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ROLE OF NEW MOLECULAR APPROACHES FOR THE EARLY DIAGNOSIS OF BLADDER CANCER IN CLINICAL PRACTICE

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1. INTRODUCTION

1.1 BLADDER CANCER

Bladder cancer is any of several types of malignancy arising from the epithelial lining (i.e., the urothelium) of the urinary bladder. Rarely the bladder is involved by non-epithelial cancers, such as lymphoma or sarcoma, but these are not ordinarily included in the colloquial term "bladder cancer." It is a disease in which abnormal cells multiply without control in the bladder. The bladder is a hollow, muscular organ that stores urine; it is located in the pelvis. The most common type of bladder cancer recapitulates the normal histology of the urothelium and it is known as transitional cell carcinoma or more properly urothelial cell carcinoma. It is estimated that there are 383.000 cases of bladder cancer worldwide[1]. Detection and monitoring of bladder cancer is usually done by urine cytology, cystoscopy and histology[2]. However these methods are highly subjective, expensive, invasive and often are not able to reveal low-grade bladder cancer at the first stage of transformation (urine cytology), flat bladder cancer in situ and bladder cancer which remain below the mucosa surface (cystoscopy). Non-invasive methods which are able to compete with cystoscopy and to implement cytology diagnostic accuracy are still needed. In recent years some non-invasive test performed on voided urine have been developed for the early detection of bladder cancer. However these biomarkers require to be validated in further studies. The aim of this study was to verify the accuracy of new molecular non invasive test in the diagnosis of bladder cancer.

1.2 EPIDEMIOLOGY

In the United States, bladder cancer is the fourth most common type of cancer in men and the ninth most common cancer in women[3]. Fifty thousand new cases with bladder cancer are diagnosed each year, even if the mortality is not so high (Figure 1). Smoking can only partially explain this higher incidence in men[4]. One other reason is that the androgen receptor, which is much more active in men than in women, plays a major part in the development of the cancer[5].

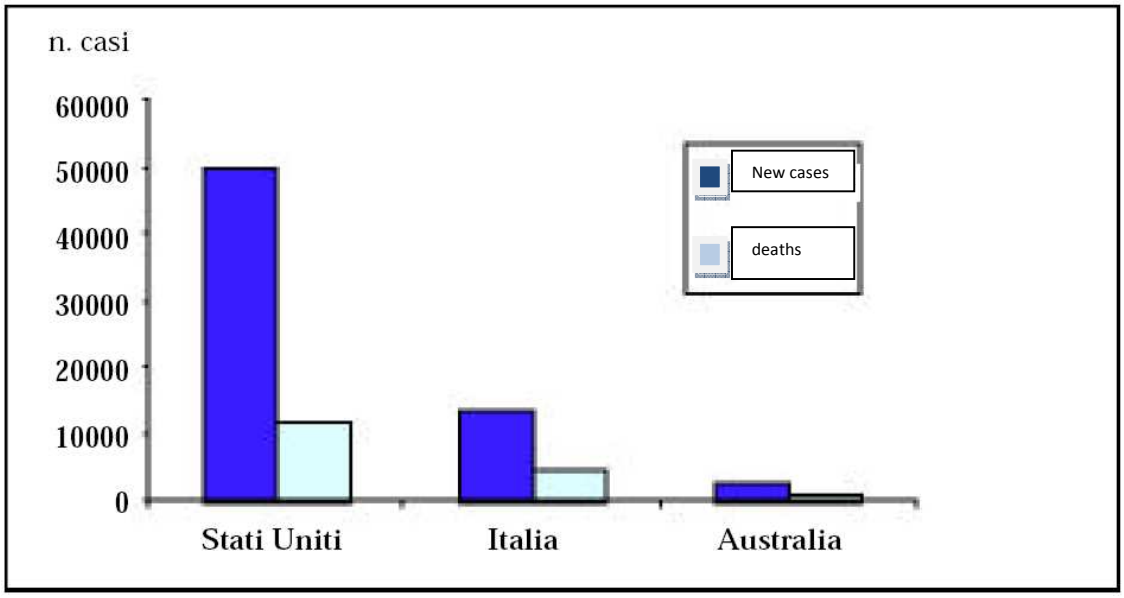

Figure 1. Bladder cancer incidence and mortality (yr 2000).

1.3 RISK FACTORS

Tobacco smoking is the main known contributor to urinary bladder cancer; in most populations, smoking is associated with over half of bladder cancer cases in men and one-third of cases among women[6]. There is a linear relationship between smoking and risk, and

quitting smoking reduces the risk[7]. Passive smoking has not been proven to be involved[8]. In a 10-year study involving almost 48.000 men, researchers found that men who drank 1.5 liters of water per day had a significantly reduced incidence of bladder cancer when compared with men who drank less than 240 mL (around 1 cup) per day. The authors proposed that bladder cancer might partly be caused by the bladder directly contacting carcinogens that are excreted in urine, although this has not yet been confirmed in other studies[9]. Thirty percent of bladder tumours probably result from occupational exposure in the workplace to carcinogens such as benzidine. 2-Naphthylamine, which is found in cigarette smoke, has also been shown to increase bladder cancer risk. Occupations at risk are bus drivers, rubber workers, motor mechanics, leather (including shoe) workers, blacksmiths, machine setters, and mechanics[10]. Hairdressers are thought to be at risk as well because of their frequent exposure to permanent hair dyes. It has been suggested that some gene mutations (i.e HRAS, KRAS2, RB1, and FGFR3) may be associated with bladder cancer in some cases[11].

1.4 ANATOMO-PATHOLOGICAL FEATURES OF BLADDER CANCER

The urinary bladder leis within the pelvic cavity. The bladder lies behind the symphysis pubis. It is below the parietal peritoneum. Urine produced in the kidneys flows into the bladder via the ureters. The urine is ultimately excreted from the bladder through the urethra. The innermost portion of the urinary bladder is the mucosa (Figure 2). The histology of the mucosa is that it is composed of transitional epithelium and connective tissue. The epithelium lies upon connective tissue called the lamina propria. The lamina propria is composed of areolar connective tissue. It contains blood vessels, nerves, and, in some regions, glands. Ninety percent of bladder cancers derived from transitional epithelium and then they are called transitional cell carcinoma (TCC). The other 10% are squamous cell carcinoma, adenocarcinoma, sarcoma, small cell carcinoma, and secondary deposits from cancers elsewhere in the body. The pattern of growth of bladder cancer can be papillary, sessile (flat) (infiltrating or not infiltrating) or carcinoma in situ (CIS).

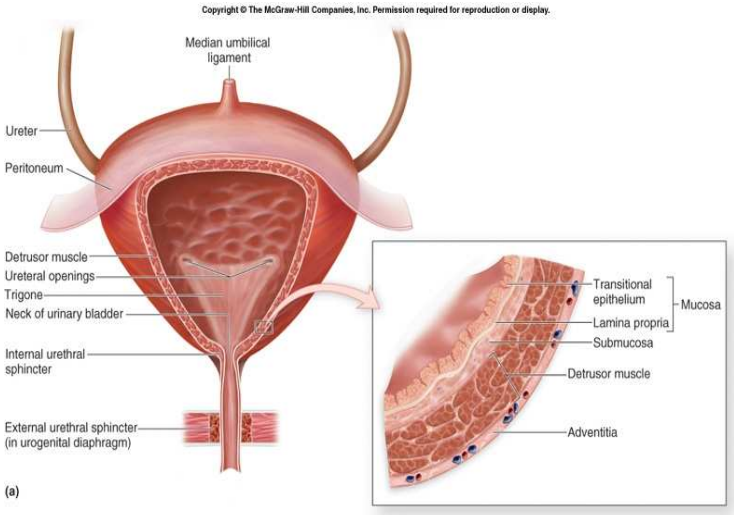

Figure 2. Conformation and bladder layers.

1.4.1 GRADING AND WHO CLASSIFICATION

Some controversies arose following the introduction of the 1998 WHO/ISUP classification of bladder tumours, mainly because of lack of validation, reproducibility, and translation studies. In particular, no sound translation scheme comparing the 1973 with the 1998 (and 2004) classifications has been put forward. Basically, grade 1 (WHO 1973 classification) tumours should be subdivided into PUNLMP and low-grade carcinomas, whereas most grade 2 and all grade 3 cases are defined as high grade carcinomas. In this scheme, grade 2 tumours, whose morphology borders with that of grade 1 tumours, become low-grade carcinomas[12] (Figure 3).

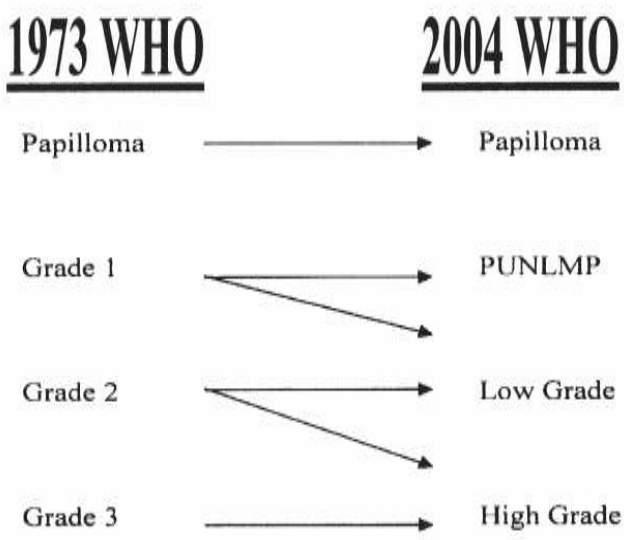

Figure 3. Comparison between 1973 and 2004 WHO classifications.

WHO classification[12]

Flat Intraepithelial Lesions

Urothelium, the dominant type of epithelium lining the urinary bladder, ureter, and renal pelvis, has unique characteristics that separate it from all other type of epithelia. The 2004 WHO classification of the flat lesions includes urothelial hyperplasia, reactive urothelial atypia, atypia of unknown significance, dysplasia, and CIS.

Urothelial Hyperplasia, Reactive Urothelial Atypia, and Atypia of Unknown Significance

Urothelial hyperplasia is defined as markedly thickened mucosa without cytologic atypia. Flat urothelial hyperplasia historically has been defined as urothelium greater than 7 cells layers thick. In practice, counting the number of urothelial cell layers is not reproducible, as urothelial cells do not line up in neat rows.

Urothelial Dysplasia

Dysplasia is an intraurothelial lesion with appreciable cytologic and architectural changes felt to be preneoplastic but that fall short of *CIS*. The morphology of dysplasia generally shows cohesive cells characterized by mild nuclear/nucleolar changes that focally include irregular nuclear crowding and slight hyperchromasia.

Urothelial Carcinoma in situ

Carcinoma in situ is a nonpapillary flat lesion in which the surface epithelium contains cells that are cytologically malignant. The morphologic diagnosis of CIS requires the presence of severe cytologic atypia (nuclear anaplasia).

Noninvasive Papillary Urothelial Lesions

This group comprises papilloma, inverted papilloma, papillary urothelial neoplasm of low malignant potential, and noninvasive low-grade and high-grade papillary urothelial carcinoma.

Urothelial Papilloma

Urothelial papilloma is a benign exophytic neoplasm composed of a delicate fibrovascular core covered by normal looking urothelium.

Inverted Papilloma

Inverted papilloma is a benign urothelial tumour that has an inverted growth pattern with normal to minimal cytologic atypia of the cells.

Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP)

This is a noninvasive papillary urothelial tumour that resembles the exophytic urothelial papilloma but shows increased cellularity exceeding the thickness of normal urothelium.

Noninvasive Low-Grade Papillary Urothelial Carcinoma

This is a neoplasm of urothelium lining papillary fronds that shows an orderly appearance but easily recognizable variations in architecture and cytologic features.

Noninvasive High-Grade Papillary Urothelial Carcinoma

This is a neoplasm of urothelium lining papillary fronds that shows a predominant pattern of disorder with moderate-to-marked architectural and cytologic atypia.

<u>Invasive Urothelial Neoplasms</u>

Lamina Propria Invasion

Lamina propria invasion is characterized by the presence of neoplastic urothelial nests, clusters, or single cells within the lamina propria, often associated with prominent retraction artifact.

Muscularis Propria (Detrusor Muscle) Invasion

The distinction on transurethral resection of muscularis mucosae from muscularis propria invasion may occasionally be difficult.

1.4.2 STAGING

The definition of the clinical and pathological stage of bladder cancer is the evaluation of the extent of the tumour on the surface, the local progression within the mucosa, submucosa or muscular layers of the bladder, and the distance to adjacent areas (Figure 4). Staging is particularly critical because of the many prognostic variables.

The correct staging allows to:

- determine the spread of the disease to other organs and systems
- propose the best therapeutic strategy
- provide prognostic information
- compare the results of different treatment options

It is imperative to differentiate between two groups: superficial and infiltrating bladder cancer[12]. In fact, the first group, which comprises 70-80 % of cancers, tends to a local disease with a chronic high chance of local recurrence and, except in the forms G3, with low tendency to progress. The second group comprises the remaining 20-30% and it is a systemic disease that tends to pose a risk to the patient's life and requires staging and different therapies.

Clinical staging requires manual palpation under anesthesia before and after resection of neoplastic masses, and urography, for advanced forms is required chest x-ray, ultrasound of the liver, abdominal and pelvic CT, bone scintigraphy. Urinary cytology is important as it is able to highlight, with a diagnostic accuracy of around 90%, the in situ carcinoma. There are three classifications used: the Jewett-Strong-Marshall, the Union Internationale

Contre le Cancer (TNM) and the American Joint Committee, although currently the TNM system is the most used in all countries. The classification of Jewett-Strong- Marshall defines the extent of the disease according to five categories (0 to D), while the other two, with slight variations, define the parameters (T, N, M).

T (Primary tumour)

- TX Primary tumour cannot be assessed
- T0 No evidence of primary tumour
- Ta Non-invasive papillary carcinoma
- Tis Carcinoma in situ (‘flat tumour’)
- T1 Tumour invades subepithelial connective tissue

- T2a Tumour invades superficial muscle (inner half)
- T2b Tumour invades deep muscle (outer half)

- T3 Tumour invades perivesical tissue:
- T3a Microscopically
- T3b Macroscopically (extravesical mass)

- T4a Tumour invades prostate, uterus or vagina
- T4b Tumour invades pelvic wall or abdominal wall

N (Lymph nodes)

- NX Regional lymph nodes cannot be assessed
- N0 No regional lymph node metastasis
- N1 Metastasis in a single lymph node 2 cm or less in greatest dimension
- N2 Metastasis in a single lymph node more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension
- N3 Metastasis in a lymph node more than 5 cm in greatest dimension

M (Distant metastasis)

- MX Distant metastasis cannot be assessed
- M0 No distant metastasis
- M1 Distant metastasis.

T CATEGORIES OF TNM CLASSIFICATION

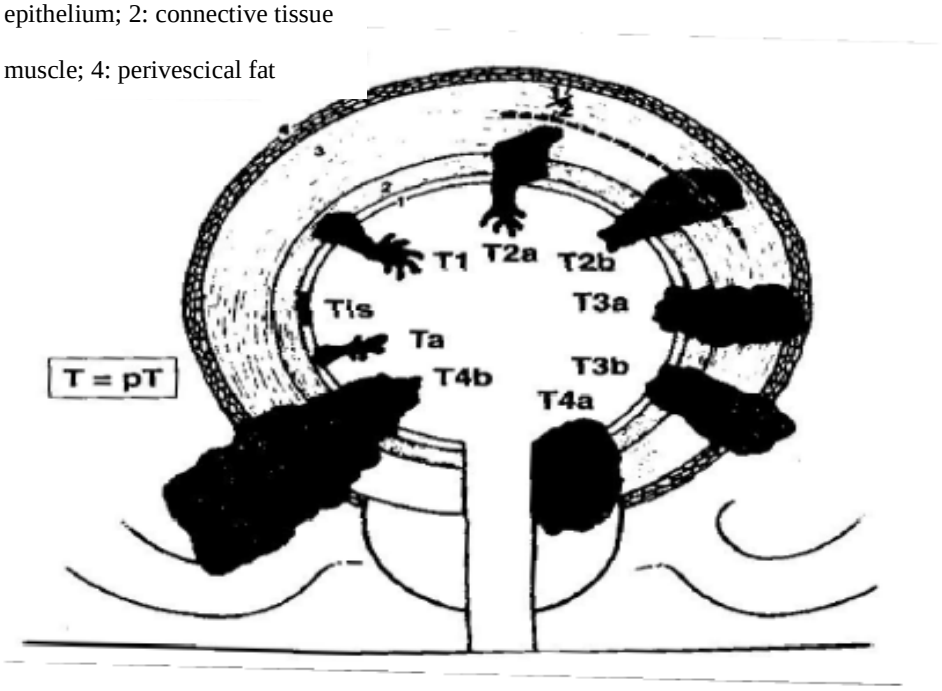

Figure 4. T Categories proposed by TNM classification.

1.5 MOLECULAR GENETICS

The molecular mechanisms of occurrence and development of transitional cell carcinomas of the bladder include interactions between tumour suppressor genes, oncogenes, growth factors, adhesion molecules and angiogenic factors. The resultant of these interactions is the acquisition of the malignant phenotype by normal transitional cells. The initiation and the development of the tumour is the result of an initial damage that involves a genetic disorder both as negation of a tumour suppressor gene is as induction of oncogenes. Tumour progression follows the acquisition by the normal cell transformed further development

capability (growth factors and angiogenic), as well as the ability to invade and overcome the lamina propria (adhesion molecules and motility factors) with the result of the diffusion distance. Repeated and sequential stages of initiation and promotion to the selection of cell populations increasingly affected. It is thought that the development of bladder cancer is a process resulting from the evolutionary stages, passing through the stages of hyperplasia and dysplasia to malignant urothelial cell transformed[13].

Cytogenetic studies have revealed both chromosomal abnormalities and specific correlations between genetic alterations and biological behavior of the tumour. The most frequent combinations of genetic and epigenetic alterations that lead to the development of bladder cancer have been already acquired. Molecular pathology could have a role in the further refinements of the classification system. The genetic studies so far published have classified tumours according to the 1973 WHO scheme, and further studies are needed to link available genetic information to the 2004 WHO scheme. Current data suggest 2 genetic subtypes/pathways that correspond to morphologically defined entities (Figure 5).

The genetically stable category includes low-grade noninvasive papillary tumours (pTa, G1, and G2), whereas the genetically unstable category contains high-grade (including pTa, G3, and CIS) and invasively growing carcinomas (stage pT1-4). Noninvasive low-grade bladder neoplasms have only few genomic alterations and are therefore viewed as genetically stable. Invasively growing and high-grade neoplasms appear to be genetically unstable and have several chromosomal aberrations. There are two distinct molecular pathways that lead to the development of low-grade non-invasive and muscle-invasive tumours.

The origin of high-grade papillary Ta tumours is not yet clear, it is thought to be caused by dysplasia or low-grade papillary tumours. Similarly, the way of the development of T1

tumours is unclear, but it could be the acquisition of invasive capacity on the part of high-grade Ta or carcinoma in situ (Figure 5).

The most common alterations of low-grade, non-invasive tumours include the deletion of chromosome 9 and the point mutation of the receptor 3 fibroblast growth factor (FGFR3).

The muscle-invasive bladder tumours show a broad spectrum of genetic alterations: some implicated known genes but also a large number of genetic alterations and changes in the number of copies in the regions of the genome yet unknown. Many studies have identified the RB1 and TP53 tumour suppressor genes, which are altered in the majority of these tumours. The involvement of TP53 is perhaps the main difference between this group of tumours and the low-grade Ta, in fact, have been identified many different mutations of TP53[14].

In conclusion numerous deletions on chromosome arms (9q, 11p, 17p) characterize bladder carcinomas. There are also correlations between the degree of malignancy and specific deletions (3p, 17p). The stadium is associated with deletions of 3p, 17p , and with the altered expression of the gene RB (retinoblastoma), and vascular invasion correlates with the 17p deletion. Recent studies demonstrated that in the literature p53/p21 alterations appear to play a prognostic predictor of recurrence and disease progression[15]although the results obtained so far are still controversial, and further studies are therefore needed.

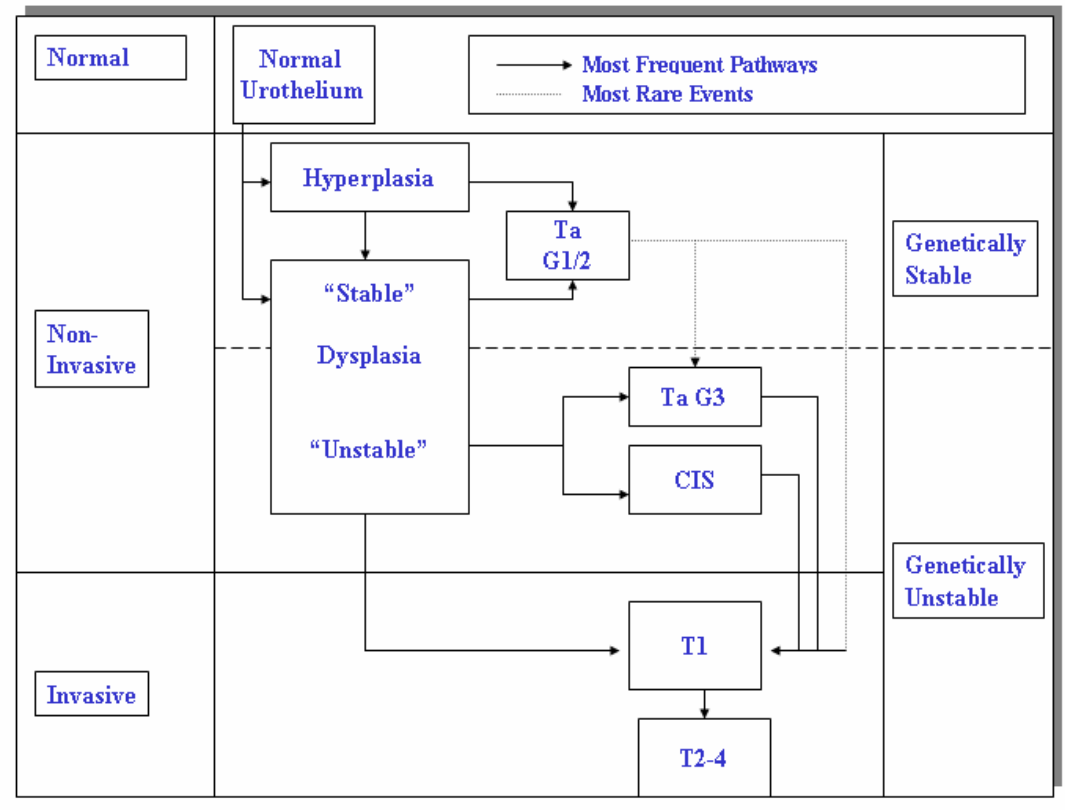

Figure 5. Current genetic data suggest two major subtypes/pathways of bladder urothelial tumours.

1.6 SIGNS AND SYMPTOMS

Bladder cancer characteristically causes blood (redness) in the urine. This blood in the urine may be visible to the naked eye (gross/macroscopic hematuria) or detectable only by microscope (microscopic hematuria)[16]. Hematuria is the most common symptom in bladder cancer. Other possible symptoms include pain during urination, frequent urination, or feeling the need to urinate without being able to do so. These signs and symptoms are not specific to bladder cancer, and are also caused by non-cancerous conditions, including prostate infections, over-active bladder and cystitis. There are many other cause of hematuria, such as bladder or ureteric stones, infection, kidney disease, kidney cancers and vascular malformations.

1.7 PREVENTION

A 2008 study commissioned by the World Health Organization concluded that "specific fruit and vegetables may act to reduce the risk of bladder cancer"[9,17]. Fruit and yellow-orange vegetables, particularly carrots and those containing selenium[18], are probably associated with a moderately reduced risk of bladder cancer. Citrus fruits and cruciferous vegetables were also identified as having a possibly protective effect. However, an analysis of 47.909 men in the Health Professionals follow-up study showed little correlation between cancer reduction and high consumption of fruits and vegetables overall, or yellow or green leafy vegetables specifically, compared to the statistically significant reduction among those men who consumed large amounts of cruciferous vegetables.

Several studies have suggested that the bladder is one of the most responsive organs to induction of detoxification enzymes by extract. This was evident in human bladder cancer cells *in vitro* and in animal models *in vivo*. Sulforaphane and broccoli sprout extract were also observed to induce apoptosis and cell cycle arrest in human bladder cancer cells *in vitro,* an anti-cancer effect attributed to the sulforaphane potential in the sprouts[19,20].

1.8 DIAGNOSIS

Many patients with a history, signs, and symptoms suspicious for bladder cancer are referred to a urologist or other physician trained in cystoscopy, a procedure in which a flexible tube bearing a camera is introduced into the bladder through the urethra. Suspicious lesions may be biopsied and sent for pathologic analysis. The gold standard for for the diagnosis of bladder cancer is biopsy obtained during cystoscopy. Sometimes it is an incidental finding during cystoscopy. Urine cytology can be obtained in voided urine or at the time of the cystoscopy ("bladder washing"). Cytology is not very sensitive especially for low grade and stage tumours (a negative result cannot reliably exclude bladder cancer)[21](Table 1). The diagnosis of bladder cancer can also be done with a Hexvix/Cysview guided fluorescence cystoscopy (blue light cystoscopy, Photodynamic diagnosis), as an adjunct to conventional white-light cystoscopy. This procedure improves the detection of bladder cancer and reduces the rate of early tumour recurrence, compared with white light cystoscopy alone. Cysview cystoscopy detects more cancer and reduce recurrence. Cysview is marketed in Europe under the brand name Hexvix[22]. However, visual detection in any form listed above, is not sufficient for establishing pathological classification, cell type or the stage of the present tumour. A so-called cold cup biopsy during an ordinary cystoscopy (rigid or flexible) is not

sufficient for pathological staging. Hence, a visual detection needs to be followed by transurethral surgery. The procedure is called transurethral resection (TUR). Further, bimanual examination should be carried out before and after the TUR to assess whether there is a palpable mass or if the tumour is fixed ("tethered") to the pelvic wall. The pathological classification obtained by the TUR-procedure, is of fundamental importance for making the appropriate choice of ensuing treatment and/or follow-up routines[23].

Table 1. Diagnostic accuracy of cytology in the different studies[21].

	Number of Cases	Sensitivity (%)	Specificity (%)
Case-control studies			
Weikert et al. [38]	400	34	93
Halling et al. [12]	265	58	98
Babjuk et al. [22]	218	33	100
Eissa et al. [55]	200	75	94
Sarosdy et al. [11]	176	26	-
Eissa et al. [26]	168	44	100
May et al. [15]	166	71	84
Saad et al. [23]	120	48	87
Adb El Gawad et al. [21]	86	54	100
Placer et al. [13]	86	64	86
Varella-Garcia et al. [14]	19	43	100
Symptomatic patients			
Grossman et al. [27]	1331	16	99
Sarosdy et al. [20]	497	38	-
Laudadio et al. [19]	300	34	93
Sharma et al. [24]	278	56	93
Kavaler et al. [45]	151	51	98
Landman et al. [25]	77	40	94

1.9 TREATMENT

The treatment of bladder cancer depends on how deep the tumour invades into the bladder wall. Superficial tumours (those not entering the muscle layer) can be "shaved off" using an electrocautery device attached to a cystoscope, which in that case is called a resectoscope. TUR serves primarily for pathological staging. In case of non-muscle invasive bladder cancer the TUR is in itself the treatment, but in case of muscle invasive cancer, the procedure is insufficient for final treatment[24]. Immunotherapy by intravesicular delivery of Bacillus Calmette–Guérin is also used to treat and prevent the recurrence of superficial tumours[25]. Bacillus Calmette–Guérin is a vaccine against tuberculosis that is prepared from attenuated (weakened) live bovine tuberculosis bacillus, Mycobacterium bovis, that has lost its virulence in humans. BCG immunotherapy is effective in up to 2/3 of the cases at this stage, and in randomized trials has been shown to be superior to standard chemotherapy[26]. The mechanism by which BCG prevents recurrence is unknown, but the presence of bacteria in the bladder may trigger a localized immune reaction which clears residual cancer cells. Instillations of chemotherapy, such as valrubicin (Valstar) into the bladder, can also be used to treat BCG-refractory CIS disease when cystectomy is not an option [27]. The drug Urocidin is currently in Phase III of trials for this procedure [28]. Patients whose tumours recurred after treatment with BCG are more difficult to treat. Many physicians recommend cystectomy for these patients (Figure 6). This recommendation is in accordance with the official guidelines of the European Association of Urologists (EAU)[24] and the American Urological Association (AUA)[29]. However, many patients refuse to undergo this life changing operation, and prefer to try novel conservative treatment options before opting to this last radical resort. Device assisted chemotherapy is one group of novel technologies used to treat superficial bladder

cancer[30]. These technologies use different mechanisms to facilitate the absorption and action of a chemotherapy drug instilled directly into the bladder. Another technology uses an electrical current to enhance drug absorption[31]. Another technology, thermotherapy, uses radio-frequency energy to directly heat the bladder wall, which together with chemotherapy shows a synergistic effect, enhancing each other's capacity to kill tumour cells. This technology has been studied by different investigators[32,33]. Untreated, superficial tumours may gradually begin to infiltrate the muscular wall of the bladder. Tumours that infiltrate the bladder require more radical surgery where part or all of the bladder is removed (cystectomy) and the urinary stream is diverted into an isolated bowel loop (ileal conduit or urostomy). In some cases, skilled surgeons can create a substitute bladder (neobladder) from a segment of intestinal tissue, but this largely depends upon patient preference, age of patient, renal function, and the site of the disease. A combination of radiation and chemotherapy can also be used to treat invasive disease. It has not yet been determined how the effectiveness of this form of treatment compares to that of radical ablative surgery. Photodynamic diagnosis may improve surgical outcome on bladder cancer[34]. For muscle invasive urothelial urinary bladder cancer there are a number of treatment options. Gold standard is radical cystectomy as mentioned. In males this usually includes also the removal of the prostate and in females, ovaries, uterus and parts of the vagina. In order to address the problem of micrometastatic disease which in itself has implications on longtime survival, new treatment options are dearly needed. Micrometastatic dissemination is often not treatable with only major surgery and the concept of neoadjuvant chemotherapy has evolved.

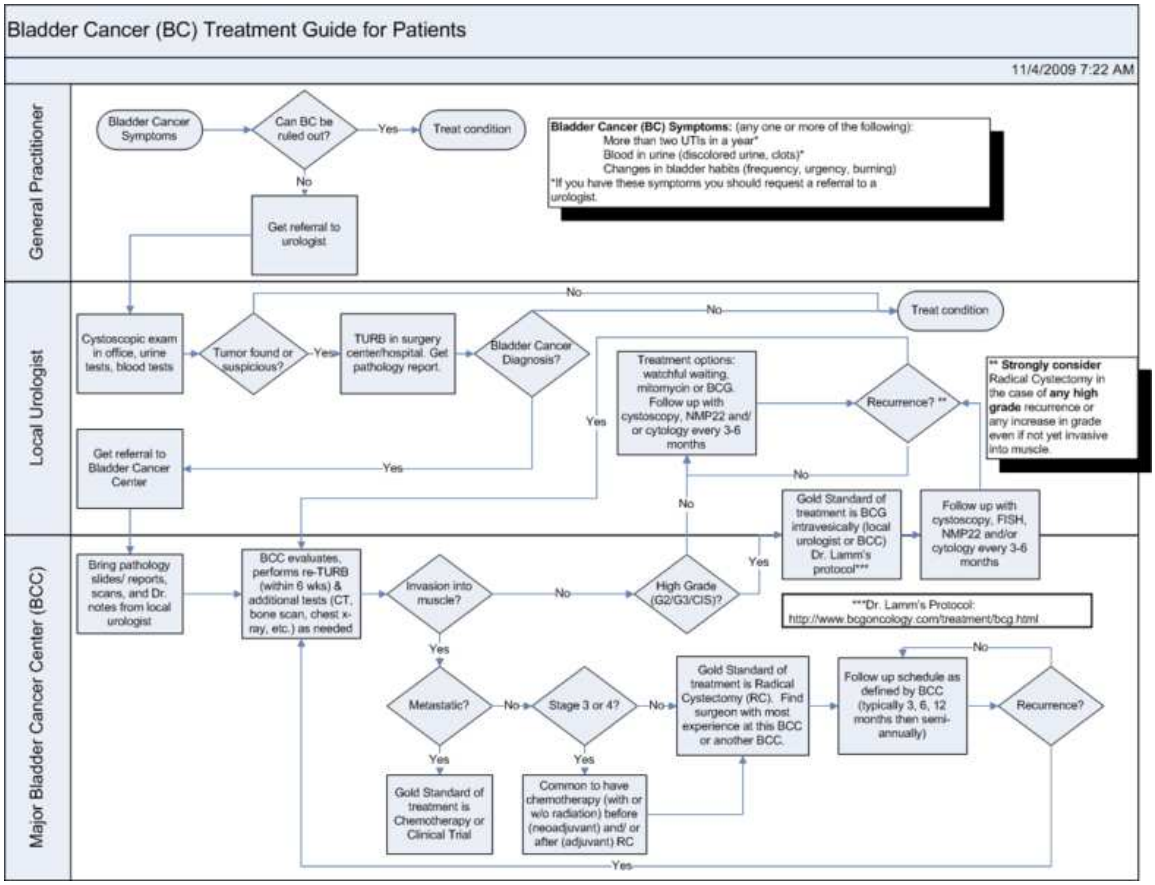

Figure 6. Flow chart of the Bladder Cancer Treatment Guide.

2. NEW MOLECULAR NON INVASIVE APPROACHES

2.1 TUMOUR MARKERS

The availability of more accurate diagnostic and possibly non-invasive tests has been a major objective pursued intensively in recent years. An ideal diagnostic marker should have both a high sensitivity and specificity, and also be able to detect well-differentiated and early stage tumours. The method must also be simple, and sufficiently inexpensive to facilitate the analysis of a large number of urine samples in a reasonable amount of time. In recent years, several markers of diagnostic relevance have been identified and a number of reagents directed against molecular targets have been developed commercially[21] (Table 2). The most intensively investigated are chromosome alterations detected by fluorescence in situ hybridization (FISH), urinary human complement factor H related protein (BTA stat and BTA TRAK), nuclear matrix protein (NMP22) and cytocheratin 8 and 18 fragments (UBC rapid, and UBC immunoradiometric assay, UBC ELISA). With regard to the most intensively investigated markers, consistent results have been obtained for FISH, with a sensitivity of approximately 80%, and a specificity between 90 and 100% in case-control studies. However, the test is expensive, cannot be performed in all laboratories, and accuracy strongly decreases when it is used for symptomatic patients. FISH requires specialized personnel to ensure a correct morphologic evaluation. Similar sensitivity and specificity have been reported for NMP22 in case control studies, albeit with lower accuracy, especially in terms of sensitivity in symptomatic patients. For all these molecular tests, sensitivity ranges from 40 to 100% in different case-control studies, and from 40 to 82% in symptomatic

patient series. Specificity also varies markedly, from 64 to 100% in the former, and from 65 to 86% in the latter subgroups (Table 2)[21].

Moreover, intra-assay variability is often higher than inter-assay variability, indicating a potential lack of standardization of technical aspects and pre-analytical phases.

Indeed, specific protocols and standards often adopted by individual laboratories determine a wide range of results which are not easily comparable.

Table 2. Diagnostic accuracy of different non-invasive assay.

	Number of cases	Type of assay	Sensitivity (%)	Specificity (%)
Case-control studies				
Halling *et al.*[12]	265	FISH	81	96
Skacel *et al.*[16]	120	FISH	85	97
Placer *et al.*[13]	86	FISH	80	85
Riesz *et al.*[17]	55	FISH	87	100
Varella-Garcia *et al.*[14]	19	FISH	87	100
Halling *et al.*[18]	265	FISH	81	96
		BTA stat	78	74
Sarosdy *et al.*[11]	176	FISH	71	100
		BTA stat	50	-
Saad *et al.*[23]	120	NMP22	81	87
		BTA stat	63	82
Babjuk *et al.*[22]	218	BTA stat	74	87
		BTA TRAK	76	73
		UBC rapid	49	79
		UBC IRMA	70	64
May *et al.*[15]	166	FISH	53	74
		UBC	40	75
Eissa *et al.*[26]	168	NMP22	85	91
		UBC	67	81
Adb El Gawad *et al.*[21]	86	NMP22	91	87
		BTA	100	92
Symptomatic patients				
Sarosdy *et al.*[20]	497	FISH	69	78
Laudadio *et al.*[19]	300	FISH	73	65
Grossman *et al.*[27]	1331	NMP22	56	86
Sharma *et al.*[24]	278	NMP22	82	82
		BTA stat	68	82
Atsü *et al.*[28]	82	NMP22	78	66
Landman *et al.*[25]	77	BTA	40	73
		NMP22	81	77

FISH = fluorescence *in situ* hybridization, BTA = bladder tumor antigen, NMP22 = nuclear matrix protein, UBC = urinary bladder cancer
Tabulated according to size of case-series within each marker

2.2 THE ROLE OF TELOMERASE

Among the markers most recently proposed to improve diagnostic accuracy, especially in terms of sensitivity, increasing attention has been focused on the role of the ribonucleoprotein, telomerase. This enzyme consists of three subunits: an RNA component (hTR), which acts as a template for DNA replication[35], a telomerase associated protein (TP1)[36] of as yet unknown function, and the telomerase reverse transcriptase (hTERT), which is responsible for catalytic activity[37]. Telomerase activity (TA) has been detected in almost all malignant cells and tissues, and only very occasionally in normal somatic cells[38-40]. The telomeric repeat amplification protocol assay (TRAP), a polymerase chain reaction (PCR) based method for detection of TA, has been available since 1994[38].The introduction of this method is an important milestone in telomerase research and has become the standard method for studying the diagnostic relevance of this enzyme (Table 3)[39-43]. TA has also been determined qualitatively and quantitatively using modified TRAP assays, for example TRAP scintillation proximity assay, TRAP-ELISA, fluorescent TRAP assay, TRAP hybridization assay, and bioluminescence linked with TRAP. Other methods have focused on the detection of the telomerase subunits, hTR and hTERT, using the reverse transcriptase polymerase chain reaction (RT-PCR). Real-time PCR methods have also permitted a quantitative and reproducible determination of these subunits.

Expression of the hTERT protein has also been analyzed by immunocytochemistry using anti-hTERT monoclonal [44,45] and polyclonal antibodies.

Table 3. Diagnostic accuracy of telomerase-based assays.

	Number of cases	Type of marker	Sensitivity (%)	Specificity (%)
Case-control studies				
Halling *et al.*[16]	265	TA*	46	91
Sanchini *et al.*[37]	218	TA*	90	88
Bravaccini *et al.*[47]	212	TA*	87	66
Sanchini *et al.*[36]	200	TA*	92	81
Saad *et al.*[23]	120	TA*	84	93
Fedriga *et al.*[35]	106	TA*	89	68
Adb El Gawad *et al.*[21]	86	TA*	80	95
Eissa *et al.*[55]	200	TA*	75	92
		hTERT	96	96
		HTR	92	89
Weikert *et al.*[38]	400	hTR	77	72
		hTERT	55	85
Symptomatic patients				
Kavaler *et al.*[45]	151	TA*	85	66
Landman *et al.*[25]	77	TA*	80	80

*TA performed by TRAP assay

2.2.1 TELOMERASE EVALUATION

2.2.1.1 ENZYMATIC ACTIVITY

TRAP assay

The detection of TA in bladder washing and voided urine has been investigated for its diagnostic potential. Since this technique detects TA, and not only the presence of the enzyme, viable cells are a prerequisite. In fact, a possible limitation of the TRAP assay is the potential vulnerability and inactivation of the enzyme by external factors. Bladder washings are obtained by mechanical irrigation of the empty urinary bladder using saline solution at physiological pH. However, in native urine, suspended tumour cells are exposed to destructive substances such as proteases, urea, salts and, usually, acid pH, for variable times. All of these factors may lead to early inactivation or degradation of the enzyme that could

explain the lack of reproducibility of results among the different studies. Moreover, bladder washings are obtained through the use of a catheter or cystoscope, which are both invasive instruments. For this reason, voided urine has been the most widely used biological sample for the TRAP assay. The first reported TRAP assay studies were based on qualitative, and thereafter with semi-quantitative TA determinations[46]. To obtain more accurate and reliable results, a quantitative TRAP assay was developed in bladder washings and voided urine, based on exponential amplification of the primer-telomeric repeats generated in the telomerase reaction[41,47-50]. Several case-control studies have also confirmed that this test is more accurate in males than females[41], with a higher specificity in younger than older individuals[42]. A recent study by the same authors suggested that these results could be due to the presence of inflammatory cells, which are almost always positive to telomerase[21]. Furthermore, the diagnostic accuracy of TA was not related to the tumour stage or grade, and was as high in both early stage and low grade tumours, including in situ carcinomas[41], in contrast to what has been reported by other authors[51]. However, before introducing this test in routine clinical practice, in combination with, or as an alternative to invasive cytoscopy, its potential, in terms of sensitivity and specificity, must be further investigated and defined in a consecutive series of symptomatic individuals[52].

2.2.1.2 EXPRESSION OF hTR AND hTERT

RT-PCR

It has been shown that transcriptional regulation of the catalytic component of the telomerase complex is a major determinant in the control of TA[41,42]. Meanwhile, hTR seems to be ubiquitously expressed in most cells independent[21] of enzyme activity. Studies have pointed

out that high hTERT mRNA expression is associated with malignancy in many tumour histotypes, and has shown great potential for early cancer detection in body fluids[5]. Indeed, the expression of hTERT and hTR mRNA, both in tissues[53] and in voided urine samples, seems to correlate positively with tumour stage and grade, even if these data have not, as yet, been confirmed[54]. Moreover, a good concordance has been shown between mRNA of both telomerase subunits and telomerase activity[54,55].

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Many studies have shown that the TRAP assay does indeed have some drawbacks, the most important being the rate of false positives due to the presence of inflammatory non-tumour cells in voided urine and bladder washings[13,21]. It is therefore important to carry out a morphological analysis to identify the true nature of urothelial telomerase expressing cells (Figure 7) and to unmask any false TRAP positives (Figure 8). The availability of both monoclonal (Mab tel 3 36-10 DIESSE Diagnostica Senese Italy, commercialized by the Alexis Corporation, Lausanne, Switzerland; NCL hTERT Novocastra, Newcastle- upon Tyne UK) and polyclonal antibodies (TERT H-231: sc-7212, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA; hTERT EST21A Alpha Diagnostic International, San Antonio, TX), able to detect hTERT protein expression, has opened up the possibility of studying the different cell components. The diagnostic accuracy of the TRAP assay could be improved by considering the percentage of non-tumour hTERT-expressing cells in the same urine sample. However, it still needs to be demonstrated that the two markers are equivalent. In fact, there is evidence that some tissues may be positive for hTERT mRNA, but not for TA.

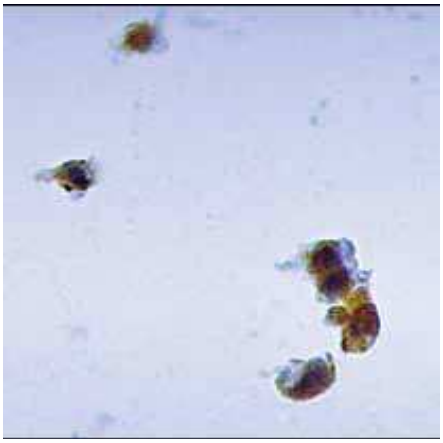


Figure 7: Immunoreactivity of bladder tumour cells to Mab anti-hTERT tel 3 36-10 Diesse[21].

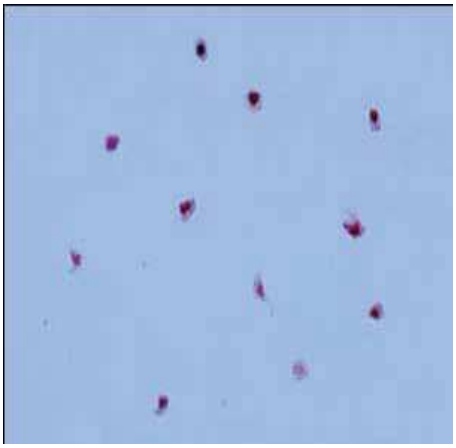


Figure 8: Immunoreactivity of inflammatory cells to Mab anti-hTERT tel 3 36-10 Diesse[21].

2.3 FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Bladder cancer progression is accompanied by increased chromosomal instability and aneuploidy[56]. Cytogenetic studies reveal frequent alterations of a variety of chromosomes including chromosomes 1, 3, 7, 9, 17, and others[56]. These chromosomal alterations can be detected with fluorescence in situ hybridization (FISH)[57,58]. FISH utilizes fluorescently labeled DNA probes to chromosomal centromeres or unique loci to detect cells with numerical or structural abnormalities indicative of malignancy. Studies have demonstrated that FISH, using the UroVysion® probe set, has similar specificity to and better sensitivity than routine cytology for detecting bladder cancer in urine. Vysis UroVysion® is a molecular cytology test that detects aneuploidy of chromosomes 3, 7, and 17 and deletion of the 9p21 locus via fluorescence in situ hybridization (FISH) in urine specimens. Presently, as approved by the US Food and Drug Administration (FDA), the UroVysion® assay is simply reported qualitatively as 'positive' or 'negative' for abnormality. However, recent studies indicate that assessing FISH quantitatively (i.e., pattern of chromosomal abnormality and percentage of abnormal cells) may help better predict which patients will recur and progress to muscle invasive disease[59]. Researchers have found that increased chromosomal instability and aneuploidy, such as that detected by the Vysis UroVysion test, are characteristic of bladder tumour progression. Due to its high specificity (~96%) and increased sensitivity (Table 2), the Vysis UroVysion test is useful for early detection of bladder cancer recurrence when used in conjunction with cystoscopy. When the Vysis UroVysion test is positive and cystoscopy is negative, cancer recurs on average 4 months earlier than when both tests are negative; thus, a positive Vysis UroVysion test may indicate a need for increased surveillance in these cases.

Given this quantitative information, patients with an anticipated uneventful clinical course and/or recurrent low-grade papillary tumours can be distinguished from those who are at a high risk of tumour progression. Accordingly, surveillance strategies could be tailored for individual patients on the basis of the biological properties of their disease.

2.4 DNA INTEGRITY

The presence of circulating cell-free DNA in plasma or serum has been reported to be a suitable marker of prostate [60] and lung cancer [61,62] but few studies have focused on the potential of urine cell-free (UCF) DNA to detect bladder cancer [63-65]. It is very important to assess whether DNA integrity is able to distinguish bladder cancer patients from from healthy individuals or from those with benign urologic disease. This hypothesis is based on the well-known fact that DNA from normal cells in apoptosis is highly fragmented, while DNA from necrotic cancer cells maintains its integrity [66]. It has been demonstrated that urine cell-free DNA fragments longer than 250 bp of 3 biomarkers frequently amplified in bladder cancer are: *c-Myc* (8q24.21), *HER2* (17q12.1), and *BCAS1* (20q13.2) [67,69].

3. AIM OF THE STUDY

RATIONAL AND INTRODUCTION FOR THE EXPERIMENTAL WORK

The early diagnosis of urogenital tumours has a strong impact on the probabilities of recovery and survival of patients affected with these pathologies. Therefore the identification of accurate and sensitive tumour markers able to diagnose these neoplasms early is essential for non invasive diagnostic approaches.

Cystoscopy is the standard approach to the diagnostic work-up of urinary symptoms. It has high sensitivity and specificity for papillary tumours of the bladder but suboptimal sensitivity and specificity for flat carcinomas. It is also expensive and may cause discomfort and complications. Urine cytology, in contrast, has the advantage of being a noninvasive test with high specificity but suffers from low sensitivity possibly due to the low number of exfoliated cells in urine, and particularly in low-grade and early-stage tumours. Numerous new noninvasive tests have been proposed but up to now there are no diagnostic markers in clinical practice for non-invasive urogenital cancers that are sufficiently accurate in terms of sensitivity and specificity. For bladder cancer, there are numerous studies aimed at identifying new molecular markers in urine of diagnostic relevance, but, so far, none of these have an optimal accuracy (both in terms of sensitivity and specificity).

Important results on the diagnostic relevance of urine telomerase have been published by our group in pilot and confirmatory case-control studies (Table 4) but its role in symptomatic individuals is not well known.

In particular, the objective of the **first study**, was to determine whether combining cytology and the TRAP assay for the detection of TA with FISH analysis improves the diagnostic performance of cytology alone in patients with urological symptoms consecutively enrolled (symptomatic patients), which represent the target subset for the activation of screening programs[70].

It would also be interesting to consider other approaches to see whether diagnostic accuracy and cost-effectiveness can be further improved. The presence of circulating cell-free DNA in plasma or serum has been reported to be a suitable marker of prostate [60] and lung cancer[61,62] but few studies have focused on the potential of urine cell-free (UCF) DNA to detect bladder cancer[63-65].

In the **second study**, the capability of DNA integrity in urine supernatant in distinguishing cancer patients from healthy individuals or from those with benign urologic diseases was assessed. This hypothesis is based on the well-known fact that DNA from normal cells in apoptosis is highly fragmented, while DNA from necrotic cancer cells maintains its integrity[66]. To this end urine cell-free DNA fragments longer than 250 bp of 3 biomarkers frequently amplified in bladder cancer: *c-Myc* (8q24.21), *HER2* (17q12.1), and *BCAS1* (20q13.2)[67,69] were analyzed.

Table 4. Studies published by our research group on telomerase.

	N° tot Cases	N° Cases		Males		Females		Global Series	
		Males	Females	% Sens.	% Spec.	% Sens.	% Spec.	% Sens.	% Spec.
Neoplasia Sanchini 2004	200	140	60	93	90	86	71	92	81
JAMA Sanchini 2005	218	218	-	90	88	-	-	90	88
J UROL Bravaccini 2007	212	-	212	-	-	87	66	87	66
IJBM Casadio 2009	515	465	50	88	70	79	62	87	70
IJU (review) Bravaccini 2009	-	-		-		-		-	

Sens.: Sensitivity

Spec.: Specificity

4. MATERIALS AND METHODS

4.1 Case series

First study

The following diagnostic procedures were compared for sensitivity and specificity with cytology alone: TRAP assay alone; cytology and TRAP assay in parallel, with subjects positive at either test being classified as positive; cytology in parallel with the in-series combination of TRAP assay and FISH analysis, with subjects positive at cytology and those positive at both TRAP and FISH being classified as positive; and the in-series combination of TRAP and FISH, with subjects positive at both tests being classified as positive. The use of TRAP assay alone was aimed to confirm that this technique is more sensitive than cytology[41,42]. Combining cytology and TRAP in parallel was designed to increase the sensitivity of cytology. The use of cytology in parallel with the in-series combination of TRAP assay and FISH analysis reflected the assumptions that cytology is fairly specific and does not need in-series testing; the specificity of TRAP may be improved with an in-series combination with the FISH analysis[71] and thus, cytology in parallel with the TRAP–FISH sequence could maximize sensitivity while retaining an acceptable specificity. Finally, the in-series combination of TRAP and FISH was expected to produce the greatest specificity. All study patients were submitted to cytology testing and TRAP assay. TRAP-positive patients underwent FISH analysis. All patients underwent cystoscopy, and biopsy if appropriate. cystoscopy (and biopsy, if any) was considered the gold standard.

Patients who were seen in the Urology Department of the Morgagni-Pierantoni Hospital in Forlı` (the only public general hospital in a catchment area of about 80.000 people in

northern Italy) between January 2007 and June 2008, presented with urinary tract symptoms, reported no history of bladder cancer, underwent cystoscopy, and had conclusive results for cytology, TRAP assay, and FISH analysis were eligible for the study.

Three hundred consecutive patients were enrolled. Of the 120 TRAP positive patients submitted to FISH analysis, 11 (9%) (9 with cancer) had inconclusive results and were excluded from analysis, leaving 289 assessable subjects (238 men, 51 women; median age 70 years; range 28–92 years).

One hundred thirteen patients reported micro/macrohematuria, and 176 (61%) irritative symptoms alone. Seventy-four patients (62 men, 12 women; median age 74 years; range 47–92 years) were diagnosed with bladder cancer, 39(53%) of whom had micro/macrohematuria. Urine test was carried out blindly to histological diagnosis. Eighty patients underwent biopsy. All cases of bladder cancer were histologically confirmed. In accordance with the 2004 World Health Organization guidelines, tumour samples were classified as high or low grade. Patients diagnosed with benign disease were followed-up for at least one year using clinical data from hospital records, and their status was confirmed. There were no losses to follow-up. The local ethics committee approved the study protocol and all participants provided written informed consent to take part in the study.

Second study

A total of 129 individuals (51 bladder cancer patients at first diagnosis, 46 symptomatic patients with benign urogenital diseases, and 32 healthy individuals; Table 5) were recruited from the Urology Department of Morgagni-Pierantoni Hospital (Forlì). All participants provided written informed consent to take part in the study and the local Ethics Committee reviewed and approved the study protocol.

Patients with bladder cancer or benign urogenital diseases were submitted to cystoscopy, after which those with malignancies or suspicious lesions underwent transurethral resection of the bladder (TURB). The final diagnosis of cancer was based on histologic results from TURB. Tumour type and differentiation were based on 2004 World Health Organization criteria. Symptomatic patients were diagnosed with different benign diseases: prostatic adenomas or benign hyperplasia (14 cases), kidney stones (3), cysts (13), lithiasis (3), prostatitis (3), bladder edema (1), and bladder diverticula (1). Eight patients did not have any disease.

Twenty-eight percent of symptomatic patients had hematuria at the time of urine collection. The control group of healthy individuals with no previous urogenital diseases or cancer was matched to patients by gender and age. UCF DNA integrity analysis was performed blindly in all individuals and was evaluable for the entire case series. Cytologic results were available for 76 patients (41 cancer cases and 35 symptomatic individuals).

Table 5. Case series.

		Sex		Age (years)		Grade		Stage	
	n	Male	Female	<70	≥70	High	Low	Ta-T1	T2-T3
Healthy individuals	32	21	11	16	16	—	—	—	—
Symptomatic patients	46	33	13	27	19	—	—	—	—
Bladder cancer patients	51	36	15	25	26	22	21	36	4

4.2 Urine collection

First study

Urine samples were collected immediately before cystoscopy. For the TRAP assay, 3 ml of urine was used and cells were pelleted by centrifugation (850 g for 10 min at 4°C) within 3 h of urine collection, washed once in phosphate-buffered saline (1X), resedimented by centrifugation (2300 g for 5 min at room temperature), and stored at -80°C until use

(maximum of 12 months). Pelleted cells were then resuspended in 200 µl of lysis reagent and left on ice for 30 min. Cell lysates were centrifuged at 10.000 g for 20 min at 4°C and the supernatant was stored at -20°C. The results of the cytological evaluation (Pathology Department, Morgagni-Pierantoni Hospital, Forlì) were reported in a descriptive manner and were classified as negative and positive by laboratory biologists at our institution. Dubious findings were considered a positive result.

For the FISH test, in accordance with the manufacturer's instructions[57] (UroVysion™; Abbott/Vysis, Downers Grove, IL), at least 33 ml of urine was used and cells were collected by cytospin centrifugation (1200 g for 5 min). Cells were fixed in Carnoy's fixative for 20 min and dehydrated for 2 min each in ethanol scalar dilutions (70%, 85%, 100%), after which the slides were heated at 45°C–50°C for 2–3 min and stored at 4°C for a maximum of 1 month.

Second study

First morning voided urine samples for UCF DNA analysis were collected from healthy individuals about 1 week after cytologic examination, and from symptomatic individuals and bladder cancer patients immediately before cystoscopy. All patients and controls were instructed to give clean-catch urine samples, maintained at 4°C for a maximum of 3 hours. Thirty milliliter aliquots of urine were centrifuged at 850 *g* for 10 minutes and the supernatants were transferred to cryovials and immediately stored at -80°C until use.

First study

4.3 TRAP assay

Urine sample aliquots containing 1 µg of protein lysate were used for the TRAP assay. As previously[41,42], telomerase products were evaluated on fluorescent electropherograms and the area underlying the different peaks was calculated. An internal telomerase assay standard (ITAS; 25 attograms) amplified by the same two primers used for TA determination was included in the TRAP buffer to obtain a semiquantitative evaluation. Protein concentrations corresponding to 10, 30, 100, 300, 1000, and 3000 cells of a human bladder cancer cell line (MCR) were analyzed in each assay and used as the reference curve. The areas of each sample were also normalized to the 150-base pair ITAS peak to obtain quantitative TA evaluations. The relative TA per cell for each sample is expressed as the percentage of the ratio of TRAP ladder/ITAS per cell versus the value of MCR and expressed in arbitrary enzymatic units.

TRAP assay was carried out on the entire case series (100% feasibility). All experiments were carried out blindly in duplicate and, when variations were >15% (observed in about 10% of cases), a third analysis was carried out. TA was expressed as a continuous variable.

4.4 FISH analysis

Hybridization was carried out according to the manufacturer's instructions[57] (UroVysion; Abbott/Vysis). The probe mixture consisted of fluorescently labeled probes targeted to the pericentromeric regions of chromosomes 3 (CEP3 Spectrum Red), 7 (CEP7 Spectrum Green), and 17 (CEP17 Spectrum Aqua), and to band 9p21 locus (LSI 9p21 Spectrum Gold) over each target area (Figure 9); 3 µl of the probe mix solution was applied. Target DNA and

FISH probes were co-denatured at 73°C for 2 min using a commercial hybridization system (HYBrite; Abbott/Vysis) and subsequently incubated at 39°C overnight. 4’,6-diamidino-2-phenylindole was used for counterstaining and cells were viewed using a fluorescent microscope (Axioscope; Zeiss, Göttingen, Germany). All slides were scanned and the number of CEP3, CEP7, CEP17, and 9p21 signals in the cells was determined. The presence of at least 25 cells per slide was required to consider a sample as evaluable, and two signals for each chromosome were indispensable to consider a cell as normal. The presence of four or more cells with gains for two or more chromosomes (3, 7, or 17) in the same cell, or 12 cells or more with zero 9p21 signals was the criterion to consider a specimen as positive. Slides were evaluated independently by two experts blinded to the patient’s history and histological findings.

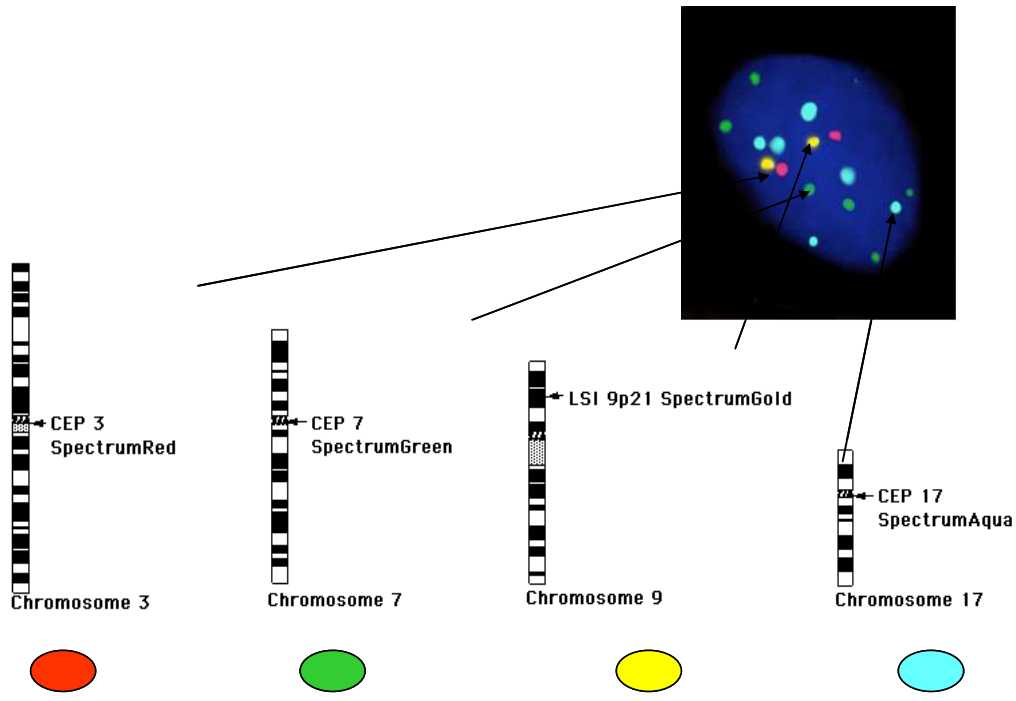


Figure 9. Correspondence between fluorescent signals and chromosome.

Second study

4.5 UCF DNA analysis

DNA was extracted and purified from 2 ml of supernatant by Qiamp DNA minikit (Qiagen, Milan, Italy) according to the manufacturer's instructions. At the same time, DNA was extracted from a human bladder cancer cell line (MCR) using the same minikit. DNA was quantified by spectrophotometry (NanoDrop ND-1000; Celbio, Milan, Italy). Real Time PCR reactions were carried out by Rotor Gene 6000 detection system (Corbett Research, St. Neots, UK) using IQ SYBR Green (Bio-Rad, Milan, Italy). Sequences longer than 250 bp, corresponding to 3 oncogenes were analyzed: *c-Myc* (locus 8q24 and 21,amplification product 264 bp), *BCAS1* (locus 20q.13.2, amplification product 266 bp), *HER2* (locus 17q12 and 1, amplification product 295 bp). A short 125 bp fragment of *STOX1* (locus 10q21 and 3) was analyzed to check for potential PCR inhibition. Primer sequences were as follows:

c-Myc fw TGGAGTAGGGACCGCATATC, rev ACCCAACACCACGTCCTAAC;

BCAS1 fw GGGTCAGAGCTTCCTGTGAG,rev CGTTGTCCTGAAACAGAGCA;

HER2 fw CCAGGGTGTTCCTCAGTTGT, rev TCAGTATGGCCTCACCCTTC;

STOX1 fw GAAAACAGGGCAGCAAGAAG, rev CAGACAGCATGGAGGTGAGA.

PCR conditions for the oncogenes were as follows: 95°C for 3 minutes, then 45 cycles at 94°C for 40 seconds, 56°C for 40 seconds, and 72°C for 1 minute. PCR conditions for the short *STOX1* sequence were as follows: 95°C for 90 seconds, then 45 cycles at 94°C for 40 seconds, and 54°C for 1 minute. All real time PCR reactions were performed in duplicate on 10 ng of each UCF DNA sample.

Various amounts of DNA from the MCR cell line (0.01, 0.1, 1, 5, 10 and 20 ng) were also analyzed to construct a standard curve. The UCF DNA value for each sample was obtained

by Rotor Gene 6000 detection system software using standard curve interpolation. The analysis was repeated if the difference between duplicate samples was greater than 1 cycle threshold. The final UCF DNA integrity value was obtained by the sum of the 3 oncogene values. Real time experiments were performed independently in duplicate on the same 8 samples to test assay imprecision. The coefficients of variation (CV) were then calculated for *c-Myc*, *HER2*, *BCAS*, and *STOX1*.

5. STATISTICAL ANALYSIS

First study

The study end points were sensitivity (the proportion of cancer patients who were correctly identified by the test or procedures) and specificity (the proportion of healthy individuals who were correctly identified)[72], with their 95% confidence intervals (CIs).

The negative and positive predictive values and overall accuracy are not presented for reasons of brevity. However, the reference diagnosis and the results of all tests are cross-tabulated to allow the reader to calculate these measures (Table 6).

Table 6. Distribution of patients according to reference diagnosis, cytology, telomeric repeat amplification protocol (TRAP) assay, and FISH analysis.

	Bladder cancer,[a] FISH				Benign disease,[a] FISH				Total
	Negative	Positive	Not carried out[b]	Total	Negative	Positive	Not carried out[b]	Total	
TRAP, negative cytology									
Negative	0	0	16	16	0	0	129	129	145
Positive	0	29	0	29	39	10	0	49	78
Total	0	29	16	45	39	10	129	178	223
TRAP, positive cytology									
Negative	0	0	9	9	0	0	26	26	35
Positive	1	19	0	20	5	6	0	11	31
Total	1	19	9	29	5	6	26	37	66
Total	1	48	25	74	44	16	155	215	289

a. Diagnosis based on cystoscopy and biopsy (if deemed appropriate).
b. In accordance with the study protocol, FISH analysis was carried out only if the result of the TRAP assay was positive.

Subgroup analyses were carried out to determine whether the results differed by patient and tumour characteristics. Urinary bleeding was considered to encompass microhematuria and macrohematuria.

The McNemar test was used to compare sensitivity and specificity rates. A P value of <0.05 was considered significant. To quantify the difference between two rates, their ratio and 95% CIs were calculated[73].To compare changes in sensitivity or specificity, the ratio of the above ratios was calculated[70,73].

Second study

The relationship between UCF DNA integrity value and the different subgroups (healthy individuals, symptomatic individuals, and cancer patients) was analyzed using a nonparametric ranking statistic test. The most discriminating cutoff values between healthy individuals, symptomatic individuals, and cancer patients were identified using receiver operating characteristic (ROC) curve analysis. The true positive rates (sensitivity) were plotted against the false positive rates (1 specificity) for all classification points. Accuracy was measured by the area under the ROC curve (AUC), which represents an average probability of correctly classifying a case chosen at random. Study endpoints were sensitivity (the proportion of cancer patients who were correctly identified by the test or procedures) and specificity (the proportion of healthy individuals who were correctly identified), with their 95% confidence intervals (CIs)[72]. Spearman's nonparametric test was used to evaluate the correlation between total DNA value and DNA integrity.

The McNemar test was used to compare sensitivity and specificity rates. *P* values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS statistical software (ver. 12.0; SPSS GmbH Software).

6. RESULTS

First study

Total sensitivity and specificity

Of the 300 enrolled patients, 120 were TRAP positive and FISH was performed as second level approach. FISH had inconclusive results on 11 cases (Figure 10).

The TRAP assay as well as the combination of cytology and TRAP and of cytology, TRAP, and FISH were more sensitive and less specific than cytology (Table 7). The combination of cytology, TRAP, and FISH provided the best trade-off between sensitivity and specificity. It showed the same high level of sensitivity as the combination of cytology and TRAP in parallel (0.78) and a more moderate decrease in specificity (0.78 versus 0.60, P = 0.000). It also had greater sensitivity than the TRAP assay alone (0.78 versus 0.66, P = 0.004) and a comparable level of specificity (0.78 versus 0.72, P = 0.136).

The combination of TRAP and FISH exhibited the highest level of specificity, with sensitivity almost identical to that of TRAP alone.

The figures 11 and 12 represent urothelial cells with chromosomal aneuploidy and normal cells form healthy individuals on fluorescent microscope (Zeiss Germany).

Figure 10. Flow chart of the first study.

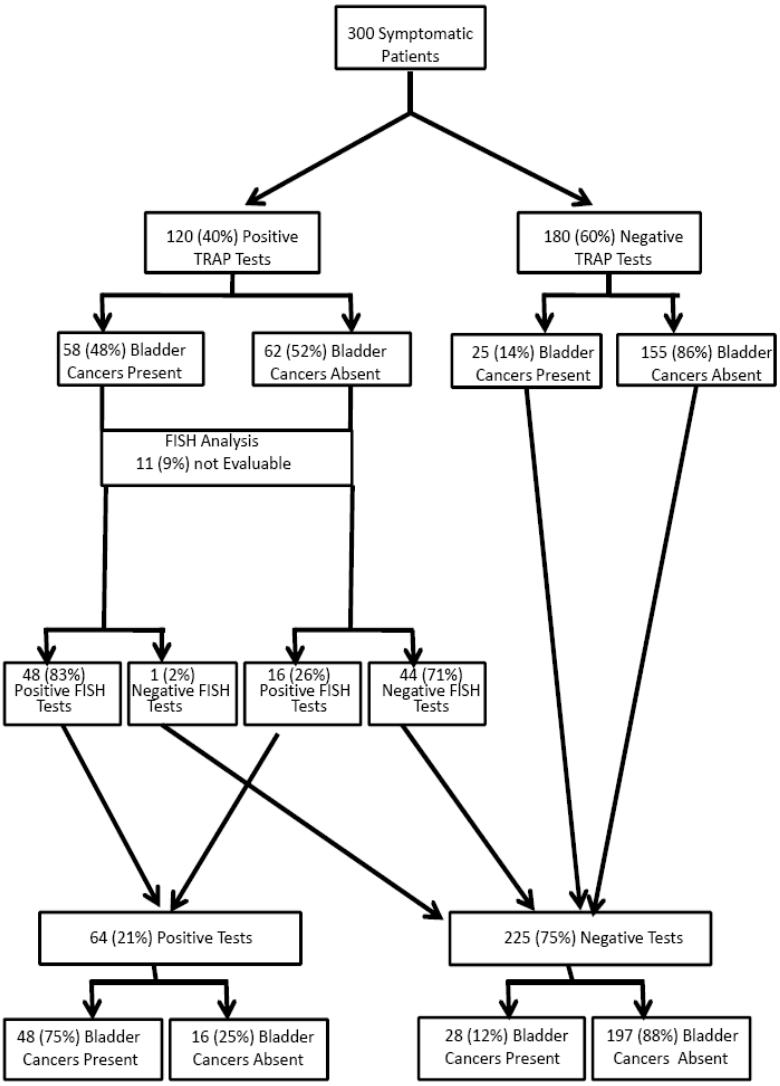
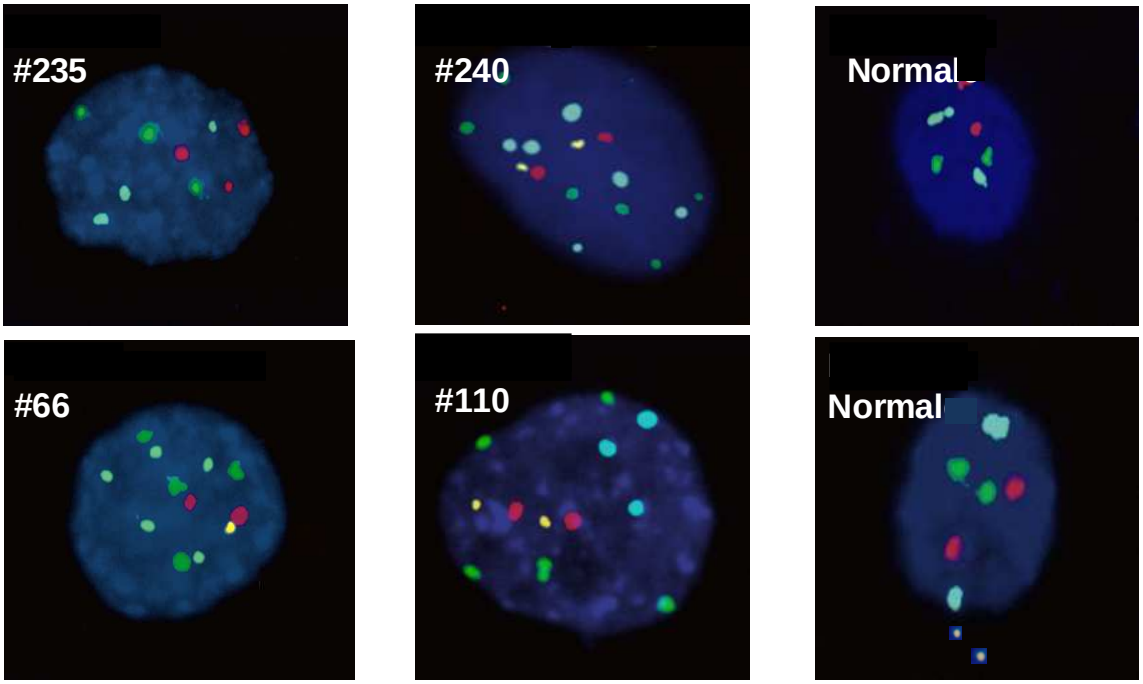


Table 7. Total sensitivity and specificity of the diagnostic procedures.

Procedure	Sensitivity	Specificity
Cytology alone		
n	29/74	178/215
Rate (95% CI)	0.39 (0.28–0.51)	0.83 (0.78–0.88)
TRAP alone		
n	49/74	155/215
Rate (95% CI)	0.66 (0.54–0.77)	0.72 (0.66–0.78)
P value[a]	0.002	0.011
Cytology + TRAP[b]		
n	58/74	129/215
Rate (95% CI)	0.78 (0.67–0.87)	0.60 (0.53–0.66)
P value[a]	0.000	0.000
Cytology + TRAP + FISH[c]		
n	58/74	168/215
Rate (95% CI)	0.78 (0.67–0.87)	0.78 (0.73–0.84)
P value[a]	0.000	0.002
TRAP + FISH[d]		
n	48/74	199/215
Rate (95% CI)	0.65 (0.53–0.76)	0.93 (0.88–0.96)
P value[a]	0.003	0.001

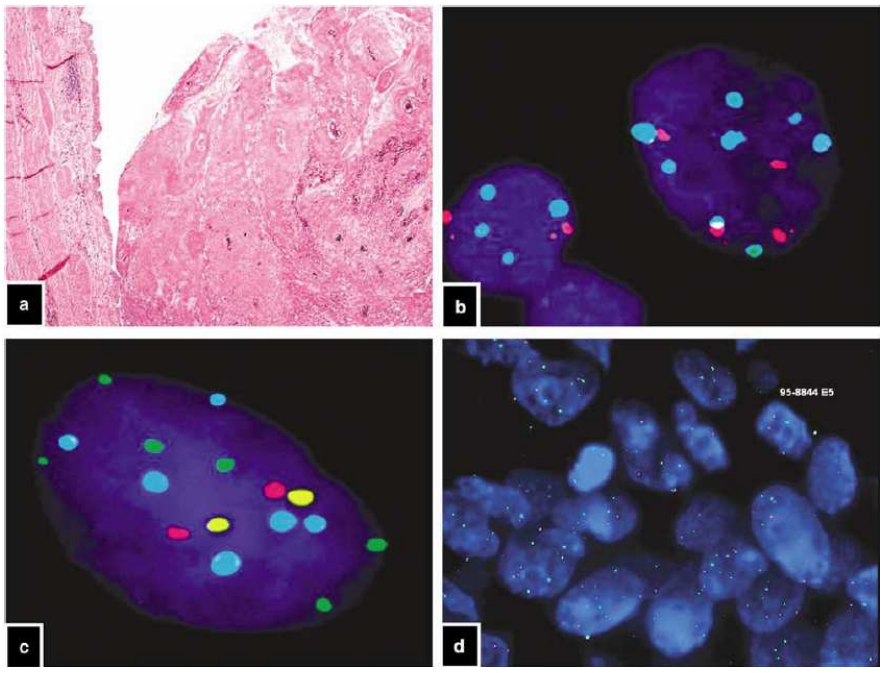
a. For comparison with cytology alone (McNemar test).
b. Cytology and TRAP in parallel. Subjects positive at either test were classified as positive
c. Cytology in parallel with the in-series combination of TRAP and FISH.
Subjects positive at cytology plus those positive at both TRAP and FISH were classified as positive.
d. TRAP and FISH in series. Subjects positive at both tests were classified as positive.
CI, confidence interval; TRAP, telomeric repeat amplification protocol.

Figure 11. Urothelial Cells (big magnification) of patients with chromosomal aneuploidy and normal cells of healthy individuals.



= patient N°

Figure 12. a) invasive squamous cell carcinoma of the bladder (hematoxylin-eosin). b) d) urothelial tumour cells of patients FISH positive (note the loss of the gold signal corresponding to the locus 9p21 and numerous aneuploidies of chromosomes 3,7,17). c) urothelial cell tumour of patient FISH positive (at higher magnification) with aneuploidy of chromosome 7 and chromosome 17 (note 6 copies of the spots green and blue).



Sensitivity and specificity according to patient characteristics

Subsequent analyses were focused on the combinations of tests that included both TRAP and FISH. The relative increase in sensitivity associated with the combination of cytology, TRAP, and FISH was greater among patients with irritative symptoms because non-bleeding cancers were especially difficult to detect by cytology alone (Table 8). A 3.57-fold increase (from 0.20 to 0.71) in sensitivity was observed among patients with irritative symptoms, and a 1.50-fold (from 0.56 to 0.85) increase among patients who reported bleeding. Conversely, the relative loss in specificity was virtually the same in both groups, i.e. 0.95 (from 0.88 to 0.84) among patients with irritative symptoms versus 0.93 (from 0.73 to 0.68) among those who experienced bleeding.

The same pattern of improvement in sensitivity in relation to patient clinical status was observed for the combination of TRAP and FISH, albeit at lower absolute levels. This combination brought about an increase in specificity of comparable magnitude among patients in both groups.

Changes in sensitivity and specificity were also compared between age (<70 versus ≥ 70 years) and gender groups, with nonsignificant differences observed.

Table 8. Sensitivity and specificity of cytology alone and of combinations of cytology, telomeric repeat amplification protocol (TRAP) assay, and FISH analysis in patients reporting irritative symptoms alone and those with urinary bleeding.

	Sensitivity		Specificity	
	Irritative symptoms	Urinary bleeding[a]	Irritative symptoms	Urinary bleeding[a]
Cytology alone				
n	7/35	22/39	124/141	54/74
Rate (95% CI)	0.20 (0.08–0.37)	0.56 (0.40–0.72)	0.88 (0.83–0.93)	0.73 (0.61–0.83)
Cytology + TRAP + FISH[b]				
n	25/35	33/39	118/141	50/74
Rate (95% CI)	0.71 (0.54–0.85)	0.85 (0.69–0.94)	0.84 (0.78–0.90)	0.68 (0.56–0.78)
P value[c]	0.000	0.001	0.031	0.125
TRAP + FISH[d]				
n	22/35	26/39	134/141	65/74
Rate (95% CI)	0.63 (0.45–0.78)	0.67 (0.50–0.81)	0.95 (0.90–0.98)	0.88 (0.78–0.94)
P value[c]	0.001	0.48	0.052	0.019

a. Including microhematuria and macrohematuria.
b. Cytology in parallel with the in-series combination of TRAP and FISH. Subjects positive at cytology plus those positive at both TRAP and FISH were classified as positive.
c. For comparison with cytology alone (McNemar test).
d. TRAP and FISH in series. Subjects positive at both tests were classified as positive.
CI, confidence interval.

Sensitivity according to tumour characteristics

Table 9 shows the relation between tumour grade and stage and the sensitivity increase obtained using the two test combinations above. Since the sensitivity of cytology was greater for high-grade cancers, the gain obtained by combining cytology with TRAP and FISH was higher among low-grade cancers (ratio 2.47; 95 CI 1.38–4.53). There was also a significant increase in sensitivity among early-stage cancers, which was not observed among late-stage cancers. The relative sensitivity performance of the combination of TRAP and FISH followed a similar pattern.

Table 9. Sensitivity of cytology alone and of combinations of cytology, telomeric repeat amplification protocol (TRAP) assay, and FISH analysis according to tumour grade and stage.

	Grade		Stage	
	Low	High	Ta, Tis, T1	T2–3
Cytology alone				
n	11/41	16/29	23/64	6/9
Rate (95% CI)	0.27 (0.14–0.43)	0.55 (0.36–0.74)	0.36 (0.24–0.49)	0.67 (0.30–0.92)
Cytology + TRAP + FISH[a]				
n	34/41	20/29	51/64	7/9
Rate (95% CI)	0.83 (0.68–0.93)	0.69 (0.49–0.85)	0.80 (0.68–0.89)	0.78 (0.40–0.97)
P value[b]	0.000	0.125	0.000	1.000
TRAP + FISH[c]				
n	30/41	15/29	44/64	4/9
Rate (95% CI)	0.73 (0.57–0.86)	0.52 (0.32–0.71)	0.69 (0.56–0.80)	0.44 (0.14–0.79)
P value[b]	0.000	1.00	0.001	0.62

Tumour grade was unknown for 4 of the 74 cancers in the case series, and tumour stage for one.
a. Cytology in parallel with the in-series combination of TRAP and FISH. Subjects positive at cytology plus those positive at both TRAP and FISH were classified as positive.
b. For comparison with cytology alone (McNemar test).
c. TRAP and FISH in series. Subjects positive at both tests were classified as positive.
CI, confidence interval.

Second study

Total free DNA was quantifiable by spectrophotometry, showing a median value of 6 ng/µl (range 2–138 ng/µl).UCF DNA integrity analysis was feasible and results were evaluable for all 129 individuals. The 125 bp *STOX1* sequence was amplified in all samples, excluding the presence of PCR inhibitors. CVs were calculated, considering 2 measurements of each gene in a series of 8 samples, to test the interim imprecision of each assay. CVs were $\leq$0.12 for *HER2*, *BCAS1*, and *STOX1*, and $\leq$0.23 for *c-Myc*. No significant correlation was found between total free DNA and DNA integrity, thus suggesting that they are independent variables. Median values of UCF DNA integrity were significantly different in the

subgroups; the value was significantly ($P < 0.0001$) lower (0.004 ng/μl, range 0–1.883) in healthy individuals, more than 3-fold higher in symptomatic individuals (0.014 ng/μl, range 0–0.864), and about 40-fold higher (0.15 ng/μl, range 0–4.460) in cancer patients ($P < 0.0001$). The AUC for UCF DNA integrity was 0.834 (95% CI 0.739–0.930) for cancer patients and healthy individuals, and 0.796 (95% CI 0.707– 0.885) for cancer patients and symptomatic individuals (Figure 13 and Figure 14). Analysis of sensitivity and specificity at different UCF DNA integrity cutoff values (0.04–0.14 ng/μl) showed a sensitivity ranging from 0.78 (95% CI 0.67– 0.89) to 0.53(0.39–0.67); specificity varied from 0.81 (95% CI 0.67–0.95) to 0.91 (95% CI 0.81–1.00) in healthy individuals and from 0.63 (95% CI 0.49–0.77) to 0.87 (95% CI 0.77– 0.97) in symptomatic patients (Table 10). The best cutoff value was 0.1 ng/μl, providing the best trade-off between loss in sensitivity (0.73, 95% CI 0.61–0.85) and gain in specificity in both healthy individuals (0.84, 95% CI 0.71– 0.97) and symptomatic patients (0.83, 95% CI 0.72–0.94). A comparison of diagnostic performance between conventional cytology and UCF DNA integrity showed an increase in sensitivity from 0.53 (95% CI 0.35–0.71) to 0.70 (95% CI 0.62– 0.68) ($P = 0.132$), while specificity was slightly but not significantly reduced from 0.92 (95% CI 0.81–1.03) to 0.75 (95% CI 0.72–0.94) ($P = 0.102$) (Table 11). In a breakdown analysis of the difference in clinical pathologic subgroups, we observed an improved performance of UCF DNA integrity compared with that of cytology, more evident in males and independent of age. More importantly, sensitivity of UCF DNA integrity increased for low grade tumours, from 0.15 (0–0.34) to 0.72 (0.53–0.91) ($P=0.008$), and for early stage Ta-T1 tumours, from 0.50 (0.30–070) to 0.72 (0.57–0.87) ($P=0.058$). There were too few patients with advanced tumours to permit such analysis. Specificity of UCF DNA integrity did not vary significantly with respect to cytology as a

function of gender, age, or any other clinical pathologic variables. To improve diagnostic performance, we constructed a flow diagram for a combined analysis of cytology and UCF DNA integrity (Figure 15). In the diagram we assumed that all cytology-positive individuals would not be subjected to further diagnostic evaluations and would therefore be considered as "positives." Conversely, all individuals with dubious or negative cytology would be submitted to UCF DNA integrity analysis. With this combined analysis, we obtained 0.81 sensitivity (95% CI 0.69–0.93), higher than that obtained with cytology (0.53) or UCF DNA integrity alone (0.73), and 0.77 specificity (95% CI 0.63– 0.91).To verify the role of each single gene in determining test accuracy, we also performed ROC curve analysis for each gene and obtained the following AUCs: 0.762 for *c-Myc*, 0.861 for *BCAS*1, and 0.757 for *HER2*. In symptomatic patients vs. cancer patients, AUCs were as follows: 0.763 for *c-Myc*, 0.764 for *BCAS1*, and 0.708 for *HER2*. *c-Myc* and *HER2* amplification in a subgroup of 36 patients was also analyzed. FISH analysis showed a high correlation between the quantity of urinary DNA fragments and amplification in tissue for *HER2* gene ($P = 0.004$), but no correlation for *c-Myc* ($P = 0.140$) (data not shown). As far as we know there is no commercially available probe for FISH analysis of *BCAS1*; an immunohistochemical approach which highlighted high expression of the gene in all tumour samples, confirming the potential role of this marker in bladder carcinogenesis was used.

Figure 13. ROC curve of UCF DNA integrity for cancer patients and healthy individuals.

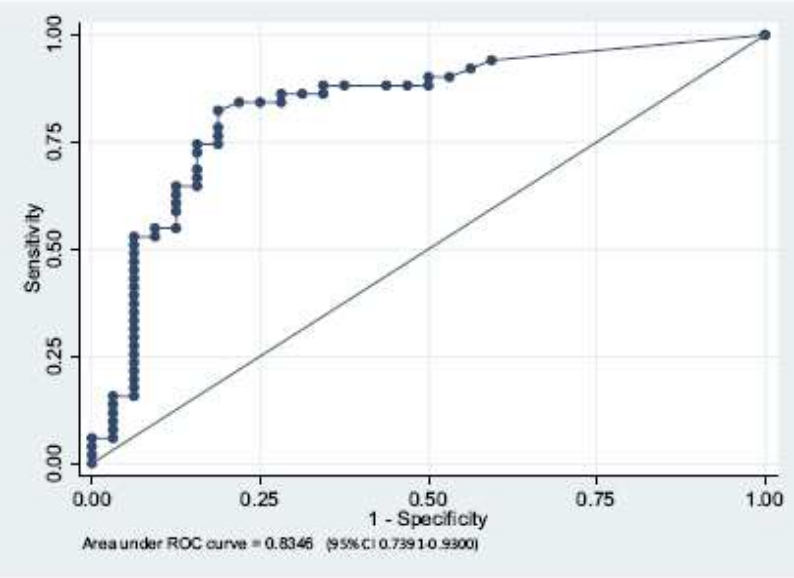


Figure 14. ROC curve of UCF DNA integrity for cancer patients and symptomatic patients.

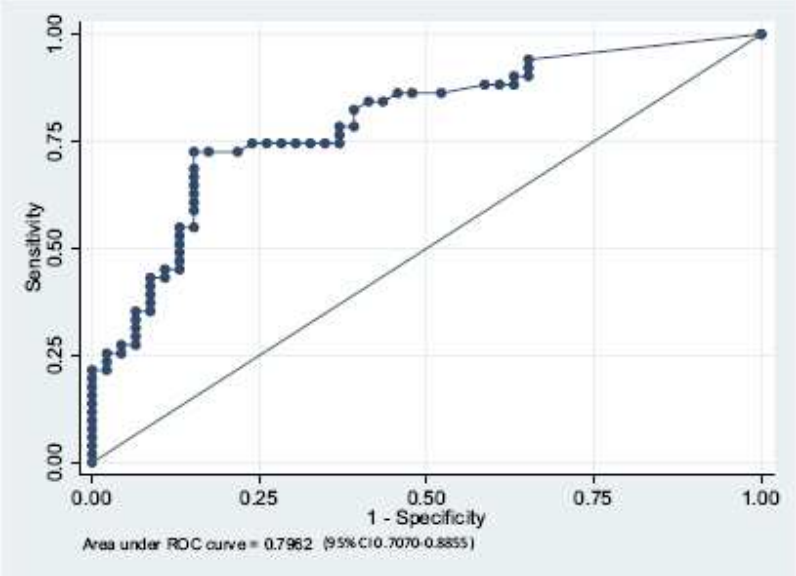


Table 10. Sensitivity and specificity of different cutoffs of UCF DNA integrity in detecting bladder cancer.

Cutoff (ng/μl)	Sensitivity	Specificity	
		Healthy individuals	Symptomatic patients
0.04			
n	40/51	26/32	29/46
Rate (95% CI)	0.78 (0.67–0.89)	0.81 (0.67–0.95)	0.63 (0.49–0.77)
0.06			
n	38/51	26/32	33/46
Rate (95% CI)	0.75 (0.63–0.83)	0.81 (0.67–0.95)	0.72 (0.59–0.85)
0.08			
n	37/51	27/32	38/46
Rate (95% CI)	0.73 (0.61–0.85)	0.84 (0.71–0.97)	0.83 (0.72–0.94)
0.10			
n	37/51	27/32	38/46
Rate (95% CI)	0.73 (0.61–0.85)	0.84 (0.71–0.97)	0.83 (0.72–0.94)
0.12			
n	32/51	28/32	39/46
Rate (95% CI)	0.63 (0.3–0.21)	0.88 (0.77–0.99)	0.85 (0.75–0.95)
0.14			
n	27/51	29/32	40/46
Rate (95% CI)	0.53 (0.39–0.67)	0.91 (0.81–1.00)	0.87 (0.77–0.97)

Table 11. Sensitivity and specificity of cytology and UCF DNA integrity in detecting bladder cancer.

	Sensitivity	Specificity	
		Healthy individuals	Symptomatic patients
Cytology*			
n	16/30	nd	22/24
Rate (95% CI)	0.53 (0.35–0.71)		0.92 (0.81–1.00)
UCF DNA integrity†			
n	21/30	27/32	18/24
Rate (95% CI)	0.70 (0.62–0.78)	0.84 (0.71–0.97)	0.75 (0.66–0.84)
P‡	0.132	—	0.102

Sensitivity and specificity were evaluated in samples for which the results of both cytology and UCF DNA integrity analysis were available (30 cancer patients and 24 symptomatic individuals).

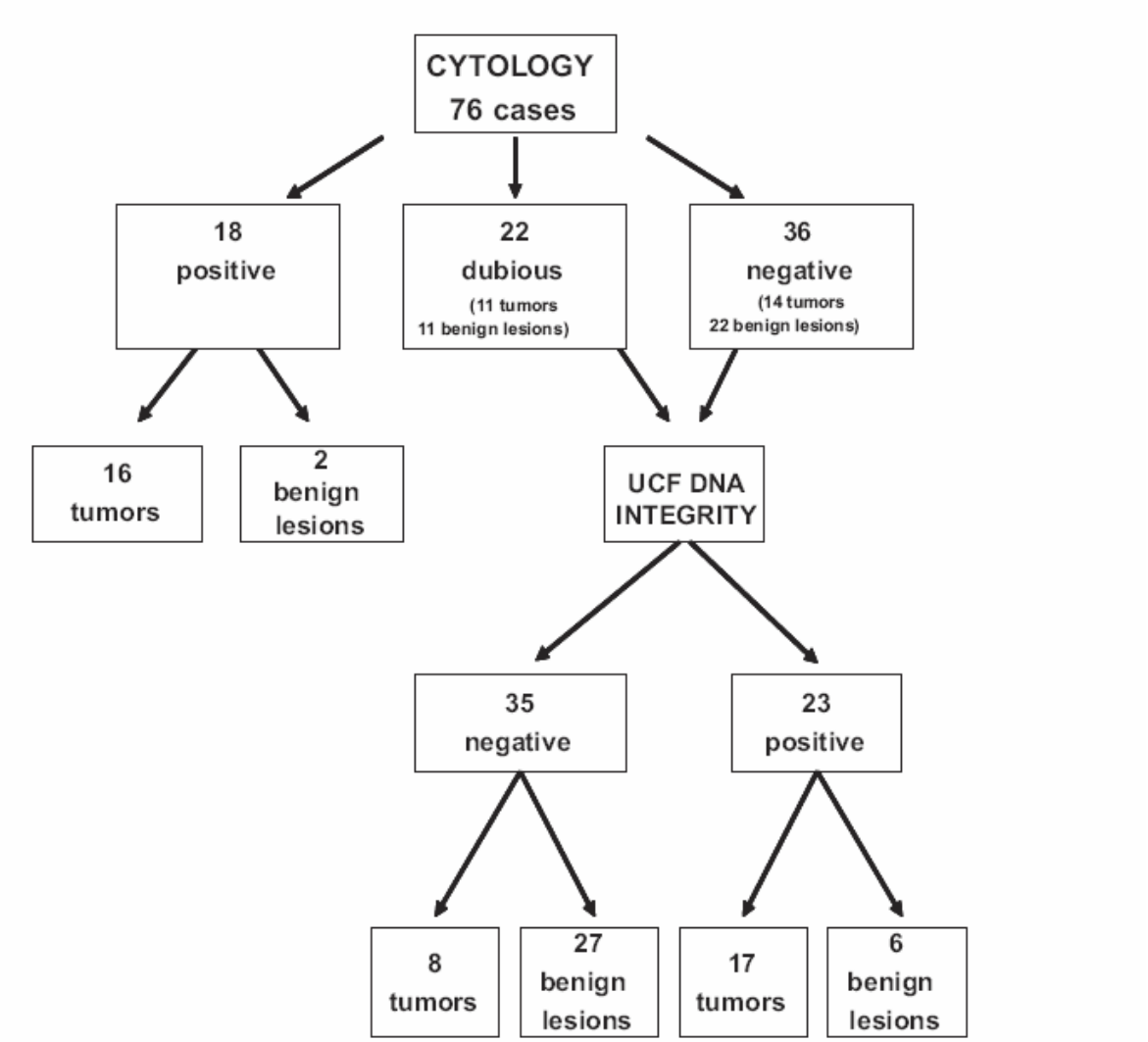
nd = not done; UCF = urine cell-free.

* Suspicious results were not included.

† Cutoff 0.1 ng/μl.

‡ McNemar test.

Figure 15. Flow diagram of sequential cytology and UCF DNA analysis.



7. DISCUSSION

First study

The rationale for this study was the recognized need to improve the performance of urine cytology in the diagnosis of bladder cancer. Using a multimodal approach, we combined cytology with TRAP assay and FISH analysis on the assumption that while using tests in parallel increases sensitivity but at the expense of specificity, an in-series combination results in the opposite. The sensitivity of cytology and TRAP assay in parallel was greater than that of cytology alone. As expected, however, this diagnostic algorithm brought about a marked loss in specificity. We thus designed a mixed procedure including cytology in parallel with the in-series combination of TRAP assay and FISH analysis, which provided the best trade-off between increase in sensitivity and loss in specificity. The increase in sensitivity was twofold, whereas specificity decreased only marginally.

Two essential findings suggested that TRAP assay and FISH analysis may play a role in the diagnosis of bladder cancer. First, it rarely happens that carrying out two tests in series improves specificity while leaving unchanged sensitivity. This was the case for the TRAP assay and the FISH analysis. All cancer patients with negative cytology who were TRAP positive (n = 29) were also FISH positive (Table 6), which means that the FISH analysis is an ideal adjunct to the TRAP assay. The TRAP assay has the basic function of increasing the sensitivity of cytology[41,42,52] and the FISH analysis decreases the false-positive TRAP results without missing any cancer.

Secondly, we found that the above combination led to a greater increase in sensitivity among patients with irritative symptoms versus those reporting urinary bleeding, and among low-

grade and early-stage cancers. In other words, the combination of cytology, TRAP assay, and FISH analysis had a greater impact on results in those conditions in which the sensitivity of cytology was lower.

The ability of the FISH analysis to complement the TRAP assay was also evident from the results of their in-series combination without cytology, which reached a specificity as high as 0.93. Furthermore, as all but one of the 49 TRAP-positive cancer patients were also FISH positive, the sensitivity of the combination remained virtually the same as that of TRAP alone (0.65 versus 0.66).

Some methodological issues in this study need to be addressed. First, we excluded 11 patients with inconclusive FISH analysis. As such results do not modify the result of the basic test (TRAP), patients with inconclusive FISH data could theoretically be considered as positive at the combination of cytology, TRAP assay, and FISH analysis. In this way, however, the rate of patients with inconclusive FISH analysis would become a direct determinant of sensitivity and an inverse determinant of specificity. To overcome this bias, we evaluated only those patients with valid results.

Secondly, it is worthy of note that the specificity of routine cytology in this study was lower than expected. We can exclude that there were patients reported to have benign bladder disease who were subsequently diagnosed with cancer. The status of al of such patients was checked and confirmed after at least 1 year of follow-up. Low cytology specificity was most likely due to dubious results being reclassified as positive. Suspect cytology in Italian patients with urinary bladder symptoms, albeit poorly predictive of cancer, is generally taken seriously from a clinical point of view.

The study design was not suitable for drawing conclusions on the potential relevance of the FISH analysis as a basic diagnostic test for bladder cancer. This technique is very expensive, especially if both direct and indirect costs are taken into account. Thus, cost–utility considerations would seem to limit, a priori, its routine use for the characterization of the disease. Having said that, data presented in Table 1 are of interest on theoretical grounds. FISH analysis was carried out in 109 patients, 49 of whom had cancer and 60 benign disease. Although this was a small, highly selected minority of patients, sensitivity of 0.98 (48 of 49) and specificity of 0.73 (44 of 60) were observed. The biological and medical implications of these circumstantial observations warrant further research using a formal study design.

In conclusion, the present study provides further evidence of the fact that TRAP assay and FISH analysis play a potential role in the diagnostic work-up of urinary symptoms. Further research is needed to develop an effective multimodal approach.

Second study

Numerous articles have been published on the role of DNA alterations, such as genetic and epigenetic modifications in urine sediment, as biomarkers for early bladder cancer diagnosis[74]but very few studies have investigated the potential role of DNA in urine supernatant[63-65].

Cell-free DNA can easily be detected in very small volumes of urine and, unlike RNA or enzymes, has good stability. Secondly, cell-free DNA in body fluids is suitable for multiple analyses[74], such as mutations, microsatellite loss of heterozygosity (LOH), and epigenetic alterations[60] . In this study, DNA integrity, a specific marker of nonapoptotic cancer cells was evaluated. As demonstrated by Chang and coworkers in 2007[64] and confirmed by Zancan et al. in 2009[65] cell-free DNA is not sufficiently accurate to correctly distinguish

between bladder cancer patients and healthy individuals. Then, DNA integrity a specific marker of nonapoptotic cancer cells, was concentrated, and only long DNA fragments was analyzed. Moreover, to increase the diagnostic accuracy of the test, the analysis on 3 specific sequences longer than 250 bp located at loci: 8q24.21 (*c-Myc*), 17q12.1 (*HER2*), and 20q13.2 (*BCAS1*) was performed. These regions were selected because they are known to be amplified during bladder cancer development and progression[74,75]. UCF DNA integrity in urine is a determining feature for bladder cancer detection, with a higher sensitivity than conventional noninvasive cytology, especially for low-grade tumours. Such a characteristic is important for a new marker aimed at early bladder cancer diagnosis[76]. This line of research has already been pursued by our group in healthy individuals and cancer patients[21,41,42] and in a clinical setting of symptomatic patients[70]. The symptomatic subgroup and individuals exposed to industrial chemicals are the main candidates for a screening program of low prevalence tumour histotypes[77,78]. Interestingly, in the present study, we found similar specificity in healthy individuals and symptomatic patients, suggesting that the test can also accurately identify tumours among benign disease with a low number of false positives. The high percentage of true positives is probably ascribable to the analysis of DNA integrity at 3 genomic regions specifically amplified in bladder cancer.

DNA status index (DSI) i.e., the ratio between DNA integrity of the 3 genes (*c-Myc*, *HER2*, *BCAS1*) and the short fragment *STOX1* were analyzed. As expected, a positive correlation was found between the DSI and the presence of bladder cancer (data not shown). These results seem to indicate that in cancer samples, DNA predominantly exists as long fragments, suggesting that its origin is mainly from nonapoptotic cells.

It has recently been demonstrated that the combination of 2 or more urine assays improves the detection of urothelial cancers[79]. Therefore, in an attempt to increase sensitivity, a diagnostic algorithm in which cytology and UCF DNA integrity were tested sequentially was constructed. In our series of symptomatic individuals, this combination demonstrated improved sensitivity compared with that of each test alone. Moreover, sensitivity and specificity were similar to those obtained using the combination of urine telomerase activity, cytology, and FISH. Our results have an important additional value in terms of the cost/benefit ratio. In fact, in the present study, only 2 tests were combined rather than 3; secondly, UCF DNA integrity analysis is easier to perform and to interpret than FISH UroVysion, which requires the consensus of 2 experts. Results for UCF DNA integrity are obtainable in a relatively short time (about 2 working days) and, most importantly, this approach costs less than one-third of the cost of FISH UroVysion. In addition, only small volumes (2 ml) of urine are required for the evaluation of cell-free DNA integrity and this analysis, unlike TRAP assay[70], is not affected by the presence of contaminants, such as inflammatory cells, as shown by the high specificity in symptomatic patients. Important limitations of the present work are the relatively small case series and the fact that both cytology and UCF DNA results were not available for all patients. However, such limitations are acceptable in a pilot study of a new diagnostic approach. A confirmatory study on a larger case series is ongoing to validate these results.

8. CONCLUSIONS

In conclusion, these preliminary results are promising and pave the way towards new molecular approaches for the early noninvasive diagnosis of bladder cancer, which could be used in combination with or as an alternative to voided cytology. In particular, these diagnostic biomarkers appear to show great potential if used in high risk subgroups such as symptomatic patients.

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