Molecular prognostic factors in bladder cancer

Jacqueline Vet

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Contents

Chapter 1	Introduction: Molecular basis of bladder cancer development Implications for molecular prognostic factor studies.	7
	Scope of this thesis	24
Chapter 2	Comparison of p53 protein overexpression with p53 mutation in bladder cancer: clinical and biological aspects.	25
Chapter 3	p53 mutations have no additional prognostic value over stage in bladder cancer.	39
Chapter 4	Predictive value of p53 mutations analyzed in bladder washings for progression of high risk superficial bladder cancer.	49
Chapter 5	Comparative analysis of p53 mutations in bladder washings and histological specimens.	63
Chapter 6	Differential expression of ferritin Heavy chain in a rat transitional cell carcinoma progression model.	75
Chapter 7	Summary and general discussion.	85
References		91
Samenvatting en algemene discussie		109
Dankwoord		115
Curriculum vitae		117

Chapter 1

Introduction

**Molecular basis of bladder cancer development
Implications for molecular prognostic factor studies.**

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In this chapter, the molecular changes relevant for bladder cancer development and progression will be outlined. Furthermore, the prognostic value of these changes will be discussed. Finally, the scope of this thesis is described.

Bladder cancer

Bladder cancer is the fifth most common cancer in the western male population; for women the incidence is about four times lower (173). In the Netherlands (15 million inhabitants) 3400 new cases of bladder cancer are diagnosed and approximately 1100 patients die each year from this disease (38). Bladder cancer usually presents in the elderly (215) and is strongly correlated with smoking. Indeed, one-half of bladder carcinomas in men and one-third in women are attributable to smoking (85). Other agents associated with bladder cancer include the aromatic amines, aniline dyes, cyclophosphamide, and phenacetin (215).

Transitional cell carcinoma (TCC) is the most common histologic type of bladder cancer, whereas squamous-cell carcinoma, adenocarcinoma, and undifferentiated carcinoma of the bladder account only for a minority of cases. TCC occurs in a wide spectrum of clinical and pathological appearances (204).

Figure 1. Different histological stages in TCC (36).

In Figure 1 a schematic drawing of the different histological stages of TCC is shown. About 80% of bladder tumors will present as superficial TCC, defined as tumor extension restricted to the mucosa (papillary shaped pTa tumors and flat carcinoma in situ (CIS)) or the lamina propria (pT1) of the bladder wall. Muscle-invasive (pT2-pT4) tumors account for 20% of bladder tumors, usually present *de novo* and are associated with a worse prognosis and a propensity to metastasize. Most patients with superficial bladder cancer have a good prognosis (173,226). Although 30 to 90% of the tumors will recur depending on stage, grade, multifocality and treatment modality, only 10-25% become invasive or metastasize during the course of the disease (74,101,102). The highest recurrence and progression rates are observed in pT1G3 tumors while pTaG3 and pT1G2 tumors show similar rates (108).

A significant problem in the management of patients with superficial TCC is the identification of those patients who are at risk for progression to invasive disease, so that an early change in treatment strategy (conservative to radical) can be made to potentially prevent progression. For invasive bladder cancer, the identification of the metastatic potential of a given tumor is very important because 50% of patients even if treated with radical cystectomy will die of systemic disease. To address these problems many investigators have tried to identify markers associated with the progression from superficial to invasive or metastatic disease. The recent advances in the molecular approach to tumor biology have revolutionized our knowledge of cancer genetics. Hopefully, a better understanding of the molecular genetic changes involved in bladder cancer progression will provide diagnostic and prognostic information.

Molecular basis of bladder cancer

Genetic studies

In general, abnormal DNA content (aneuploidy) is recognized as a marker of malignancy. In bladder cancer, the overall incidence of aneuploidy rises with increasing stage and grade (29,40,79,219,248). Several techniques can be applied to study genetic aberrations during tumor development and progression. DNA content analysis is not able to discriminate between small differences in DNA content, and therefore not suitable to identify specific genetic alterations. Cytogenetic studies revealed an

association between more specific chromosomal changes and carcinogenesis and disease progression.

Conventional karyotype analyses have shown non random chromosomal changes in bladder cancer involving chromosomes 1, 5, 7, 9 and 11 (3,12,70,71,178,202,231). Gibas et al. (71) designated deletion from chromosome 11 as a secondary event in bladder cancer development associated with muscle invasion. Hopman and coworkers performed interphase cytogenetics using centromere-associated probes to study numerical and structural chromosomal changes in non mitotic cells (88,89,90,168). These studies showed chromosome 9 to be under represented as compared to other chromosomes in low stage/low grade TCC, suggesting the loss of this chromosome as one of the primary events in the development of TCC. A new *in situ* hybridization technique (ISH), comparative genomic hybridization (CGH) allows screening of a tumor genome for chromosomal imbalances (97). The use of CGH to identify DNA alterations in a limited number of TCCs revealed besides known chromosomal losses, distinct amplifications on 1p22, 1q31, 3q24-q26, 8q21, 10p13-14, 12q13-15, 13q21-q34, 17q22-23, 18p11 and 22q11-13 (98,239).

The technique of Restriction Fragment Length Polymorphism (RFLP) allows an even more detailed analysis of chromosomal changes. The most important allelic losses have been observed on chromosomes 3p (77), 4p (105), 8p (103), 9q and 9p (77,104,221), 11p (51,77,221), 13q (77); 17p (77,159,221) and 18q (17,105). Loss of heterozygosity (LOH) of chromosomes 3p, 8p, 11p, 13q, 17p and 18q was associated with high stage and high grade tumors. Habuchi and coworkers (77) showed that the loss of chromosome 9q was equally observed in low stage/low grade and high stage/high grade tumors. Furthermore, they found that although the loss of 11p was more frequently associated with invasive phenotype, the LOH of 11p was detected in 35% of non-invasive tumors. Therefore they concluded that the loss of 11p might occur at an earlier stage before the loss of 17p, 13q, and 3p in tumor progression. The most important chromosomal changes found in bladder cancer so far are summarized in Figure 2.

Putative molecular pathways in bladder cancer progression

Figure 2 also shows the two molecular pathways thought to be involved in bladder cancer progression. Initially, the loss of chromosome 9 was shown to be an early event in bladder cancer progression, whereas the loss of chromosome 17p occurred at a later stage. The tumor suppressor gene located on 17p13.1 is p53 (11) and mutations in the p53 gene were associated with high stage tumors (199). It was suggested that loss of chromosome 9 may cause proliferative behavior and that p53 mutations were necessary to initiate progression to invasive disease. However, it has recently been shown that in 65% of CIS, the p53 gene is mutated, whereas chromosome 9 deletions occur significantly less frequent than in invasive tumors (146,207). These results suggest the existence of a second molecular progression pathway to invasive disease, whereby the p53 mutation is the initial event and chromosome 9 loss occurs later.

Figure 2. The two putative molecular pathways in bladder cancer progression.

Candidate oncogenes and tumor suppressor genes

Many of the regions involved in allelic loss are likely to harbor tumor suppressor genes, whereas the chromosomal amplifications could indicate the presence of proto-oncogenes. Table 1 summarizes the chromosomal regions that may harbor genes involved in bladder cancer development or progression. The gene for the retinoic acid receptor is located on chromosome 3p and it is of interest since the loss of this receptor is correlated with a poor prognosis in bladder cancer (37,138). The von Hippel-Lindau (VHL) disease tumor suppressor gene is also located on chromosome 3p but its relevance for bladder cancer is not yet determined (113). DNA polymerase β is an interesting candidate gene which maps within the region of deletion on 8p21-8q11.2 that

might have an effect on the mutation rate (105). At least two tumor suppressor genes are likely to be mapped on chromosome 9 (100,161,175). Region 9p21-22 is of particular interest because two studies independently reported homozygous deletions in this region (23,41). The CDKN2 (MTS1) gene is located at 9p21, its product, p16, inhibits the cyclinD/cdk4 complex that phosphorylates pRB, thus negatively regulating cell cycle progression (193). The deleted area on chromosome 11p contains the ras oncogene (165), the two Wilms' tumor suppressor loci (WT1 and WT2, at 11p13 and 11p15, respectively) (25,107) and the CAT locus, that is thought to be involved in DNA repair processes (197). The retinoblastoma gene, the first identified tumor suppressor gene, is the candidate gene on 13q14 (206). As addressed earlier chromosome 17p harbors the tumor suppressor gene p53. The DCC gene (18q21), known to be involved in colorectal cancer (52), might be the target of deletions in a subset of bladder tumors.

Table 1. Chromosomal deletions in bladder cancer and candidate oncogenes and tumor suppressor genes.

Locus	Candidate gene
3p	retinoic acid receptor, VHL
8p21-8q11.2	DNA polymerase β
9p21-22	CDKN2
11p	H-ras; WT1 (11p13); WT2 (11p15); CAT locus
13q14	RB
17p13.1	p53
18q21	DCC

The exact biological role of these chromosomal aberrations and the genes involved is subject for on-going investigations and once these genes are identified, a more complete view of the genetic events that determine progression will be possible.

Differential gene expression

Another approach in the search for new molecular markers with prognostic value is to compare different stages of progression at the level of gene expression by means of differential or subtraction hybridization analysis of cDNA libraries. These methods are

based on comparison of steady state mRNA populations. A drawback of these techniques is that genes that are aberrantly expressed (i.e. different size of transcript) can not be identified, unless the level of steady state mRNA has changed. Differences in post-translational regulation cannot be identified using this approach. However, the advantages of differential/substraction hybridization analyses are, that relevant but unknown genes can be isolated. Moreover, genes that are either upregulated (e.g. oncogenes) or down modulated (e.g. tumor suppressor genes) can be identified. The cDNA clones identified can be routinely characterized by DNA sequence analysis and comparison of the resulting DNA sequence with nucleotide sequence data bases. If the isolated cDNA clone is of interest it can be immediately evaluated for its use in diagnosis by RNA *in situ* hybridization analysis (211). The selection of the method for comparing mRNA populations depends on the relative abundance of the mRNA population of interest. To study high and low abundance transcripts ($>0.01\%$), the differential hybridization analysis is a valid approach (180,189). The identification of very low abundance ($<0.01\%$) differentially expressed genes, requires a more sophisticated approach, i.e. subtraction hybridization analysis which is based on the subtraction of common mRNAs from a given population, thus leaving unique mRNAs (at very low levels, 0.001%) (218). Both differential and subtraction hybridization are technically difficult and require relative large amounts of approx. $5\ \mu\text{g}$ poly-A⁺ RNA. A recently developed alternative approach, named mRNA differential display offers the important advantages that much less mRNA (approx. $10\ \text{ng}$ /reaction) is required. Furthermore, it is quicker and technically simpler (120,121,122). The most essential part of these three "gene-hunting" techniques is the choice of the mRNA populations that are compared which should be homogeneous and well characterized. At present the above mentioned techniques have succeeded in isolating only a small number of important genes, i.e., NM23 (208), stromelysin-3 (8), EMS1 (191) and WAF-1 (45), which were all identified in very precisely defined populations.

In the following paragraphs the value of the most relevant known molecular prognostic markers for bladder cancer will be discussed.

Molecular prognostic factors

DNA ploidy

By DNA flow cytometry or image cytometry insight can be obtained in the extent of genetic damage. In a few studies the biological significance of aneuploidy in tumor progression has been addressed. It has been hypothesized that tumor cells first acquire a tetraploid status, whereafter cells can evolve by chromosome loss without lethal effect (42,69). Thus, more aggressive clones could emerge at a higher rate in these tumors than in diploid tumors. Aneuploidy in bladder tumors has been extensively studied and correlates with a high propensity to progress to invasion and metastasis as well as with poor survival (14,106). In muscle invasive operable bladder cancer, DNA ploidy does not represent a prognostic parameter (58). For superficial bladder cancer (Ta/T1) the 2c deviation index appeared to have significant predictive value for survival (15,190). However, the DNA content may be a differential prognostic marker only for grade 2 tumors, since most grade 1 tumors are diploid and most grade 3 tumors are aneuploid.

Epidermal Growth Factor Receptor

The interest in studying Epidermal Growth Factor (EGF) and its receptor (EGFR) was based on the finding of high EGF (urogastron) levels in urine. Messing et al. reported in 1984 that growth and division of certain TCC cell lines responded to EGF (140). Several investigators have studied the epidermal growth factor receptor (EGFR), the product of c-erbB-1 (located on chromosome 7), and found a significant correlation between EGFR overexpression and both grade and stage (13,141,153,174). The correlation between EGFR positivity and clinical outcome was studied immunohistochemically in 101 bladder tumors (125,154,155). EGFR overexpression appeared to be a significant prognostic factor for the whole group studied. The difference in survival between EGFR-positive tumor and EGFR-negative tumor bearing patients was not significant when the data from patients with muscle invasive disease were analyzed separately. However, in patients with superficial TCC (pTa, pT1), EGFR overexpression is correlated with recurrence rate, time to recurrence and tumor progression.

EGFR gene amplification has been studied by Southern blot analysis but was found in only one out of 31 bladder tumors (13). When the polymerase chain reaction was used to

assess EGFR amplification in carcinoma cells from bladder washings, an amplification in 2 out of 47 patients was observed (131). A more recent study showed the same rate of EGFR gene amplification (5 out of 107 tumors) (187). Apparently, EGFR gene amplification is not a predominant mechanism for EGFR overexpression.

EGFR expression can be induced by overexpression of the H-ras gene. This was found to occur both at the protein and mRNA levels and analysis of EGFR promotor/enhancer sequences has revealed a putative AP-1 site which may possibly serve as a ras responsive element (212). These results suggest a role for overexpression of H-ras, whether wild type or mutant, in upregulating the EGFR. Mutated H-ras in bladder cancer has been reported at various frequencies ranging from 6%-44% in different studies, but no correlation with grade or stage was observed (20,35,57,104,116). This range in mutation frequencies could be caused by the use of different detection methods and variable tumor specimens. The exact role of H-ras in bladder cancer remains to be elucidated.

In conclusion, overexpression of EGFR might be useful as a prognostic marker in the group of superficial bladder tumors.

C-erbB-2

C-erbB-2 is a human proto-oncogene located on chromosome 17q21 encoding a transmembrane growth factor receptor with significant sequence homology to the EGFR (252). Different studies showed varying rates of c-erbB-2 gene amplification ranging from 8%-46%. C-erbB-2 protein overexpression was shown to be more frequent than the gene amplifications (32,186,256). Hence, for c-erbB-2 overexpression, gene amplification also does not seem to be the predominant mechanism. Moreover, Underwood and coworkers (227) concluded that c-erbB-2 gene amplification was of no value as an independent marker for the prediction of disease recurrence or progression.

Sato et al. (185) showed c-erbB-2 protein expression to be correlated with tumor grade and survival. Multivariate analysis showed that c-erbB-2 expression was a prognostic factor independent of grade and stage of the tumors. In contrast to the findings of other recent immunohistochemical studies (114,125,227). Although the study of Lipponen (125) showed a correlation between c-erbB-2 expression, grade, DNA aneuploidy, high S-phase fraction and decreased survival, no additional significant prognostic value over

already established prognostic parameters in TCC was found. In the other two studies no significant correlation with either grade, stage or survival was observed.

At present it appears that c-erbB-2 gene amplification and protein overexpression have no additional predictive value for disease recurrence or progression. The disparity between the different immunohistochemical studies emphasizes the need for further detailed investigation.

The Retinoblastoma gene

Another tumor suppressor gene potentially involved in bladder cancer is the Retinoblastoma (RB) gene located on 13q14. Restoring the wild-type RB function in a RB-negative cell line (HTB9) resulted in complete suppression of growth in soft agar and decreased tumorigenicity in nude mice, suggesting a key role for the RB gene in bladder cancer (209). The RB gene encodes a nuclear phosphoprotein and several investigators have hypothesized that it functions as a cell cycle control protein (72,115, 244). More recently, it has been suggested that pRB functions downstream of p53 in the same regulatory pathway. HPV proteins E6 that targets p53 for destruction and E7 that binds to pRB, undermine the p53-pRB-dependent damage checkpoint (39,201). p53 influences the activity of cyclin-dependent kinases that phosphorylate pRB, via p21 production (45). High levels of p21 inhibit cyclinE-Cdk2, which usually phosphorylates pRB in G1/S of the cell cycle, thereby resulting in hypo-phosphorylated pRB. This form of pRB blocks the transcription of genes required for subsequent stages of the cell cycle and arrests the cell cycle.

Loss of RB function at the protein level occurs in approximately one-third of human bladder cell lines (92). Gross structural deletions of the RB gene as well as point mutations have been documented in many of these cell lines (91,92). More recently, loss of RB protein expression and deletions at the RB locus have been identified in primary bladder cancers, and it appeared that altered RB expression is correlated with high grade and high stage tumors (22,33,68,73,95,128,130,251). A strong correlation was found between LOH at the RB locus and the absence of normal RB protein expression (251). Rb gene mutations have been shown to occur in both low grade superficial as in high grade invasive bladder cancers (145). Two immunohistochemical studies have shown that a decreased expression of the RB protein is associated with a more aggressive

biological behavior (33,130). Decreased Rb expression correlated with shortened survival in the group with invasive tumors. Based on current knowledge it can be concluded that altered RB protein expression may be a prognostic marker in patients with invasive bladder cancer.

E-Cadherin

To metastasize, cancer cells must be released into the blood or lymphatic stream. Decreased intercellular adhesiveness favors detachment of tumor cells and may play a role in the early steps of the metastatic process. Although cell-cell adhesion is complex involving at least four families of adhesion molecules (integrin, immunoglobulin, selectin, and cadherin), several lines of evidence indicate that the Ca^{2+} -dependent, E-cadherin-mediated adhesiveness is critically important for epithelial integrity (210). Modulation of E-cadherin function of several tumor cell lines either by blocking antibodies or by transfection experiments revealed an invasion suppressor role for E-cadherin-mediated cell-cell adhesion *in vitro* (10,63,235). The correlation between E-cadherin expression and *in vitro* invasiveness has been tested for 3 bladder cell lines. The RT4 and RT112 cell lines express E-cadherin and are not invasive, whereas the EJ24 cell line does not express E-cadherin and is invasive (63). E-cadherin expression has been analyzed in 49 bladder tumors with anti-E-cadherin monoclonal antibodies (18). In this immunohistochemical study decreased E-cadherin expression correlated with both increased grade and stage whereas abnormal E-cadherin expression correlated with shorter survival for the whole group studied as well as for the group with invasive tumors. No conclusions can be drawn for the patients with superficial TCC. These data were recently confirmed by Otto et al. (162) who also observed a concomitant association between increased overexpression of the autocrine motility factor and progression of disease. In conclusion, E-cadherin seems to have potential as a prognostic marker although its precise clinical relevance has to be assessed in larger clinical trials.

Potential prognostic factors

Besides the most relevant prognostic parameters, there is an additional panel of molecular factors with potential prognostic relevance but only the most important parameters will be discussed.

Scatter factor (SF) has been shown to be overproduced by bladder carcinomas (96) and is involved in invasion and angiogenesis. Both are characteristics of biologically aggressive tumors suggesting that the accumulation of SF within tumors might promote progression to a more malignant phenotype. Its exact clinical relevance remains to be determined. Fradet and coworkers raised monoclonal antibodies against tumor-associated antigens (59,60,61). The most relevant ones are the tumor-associated antigens M344 and T138. M344 is predominantly expressed on superficial TCC and its expression decreases with increasing grade of the tumors (62). Preliminary data suggest that M344 positive tumors rarely progress. T138 was found to have an independent significant prognostic value for bladder cancer (174). Larger clinical studies, in which also the separate groups of superficial and invasive tumors are studied, are necessary to reveal the exact clinical usefulness of T138.

Altered expression of MDM2 is reported in bladder cancer (78,119) and overexpression is associated with low grade, low stage bladder tumors and has been implicated in the progression of bladder cancer. Whether MDM2 overexpression represents an alternative to p53 mutation in inactivating the p53 regulatory pathway is still unclear.

The p53 tumor suppressor gene

The p53 gene

The most extensively studied tumor suppressor gene is p53, often referred to as "the guardian of the genome" (111). Since the main part of the present thesis concerns the value of p53 as a prognostic marker in bladder cancer, p53 will be discussed more extensively.

The p53 gene is located on chromosome 17p13.1 (11) and it is mutated in over half of all human tumors (87). Frequently one of the two alleles is lost and the other is mutated (6,87). Most mutations are found in four conserved domains (II: aa 117-142; III: aa 171-181; III: aa 234-258; IV: aa 270-286), located in the DNA binding domain of the p53

protein (205). The protein encoded by the p53 gene is a 53-kd nuclear phosphoprotein consisting of a transactivation-, a DNA binding- and an oligomerization domain (Figure 3).

Figure 3. Structural domains of human p53. Boxes II to V represent the conserved regions containing the frequent sites of mutation. Above the p53 sequence the different domains of p53 activity and the positions of defined phosphorylation are indicated. Below the p53 sequence, the main nuclear localization signal (NLS) and the interaction of various factors with p53 are shown. Abbreviations: dsDNA-PK, double-stranded protein kinase; CKI, casein kinase I; cdc-2, p34^{cdc2} kinase; CKII, casein kinase II; TBP, TATA binding protein; mdm-2, murine double minute-2; E1b, adenovirus 5 E1b protein; E6, human papilloma virus E6 protein, SV40 Tag, simian virus 40 large T antigen; NLS, nuclear localization signal (adapted from: Harris et al. (84); Prives et al. (170)).

Transcriptional regulation by p53

The tumor suppressor protein p53 plays a role as a transcriptional regulator (49,99,129, 166,238,253) and is involved in the control of the cell cycle at G1 to S phase transition (54,134). When DNA is damaged, p53 can delay the progression through the cell cycle allowing DNA repair (134) or can initiate programmed cell death (apoptosis) (132). It should be stressed that p53 is more involved in regulating and controlling the cell cycle, than in playing a direct mechanistic role in cell cycle progression. Studies using knock-out mice have demonstrated that functional p53 is not essential for mitotic or meiotic cell cycles, but the high incidence of tumor development in p53-null mice (43) strongly suggests that p53 provides an important checkpoint that prevents aberrant growth, division and neoplastic transformation. Loss of wild type p53 function results in an altered G1-S cell cycle checkpoint which can lead to replication of incorrect DNA, resulting in genetic instability (129,253).

p53 acts both through transcriptional suppression and transcriptional activation. The p53 protein interacts with the transcription factors Taf_{II}40 and Taf_{II}60 (216) and possibly also with the TATA binding protein (TBP) (28,195,220). These interactions are thought to contribute to the ability of p53 to repress general transcription. p53 can activate transcription by binding to repeats of the DNA consensus site PG (44). Viral oncogenes that overcome p53-mediated growth suppression abrogate its ability to transactivate (142). A few targets of p53 transcriptional activation have been identified that might account for the G1 arrest in response to DNA damage (Figure 4).

Figure 4. The p53 mediated response to DNA damage (177).

The p53 mediated responses to DNA damage

DNA repair

WAF-1 (wild-type p53 activated fragment), also known as CIP1 (83) and Sdi (156), was isolated by subtractive hybridization screening from cells overexpressing p53 (45). The protein p21 encoded by WAF-1 is an inhibitor of G1 cyclin dependent kinases and complexes with cyclin, Cdk and PCNA (proliferating cell nuclear antigen) in normal cycling cells (250). The p21 protein is known to interact directly with PCNA thereby blocking its replication, but not its repair activity (118,198,240,242). Although upregulation of p21 by p53 appears to be the most important mediator of p53 mediated growth arrest, other genes that are transcriptionally activated by p53 may also play a role in DNA damage-dependent cell cycle arrest.

For example, cyclin G1, a gene, whose expression is induced by wild-type p53 (157), might be a candidate although its exact role in cell cycle arrest or apoptosis is not clear (21). The excision repair ERCC3 gene may also be transcriptionally induced by p53 (241). Smith and coworkers showed that GADD45 (growth arrest and DNA damage gene) which is also upregulated by p53, probably serves as a link between the p53-dependent cell cycle checkpoint and DNA repair (203) (Figure 4). GADD45 can interact directly with the essential replication factor PCNA (203), thereby blocking DNA replication and possibly enhances nucleotide excision repair of damaged DNA.

Apoptosis

The responses mentioned above presumably favor enhanced survival. However, in some situations, p53 activates a suicide pathway (apoptosis) following DNA damage. Recently, Miyashita and Reed (147) suggested a mechanism by which p53 might induce apoptosis. Bax and Bcl-2 are homologous proteins that have opposing effects on apoptosis (Figure 4). Bcl-2, the product of a proto-oncogene, protects the cell from apoptosis, while Bax acts as an apoptotic signal (158). The upregulation of p53 in some cell types is concomitant with the down-regulation of Bcl-2, and the upregulation of Bax. The induction of the Bax protein increases the concentration of Bax:Bax homodimers and Bax:Bcl-2 heterodimers in the cell, thereby potentiating apoptosis under certain circumstances (158). Recent evidence indicates that a functional p53

pathway is required for efficient cell kill by chemotherapeutic agents (56,133), although conflicting findings have also been reported (16,65).

The p53 protein controls the maintenance of DNA integrity by the mechanisms mentioned above. In addition to mutation, loss of wild type p53 function can be the result of complexation with viral oncoproteins, e.g., simian virus 40 (SV40) Large T antigen (110,124), adenovirus 5 E1b (184) and E6 protein of human Papillomavirus 16 and 18 (243). The cellular oncoprotein, mdm2 also interacts directly with p53 and functionally inactivates it (148).

Prognostic value of p53

The p53 tumor suppressor gene can be studied directly by analyzing p53 gene mutations using e.g. Single Strand Conformation Polymorphism (SSCP) or direct sequencing. The occurrence of p53 mutations can lead to conformational changes of the p53 protein, resulting in a prolonged half-life and subsequently, in accumulation of the protein (55). The extended half-life of the protein is the basis for immunohistochemical detection of p53. Despite the good concordance between p53 mutation and p53 overexpression, discrepancies are observed in a substantial number of tumors. (34,46,214; this thesis).

A number of studies have investigated p53 genetic alterations in bladder cancer. The loss of heterozygosity of chromosome 17p is associated with high grade and high stage bladder carcinomas (159). By means of subcloning and sequencing of exon 5 through 9 of the p53 gene, it has been shown that 17p allelic loss is strongly associated with p53 mutation in 9 out of 10 cases and that p53 mutation is associated with high stage tumors (199). The correlation between p53 mutations and stage as well as grade was confirmed in four other studies using PCR-SSCP (64,207,245; this thesis). Analysis of p53 mutations in carcinoma *in situ* showed a high prevalence of 65% of p53 mutations similar to that seen in invasive bladder cancer (146,207) (see Figure 2).

The relatively high frequency of p53 mutations in T2-T4 tumors compared to T1 tumors suggest their involvement in the progression of T1 tumors to invasive disease (207). However, the exact point at which p53 mutations occur during tumor progression from Ta to T1 and T2-T4 tumors is still unclear. p53 mutations were reported to have prognostic significance for bladder cancer (225; this thesis), although it offered no additional prognostic value over stage for invasive bladder cancer (T2-T4) (this thesis).

The exact prognostic value of p53 mutations for superficial bladder cancer remains to be determined.

Overexpression of the p53 protein has been reported to have a clear prognostic significance for superficial bladder cancer (47,181,182,194), although conflicting data were also reported (66,125). Differences in antibody, methodology, treatment of the patients and the scoring systems used might explain these differences. The prognostic value of p53 overexpression is also ambiguous for invasive tumors (47,125,183; this thesis). Apparently, as long as the tumors are confined to the bladder (pT $\leq$ 3A, confined to the detrusor muscle), p53 immunohistochemistry has prognostic value. With more extensive disease, p53 alterations have no additional prognostic value.

Scope of this thesis

In this thesis two approaches are used to investigate the predictive value of molecular markers for bladder cancer progression

First, the prognostic value of a known gene, the tumor suppressor gene p53, was studied. Alterations of p53 can be studied by various methods. In chapter 2, the direct detection of mutations in the p53 gene by PCR-SSCP and the immunohistochemical detection of p53 protein overexpression were compared to assess their usefulness. To evaluate the prognostic value of p53 alterations in bladder cancer, we studied the association of both p53 overexpression (Chapter 2) and p53 mutations (Chapter 3) with grade, stage and survival. Furthermore, the association of p53 mutations with frequency of allelic loss as a putative indicator of genetic instability, was investigated. To assess the value of p53 mutations (PCR-SSCP) in predicting the progression of superficial TCC, consecutive bladder washings from high risk superficial TCC patients (indicated by quantitative karyometric analysis) were examined (Chapter 4). To determine the actual value of bladder washing specimens to assess the p53 status of histological specimens we analyzed the bladder washings and their synchronous tumors for the presence of p53 mutations (Chapter 5).

As a second approach we used differential hybridization analysis to identify new molecular prognostic markers. We used a rat bladder tumor model system (RBT), in which the different stages of tumor progression are represented. In the differential hybridization analysis the mRNA populations of low and high metastatic lines were compared (Chapter 6).

Chapter 2

Comparison of p53 protein overexpression with p53 mutation in bladder cancer: Clinical and biological aspects.

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Abstract

BACKGROUND Alterations of the tumor suppressor gene p53 are known to occur in bladder cancer. Although p53 overexpression is associated with mutation of the p53 gene, a substantial discrepancy between molecular genetic alteration in p53 and overexpression of the protein has been found.

EXPERIMENTAL DESIGN Tumor specimens of 39 bladder cancer patients were immunohistochemically analyzed for p53 overexpression, and the results were compared with the presence of a mutation as assessed by Single Strand Conformation Polymorphism (SSCP) and direct sequencing. Both clinical and biologic aspects were studied.

RESULTS A significant correlation between p53 overexpression and poor survival in the whole group studied was found ($p < 0.01$). No association between p53 overexpression and decreased survival was found for invasive tumors in contrast with other studies. Differences in treatment of the patients and different antibodies and scoring systems used might explain these differences. In our study, the Kaplan Meier curves showed the same result for p53 overexpression and p53 mutation when the whole group and the invasive tumors were studied. However, in the group of superficial tumors, which was unfortunately too small for statistical analysis, we found p53 overexpression in three tumors while no p53 mutations were found.

A good concordance between p53 mutation and p53 overexpression was found ($p < 0.02$). However, two out of eight tumors with an SSCP proven p53 mutation showed no p53 immunoreactivity, probably as a result of loss of the nuclear localization signal. Twenty three percent (7/31) of the tumors showed p53 overexpression without any sign of a mutation.

CONCLUSION Our results indicate that, despite a good concordance between p53 mutation and p53 overexpression, there is no direct causal relationship between mutation and protein accumulation. Apparently other events than mutation can trigger p53 stability.

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Introduction

The tumor suppressor gene p53 is located on chromosome 17p13.1 and encodes a 53 kD nuclear phosphoprotein with specific DNA binding properties. Chromosomal losses of 17p13 occur during tumor progression in a variety of human tumors (87). In bladder cancer, loss of 17p13 is associated with high grade tumors and invasive disease (159,169). In accordance with the classic tumor suppressor theory, the loss of heterozygosity (LOH) of 17p13 is often accompanied by a mutation of the remaining allele (6). In bladder cancer, p53 mutations correlate with grade and stage (64,199,233) and probably play a role in the progression of this disease.

Wild-type p53 acts as a cell cycle control protein at the level of G1 to S phase transition (99). If DNA damage occurs, p53 levels rise and block cells in the G1 phase. The DNA damage can subsequently be eliminated either by DNA repair or by initiation of apoptosis (132). The up-regulation by p53 of p21, an inhibitor of G1 cyclin-dependent kinases, appears to be responsible for p53 mediated growth arrest (94). Recently, Smith et al. showed that Gadd45 which is upregulated by p53, probably serves as a link between the p53-dependent cell cycle checkpoint and DNA repair (203). p53 seems to function through modulation of transcriptional activity, enhancing the expression of genes containing p53-binding sites and interacting with a variety of transcription factors to inhibit the expression of other genes (166,254). Cells that lose this wild type p53 function fail to show growth arrest if DNA damage occurs which can lead to replication of incorrect DNA, resulting in genetic instability (129,253).

In addition to mutation, loss of wild type p53 function can be the result of complexing with viral oncoproteins, e.g., simian virus 40 (SV 40) large T Ag (110,124), adenovirus 5 E1b protein (184) and E6 protein of human Papillomavirus 16 and 18 (243). The cellular oncoprotein mdm2 also interacts directly with p53 and functionally inactivates it (148).

The occurrence of p53 mutations leads to conformational changes of the protein, resulting in a prolonged half-life and subsequently in accumulation of the protein (55). The extended half-life of the protein is the basis for immunohistochemical detection of p53. Although there is a good concordance between p53 overexpression and mutation of the p53 gene, several studies have shown a considerable discrepancy between molecular genetic alteration in p53 and overexpression of the protein (26,81,137,249).

In this study we compared the immunohistochemical detection of p53 overexpression with p53 mutation as assessed by Single Strand Conformation Polymorphism (SSCP).

Materials and methods

Tumor specimens

Twenty-three snap frozen, superficial bladder carcinomas (pTa-pT1) and 24 muscle-invasive bladder carcinomas (pT $\geq$ 2) obtained from 45 patients were used for SSCP analysis, as described previously (233). The transitional cell carcinomas were classified according to the World Health Organisation criteria (149). For immunohistochemistry the same specimens were investigated with the exception of 2 superficial and 2 invasive carcinomas that could not be analyzed because of poor quality of the frozen tissue. Moreover, the two squamous cell carcinomas were removed from this study because of their different pathologic background. The clinical and pathologic data are shown in Table 1. Genomic DNA was extracted from step-sectioned tumors (>70% tumor cells) using a method described by Miller and coworkers (144).

Immunohistochemistry

Cryostat sections (5 μ m) were dried overnight. Tissue sections were fixed in acetone for 10 minutes and then incubated overnight at 4°C with the mouse mAb DO-7 (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:100. This Ab recognizes both wild-type and mutant p53. Sections were subsequently incubated for 30 minutes with biotinylated sheep anti-mouse Ig antibody (1:200, Amersham, Buckinghamshire, UK) and then incubated for 30 minutes with streptavidin biotinylated-horseradish peroxidase complex (1:100, Amersham). After washing with PBS, 3,3'- Diaminobenzidine (Sigma Chemical Company, St. Louis, MO, USA) was used as a chromogen and hematoxylin for counterstain.

Analysis of the immunohistochemical results was performed by two investigators (J.V., P.P.B). The pattern of p53 nuclear overexpression was classified in four categories by estimating the percentage of stained tumor cells: -, no cells positive; +, 1 to 10% positive tumor cells; ++, 10 to 50% positive tumor cells and +++, > 50% positive tumor cells. Cytoplasmic staining was not scored.

PCR-SSCP

PCR-SSCP analysis (160) was performed to investigate p53 mutations in exons 5 to 8. The intron primers for amplification of exons 5 to 8 were: 5S (5'-tca-ctt-gtg-ccc-tga-ctt-3'), 5AS (5'-gag-gaa-tca-gag-gcc-tgg-3'), 6S (5'-gag-acg-aca-ggg-ctg-gtt-3'), 6AS (5'-gag-acc-cag-ttg-caa-acc-3'), 7S (5'-cca-agg-cgc-act-ggc-ctc-3'), 7AS (5'-gag-gca-agc-aga-ggc-tgg-3'), 8S (5'-cct-tac-tgc-ctc-ttg-ctt-c-3'), 8AS (5'-tga-atc-tga-ggc-ata-act-3').

Genomic DNA (250 ng) was subjected to 35 cycles of PCR (95, 57 and 72°C for 0.5, 2 and 1.3 minutes, respectively). Exons 5, 6 and 8 were amplified in 50 µl containing: 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.75 mM MgCl₂, 250 µM deoxynucleotide triphosphates, 10 pmol of each 5'-end-labeled primer and 1.5 U of Taq polymerase (Perkin Elmer/Cetus, Norwalk, Connecticut, USA). Exon 7 was amplified in the same buffer containing 1.5 mM MgCl₂.

Five microliters of the PCR product was diluted in 15 µl loading buffer (96% formamide, 20 mM EDTA, 0.05% bromophenol blue and xylene cyanol), boiled for 3 minutes and then quenched (10 minutes) on ice before loading (2 µl/lane). Each sample was applied to a 5% polyacrylamide (49:1)/Tris-Borate EDTA (0.5x) gel with and without 10% (v/v) glycerol. Subsequently, electrophoresis was performed at room temperature for 16 hours at 6 or 3W respectively.

Sequence Analysis

Direct sequencing of the double stranded PCR products that showed a shift on the SSCP gels was performed as described previously (109). Amplified PCR products were purified using the magic PCR preps[™] DNA purification system (Promega, Madison, WI, USA). The PCR primers were used for sequencing in the dsDNA cycle sequencing system (Life Technologies, Inc, Gaithersburg, MD, USA). Electrophoresis was performed on 6% polyacrylamide (19:1) gels containing 7 M ureum.

Statistical Analysis

The Kaplan-Meier method was used to estimate survival probability as a function of time. Differences in survival were analyzed by a log-rank test. The Chi-square test (with Yates correction if relevant) was used for the other correlations.

Table 1. p53 Overexpression, clinical and pathological data for each patient.

Case no.	Age (yr)	Stage/Grade	p53 Immuno-positivity ^A	Recurrences	Survival (months)	Treatment ^B
1	77	a/1-2	++	no	31	TURT
2	81	a/1	-	a/2, 8 months	>65	TURT
3	77	a/1	-	no	>58	TURT
4	73	a/1	-	no	>55	TURT,Ch
5	66	a/3	+++	a/2, 8 months	23	TURT,Ct,R,Ch
6	55	a/1-2	-	a/2, 4 months	>68	TURT,BCG,Ct
6 rec.		a/2	+			
7	54	a/1	-	no	>46	TURT
8	50	a/1	-	a/1, 8 months	>84	TURT,BCG,Ch
9	88	a/2	-	no	52	TURT,Ch
10	50	a-1/2	++	no	>69	TURT
11	68	a-1/1	-	1/2, 4 months	>37	TURT
12	75	1/3	-	a/2, 6 months	>72	TURT,BCG
13	92	1/2	+	a/2, 11 months	73	TURT,Ch
14	62	1/2	-	no	>40	TURT,Ch
15	58	1/2	+	a/1, 9 months	>50	TURT,BCG
16	71	1/1	-	a/2, 4 months	>75	TURT
17	79	1/2	+	a/3, 3 months	49	TURT,Ch
17 rec.		a/3	-			
18	77	1/2	+	no	>58	TURT,Ch
19	72	1/2	+	a/2, 6 months	>44	TURT,Ch,BCG
20	81	2/2	-		9	TURT
21	73	2/2	+		8	TURT,Ch,Ct
22	53	2/3	+		>50	TURT,R,Iridium
23	79	2/3	++		4	Ct
24	81	2/2	-		6	TURT
25	53	2/3	++		7	TURT,Ch
26	70	2/3	++		3	TURT,Ch
27	47	2/3	-		8	TURT,Ch
28	67	≥2/3	-		>20	TURT,Ch,R
29	72	≥2/3	+++		>85	TURT,Ch
30	79	≥2/3	+		10	TURT,Ct
31	59	≥2/3	++		3	TURT,R
32	73	2-3/3	++		2	TURT,R
33	68	2-3/3	+++		2	TURT
34	63	3/3	++		5	TURT,R
35	74	3/3	+++		10	Ct,Ch
36	74	3b/2	++		>39	Ct
37	80	3b/3	-		>53	TURT,R,Ct
38	44	4/3	-		29	TURT,Ct
39	82	4/2	-		5	TURT

^A Number of positive cells: -, 0%; +, 1-10%; ++, 10-50%; +++, >50%. rec., recurrence;^B Ch, chemotherapy; Ct, cystectomy; R, radiotherapy; TURT, transurethral resection of the tumor, BCG, bacillus calmette-guerin instillations.

Results

Immunostaining patterns

The DO-7 staining patterns of the four groups are shown in Figure 1. We defined a tumor as p53 positive if more than 10% of the tumor cells showed nuclear p53 expression. Of the 41 tumors studied, 13 showed p53 overexpression (Table 1).

Figure 1. Immunohistochemical staining of p53 expression in transitional cell carcinomas using p53 antibody DO-7 A. Stage Ta grade 2 tumor, no tumor cells stained (0% = -) B. Stage T2 grade 2 tumor, a few tumor cells are stained (1-10% = +) C. Stage T2 grade 3 tumor, heterogeneous staining (10-50% = ++) D. Stage T3 grade 3 tumor, clearly more than 50% of tumor cells show nuclear p53 overexpression (>50% = +++) Original magnification, times 400.

Correlation between tumor grade/stage and p53 overexpression

p53 overexpression was found in 9% of the grade 1, 23% of the grade 2 and 60% of the grade 3 tumors. Overexpression of p53 was found in 16% of the superficial tumors and in 50% of the invasive tumors ($p < 0.05$) (Table 2,3). p53 Mutations found by SSCP analysis showed a high correlation with both increasing grade ($p < 0.001$) and stage ($p < 0.001$) (233).

Table 2. Correlation between p53 overexpression and grade.

Grade	p53 immuno-positivity ^A (n)				% immuno-positivity
	-	+	++	+++	%
1	9	1	1	0	9%
2	6	4	3	0	23%
3	4	2	6	3	60%

^A Number of positive cells: -, 0%; +, 1-10%; ++, 10-50%; +++, >50%.

Table 3. Correlation between p53 overexpression and stage.

Stage	p53 immuno-positivity ^A (n)				% immuno-positivity
	-	+	++	+++	%
superficial	10	6	3	0	16%
invasive	7	3	7	3	50%

^A Number of positive cells: -, 0%; +, 1-10%; ++, 10-50%; +++, >50%.

Correlation between p53 overexpression and survival

The survival according to p53 overexpression is shown in Figure 2. For the whole group, a shorter survival time of patients with p53 overexpression was observed (Chi-square: 8.00, $p < 0.01$, Figure 2a). Although statistical analysis was not possible, because of the small number studied, we observed a shorter survival time for patients with superficial bladder cancer that showed p53 overexpression (Figure 2b). Within the group with invasive disease, no significant difference in survival time between the patients with and without p53 overexpression was seen (Figure 2c).

Figure 2. Kaplan Meier survival curves of bladder cancer patients according to the presence or absence of overexpression of the p53 protein.

A. All patients ($P < 0.01$, log rank test). B. Patients with superficial (pTa, pT1) tumors (not significant). C. Patients with muscle invasive disease (not significant).

Table 4. Relationship between p53 mutations analyzed by PCR-SSCP and p53 overexpression by immunostaining.

	Immunostaining [^] (n)				< 10% (%)	> 10%
	-	+	++	+++		
P53 mutation/SSCP						
No mutation	18	8	7	0	77	23
Mutation	2	0	3	3	25	75
Exon 5	1	0	1	1		
Exon 6	0	0	0	1		
Exon 7	0	0	1	0		
Exon 8	1	0	1	1		

[^] Number of positive cells: -, 0%; +, 1-10%; ++, 10-50%; + + +, >50%.

Comparison between p53 overexpression with p53 mutations found by PCR-SSCP

The overall comparison of p53 mutations analyzed by PCR-SSCP and p53 overexpression assessed by immunohistochemistry (IHC) is shown in Table 4. The sensitivity of IHC, defined as percentage of IHC-positive tumors among tumors with identified mutation, was 75%. The specificity of IHC, defined as percentage of IHC-negative tumors among neoplasms without a p53 mutation, was 77%. Despite the good concordance between p53 mutation and p53 protein overexpression ($p < 0.02$), 23% (7/31) of the tumors without a p53 mutation as assessed by SSCP analysis showed p53 overexpression.

Table 5. Comparison immunostaining and PCR-SSCP analysis for mutants.

Histopathology		p53 Mutation/SSCP			p53 Overexpression [^]
Case no.	Stage/Grade	Exon	Codon	Amino acid change	
1	> 2/3	5	179	CAT → TAT (<i>His</i> → <i>Tyr</i>)	+ + +
2	3/3	8	285	GAG → AAG (<i>Glu</i> → <i>Lys</i>)	+ + +
3	4/3	5	166	TCA → TGA (<i>Ser</i> → <i>umber</i>)	-
4	2/3	8	282	del. G → frameshift	-
5	2/3	5	158	CGC → CTC (<i>Arg</i> → <i>Leu</i>)	+ +
6	2-3/3	8	285	GAG → AAG (<i>Glu</i> → <i>Lys</i>)	+ +
7	2-3/3	6	215	AGT → GGT (<i>Ser</i> → <i>Gly</i>)	+ + +
8	> 2/3	7	259	GAC → GTC (<i>Asp</i> → <i>Val</i>)	+ +

[^] Number of positive cells: -, 0%; +, 1-10%; ++, 10-50%; + + +, >50%.

Table 5 shows the eight p53 mutants found by PCR-SSCP analysis. They all showed p53 overexpression except for cases 3 and 4, which showed no p53 immunoreactivity at all. These two cases contained a C to G transversion at codon 166 leading to a stopcodon and a deletion of a guanine nucleotide in codon 282, resulting in a frameshift.

Discussion

This study compares the overexpression of the p53 protein assessed by IHC with p53 mutation determined by means of SSCP analysis in bladder cancer. We looked at both clinical and biologic aspects of p53 alterations. We chose a cut off of 10% p53 positive tumor cells for p53 overexpression. The appearance of occasionally stained cells in tumors, which we scored as negative, can be explained by the existence of clones of cells with a p53 mutation or by the occurrence of a genotoxic event that raises levels of wild type p53 protein in normal cells, resulting in cell cycle arrest (80,111). More detailed examination of microdissected focally stained areas by means of mutation analysis (SSCP) has to be performed to address this problem and to check the biologic importance of this phenomenon.

The overexpression of p53 as assessed by IHC correlates with grade and stage ($p<0.05$). However, correlation between mutations in the p53 gene as determined by SSCP analysis and grade and stage was higher ($p<0.001$) (233). In concordance with other IHC studies (47,126) we demonstrated that p53 overexpression is an unfavorable prognostic factor for bladder cancer patients ($p<0.01$). No association between p53 overexpression and decreased survival was found for invasive tumors, in contrast with other studies. Differences in the treatment of the patients and the different Ab and scoring systems used might explain these differences. Esrig and coworkers (47) showed that p53 overexpression is a significant predictor of tumor progression if the disease was confined to the bladder. However, no association between p53 overexpression and tumor progression was found if the disease is not confined to the bladder (pT3b, pT4). Because of the relatively small number in our study, we can not compare these two groups. The results of our study show that p53 overexpression has no additional prognostic value over stage, whereas for this same group of patients we found that E-cadherin may have

additional prognostic value over stage (18). Apparently, other mechanisms can lead to tumor progression and they may override or bypass the function of p53.

The Kaplan Meier curves show the same result for p53 overexpression and p53 mutation (233) when the whole group and the invasive tumors are studied. For superficial tumors, however, three patients showed p53 overexpression without a mutation confirmed by SSCP analysis. Although this was not statistically significant, a trend was observed toward worse survival for the patients showing p53 overexpression. This was not observed when p53 mutations were analyzed, because we found no mutations at all in the group of superficial tumors. In high grade pT1 tumors, p53 overexpression had predictive value for progression of disease (181). These results, combined with the correlation of p53 overexpression with increasing grade and stage as shown here, imply that p53 overexpression plays a role in the progression of bladder cancer.

Analysis of p53 mutations at the molecular genetic level is rather difficult and time consuming and therefore is not suitable for routine use. IHC, however, is a standard technique in pathology laboratories. There is a good concordance between p53 mutation and p53 overexpression in relation to grade, stage and survival, so IHC detection of p53 alterations is preferable as a predictor of prognosis. Furthermore, immunohistochemistry might reveal anomalies in the p53 pathway other than p53 mutations (see below).

A good concordance between p53 mutation by SSCP and p53 overexpression by IHC ($p < 0.02$) was observed. This strong correlation between high levels of p53 protein and mutation in the p53 gene has previously been described for a number of tumor types, and this led to the hypothesis that mutant p53 gene products are characterized by conformational changes of the protein, resulting in a higher stability and consequently in accumulation of the protein. However, we have observed discrepancies between p53 mutation and p53 overexpression.

Of the eight tumors with a p53 mutation as assessed by SSCP, two tumors showed no p53 immunoreactivity at all. In one tumor sequence analysis showed a transversion in codon 166 of exon 5 generating a stop codon and thereby a truncated protein, which does not contain the nuclear localization domain at amino acids 316 to 325 (238). In the other tumor a deletion of a guanine nucleotide found in codon 282 of exon 8 gave rise to a frameshift, which also affected the downstream nuclear localization sequence. In both cases, the loss of the nuclear localization signal most likely prevented nuclear accumulation, and therefore IHC detection was impossible.

In addition to these "false negatives", which can still be explained by the proposed theory of extended stabilization, we observed p53 overexpression in 23% of the tumors without any sign of p53 mutation as assessed by SSCP. This p53 overexpression without a concomitant mutation was also found in a considerable number of bladder tumors in two other studies (34,46). Although we studied exons 5 to 8, which are known to contain the majority of the p53 mutations (169) mutations outside this region and intron mutations might explain this result. Moreover, although SSCP is a sensitive method for detecting mutations (31), not all mutations are detected by this technique. The presence of nonneoplastic tissue, although reduced to $\leq 30\%$, can lead to a negative SSCP result if the percentage of tumor cells overexpressing p53 is approximately 10%. This cannot be the explanation for p53 overexpression in the 23% of the tumors without a SSCP proven p53 mutation, because most of these tumors contained at least 20% of p53 overexpressing tumor cells.

All off the mechanisms leading to p53 overexpression are not yet fully understood. According to the already mentioned hypothesis, mutant p53 would always be stable and wild type p53 would be unstable. Thus the tight correlation between mutation and overexpression could indicate a causal relationship. However, this is not always true. In fibroblasts obtained from Li-Fraumeni patients who carried heterozygote germ-line p53 mutations, the mutant p53 protein did not accumulate to a high level and was unstable like the wild type p53 (112). By contrast, in the tumors of these patients, when the wild type allele was lost, the mutant p53 protein did accumulate. Clearly, p53 mutation per se does not cause p53 protein accumulation.

Alternatively, wild type p53 can accumulate in some circumstances, e.g., as a result of complexing with viral oncoproteins (110,124,184) and also with the cellular oncoprotein mdm2 (148). Accumulation of wild type p53 in normal tissue was shown to occur in a novel cancer family syndrome (7). One can hypothesize that anomalies elsewhere in the p53 pathway can result in stabilization of p53 proteins as well as in the ability to ignore its growth-suppressive commands.

We found p53 overexpression in 23 % of the cases without any sign of a mutation, which is in concordance with two other studies (34,46), so we suspect that some other event(s) in addition to mutation plays a role in stabilization. The viral oncoproteins are not relevant for the human system. The mdm2 oncoprotein has been implicated in the progression of bladder cancer (119). Whether mdm2 overexpression represents an

alternative to p53 mutation in inactivating the p53 regulatory pathway is still unclear. Events other than mutation that lead to stability remain to be elucidated. It is noteworthy that p53 can accumulate in normal cells upon DNA damage. Thus some tumor cells behave as if they were in a permanent state of DNA damage (112). Additional research is necessary to characterize the signals that trigger this state and to understand the mechanisms through which this overexpression reflects alteration of p53 function and consequently gives rise to an altered phenotype in cancer cells.

Chapter 3

**P53 mutations have no additional prognostic value
over stage in bladder cancer.**

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Abstract

Evidence is accumulating that the tumor suppressor gene p53 is involved in the development of bladder cancer. Therefore we studied p53 mutations in 47 bladder cancers obtained from 45 patients using polymerase chain reaction/ single-strand conformation polymorphism (PCR-SSCP) analysis. Eight out of 24 invasive tumors appeared to have a p53 mutation while no p53 mutations were found in the superficial tumors. All the p53 mutations were found in grade 3 tumors.

The tumors with altered p53 showed a higher frequency of allelic loss (FAL) compared to the tumors without a mutation (55.8% versus 21.1%, $p < 0.05$, by Chi-square test). This increase in FAL suggests a correlation between p53 mutations and genetic instability.

A significant correlation between mutated p53 and poor survival in the whole group studied was found ($p < 0.001$, by log rank test). However, within the group of muscle-invasive tumors the occurrence of p53 mutations had no additional prognostic value. Therefore, even though p53 mutations were found in aggressive tumors, the clinical usefulness of its detection seems limited.

Nevertheless, these results implicate that p53 is involved in the clinical behavior of bladder cancer, and especially its role in the progression of superficial cancer to invasive disease needs further attention.

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Introduction

Bladder cancer is the fifth most common cancer in the western male population, with an annual incidence of 20 cases per 100,000. The incidence in women is lower, about 5 cases per 100,000 are diagnosed annually (173). Transitional cell carcinoma of the bladder is divided in two groups: A) superficial (pTis, pTa, pT1) and B) muscle invasive (pT2, pT3, pT4) disease. Most superficial bladder cancers have a good prognosis, however 10-25% clinically progress to a more aggressive state, showing an increase in grade and/or infiltration into the muscle layer. pT1 tumors invade the lamina propria and have a higher incidence of progression than the pTa tumors, which are confined to the urothelium. Patients with invasive tumors usually present *de novo* and have a worse prognosis. Non random chromosomal changes were observed in bladder cancer, for example, in cytogenetic studies monosomy of chromosome 9 has been reported (70,88,202,231). Using restriction fragment length polymorphism analysis (RFLP), allelic loss of chromosomes 9, 11 and 17 in bladder cancer has been demonstrated (221). Abnormalities of chromosome 11p appeared to be more frequent in invasive- than in superficial tumors and monosomy of chromosome 9 was not correlated with grade or stage. Loss of heterozygosity (LOH) of chromosome 17p occurred only in high-grade (G3) tumors (159). The p53 gene is considered the candidate tumor suppressor gene on chromosome 17p, since in cancer development, frequently one of the two alleles is lost and the remaining is mutated (6,87). Recently, it has been suggested that p53 acts as a cell cycle control protein at the level of G1 to S phase transition (99,129,253). The loss of this function may result in an increased genetic instability (253). In bladder cancer it has been shown by means of subcloning and sequencing of exon 5 through 9 that 17p allelic loss is strongly associated with p53 mutation (9 out of 10 cases) and that out of 18 invasive tumors 61% have a p53 mutation (199). This association of p53 mutations and invasive tumors was confirmed in another study by using the technique of PCR-SSCP (64).

We studied p53 mutations in a group of 45 patients with bladder cancer, using PCR-SSCP analysis. Besides a correlation with grade and stage we investigated the prognostic significance of p53 mutations. Since p53 mutations are thought to be associated with genetic instability (99,129,253) we also studied the correlation of p53 mutations and genetic instability evaluated by the frequency of allelic loss.

Materials and methods

Tumor specimens

Twenty-three snap frozen superficial carcinomas (pTa-pT1) and 24 muscle invasive carcinomas (pT $\geq$ 2) obtained from 45 patients have been analyzed. Superficial tumors comprised 2 recurrences of previously analyzed tumors. Among the invasive tumors 2 were squamous cell carcinomas. All the other tumors were transitional cell carcinomas. Pathological and clinical data for the patients are summarized in Table I. Genomic DNA was extracted from step-sectioned tumors (>70% tumor cells) (144).

PCR-SSCP and sequence analysis

PCR-SSCP and sequence analysis were performed as described in chapter 2.

Restriction fragment length polymorphism

DNA probes used : chromosome 9q: EFD 126 (152), chromosome 11p: H-ras (172), chromosome 16q: pV962 (135) and 79.2.23 (19), chromosome 17p: 144D6 (192), chromosome 18q: 15.65 (50). RFLP analysis was performed on the 24 patients from whom normal DNA was available (168). The RFLP results were used to determine the frequency of allelic loss (FAL), also called fractional allelic loss (236).

Statistical analysis

The Kaplan-Meier method was used to estimate survival probability as a function of time. Differences in survival were analyzed by a Log-rank test. Chi-square test was used for the other correlations.

Table 1. p53 Mutations, clinical and pathological data for each patient.

Case no	Age (yr)	Stage/Grade	p53 Mutations		Survival (months)	Treatment ^A
			Exon/Codon	Amino acid change		
1	77	a/1-2	(p)		31	TURT
2	75	a/1			>41	TURT
3	72	a/1			>38	TURT
4	68	a/1			>41	TURT,C
5	66	a/3			23	TURT,Ct,R,CH
6	66	a/2			>57	TURT,Ct
7	49	a/2			>45	TURT,BCG,Ct
7 rec.		a/2				
8	62	a/1			>64	TURT
9	53	a/1			>61	TURT,BCG
10	42	a/1			>65	TURT,BCG,Ch
11	88	a/2			52	TURT,Ch
12	44	a-1/2			>39	TURT
13	65	a-1/1			>22	TURT
14	68	1/3			>55	TURT,BCG
15	86	1/2			>58	TURT,Ch
16	76	1/2			9	TURT,Ch
17	53	1/2			>37	TURT,BCG
18	64	1/1			>55	TURT
19	85	1/2	(p)		>45	TURT,Ch
19 rec		a/3	(p)			
20	71	1/2	(p)		>36	TURT,Ch
21	68	1/2			>36	TURT,Ch,BCG
22	81	2/2			9	TURT
23	73	2/2			8	TURT,Ch,Ct
24	49	2/3			>36	TURT,R,Iridium
25	79	2/3	5/158	CGC → CTC (<i>Arg</i> → <i>Leu</i>)	4	Ct
26	81	2/2			6	TURT
27	53	2/3			7	TURT,Ch
28	70	2/3			3	TURT,Ch
29	47	2/3	8/282	del. G → frameshift	8	TURT,Ch
30	62	≥2/3			>27	TURT,Ch,R
31	65	≥2/3	5/179	CAT → TAT (<i>His</i> → <i>Tyr</i>)	>66	TURT,Ct
32	83	≥2/3			21	TURT,R
33	79	≥2/3			10	TURT,Ct
34	59	≥2/3	7/259	GAC → GTC (<i>Asp</i> → <i>Val</i>)	3	TURT,R
35	73	2-3/3	8/285	GAG → AAG (<i>Glu</i> → <i>Lys</i>)	2	TURT,R
36	73	2-3/3			9	TURT
37	76	2-3/2,SCC			32	TURT,Ct,R
38	68	2-3/3	6/215	AGT → GGT (<i>Ser</i> → <i>Gly</i>)	2	TURT
39	63	3/3			5	TURT,R
40	74	3/3	8/285	GAG / AAG (<i>Glu</i> → <i>Lys</i>)	10	Ct,Ch
41	50	3/2,SCC			5	TURT
42	70	3b/2			>28	Ct
43	75	3b/3			>40	TURT,R,Ct
44	44	4/3	5/166	TCA → TGA (<i>Ser</i> → <i>umber</i>)	29	TURT,Ct
45	82	4/2			5	TURT

SCC, squamous cell carcinoma; (p), polymorphism codon 213; rec., recurrence,

^A Ch, chemotherapy; Ct, cystectomy, R, radiotherapy; TURT, transurethral resection of the tumor, BCG, bacillus calmette-guerin instillations.

Results

p53 mutations in bladder cancer

Table 1 summarizes the results of the PCR-SSCP and sequence analysis of the p53 gene in bladder tumors of 45 patients. Eight mutations were found in invasive tumors: three in exon 5, one in exon 6, one in exon 7 and three in exon 8. Seven out of the eight mutations found were point mutations, while one appeared to be a deletion of a G, leading to a frameshift. We found four transitions: two G to A, one A to G, one C to T and three transversion: one C to G, one G to T and one A to T. In three patients with a superficial tumor a similar shift in the SSCP pattern of exon 6 was observed. One of these also showed loss of heterozygosity of chromosome 17p. After sequencing it appeared that the shift in the SSCP pattern was due to a silent alteration of CGA to CGG at codon 213. This polymorphism was already described before (139). No mutations were found in the squamous cell carcinomas.

Correlation between tumor stage/grade and p53 mutations

All the p53 mutations are found in grade 3 tumors, as is shown in Table 2a ($p < 0.001$). In the group of superficial tumors no mutations have been found, while the invasive tumors have in 8 out of 24 (33%) cases a p53 mutation ($p < 0.001$) (Table 2b). This is in good agreement with previous observations (64,199).

Table 2a. Relationship between p53 mutation and grade.

Grade	p53 mutation		% mutations
	negative	positive	
1	11	0	0
2	15	0	0
3	11	8	42

Table 2b. Relationship between p53 mutation and stage.

Stage	p53 mutation		% mutations
	negative	positive	
Superficial	21	0	0
Invasive	16	8	33

Table 3a. Allelic loss of chromosomes 9q,11p,16q,17p,18q and p53 mutations in bladder tumors.

Case no.	Stage/Grade	p53 Mutation	RFLP analysis Chromosomal region				
			9q	11p	16q	17p	18q
1	a/1	-	●	○	●	○	○
2	a/1	-	○	○	○	○	NI
3	a/1	-(p)	○	○	○	○	○
4	a/1	-	○	○	○	○	NI
5	a/1	-	○	NI	○	NI	○
6	a/1-2	-	○	○	○	○	○
7	a/1-2	-	●	●	○	○	○
7 rec.	a/1-2	-	●	●	○	○	○
8	1/2	-(p)	●	○	○	●	ND
8 rec.	a/3	-(p)	●	○	○	●	●
9	a/2	-	○	NI	○	●	●
10	a/2-3	-	NI	○	○	○	NI
11	1/2	-(p)	●	○	○	○	NI
12	1/2	-	●	●	○	○	NI
13	1/2	-	○	○	○	○	NI
14	1/2	-	○	○	○	○	○
15	1/2	-	NI	●	○	○	●
16	1-2/1-2	-	○	NI	○	○	○
17	2/3	-	○	○	●	○	NI
18	2/3	+	NI	NI	○	●	○
19	> 2/3	-	NI	NI	○	○	NI
20	> 2/3	+	●	○	○	●	○
21	3/3	-	●	○	○	NI	ND
22	3/3	-	NI	○	○	○	ND
23	3/3	+	NI	NI	●	●	ND
24	4/3	+	●	NI	○	●	○

-, no p53 mutation; +, p53 mutation; ○, no LOH; ●, LOH; NI, not informative; ND, not determined; rec., recurrent tumor; (p), polymorphism at codon 213.

Table 3b. Frequency of allelic loss according to p53 mutation.

p53 Mutation	Number of tumors	FAL%
negative	22	21.1
positive	4	55.8

Relation between p53 mutations and frequency of allelic loss

As a measure for genetic instability we used frequency of allelic loss (FAL). FAL was based on RFLP analysis using probes for chromosomes 9q, 11p, 16q, 17p and 18q

(Table 3a). LOH of 17p was found in 7 tumors. Four tumors showed a p53 mutation, while in two a polymorphism at codon 213 was found. No mutations were found in the tumors without 17p LOH. The tumors with a p53 mutation show a FAL of 55.8%, those without a mutation have a FAL of 21.1% ($p < 0.05$) (Table 3b).

Correlation of p53 mutation and survival

The survival according to p53 mutation for the whole group of 45 patients is shown in the Kaplan-Meier curve (Figure 1a). The patients with a p53 mutation survived for a shorter period of time ($\chi^2 = 11.25$, $p < 0.001$). There was no significant association between the presence of a p53 mutation and decreased survival among patients with invasive disease ($\chi^2 = 1.46$, n.s.) (Figure 1b).

Figure 1. Three year survival (%) of bladder cancer patients according to the presence or absence of p53 mutations. A. All patients ($p < 0.001$ by log rank test) B. Patients with muscle invasive tumors ($p =$ not significant)

Discussion

In this study, we examined mutations in the p53 gene by PCR-SSCP analysis. We show that the occurrence of p53 mutations correlates with grade and stage, which is in concordance with previous studies (64,199). A p53 mutation was found in 8/24 (33%) of the invasive tumors, which were all grade 3.

The p53 tumor suppressor gene is known to be mutated in many types of cancer (87) and during tumor progression one of the two alleles is often lost resulting in a reduction of growth control (6). However, some p53 mutations are known to be dominant negative: the protein produced by the mutated allele has the ability to bind and inactivate the remaining wild-type product (237). In our study we did not find any mutation without LOH of 17p. On the contrary one patient with 17p LOH has no p53 mutation. It could be that the mutation is outside the region of the p53 gene studied or that a second locus distinct from p53 is involved (188).

Wild-type p53 has been suggested to be a cell cycle control protein since progression from G1 to S phase is often blocked in cells expressing high levels of this protein (99,129,253). It has been shown that cells without wild-type p53 protein failed to show growth arrest (when placed in a condition unfavorable for the S-phase completion) and gene amplification occurs (253), which can be considered as a form of genetic instability. As p53 mutations are known to occur in bladder cancer (64,199), we used frequency of allelic loss as an indicator for genetic instability (236). The high frequency of allelic loss found in tumors with a p53 mutation (55.8%, $p<0.05$) suggests a correlation with genetic instability. This increase in allelic loss in tumors with a p53 mutation could be explained by an altered G1-S cell cycle checkpoint which can be the result of the loss of the wild-type p53 function. The loss of certain alleles, e.g. those harboring tumor suppressor genes, could provide selective advantages during tumor progression by generating variants with a more aggressive phenotype (52). FAL showed no significant correlation with grade or stage.

In breast cancer p53 mutations are inversely correlated with survival (2). This study is the first report investigating the prognostic significance of p53 mutations in bladder cancer. The results demonstrate that p53 mutations are an unfavorable prognostic factor ($p<0.001$) for the whole group studied. However, among patients with invasive tumors there was no significant association between the presence of a p53 mutation and a

decreased survival. Therefore patients without a p53 mutation are not ensured to have a good prognosis. There are other features that can lead to an inactive p53 protein: such as complexing with other proteins (148), or other genetic events can occur that result in a poor prognosis. EGFR positivity is shown to be associated with tumor progression and decreased survival (154). Also no significant difference between EGFR positivity and survival among patients with invasive tumors was found. Furthermore, recently it has been shown that decreased E-cadherin expression correlates with the clinical aggressiveness of bladder cancer (18).

The variable order of appearance of genetic alterations in oncogenesis suggests that rather accumulation, than the order of occurrence, is important for tumor progression (52). The increased frequency of allelic loss we find in the tumors with a p53 mutation can lead to tumor progression and poorer prognosis. But as mentioned above there are other mechanisms which can lead to tumor progression and these may overrule or bypass the function of p53.

Recently immunohistochemical studies have shown p53 overexpression to be correlated with poor survival (126,181). In pT1 bladder tumors Sarkis et al. found a clear correlation between nuclear overexpression of p53 protein and disease progression. The study of Lipponen showed a significant correlation between p53 overexpression and decreased survival for the entire cohort and for the muscle-invasive tumors. However for the pTa and pT1 tumors only a trend was found. In muscle-invasive tumors, we found no association between p53 mutations and decreased survival. This difference stresses again the discordance of p53 immunohistochemistry and p53 mutation analysis (214). The discrepancy in prognostic value for p53 mutations in superficial disease found in the two immunohistochemical studies, can be the result of the use of different antibodies and the scoring system used. We found no p53 mutations in superficial tumors, which can be explained by the lack of grade 3 tumors in this group.

The results of our study indicate that the presence of a p53 mutation is an unfavorable prognostic factor for the whole group studied, but it has no additional prognostic value for the group of muscle invasive tumors. The step at which p53 mutations occur in the tumor progression cascade of bladder cancer is still unclear. In this respect grade 3 superficial tumors deserve more attention. In general superficial bladder cancer has a good prognosis, however, 10-25% of these tumors progress to an invasive stage.

Therefore the correlation between prognosis and p53 mutation in this subgroup is of particular interest.

Furthermore the discordance of immunohistochemical and p53 mutation analysis indicate that comparative analysis of p53 mutations and overexpression of the p53 oncoprotein in progression of the pTa and pT1 tumors are necessary.

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Chapter 4

**Predictive value of p53 mutations analyzed in bladder washings
for progression of high risk superficial bladder cancer.**

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Abstract

To assess the value of p53 mutations in predicting the progression of superficial bladder cancer (TCC) and to define exactly when p53 mutations occur in the process of tumor progression, 80 consecutive bladder washings from 26 high risk (indicated by quantitative karyometric analysis) superficial TCC patients were examined by Single Strand Conformation Polymorphism (SSCP). Six out of 13 patients who experienced clinical progression (to pT $\geq$ 2) were found to have a p53 mutation in one or more of their bladder washings. In the control group (no progression to invasive disease) only 1 out of 13 patients had a p53 mutation. For these high risk superficial TCC patients the occurrence of a p53 mutation has a positive predictive value of 86% for the progression of disease. A negative predictive value of 63% was observed. Moreover, since p53 mutations were found in samples prior to progression (with a mean of 8 months) it could identify patients who need a change in the treatment strategy in order to prevent progression to invasive disease. In spite of these promising results, it is obvious that to increase not only the positive predictive value but especially the negative predictive value of this procedure to predict progression, additional prognostic markers are still needed.

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Introduction

Transitional cell carcinoma (TCC) is the most common histologic type of bladder cancer, presenting with a wide spectrum of clinical and pathological appearances. Superficial TCC accounts for approximately 80% of all newly diagnosed tumors in the bladder (204). Thirty to 90% of these tumors will recur depending on stage, grade, multifocality and treatment modality, however, only 10-25% become invasive or metastasize during the course of the disease (74,102). Early identification of patients whose superficial bladder tumors will progress is very important, since a change in their treatment strategy can have a major impact on the outcome of disease. The morbidity of cystectomy, which at the moment is the only treatment to prevent progression to invasive disease, emphasizes the urgent need for markers that identify patients at risk for tumor progression.

The p53 tumor suppressor gene, located on chromosome 17p13.1, is known to be involved in the development of bladder cancer. Allelic deletions of 17p were predominantly observed in invasive tumors and to a lesser extent in high grade superficial TCC, but were absent in low grade Ta tumors (36,77,159). Mutations in the p53 tumor suppressor gene frequently occur in bladder cancer and appear to correlate with grade and stage (64,199,233). The relatively high frequency of p53 mutations in T2-T4 tumors compared to T1 tumors suggests their involvement in the progression of T1 tumors to invasive disease (207). However, the exact point at which p53 mutations occur during tumor progression from superficial (Ta) tumors to lamina propria invasive (T1) and muscle invasive (T2-T4) tumors is still unclear.

The tumor suppressor protein p53 appears to play a role as transcriptional regulator (54,134) and acts as a cell cycle control protein at the level of G1 to S phase transition (99,129). In case of DNA damage, p53 can delay the progression through the cell cycle, to permit DNA repair (134) or can initiate programmed cell death (apoptosis) (132). The upregulation by p53 of p21, an inhibitor of G1 cyclin-dependent kinases, appears to be responsible for p53 mediated growth arrest (94). Recently, Smith and coworkers showed that Gadd45 which is also upregulated by p53, probably serves as a link between the p53-dependent cell cycle checkpoint and DNA repair (203). The loss of these p53 regulatory functions can result in replication of incorrect DNA, leading to increased genetic instability (253).

From a large prospective study in which approximately 3000 bladder washings were investigated by quantitative karyometric analysis (228) we selected 26 patients who were indicated to be high risk (i.e. suspected for recurrences) on basis of this analysis. We studied the value of p53 mutations as a additional prognostic marker in this group of high risk superficial TCC patients, using PCR-SSCP analysis. In order to define when p53 mutations occur during natural history of these tumors we analyzed consecutive bladder washings from the same patients.

Materials and methods

Specimens

Eighty consecutive bladder washing specimens from 13 patients with high risk superficial TCC who underwent clinical progression to invasive disease ($pT \geq 2$) and 13 patients (age/sex/follow-up matched) who did not experience progression were analyzed. As a control group 35 bladder washings from 11 patients with a low risk karyometric score and no progression of disease were analyzed. The bladder washings were obtained from a large prospective study in which bladder washings of patients were collected for quantitative karyometric analysis (228). If patients were diagnosed with superficial TCC, bladder washings were collected and analyzed with three to twelve months intervals. All patients were designated as high risk superficial TCC on basis of quantitative karyometric analysis (228) and intermediate or high risk by calculating their prognostic index score based on tumor stage, multicentricity, recurrence rate and number of bladder areas involved (101). All patients received optimal intravesical therapy (e.g. Bacillus Calmette-Guerin instillations) as soon as they were considered to have a high risk superficial bladder tumor. Clinical and pathological data for the high risk patients are summarized in Table 1.

The bladder was washed with 50cc saline that was immediately mixed with an equal volume Carbowax (50% ethanol, 2% polyethyleneglycol (PEG 1500)) for fixation. After centrifugation, the supernatant was decanted and the pellet resuspended in 3 ml of Carbowax and stored at -20°C .

For DNA isolation 150 μl of bladder washing material was centrifuged for 5 minutes at 13000 rpm. After decanting the supernatant, the pellet was washed two times with a

mixture of acetone/methanol (1:1). Subsequently, the cells were lysed in 50 µl lysis buffer (10 mM TrisHCl pH 8.8, 400 mM NaCl, 2 mM EDTA, 0.45% Tween 20[™] and 0.45% Nonidet P40) with 0.5 µg/µl proteinase K, for 1 hour at 60°C. Proteinase K was inactivated by boiling the sample for 5 minutes.

PCR-SSCP

PCR-SSCP analysis was performed to investigate p53 mutations in exons 5 through 9 (160). Exon 8 and 9 were amplified together. The intron primers for amplification were 5S (5'-tca-ctt-gtg-ccc-tga-ctt-3'), 5AS (5'-gag-gaa-tca-gag-gcc-tgg-3'), 6S (5'-gag-acg-aca-ggg-ctg-gtt-3'), 6AS (5'-gag-acc-cca-gtt-gca-aac-3'), 7S (5'-cca-agg-cgc-act-ggc-ctc-3'), 7AS (5'-gcg-gca-agc-aga-ggc-tgg-3'), 8/9S (5'-cct-tac-tgc-ctc-ttg-ctt-c-3'), 8/9AS (5'-aaa-ctt-tcc-act-tga-taa-3').

A 2 µl aliquot of bladder washing DNA solution was subjected to 40 cycles of PCR, 0.5 minutes at 95°C, 2 minutes at 55°C and 1.3 minutes at 72°C.

Exons 5, 6, 8/9 were amplified in 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.75 mM MgCl₂, 250 µM deoxynucleotide triphosphates, 10 pmol of each primer, 3 µCi α³²P-dATP and 1.5 units of Taq polymerase (Perkin Elmer/Cetus, Norwalk, Connecticut, USA). Exon 7 was amplified in the same buffer containing 1.4 mM magnesium chloride.

Five microliters of the PCR product were diluted in 15 µl of loading buffer (96% formamide, 20 mM EDTA, 0.05% bromophenol blue and xylene cyanol), boiled for 3 minutes and then quenched (10 min) on ice before loading (2 µl per lane). Each sample was applied to a 5% polyacrylamide/Tris-borate EDTA (0.5x) gel with 10% (v/v) glycerol. Subsequently, electrophoresis was performed at room temperature for 16 hours at 6W (exon 8/9) and 5W (exon 5, 6, 7) respectively.

Sequence analysis

Sequencing of the double stranded PCR products that showed a mobility shift on the SSCP gel was performed as previously described (109). Direct sequencing of the amplified product was performed in cases where the intensity of the mutant DNA fragment was ≥50% of the wild-type DNA fragment. Whenever the intensity of the shifted DNA fragment was ≤50%, we excised the mutant fragment from the polyacrylamide gel and the gel piece was immersed in 100 µl of a 20 mM Tris (pH 8.0)

1 mM EDTA solution and heated at 80°C for 15 minutes. The extract was briefly vortexed and incubated overnight at 4°C. After brief centrifugation 10 µl of the supernatant was subjected to PCR reamplification for 45 cycles.

Before sequencing, the amplified PCR products were subjected to electrophoresis on an 2% low-melt agarose gel and further purified with the Magic Preps[™] DNA purification system (Promega, Madison, WI, USA). For sequencing in the AmpliCycle[™] system (Perkin Elmer/Cetus, Norwalk, Connecticut, USA) we used internal primers: 5S (5'-tgt-ctc-ctt-cct-ctt-cct-ac-3'), 6S (5'-ggg-ccc-cag-gcc-tct-gat-tc-3'), 7S (5'-tct-tgg-gcc-tgt-gtt-gtc-tc-3'), 8S (5'-ttg-ctt-ctc-ttt-lcc-tat-cc-3'), 9S (5'-cac-ctt-tcc-ttg-cct-ctt-tc-3'). Electrophoresis was performed on a 6% polyacrylamide (19:1) gel containing 7M urea.

Table 1. Clinical and pathological[^] data correlating with bladder washings of high risk superficial bladder cancer patients, respectively with and without progression of disease.

	Progression (n)	No progression (n)
Stage		
Ta	6	5
Ta + CIS	-	1
T1	3	4
T1 + CIS	2	2
CIS	2	1
Grade		
1	1	-
2	4	6
3	6	6
Age (mean in years)	69.1	65
Sex ratio (M/F)	11/2	11/2
follow-up		
(mean in months)	50.5	53.9
(range in months)	(13-91)	(31-83)

[^] Pathological data at the moment of first quanticyt sample, in case no pathological data are present previous diagnosis is taken into account, M, male, F, female

Results

SSCP analysis

PCR-SSCP analysis of exons 5 through 9 was performed on DNA isolated from bladder washings. Figure 1A shows in lane 5 and 6, next to the wild type DNA fragments corresponding to exon 5, a clear mobility shift. The intensity of the wild type DNA fragments compared to the mutated fragment could most likely be explained by the presence of normal cells (wild type p53 phenotype) in the bladder washing. Figure 1B shows in lane 6, a clear mobility shift of the DNA fragment corresponding to exons 8/9 that were amplified together. The intensity of the mutated DNA fragment implies that in most cells in this bladder washing the p53 gene is mutated.

Figure 1A. PCR-SSCP analysis of exon 6 in bladder washings of patients with superficial TCC. Mobility shifts are observed in lanes 5 and 6.

Figure 1B. PCR-SSCP analysis of exon 8/9 in bladder washings of patients with superficial TCC. A mobility shift is observed in lane 6.

Figure 1C shows the sequence analysis result of the antisense strand of exon 6 (case #3); a transition from a T to a C is shown at codon 205 resulting in a change of a tyrosine to a lysine. Mobility shifts were observed in eight patients (Table 2). Mutations were found once in exon 5, two times in exon 6 and four times in exon 8. In one patient (case #1) two different mutations were detected in the consecutive bladder washings analyzed. A different mobility shift was observed by SSCP-analysis, in concordance with the different nucleotide change. We found four transitions; twice a G to A, once a A to G

and three transversions; a T to G and twice a G to C. In case #5 the known polymorphism in codon 213 of exon 6 (139) was detected. The identified point mutation, i.e. an A to G transversion did not result in an amino acid change (Table 2).

Figure 1C. Sequence analysis of the antisense strand of exon 6 (case #3); a mutation from T to C is shown at codon 205 resulting in a change of a tyrosine to a lysine.

Correlation p53 mutation and progression of disease

To assess the additional prognostic value of p53 mutations for superficial TCC indicated to be high risk on basis of quantitative karyometric analysis, p53 mutations were analyzed in bladder washings of 26 patients (Table 2a and b). p53 Mutations were found in 6 out of 13 patients (46%) with progressive superficial bladder cancer (Table 2a). One out of 13 patients (7.7%) with no progression of disease (23 month follow up) (Table 2b) showed a p53 mutation. The positive predictive value of p53 mutations for progression of high risk superficial TCC was 86% (6/7). The observed negative predictive value was 63% (12/19). To assess when p53 mutations occur during the process of tumor progression, consecutive bladder washings from the same patient were analyzed.

Table 2. p53 Mutations analyzed (PCR-SSCP) in consecutive bladder washings of patients with superficial bladder cancer.

A. Patients with progression of disease (high risk)

Case no.	Year ^A	Stage/Grade ^B	p53 Mutation	Amino acid change
1	90	a/1	exon 8	codon 285 / GAG → AAG / <i>Glu</i> → <i>Lys</i>
	92	2/3	exon 8	codon 281 / GAC → CAC / <i>Asp</i> → <i>His</i>
2	92	1/3	exon 5	nd
	93	1/2b, CIS	normal	
	93	2/2	nd	
3	90	a/2	normal	
	91	a/2	normal	
	91	1/2b	normal	
	91	1/3, CIS	exon 6	codon 205 / TAT → TGT / <i>Tyr</i> → <i>Lys</i>
	92	4/3	exon 6	codon 205 / TAT → TGT / <i>Tyr</i> → <i>Lys</i>
4	93	1/3, CIS	exon 8	codon 273 / CGT → CAT / <i>Arg</i> → <i>His</i>
	94	> 2/3	exon 8	codon 273 / CGT → CAT / <i>Arg</i> → <i>His</i>
5	90	CIS	exon 6 ^C	codon 213 / CGA → CGG / <i>Arg</i> → <i>Arg</i>
	91	NR	exon 6	nd
	92	atypia	exon 6	codon 213 / CGA → CGG / <i>Arg</i> → <i>Arg</i>
	92	4a/2-3	exon 6	codon 213 / CGA → CGG / <i>Arg</i> → <i>Arg</i>
6	91	CIS	exon 6	codon 194 / CTT → CGT / <i>Leu</i> → <i>Arg</i>
	91	2/3	normal ^D	
7	92	NR	normal	
	93	NR	normal	
	94	CIS	normal	
	94	1/3, CIS	normal	
	94	4a/3	nd	
8	91	NR	normal	
	92	1/3	normal	
	92	2/3	normal	
9	93	1/3, CIS	normal	
	93	NR	normal	
	94	CIS?	exon 8	nd
	94	CIS?	exon 8	codon 280 / AGA → AAA / <i>Arg</i> → <i>Lys</i>
	94	NR	normal	
	94	2/3	normal	
10	92	NR	normal	
	94	NR	normal	
	94	≥2	normal	
11	91	a/2	normal	
	93	sq metaplasia	nd	
	93	(CT CIS + positive lymph nodes)		
12	94	1/3	normal	
	95	4/3	normal	
13	92	a/2	normal	
	94	2/3	nd	

^A year of bladder wash sampling, ^B of concomitant tumors, ^C known polymorphism (139),^D not representative sample; CT, cystectomy; NR, no abnormalities seen by cytосcopy,

nd, not determined, sq, squamous; CIS?, suspicion for presence of CIS

Table 2.

B Patients without progression of disease (high risk).

Case no	Year ^A	Stage/Grade ^B	p53 Mutation	Amino acid change
14	91	1/3	normal	codon 280 / AGA → ACA / <i>Arg</i> → <i>Thr</i> codon 280 / AGA → ACA / <i>Arg</i> → <i>Thr</i>
	92	NR	normal	
	93	NR	exon 8	
	94	a/3, CIS	exon 8	
	94	NR	normal	
15	91	recurrences	normal	
	91	recurrences	normal	
	92	a/1	normal	
	94	papillary multiple	normal	
16	90	a/2b	normal	
	91	a/3, CIS	normal	
	92	1/3	normal	
17	90	a/2a	normal	
	91	1/3	normal	
	93	a/2b	normal	
	94	a/2a, CIS	normal	
	94	papillary multiple	normal	
18	90	a/2b	normal	
	91	NR	normal	
	93	1/2, CIS	normal	
19	91	a/2a	normal	
	93	NR	normal	
20	90	1/2b	normal	
	91	1/2b	normal	
21	91	1/2b, CIS	normal	
	92	NR	normal	
	94	a/3	normal	
22	92	1/2, CIS	normal	
	92	1/2, CIS	normal	
23	92	a/3, CIS	normal	
	93	NR	normal	
	93	NR	normal	
24	92	NR	normal	
	92	tumor	normal	
	93	NR	normal	
	94	NR	normal	
25	92	NR	normal	
	94	NR	normal	
	94	papillary multiple	normal	
26	92	NR	normal	
	93	NR	normal	
	94	NR	normal	
	94	papillary lesion	normal	

^A year of bladder wash sampling, ^B of concomitant tumors sample,
NR, no abnormalities seen by cytосcopy

The occurrence of a p53 mutation in the course the disease in individual patients (n=7) is summarized in Figure 2. Out of these 7 patients, 6 showed progression. It is noteworthy that the p53 mutations were detected in bladder washings with a mean of 8 months (range 4-14) before progression to invasive disease. From two patients (case #2 and #4) no bladder washings were available from the first period of disease (resp., 58 and 6 months). The first analyzed sample showed in both cases a mutation, followed by progression to invasive disease after respectively 9 and 5 months. In two patients (case #3 and #9) first the wild type genotype was found. Within 12 and 9 months respectively, a mutation was detected followed by the occurrence of an invasive tumor 7 and 9 months later. In two patients (case #1 and #6) mutated p53 was detected at first diagnosis followed by progression to invasive disease after respectively 14 and 4 months. In only one patient (case #15) a p53 mutation was not followed by progression to invasive disease; 2 years after diagnosis the mutated p53 was observed in the bladder washings, but until now (23 months later) no progression to invasive disease has been observed. The histologic data of the tumors resected from the bladder at the moment of the first appearance of a p53 mutation in the bladder washings are shown in Table 2.

Figure 2. Timing p53 mutations in tumor progression cascade. 0 = first diagnosis; ? = no bladder wash available; WT = wild type p53; MUT = mutated p53; INV = occurrence of invasive tumor (pT $\geq$ 2); A = alive; D = dead; time in months.

Discussion

Compared to superficial TCC, invasive disease constitutes an considerably greater threat to the patient's health. Since 10-25% of superficial TCC progress to invasive disease (74,101), prediction of tumor progression is an important issue in bladder cancer management. Multifocal occurrence of bladder tumors suggests a general disease of the urothelium rather than a localized process. In this respect both voided urine and bladder washings have the advantage over histological biopsies in that they reflect the general status of the bladder mucosa since cells from the entire bladder are sampled. In 1945 Papanicolaou had already shown that exfoliation of tumor cells in the urine is a powerful diagnostic tool for detection and grading of patients with TCC of the bladder (164). However, the sensitivity for detecting low grade tumors and the reproducibility of this cytological grading system is disturbingly low (196). Since bladder washings enable the harvest of more and better preserved material compared to voided urine (1,255), van der Poel and coworkers applied quantitative karyometric analysis (2c Deviation Index and nuclear shape) to bladder washings in order to increase both the reproducibility and sensitivity of the cytological analysis (228). The sensitivity for detecting low grade tumors increased substantially by using this analysis and additionally 86% of all tumor recurrences were preceded by a cytoscopically negative sample with positive karyometric score (Van der Poel et al., unpublished data). Notwithstanding this improvement, we attempted to enhance the clinical usefulness of this screening procedure by adding an additional marker. Although mutations in the p53 tumor suppressor gene frequently occur in bladder cancer and correlate with grade and stage (64,199,233,207), until now it has remained unclear exactly when during the tumor progression process p53 mutations occur. We choose to analyze mutations in the p53 gene directly because of the more objective nature of this analysis compared to immunohistochemical studies. Variation between immunohistochemical studies caused by different antibodies and scoring systems (5,233) can make comparison difficult. For superficial bladder cancer a clear prognostic significance for p53 overexpression has been shown (47,181,182,194), however conflicting data were also reported (66,126). Despite the fact that p53 accumulation and mutation of the p53 gene are well correlated, in 20-30% of the cases they appear to be separate events (46,163,234), so there is no obligate causal relationship between mutation and protein overexpression.

Last year Hruban and coworkers showed that in the case report of Hubert H. Humphrey, a p53 mutation was present in a urine-cytology specimen 9 years before an infiltrating carcinoma of the bladder occurred (93). It should be noted that in this study the mutation was first analyzed in the histological tissue and then retrospectively the known mutation was detected in the urine specimen. Although the method used is very sensitive it has limited clinical relevance. In our study we used PCR-SSCP analysis and applied it directly to bladder washings in order to define when p53 mutations take place during the tumor progression process and moreover assess the additional prognostic value of p53 mutation analysis for patients who were indicated to be at high risk for recurrences on basis of their quantitative karyometric analyses (228).

In the present study we found p53 mutations in the samples of all patients prior to progression, with a mean of approximately 8 months (range 4-14). Of two patients (case #2 and #4) however, in the earlier course of their disease, no bladder washings were done. Case #4 was diagnosed 6 months before the first mutation was found so the period between the first p53 mutation and progression could have been at most, 11 months. Case #2 could have had the mutation much longer since we have no bladder washing samples from the first 58 months of disease. In only one patient (case #15) the appearance of a p53 mutation has not yet been followed by progression to invasive disease (23 month follow up). A drawback of analyzing p53 mutations in bladder washings is illustrated by the absence or low number of mutated p53 cells in the bladder washing specimens of case #6 and #9 in the presence of a concomitant invasive tumor. The existence of the p53 mutation in the invasive tumor was confirmed by analyzing the histological material (data not shown). It is obvious that relevant cells are not sampled and a mutation can be missed if a tumor grows into the bladder wall or is covered by normal urothelium.

We observed identical p53 mutations in the consecutive bladder washings of four patients, which is in favor with the theory of the clonal origin of bladder tumors (200). However, there was one exception, case #1, in which we found different p53 mutations in the individual bladder washings, implicating that the tumors resulted from two independent transforming events. These results are in agreement with the observed different p53 gene mutations in CIS or dysplasia and consecutive invasive tumors in two patients in a previous study (207).

Six out of 13 patients that clinically progressed (to $pT \geq 2$) showed a p53 mutation in one or more of their bladder washings. In the control group (no progression) only 1 out of 13 patients showed a p53 mutation. In the group of superficial TCC patients at risk for recurrence, the occurrence of a p53 mutation has a positive predictive value for progression to invasive disease of 86%.

We observed a negative predictive value of 63%. This low negative predictive value implicates that, although a p53 mutation indicates a malignant potential for the tumor, the absence of a p53 mutation does not guarantee a good prognosis, which we have already demonstrated for invasive bladder tumors (233). Other mechanisms as well can lead to tumor progression and these may override or bypass the function of p53. For example, overexpression of the epidermal growth factor receptor (EGFR) in patients with superficial TCC has been shown to correlate with several malignant features and a worse prognosis (127,154,155). E-cadherin is a potential candidate with predictive value for progression of superficial TCC, but its clinical usefulness remains to be assessed (18,162). Inactivation of wild type p53 by complexing with the cellular proto-oncogene MDM2 could also disrupt p53 function (148). It has also been recently shown that MDM2 enhances proliferation by stimulating the S-phase inducing transcription factor E2F/DP1 (136). Both mechanisms could participate in progression of disease. For bladder cancer it has been shown that MDM2 overexpression is strongly correlated with low-stage, low-grade tumors and that a strong association with p53 overexpression exists. The precise clinical relevance of MDM2 overexpression in bladder cancer remains to be proven (119).

In spite of the positive predictive value of 86%, the occurrence of a p53 mutation is not an absolute sign for progression of disease. Whenever a high karyometric score is observed in a bladder washing of a superficial TCC patient this is an indication for both a careful follow up (at least every three months) and p53 mutation analysis. As a control experiment we also analyzed thirty-five bladder washings with a low karyometric score for the presence of a p53 mutation; in this group no p53 mutations were detected (data not shown). The occurrence of all p53 mutations in the group of patients with a high 2cDI value (abnormal DNA content) suggests a link between p53 dysfunction and genomic instability (213,229,233). The combination of a high karyometric score and a p53 mutation identifies patients with a high probability of progression to invasive disease, within a relatively short time period (approximately 8 months). However, 54%

of the high risk patients without a demonstrable p53 mutation progress to invasive disease. Summarizing these data, it is obvious that more prognostic markers are needed to increase not only the positive predictive value but especially the negative predictive value of this screening procedure. Our choice to study p53 mutations by SSCP, does not exclude the usefulness of p53 immunohistochemistry on bladder washings, especially since it is more accessible as a diagnostic tool. We are currently studying the predictive value of p53 overexpression analyzed by immunohistochemistry in bladder washings for progression of superficial TCC.

In conclusion, for a group of superficial TCC patients indicated to be high risk based on karyometry (228), the occurrence of a p53 mutation has positive predictive value for disease progression. Moreover, since p53 mutations were found in samples prior to documented progression their presence could identify patients who need a change in their treatment strategy in order to prevent progression to invasive disease. The clinical usefulness of p53 mutations as a marker with additional predictive value should now be assessed in a large prospective study.

Chapter 5

**Comparative analysis of p53 mutations in bladder washings
and histological specimens.**

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Abstract

Bladder washings have the advantage over histological specimens in that they reflect the general status of the urothelium and since it is a simple outpatient procedure, patients can be monitored more carefully. Mutations in the tumor suppressor gene p53, analyzed in bladder washings, have been reported to be of significant prognostic value for the progression of superficial bladder cancer to invasive disease. To determine the actual value of bladder washing specimens to assess the p53 status of histological specimens, we analyzed bladder washings and the synchronous tumors of 15 patients for the presence of p53 mutations using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP). A significant correlation ($p < 0.05$, Fischer Exact, two-tail test) between the p53 status of bladder washings and histological specimens was observed if these were compared among the specimens of a single patient. Overall, in 2 patients, the mutation present in the tumors was not detected in the bladder washings and in one patient the mutation in the bladder washing was not detected in the histological specimens. These conflicting results between bladder washings and histological specimens could partly be explained by the architecture of the tumors. The observed specificity of 86% and sensitivity of 75% emphasizes that although there is a good correlation between the two methods in a number of cases they are complementary to one another. From these findings we conclude that the analysis of p53 mutations in consecutive bladder washings of a patient does generally reflect the p53 status of the synchronous tumors.

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Introduction

Bladder cancer is, with an annual incidence of 20 cases per 100.000, the fifth most prevalent cancer in the western male population. In women a lower incidence of five cases per 100.00 is observed (173). Superficial TCC, which accounts for 80% of all newly diagnosed TCCs, in general has a good prognosis. However, 10-25% of these tumors progress to invasive disease (74,101). To identify patients at risk for progression to invasive disease, reliable prognostic markers are urgently needed.

Multifocal occurrence of bladder tumors is consistent with field disease of the urothelium rather than a focal process. In this respect bladder washings have an advantage over histological biopsies in that they reflect the general status of the entire urothelium of the bladder. In a large prospective study in our hospital, bladder washings were studied by quantitative karyometric analysis in which two nuclear features, 2c Deviation Index and nuclear shape were analyzed (228) . This karyometric analysis on exfoliated cells in bladder washings resulted in a substantial increase of the reproducibility and sensitivity of the standard cytological analysis. Moreover, it offers additional prognostic information to classical factors (unpublished observations). In a previous study we attempted to enhance the clinical usefulness of this procedure to predict progression, by adding an additional prognostic marker: the tumor suppressor gene p53. The p53 gene is located on chromosome 17p13.1 and encodes a nuclear phosphoprotein involved in the maintenance of DNA integrity and is the most common mutated gene in human malignant tumors (87,117). Our findings indicate that for a group of superficial TCC patients at risk for recurrence as indicated by karyometric analysis (228), the occurrence of a p53 mutation detected in their bladder washings has a positive predictive value of 86% for the progression to invasive disease (unpublished observations). In order to determine the actual value of bladder washing specimens to assess the p53 status of histological specimens, we analyzed the bladder washings and their synchronous tumors of 15 patients for the presence of p53 mutations.

Material and Methods

Specimens

Histological samples

Sixteen paraffin-embedded superficial bladder carcinomas (pTa-pT1), three superficial carcinomas with associated carcinoma in situ (CIS), eight muscle-invasive carcinomas (pT $\geq$ 2), two CIS, 3 metastases obtained from 15 patients with high risk superficial TCC were used for PCR-SSCP analysis. All patients were indicated to be at high risk for superficial TCC progression on the basis of quantitative karyometric analysis (228) and as intermediate or high risk by calculating their prognostic index score based on tumor stage, multicentricity, recurrence rate and number of bladder areas involved (101). All patients received optimal intravesical therapy (e.g. Bacillus Calmete-Guerin instillations) as soon as they were found to have a high risk superficial bladder tumor.

For DNA isolation four 20 μ m sections of paraffin embedded tissue were used. Using an adjacent 5 μ m H/E stained section as a guide, the tissue of interest ($\geq$ 70% tumor cells) was dissected from the paraffin section and transferred to an eppendorf tube. Depending on the amount of tissue 100-500 μ l lysis buffer was added. After vortexing the tube was briefly centrifuged. For each 100 μ l lysis buffer (10 mM TrisHCl pH 8.8, 400 mM NaCl, 2 mM EDTA, 0.45% Tween 20[™] and 0.45% Nonidet P40) 5 μ l proteinase K (10 mg/ml) was added. The tube was gently mixed and incubated for 1 hour at 60 °C.

The proteinase K was inactivated by boiling for 5 minutes. After brief centrifugation, the DNA suspension was removed from the remaining paraffin and transferred to a new tube. A phenol extraction and an alcohol precipitation were performed and the samples were resuspended in one-half of the original volume. A 2.5 - 5 μ l aliquot of DNA solution, depending on the original amount of tissue, was subjected to 45 cycles of PCR.

Bladder washing samples

Twenty-six bladder washing specimens from the same 15 patients were analyzed for p53 mutations. DNA isolation was performed as described in chapter 4.

PCR-SSCP and sequences analysis

PCR-SSCP and sequence analysis were performed as described in chapter 4.

p53 immunohistochemistry

Paraffin embedded tissue of 29 bladder tumors and 3 metastases were studied for the occurrence of p53 overexpression. For immunohistochemistry 5 µm sections were deparaffinized, rehydrated and covered with citrate buffer (10 mM, pH6) and underwent a microwave-retrieval treatment (setting at 450W) for 15 minutes (75). The slides were then washed twice with PBS, and were rinsed thoroughly with PBS between each of the following steps. The sections were first blocked with undiluted rabbit serum, for 10 minutes at 37°C. The sections were then incubated overnight with a 1:400 dilution of D0-7 (DAKO) at 4°C. Subsequently the sections were incubated with biotinylated rabbit anti-mouse (1:1000 in PBS/1% BSA) (DAKO) for 30 minutes at room temperature. Preformed peroxidase-conjugated streptavidin-biotin complex (DAKO) was then applied for 30 minutes at room temperature, and peroxidase was demonstrated by applying 0.05% 3,3'-Diaminobenzidine containing 0.6% hydrogen peroxide for 5 minutes. The sections were then thoroughly washed in water and nuclei were lightly counterstained with hematoxylin. The pattern of nuclear overexpression was classified in four categories by estimating the percentage of stained tumor cells: -, no positive cells; +, 1 to 10% positive tumor cells; ++, 10 to 50% positive tumor cells and +++, >50% positive tumor cells.

Statistical analysis

The Chi-square test (with Yates correction if relevant) and the Fischer Exact test were used to demonstrate statistical difference between groups.

Results

p53 mutations in histological specimens

Thirty two histological specimens from 15 patients were analyzed by PCR-SSCP. Mutations were found in 7 out of 8 (87.5%) muscle-invasive tumors, 6/21 (28.5%) superficial tumors (and associated CIS) and in one of the three metastases studied (Table 1). Mutations were found four times in exon 6 and eleven times in exon 8.

Table 1. Comparison of p53 mutations analyzed (PCR-SSCP) in bladder washings and concomitant histological specimens.

Case no	p53 Alterations							
	Histological material				Bladder washings			
	Year	Stage/Grade	Mutation	Immunopositivity ^A	Year	Mutation	Amino acid change ^B	
1	051290	a/1	normal	+	051290	exon 8	codon 285 / GAG → AAG / <i>Glu</i> → <i>Lys</i>	
	140292	2/3	exon 8	+	140292	exon 8	codon 281 / GAG → GAC / <i>Glu</i> → <i>Asp</i>	
2	091292	1/3	normal	+	011292	exon 5		
	160393	1/2b	normal	-	160392	nd		
	101193	1/2	normal	-	210993	normal		
3	181290	a/2	normal	+	181290	normal		
	081191	1/2b	normal	+	071191	normal		
	311291	1/3,CIS	exon 6	+++	301291	exon 6	codon 205 / TAT → TGT / <i>Tyr</i> → <i>Lys</i>	
	150792	≥2/3	exon 6	+++	150792	exon 6	codon 205 / TAT → TGT / <i>Tyr</i> → <i>Lys</i>	
4	221093	1/3	exon 8	+	131093	exon 8	codon 273 / CGT → CAT / <i>Arg</i> → <i>His</i>	
	090594	4/3	exon 8	+	290394	exon 8	codon 273 / CGT → CAT / <i>Arg</i> → <i>His</i>	
	090594	(meta)	exon 8	+			codon 273 / CGT → CAT / <i>Arg</i> → <i>His</i>	
5	060892	4/2b	exon 6 ^P	-	011092	exon 6 ^P	codon 213 / CGA → CGG / <i>Arg</i> → <i>Arg</i>	
6	230891	CIS	exon 6	+	230891	exon 6	codon 194 / CTT → CGT / <i>Leu</i> → <i>Arg</i>	
	111291	≥2/3	exon 6	+	101291	*	codon 194 / CTT → CGT / <i>Leu</i> → <i>Arg</i>	
7	021287	a/1	exon 8	-	nd			
	270592	1/3	exon 8	+	200592	normal		
	150992	2/3	exon 8	+	140992	normal		
	301192	(meta)	normal	+				
8			nd		180594	exon 8	codon 280 / AGA → AAA / <i>Arg</i> → <i>Lys</i>	
	281194	2/3	exon 8	+	161194	normal	codon 280 / AGA → AAA / <i>Arg</i> → <i>Lys</i>	
	020195	4/3	exon 8	+	nd		codon 280 / AGA → AAA / <i>Arg</i> → <i>Lys</i>	
	020195	(meta)	normal	+				
9	260690	1/3,CIS	normal	+		nd		
			nd		211191	normal		
	040393	CIS	exon 8	+++		nd	nd	
10			nd		290993	exon 8	codon 280 / AGA → ACA / <i>Arg</i> → <i>Thr</i>	
	230889	a/2b	normal	+		nd		
	061192	a/2b	normal	+	211092	normal		
11	270691	a/3	normal	+	270691	normal		
	100192	1/3	normal	-		nd		
12	081093	a/2b	normal	-	290993	normal		
13	011290	a/2b	normal	-	011290	normal		
14	061191	a/2b	normal	-	051191	normal		
15	161091	1/2b,CIS	exon 8	+++	151091	normal		
			nd		221092	normal		
			nd		190892	normal		

^A number of positive cells: -, 0%; +, 1-10%; ++, 10-50%; +++, > 50%; ^P polymorphism codon 213,^B mutations confirmed in both bladder washing and histological specimen (when both specimens were available).

*, not representative sample, nd, not determined.

In case #5 the previously described polymorphism in codon 213 of exon 6 (139) was detected. The identified nucleotide change, i.e. an A to G transversion did not result in an amino acid change (Table 1).

p53 mutations in bladder washings

Twenty-six bladder washings of 15 patients from this group were compared with the concomitant histological specimens. Mutations were found in 10/26 (38.5%) bladder washings (Table 1).

Mutations were found once in exon 5, three times in exon 6 and six times in exon 8 (Table 1). In case #5 again the known polymorphism in exon 6 was detected (139).

Table 2. Relationship between the overall p53 status of bladder washings and synchronous histologic specimen summarized for each individual patient.

	Histo / p53mut	Histo / p53wt	Total number
BW / p53mut	6	1	7
BW / p53wt	2	6	8
Total number	8	7	15

Histo, histological specimen; BW, bladder washing;
p53mut, mutated p53 gene; p53wt, normal p53 gene.

Comparison p53 mutations in bladder washings and synchronous histological material.

To investigate the usefulness of bladder washings to determine the p53 status of the synchronous tumors, we compared both specimens for these 15 patients (Table 1).

If the results for each individual patient are summarized, the p53 analysis of the bladder washings and histological specimens showed a significant correlation ($p < 0.05$, Fischer's exact test, two-tail) (Table 2). A specificity of 86% (6/7) and a sensitivity of 75% (6/8) was observed. Overall in two patients (case #7 and #15) the mutation found in the histological specimen could not be detected in one of the consecutive bladder washings and in one patient (case #2) the mutation observed in the bladder washing is not detected in any of the histological specimens analyzed. These conflicting results between histological specimens and bladder washings can partly be explained by the architecture of the tumors as is shown in Figure 1. Both the pT1G3 and the pT2G3 tumor of case #7

were covered by normal urothelium, as is clearly illustrated by the immunohistochemical staining of these histological specimens (Figure 1A and 1B). In case #15 the p53 overexpressing cells are present in a CIS lesion that is in contact with the luminal site, but the number of cells is very small (Figure 1C). The concomitant pT1G2b tumor is growing in brunns cell nests (Figure 1D). In two out of the three metastases (case #7 and #8) the mutation observed in the original tumor is not detected. The relatively small number of p53 overexpressing tumor cells in these metastases is the most likely explanation (Figure 1E). The PCR-SSCP analysis of the other metastasis (case #4) revealed the same mutation as observed in the original tumor. As is shown in figure 1F the relative amount of p53 overexpressing tumor cells in this metastasis is much higher compared to case #7 and #8.

Individual comparison of bladder washings and their synchronous histological specimens revealed some additional discrepancies as is shown in Table 1. In case #1 the mutation in the first analyzed bladder washing was not found in the concomitant tumor. The next histological specimen, however, revealed a different mutation, that was confirmed in the concomitant bladder washing. In case #8 a mutation was found in the histological specimen but not in the concomitant bladder washing. However, the same mutation was found in a prior analyzed bladder washing.

p53 immunohistochemistry

We defined a tumor as p53 positive if more than 10% of the tumor cells showed p53 overexpression. A significant correlation between p53 mutation and p53 overexpression is observed ($p < 0.01$, chi-square = 7.18) (Table 1). However, 3 out of 15 tumors (20%) with a proven p53 mutation show no p53 overexpression, whereas of the 18 tumors with p53 overexpression 6 tumors (33%) have no identified p53 mutation.

Comparison specific nature mutations confirmed by sequence analysis

Sequence analysis revealed 9 transitions and 4 transversions (Table 1). Of the 5 patients of which sequence data were available from both the mutation observed in the bladder washing as well as in the synchronous tumor, 4 showed the same mutation. However, in one patient (case #1), different mutations were observed in the two bladder washings analyzed.

Figure 1: Immunohistochemical staining of p53 expression using p53 DO-7 monoclonal antibody. A. pT1G3 tumor of case #7; B. pT2G3 tumor of case #7; C. CIS of case #15; D. pT1G2b tumor of case #15; E. lymph-node metastasis of case #8; F. lymph-node metastasis of case #4; Original magnification: 100x (B), 200x (A,D,E,F) and 400x (C).

Discussion

A significant problem in the management of patients with superficial TCC is the identification of those patients who are at risk for progression to invasive disease. Early identification could make a change in the treatment strategy possible when the disease is still confined to the bladder. In our institution the prognostic index score (based on stage, grade, multicentricity and number of bladder areas involved) (101) and the quantitative karyometric analysis of bladder washings (228) are used as prognostic factors. These markers are useful to stratify patients with superficial TCC into low or high risk categories. However, the morbidity associated with cystectomy, which at the moment is the only treatment to prevent progression to invasive disease, emphasizes the need for additional markers to increase both the specificity and sensitivity of this procedure to predict progression. In a previous study we showed the positive predictive value of p53 mutation analysis in bladder washings for progression of high risk (101,228) superficial TCC (unpublished observations). We chose to use bladder washings, because it is a simple outpatient procedure and patients can be monitored more carefully. Moreover, since bladder cancer is hypothesized to be a field disease of the urothelium, bladder washings have the advantage of reflecting the status of the entire bladder mucosa. The use of bladder washings is preferred over urine specimens, since more representative and better preserved material is harvested (1,255).

PCR-SSCP analysis of the p53 gene in our histological specimens revealed an association of p53 mutations with invasive tumors as was previously shown (64,199,233). The pre-selected nature of the tumors in this study explains the observed higher percentage p53 mutations in both the superficial as well as the invasive tumors.

The importance of analyzing p53 mutations in bladder washings results from the possibility of preventing disease progression by increased surveillance. The clinical relevance of the analysis is due to the ability to detect a p53 mutation in the bladder washing before progression to invasive disease has occurred and not per se the absolute concordance between the p53 status of each histologic specimen and its paired bladder washing. We found a significant correlation ($p < 0.05$, Fischer Exact test, two-tail) between the p53 status of bladder washings and histological specimens if these were compared among the specimens of a single patient. However some discrepancies were observed. In one patient (case #2) a mutation was observed in the bladder washing, but

could not be detected in any of the histological specimens analyzed. The existence of minor lesions that have already acquired the mutant p53 status (207) but were too small to be observed by cystoscopy could explain the presence of mutated p53 cells in these bladder washings. This phenomenon emphasizes the advantage of bladder washings which makes analysis of cells from the entire bladder mucosa possible. In two patients (case #7 and #15) the observed mutation in the histological specimen(s) was not detected in any of the bladder washings analyzed. The absence of mutated cells in the bladder washings of patient #7 could be explained by the histologic composition of the tumors. Both tumors were clearly covered by 'normal' (no p53 overexpression) urothelium which most likely prevented the release of mutated p53 tumor cells into the bladder washing. Although the clear presence of CIS in patient #15 (all of the cells showed p53 overexpression) would predict that mutated cells should be present in the bladder washing, most likely the number of cells was too small to detect the mutation. The concomitant pT1G2b tumor is growing in brunns cell nests, preventing the major part of the p53 overexpressing cells from releasing in the bladder washing.

Individual comparison of the synchronous bladder washings and histological specimens showed some additional discrepancies. In one patient (case #8) the mutation detected in the histological specimen (pT2G3) was not found in the concurrent bladder washing, but was observed in a previous bladder washing. The invasive nature of the tumor could have prevented the release of the mutated cells into the bladder washing. In one patient (case #1) a mutation was observed in the bladder washing but could not be detected in the synchronous tumor. The subsequently resected tumor (14 months later), showed a mutation. This mutation however differed from the first observed mutation in the bladder washing, but was identical to the mutation found in the concomitant bladder washing. This occurrence of different p53 gene mutations in bladder tumors of one patient has also been reported in two TCC patients in a previous study (207). The other four patients showed identical p53 mutations in the consecutive bladder washings and the synchronous tumors, which is in agreement with the theory of the clonal origin of bladder tumors (200).

PCR-SSCP analysis using histologic specimens can despite microdissection of tumor give false-negative results caused by the presence of a relatively high percentage of normal cells as is shown in this study, by the high number of lymphocytes in two of the three metastases analyzed (247). Although the good concordance between p53 mutations

and p53 overexpression, in a substantial number of tumors discrepancies are observed, which is in agreement with previous studies (81,234,249).

In conclusion, in two patients, the mutation present in the tumors was not detected in the bladder washings and in one patient the mutation in the bladder washing was not detected in the histologic specimens. The observed specificity of 86% and sensitivity of 75% emphasizes that although there is a good correlation between the two methods in a number of cases they are complementary to one another. More regular examination of bladder washings for the presence of a p53 mutation could lead to early identification of patients who need a change in their treatment strategy (conservative to radical) and thereby potentially prevent disease progression.

From these findings we conclude that the analysis of p53 mutations in consecutive bladder washings of a patient does generally reflect the p53 status of the synchronous tumors. We are currently assessing the clinical usefulness of p53 mutation analysis in bladder washings as a prognostic marker in a prospective study.

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Chapter 6

**Differential expression of ferritin Heavy chain in a rat
Transitional Cell Carcinoma progression model.**

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Abstract

To identify molecular markers with predictive value for the progression of superficial bladder cancer we used the differential hybridization analysis approach. Since primary tumor material is heterogeneously composed of subpopulations that are poorly characterized, we used in this study a rat progression model system that phenotypically and cytogenetically resembles human superficial bladder cancer. In the differential hybridization analysis we compared the mRNA populations of low and high metastatic tumor lines. We observed an overexpression of ferritin Heavy chain (ferritin H) in the tumor line with the lower metastatic capacity and better differentiated phenotype. The exact clinical relevance for the differential expression of ferritin H in human bladder cancer remains to be determined.

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Introduction

An important issue in the management of superficial transitional cell carcinoma (TCC) of the bladder is to identify those patients that are at risk for progression to invasive disease. Since primary tumor material is usually heterogeneously composed of subpopulations that are poorly characterized, it is not suitable to identify TCC progression markers. The identification of such parameters requires a tumor model system in which the different stages of tumor progression are represented.

In this study a rat bladder tumor model system (RBT) is used that phenotypically and cytogenetically resembles human superficial TCC (230). In order to identify genes that are associated with the progression of superficial bladder cancer we applied the technique of differential hybridization analysis to compare the mRNA levels of the RBT 323 with the RBT 157 line, two lines that differ in their metastatic capacity. Both tumor lines initially resembled grade 2 TCC. However, while the histological pattern of the RBT 157 remained essentially unchanged, the RBT 323 tumor progressed to a grade 3 tumor in the third passage. The cDNA clones that detected differentially expressed genes were further evaluated for their relationship to the metastatic phenotype by means of Northern blot analyses of all the passages of the rat TCC progression model. The cDNA clones of interest were sequenced and the resulting nucleotide sequences were compared to nucleotide database to search for homology with known genes.

Materials and methods

Tumor specimens

Tumor material from a rat bladder tumor model system (RBT) (230) was used in this study (Table 1). Transplant passage #10 of the highly metastatic line RBT 323 and passage #1 of the moderate metastatic line RBT 157 were used for the differential hybridization experiments. In order to check the relevance of the selected clones passages 1, 2, 8, 9, 15 and 16 of the RBT 323 line and passage 1 through 6 of the RBT 157 line were used.

Table 1. Growth characteristics of rat bladder tumor lines.

	Tumor passage	Tumor doubling time ^A	Metastases ^B
RBT323		ND	0/1
	Passage 1	ND	0/6
	Passage 2	13.4 (1.4)	0/8
	Passage 3	11.8 (1.2)	1/10
	Passage 4	5.9 (1.0)	0/9
	Passage 5	5.7 (0.3)	3/7
	Passage 6	4.4 (0.1)	11/13
	Passage 8	3.8 (0.2)	15/15
	Passage 10	3.7 (0.2)	9/9
	Passage 12	4.2 (0.3)	19/22
	Passage 13	3.3 (0.3)	6/6
	Passage 15	4.3 (0.3)	5/6
RBT157		ND	0/1
	Passage 1	11.2 (0.8)	0/4
	Passage 2	ND	1/7
	Passage 3	10.5 (1.1)	2/9
	Passage 4	8.2 (0.3)	5/8
	Passage 6	9.5 (1.8)	2/6

^A Tumor doubling time in days, standard error of the mean in brackets. ^B Number of rats with lung metastases/total number of rats implanted with respective tumor passage. ND, not determined. [RJA van Moorselaar et al.(230)]

Construction of cDNA libraries

A directionally cloned cDNA library was constructed using polyadenylated cytoplasmic RNA from the highly metastatic RBT323 line, passage #10. Poly(A⁺)-RNA (5 µg) was fractionated from total RNA by oligo(dT)-cellulose chromatography and total RNA was isolated using the lithium chloride/urea procedure (4). First strand cDNA was synthesized by using an oligo(dT) primer-adaptor (Promega) containing a NOT-I site 5' to the oligo(dT) tail. Second strand cDNA synthesis was performed using the procedures described by Gubler and Hofman (76). The cDNA was blunt ended by using T4-DNA polymerase (10 U). After purification using micro collodion bags (Sartorius GmbH), EcoRI linkers (1 µg, Stratagene) were ligated to the blunt ended cDNA by means of T4-DNA ligase, followed by digestion with NOT-I and Eco-R1 restriction endonucleases. The digested linkers were separated from the cDNA by means of PurElute[™] agarose gel electrophoresis (Invitrogen) and the cDNAs with the desired size range (0.5-10 kB) were

isolated from the agarose. Electroelution of the cDNA from the agarose was performed using the Geneluter[™] electroelution system (Invitrogen). Subsequently the ds cDNA was ligated in the Lambda Zap-II cloning vector (Stratagene), inserted into the phage particles using Promega Packagene Lambda DNA Packaging System and transformed to XL-1 Blue E. coli bacteria.

Differential screening of the cDNA library

For the differential screening of the RBT 323/10 cDNA library, 36,000 recombinant phages were plated. After overnight incubation, 4 replicas of each plate were taken on nitrocellulose by incubation during 1, 3, 10 and 30 minutes respectively. The filters were next lysed according to Sambrook et al. (1976). Hybridization was performed according to Hanahan and Meselson (82) in 40% formamide at 42°C during 60 hours. For the differential screening, probes representative for the RBT 323/pass.10 and RBT 157/pass.1 mRNA populations were prepared by random prime labeling (53) of first strand cDNA. For hybridization of the filters, 1.10^6 dpm/ml hybridization solution was used. The replica's 1 and 10 minutes were hybridized with probe derived from the RBT 157/pass.1 and the replica's 3 and 30 minutes were hybridized with probe derived from the RBT 323/pass.10. The blots were washed according to Hanahan and Meselson (82). The filters were exposed for autoradiography during three days. Extensive comparison of the autoradiograms resulted in selection of differentially expressed clones.

Phagemid isolation

Putative differential expressed cDNA clones were purified and phagemids were isolated according to the Stratagene protocol.

Northern blot analysis.

For the northern blot analysis, 10 micrograms of total RNA was glyoxylated, size fractionated on 1% agarose gels and transferred to Hybond-N+ nylon membranes (Amersham). Probes were made of the differentially expressed cDNA clones, obtained from small scale plasmid isolations or of purified inserts of the cDNA clones, by random prime labeling reactions (53). Hybridization experiments were performed according to Church and Gilbert (30).

Sequence analysis and computer analysis

Sequence analysis was performed with the Sequenase Version 2.0 System (United States Biochemical). M13 and reverse primers were used allowing sequence analysis from both ends of the clones. Computer assisted comparison studies of the sequences were performed with the EMBL and Genbank nucleotide sequence databases.

Results

Differential screening

A cDNA library was constructed from the mRNA of the highly metastatic line RBT 323/passage 10. The complexity of the library was 4.3×10^5 recombinant clones and 36,000 recombinant phages from the primary library were plated. Replica filters were differentially screened using radioactively labeled cDNA probes derived from poly-A⁺RNA of the RBT 323/pass.10 (high metastatic) and the RBT 157/pass.1 (low metastatic).

Figure 1. Differential screening of the RBT 323/10 cDNA library. Replicas were hybridized to cDNA probes prepared using poly-A⁺-RNA from the RBT 157/1 line (A + C) or from the RBT 323/10 line (B + D).

Extensive comparison of the resulting autoradiograms (Figure 1) revealed 22 cDNA clones that seemed to be differentially expressed; 16 overexpressed in the RBT 323 line and 6 overexpressed in the RBT 157 line. These 22 selected cDNA clones were further evaluated using Northern blots containing 10 μ g of RBT 157/1, RBT 323/1 and RBT 323/10 RNA. The expression patterns of the most significant clones are shown in Figure 2.

Figure 2. Secondary screening of the selected cDNA clones on the passages RBT 157/1 (1), RBT 323/1 (2) and RBT 323/10 (3). 10 μ g of total RNA was glyoxylated, size fractionated on 1% agarose gels and transferred to Hybond-N+ nylon membranes. Probes were made of the differentially expressed cDNA clones by random prime labeling reactions

Expression pattern of pV13

Clone pV13, is clearly overexpressed in RBT 157/1, the line with the lowest metastatic capacity. pV13 detects a single transcript of approximately 1.1 Kb (Figure 2). The correlation of clone pV13 expression with metastatic capacity was further studied by means of Northern blot analysis of all passages of the rat progression model (Figure 3). The RBT 157 line, with the moderate metastatic capacity, shows an higher expression level compared to the RBT 323 line, with the high metastatic capacity. The right panel of figure 3 illustrates that the higher expression is retained in all the passages of RBT 157. The other clones did not retain a significant differential expression on further evaluation or appeared to originate from ribosomal RNA.

Figure 3. Correlation of clone pV13 with metastatic phenotype. 10 μ g of total RNA was glyoxylated, size fractionated on 1% agarose gels and transferred to Hybond-N+ nylon membranes. The probe was made, using the isolated insert of cDNA clone pV13, by random labeling reaction

DNA sequence analysis of pV13

To obtain further information on the cDNA clone, the nucleotide sequences were determined using the Sequenase Version 2.0 sequence system. Computer-assisted comparison of the resulting nucleotide sequences with the EMBL and Gen bank nucleotide sequence databases revealed that pV13 is highly homologous to the Rat Ferritin Heavy chain gene (97%) (151) and the Mouse Ferritin Heavy chain (94%) (217).

Discussion

In search of new molecular markers with predictive value for the progression of superficial TCC, we compared two different tumor lines of a rat TCC model system at the level of gene expression using differential hybridization analysis. We observed an overexpression of ferritin H in the RBT 157 tumor line which displays the lowest metastatic capacity and the best differentiated phenotype.

Ferritin H combines with the ferritin L subunit to form the iron storage protein ferritin (143). Ferritin H-rich molecules take up and release iron more readily than L-rich molecules, that are more suited for long-term storage (150). A relationship between ferritin and cancer has been proposed, since serum ferritin is frequently elevated in patients with cancer (48,246). Some neoplastic cells also exhibit a remarkably altered ferritin subunit composition (i.e. enrichment for the heavy chain subunit) as compared to their normal counterparts (24,232). A direct association between oncogenic transformation and ferritin synthesis has been suggested, since the adenovirus E1A oncogene specifically represses ferritin H expression (223). The observed downregulation of ferritin H expression in the more malignant tumor line RBT 323 is in concordance with this observation. The correlation between ferritin H expression and a number of differentiation processes shown by others (9,27), is corroborated in this study by the overexpression of ferritin H in the better differentiated tumor line RBT 157. In smooth muscle cells it is proposed that cAMP promotes ferritin H expression resulting in both growth arrest and an enhanced differentiated phenotype (123).

The exact biological consequences of altered ferritin subunit composition are not well understood. It may result in modulation of the iron binding capacity of ferritin and ultimately, the regulatory pools of free iron (143,222). An enrichment for the ferritin H subunit leads to more efficient iron detoxification and cellular protection against free radical formation (179).

It should be emphasized that the mechanisms underlying the altered subunit composition of ferritin in neoplastic cells are not elucidated. Ferritin H is located on chromosome 11 (86), near 11q13 (67), a region that is sometimes amplified in bladder cancer (171). We have determined that the ferritin H gene is not within the amplicon on 11q13 (data not shown).

We are currently investigating the value of this differential expression of ferritin H as a prognostic marker in human bladder cancer using quantitative comparative Polymerase Chain Reaction.

Chapter 7

Summary and general discussion

A significant problem in the clinical management of transitional cell carcinoma of the bladder (TCC) is the identification of those patients that are at risk for progression of their disease. In addition to the "classic" histopathologic diagnosis and clinical parameters, new markers identifying patients at risk for tumor progression are urgently needed. The advances in the molecular approach to tumor biology of the last years resulted in a better understanding of cancer genetics and new potential prognostic markers. This thesis describes two approaches to evaluate molecular prognostic markers: (i) the major part focuses on the prognostic value of the known tumor suppressor gene p53 and its significance as a diagnostic tool, (ii) the use of differential hybridization analysis as a method to identify new molecular prognostic factors.

Alterations of p53 can be identified directly at the genetic level, by detecting mutations in the p53 gene using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) analysis or indirectly by the detection of p53 protein overexpression using immunohistochemistry (IHC). These two methods were compared in **chapter 2** to assess their reliability. Although a good concordance ($p < 0.02$) between p53 mutation by SSCP analysis and p53 overexpression by IHC was observed, in a substantial number of tumors discrepancies were found. Two out of eight tumors with a SSCP proven p53 mutation showed no p53 immunoreactivity at all, most likely as a result of loss of the nuclear localization signal. In addition to these "false negatives", p53 overexpression was observed in 23% of the tumors without any sign of p53 mutation as assessed by SSCP analysis. Therefore, despite the tight correlation between p53 mutation and p53 overexpression, there is no direct causal relationship between mutation and overexpression. Apparently other events than mutation can trigger p53 stability. Additional research is necessary to characterize these events and to understand the mechanisms through which this overexpression without a concomitant mutation reflects alteration of p53 function and consequently gives rise to an altered phenotype in cancer cells.

Clinical parameters were compared with p53 overexpression (IHC) and p53 mutation (PCR-SSCP) (**Chapter 2 and 3**). A significant correlation between p53 alteration and grade as well as stage was observed ($p < 0.05$ vs. IHC and $p < 0.001$ vs. SSCP, respectively). Moreover, p53 alteration is an unfavorable prognostic factor for the whole group of bladder cancer patients ($p < 0.01$ vs. IHC and $p < 0.001$ vs. SSCP, respectively). No association between p53 alteration and decreased survival was found for invasive

tumors. The immunohistochemical results for invasive TCC are in contrast with other studies. Differences in treatment of patients, antibody, methodology and scoring systems used might explain these differences. However, a recent study has shown that p53 overexpression is a significant predictor of tumor progression for the group of muscle-invasive tumors, only when the disease is confined to the bladder ($pT \leq 3a$). With more extensive disease ($pT \geq 3b$), p53 overexpression has no additional prognostic value. Because of the relatively small number of invasive tumors in our study, we could not compare these two groups. In superficial TCC a trend was observed towards poorer survival for the patients showing p53 overexpression. No p53 mutations were observed in this group of superficial tumors. This difference stresses again the discordance of p53 immunohistochemistry and p53 mutation analysis.

In **chapter 3** also the association between p53 mutations and frequency of allelic loss, as a putative indicator of genetic instability, was investigated. A high frequency of allelic loss was found in tumors with a p53 mutation, suggesting a correlation with genetic instability, which could be explained by an altered G1-S cell cycle checkpoint, resulting from the loss of wild type p53 function.

It is critically important to define if, and when p53 mutations occur during tumor progression from superficial (Ta) tumors to lamina propria invasive (T1) tumors and muscle invasive (T2-T4) tumors. To assess the value of p53 mutations in predicting the progression of superficial TCC, consecutive bladder washings from high risk superficial TCC patients (indicated by quantitative karyometric analysis) were examined (**Chapter 4**). We chose for direct analysis of mutations because of the more objective nature of this method compared to IHC. Bladder washings have the advantage over histological biopsies in that they reflect the general status of the bladder mucosa since cells from the entire bladder are sampled. Moreover, this is a simple outpatient procedure and patients can be monitored more frequently. Six out of 13 patients that clinically progressed (to $pT \geq 2$) showed a p53 mutation in one or more of their bladder washings prior to progression. In the control group (no progression) only one out of 13 patients showed a p53 mutation. In high risk patients, as indicated by quantitative karyometric analysis, the occurrence of a p53 mutation has a positive predictive value for progression to invasive disease of 86%. The low negative predictive value (63%) implicates that, although a p53 mutation indicates a malignant potential for the tumor, the absence of a p53 mutation does not guarantee a good prognosis. Since p53 mutations were found in samples prior

to documented progression their presence could identify patients who need a change in their treatment strategy to potentially prevent progression to invasive disease. The clinical usefulness of p53 mutation analysis as a diagnostic tool should now be assessed in a large prospective study.

Chapter 5 describes the comparative p53 mutation analysis of bladder washings and their synchronous tumors to determine the actual value of bladder washings to assess the p53 status of the histological specimens. A significant correlation between the p53 status of bladder washings and histological specimens was observed if these were compared among the specimens of a single patient ($p < 0.05$). Overall, in two patients (2 out of 15) the mutation present in the tumors was not detected in the bladder washings and in one additional patient the mutation in the bladder washing was not detected in the histological specimens. These conflicting results between bladder washings and histological specimens could partly be explained by the architecture of the tumors. This study shows that the analysis of p53 mutations in consecutive bladder washings of a patient does generally reflect the p53 status of the synchronous tumors. As addressed earlier, washing of the bladder is a simple outpatient procedure, whereby monitoring of patients on more frequent basis is possible. This could lead to an increased detection of TCC patients at high risk for progression and thereby potentially prevent this progression to invasive disease.

In addition to the study of the prognostic value of the known tumor suppressor gene p53, we used differential hybridization analysis to identify new molecular markers that are associated with the progression of superficial bladder cancer (**Chapter 6**). Primary tumor material is usually composed of subpopulations with different malignant potential and is therefore not suitable to identify TCC progression markers. In this study a rat bladder tumor model system was used, that phenotypically and cytogenetically resembles human superficial TCC. The mRNA populations of low and high metastatic tumor lines were compared. Ferritin Heavy (H) chain was identified to be overexpressed in the tumor line with lowest metastatic capacity and a more differentiated phenotype. Ferritin H combines with the ferritin light subunit to form the iron storage protein ferritin. A relationship between ferritin and cancer has been proposed and some neoplastic cells exhibit a remarkably altered ferritin subunit composition compared to their normal counterparts. The overexpression of ferritin H in the more differentiated tumor line is a corroboration of the observed association between ferritin H and a

number of differentiation processes. The observed lower expression of ferritin H in the more malignant tumor line is in concordance with the observation that during oncogenic transformation, the adenovirus E1A oncogene specifically represses ferritin H expression. Since we used a rat bladder tumor model system for the differential hybridization experiments, the value of the differential expression of ferritin H as a prognostic marker in the human system remains to be elucidated.

The studies described in this thesis emphasize that alterations in the tumor suppressor gene p53 are important in the clinical behavior of bladder tumors. Although a good overall concordance between p53 gene mutation and p53 protein overexpression in relation with grade, stage and survival exists, there is no direct causal relationship between mutation and protein overexpression. In the group of high risk superficial TCC patients, identified by quantitative karyometric analysis, the occurrence of a p53 mutation as examined in bladder washings, appears to be of value as an additional marker to predict progression to invasive disease. In spite of these promising results, it is obvious that more prognostic markers are needed to increase not only the positive predictive value, but especially the negative predictive value of this screening procedure. The technique of differential display could be useful as a tool to identify new prognostic markers, provided that the compared mRNA populations are very precisely defined. Good candidates for the panel of prognostic markers that are available at this moment, are Epidermal Growth Factor Receptor and E-cadherin. Together with the "classic" histopathologic diagnosis and clinical parameters this panel of prognostic markers should be evaluated in a large prospective study, with well defined patient populations and standardized technical procedures to assess their exact clinical relevance.

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Samenvatting en algemene discussie

Een belangrijk probleem bij de klinische behandeling van transitioneel cel carcinoma van de blaas (TCC), is de identificatie van patienten met een risico voor progressie van hun ziekte. Naast de "klassieke" histopathologische diagnose en klinische parameters is er dringend behoefte aan nieuwe markers, die patienten met een risico voor tumorprogressie kunnen identificeren. De laatste jaren heeft de vooruitgang in de moleculaire benadering van tumorbiologie geresulteerd in een beter begrip van kankergenetica en nieuwe potentiële prognostische markers. Dit proefschrift beschrijft twee benaderingen om moleculaire prognostische factoren te evalueren: (i) de prognostische waarde van het bekende tumorsuppressor gen p53 en zijn belang als diagnostisch hulpmiddel, (ii) het gebruik van de differentieel hybridisatie analyse als een methode om nieuwe moleculaire prognostische factoren te identificeren.

Op genetisch nivo kunnen p53 veranderingen direct geïdentificeerd worden door mutaties in het p53 gen te detecteren, gebruik makend van de Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) analyse, of indirect door detectie van overexpressie van het p53 eiwit, gebruik makend van immunohistochemie (IHC). Om hun betrouwbaarheid te bepalen zijn deze twee methoden onderling vergeleken in **hoofdstuk 2**. Ondanks de goede correlatie ($p < 0.02$) tussen p53 mutatie, bepaald door SSCP analyse, en p53 overexpressie, bepaald door IHC, zijn in een aanzienlijk aantal tumoren verschillen gevonden. Twee van de acht tumoren met een p53 mutatie (SSCP) vertoonden in het geheel geen p53 immunoreactiviteit, waarschijnlijk veroorzaakt door verlies van het kern localisatie signaal. Naast deze "onjuist negatieven", is p53 overexpressie waargenomen in 23% van de tumoren waarbij geen p53 mutatie aangetoond kon worden door SSCP analyse. Daarom is er, ondanks de sterke correlatie tussen p53 mutatie en p53 overexpressie, geen direct causaal verband tussen mutatie en overexpressie. Blijkbaar kunnen behalve mutaties ook andere gebeurtenissen stabiliteit van p53 veroorzaken. Aanvullend onderzoek is nodig om deze gebeurtenissen te karakteriseren en de mechanismen te begrijpen waardoor deze overexpressie, zonder een gelijktijdige mutatie, verandering van p53 functie weerspiegelt en vervolgens een ander fenotype veroorzaakt in kankercellen.

Klinische parameters zijn vergeleken met p53 overexpressie (IHC) en p53 mutatie (PCR-SSCP) (**Hoofdstuk 2 en 3**). Een significante correlatie tussen p53 verandering en zowel graad als stadium is waargenomen ($p < 0.05$ vs IHC en $p < 0.001$ vs SSCP, respectievelijk). Bovendien is p53 verandering een slechte prognostische factor voor de hele groep van blaaskanker patiënten ($p < 0.01$ vs IHC en $p < 0.001$ vs SSCP, respectievelijk). Voor invasieve tumoren is er geen associatie gevonden tussen p53 verandering en een verkorte overlevingsperiode. De immunohistochemische resultaten voor invasieve tumoren zijn in tegenspraak met andere studies. Verschillen in de behandeling van patiënten, het antilichaam, methode en scoringssysteem dat gebruikt is, zouden deze discrepanties kunnen verklaren. Echter, een recente studie heeft laten zien dat voor de groep van spier-invasieve tumoren, p53 overexpressie slechts significant tumorprogressie voorspelt, wanneer de ziekte zich beperkt tot de blaas ($pT \leq 3a$). Indien de ziekte meer uitgebreid is ($pT \geq 3b$), heeft p53 overexpressie geen additionele prognostische waarde. Vanwege het relatief kleine aantal invasieve tumoren in onze studie hebben we deze twee groepen niet kunnen vergelijken. In oppervlakkig TCC is er een verschuiving waargenomen naar een kortere overlevingsperiode voor de patiënten met p53 overexpressie. Er zijn geen p53 mutaties waargenomen in deze groep van oppervlakkige tumoren. Dit verschil benadrukt opnieuw het onderscheid tussen p53 immunohistochemie en p53 mutatie analyse.

In **hoofdstuk 3** is ook de associatie tussen p53 mutaties en frequentie van allelisch verlies, als een mogelijke indicator voor genetische instabiliteit, onderzocht. Er is een hoge frequentie van allelisch verlies gevonden in tumoren met een p53 mutatie, hetgeen wijst op een correlatie met genetische instabiliteit. Dit kan worden verklaard door een veranderd G1-S cel cyclus controlepunt, als gevolg van het verlies van de wild type p53 functie.

Het is zeer belangrijk om te definiëren of en wanneer p53 mutaties optreden tijdens tumorprogressie van oppervlakkige (Ta) tumoren naar lamina propria invasieve (T1) tumoren en spier-invasieve (T2-T4) tumoren. Om de waarde van p53 mutaties te bepalen voor het voorspellen van progressie van oppervlakkig TCC zijn opeenvolgende blaasspoelingen van oppervlakkige TCC patiënten met een hoog risico (geïdentificeerd door kwantitatieve karyometrische analyse) onderzocht (**Hoofdstuk 4**). We hebben gekozen voor directe analyse van mutaties vanwege het meer objectieve karakter van deze methode vergeleken met IHC. Omdat cellen van de gehele blaas worden geoogst,

hebben blaasspoelingen het voordeel ten opzichte van histologische biopsies dat ze de algemene status van de blaas mucosa weergeven. Bovendien is dit een eenvoudige poliklinische procedure zodat patiënten vaker kunnen worden gecontroleerd. Zes van de 13 patiënten met klinische progressie (naar $pT \geq 2$) vertoonden een p53 mutatie in één of meer van hun blaasspoelingen voordat progressie optrad. In de controle groep (geen progressie) vertoonde maar één van de 13 patiënten een p53 mutatie. In patiënten met een hoog risico, geïdentificeerd door kwantitatieve karyometrische analyse, heeft het verschijnen van een p53 mutatie een positief voorspellende waarde voor progressie naar invasieve ziekte van 86%. De lage negatief voorspellende waarde (63%) impliceert dat, ondanks het feit dat een p53 mutatie de kwaadaardige potentie van een tumor aangeeft, de afwezigheid van een p53 mutatie geen garantie is voor een goede prognose. Omdat p53 mutaties zijn gevonden in samples voor gedocumenteerde progressie, kan hun aanwezigheid patiënten identificeren die een verandering in hun behandeling nodig hebben om mogelijk progressie naar invasieve ziekte te voorkomen. De klinische bruikbaarheid van p53 mutatie analyse als diagnostisch hulpmiddel zal nu bepaald moeten worden in een grote prospectieve studie.

Hoofdstuk 5 beschrijft de vergelijkende p53 mutatie analyse van blaasspoelingen en hun vergezellende tumoren om de werkelijke waarde van blaasspoelingen te bepalen voor het weergeven van de p53 status van het histologische materiaal. De p53 status van de blaasspoelingen en het histologische materiaal vertonen een significante correlatie, wanneer deze vergeleken werden voor alle samples van een enkele patient ($p < 0.05$, Fischer exact test, two tail). Samenvattend, in twee patienten (2 van de 15) is de mutatie die aanwezig was in de tumoren niet gedetecteerd in de blaasspoelingen. In een derde patient is de mutatie in de blaasspoeling niet gedetecteerd in het histologische materiaal. Dit tegenstrijdige resultaat tussen de p53 status van de blaasspoelingen en het histologisch materiaal kan gedeeltelijk worden verklaard door de architectuur van de tumoren. Deze studie laat zien dat de analyse van p53 mutaties in opeenvolgende blaasspoelingen van een patient in het algemeen de p53 status van de vergezellende tumor weergeeft. Zoals reeds vermeld is het wassen van de blaas een eenvoudige poliklinische procedure waardoor controle van patiënten op een meer frequente basis mogelijk is. Dit zou kunnen leiden tot een verhoogde detectie van TCC patienten met een hoog risico voor progressie waardoor mogelijk deze progressie naar invasieve ziekte voorkomen zou kunnen worden.

Naast de studie van de prognostische waarde van het bekende tumorsuppressor gen p53, hebben we gebruik gemaakt van differentiële hybridisatie analyse om nieuwe moleculaire prognostische markers te identificeren die geassocieerd zijn met progressie van oppervlakkig blaaskanker (**Hoofdstuk 6**). Primair tumormateriaal is meestal samengesteld uit subpopulaties met verschillende kwaadaardige potentie en is daarom niet geschikt om TCC progressie markers te identificeren. In deze studie is een rat blaas tumor model systeem gebruikt, dat phenotypisch en cytogenetisch lijkt op humaan oppervlakkig TCC. De mRNA populaties van laag en hoog metastaserende lijnen zijn vergeleken. Er is overexpressie van ferritine Heavy (H) chain waargenomen in de tumorlijn met de laagst metastaserende capaciteit en beter gedifferentieerd phenotype. Ferritine H vormt samen met de ferritine Light subunit het eiwit ferritine, dat zorgt voor de opslag van ijzer. Er is een relatie tussen ferritine en kanker voorgesteld en sommige neoplastische cellen vertonen een opmerkelijk veranderde samenstelling in ferritine subunits, vergeleken met hun normale tegenhangers. De overexpressie van ferritine H in de beter gedifferentieerde tumorlijn is een bevestiging van de waargenomen associatie tussen ferritine H en een aantal differentiatie processen. De waargenomen lagere expressie van ferritine H in de meer kwaadaardige tumorlijn stemt overeen met de waarneming dat tijdens oncogene transformatie, het adenovirus E1A oncogen specifiek de expressie van ferritine H remt. Omdat we gebruik hebben gemaakt van een rat blaas tumor model systeem voor de differentiële hybridisatie experimenten, zal de waarde van de differentiële expressie van ferritine H als een prognostische marker in het humane systeem nog opgehelderd moeten worden.

De studies die in dit proefschrift beschreven zijn, benadrukken dat veranderingen in het tumorsuppressor gen p53 belangrijk zijn voor het klinische gedrag van blaastumoren. Ondanks de goede overall overeenkomst tussen p53 gen mutatie en p53 eiwit overexpressie in relatie tot graad, stadium en overlevingsperiode, is er geen causaal verband tussen mutatie en overexpressie. In de groep van oppervlakkig TCC patiënten met een hoog risico, geïdentificeerd door middel van kwantitatieve karyometrische analyse, lijkt de verschijning van een p53 mutatie geanalyseerd in blaasspoelingen van waarde te zijn als een extra marker om progressie naar invasieve ziekte te voorspellen. Ondanks deze veelbelovende resultaten is het duidelijk dat er meer prognostische markers nodig zijn om niet alleen de positief voorspellende waarde, maar vooral de

negatief voorspellende waarde van deze screenings procedure te verhogen. De differential display techniek zou bruikbaar kunnen zijn als een middel om nieuwe prognostische markers te identificeren, gesteld dat de te vergelijken mRNA populaties zeer precies worden gedefinieerd. Voor het panel van prognostische markers zijn op dit moment de Epidermal Growth Factor Receptor en E-cadherin goede kandidaten. Samen met de "klassieke" histopathologische diagnose en klinische parameters, zou dit panel van prognostische markers geëvalueerd moeten worden in een grote prospectieve studie met goed gedefinieerde patiënten populaties en gestandaardiseerde technische procedures, zodat de preciese klinische relevantie bepaald kan worden.

Dankwoord

Aan het einde van dit proefschrift wil ik benadrukken dat tijdens een promotieonderzoek de hulp van, en de samenwerking met, andere mensen onmisbaar is. Naar mijn mening is samenwerken de basis voor goed onderzoek en datgene waardoor het onderzoek leuk blijft. Allen die op welke wijze dan ook betrokken zijn geweest bij de totstandkoming van dit proefschrift wil ik hiervoor hartelijk danken. Het leven is meer dan een promotieonderzoek alleen, al leek het er de laatste maand soms niet op.

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(vrij naar: Sine amicitia vitam esse nullam. Cicero, Laelius de amicitia dialogus, 23,86).

Curriculum vitae

Jacqueline Vet werd geboren op 28 maart 1963 te Gendringen. In 1981 behaalde zij het Atheneum diploma aan het Isala College te Silvolde. In hetzelfde jaar werd begonnen met de opleiding tot analist, specialisatie botanie, aan de Rijks Hogere Agrarische School te Wageningen. Na de succesvolle afronding van deze studie in 1985, werkte zij van mei 1985 tot april 1991 als analiste op het Centraal Hematologisch Laboratorium van het Academisch Ziekenhuis Nijmegen. In 1986 werd begonnen met de studie Biologie aan de Katholieke Universiteit te Nijmegen en deze studie werd in 1988 in deeltijd voortgezet aan de Rijks Universiteit van Utrecht. In februari 1991 behaalde zij het doctoraal examen Biologie met als specialisaties Microbiologie en Haematologie. In april 1991 volgde een aanstelling als wetenschappelijk medewerkster op de afdeling Urologie van het Academisch Ziekenhuis Nijmegen waar werd gewerkt aan het onderzoek waarvan de resultaten in dit proefschrift beschreven staan. Sinds 1 maart 1996 is zij werkzaam als post-doctoral fellow op de afdeling Molecular Genetics van het Public Health Research Institute te New York.

*Het Ene is in alles
alles is in het Ene
Als je dit beseft
ben je niet langer bezorgd
over je tekortkomingen.*

(Hsin Hsin Ming, oude Chinese Zen-tekst)