to detect cancer cells in the peripheral blood of urothelial patients, it detected cells of 10 bladder cancer cell lines in a sample of ten million peripheral-blood mononuclear (PBMN) cells. CK20-specific signals were detected in 9 (22.5%) of 40 PBMN cell samples prepared from 40 patients with urothelial cancer, in relation to the tumor stage, including 0/13 patients with a superficial tumor, 4/21 (19%) with a regionally invasive tumor and 5/6 (83%) with metastases. No signals were detected in any of 25 PBMN cell samples from healthy donors. CK20 RT-PCR assay is, therefore, applicable to the detection of urothelial cancer cells in peripheral blood and also confirms that hematogenic dissemination occurs in invasive urothelial cancers but rarely in superficial ones (Fujii Y, etal, Jpn J Cancer Res, Jul 1999;90(7):753-7).

The suitability of CK20 mRNA expression as a marker for the detection of minimum residual disease in patients with cancer of epithelial origin was evaluated using a sensitive nested RT-PCR assay with multiple replicates optimized to detect a minimum number of circulating tumor cells expressing CK20 mRNA. CK20 mRNA expression was examined in 10 epithelial and 7 leukemic cell lines, in 8 bladder tumors, in peripheral blood samples from 18 tumor patients and from 29 healthy controls, and in 8 bone marrow samples from healthy donors. CK20 mRNA was found in 13/18 (72%) blood samples from patients with cancer of epithelial origin and in all the epithelial tumor cells tested. However, CK20 mRNA was also detected in 21/29 (72%) blood samples, in 8/8 (100%) bone marrow samples from healthy donors, and in 4/7 (57.1%) leukemic cell lines. These results highlight a requirement for either determination of threshold levels of CK20 normal expression, or the development of quantitative techniques to distinguish between a tumor-specific CK20 gene expression, and a low level background transcription of this marker. These results would also advise caution in using CK20 as a tumor specific marker in clinical investigations (Champelovier P, etal, Anticancer Res, May-Jun 1999;19(3A):2073-8).

In a prospective study, immunocytochemistry for CK20 was performed on tumors of all patients who presented for the first time with noninvasive papillary bladder tumors. The expression pattern of CK20 was classified as normal or abnormal at the time of initial diagnosis, and the time of first biopsy-proven recurrence, or length of follow-up when no recurrence was observed, was recorded. Among 58 consecutive patients, none of 10 patients with tumors with a normal pattern of CK20 expression developed further tumors during a median follow-up of 18 months. By contrast, tumors recurred in 30 (73%) of the 41 evaluable patients with tumors that showed abnormal CK20 expression with the median time to a second tumor being 6 months. The only factor that had a significant effect on the outcome of patients in terms of recurrence was expression of CK20. Parenthetically, normal urothelial differentiation, as evidenced by a normal pattern of CK20 expression, is retained in a proportion of noninvasive papillary urothelial tumors justifying use of the term urothelial papilloma (Harnden P, etal, Lancet, 20 Mar 1999;353 (9157):974-7).

Tests have also been developed that detect other cytokeratins in various media. Tissue polypeptide antigen (TPA) is a serological tumor marker, that measures CK 8, 18 and 19, used in the follow-up of nonsquamous epithelium-derived neoplasms. It has been demonstrated that TPA is reliable in the monitoring of the efficacy of a curative or palliative treatment of bladder cancer. Recently, a monoclonal antibody-based assay for TPA (TPA-M) has been developed, which seems to be equivalent to the polyclonal-based assay. In a study to compare the MAb to the polyclonal antibody test in patients with bladder carcinoma, the value of TPA was measured both with TPA and TPA-M IRMA. A correlation coefficient of 0.96 was obtained. Precision testing showed a lower overall variability with TPA-M. Although both tests correlated well, TPA-M testing was more precise, faster and easier to perform (Bennink R, etal, Anticancer Res, Jul-Aug 1999;19(4A):2609-13).

Another CK-based test, UBC, that detects and/or measures fragments of cytokeratin 8 and 18 in the urine, has also been developed for monitoring bladder cancer patients. IDL Biotech (Bromma, Sweden) has introduced several formats of this test, including the IDeaL UBC Rapid test, a 10-minute semiquantitative test for the determination of antigens in urine from urothelial tumors, and the IDeaL UBC ELISA and UBC IRMA quantitative tests. The IDeaL UBC Rapid test could be used as a sensitive monitoring test for the follow-up of treatment of urinary bladder cancer, and also as the first alternative determination of UBC antigens, checking if a quantitative UBC IRMA/ELISA should be performed. UBC ELISA and IRMA are sensitive indicators of the aggresiveness of early-stage superficial baldder tumors, and can be used as a prognostic, monitoring and follow-up test.

In a study conducted at Hospital General Universitario de Alicante, in Spain, to evaluate the diagnostic performance of the UBC Rapid test for the detection of TCC of the bladder, and to assess the differential sensitivity of the biomarker regarding the most relevant histologic and clinical parameters of bladder cancer, 267 subjects were

entered into the study and classified into five groups comprising 111 patients with active TCC of the bladder (group 1); 76 follow-up patients with previously diagnosed TCC free from disease as confirmed by cystoscopy (group 2); 25 patients with other benign urologic diseases (group 3); 25 patients with other malignant pathologic conditions (group 4); and 30 healthy subjects (group 5). The sensitivity in group 1 was 78.4% and the specificity in group 2 was 97.4%. Positive and negative predictive values in groups 1 and 2 were 97.4% and 79.0%, respectively, with a global accuracy of 86.1%. False-positive rates were 20.0% and 44.0% for groups 3 and 4, respectively. Although the sensitivity of the UBC rapid test was associated with the histologic and clinical characteristics of bladder cancer, it did not reach statistical significance. The UBC Rapid test appears to be a promising noninvasive adjunct that might guide the urologist in the decision to perform cytoscopy for the detection of TCC of the bladder (Sanchez-Carbayo M, etal, Urology, Oct 1999;54(4):656-61).

However, when urine samples from patients with histologically proven TCC and healthy controls were examined for the presence of UBC antigen, it was concluded that, in its current format, this test is not clinically useful for the detection of bladder cancer. Urine samples of 355 individuals (healthy volunteers=77, patients with benign urologic disorders=111, patients with histologically proven TCC=167) were examined at Freie Universitat, Berlin, for the presence of UBC antigen prior to therapy. Compared to healthy volunteers or patients with benign urologic disease, patients with TCC had significantly higher median urinary levels of UBC antigen which correlated positively with tumor grade and stage. Levels of UBC antigen were significantly higher in cases of invasive than superficial TCC. Elevated levels of UBC antigen were also found in patients with benign urologic conditions. Using a cutoff of 14.06 mg/g creatinine, corresponding to 95% specificity in the group of healthy individuals, sensitivity of UBC antigen ranged between 21.6% (Ta tumors) and 75% (T4). Overall specificity was 76.6% (Heicappell R, etal, Scand J Clin Lab Invest, Jul 2000;60(4):275-82).

UBC tests fared better when compared to other tests for the diagnosis and monitoring of urothelial cell carcinoma. Investigators at the General Hospital of Bolzano, in Italy, compared the diagnostic value of BTA Stat and UBC Rapid test, and the NMP-22 and UBC ELISA tests in voided-urine samples of patients having symptoms suggestive of urothelial cell carcinoma and those being followed-up after TUR. All patients underwent subsequent cystoscopic evaluation, and biopsy of any suspicious lesion. In the BTA and UBC rapid test comparison among 180 patients (57 had symptoms suggestive of urothelial cancer and 123 were being followed-up after complete TUR for such cancer), in 53 patients with histologically proven urothelial cell carcinoma, the sensitivity and specificity of UBC Rapid test (Exhibit 3) was superior to BTA Stat (Mian C, etal, Urology, 1 Aug 2000;56(2):228-31). The comparison between NMP-22 ELISA and UBC ELISA, produced similar results (Exhibit 3). In a retrospective study of 240 patients, 81 with symptoms suggestive of urothelial cell carcinoma, and 159 under follow-up after TUR, these tests were performed on previously frozen urine samples. The cutoff levels for bladder cancer positivity were 10 U/ml for the NMP-22 test and 12 mg/l for the UBC test. In 54 patients with histologically proven cancer, the UBC ELISA test was superior to the NMP-22 test in terms of both sensitivity and specificity, but neither test could replace cystoscopy (Mian C, etal, Urology, Feb 2000;55(2):223-6).

Hyaluronic acid (HA) and hyaluronidase (HAase), that are secreted in urine, are associated with the biology of bladder tumor angiogenesis and metastasis. In Grade II and III bladder tumors, HA is degraded by HAase, resulting in the generation of angiogenic HA fragments, which, in turn, are secreted in urine. An elevated urinary HA level (≥500 ng/mg) producing a positive IIA test, suggests the presence of bladder cancer regardless of tumor grade. Levels of urinary HAase which promotes the growth of new blood vessels, correlate with the malignant potential of bladder cancer and are elevated (≥10 mU/mg) in the urine of patients with Grade II and III bladder cancer. A HA-HAase test was developed at the University of Miami School of Medicine (Miami, FL) that actually comprises two separate urine tests, one that detects the presence of HA, and the other the enzyme HAase. Use of the HA-HAase test may detect bladder cancer and establish its grade. The overall 92% sensitivity of the combined HA-HAase test in detecting bladder cancer is higher than the sensitivity of the individual tests with little compromise in specificity. The HA-HAase test is equally sensitive for monitoring tumor recurrence. Also, when compared with existing noninvasive tests, the HA-HAase test may be significantly less expensive and more accurate (Lokeshwar VB and Block NL, Urol Clin North Am, Feb 2000;27(1):53-61).

Cystoscopy/Biopsy

Whenever the presence of TCC is suspected, a full urologic evaluation consisting of cystoscopy, urinary cytology, and IVP, is mandatory. This evaluation allows for assessment of the whole urinary tract since tumor lesions may be located anywhere along the upper urinary tract (calyces, renal pelvis, ureters), or lower urinary tract (bladder and proximal urethra).

Cystoscopy is considered the "gold standard" for identifying bladder cancer. The procedure involves the insertion of a cystoscope, a thin, lighted tube, directly into the bladder through the urethra, and may be performed under local or general anesthesia. During cystoscopy the operator examines the lining of the bladder, and may obtain biopsy samples for pathologic examination. In some cases, the entire tumor may be removed during biopsy by TUR. When a lesion is detected by cystoscopy, its configuration (flat, sessile, or papillary), location (trigone, base, right lateral wall, left lateral wall, dome), size (in centimeters), is noted, as well as the number of lesions found.

A biopsy is indicated in late-stage tumors. However, biopsy results do not contribute to staging, or to the choice of adjuvant therapy after TUR. According to a report from the Superficial Bladder Committee of the EORTC Genito-Urinary Tract Cancer Cooperative (EORTC-GU) Group, biopsies of normal-appearing urothelium in Ta and T1 bladder cancer patients show no abnormalities in about 90% of cases. In EORTC protocol 30863 involving patients with low-risk tumors, 393 patients underwent a biopsy of normal-appearing urothelium. In protocol 30911, involving patients with intermediate- and high-risk tumors, multiple biopsies were taken from normal appearing urothelium in 602 patients. No abnormalities were found in the random biopsies of 376 (95.6%) patients with low-risk tumors, and in 532 (88.4%) patients with intermediate- and high-risk tumors. CIS was detected in random biopsies of 6 (1.5%) patients with low-risk tumors, and in at least 21 (3.5%) with higher-risk tumors. No invasive tumors (T2) were detected among patients in the low-risk group, and in only 1 (0.2%) patient in the higher-risk group (van der Meijden A, etal, Eur Urol, Apr 1999;35(4):267-71).

Cystoscopy is typically billed at $300, is invasive and causes some patient discomfort. Despite current efforts, however, urine bound diagnostic tests cannot replace cystoscopy. Also, although immunologic markers, such as BTA Stat and NMP-22, are superior to cytologic evaluation, and image analysis using Quanticyt is an improvement over cytology in detecting low-grade TCC, they all have low specificity and sensitivity in Grade III TCC. When voided urine and/or bladder-wash specimens from 291 patients with symptoms suggestive of TCC, or who were being followed after treatment for that disease, were prospectively evaluated, in 91 patients with histologically proved TCC (Wiener HG, etal, J Urol, Jun 1998;159(6): 1876-80), results were as follows:

Test	Sensitivity (%)				Specificity (%)
	Overall	Grade I	Grade II	Grade III	
NMP-22	48	52	45	50	70
BTA Stat	57	48	58	63	68
Rapid staining cytology of bladder-wash/ voided-urine samples	58/57	17	61	90	100
Image analysis	59				93

HISTOPATHOLOGY AND BLADDER CANCER TYPES

On histopathologic examination, in the USA, 93% of bladder cancers are TCC, 5% are squamous cell carcinomas, and 2% are adenocarcinomas (see Exhibit 4). Other rare types of bladder cancer, also referred to as primitive tumors, include small-cell carcinoma, a tumor whose cells have endocrine-like characteristics and is sometimes metastasized from a primary site in the lung; rhabdomyosarcoma; sarcomatoid carcinoma; pure or mixed epidermoid carcinoma (Budia Alba A, etal, Actas Urol Esp, Feb 1999;23(2):111-8); undifferentiated carcinoma, a rare tumor (<1% of cases) that shows no mature epithelial (bladder lining) cells; and leiomyosarcoma, a malignancy of smooth muscle origin representing a very rare (<0.5% of cases) and aggressive bladder tumor. Generally, tumors with nontransitional cell histologies behave very aggressively, and are less responsive to treatments other than extirpative surgery.

Transitional Cell Carcinoma (TCC)

Transitional cell carcinoma (TCC), the most common bladder tumor, is so called because tumor cells can undergo changes in size from cubical when the bladder is empty, to flat when the bladder is full. TCC rarely (<5% of cases) originates in the kidneys or ureters. In some cases TCC is mixed with other bladder tumor types.

Squamous Cell Carcinoma

Squamous cell carcinoma of the bladder is often associated with chronic inflammation and, in tropical climates, with bilharzial parasites. Squamous cell carcinomas are approximately twice as likely to occur in women than in men, and in individuals with *Schistosoma hematobium* infections of the bladder, or with histories of long-term indwelling urinary catheters, bladder stones, or recurrent bladder infections.

Adenocarcinoma

Adenocarcinoma involves cells from the lining of the walls of the bladder. The cells have glandular characteristics and are manifested as solid or papillary (wart-like) tumors. Adenocarcinoma may be either of urachal or nonurachal origin. Urachal adenocarcinoma is a rare tumor that involves the urachus, an outer, fetal bladder canal, and is thought to arise from metaplasia of chronically irritated transitional epithelium. It may be composed of tissue classified as adenocarcinoma, squamous cell carcinoma (SCC), or even sarcoma. Urachal tumors may cause mucous or bloody discharges in the urine, and they may produce dotted or "stippled" images on X-ray. Urachal tumors often are wider and deeper than expected, difficult to treat, and they tend to metastasize and/or recur. Other types of bladder adenocarcinoma are primary vesical, involving the bladder proper, and metastatic. A rare tumor (<0.7% of cases) that may also be a a variant of adenocarcinoma is micropapillary bladder carcinoma, that under the microscope appears strikingly similar to ovarian papillary serous carcinoma. This tumor is associated with a poor prognosis (Johansson SL, etal, J Urol, Jun 1999;161(6):1798-802).

STAGE AND GRADE

Staging of bladder cancer is described in Exhibit 5, and incidence by stage is estimated in Exhibit 6. At diagnosis, bladder cancer is either superficial (Tis, Ta, T1), account-

Exhibit 3
Comparisons of UBC, BTA and NMP-22 Tests

Test	Sensitivity (%)							Specificity (%)
	Overall	Ta	T1	≥T2	Grade I	Grade II	Grade III	
UBC Rapid	66.0	60.7	69.2	80.0	44.4	78.9	75.0	90.0
BTA Stat	52.8	42.8	61.5	70.0	38.8	52.6	68.7	70.0
UBC MAb-based ELISA	64.8	62.1	53.8	80.0	66.6	60.0	68.7	92.0
NMP-22 ELISA	55.5	51.7	46.1	70.0	50.0	50.0	68.7	79.0

ing for 80% of cases, invasive (T2, T3-4, N+), accounting for 15% of cases, or metastatic (M+), accounting for 5%. About 10% of those diagnosed with superficial disease will fail treatment, or progress to invasive disease. Staging is performed by either invasive procedures such as pelvic lymph node dissection to detect malignancy in the regional lymph nodes, or by noninvasive approaches such as CT, MRI and, lately, PET to detect regional and distant metastases.

In a study to evaluate whether pelvic lymph node metastases in bladder or prostate cancer can be detected with 2-[(18)F]-2-deoxy-D-glucose (FDG)-PET, 8 patients with bladder cancer and 17 with prostate cancer were examined by FDG-PET before pelvic lymph node dissection. Results of PET were then compared to histology of pelvic lymph nodes obtained at surgery. Lymph node metastases were detected by histopathologic examination in 3 patients with bladder cancer, and in 6 patients with prostate cancer. At the sites with histologically proven metastases, increased FDG uptake suspicious of metastatic disease was found in 2/3 and 4/6 patients, respectively. The smallest detected metastasis was a micrometastasis with a diameter of 0.9 cm. In 3 additional patients with histopathologically proven micrometastases (</=0.5 cm), FDG uptake was within the normal range. There were no false-positive results (Heicappell R, etal, Eur Urol, Dec 1999;36(6):582-7).

Bladder cancer is also classified by grade which refers to the degree of abnormality of tumor cells. Grade is a pathologic diagnosis/finding, and essentially indicates how abnormal (less differentiated) the cancer cells are in comparison to normal cells of the same organ type. The current system of grading, designated as II/III, uses only three different grades, i.e., well differentiated (Grade I), moderately differentiated (Grade II), and poorly differentiated (Grade III). Some pathologists may use a 4-level grading system (Grade I to IV) designated as II/IV. As a general rule, a tumor's grade corresponds to its rate of growth or aggressiveness. An undifferentiated or high-grade tumor grows more quickly than a well differentiated or a low-grade one. Tumor size or depth are not directly related to tumor grade. For instance, although CIS is a superficial tumor, it is a potentially dangerous and usually high-grade. Tumor grading, although important, is less meaningful than staging regarding prognosis and treatment options. Incidence by tumor grade in the USA is presented in Exhibit 7.

One less commonly used test that helps grade bladder cancer is flow cytometry that counts the number of chromosomes (ploidy) in cancer cells. Normal diploid cells have 46 chromosomes comprising 23 pairs. However some cancer cells have an extra chormosome (aneuploid). Aneuploid cancers tend to spread more quickly and often have a worse prognosis.

When BTA Trak, p53, epidermal growth factor receptor (EGFr), microvessel density (MVD), and cytology, were evaluated to determine if they were predictive of clinical stage in patients newly diagnosed with bladder cancer, based on multivariate analysis, tumor grade was the single best predictor of clinical stage. BTA Trak and MVD contributed incremental information to tumor grade in predicting clinical stage. These findings are from a prospective pilot study conducted at the University of Virginia Health Sciences Center (Charlottesville, VA), that enrolled, between December 1997 and September 1998, 22 men and 7 women with a cystoscopic diagnosis of urothelial bladder cancer; 21 patients had sufficient tissue for all immunohistochemical assessments and comprised the study group. Urine was collected for cytologic and BTA Trak evaluation before TUR, and tumor grade and clinical stage were obtained from the TUR specimen. Univariate and multivariate comparisons were carried out to determine the contribution of each variable to the prediction of clinical stage. Although there was a trend, cytologic analysis and p53 and MVD immunoreactivity did not significantly correlate with clinical stage, while tumor grade, BTA Trak and EGFr immunoreactivity did. In a univariate analysis, tumor grade and BTA Trak were related to clinical stage, and addition of BTA Trak and MVD information to grade incrementally improved its predictive ability (Krupski T, etal, BJU Int, Jun 2000;85(9):1027-32).

Editor's note: *The next issue of FUTURE ONCOLOGY comprises Part III of the bladder cancer series covering current treatment approaches, including a database of combination and multimodality clinical trials.*

TECHNOLOGY UPDATE

SYNTHETIC NUCLEIC ACID SEQUENCE CONSTRUCTS AS ONCOLOGY THERAPEUTICS — PART II

TYPES OF SYNTHETIC NUCLEIC ACID CONSTRUCTS IN DEVELOPMENT AS ONCOLOGY THERAPEUTICS

There are many different types of synthetic nucleic acid constructs, representing true antisense ODN, triplex-forming oligos, catalytic nucleic acids or ribozymes, chimeric nucleotides, antisense RNA, and aptamers; a number of these are being investigated in preclinical or clinical studies in oncology and many other indications.

Antisense Oligos

An antisense oligonucleotide is a chemically synthesized single-stranded RNA fragment that is complementary to the nucleotide sequence of a known mRNA of interest. Traditionally 15-25 bases long, this synthetic oligomer can hybridize with the mRNA through Watson-Crick base pairing, thereby selectively and transiently blocking normal mRNA-directed protein synthesis (translation). This blocking effect, however, becomes irreversible if a single-stranded DNA fragment is targeted against a specific mRNA. In this case, the resultant DNA/mRNA hybrid serves as a substrate for RNase H, an enzyme involved in DNA replication, which cleaves the RNA moiety, making the antisense effect irreversible. Although complementary base-pairing between target mRNA and RNA strands (ORN) as well as DNA strands (ODN) represent antisense approaches, it is the latter that is considered the classic antisense mechanism (Varga LV, etal, Immunol Lett, 3 Aug 1999;69(2):217-24). Fomivirsen, approved in 1998 for the treatment of CMV retinitis, is a P=S antisense DNA ODN acting through the classic mechanism, as do most of the agents in current clinical development; however, there are some newer oligonucleotides in development, including the M=P, P=A, morpholino, and PNA ODN, that inhibit mRNA translation through highly sequence-specific antisense steric blockade, rather than classical RNase H activation.

In addition to their effects on mRNA translation, antisense oligomers have been introduced into cells as decoy *cis*-elements to alter gene expression by inhibiting specific transcriptional regulatory proteins. The CRE, 5'-TGACGT-CA-3', is the consensus sequence for the *cis*-element that directs cAMP-regulated gene expression; the CRE complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes. Because the CRE *cis*-element is palindromic, a synthetic single-stranded oligonucleotide composed of the CRE sequence self-hybridizes to form a duplex/hairpin. The CRE-palindromic oligonucleotide has been shown to be capable of penetrating into cells, competing with CRE enhancers for binding transcription factors, and specifically interfering with CRE- and AP-1-directed transcription. These constructs have demonstrated antiproliferative effects on tumor cells *in vitro* and *in vivo*, apparently through the stabilization and activation of p53, without affecting the growth of nonmalignant cells (Park YG, etal, J Biol Chem, 15 Jan 1999;274(3):1573-80, and Lee YN, etal, Biochemistry, 25 Apr 2000;39(16):4863-8).

Triplex-Forming ODN (TFO)

Triplex-forming ODN (TFO) are antisense oligomers, usually synthetic DNA homologs, that are complementary to specific nucleic acid sequences of the genomic DNA duplex itself and bind by strand invasion; by selectively hybridizing with their target DNA, TFO can modulate or repress mRNA transcription initiation at the level of the gene, possibly by interfering with the attachment of control proteins or enzymes necessary for transcription. Unlike antisense oligomers targeting mRNA, that might have to intercept literally thousands of frequently regenerated targets to be effective, TFO would need to bind to only a small number of operating gene copies per cell to inactivate the target. This approach may also provide for selective gene activation or destruction; the former by antagonizing a repressor of transcription initiation, the latter by affixing a reactive chemical to the TFO. Although referred to as "antisense," given their sequence-specific complementarity, TFO are more properly considered an "antigene" approach.

Several ODN recognize duplex homopurine sequences of DNA, including:

- PNA ODN, which form a stable PNA-DNA-PNA triplex with a looped-out DNA strand (Hanvey JC, etal, Science, 27 Nov 1992;258(5087):1481-5, Knudsen H and Nielsen PE, Nucleic Acids Res, 1 Feb 1996;24(3):494-500, and Ray A and Norden B, *ibid*)
- P=A ODN that can form extremely stable triple stranded complexes with single or duplex DNA under near physiological salt and pH conditions (Gryaznov SM, *ibid*)
- morpholino ODN (Lacroix L, etal, Biochem Biophys Res Commun, 13 Apr 2000;270(2):363-9)
- G-rich ODN (Cheng AJ and Van Dyke MW, *ibid*).

Unfortunately, like other ODN, TFO suffer from inefficient intracellular localization, and may require uptake facilitators for efficient entry into the cytosolic/nuclear compartment of cells. In a strategy designed to enhance nuclear uptake of TFO for gene-targeted therapeutics, scientists at the University of Medicine and Dentistry of New Jersey (UMDNJ)-Robert Wood Johnson (RWJ) Medical School (New Brunswick, NJ) and Josai University (Saitama, Japan), have achieved a 6- to 10-fold increase in the nuclear localization of a 37-mer TFO targeted to the promoter region of c-myc, in MCF-7 and MDA-MB-231 breast cancer cells by complexing it with a hexamine (Thomas RM, etal, AACR99, Abs. 3870:587). When scien-

tists at these institutions examined the effect of polyamine analogs such as BE-norspermine (BE-3-3-3) and BE-homospermine (BE-4-4-4) on the stability of the above TFO under physiological pH and ionic conditions, the analogs stabilized the TFO, and treatment of MCF-7 breast cancer cells by analog-complexed TFO showed a synergistic growth inhibitory effect, with HPLC analysis demonstrating facile transport of the analogs into the cells (Thomas TJ, etal, AACR99, Abs. 128).

In view of the fact that gene therapy has been hindered by the low frequency of homologous recombination in mammalian cells, at Yale University School of Medicine (New Haven, CT) and the National Cancer Institute (NCI), researchers are collaborating on the potential utility of TFO to stimulate recombination in a site-specific manner within cells as an alternative to standard gene transfer techniques (Chan PP, etal, J Biol Chem, 23 Apr 1999;274(17):11541-8, and Vasquez KM and Wilson JH, Trends Biochem Sci, Jan 1998;23(1):4-9).

By treating monkey COS cells with ODN linked to psoralen, targeted mutations were generated in a simian virus 40 (SV40) vector contained within the cells via intracellular triple helix formation. ODN entry into the cells and sequence-specific triplex formation within the SV40 DNA delivered the psoralen to the targeted site, and photoactivation of the psoralen by long-wavelength UV light yielded adducts and, consequently, mutations at that site (Wang G, etal, Mol Cell Biol, Mar 1995;15(3):1759-68, Faruqi AF, etal, Mol Cell Biol, Dec 1996;16(12):6820-8, and Majumdar A, etal, Nat Genet, Oct 1998;20(2):212-4).

Further work, showing that triple helices, even in the absence of associated psoralen adducts, are able to provoke DNA repair and cause mutations, has led to finding that intermolecular triplex formation itself is capable of stimulating recombination, and that this effect is dependent on a functional nucleotide excision repair (NER) pathway. However, the ability of triplex-directed psoralen cross-links to induce recombination is only partially reduced in NER-deficient cells, suggesting that NER is not a required pathway for the metabolization of targeted psoralen photoadducts into recombinagenic intermediates (Faruqi AF, etal, Mol Cell Biol, Feb 2000;20(3):990-1000).

In other work, Yale scientists have used TFO to promote recombination within a mouse LTK(-) cell line carrying two mutant copies of the herpes simplex virus (HSV) thymidine kinase (TK) gene as direct repeats in a single chromosomal locus. Recombination between these repeats that occurs at a spontaneous frequency of 4×10^{-6} under standard culture conditions, can produce a functional TK gene. When cells were microinjected with TFO designed to bind to a 30-bp polypurine site situated between the two TK genes, recombination was observed at frequencies in the range of 1%, 2,500-fold above the background. Control oligomers of scrambled sequence but identical base composition were ineffective, and no TFO-induced recombination was seen in a control LTK(-) cell line carrying an otherwise identical dual TK gene construct lacking the 30-bp polypurine target site. Examination of the TFO-induced recombinants by genomic Southern blotting revealed gene conversion events in which both TK genes were retained, but either the upstream (57%) or the downstream (43%) gene was corrected to wild type (Luo Z, etal, PNAS USA, 1 Aug 2000;97(16):9003-8).

In related work, scientists at Codon Pharmaceuticals (Gaithersburg, MD) attempted to correct chromosomal point mutations in human cells with bifunctional ODN combining two triplex-forming specificities; these ODN combine a repair domain (RD) containing the native sequence of the target region, which forms a heteroduplex leading to DNA-loop formation through Watson-Crick binding, and a third strand-forming domain (TFD) designed to form a triplex by Hoogsteen interactions. The design is based on the premise that the RD will rapidly form a heteroduplex that is anchored synergistically by the TFD. Bifunctional TFO were shown to be capable of correcting the mutant sequence of ADA-deficient human lymphocytes in 1%-2% of cells, whereas correction of p53 mutation in human glioblastoma cells induced apoptosis in 7.5% of cells; compared with published mutation correction efficiencies using traditional homologous recombination strategies, the bifunctional TFO produced greater than a 100-fold increase in correction efficiency (Culver KW, etal, Nat Biotechnol, Oct 1999;17(10):989-93). Codon, a subsidiary of Oncor, was liquidated in 1999, with all intellectual property relating to TFO in the repair of genetic mutations in living cells reverting back to Yale University, from which it had been licensed in 1995.

This work demonstrates that ODN-mediated triplex formation can provide the basis for a potential recombinagenic approach to genome modification. To improve the efficiency of this strategy, NCI investigators M.M. Seidman and A. Majumdar are using TFO to modify the gene sequences of cells which have been cultured so as to synchronize their cell cycles (NIH, Office of Technology Transfer, Selected Technologies Available for Licensing; serial # 60/191,996, filed on March 24, 2000).

Catalytic Nucleic Acids/Ribozymes

Catalytic nucleic acids, commonly referred to as ribozymes, represent a distinct class of metalloenzymes made entirely of RNA. First identified in 1981 by Thomas R. Cech and colleagues at the University of Colorado (Boulder, CO), naturally occurring ribozymes are capable of site-specific, endonuclease-mediated, reversible cleavage of phosphodiester bonds in target RNA in *cis* (Cech TR, etal, Cell, Dec 1981;27(3 Pt 2):487-96). Most ribozymes catalyze reactions at phosphorus centers, cleaving the phosphodiester bond of substrate RNA at a point where there is a triplet of nucleotide bases in the order GUC, to generate 5'-hydroxyl and 2',3'-cyclic phosphate termini (Pyle AM, Science, 6 Aug 1993;26 (5122):709-14).

Artificial ribozymes can also be engineered that promote sequence-specific catalytic cleavage of target mRNA in *trans*, a strategy that may allow alteration of the level of any RNA in a living organism (Haseloff J and Gerlach WL, Biotechnology 1992;24:264-9, Zhenodarova SM, Mol Biol (Mosk), Mar-Apr 1993;27(2):245-68, Atkins D and Gerlach WL, Antisense Res Dev, Summer 1994;4(2):109-17, and Jarvis TC, etal, J Biol Chem, 15 Nov 1996; 271(46):29107-12).

Exhibit 4
Incidence of Bladder Cancer by Histologic Type in the USA in 2000

	Incidence (#)	Total (%)
Urothelial transitional cell carinoma (TCC) of the bladder	50,400	93.0
Squamous cell carcinoma	2,709	5.0
Adenocarcinoma	1,084	2.0
All bladder cancer	54,193	100.0

Hammerhead ribozymes are perhaps the best-characterized and most widely studied catalytic RNAs. Hammerhead ribozymes form a stem-loop secondary structure called a "hammerhead" in which the catalytic (ribozyme) and substrate sequences are brought close together. In the hammerhead ribozyme, a small catalytic RNA motif of three base-paired stems that are A-form helices, is combined with a core of highly conserved, non-complementary nucleotides having two structural domains, the first being a sharp turn identical to the uridine turn of transfer RNA, and the second being a non-Watson-Crick three-base-pair duplex with a divalent-ion binding site (Pley HW, etal, Nature, 3 Nov 1994;372(6501): 68-74, Birikh KR, etal, Eur J Biochem, 1 Apr 1997;245(1):1-16, and Wedekind JE and McKay DB, Annu Rev Biophys Biomol Struct 1998;27:475-502).

Hammerhead ribozymes have been constructed against a number of molecular markers, including HER2/neu (Giannios JN, etal, ASCO00, Abs. 1878:478a), nuclear factor kB (Kashani-Sabet M, etal, ASCO00, Abs. 1805:460a), VEGF receptors Flt-1 and KDR (Parry TJ, etal, Nucleic Acids Res, 1 Jul 1999;27(13):2569-77, and Parker VP, etal, ASCO00, Abs. 703:180a), bcl-2 (Dorai T, etal, Int J Cancer, 9 Sep 1999;82(6):846-52), and telomerase (Folini M, etal, J Invest Dermatol, Feb 2000;114(2):259-267), among others.

Unfortunately, despite much effort and numerous developments that have advanced the state-of-the-art in the field of catalytic nucleic acids, progress has been slow in attaining highly efficient cleavage of target molecules in living cells. A major limitation facing conventional *trans*-acting hammerhead ribozymes is that none have demonstrated complete or nearly-complete target cleavage *in vivo.*

Several modifications have been attempted to improve cleavage efficiency of hammerhead ribozymes.

Minizymes were constructed by scientists at the University of Tsukuba (Tsukuba Science City, Japan) to achieve more efficient cleavage. A minizyme is a hammerhead ribozyme with a short oligonucleotide linker in place of the stem/loop II. Exhibiting low activity as monomers, minizymes form active dimeric structures with a common stem. When placed under the control of a human tRNA(Val) promoter, minizymes inhibited reporter gene activity by 95% compared to 55% inhibition achieved with a tRNA(Val)-embedded conventional hammerhead ribozyme (Kuwabara T, etal, Nat Biotechnol, Oct 1998;16(10):961-5).

Maxizymes are heterodimer minizymes that cleave the target substrate at two sites simultaneously by forming a cavity that can capture catalytically indispensable Mg2+ ions. Maxizymes are allosterically controllable and can be transcribed *in vivo* under the control of a human tRNA(Val) promoter (Kuwabara T, etal, Mol Cell, Nov 1998;2(5):617-27). A maxizyme has been used to specifically cleave bcr-abl fusion mRNA, the translated products of which have been associated with the development of chronic myelogenous leukemia (CML), strongly suppressing the progression of CML in murine models (Hamada M, etal, FEBS Lett, 12 Nov 1999;461(1-2):77-85, and Tanabe T, etal, ASH99, Abs. 459).

Hairpin ribozymes are small catalytic RNA constructs that are members of the hammerhead family. They were designed to improve cleavage efficiency and are capable of catalyzing reversible sequence-specific mRNA hydrolysis in *trans* (Earnshaw DJ and Gait MJ, Antisense Nucleic Acid Drug Dev, Aug 1997;7(4):403-11). Conventional hairpin ribozymes consist of two domains that interact with each other by antiparallel docking to facilitate catalysis, with a region between the two domains acting as a flexible hinge for interdomain interactions to occur. Unlike typical hammerhead ribozymes that cleave target phosphodiester bonds in a reaction dependent on divalent metal ions, the hairpin is unique in that its catalytic mechanism does not require metals for cleavage or ligation of substrate RNA (Shippy R, etal, Mol Biotechnol, Aug 1999;12(1):117-29).

Angiozyme (RPI.4610), under development by Ribozyme Pharmaceuticals (Boulder, CO), and the first ribozyme to be approved for clinical trials, is a hairpin ribozyme. At the Humboldt-Universitat zu

Berlin, in Germany, researchers have constructed hairpin-derived twin ribozymes by coupling conventional and reverse-joined hairpin ribozymes in a construct that is capable of cleaving a suitable RNA substrate at two specific sites, while maintaining the target specificity of the individual mono-ribozymes; the hairpin ribozymes with reverse-joined domains are constructed by dissecting the domains at the hinge and rejoining them in reverse order (Schmidt C, etal, Nucleic Acids Res, 15 Feb 2000;28(4):886-94).

Snorbozymes are small nucleolar RNA:ribozyme hybrids constructed in the laboratory of Maurille J. Fournier, PhD, at the University of Massachusetts (Amherst, MA), in collaboration with researchers at the Université de Montreal in Canada, and the Albert Einstein College of Medicine (Bronx, NY), to improve ribozyme-mediated RNA cleavage *in vivo*. This *trans*-acting hybrid, formed by using the U3 small nucleolar RNA (snoRNA) as a carrier to localize a hammerhead ribozyme to the yeast nucleolus, is metabolically stable and has been shown to cleave a target U3 RNA with nearly 100% efficiency *in vivo*.

This strategy may prove valuable for modulating natural snoRNA and ribosomal RNA levels, and the levels of other RNAs apparently associated with the nucleolus, including various mRNA, tRNA and splicing snRNA as well as RNA components of telomerase, the signal recognition particle, MRP RNase, and RNase P (Samarsky DA, etal, PNAS USA, 8 Jun 1999;96(12):6609-14). In the USA, a patent application has been made on this technology, which is available for licensing from the University of Massachusetts.

Deoxyribozymes or DNA enzymes (DNAzymes), constructed by investigators at the City of Hope National Medical Center (Duarte, CA), are a new form of catalytic nucleic acids that can bind to specific sequences of RNA and catalytically cleave the target site. DNAzymes are smaller and have been shown to be less expensive to synthesize, more resistant to serum, and more efficient ezymatically than conventional ribozymes. Three 5' cholesterol-conjugated P=S DNAzymes have been designed and synthesized that specifically target the mRNA transcripts of the splice 1 and splice 2 variants of the p210 bcr-abl gene and the p190 variant, associated with CML and Ph-positive acute lymphoblastic leukemia (ALL), respectively.

These constructs have been taken up efficiently by a variety of Ph-positive target cells, including pre-B lymphoblastic cells, and have demonstrated inhibition of protein expression and cell growth as well as promotion of apoptosis. This approach may prove useful in purging Ph-positive leukemic cells from stem cell populations in autologous transplantation procedures for CML and ALL (Wu Y, etal, Hum Gene Ther, 20 Nov 1999;10(17):2847-57, and Snyder DS, etal, ASH99, Abs. 1762).

Dy-Tex ribozyme analogs, created by Pharmacyclics (Sunnyvale, CA), in collaboration with the University of Texas at Austin (UTA; Austin, TX), were constructed by covalently binding a phosphoramidate derivative of dysprosium (III) texaphyrin to a synthetic ODN. Texaphyrins, discovered by Dr. Jonathan Sessler of UTA, are metal-coordinating, expanded porphyrins that are small, ring-shaped molecules that bind metals and transport ions. Metals are natural catalysts for many biochemical processes, and the enlarged binding ring of the texaphyrins permits the stable complexation of a wide variety of metal atoms, including dysprosium (III), a lanthanide metal that is capable of catalytically hydrolyzing phosphodiester bonds of RNA. The stability of the metallotexaphyrin complexes, designated Dy-Tex, makes it possible to direct the metal ions to specific nucleotide sequences by attaching the complexes to oligomers that are complementary to the target sequences. The texaphyrin ring holds the metal near or within the plane of the molecule, allowing free interaction with adjacent molecules, and efficient interaction of dysprosium with the RNA target. Still in the research stage, the Dy-Tex ribozyme analog has demonstrated cleavage of an RNA target at enhanced rates, and shown enzyme-like behavior in the presence of excess target (Magda D, etal, J Am Chem Soc 1994;116:7439-40).

Chimeric Nucleotides

The term chimeric nucleotide, although used occasionally to describe oligonucleotides of mixed-backbone, more appropriately refers to ODN consisting of both RNA and DNA residues. In a technique originally developed in the laboratory of Drs. Eric B. Kmiec, Kyonggeun Yoon, and Allyson Cole-Strauss at Thomas Jefferson University (Philadelphia, PA), self-complementary chimeric oligomers composed of a contiguous stretch of DNA and 2'-O-methyl RNA nucleotides are arranged in a duplex configuration with double-hairpin caps on the ends. The 2'-O-methyl modification adds protection against cleavage by RNase H activities, and the hairpin caps protect the molecule against destabilization or destruction by cellular helicases or exonucleases. This technique, referred to as chimeraplasty, is capable of effecting site-directed genetic repair in mammalian cells *in vitro* and *in vivo*. Apparently, the DNA strand of the chimera acts as a template for high-fidelity gene correction through nucleotide exchange, when one of its bases is mismatched to the targeted gene, while the chimeric strand, although not functioning as a template for gene repair, appears to augment the frequency of gene correction by facilitating complex formation with the target. Depending on the accuracy of the chimera design, point mutations and single base deletions can be corrected at frequencies approaching 30%-40% (Yoon K, etal, PNAS USA, 5 Mar 1996;93(5):2071-6, Kren BT, etal, Hepatology, Jun 1997;25(6):1462-8, Cole-Strauss A, etal, Nucleic Acids Res, 1 Mar 1999;27(5):1323-30, Alexeev V and Yoon K, Pigment Cell Res, Apr 2000;13(2):72-9, and Gamper HB Jr, etal, Biochemistry, 16 May 2000;39(19):5808-16).

Patented by Dr. Kmiec (USA patent #5,565,350; issued on October 15, 1996) while at Thomas Jefferson University, the chimeraplasty approach to selective gene modification has been licensed to ValiGen (formerly Kimeragen; Newtown, PA), of which Dr. Kmiec is a founder. ValiGen plans to develop and market chimeraplasty as pharmaceuticals to repair genetic abnormalities implicated in a broad array of human diseases, for plant and industrial products to enhance genetic traits in plants, and for genomic and transgenic animal technologies aimed at developing higher value applications and products for human health and veterinary care. For *in vivo* gene modification, the chimeras can be attached to organ-specific ligands (Smaglik P, The Scientist, 10 Jan 2000;14(1):13), or complexed with liposomes and synthetic polymers for delivery to the appropriate cells or tissues (Stephenson J, JAMA, 13 Jan 1999;281(2):119-21).

Use of these chimeras *in vivo*, however, suffers from some of the same limitations encountered with other ODN, including poor intracellular penetration, and the possibility of nonspecific integration if other, unknown, chemical pathways are involved in gene repair. In addition, each strand of a chimera can hybridize with double-stranded DNA in the presence of RecA protein to form a complement-stabilized D-loop, and sequencing data presented by researchers at the University of Delaware, where Dr. Kmiec recently became director of the Laboratory of Gene Therapy, suggests that these structures may be weakly mutagenic. If this is the case, a low level of random mutagenesis could potentially accompany gene repair in the vicinity of the chimera binding site (Gamper HB Jr, etal, *ibid*).

Exhibit 5
Basis for Staging of Bladder Cancer

TMN	Description
	Tumor Characteristics
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma confined to the epithelium
Tis	Carcinoma *in situ;* reddened flat tumors with high-grade histologic features, confine to the epithelium
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
T3a	Tumor invades microscopically
T3b	Tumor invades macroscopically (extravesical mass)
T4	Tumor invades any of the following, prostate, uterus, vagina, pelvic wall, or abdominal wall
T4a	Tumor invades the prostate, uterus, vagina
T4b	Tumor invades the pelvic wall, abdominal wall
	Regional Lymph Nodes (N) Within the True Pelvis (all others are distant lymph nodes)
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node, £2 cm in greatest dimension
N2	Metastasis in a single lymph node, >2 cm but £5 cm in greatest dimension; or multiple lymph nodes, none >5 cm in greatest dimension
N3	Metastasis in a lymph node >5 cm in greatest dimension
	Metastasis to Other Organs
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Intracellularly Produced Antisense RNA and DNA

Among antisense agents, antisense RNA produced intracellularly by an expression vector has been used *in vitro* and, preclinically, to inhibit target proteins, or genes, responsible for the invasive potential of tumor cells, and directly involved in cell cycle progression, including:

- calmodulin in glioblastoma (Prostko CR, etal, Oncol Res 1997;9(1):13-7)
- proliferating cell nuclear antigen (PCNA) in gastric cancer (Dai L, etal, Chung Hua Chung Liu Tsa Chih, Sep 1998;20(5):337-9)
- urokinase-type plasminogen activator receptor (uPAR) in glioblastoma (Mohan PM, etal, Clin Exp Metastasis 1999;17(7):617-21)
- insulin-like growth factor-1 (IGF-1) in hepatoma and colon carcinoma (Liu Y, etal, Cancer Gene Ther, Mar 2000;7(3):456-65)
- interleukin 8 (IL-8) in transitional cell carcinoma (Inoue K, etal, Cancer Res, 15 Apr 2000;60(8):2290-9)

- cathepsin L in myeloma (Kirschke H, etal, Eur J Cancer, Apr 2000;36(6):787-95)
- aldehyde dehydrogenase class-1 (ALDH-1) in leukemia and lung cancer (Moreb JS, etal, J Pharmacol Exp Ther, May 2000;293(2):390-6)
- glutaminase in Ehrlich ascites (Lobo C, etal, Biochem J, 1 Jun 2000;348 Pt 2:257-61)
- translation initiation factor eIF4E in head and neck squamous cell cancers (DeFatta RJ, etal, Laryngoscope, Jun 2000;110(6):928-33)
- cancer-associated Sm-like (CaSm) oncogene in pancreatic cancer (Kelley JR, etal, Surgery, Aug 2000;128(2):353-60)
- human papillomavirus 16 (HPV16) E7 gene in cervical cancer (Choo CK, etal, Gynecol Oncol, Sep 2000;78(3 Pt 1):293-301).

In this approach, the cDNA coding for the RNA sequence of interest is cloned into an expression vector in reverse orientation. The expression vector, which may be a replication-defective retrovirus or adenovirus or some other eukaryotic expression vector such as pcDNA3, is used to transfect the target cell population, resulting in the intracellular synthesis of antisense RNA. Although RNA expression vectors offer high transfection rates and can be targeted to specific tissues through incorporation of cell-specific promoter moieties, antisense RNA molecules requiring vector enveloping cannot be modified like ODN (i.e., phosphorothioated, etc.) and may be subject to significant degradation by cellular enzymes. However, intracellular production of antisense RNA continues after transfection, offering the advantage of a longer duration of action, a feature that is particularly attractive given the relatively low hybridization rates between the target and rapidly turning-over antisense RNA (Weiss B, etal, Cell Mol Life Sci, Mar 1999;55(3):334-58).

CytoGenix (Houston, TX) is developing intracellular expression systems that enable direct enzymatic synthesis of sequence-specific, single-stranded (antisense) DNA (ssDNA or ODN) in the cell, rather than RNA. The company's EnzSyn technology platform is a cassette that can be inserted into any delivery or expression vector, enabling the vector to express any desired antisense ODN at therapeutic but subtoxic levels; the ssDNA can be targeted to multiple sites on a target RNA or to multiple RNA targets. In July 2000, studies were initiated at Baylor College of Medicine (Houston, TX) to examine the effectiveness of the EnzSyn technology approach in the treatment of cancer as well as other diseases. These investigations are being conducted under a CytoGenix-sponsored research agreement in the laboratories of Dr. Bert W. O'Malley, who is working on the development of an "orally regulatable" antisense therapy for breast cancer, and Dr. Steve E. Welty, who is pursuing antisense strategies against lung inflammation and injury.

The EnzSyn technology platform expands upon CytoGenix' patented ssDNA intracellular expression vector, TroVec. CytoGenix acquired the rights to the vector technology in April 1997 from its original developer Charles A. Conrad, MD, to express ssDNA in prokaryotes for *in vitro* laboratory research. Dr. Conrad was issued a USA patent (#6,054,299) on the methods and compositions relating to the vector in April 2000. The TroVec vector was licensed in February 2000 to PharmaGenix, jointly owned by CytoGenix and Professional Compounding Centers of America, where it will be used in the development of non-regulated nutritional and topical products incorporating nucleic acid constituents. A beta test kit version of the vector was made available in March 1999 to commercial biotech companies.

Aptamers

Nucleic acid aptamers (the term aptamer, coined by Gilead Sciences, comes from the Latin *aptus*, meaning 'fit'), obtained by *in vitro* screening of large oligomer libraries containing random sequences of up to a few hundred nucleotides, are RNA or DNA sequences that are selected for high-affinity binding to a particular target, typically through the systematic evolution of ligands by exponential enrichment (SELEX) process. Although for the most part unstructured in solution, upon associating with their ligands, aptamers fold into molecular complexes of which the ligand is an intrinsic part, thereby not only binding a target, but, in many cases, inhibiting its biological function.

Aptamers have been identified for almost every kind of target molecule, including organic dyes, amino acids, biological cofactors, antibiotics, peptides and proteins, reflecting a diversity of sophisticated, three-dimensional motifs that confer specificity to the aptamer-ligand association through precise stacking of flat moieties, specific hydrogen bonding, and molecular shape complementarity, rather than through base-pair binding (Famulok M and Mayer G, Curr Top Microbiol Immunol 1999;243:123-36, Hermann T and Patel DJ, Science, 4 Feb 2000; 287(5454):820-5, Famulok M, etal, Acc Chem Res, Sep 2000;33(9):591-9).

The SELEX process allows for rapid screening of libraries containing up to one million billion (10^{15}) random oligonucleotide sequences. If required, additional aptamer stability can be conferred by using libraries of chemically modified nucleic acids, or by exploiting the nuclease resistance of the enantiomer of naturally occurring nucleic acids (Klussmann S, etal, Nat Biotechnol, Sep 1996;14(9):1112-5, and Nolte A, etal, Nat Biotechnol, Sep 1996;14(9):1116-9). Compared to naturally occurring RNA, SELEX-derived aptamers from sugar-modified nucleotide libraries are up to 200 times more stable in circulation in animal models, and furthermore, circulation time can also be increased by conjugating aptamers to higher molecular weight vehicles (Gold L, J Biol Chem, 9 Jun 1995;270(23):13581-4, Eaton BE, etal, Bioorg Med

Exhibit 6
Incidence of Bladder Cancer by Stage in the USA in 2000

AJCC Stage Groupings	TNM Stage	Incidence (#)	Total (%)
Stage 0a	Ta/N0/M0	43,354	80.0
Stage 0is	Tis/N0/M0		
Stage I	T1/N0/M0		
Stage II	T2a/N0/M0; T2b/N0/M0	8,129	15.0
Stage III	T3a/N0/M0; T3b/N0/M0; T4a/N0/M0		
Stage IV	T4b/N0/M0; any T/N1/M0; any T/N2/M0; any T/N3/M0; any T/any N/M1	2,710	5.0
All Stages		54,193	100.0

Chem, Jun 1997;5(6):1087-96, and Brody EN and Gold L, J Biotechnol, Mar 2000;74(1):5-13).

Originally developed by C. Tuerk and L. Gold at the University of Colorado (Tuerk C and Gold L, Science 1990;249:505-10), and licensed to Nexagen, which subsequently became NeXstar Pharmaceuticals (Boulder, CO), SELEX is now part of the Gilead Sciences (Foster City, CA) technology portfolio, following the merger of NeXstar into Gilead in July 1999. The SELEX process was non-exclusively licensed to Glaxo Wellcome in 1998 for the identification of specific anticancer drug candidates. Gilead is currently developing aptamers for the *in vivo* diagnosis of tumors and thromboembolic disease as part of a strategic alliance with Schering AG (Berlin, Germany) that was established by NeXstar in 1993. Under this agreement, Schering is also pursuing the development of aptamers as radiotherapeutics.

As engineered nucleic acid constructs, aptamers can be used to identify previously unknown natural ligand binding sites for a given target protein (or other molecule) that may be involved in disease processes. They can also be used to inhibit specific proteins (and other molecules) based on the conformational ordering that occurs upon ligand binding. For instance, at Rockefeller University (New York, NY), investigators using the SELEX process have isolated RNA aptamers which bind to neuron-specific RNA binding protein Nova-1, an autoantigen in paraneoplastic opsoclonus myoclonus ataxia, a neurologic disorder associated with breast cancer and motor dysfunction. For the first time, aptamers were used to identify a conserved 15-mer consensus motif found to be absolutely essential for Nova-1 binding to two neuronal pre-mRNAs, the glycine receptor α2 (GlyR α2) pre-mRNA, and the pre-mRNA encoding for Nova-1 itself. Study findings suggested that the mechanism of disease involves disruption of Nova-1 binding to GlyR α2 pre-mRNA by the autoantibody (Buckanovich RJ and Darnell RB, Mol Cell Biol, Jun 1997;17(6):3194-201).

In another study, scientists at the University of Michigan (Ann Arbor, MI) used the SELEX process to select DNA aptamers that bind to cellobiose, the disaccharide of cellulose. Cell surface oligosaccharides are essentially involved in diverse biological phenomena, including cellular adhesion, inflammation, and molecular recognition. Therefore, high affinity ligands, capable of selectively recognizing different oligosaccharides based on small surface motifs may prove useful as diagnostic tools as well as therapeutic agents in cancer and other diseases. Unlike antibodies, that often cannot discriminate between various sugar tags of cell surface receptors, the selected aptamers were capable of discriminating between sugar epimers, anomers, and other disaccharide linkages (Yang Q, etal, PNAS USA, 12 May 1998;95(10):5462-7).

Aptazymes are allosteric ribozymes produced by aptamers combined with catalytic nucleic acid motifs. The operation of cellular processes relies on strict control exerted by each cell over its metabolic pathways, with some protein enzymes being subject to allosteric regulation, in which binding sites located apart from the enzyme's active site can specifically recognize effector molecules and alter the catalytic rate of the enzyme through conformational changes. Aptazymes can be regulated by small-molecule cofactors, with the aptamer domain recognizing the presence of a high-affinity ligand and the catalytic domain effecting target disruption through endonuclease-mediated hydrolysis. Aptazymes can rival the catalytic efficiency of natural ribozymes (Robertson MP and Ellington AD, Nat Biotechnol, Jan 1999;17(1):62-6, Soukup GA, etal, J Mol Biol, 12 May 2000;298(4):623-32, and Soukup GA and Breaker RR, Curr Opin Struct Biol, Jun 2000;10(3):318-25).

At Yale University (New Haven, CT), researchers have joined an ATP-binding RNA aptamer to a self-cleaving ribozyme to create an allosteric ribozyme that has a catalytic rate that can be tightly and specifically controlled by ATP. For instance, a 180-fold reduction in rate was observed upon addition of either adenosine or ATP, but no inhibition was detected in the presence of dATP or other nucleoside triphosphates. Using a similar design approach, allosteric ribozymes that are activated in the presence of theophylline have also been created (Tang J and Breaker RR, Chem Biol, Jun 1997;4(6):453-9). Scientists at Massachusetts General Hospital have isolated AMP-activat-

ed ligase ribozymes from a sequence library constructed by conjoining a pool of long random RNA sequences to a mutagenized ATP aptamer (Hager AJ and Szostak JW, Chem Biol, Aug 1997;4(8):607-17).

Aptamers as diagnostics may surpass monoclonal antibodies (MAb) because of their capacity to recognize virtually any class of target molecules with high affinity and specificity. Aptamers mimic the properties of antibodies in a variety of diagnostic formats, and with the demand for diagnostic assays to assist in the management of existing and emerging diseases increasing, aptamers have the potential to fulfill molecular recognition needs in those assays (Jayasena SD, Clin Chem, Sep 1999;45(9): 1628-50).

With the automation of the SELEX process, arrays containing hundreds to thousands of aptamers on solid surfaces can be made in an economically feasible manner; blood and urine can be analyzed on surfaces that capture and quantitate proteins. The SELEX process has also been adapted to the use of 5-bromo and 5-iodo deoxyuridine residues, halogenated bases that can be specifically cross-linked to proteins. The use of photo-cross-linkable aptamers will allow the covalent attachment of aptamers to their cognate proteins, with very low backgrounds from other proteins in body fluids, giving the aptamer the capability of substituting for two reagents, the capture antibody and the detection antibody, in a typical sandwich array (Brody EN, etal, Mol Diagn, Dec 1999;4(4):381-8, and Brody EN and Gold L, *ibid*).

BIOLOGICAL ACTIVITY OF ODN

Despite all the potential therapeutic advantages of using antisense ODN, and the large number of studies that have been performed over the years, it is difficult to conclusively demonstrate that the biological effects observed *in vitro* and *in vivo*, including tumor growth inhibition, are attributed exclusively to sequence-specific inhibition of genetic expression. There is evidence both for a true antisense mechanism of action and for nonantisense protein-oligonucleotide interactions, the nature of which remains unclear. If ODN prove safe and effective in human clinical trials, knowledge of the exact nature of their biologic effect may be considered less important than the consequences of the effect itself. However, when used for target validation studies in drug development, it is critical to be able to differentiate between antisense and nonantisense interactions. Unfortunately, while it is clear that the affinity of an ODN for its target RNA is influenced by both base composition and sequence, knowledge of the biologic effects of possible nucleotide motifs is limited. Some motifs may cause adverse or different biological effects of equal or greater activity than the ODN. So far, biological consequences have been elucidated for only certain sequence structures, most notably CpG and G-rich (Agrawal S and Kandimalla ER, Mol Med Today, Feb 2000;6(2):72-81).

CpG-containing ODN

Oligonucleotides containing cytidine-phosphate-guanosine (CpG) dinucleotide motifs that mimic bacterial DNA exhibit potent immunostimulatory activity (Zhao Q, etal, Biochem Pharmacol, 26 Jan 1996;51(2):173-182), inducing B cells to proliferate, differentiate, and secrete cytokines, including IL-12, IL-6, IFN-γ, and TNF-α, that can in turn induce host natural killer (NK) cell lytic activity (Zhao Q, etal, Antisense Nucleic Acid Drug Dev, Oct 1997;7(5):495-502, Kranzer K, etal, Immunology, Feb 2000;99(2):170-8, Takeshita F, etal, Eur J Immunol, Jan 2000;30(1):108-16, and Takeshita F and Klinman DM, Eur J Immunol, Jul 2000;30(7):1967-76).

The triggering of B-cell proliferation and differentiation depends on the sequence, base composition, and position of the CpG motif in the sequence. CpG motifs contained in oligonucleotides as short as 15 bases in length have been shown to be quite effective at inducing NK cell lytic activity, and a definite requirement for an unmethylated CpG dinucleotide flanked by two purines on the 5'-side and two pyrimidines on the 3'-side of the oligomer has been identified. In addition, CpG oligomers bearing phosphorothioate linkages in the nucleotide backbone trigger an immune activation that is approximately 2 logs more potent than that elicited by the same sequence with a phosphodiester backbone. Phosphorodithioate linkages render even greater potency, with B-cell activation induced at concentrations approximately 1 log lower than required for phosphorothioate backbone modifications (Ballas ZK, etal, J Immunol, 1 Sep 1996;157(5):1840-5, and Krieg AM, etal, Antisense Nucleic Acid Drug Dev, Summer 1996;6(2):133-9).

The immunostimulatory activity of phosphorothioate oligonucleotides containing the CpG motif can be modulated by appropriate substitution of deoxynucleosides in the flanking region with 2'-O-methylribonucleosides, allowing the immune response stimulation engendered by these oligomers to be suppressed or enhanced as may be required for different therapeutic uses (Zhao Q, etal, Bioorg Med Chem Lett, 20 Dec 1999;9(24):3435-8). Modulation can also be accomplished by substitution of a single deoxynucleoside by a 3'-O-methylribonucleoside or a 2'-O-methoxyethoxyribonucleoside at specific sites in the flanking region to the CpG motif (Zhao Q, etal, Bioorg Med Chem Lett, 15 May 2000;10(10):1051-4).

Immune-activating ODN containing CpG motifs are currently being investigated as immunomodulators for the treatment of allergies, infectious diseases, autoimmune disorders, and cancer (Klinman DM, etal, Springer Semin Immunopathol 2000;22(1-2):173-83). For instance, CpG-containing ODN have successfully been used as immune adjuvants in murine models of malignant glioma (Connell YS and Neckers LM, AACR99, Abs. 1982:299).

These ODN may also have utility as antisense therapeutics. However, if an antisense ODN sequence of interest has a CpG motif, extreme care will be required to estab-

Exhibit 7
Incidence of Bladder Cancer by Grade in the USA in 2000

Grade	Description	Comments	Incidence (#)	Total ($)
Low-grade tumors (Grade I and II)	Well differentiated tumors that resemble normal bladder tissue and usually do not grow or spread quickly	More than 90% of patients with Grade I tumors have a benign form of bladder neoplasm, and few have truly malignant tumors (Holmang S, etal, J Urol, Sep 1999;162(3 Pt 1):702-7)	29,806	55.0
High-grade (Grade III and sometimes IV)	Poorly differentiated tumors that do not resemble normal bladder and usually grow quickly and spread to other tissues earlier		21,677	40.0
CIS	Carcinoma *in situ*			
Mixed-grade	Multiple tumors of different grades		2,710	5.0
All Grades			54,193	100.0

lish its specificity of antisense activity. In addition, sequence-independent side effects associated with these ODN include complement activation, decreased blood clotting and heart rate, and increased blood pressure (Gura T, Science, 27 Oct 1995;270(5236):575-7, and Gura T, Science, 17 May 1996;272(5264):954-6), and although typically milder than those attributable to cytotoxic drugs, are still worrisome, especially if larger ODN doses and/or longer duration of therapy become necessary.

If a CpG motif is absolutely required for antisense activity, nonantisense related effects can be reduced through chemical modification of the ODN, which may take the form of replacing the cytosine base with a 5-methyl-cytosine base, or replacing a P=S linkage between C and G with a M=P linkage (Agrawal S and Zhao Q, Antisense Nucleic Acid Drug Dev, Apr 1998;8(2):135-9). The immunostimulatory activity of ODN containing the CpG motif can also be modulated by substitution of a single deoxynucleoside at specific sites with either 2'-O-methylribonucleoside, 3'-O-methylribonucleoside or 2'-O-methoxyethoxyribonucleoside in the flanking region to the CpG motif (Zhao Q, etal, Bioorg Med Chem Lett, 20 Dec 1999;9(24):3453-8; Zhao Q, etal, Bioorg Med Chem Lett, 15 May 2000;10(10):1051-4).

G-rich ODN

Oligonucleotides containing four or more contiguous guanosines (G) that are referred to as G-rich or G4 (G-quartets), may also exhibit biological activity through nonantisense mechanisms (Saijo Y, etal, Jpn J Cancer Res, Jan 1997;88(1):26-33). Evidence suggests that this activity relates to the ability of GGGG sequences to form hyperstructures in which four hydrogen-bonded guanines are stabilized as square planar arrangements by monovalent cations. Non-antisense effects may be attributable to sequestration of intracellular or surface proteins by the folded or G-quartet-containing structures, with binding mediated by their three-dimensional shape rather than through ODN sequence recognition (Benimetskaya L, etal, Nucleic Acids Res, 1 Jul 1997;25(13):2648-56, and Stein CA, Ciba Found Symp, *ibid*).

As is the case with CpG-containing ODN, if intended for antisense uses, ODN containing G-rich motifs should be avoided. To date, with the exception of replacing the guanosines in the G-rich site with 7-deazaguanines, no chemical modification has been shown effective in preventing hyperstructure formation (Agrawal S and Kandimalla ER, *ibid*). On the other hand, certain G-rich ODN have been shown to strongly inhibit proliferation in several human tumor cell lines, including prostate (DU145), breast (MDA-MB-231, MCF-7), and cervical (HeLa) carcinomas. These nonsequence-specific effects appear to be cytostatic rather than cytotoxic, and in many cases can equal or exceed the expected biological effects of antisense ODN (Barton CM and Lemoine NR, Br J Cancer, Mar 1995;71(3):429-37, and Bates PJ, etal, J Biol Chem, 10 Sep 1999;274(37):26369-77).

The nonantisense effects of G-rich ODN on tumor cell growth may represent a useful therapeutic approach in cancer. However, until recently, the protein target(s) of these ODN that mediate nonantisense effects had not been unequivocally identified. Researchers at the University of Alabama at Birmingham have presented convincing evidence that G-rich ODN bind to nucleolin, a specific, cellular, multifunctional phosphoprotein, whose levels are related to the rate of cell proliferation, and that the biological activity of these ODN correlates with binding to this protein (Bates PJ, etal, *ibid*, and Bates PJ, etal, AACR99, Abs. 141:21).

Nucleolin is thought to be located predominantly in the nucleolus of proliferating cells, and although the relationship between nucleolin/G-rich ODN binding and antiproliferative activity has not yet been fully elucidated, the pro-

tein has been implicated as an architectural factor in ribosome biogenesis, including the control of rDNA transcription, replication, and recombination (Ginisty H, etal, J Cell Sci, Mar 1999;112(Pt 6):761-72). The top or nontemplate strand of rDNA is very guanine-rich and is able to form stabilized structures by GG pairing.

Nucleolin binds GG-paired or G4 DNA with very high affinity, apparently through its central domain, which contains four RNA recognition motifs, and through its glycine- and arginine-rich C terminus, and this binding may inhibit one or more of nucleolin's normal functions, with profound consequences for cell growth (Dempsey LA, etal, J Biol Chem, 8 Jan 1999;274(2):1066-71, and Hanakahi LA, etal, J Biol Chem, 28 May 1999;274(22):15908-12).

ODN Conjugated to Radioisotopes

Given their site-specific selectivity, ODN labeled with gamma-emitting radioisotopes are likely to find eventual application as radiopharmaceuticals, an approach under development in Dr. Donald J. Hnatowich's laboratory at the University of Massachusetts. Methods of radiolabeling single-stranded DNA oligonucleotides by chelation with radionuclides such as In-111 and Tc-99m have been developed. However, labeled phosphodiester and phosphorothioate ODN have been judged unsuitable for most radiopharmaceutical applications because of interference by the chelate with hybridization of the ODN with its complement as well as unacceptable retention of label in organs such as liver, spleen and kidney (Hnatowich DJ, Q J Nucl Med, Sep 1996;40(3):202-8).

On the other hand, the ability of PNA oligomers to hybridize with their complement appears to be unimpaired after conjugation and radiolabeling with Tc-99m through a modified MAG3 chelator. Whole-body clearance of conjugate in mice was rapid, with 50% of the label eliminated in about two hours. The highest uptake of label was in the kidneys, and was only 1.5% of the injected dose/gm after 2.5 hours; at 24 hours, less than 0.7% injected dose/gm was present in all sampled tissues. These results indicate that Tc-99m-labeled PNAs exhibit the stability and pharmacokinetic properties needed for use as radiopharmaceuticals (Mardirossian G, etal, J Nucl Med, Jun 1997;38(6):907-13, Rusckowski M, etal, Cancer, 15 Dec 1997;80(12 Suppl):2699-705, and Hnatowich DJ, J Nucl Med, Apr 1999;40(4):693-703). This technology, relating to radionuclide labeling of PNAs, is being offered for commercial licensing by the University of Massachusetts (licensing ref: UMMC 98-24).

CLINICAL CONSIDERATIONS

ODN are being clinically evaluated in a variety of settings, and although an ODN has been commercialized indicating that such constructs may play a role in the treatment of human disease, their application in the treatment of cancer is still in the experimental stage. Part III of this article will present a comprehensive review of the status of the various ODN types described in this issue in terms of their oncology targets and clinical development. ODN have been constructed against numerous oncologic targets and more than a dozen have been evaluated in clinical trials. Still, it is too early to forecast the role of ODN in the management of cancer. In addition to the issue of clinical effectiveness, questions remain as to potential toxicities, delivery issues, permanency of effect and costs.

Toxicity

Studies in animals and humans indicate that ODN can be therapeutically administered with minimal toxic side effects (Gewirtz AM, etal, Blood, 1 Aug 1998;92(3):712-36, Levin AA, Biochim Biophys Acta, 10 Dec 1999;1489(1):69-84, Cotter FE, etal, Biochim Biophys Acta, 10 Dec 1999;1489(1):97-106, Chen HX, etal, Clin Cancer Res, Apr 2000;6(4):1259-66, Pennie WD, etal, Toxicol Sci, Apr 2000;54(2):277-83, Cunningham CC, etal, Clin Cancer Res, May 2000;6(5):1626-31, Waters JS, etal, J Clin Oncol, May 2000;18(9):1812-23, and Dvorchik BH and Marquis JK, Drug Metab Dispos, Oct 2000;28(10):1255-61). Nonetheless, the possibility exists, especially with nonsequence-specific biological effects, that disruption of the regulation of nontarget proteins may lead to unknown complications. This may be particularly true with G-rich ODN, which can be difficult to synthesize and purify (Murphy M, etal, Biotechniques, Dec 1993;15(6):1004-6,1008,1010, and Agrawal S, etal, Bioorg Med Chem Lett 1996;6:2219-2224), and can be very polymorphic under physiological conditions, often resulting in structures with vastly different functional characteristics (Cheng AJ and Van Dyke MW, Gene, 15 Sep 1997;197(1-2):253-60).

In addition, although backbone modifications and uptake facilitators can protect ODN against premature degradation and improve their ability to reach their intracellular target, pharmacokinetic studies suggest that, depending on sequence, ODN can be rapidly cleared from the bloodstream, with the liver being the main site of elimination, but with scavenger receptors on bone marrow cells also contributing to this effect (Biessen EA, etal, Mol Pharmacol, Feb 1998;53(2):262-9). Such sequence-related effects on the biodistribution of ODN could require oligomers to be administered in large doses for long periods of time, and the potential consequences of long-term ODN-based regimens are not known.

Administration Route

ODN to date have been typically administered parenterally, by slow intravenous infusion, to avoid their putative plasma concentration-dependent hemodynamic side-effects. The development of alternative administration routes would help to enhance the therapeutic and commercial potential of these agents.

Oral delivery represents one of the most attractive approach for large-scale exploitation of human therapeutic agents. Unfortunately, for most oligonucleotides administration via the gastrointestinal tract results in negligible bioavailability. One exception are the hybrid mixed-back-

bone oligonucleotide (MBO). End-modified phosphorothioate ODN containing nuclease-resistant 2'-O-alkylribonucleotides or methylphosphonate internucleotide linkages at both the 3' and 5' ends, exhibit pharmacokinetic profiles similar to the parent ODN, but are significantly more stable *in vivo* and can be administered orally (see FO, p. 1233). Oligomers have also been incorporated into small hydrogel copolymeric carriers which are capable of cross-polarizing monolayers of intestinal cells, suggesting the potential usefulness of this system for oral administration of ODN (see FO, p. 1245).

Pulmonary delivery is another promising mode of nonparenteral dosing for antisense oligonucleotides. The lung is an excellent target for the direct delivery of ODN via respirable aerosol, because its surface is lined with surfactant proteins, which appear to facilitate the uptake of oligonucleotides delivered by inhalation (Kalina M, etal, Am J Respir Cell Mol Biol, Jun 1992;6(6):594-600, and Nyce JW and Metzger WJ, Nature, 20 Feb 1997;386(6618):721-5).

Scientists at the Novartis Horsham Research Centre (Horsham, West Sussex, UK) have demonstrated dose-dependent lung retention and pulmonary bioavailability for ODN using intratracheal administration of ISIS 3521, an antisense compound that binds to an mRNA sequence specific to PKC-α, and ISIS 5132, a phosphorothioate ODN complementary to C-raf kinase mRNA, in rodent models (Nicklin PL, etal, Pharm Res, Apr 1998;15(4):583-91, and Danahay H, etal, Pharm Res, Oct 1999;16(10):1542-9). These results indicate that the lung can be used as a target for pulmonary delivery of antisense molecules, with low doses retained in the lungs for local effects, and higher doses potentially suitable for the treatment of various systemic diseases The value of local delivery of antisense oligomers has been confirmed by fomivirsen, delivered by direct injection into the eye and recently approved by the FDA for the treatment of drug-refractory CMV retinitis in AIDS patients.

The first respirable antisense oligonucleotide to enter clinical testing is EpiGenesis' EPI-2010, designed to prevent asthma symptoms by targeting the adenosine A1 receptor. A phase I clinical trial of EPI-2010 was initiated in October 2000. EpiGenesis hopes to eventually apply the concept of inhalable antisense agents to the treatment of other respiratory diseases, including lung cancer.

Combination Therapy

Despite its effectiveness, conventional chemotherapy for the treatment of cancer has associated toxicities to normal tissue and organs, often representing a major dose-limiting factor. In addition, successful cancer chemotherapy can be compromised by the development of multidrug resistance (MDR) in tumor cells, characterized by overexpression of the mdr1 gene product P-glycoprotein. Although ODN offer promise as stand-alone anticancer agents, it may be that their true potential will only be realized when used in combination with standard chemotherapeutics. Antisense-based transient downregulation of genes which are involved in the transformation or perpetuation of the cancerous disease state can remove the growth and survival advantages exploited by tumor cells, contributing in an additive or synergistic fashion to the cytotoxic effects of chemotherapy, and possibly lowering the doses of conventional therapeutics required to effectively combat disease (Cucco C and Calabretta B, Cancer Res, 1 Oct 1996;56(19):4332-7, Benner E, etal, J Pharmacol Toxicol Methods, Jun 1997;37(4):229-35, and Roh H, etal, Surgery, Aug 1999;126(2):413-21).

Editor's note: *The next issue of FUTURE ONCOLOGY comprises the final installment of the synthetic nucleic acid sequence series, including a database of 71 agents in research and preclinical development, and 12 in clinical trials.*

INDEX OF COMPANIES & INSTITUTIONS

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