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Molecular and kinetic features of transitional cell carcinomas of the bladder: biological and clinical implications

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Abstract Molecular and kinetic analyses have contributed to our understanding of the biology of transitional cell carcinomas (TCC) of the bladder. The concordant pattern of X-chromosome inactivation of multiple TCCs appearing at different times and at different sites and concordant genetic abnormalities in a subset of muscle-invasive TCC strongly support a monoclonal origin and a homogeneous tumor cell selection throughout the neoplasm. However, topographic intratumor heterogeneity results from the accumulation of genetic lesions in tumor suppressor genes, predominantly neurofibromatosis (*NF*)-*1*-defective in the superficial compartment and tumor protein p53 (*TP53)*-defective in the deep one, with lower proliferation and down-regulation of apoptosis in the latter. TCCs follow the general concept of multistep carcinogenesis and proceed through two distinct genetic pathways responsible for generating different TCC morphologies. These are the inactivation of cyclin-dependent kinase inhibitors (*p15*, *p16*, and $p21^{WAF/CIP1}$) in low-grade TCC and early *TP53*-mediated abnormalities in high-grade TCC. TCC progression correlates with genetic instability and accumulation of collaborative genetic lesions mainly involving *TP53*, retinoblastoma (*RB)-1*, and growth factors. Distinctive genetic (low incidence of *RB-1* and *NF-1* abnormalities) and kinetic (slower cell turnover) profiles also correlate with a "single-file" infiltration pattern and poor survival in muscle-invasive TCCs. The underlying molecular changes of carcinoma in situ involve multiple and more extensive deletions (normally *TP53*-defective) than coexistent invasive TCC, suggesting an independent genetic evolution, while low-grade dysplasia is mainly polyclonal and shows a low rate of gene deletions.

Keywords Bladder · Transitional cell carcinoma · Oncogene · Tumor Suppressor Gene · Cell kinetics · Tumor progression · Tumor heterogeneity

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Introduction

The heterogeneity and complexity of transitional cell carcinomas (TCCs) of the urinary bladder are reflected in the number of publications. More than 700 references in the last 5 years can be retrieved from bibliography databases regarding the molecular and kinetic features of these tumors.

Two opposing theories explain multiple TCCs as multicentric (field carcinogenesis) or multifocal (spread from a single tumor). Clonality analysis has helped to resolve this disparity, but the results of any analysis must be interpreted in the appropriate setting of neoplastic transformation and cellular kinetics (tumor cell selection) [15, 17, 18]. Additionally, the association between clonality and cell kinetics closely correlates with intratumor heterogeneity, depth of invasion, and the molecular pathways [51, 52, 61]. Bladder TCCs have been classified into low-grade papillary TCC (usually superficial), high-grade TCC (normally muscle-invasive), and flat intraurothelial lesions [dysplasia, carcinoma in situ (CIS)] [19] However, this grading–staging system does not correlate with the molecular patterns and leaves undefined the relationship between low-grade dysplasia, primary CIS, and secondary CIS.

We review the role and relationship of clonality and cell kinetics in tumor progression of bladder TCC with special emphasis on intratumor heterogeneity. The molecular pathways of bladder TCCs are then considered for both invasive (low- and high-grade TCC) and intraurothelial neoplasms, with attention focusing on the ge-

netic control of proliferation and apoptosis, the two key elements in TCC development.

Tumor cell selection and kinetics in invasive TCC

Neoplasms are defined as abnormal, self-maintained proliferations, which share a common kinetic advantage and result from the accumulation of genetic lesions (multistep tumorigenesis) [7, 15, 17, 44, 53, 59, 66]. In that setting, the results of any molecular test depend on the timing between the biological feature evaluated and the specific marker and are positive only if the target participates in the molecular pathway (Fig. 1) [15, 17, 18, 39]. In addition, one must consider cell kinetics. Some mutations are, however, more important than others because they facilitate additional phenotypic or genotypic changes, thus explaining certain trends in the sequence of mutations [15, 17]. Cooperative genetic alterations in kinetically active tumor cells lead to progressive clone selection and enhance genetic diversity within a neoplasm, resulting in tumor outgrowth only if they bypass the cell repairing systems, do not activate the apoptotic pathway, and maintain proliferation [15, 18]. The lack of a distinct sequence and the unpredictability of genetic changes in malignancies preclude the extensive clinical use of those genetic markers for diagnostic and prognostic purposes.

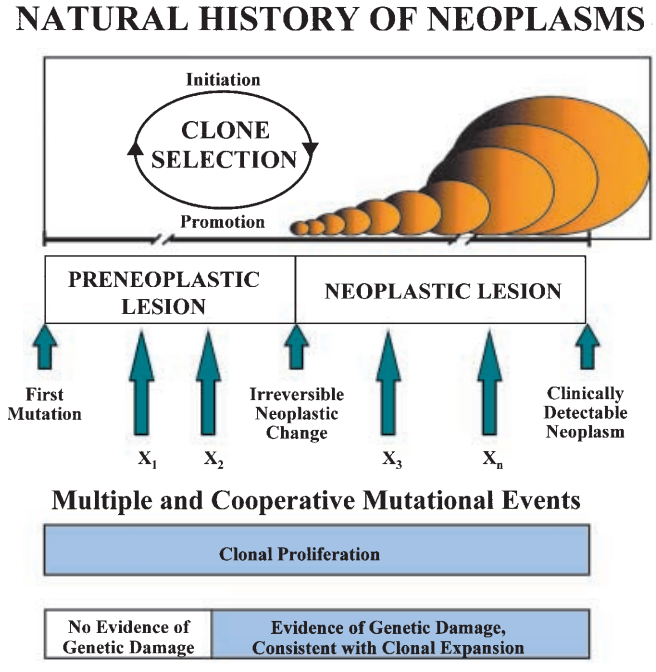


Fig. 1 Clonality analyses and tumor progression. Tumors evolve in multiple steps of initiation and promotion resulting in clone selection and eventually progression. Two main stages can be identified (preneoplastic and neoplastic), and these are separated by an irreversible genetic change. After the initial mutation, the kinetic advantage determines a clonal proliferation detectable with tests, such as X-chromosome inactivation. However, specific chromosome markers only provide positive information if mutated; they are negative before that event. This reason justifies the dual approach for clonality assays in neoplastic conditions of the bladder

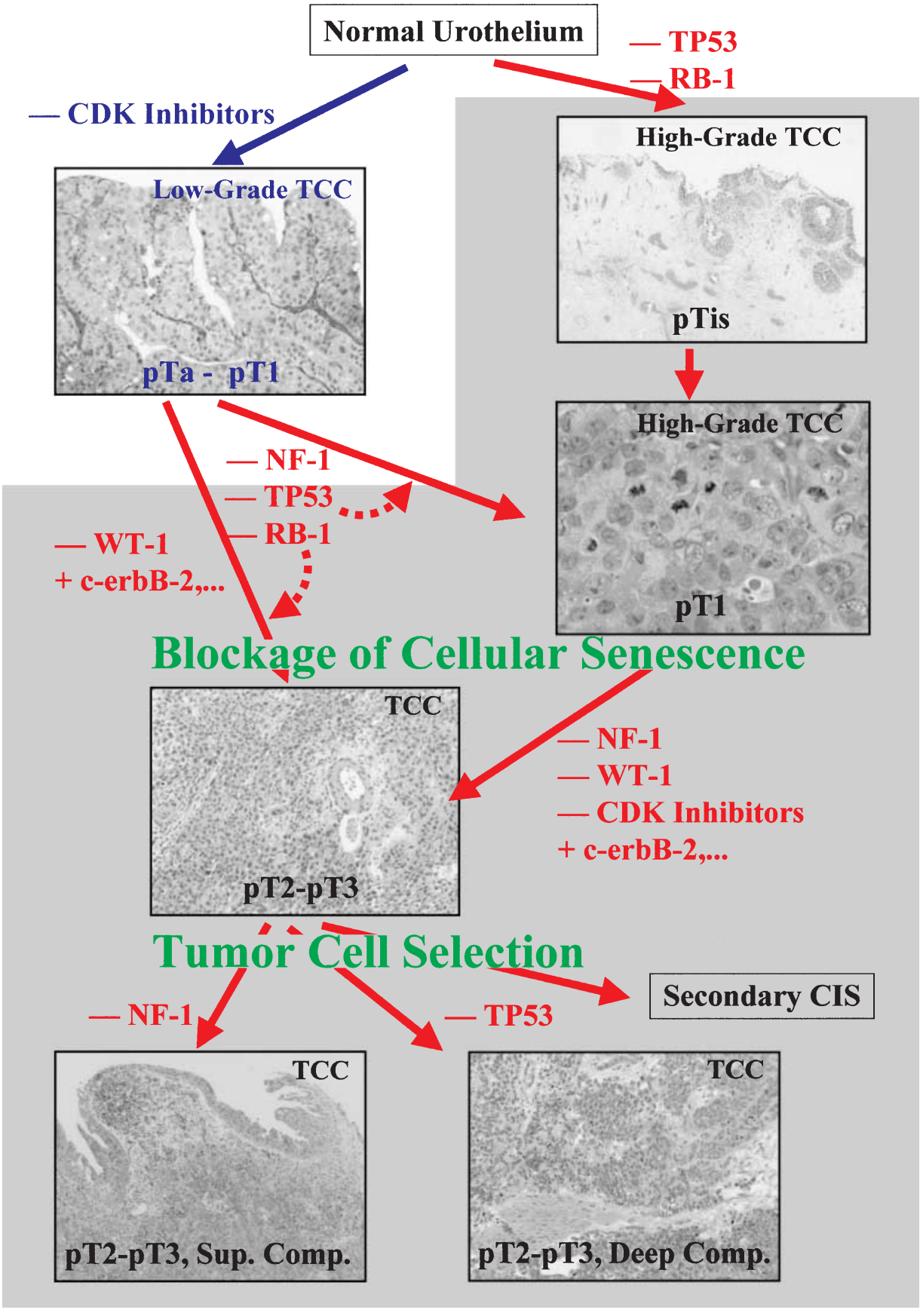
Low-grade superficial TCC often present as multiple tumors, appearing at different times and at different sites in the bladder, indicating that a "field defect" has occurred. The concordant pattern of X chromosome inactivation and microsatellite markers in multiple TCC from a single patient suggests that different TCC in a single patient derive from the same progenitor cell [59, 63]. Allele typing in each tumor from a given patient has shown loss of the same allele on chromosome 9q, suggesting that this loss preceded the spread of neoplastic cells in the bladder. Losses of 17p and 18q alleles, however, are not common to different tumors, suggesting late genetic events [59]. Similarly, the superficial and deep compartments of muscle-invasive TCCs have been demonstrated to show a concordant microsatellite pattern of tumor suppressor genes (TSG) and the same X-chromosome inactivated [4].

Coexistent genetic abnormalities involving two or more TSG loci in several samples strongly support a monoclonal origin [15, 17]. The background level of loss of heterozygosity (LOH) reported in tumors and normal tissues ranges between 4% and 20% [14, 69]. Considering all genetic lesions to be equally important and frequent, the probability of finding coexistent genetic alterations randomly would be 0.2^2 for two genetic loci, 0.2^3 for three genetic loci, and so on. Applying this principle to two separately microdissected samples (superficial and deep) from a given tumor, the probability of getting the same locus involved in both samples would be $(0.2^2)^2$ for two genetic loci or $(0.2^3)^2$ for three genetic loci. We found the same inactivated X-chromosome and concordant TSG microsatellite patterns in both compartments in 68.2% of muscle-invasive TCC, strongly supporting a monoclonal origin and a homogeneous tumor cell selection throughout the neoplasm. This finding suggests that this subset of muscle-invasive TCC arose from the uncontrolled spread of a single transformed cell and then grew through a multistep tumorigenesis that involves "common" TSG, such as tumor protein p53 (*TP53*), retinoblastoma (*RB-1*), and neurofibromatosis (*NF)-1* [16].

Intratumor heterogeneity: correlation between molecular and kinetic profiles in TCC

Histological and genetic heterogeneity is well documented in TCCs [2, 5, 28, 56, 63, 67]. After malignant transformation, tumor cells can grow independently with variable subsequent genetic alterations in each tumor com-

Fig. 2 Molecular pathways in bladder carcinogenesis. Both pathways can only be distinguished by the relative timing of genetic abnormalities. Genetic instability (*shaded area*) is mainly related to *TP53* and/or *RB-1* dysfunction that leads to tumor progression; it has been described in late stages for low-grade superficial transitional cell carcinomas (TCC) and in early phases for high-grade TCC. The biologic process of progression is determined by blockage of cellular senescence, resulting in topographic cell selection by compartments in muscle-invasive TCC [16] ▶


Normal Urothelium
— TP53
— RB-1
— CDK Inhibitors
Low-Grade TCC
pTa - pT1
High-Grade TCC
pTis
High-Grade TCC
pT1
— NF-1
— TP53
— RB-1
— WT-1
+ c-erbB-2,...
Blockage of Cellular Senescence
TCC
pT2-pT3
— NF-1
— WT-1
— CDK Inhibitors
+ c-erbB-2,...
Tumor Cell Selection
Secondary CIS
— NF-1
— TP53
TCC
pT2-pT3, Sup. Comp.
TCC
pT2-pT3, Deep Comp.

partment, explaining the heterogeneity [16, 63]. In that sense, the presence of additional clonal abnormalities in different cultures illustrates intratumoral heterogeneity [43], but tumor cell topography in the bladder wall has rarely been considered [16].

Multiple genetic lesions can be expected in muscle-invasive TCC if we consider the accumulation of genetic alterations to be an expression of molecular progression [15]. It would help to explain the relatively high incidence of genetic abnormalities reported in *RB-1* and Wilms Tumor (*WT)-1* loci [16]. Inactivated retinoblastoma protein (pRB-1) has been found in one-third of randomly selected bladder cancers [29], and *RB-1* abnormalities in 51.5% of muscle-invasive TCCs [16]. *WT-1* exon mutations were not found in any of 11 randomly selected bladder cancers using polymerase chain reaction (PCR)-single strand conformation polymorphism analysis and restriction fragment length polymorphism analysis [30]. However, more sensitive microsatellite analysis using PCR/denaturing gradient gel electrophoresis (DGGE) demonstrated WT-1 abnormalities in 53.8% of muscle-invasive TCCs [16].

Topographic genetic heterogeneity has been reported in 31.8% of muscle-invasive TCC [16], but it did not occur at the phenotypic level: the pattern of protein expression showed no relationship with LOH or single nucleotide polymorphism [4, 16]. The comparison of topographic compartments of muscle-invasive TCCs has suggested that two processes of tumor cell selection should be responsible for the topographic heterogeneity. *NF-1* was more frequently altered in tumors in the superficial compartment (75% of cases), while the deep compartment always revealed *TP53* abnormalities (Fig. 2) [16]. The *NF-1* gene has rarely been implicated in bladder carcinogenesis, and no mutations were initially reported [65]. However, the presence of *NF-1* gene abnormalities in 63.3% of muscle-invasive TCCs [16], and the decreased NF-1 mRNA and protein levels in high-grade TCCs [1] support an important role for *NF-1* in bladder carcinogenesis, especially superficial TCC compartments [16]. The NF-1 gene product has an effect on *ras* inhibition, and ras protein is expressed with the highest levels in immature and proliferating cells. The absence of the *NF-1* inhibitory effect will favor increased proliferation. *TP53* abnormalities have been reported in 67.4% of the muscle-invasive TCCs [16] and 61.1% of the invasive TCCs [58], frequently showing coexistent allelic deletions and mutations [58]. *TP53* abnormalities are more frequently found in the deep tumor compartment, a finding that may represent the consequence of tumor cell selection [16]. Similarly, the loss of chromosome 17p has been reported as a late event in tumor progression in superficial TCC [59]. Figure 2 summarizes this position [16].

The differential genetic profile by tumor compartments is also reflected on the tumor proliferation and differentiation grade. Kinetic markers (including proliferation and apoptosis) have been used as diagnostic and prognostic tools in TCC [31, 34, 37] but have revealed variability as a result of intratumor heterogeneity. Using slide cytometry, the topographic evaluation of kinetic features in 72 muscle-invasive TCCs showed significantly higher mitotic figure counts, Ki-67 index, and proliferation index in the superficial compartment than in the deep compartment. Apoptosis based on the in situ end labeling (ISEL) of fragmented DNA was revealed to be less than 1% in 63% of the superficial compartments and 86% of the deep compartments (P=0.05). These results suggest that lower proliferation activity and downregulation of apoptosis mainly define the kinetic profile in the deep compartment of muscle-invasive TCC of the bladder and correlate with the genetic profile described above [16, 51].

Molecular and kinetic patterns in urothelial dysplasia–CIS

Urothelial dysplasia is frequently identified as being associated with TCC of the urinary tract and is assumed to be the redundant precursor of invasive TCC. The Consensus Classification of Urothelial Neoplasms distinguishes low-grade urothelial dysplasia (LGUD) and high-grade urothelial dysplasia (HGUD)–CIS only [19]. Urothelial dysplasia confers a significant risk for the development of CIS and invasive TCC; cytological progression is documented but with different topography reported for LGUD and CIS in a given patient [9]. This finding supports the multifocal distribution of TCC, although does not prove the clonal identity of LGUD and CIS to sustain the sequence LGUD→CIS.

Fluorescence in situ hybridization using gene locus-specific probes for chromosomes 9q22 (*FACC*), 9p21 (*p16/CDKI2*), and 17p13 (*TP53*) has shown the same chromosome nine deletions in urothelial hyperplasias (10 of 14), coexistent low-grade papillary superficial TCCs (8 of 9), and the surrounding histologically normal urothelium (6 of 12) [27]. In contrast, 17p13 hemizygous deletion was found in the urothelial hyperplasia and papillary TCC from 1 of 12 patients, whereas the normal urothelium was always normal [27]. This genetic profile suggests a neoplastic potential for flat urothelial lesions, regardless of whether or not cytologic atypia is present. It also suggests that the earliest molecular alterations of low-grade TCC involve chromosome 9 (*p16/CDKI2*) but not chromosome 17 (*TP53*) [27]. However, this study does not analyze urothelial dysplasia and high-grade muscle-invasive TCC, which have been reported to evolve through a different molecular pathway [52, 61].

The biologic and kinetic patterns of LGUD and CIS have been revealed to be different. The analysis of dysplastic lesions associated with muscle-invasive TCC revealed monoclonal patterns in CIS (6, 100%), invasive TCC (13, 100%), and LGUD (2, 20%), whereas polyclonal patterns were observed in LGUD only (8, 80%). CIS showed aneuploid DNA content and more microsatellite loci altered (PCR/DGGE analysis) than the corresponding invasive compartments, always involving *TP53* loci and expressing abnormal p53 (12 cases) [52, 61]. In contrast, LGUD (18 cases) revealed diploid DNA content

and microsatellite abnormalities in only two cases, one monoclonal (*RB-1*) and one polyclonal (*WT-1* and *NF-1*). Opposite kinetic patterns were observed for CIS (higher Ki-67 index, lower ISEL index) and LGUD (lower Ki-67 index, higher ISEL index). These genetic findings suggest that CIS evolution is independent of muscle-invasive TCC, whereas LGUD should not be closely connected with this molecular progression. These kinetic patterns would contribute to the accumulation of genetic abnormalities in CIS but not in LGUD. LOH involving several loci have been shown in CIS, especially 9p (77%), 14q (70%), 8p (65%), 17p (60%), 13q (56%), 11p (54%), and 4q (52%), and slightly less frequently 11q (36%), 4p (32%), 3p (31%), 18q (29%), and 5q (20%) [6, 8, 23, 24, 25, 26, 57, 70]. The key molecular changes are largely unknown, but both p53 over-expression and LOH of chromosome 9 have been implicated in the progression into muscle-invasive TCC [52]. Therefore, only CIS displays the genetic alterations reported in invasive TCC, potentially accounting for the aggressive nature of these lesions [52]. However, both the differential LOH pattern found in the superficial TCC compartment (*NF-1* defective) and the accumulation of genetic damages in CIS does not support the sequence CIS→superficial TCC→deep TCC [16]. This incidence of genetic abnormalities has been revealed to be higher in CIS than in invasive TCCs, suggesting an advanced molecular stage for CIS. As presented above, several and coexistent deletions, generally involving *TP53* gene, characterize secondary CIS and suggest that CIS follows an independent progression from the invasive TCC rather than its precursor lesion.

Molecular findings of use in the classification and pathogenesis of TCC

The molecular analysis of tumors on the basis of gene expression can identify previously undetected and clinically significant subtypes of cancer. DNA microarrays are valuable in tumor classification and prognosis [21], as demonstrated in gastrointestinal tumors. This strategy will be useful in the future for discovering and predicting cancer classes, but it will require certain identification of cell populations, because it is based on gene expression. The heterogeneous differentiation normally observed in epithelial neoplasms would also limit its general application in carcinomas, although the technique can reveal useful genes for diagnosis and prognosis. In contrast, in situ hybridization allows efficient recognition of the chromosomes involved in the process of imbalance and is a valid method to assess structural chromosome aberrations in interphase nuclei of TCC [46, 50, 68].

Molecular pathways in TCC

TCC is believed to arise through a series of genetic changes affecting proliferation and apoptosis. TCC occur via multistep carcinogenesis and proceed through two distinct pathways of genetic alterations. One is associated with papillary low-grade TCC, where the initial key factor is inactivation of CDK inhibitors (*p15, p16, and* $p21^{WAF/CIP1}$). The second is associated with high-grade lesions, *TP53* abnormalities being the key element. TCC progression correlates with genetic instability and accumulation of collaborative genetic lesions, mainly involving *TP53*, *RB-1*, and growth factors (Fig. 2) [32, 61]. Papillary pTa-pT1 TCCs are often multifocal and only occasionally progress, whereas CIS frequently progress to invasive disease [19]. This distinctive morphology reflects the differential molecular background: LOH of chromosome 9 has been observed mainly in papillary low-grade TCC, while *TP53* alterations characterize high-grade TCCs. Both *TP53* and *RB-1* alterations are also known to occur in early stage bladder CIS where they are thought to indicate a poor prognosis through their propensity to progress, since these mutations are known to destabilize the genome. The presence of *TP53* mutations has been similarly reported in CIS and muscle-invasive TCCs [16, 51, 61]. Analysis of several tumor pairs involving a CIS and an invasive cancer provided evidence that chromosome 9 alteration may in some cases be involved in the progression of CIS to invasive TCC, in addition to its role in the initiation of pTa TCCs [63].

Alterations in *TP53* and *RB-1* occur in approximately 50% and 33% of bladder cancers, respectively, and are associated with high-stage, high-grade TCC [12, 13]. Amplification and/or over-expression of growth factor receptors, such as c-erbB-2 and loss of 17p and 18q sequences are associated with advanced TCC [59]. Finally, recent findings generated using in vitro transformation systems with human urothelial cells provide strong evidence that the loss of genes on 3p, reported in approximately 20% of TCC, and/or the gain of genes on 20q play an important role in blocking cellular senescence. This feature should represent a critical step in oncogenesis, because cells that do not senesce can survive to accumulate multiple genetic alterations [15], such as those associated with invasive TCC (Fig. 2) [51].

Additionally, the genetic profile has also been correlated with the infiltration pattern of muscle-invasive TCC [16]. TSG microsatellite analysis using PCR/DGGE in muscle-invasive TCCs has revealed a distinctive genetic profile in “single-file” TCCs, characterized by a low incidence of genetic abnormalities not involving the *RB-1* locus (P=0.0003) and very occasionally involving the *NF-1* locus (two cases, 13%; P=0.0023). “Single-file” TCCs showed lower cell turnover (Ki-67 index 14.94±4.28, ISEL 14.1±10.0), lower incidence of aneuploid DNA content, and shorter mean survival (20 months) than solid TCCs (Ki-67 index 20.65±4.94, ISEL 20.2±22.7, 37-month survival, respectively; unpublished observations). The relatively low cell turnover would be consistent with the low incidence of genetic alterations, especially RB-1 and NF-1, suggesting that “single-file” muscle-invasive TCCs are

related by having an alternative molecular pathway. It is not known yet whether specific alterations targeting cell adhesion molecules, thus disturbing the growth pattern, are involved.

Deregulation of the genetic control of cell kinetics in TCC

Those pathways mainly involve three basic types of genes: oncogenes, TSG, and genes involved in DNA repair. Most studies in bladder TCC have centered the attention on cell kinetic regulators of proliferation-apoptosis [*TP53*, *MDM-2*, *RB-1*, E2F-1 transcription factor, and cyclin-dependent kinase (CDK) inhibitors] and telomerase (Fig. 3).

TP53, probably the most extensively studied gene in TCC [11, 20, 51, 54, 55, 60, 61, 64], shows diverse biological functions and a heterogeneous molecular mechanism of inactivation [10]. Alterations in *TP53* have been reported to be associated with bad prognostic factors such, as high grade or stage [20, 60], or higher proliferation rates [40, 41, 48]. However, *TP53* is only one of the cell cycle regulators at the restriction point (Fig. 3) and must be considered together with other regulators, especially *MDM-2*. Its interaction with *RB-1* and its associated transcription factor (E2F-1) is also important.

The *MDM-2* gene is located on the long arm of chromosome 12 (12q13–14) and encodes for a 90 kDa nuclear protein (Mdm2) [10]. *MDM-2* links the two main regulators of the restriction point, *TP53* and *RB-1*. Mdm2 binds pRB-1 and is shown to be essential for *RB-1* to overcome both the anti-apoptotic function of Mdm2 and the *MDM-2*-dependent degradation of p53. The

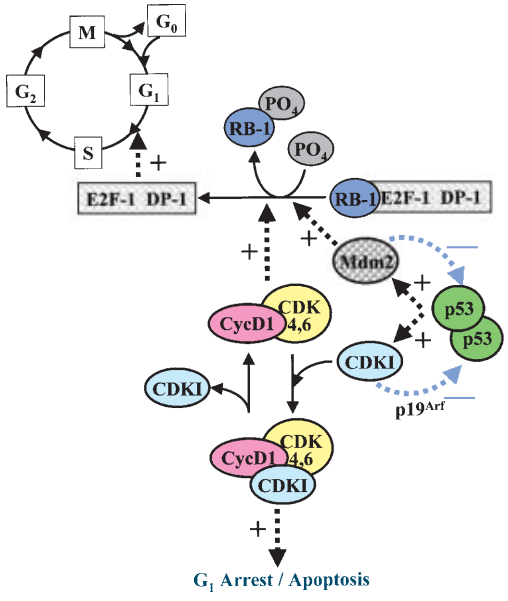


Fig. 3 Main cell cycle regulators of the G_1-S transition ('restriction point'). The final outcome (cell cycle progression vs G_1 arrest – apoptosis) is determined by the balance of several gene products

RB-MDM-2 interaction does not prevent *MDM-2* from inhibiting p53-dependent transcription, but the *RB-MDM-2* complex still binds to p53. Since *RB-1* specifically rescues the apoptotic function [22], but not the transcriptional activity of p53 from negative regulation by *MDM-2*, transactivation by wild-type p53 is not required for the apoptotic function of p53. However, a pRB-Mdm2-p53 trimeric complex is active in p53-mediated transrepression. These data link directly the function of p53 and pRB-1 and demonstrate a novel role of *RB-1* in regulating the apoptotic function of p53. TCC have demonstrated a strong statistical association between Mdm2 and p53 overexpression, with Mdm2 overexpressed in low-stage, low-grade TCC. Those results suggest that aberrant Mdm2 and p53 phenotypes are frequent events in bladder cancer and may be involved in tumorigenesis or tumor progression [35].

RB-1 plays a role similar to *TP53* in TCC: losses of functional protein correlate with aggressive behavior, high grade, and stage [38]. Both *TP53* and *RB-1* have frequently been reported to be abnormal in TCC and have prognostic significance, suggesting a cooperative mechanism in tumor progression [12, 13]. Altered p53 and undetectable pRB-1 are commonly found together and correlate with a marked increase in progression and decreased overall survival, after stratifying cases for tumor stage, tumor grade, and suspicion of vascular invasion [12]. Likewise, TCC with altered p53 and pRB-1 have shown significant increased rates of recurrence and survival, relative to patients with no alterations in either p53 or pRB-1; patients with alterations in only one of these proteins had intermediate rates of recurrence and survival [13]. These data suggest that alterations of p53 and pRB-1 have a cooperative or synergistic negative effect to promote tumor progression and in decreasing survival in primary TCC. It may be postulated that aberrant p53 and pRB-1 expression deregulates cell cycle control at the G_1 checkpoint and engenders tumor cells with reduced response to programmed cell death (Fig. 3), resulting in the aggressive clinical course of TCC harboring both p53 and pRB-1 alterations [12]. In addition, cases with undetectable and high pRB-1 reactivity had identical rates of recurrence, indicating that high levels of pRB-1 expression may reflect a dysfunctional (altered) *RB-1* pathway and do not reflect the tumor suppressor effects of the protein [13].

In the complex regulation of the restriction point, cyclin D1 and the transcription factor E2F-1 (Fig. 3) play a role and have been implicated in bladder carcinogenesis. *Cyclin D1* can also cooperate to dysregulate the G_1 checkpoint, which becomes completely abolished only if *RB-1* is lost, removing any selective advantage for cells that alter additional cell cycle proteins [42]. E2F-1 is a transcription factor that binds to pRB-1 [10]. The results reported on TCC have demonstrated occasional nucleotide polymorphism and no bandshifts in the nuclear-localization or DNA-binding domains.[49]. *E2F-1* alterations occur at the phenotypic level, rather than at the genotypic level, in TCC: the pattern of E2F-1 protein ex-

pression has shown no relationship with nucleotide polymorphism but correlated inversely with the percentage of cells showing pRB-1 reactivity. Rabbani et al. also reported that patients with lower E2F-1 reactivity had statistically significant increased risks of progression to metastases and death, suggesting a possible tumor suppressor role for E2F-1 in TCC [49].

CDK inhibitors implicated in bladder carcinogenesis include *p15*, *p16*, and *p21$^{WAF/CIP1}$*. *p16* (also known as *CDKN2, INK4a, or MTS1*) and *p15* (also described as *INK4b or MTS2*) are found in tandem on chromosome 9p21. They encode proteins that function as negative cell cycle regulators encoding inactivating polypeptides that inactivate specific cyclin-protein kinase complexes required for progression through the cell cycle (Fig. 3) [10]. The overall frequency of alteration reported for these genes in TCC is approximately 18% for each one, being significantly associated with low stage and grade TCC for *p16* gene and low stage TCC for *p15* gene alterations [45]. Overall, 70% of the TCCs showed abnormalities in one or more of the intrinsic proteins of the G_1 checkpoint [42], supporting the cooperative role of *p16* and *p15* gene alterations (especially coincident homozygous deletion) as a common event in bladder carcinogenesis [42, 45]. These CDK inhibitors cross-talk several molecular pathways, as demonstrated for the *INK4a* gene [47]. The *INK4a* gene encodes two distinct growth inhibitors – the CDK inhibitor p16Ink4a, which is a component of the *RB-1* pathway and the tumor suppressor p19Arf, which has been functionally linked to p53. p19Arf potently suppresses the oncogenic transformation in primary cells, and this function is abrogated when *TP53* is neutralized by viral oncoproteins and dominant-negative mutants but not by the *TP53* antagonist *MDM-2*. Coupled with the findings that p19Arf and Mdm2 physically interact and that p19Rrf blocks *MDM-2*-induced p53 degradation and transactivational silencing, the results reported suggest that p19Arf functions mechanistically to prevent neutralization of p53 by *MDM-2*. All of these findings together ascribe the potent tumor suppressor activity of INK4a to the cooperative actions of its two protein products and their relation to the two central growth control pathways, *RB-1* and *TP53* [47]. The action of *TP53* on cell cycle regulation is mediated, in part, through the expression of p21$^{WAF/CIP1}$, as suggested by the significant association between p21$^{WAF/CIP1}$ expression and p53 status [62]. p21$^{WAF/CIP1}$ expression has been demonstrated to be an independent predictor of TCC recurrence and of survival when assessed with tumor grade, tumor stage, lymph node status, and p53 status: patients with p53-altered/p21-negative TCC demonstrated a higher rate of recurrence and worse survival when compared with those with p53-altered/p21-positive tumors [62]. Those results also suggest that maintenance of p21$^{WAF/CIP1}$ expression appears to abrogate the deleterious effects of *TP53* alterations on TCC progression.

Telomerase activation may be a critical step in TCC pathogenesis.[33, 36, 71]. Unexpectedly, no significant correlation was observed between levels of telomerase expression and the clinicopathologic features of the tumors, including clinical stage, pathologic grade, tumor multiplicity, and status of recurrence [33]. However, proliferating human urothelial cells in tissue culture reveal telomerase activity, which is readily detected usually at lower levels than in TCC, and telomeres did not shorten [3]. Notably, telomerase activity was relatively low or undetectable in non-proliferating cultures. These data support a model in which the detection of telomerase in TCC biopsies reflects differences in proliferation between tumor and normal cells in vivo [3].

In conclusion, the molecular analysis of bladder TCC has improved the classification and our knowledge on the pathogenesis of these neoplasms. Two main molecular pathways have been clearly demonstrated, which closely correlate with the morphological appearance. These are the inactivation of cyclin-dependent kinase inhibitors in low-grade TCC and early *TP53*-mediated abnormalities in high-grade TCC. However, the heterogeneity associated with advanced neoplasms needs a careful correlation of genetic and kinetic features to understand cellular progression and tumor cell selection in TCC. This combined analysis becomes more powerful when coupled with reliable topographic features, because it will allow the identification of aggressive tumor cell clones susceptible of genetic therapy.

References

1. Aaltonen V, Bostrom PJ, Soderstrom KO, Hirvonen O, Tuukkanen J, Nurmi M, Laato M, Peltonen J (1999) Urinary bladder transitional cell carcinogenesis is associated with down-regulation of NF1 tumor suppressor gene in vivo and in vitro. Am J Pathol 154:755–765
2. Baithun S, Daruwala P, Oliver RTD (1998) Squamous change in bladder cancer and its relevance to understanding clonal evolution in development of bladder cancer. Cancer Surveys 31:17–27
3. Belair CD, Yeager TR, Lopez PM, Reznikoff CA (1997) Telomerase activity: a biomarker of cell proliferation, not malignant transformation. Proc Natl Acad Sci USA 94:13677–13682
4. Blanes A, Rubio J, Garcia I, Sanchez-Carrillo JJ, Matilla A, Diaz-Cano SJ (1999) Genetic changes of cell cycle regulators define topographic compartments in muscle-invasive transitional cell carcinoma of the urinary bladder (abstract). J Pathol 187:12
5. Brown JL, Russell PJ, Philips J, Wotherspoon J, Raghavan D (1990) Clonal analysis of a bladder cancer cell line: an experimental model of tumour heterogeneity. Br J Cancer 61:369–376
6. Cairns P, Proctor AJ, Knowles MA (1991) Loss of heterozygosity at the RB locus is frequent and correlates with muscle invasion in bladder carcinoma. Oncogene 6:2305–2309
7. Califano J, van der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W, Sidransky D (1996) Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res 56: 2488–2492
8. Cappellen D, Gil Diez de Medina S, Chopin D, Thiery JP, Radvanyi F (1997) Frequent loss of heterozygosity on chromosome 10q in muscle-invasive transitional cell carcinomas of the bladder. Oncogene 14:3059–3066
9. Cheng L, Cheville JC, Neumann RM, Bostwick DG (1999) Natural history of urothelial dysplasia of the bladder. Am J Surg Pathol 23:443–447

10. Cordon-Cardo C (1995) Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 147:545–560
11. Cordon-Cardo C, Reuter VE (1997) Alterations of tumor suppressor genes in bladder cancer. Semin Diagn Pathol 14: 123–132
12. Cordon-Cardo C, Zhang ZF, Dalbagni G, Drobnjak M, Charytonowicz E, Hu SX, Xu HJ, Reuter VE, Benedict WF (1997) Cooperative effects of p53 and pRB alterations in primary superficial bladder tumors. Cancer Res 57:1217–1221
13. Cote RJ, Dunn MD, Chatterjee SJ, Stein JP, Shi SR, Tran QC, Hu SX, Xu HJ, Groshen S, Taylor CR, Skinner DG, Benedict WF (1998) Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. Cancer Res 58:1090–1094
14. Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS (1996) Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 274:2057–2059
15. Diaz-Cano SJ (1998) Clonality studies in the analysis of adrenal medullary proliferations: application principles and limitations. Endocr Pathol 9:301–316
16. Diaz-Cano SJ, Blanes A, Rubio J, Matilla A, Wolfe HJ (2000) Molecular evolution and intratumor heterogeneity by topographic compartments in muscle-invasive transitional cell carcinoma of the urinary bladder. Lab Invest 80:279–289
17. Diaz-Cano SJ, Blanes A, Wolfe HJ (2000) PCR-based techniques for clonality analysis of neoplastic progression. Bases for its appropriate application in paraffin-embedded tissues. Diagn Mol Pathol (in press)
18. Diaz-Cano SJ, de Miguel M, Blanes A, Tashjian R, Galera H, Wolfe HJ (2000) Clonality as expression of distinctive cell kinetic patterns in nodular hyperplasias and adenomas of the adrenal cortex. Am J Pathol 156:311–319
19. Epstein JI, Amin MB, Reuter VR, Mostofi FK (1998) The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. Am J Surg Pathol 22:1435–1448
20. Esrig D, Spruck CHd, Nichols PW, Chaiwun B, Steven K, Groshen S, Chen SC, Skinner DG, Jones PA, Cote RJ (1993) p53 nuclear protein accumulation correlates with mutations in the p53 gene, tumor grade, and stage in bladder cancer. Am J Pathol 143:1389–1397
21. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286:531–537
22. Haas-Kogan DA, Kogan SC, Levi D, Dazin P, T'Ang A, Fung YK, Israel MA (1995) Inhibition of apoptosis by the retinoblastoma gene product. Embo J 14:461–472
23. Habuchi T, Ogawa O, Kakehi Y, Ogura K, Koshiba M, Sugiyama T, Yoshida O (1992) Allelic loss of chromosome 17p in urothelial cancer: strong association with invasive phenotype. J Urol 148:1595–1599
24. Habuchi T, Ogawa O, Kakehi Y, Ogura K, Koshiba M, Hamazaki S, Takahashi R, Sugiyama T, Yoshida O (1993) Accumulated allelic losses in the development of invasive urothelial cancer. Int J Cancer 53:579–584
25. Habuchi T, Devlin J, Elder PA, Knowles MA (1995) Detailed deletion mapping of chromosome 9q in bladder cancer: evidence for two tumour suppressor loci. Oncogene 11:1671–1674
26. Habuchi T, Yoshida O, Knowles MA (1997) A novel candidate tumour suppressor locus at 9q32–33 in bladder cancer: localization of the candidate region within a single 840 kb YAC. Hum Mol Genet 6:913–919
27. Hartmann A, Moser K, Kriegmair M, Hofstetter A, Hofstaedter F, Knuechel R (1999) Frequent genetic alterations in simple urothelial hyperplasias of the bladder in patients with papillary urothelial carcinoma. Am J Pathol 154:721–727
28. Hierro Martin I, Alvarez Perez M, Blanes Berenguel A, Quinonero Diaz A, Diaz Cano S, Garcia Gonzalez I, Matilla Vicente A (1999) Contribucion a la categorizacion de los CCT de vejiga, sistematizacion de la gradacion y utilidad de la ploidia de ADN. Actas Urol Esp 23:489–496
29. Horowitz JM, Park SH, Bogenmann E, Cheng JC, Yandell DW, Kaye FJ, Minna JD, Dryja TP, Weinberg RA (1990) Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. Proc Natl Acad Sci USA 87:2775–2779
30. Kageyama Y, Yamamura Y, Oshima H, Ikawa Y (1995) Infrequent mutations of the WT1 gene in primary cancers of the adult urinary tract. Jpn J Clin Oncol 25:173–178
31. King ED, Matteson J, Jacobs SC, Kyprianou N (1996) Incidence of apoptosis, cell proliferation and bcl-2 expression in transitional cell carcinoma of the bladder: association with tumor progression. J Urol 155:316–320
32. Kroft SH, Oyasu R (1994) Urinary bladder cancer: mechanisms of development and progression. Lab Invest 71:158–174
33. Kyo S, Kunimi K, Uchibayashi T, Namiki M, Inoue M (1997) Telomerase activity in human urothelial tumors. Am J Clin Pathol 107:555–560
34. Lara PC, Perez S, Rey A, Santana C (1999) Apoptosis in carcinoma of the bladder: relation with radiation treatment results. Int J Radiat Oncol Biol Phys 43:1015–1019
35. Lianes P, Orlow I, Zhang ZF, Oliva MR, Sarkis AS, Reuter VE, Cordon-Cardo C (1994) Altered patterns of MDM2 and TP53 expression in human bladder cancer. J Natl Cancer Inst 86:1325–1330
36. Linn JF, Lango M, Halachmi S, Schoenberg MP, Sidransky D (1997) Microsatellite analysis and telomerase activity in archived tissue and urine samples of bladder cancer patients. Int J Cancer 74:625–629
37. Lipponen PK, Aaltomaa S (1994) Apoptosis in bladder cancer as related to standard prognostic factors and prognosis. J Pathol 173:333–339
38. Lipponen PK, Liukkonen TJ (1995) Reduced expression of retinoblastoma (Rb) gene protein is related to cell proliferation and prognosis in transitional-cell bladder cancer. J Cancer Res Clin Oncol 121:44–50
39. Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D (1994) Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci USA 91: 9871–9875
40. Moch H, Sauter G, Mihatsch MJ, Gudat F, Epper R, Waldman FM (1994) p53 but not erbB-2 expression is associated with rapid tumor proliferation in urinary bladder cancer. Hum Pathol 25:1346–1351
41. Nakopoulou L, Constantinides C, Papandropoulos J, Theodoropoulos G, Tzonou A, Giannopoulos A, Zervas A, Dimopoulos C (1995) Evaluation of overexpression of p53 tumor suppressor protein in superficial and invasive transitional cell bladder cancer: comparison with DNA ploidy. Urology 46:334–340
42. Niehans GA, Kratzke RA, Frober g MK, Aeppli DM, Nguyen PL, Geradts J (1999) G1 checkpoint protein and p53 abnormalities occur in most invasive transitional cell carcinomas of the urinary bladder. Br J Cancer 80:1175–1184
43. Nordenson I, Ljungberg B, Roos G (1988) Chromosomes in renal carcinoma with reference to intratumor heterogeneity. Cancer Genet Cytogenet 32:35–41
44. Nowell PC (1976) The clonal evolution of tumor cell populations. Science 194:23–28
45. Orlow I, Lacombe L, Hannon GJ, Serrano M, Pellicer I, Dalbagni G, Reuter VE, Zhang ZF, Beach D, Cordon-Cardo C (1995) Deletion of the p16 and p15 genes in human bladder tumors. J Natl Cancer Inst 87:1524–1529
46. Poddighe PJ, Ramaekers FC, Smeets AW, Vooijs GP, Hopman AH (1992) Structural chromosome 1 aberrations in transitional cell carcinoma of the bladder: interphase cytogenetics combining a centromeric, telomeric, and library DNA probe. Cancer Res 52:4929–4934
47. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW, Cordon-Cardo C, DePinho RA (1998) The Ink4a tumor suppressor

gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 92:713–723
48. Popov Z, Hoznek A, Colombel M, Bastuji-Garin S, Lefrere-Belda MA, Bellot J, Abboh CC, Mazerolles C, Chopin DK (1997) The prognostic value of p53 nuclear overexpression and MIB-1 as a proliferative marker in transitional cell carcinoma of the bladder. Cancer 80:1472–1481
49. Rabbani F, Richon VM, Orlow I, Lu ML, Drobnjak M, Dudas M, Charytonowicz E, Dalbagni G, Cordon-Cardo C (1999) Prognostic significance of transcription factor E2F-1 in bladder cancer: genotypic and phenotypic characterization. J Natl Cancer Inst 91:874–881
50. Reeder JE, O'Connell MJ, Yang Z, Morreale JF, Collins L, Frank IN, Messing EM, Cockett AT, Cox C, Robinson RD, Wheeless LL (1998) DNA cytometry and chromosome 9 aberrations by fluorescence in situ hybridization of irrigation specimens from bladder cancer patients. Urology 51:58–61
51. Reznikoff CA, Belair CD, Yeager TR, Savelieva E, Blelloch RH, Puthenveettil JA, Cuthill S (1996) A molecular genetic model of human bladder cancer pathogenesis. Semin Oncol 23:571–584
52. Rosin MP, Cairns P, Epstein JI, Schoenberg MP, Sidransky D (1995) Partial allelotype of carcinoma in situ of the human bladder. Cancer Res 55:5213–5216
53. Ruppert JM, Tokino K, Sidransky D (1993) Evidence for two bladder cancer suppressor loci on human chromosome 9. Cancer Res 53:5093–5095
54. Sarkis AS, Dalbagni G, Cordon-Cardo C, Zhang ZF, Sheinfeld J, Fair WR, Herr HW, Reuter VE (1993) Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. J Natl Cancer Inst 85:53–59
55. Sarkis AS, Dalbagni G, Cordon-Cardo C, Melamed J, Zhang ZF, Sheinfeld J, Fair WR, Herr HW, Reuter VE (1994) Association of P53 nuclear overexpression and tumor progression in carcinoma in situ of the bladder. J Urol 152:388–392
56. Schapers R, Smeets W, Hopman A, Pauwels R, Geraedts J, Ramaekers F (1993) Heterogeneity in bladder cancer as detected by conventional chromosome analysis and interphase cytogenetics. Cancer Genet Cytogenet 70:56–61
57. Shipman R, Schraml P, Colombi M, Raefle G, Ludwig CU (1993) Loss of heterozygosity on chromosome 11p13 in primary bladder carcinoma. Hum Genet 91:455–458
58. Sidransky D, Von Eschenbach A, Tsai YC, Jones P, Summerhayes I, Marshall F, Paul M, Green P, Hamilton SR, Frost P, Vogelstein B (1991) Identification of p53 gene mutations in bladder cancers and urine samples. Science 252:706–709
59. Sidransky D, Frost P, Von Eschenbach A, Oyasu R, Preisinger AC, Vogelstein B (1992) Clonal origin bladder cancer. N Engl J Med 326:737–740
60. Soini Y, Turpeenniemi-Hujanen T, Kamel D, Autio-Harmainen H, Risteli J, Risteli L, Nuorva K, Paakko P, Vahakangas K (1993) p53 immunohistochemistry in transitional cell carcinoma and dysplasia of the urinary bladder correlates with disease progression. Br J Cancer 68:1029–1035
61. Spruck CH 3rd, Ohneseit PF, Gonzalez-Zulueta M, Esrig D, Miyao N, Tsai YC, Lerner SP, Schmutte C, Yang AS, Cote R, et al. (1994) Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res 54:784–788
62. Stein JP, Ginsberg DA, Grossfeld GD, Chatterjee SJ, Esrig D, Dickinson MG, Groshen S, Taylor CR, Jones PA, Skinner DG, Cote RJ (1998) Effect of p21WAF1/CIP1 expression on tumor progression in bladder cancer. J Natl Cancer Inst 90:1072–1079
63. Takahashi T, Habuchi T, Kakehi Y, Mitsumori K, Akao T, Terachi T, Yoshida O (1998) Clonal and chronological genetic analysis of multifocal cancers of the bladder and upper urinary tract. Cancer Res 58:5835–5841
64. Tetu B, Fradet Y, Allard P, Veilleux C, Roberge N, Bernard P (1996) Prevalence and clinical significance of HER/2neu, p53 and Rb expression in primary superficial bladder cancer. J Urol 155:1784–1788
65. Uchida T, Wada C, Ishida H, Egawa S, Ao T, Yokoyama E, Koshiba K (1995) Infrequent involvement of mutations on neurofibromatosis type 1, H-ras, K-ras and N-ras in urothelial tumors. Urol Int 55:63–67
66. Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R (1989) Allelotype of colorectal carcinomas. Science 244:207–211
67. Voorter C, Joos S, Bringuier PP, Vallinga M, Poddighe P, Schalken J, du Manoir S, Ramaekers F, Lichter P, Hopman A (1995) Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization. Am J Pathol 146:1341–1354
68. Watters AD, Ballantyne SA, Going JJ, Grigor KM, Bartlett JM (2000) Aneusomy of chromosomes 7 and 17 predicts the recurrence of transitional cell carcinoma of the urinary bladder. BJU Int 85:42–47
69. Wolman SR, Heppner GH (1992) Genetic heterogeneity in breast cancer. J Natl Cancer Inst 84:469–470
70. Xu HJ, Cairns P, Hu SX, Knowles MA, Benedict WF (1993) Loss of RB protein expression in primary bladder cancer correlates with loss of heterozygosity at the RB locus and tumor progression. Int J Cancer 53:781–784
71. Yoshida K, Sugino T, Tahara H, Woodman A, Bolodeoku J, Nargund V, Fellows G, Goodison S, Tahara E, Tarin D (1997) Telomerase activity in bladder carcinoma and its implication for noninvasive diagnosis by detection of exfoliated cancer cells in urine. Cancer 79:362–369