PREFACE

Bladder Cancer: Current Diagnosis and Treatment has been designed to present state-of-the-art information on and understanding of the many aspects of bladder cancer that factor into decisions regarding its assessment and treatment. The guiding byword in this is the term *"understanding,"* and the authors were thus asked to encapsulate their comprehensive knowledge of their specific topics, offer their critical perspectives on the ever increasing amount of fresh data, and provide their insights regarding precisely what meaningfully advances our knowledge and understanding of bladder cancer.

The critical task in working toward this objective was to identify those who could provide all of these attributes—knowledge, perspective, and insight—in addressing the various areas that comprise this volume. In addition, because a substantial number of issues might still be unresolved, or in many instances be controversial, an attempt was made to recruit contributors who might offer differing perspectives on a given topic. This permitted an exposition on both sides of a given issue. Although this made the draft of each chapter a more onerous undertaking, the final result was a more complete compendium of reliably and objectively presented information.

I am indebted to each of our contributors for having undertaken their assignments with enthusiasm and with a productive application of their uniquely valuable knowledge and perspectives. To those who tired of my frequent calls and obsessive editing, I apologize. I do hope, however, that the final product reflects the focused attention and tremendous effort that was invested.

I am grateful to each contributor for having educated me to an extent that I had not anticipated. It is now time to share this with our readership with the hope that the information contained in *Bladder Cancer: Current Diagnosis and Treatment* will prove useful both as an information base and as a stimulant for further study.

Finally, I am grateful to Dr. Eric Klein of the Department of Urology, the Cleveland Clinic Foundation, for having permitted me to organize and edit this monograph; and to Mr. Paul Dolgert, Craig Adams, and the Humana Press for all of their assistance in bringing this project to fruition.

***Michael J. Droller,* MD**

2 Biology and Molecular Aspects of Development and Progression of Bladder Cancer

Dan Theodorescu, MD, PHD
and William A. See, MD

CONTENTS

INTRODUCTION AND OBJECTIVES

Carcinoma of the urinary bladder is the second most common urologic malignancy *(1)*. Not only are neoplasms of the urinary bladder of high clinical relevance, but they represent one of the best understood of the genito-urinary (GU) neoplasms. Relative to other tumors, the etiology, natural history, tumor biology, treatment options and outcome for the spectrum of bladder malignancies are well defined. This level of understanding arises as a consequence of multiple factors and represents a convergence of knowledge from diverse scientific disciplines. Insight provided by these disciplines, coupled with unique features of this neoplasm which make it assessable for detection, monitoring and treatment, combine to make this disease a model system for modern oncology.

From: *Current Clinical Urology: Bladder Cancer: Current Diagnosis and Treatment*
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The intent of this chapter is to provide the reader an overview of our current understanding of this tumor from the standpoint of its clinical and molecular biology. To this end, a brief review of the clinical biology will be provided to serve as a backdrop against which our molecular understanding of this tumor has proceeded. Indeed the process through which our insights into this neoplasm have evolved, "hypothesis generating" clinical observation followed by scientific investigation, can serve as a paradigm for our work in other GU tumors.

Proceeding from a brief review of the clinical biology it is the author's intent to present our current understanding of the molecular basis for the biology of this disease. Finally in closing, we will provide a brief consideration of what relevance these molecular insights may hold for the future clinical management of patients with bladder neoplasms in terms of detection, monitoring, treatment and prognosis.

CLINICAL BIOLOGY AND NATURAL HISTORY

Histologic Types of Bladder Cancer

Neoplasms involving the urinary bladder encompass a spectrum of histologic types *(2)*. Any of the cellular elements composing the bladder wall and its lining can undergo malignant transformation. Furthermore, specific elements may de-differentiate into more primitive phenotypes under the influence of specific etiologic factors. In order of prevalence, the histologic variants comprising bladder neoplasms are transitional, squamous cell, adenocarcinoma, and finally sarcoma. While there are significant geographic variations in the relative incidence of these different histologic types, particularly as it pertains to squamous cell carcinoma, in the United States, transitional cell carcinoma in its variants are by far the most common type *(2)*. In addition to marked differences in their incidence, squamous cell carcinoma and adenocarcinomas involving the urinary bladder are distinct from transitional cell carcinomas in multiple respects including their etiology, epidemiology, and clinical biology. It is beyond the scope of this chapter to attempt to exhaustively deal with these differences. Consequently this work will focus exclusively on transitional cell carcinoma of the urinary bladder.

As a point of histologic clarification it is important to understand that it is not uncommon for transitional cell malignancies to have minor elements which have dedifferentiated along adenomatous or squamous cell lines. However, from the clinical management standpoint, urinary neoplasms with minor components of these two histologic types are treated for their primary component. The clinical relevance of these minor

components or the percentage at which a minor component becomes clinically significant is poorly defined at this time.

Clinical Biology: Superficial Tumors

In the transformation pathway which leads to the development of superficial transitional carcinoma the most striking biologic feature is the propensity for tumor recurrence. Recurrence polychronotropism (multiple in space and time) in superficial bladder tumors is uniquely high relative to any other organ site. Up to 70% of patients ultimately suffer disease recurrence *(3,4)*. While in the absence of progression recurrence per se is not life threatening, this phenomenon nonetheless constitutes a cause of significant morbidity and treatment expense. While less common, the progression of superficial tumors to muscle invasion has potential mortal consequence. Progression risks vary widely by stage, and grade ranging from less than 5% for TA grade 1 up to 50% for T1 lesions with associated carcinoma *in situ (5)*.

Clinical Biology: Invasive Tumors

For tumors in the invasive pathway, the pivotal issue dictating patient survival is tumor metastasis. The propensity for invasive TCC to disseminate by both lymphatic and hematogenous routes is clearly established. At some point in their disease history 40–50% of patients in this group will manifest metastatic disease *(6,7)*. Liver, lung, and regional lymph nodes represent the sites most commonly involved by metastatic disease.

Predictors of Clinical Behavior

The management of urothelial neoplasms is undergoing a period of transition where a number of new markers are being assessed for their clinical utility relative to the conventional prognostic benchmarks of tumor stage and grade. As an example, there is an active phase III trial which stratifies patient management according to *P53* status *(8)*. However, at this juncture, the "historic" parameters remain the proven and widely utilized prognosticators upon which clinical decision making is based. Even so, the current classification system has shortcomings. The staging system for superficial bladder tumors illustrates some of these limitations.

Superficial bladder tumors are defined as limited to the bladder layers above the muscularis propria. While not a surprising consequence of the "superficial" vs "invasive" categorization, the tendency of some clinicians to view so called superficial disease as a single entity represents

a serious shortcoming of the present staging system. In reality, tumors within the superficial category are far more complex and represent a spectrum of malignancy encompassing no less than 7 stage/grade categories. Each of these has a potentially different relevant clinical endpoint. Despite this seeming complexity, these seven categories can be broadly divided into two subcategories based upon their relevant clinical endpoints of either recurrence or progression. As a high recurrence risk is a common feature of all categories of superficial bladder cancer it is progression risk that primarily distinguishes them. We propose that for the purpose of clinical decision making, superficial bladder cancer should therefore be divided into low and high risk categories based upon progression risk. Low risk tumors would encompass grade 1 and 2 Ta lesions with high risk tumors including carcinoma *in situ*, T1, and any grade 3 tumor. The above proposal is not intended to supplant the need for TNM staging but rather to be complimentary and highlight to the clinician the distinct biology of the different stage/grade classifications of so called superficial tumors.

The current stage and grading system works well for clinical management of the invasive categories of bladder neoplasms. In general, the staging system distinguishes organ confined vs metastatic neoplasms. These categorizations provide good correlation with prognosis and treatment outcome. The recognition that extravesical tumor spread, and node positivity portend poorer outcome, are currently being used as the basis for patient stratification into studies of adjuvant chemotherapy.

Stage and grade have been and remain important clinical tools for patient management. However, the current system functions best at the extremes of the neoplastic process, specifically low grade/stage tumors and high grade/stage tumors. Regrettably, a significant portion of patients will fall between these boundaries. For these patients stage and grade classification remain relatively crude indicators of individual tumor biology. The next section will discuss exciting work, which is making progress towards a more sophisticated understanding of the molecular level cellular functions, which constitute the very soul of tumor biology.

TUMOR DEVELOPMENT

The first insight into the etiology of transitional cell carcinomas of the urinary bladder began with the observation of an increased incidence associated with industrial development. Workers in the aniline dye industry in Germany were noted to be at increased risk for the development

of this tumor *(9)*. This association made bladder neoplasms the first of what would subsequently be recognized as many chemically induced tumors. Subsequent understanding has come to identify the process of uroepithelial transformation as one of contact carcinogenesis. Carcinogens ingested by one of multiple routes, either inhaled, consumed, or absorbed through the skin, are concentrated in the urine and subsequently come in contact with the lining of the urinary tract. This diffuse exposure predisposes to what has come to be known as field change. Thus the entire uroepithelium to which urine has been exposed may have multiple areas of frank or preneoplastic transformation.

Early clinical observations regarding the biology of the "at risk" field suggested that sites of preneoplastic changes could follow several distinct clinical courses. It is possible that areas of dysplasia remain simply dysplastic. Alternatively, the urinary epithelium can progress either to superficial bladder neoplasms, characterized by recurrence but rare life threatening progression, or along the path towards invasion with its well recognized risk of mortality. Evidence in support of these disparate pathways comes from the low progression rate of the majority of superficial bladder tumors, coupled with the fact that many invasive neoplasms present as such initially. An example of a clinical evidence based pathway detailing these distinctions in tumor biology is illustrated in Fig. 1 *(10)*. Insight afforded by these clinical observations has played a central role in generating hypotheses, developing models, and directing basic research in bladder cancer. Not only have these clinical observations served as the basis for research undertakings, but these subsequent research activities have in turn provided strong evidence to support the validity of these clinical models.

Carcinogenesis

Models of molecular carcinogenesis must explain the relevant clinical natural history and aspects of tumor behavior such as uncontrolled cellular proliferation, neovascularization, and altered apoptosis. In addition, models of neoplastic transformation should account for other clinically relevant features of the neoplasm in question. For superficial and invasive transitional cell carcinomas of the urinary bladder, these would include tumor recurrence and tumor metastasis respectively.

The historic view of two stage carcinogenesis in which tumor initiation (mutation) is followed by tumor promotion (epigenetic changes) has been conceptually important but is currently thought to be too simplistic. It is now believed that there may be six or more independent mutational events *(11,12)* necessary for carcinogenesis. Furthermore,

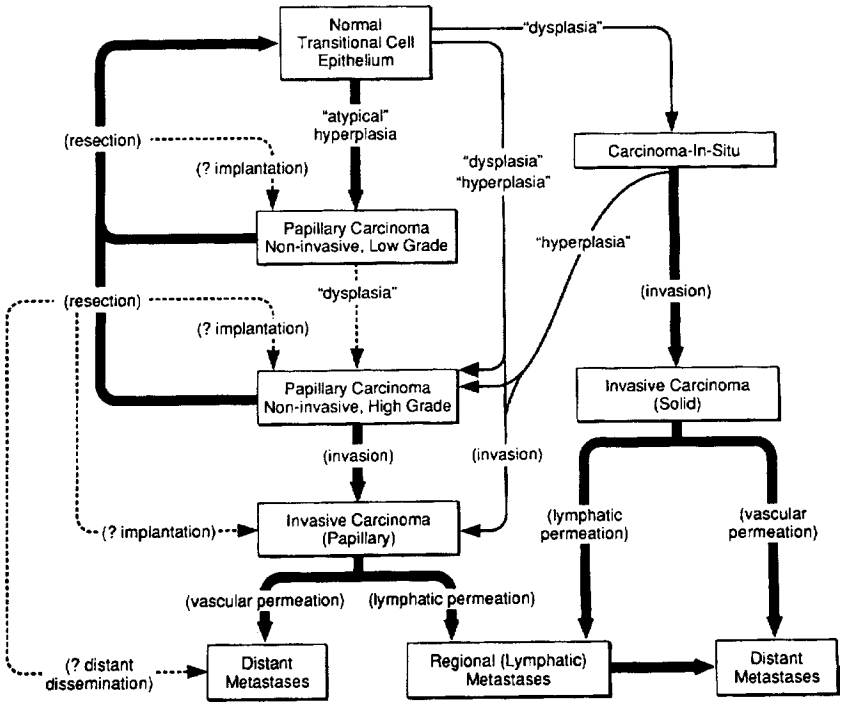

Fig. 1. Proposed pathway for bladder tumor development derived from clinical observation. Note that the superficial and invasive pathways are distinct, with divergence early in the process of tumorigenesis. (From Jones PA, Droller MJ. Pathways of development and progression in bladder cancer: new correlations between clinical observations and molecular mechanisms. Semin Urol 1993; 11: 177–192).

chemical carcinogens may be genotoxic, non-genotoxic *(13)* or induce epigenetic effects *(14)* with dose response relations being linear or non-linear *(15,16)*. Endogenous mutagenic mechanisms such as DNA oxy-radical damage, de-purination and polymerase infidelity also contribute to carcinogenesis *(12,17–19)* leading to a debate regarding the relative importance of endogenous versus exogenous mutagenic events and the value of animal bioassays or short term mutagenic assays for assessment of human cancer risks *(11,18,20,21)*. In the section below we will discuss two of the best characterized molecular paradigms leading to transitional cell carcinoma. Together, these will highlight how the effect of a chemical carcinogen may be altered by the characteristics of the host and serve as both a model system and framework for further research in this area.

Many different exposures and risk factors have been identified in bladder cancer. In the late nineteenth century, the German physician Rehn observed an association between the occurrence of bladder cancer and exposure of workers to aromatic amines (arylamines) and polycyclic aromatic hydrocarbons (PAH) compounds found in the dyestuff industry. In addition to these environmental exposures, tobacco smoking has also been associated with an elevated risk for multiple types of human cancers *(22)*. Several of the chemicals identified in tobacco smoke have been shown to cause cancer in laboratory animals *(23)*. The property that is common to all of the diverse types of chemical carcinogens is that they can form directly or are metabolized to highly reactive electrophilic forms *(24)*. These electron deficient species can attack the many electron rich or nucleophilic sites in molecules such as proteins and nucleic acids to form covalent adducts or induce mutagenesis *(25)*. There is considerable evidence to suggest that DNA is the molecular target of these agents. Damage to DNA induced by these adducts is hypothesized to lead to mutations in proto oncogenes and/or tumor suppressor genes. Two components of tobacco smoke, benzopyrene, a PAH, and 4-aminobiphenyl, an arylamine, form adducts with DNA, suggesting that these components may be direct mutagens contributing to the development of bladder cancer.

Interestingly, neither PAHs nor arylamines are direct carcinogens and therefore it would seem that additional steps are necessary for their activation and metabolism (Fig. 2A). The normal role of the host enzymes which act on chemical carcinogens is to convert these foreign lipophilic compounds into more hydrophilic forms that can be readily excreted. However, in attempting to create a hydrophilic product, these enzymes inadvertently form a reactive product. Most of these reactions are catalyzed by cytochrome P450 dependent mono oxygenases located predominantly in the liver. In the case of carcinogenic arylamines, the first step in this process is N-oxidation catalyzed by hepatic cytochrome P450 1A2 isoenzyme (CYP1A2) *(26)*. This enzyme has been shown to be inducible by several environmental factors including cigarette smoke, which has resulted in significant individual and population variability when the activity of this enzyme is measured *(27)*. Due to its critical role, it is not surprising to find indirect evidence that a phenotype associated with enhanced CYP1A2 activity, may be a risk factor for bladder cancer *(28)*. These electrophilic metabolically active forms of arylamines or hydroxylamines can form adducts with hemoglobin or circulate freely as glucuronide conjugates and be excreted in the urine *(29)*. Hydroxylamines are then hydrolyzed in the acidic urinary environment allowing formation of adducts with nucleophilic sites in the transitional bladder mucosa.

Fortunately, alternative processing of arylamines can occur by detoxifying pathways (Fig. 2A), with the most studied of these pathways being *N*-acetylation. Two isoenzymes of *N*-acetyltransferase (NAT 1 and NAT 2) have been identified in humans *(25)*. The NAT2 enzyme is encoded by a single polymorphic gene, with individuals having any two of several possible mutant alleles display a slow acetylator phenotype and hence exhibit impaired detoxification of carcinogenic arylamine *(30)* (Fig. 2B). Several recent case control studies have investigated the relationship of NAT2 phenotype or genotype and bladder cancer risk *(31–33)* and have demonstrated that "slow aceltylators", namely, individuals who detoxify arylamines slowly due to decreased activity of these pathways, have substantially higher risk of bladder cancer. On the other hand, NAT2 does not appear to play a role in bladder carcinogenesis induced by PAH *(34)*. In addition to NAT2, glutathione S transferase M1 (GST-M1), a family member of a class of enzymes which detoxify reactive chemicals by promoting their conjugation to glutathione *(35)* has also been studied in relation to bladder cancer risk. Metabolites of several PAH that are present in cigarette smoke as well as arylamines are known or potential substrates of GST-M1 *(35,36)*. Thus, NAT2 and GSTM1 likely play key roles in the risk for bladder cancer development in individuals exposed to similar doses/durations of carcinogens. In addition, the status of these enzymes may explain in part the wide variation in bladder cancer risk in different ethnic and racial groups *(37,38)*. Both NAT2 and GSTMI have shown racial/ethnic variations which may explain in part why similar smoking habits result in different risks of bladder cancer *(31,36,39)*.

A number of specific genes are known to be mutated by chemical carcinogens. Two of the genes, *HRAS* and *P53*, have also been implicated in bladder tumorigenesis and progression. The *HRAS* gene codes for p21Ras, a small GTPase involved in signal transduction *(40)*, which was the first proto oncogene found to be mutated in the T24 bladder cancer cell line *(41)*. Evidence from clinical studies using immunohistochemical techniques has demonstrated a correlation between the levels of the Ras protein and the degree of tumor invasiveness and that *HRAS* expression is an independent prognostic variable for tumor invasion *(42)*. In addition, an in vivo *(43)* study has implicated this molecule in several of the steps involved in tumor invasion, supporting the notion that *HRAS* overexpression is causally related to tumor progression and not merely epiphenomenon. Detailed staining for *HRAS* in normal bladder tissue has revealed that the basal (progenitor) cells of the multilayered transitional epithelium stain with the highest intensity while more superficial (differentiated) compartments stain to a much lesser degree. Thus the level of normal *HRAS* protein diminishes considerably with differentia-

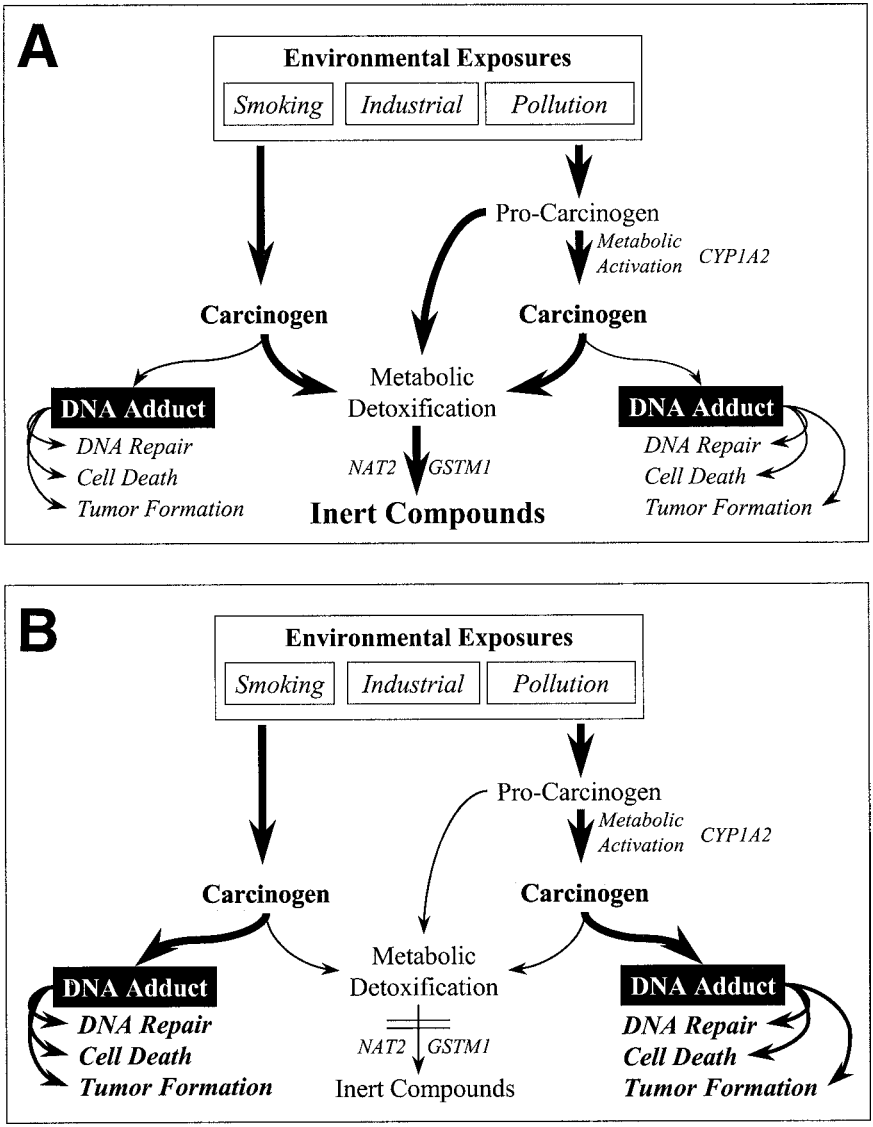

Fig. 2. Hypothetical model of carcinogen activation and detoxification and resulting cellular consequences in (**A**) patients with normal detoxification, and (**B**) in individuals with abnormal detoxification mechanisms. Abbreviations: CYP1A2, hepatic cytochrome P450 1A2; NAT 2, *N*-acetyltransferase 2; GST-M1, glutathione S transferase M1.

tion. However, *HRAS* overexpression per se is not restricted to the malignant state in bladder tissue. It is thus conceivable that a deregulation of *HRAS* gene expression *(42)* or expression of a mutant protein *(41)*

can occur and result in the induction of bladder cancer. Support for this idea comes from results demonstrating that transfection of an *HRAS* gene will convert SV40 immortalized human urothelial cells into invasive transitional cell carcinomas *(44,45)*. Recent reports *(46,47)* utilizing PCR-based methods, revealed that approximately 40% of bladder tumors harbor *HRAS* codon 12 mutations.

For genotoxic carcinogens, the interaction with DNA is likely not to be random, and each class of agents reacts selectively with purine and pyrimidine targets *(48,49)*. In addition, targeting of carcinogens to particular sites in DNA is determined by the nucleic acid sequence *(50)*, by specific DNA repair processes and host cell type, making some genetic sequences more at risk than others. As expected from this chemistry, genotoxic carcinogens are potent mutagens, able to cause base mispairing or small deletions, leading to missense or nonsense mutations *(48)*, but the spectra of mutations seems to be dependent on the agent. For example, the mutations found in activated *RAS* protooncogenes associated with tumors of animals exposed to *N*-nitroso compounds are predominantly G:C to A:T base substitutions *(51)*. Although there are several guanine residues in *RAS* codons that would generate a transforming protein if substituted by adenine, these experiments have revealed that the mutations detected in tumors occur overwhelmingly at only one of the possible mutation sites. PAHs, on the other hand, produce a different mutation spectrum *(52)*, and other chemical classes, such as tobacco-specific nitrosamines, have yet other spectra *(53)*. In vitro studies using either prokaryotic or human cells, indicate that human exposure to mutagens may result in a narrow non-random spectrum of mutations *(54)*. Finally, adding another layer of complexity in humans, the spectra of *KRAS* gene mutations in adenocarcinomas vary according to tissue sites, indicating that mutational spectra may be dependent on the causal agent, the target gene and the tissue involved.

Another important genetic target for chemical carcinogenesis is the *P53* tumor suppressor. This gene is of particular relevance in bladder cancer because of its putative roles in both transformation *(55)* and progression *(8)*. Mutations in the *P53* tumor suppressor gene are a frequent event in both transitional cell and squamous cell carcinomas of the bladder *(56)* with up to 40% of bladder cancers harboring such lesions. Especially valuable have been studies of the timing of occurrence of these mutations during different stages of bladder cancer pathogenesis. Mutations are rare in low-grade papillary tumors but are common in CIS and more invasive high-grade bladder cancers, suggesting that *P53* may play a role in both transformation *(55)* and progression *(8)*. Recent immuno-

histochemical studies of patients with bladder TCC have revealed a significant correlation between the number of cigarettes smoked and the incidence of positive *P53* immunohistochemistry. Studies comparing cases of bladder cancer from smoking and nonsmoking patients showed an increased frequency of G:C to C:G transversions in both groups. While smokers did not have a different mutational spectrum than nonsmokers, they did exhibit a higher frequency of double mutation events *(57,58)*. Mutations in *P53* are particularly detrimental due to this gene's multiple cellular regulatory and supervisory roles *(59)*.

Molecular Basis of Tumor Development

The molecular basis of urothelial transformation and progression can be deduced from numerous studies carried out over the last several years. Using cytogenetic, molecular genetic and immunohistochemical methods, a general pattern seems to be emerging as to which genes and/or chromosomal locations are important for tumor development and progression. In this section we will highlight the genetic abnormalities associated with neoplastic transformation and focus on those associated with progression later on. Multistage carcinogenesis is regarded as a consequence of the accumulation of somatic genetic alterations which include activation of cellular proto oncogenes, and the inactivation of tumor suppressor genes. As outlined above for Ras and *P53*, environmental carcinogens can induce alterations of both gene types. In addition, to these studies, a large number of reports have surveyed the cytogenetic changes found in TCC *(60)*. Studies of TCC revealed consistently high incidence of chromosomal abnormalities in chromosome 9 *(61)* and 17p *(62)*.

Currently, it would appear that chromosome 9 *(63)* and *P53 (64)* changes may occur relatively early in the genesis of TCC while other changes such as EGFR and E-Cadherin are associated with progression. Chromosome 9 deletions are often found early in bladder tumor development, a finding also observed in other cancers such as lung *(65)*, ovary *(66)* and kidney *(67)*. A candidate tumor suppressor gene *CDKN2A*:p16 was recently identified in the 9p21 region *(68)*, an area commonly altered in bladder cancer *(60)*. *CDKN2A* encodes a protein which is part of a new group of cell cycle inhibitory molecules known as cyclin dependent protein kinases (CDK) *(69)*. Among these are also p15 (*INK4B/MTS2*) which together with p16 can inhibit the phosphorylation of the retinoblastoma protein (*RB*), thereby inhibiting the cell cycle. Loss of either of these genes may have profound implications on the cell cycle and result in uncontrolled growth and tumor formation. The loss of p16, often accompanied by p15 loss is a very frequent occurrence in bladder

cancer, occurring in up to 40% of cases *(70)*. The importance of *P53* in bladder tumorigenesis was suggested by the high frequency of LOH of chromosome 17p where this gene is located (17p13.1). *P53* codes for a 53kDa phosphoprotein with DNA binding properties which is involved in multiple cell functions including gene transcription, monitoring the fidelity of DNA synthesis and apoptosis *(71)*. *P53* mutations may be induced by carcinogens as outlined above, resulting in a selective growth advantage of cells harboring these defects. The role of *P53* as a target for chemical carcinogenesis was discussed earlier. While there is significant evidence to support the role of *P53* in bladder tumor progression, the role of *P53* has only recently been clarified in tumorigenesis of TCC. Recent genetic evidence has suggested that different clinical forms of TCC may result from different genetic lesions *(55)*. A model has been recently proposed which hypothesizes two different pathways leading to the development of superficial bladder tumors including carcinoma in situ (Fig. 3). This model postulates that chromosome 9 alterations in normal cells lead to papillary superficial TCC while *P53* mutations lead to carcinoma *in situ* (CIS/Tis). Both *P53* and chromosome 9 losses can play a complimentary role further downstream in tumor progression in concert with other genetic changes.

In addition to these changes, microsatellite instability at loci on chromosome 9, was found in TCC *(72)*. Microsatellites are sequences of polymorphic nucleotide repeats found throughout the human genome *(73,74)*, which are routinely used in the analysis of loss of heterozygosity (LOH) in human cancers. In addition, abnormalities or instabilities consisting of alterations of the number of repeats of a specific microsatellite in tumor DNA when compared to normal tissue DNA, indicate that replication errors have occurred *(75)*. The persistence of these errors is an indication of the reduced ability of cancers to repair mutations. The greater the instability, the less the capacity of repair the greater the potential for the generation of heterogenous populations some of which exhibiting novel and more malignant attributes such as enhanced growth, growth factor independence and drug resistance among many others. In colon cancer, microsatellite instability has been linked to alterations in the *MSH2* gene, located on 2p16 *(76)*, which codes for an enzyme involved in DNA repair.

Since microsatellite abnormalities found in TCC appear to be early changes *(61,72,77)*, they may be reflecting severe deregulation of cellular DNA which if left unchecked may lead to unrepaired mutations in key regulatory genes such as p53. In addition, genes such as *MSH2* may themselves be targets of carcinogenic insults. Finally, a case study by Schoenberg et al. *(78)* describes a patient who developed TCC of the

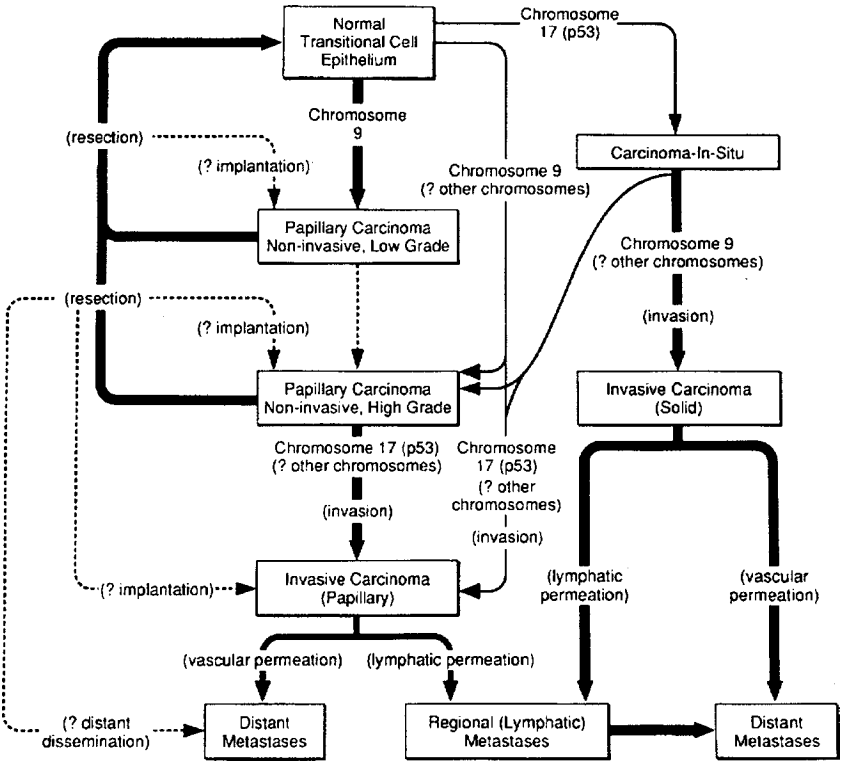

Fig. 3. Proposed pathway for bladder tumor development derived from molecular epidemiological data. The divergent, distinct pathways for superficial and invasive tumors parallels the model developed from observations of clinical biology (Fig. 1). (From Jones PA, Droller MJ. Pathways of development and progression in bladder cancer: new correlations between clinical observations and molecular mechanisms. Semin Urol 1993; 11: 177–192).

bladder and renal pelvis at an early age. The patient was found to have the germline translocation t(5;20)(p15;q11), which may have been an initiating factor in the disease. A recent literature review by Kiemeney and Schoenberg *(79)* examined case reports and epidemiological studies on TCC and concludes that there is evidence for a familial bladder cancer gene, which is a distinct entity from the known cancer predisposition syndromes.

MOLECULAR BASIS OF SUPERFICIAL TUMOR RECURRENCE

A central feature of the clinical biology of superficial bladder cancer is its idiosyncratic rate of recurrence. Its uniquely high metachronous

recurrence rate, distinguishing it from all other organ sites involved by contact carcinogenesis, has served as the basis for a longstanding debate in the urologic literature. While a number of theories have been proposed to account for this unique feature of superficial bladder cancer, two fundamental theories have received greatest attention. The concept of urothelial field change following exposure to a urinary carcinogen is both intuitively appealing and supported by multifocality and associated dysplasia in this disease *(80,81)*. Nonetheless other contact carcinogen induced tumors should have similar risks and yet fail to have metachronous recurrence rates approximating those associated with superficial bladder neoplasms. For this reason, and given the unique nature of the lower urinary tract, other authors have proposed intraepithelial tumor dissemination and or treatment induced implantation as a phenomenon accounting for the idiosyncrasy of superficial bladder cancer recurrence biology *(82–85)*. Anecdotal evidence in support of this concept in addition to the unusual recurrence rate include the temporality of recurrence in relation to surgical removal of a primary lesions and the location of recurrences in relation to the index lesion *(86)*.

Debate on this issue is traceable to the turn of the century when Albarran first proposed implantation as a mechanism accounting for bladder tumor recurrence *(87)*. The pendulum swung several times in the ensuing years. In the 1950s, Melicow, and Kaplan clearly demonstrated associated areas of dysplasia and pre-neoplasia in the urothelium intervening between sites of frank neoplasia *(80,81)*. However subsequent work by McDonald showed that urothelial malignancies could be implanted into and grow on sites of urothelial trauma even given relatively crude immunosuppression and understanding of transplant rejection in that era *(84)*. These observations were later expanded on by Soloway and the specific mechanisms involved in tumor implantation delineated by See *(85,88,89)*.

A definitive answer to the issue of the mechanism of bladder tumor recurrence was not provided until early in the 1990s. Using a molecular analysis of X chromosome inactivation in women with multifocal bladder tumors Sidransky et al. provided strong evidence to suggest that the multifocal tumors were clonal in origin *(90)*. Subsequently, Habuchi demonstrated that heterotopic urothelial recurrence was associated with identical mutations in *P53* at both the upper and lower track sites of occurrence *(91)*. Most recently this same group did microsatellite analysis on patients with multifocal metachronous tumor recurrence *(92)*. They found identical microsatellite alterations on multiple chromosomes in 80% of patients with multifocal recurrences. Overall this combination of data provides virtually conclusive evidence that the majority of superficial

bladder recurrences are clonal in their etiology. Nonetheless some minor issues related to the precise mechanism of recurrence remain unresolved. Tsai found mosaicism in the human uroepithelium which suggested that clonal heterogeneity within the bladder was more limited than previously thought *(93)*. Indeed further evidence suggested that the bladder could develop from as few as 200 primordial cells and that the risk of tumor development and recurrence might be a consequence of limited diversity within the progenitor cell population.

While the etiologic debate regarding the mechanism of tumor recurrence has been largely resolved, the molecular mechanisms underlying the ability of superficial bladder tumors to implant and grow at sites different from the primary are largely undefined. See et al. outlined the requisite steps necessary for tumor implantation and or intraepithelial tumor dissemination to occur *(94)*. In the case of implantation the obvious first step is the presence of free floating tumor cells on the luminal surface of the bladder. These tumor cells must remain viable in the detached state and subsequently be able to adhere to sites on the urothelial surface. Following adherence, the local milieu must be conducive to the ultimate outgrowth of the adherent cell or cells. This would include an ability for the cells to divide, proliferate, and develop a vascular support structure.

Clinical observation and basic science research has provided some insight into factors associated with certain of the aforementioned steps. The mechanism of bladder tumor ablation, that is electrosurgical disruption into a fluid-filled medium, frees tumor cells from their underlying site of origin and effectively disseminates them throughout the luminal surface of the bladder. Surgical injury associated with the process of electrosurgical resection of bladder tumors results in sites of urothelial injury which selectively predisposes to tumor cell adherence via the formation of fibrin clots and effective entrapment/adherence of tumor cells at these sites. Given the central role of cellular adherence to clots at the site of urothelial injury, several studies have suggested that tumor intrinsic pericellular proteolysis through one of several fibrinolytic pathways may be a regulator of tumor cell adherence and ultimate outgrowth *(95–97)*. However, little work has been done to define whether specific molecular alterations in pericellular proteolysis might account for patterns of recurrence.

Other facets of the implantation process, such as proliferation and neovascularization, have been alluded to in other work. The epidermal growth factor/TGF alpha autocrine and paracrine loops have been suggested to predispose to recurrence *(98)*. Subsequently, cellular production of vascular endothelial growth factor, allowing for the establishment

of a vascular support structure, has been suggested as a prognostic feature correlating with recurrence risk *(99)*. While a number of associated factors have been identified, these studies are at a very preliminary stage. The precise mechanisms responsible for dysregulation of cellular expression of these various proteins remain to be clarified.

MOLECULAR BASIS OF BLADDER CANCER PROGRESSION

While less common than tumor recurrence, progression of superficial tumors to muscle invasion has profound consequences with respect to prognosis and treatment. In fact, tumor progression encompasses a spectrum of clinical and biological changes in both the tumor and the host *(100)* from early invasion of the basement membrane to widely metastatic disease. In this section we will focus and highlight the changes occurring when superficial bladder cancers become muscle invasive.

In general, organs are composed of a series of tissue compartments separated from each other by two types of extracellular matrix: basement membranes and interstitial stroma *(101)*. The extracellular matrix determines tissue architecture, has important biologic functions, and is a mechanical barrier to tumor cell invasion. The nuances of what is meant by invasive and superficial bladder cancer are worth mentioning here, since they are somewhat at odds with the pure definition of tumor invasion which is the penetration of normal tissue barriers such as the basement membrane. In the purest sense only stage Ta and CIS tumors are truly "superficial," thus not penetrating the basement membrane of the bladder wall. Historically however, urologists have also considered T1 tumors as superficial despite their invasion of the lamina propria. Tumors labeled as "invasive" on the other hand are those penetrating the true muscle of the bladder wall. As a group, most stage T1 lesions are more prone eventually to invade the detrusor during subsequent recurrences than are Ta tumors. Conversely, despite being truly superficial, CIS is more aggressive and behaves more akin to T1 than Ta tumors. This may be the result of the differing genetic lesions that led to its formation compared to those leading to Ta/T1 cancers *(102)*. Due to the significant drop in a patients' prognosis with any step in tumor progression, the genetic basis of this phenomenon is therefore a subject of considerable clinical importance. In the current section we will highlight the cytogenetic, molecular genetic and immunohistochemical evidence supporting the role of specific genetic changes in the progression of bladder cancer to muscle invasive disease.

Cytogenetic Changes Associated with TCC Progression

Several recent studies have examined the common regions of deletion in human bladder tumors *(60,102)*. In a recent series, Knowles *(60)* and associates screened 83 cases of transitional cell carcinoma for loss of heterozygosity (LOH) on all autosomal chromosome arms. The most frequent losses were monosomies of chromosome 9 (57%), losses on chromosomes 11p (32%), 17p (32%), 8p (23%), 4p (22%), and 13q (15%). This series was composed of a majority of superficial low grade lesions and thus the incidence of the various losses would be reflective of the genetic alterations specifically present in this cohort of patients. Other groups have focused on identifying the common deletions specifically associated with tumor progression. In these cases, a somewhat different spectrum of abnormalities was observed, involving alterations at chromosomal locations 3p *(103)*, 4q *(104)*, 8p *(105)*, 18q *(106)*, 10 *(107, 108)*, 15 *(109,110)*, and 17p *(64)*. Some of these changes have also been observed in a recently characterized highly tumorigenic variant of the T24 human bladder cell line *(111)*.

Previous studies on predominantly superficial bladder cancer specimens *(60)* indicated an overall low frequency of chromosome 10 allele losses and deletions in bladder cancer. However when cohorts with significant proportions of invasive tumors were investigated *(108)*, the incidence of LOH on this chromosome was found in 40% of tumors for at least one locus. Remarkably, LOH on chromosome 10 was observed mainly in muscle-invasive or high grade tumors, the latter of which were most likely invasive or to have high chance of future progression to invasive disease. Confirming these findings, Kagan and colleagues *(107)* found LOH with at least one allele lost on the long arm of chromosome 10 in 9/20 (45%) invasive transitional cell carcinomas. Recently, LOH studies have also suggested that human chromosome 15 may harbor a novel putative tumor suppressor gene which appears to play a role during metastasis in breast and bladder *(110)* cancer. This observation supported other studies where fluorescence in situ hybridization (FISH) for chromosome 15 specific centromeric repeat sequences, revealed loss of this chromosome in 67% of specimens from patients with histologically confirmed transitional cell carcinoma *(109)*.

Molecular and Immunohistochemical Changes Associated with TCC Progression

Studies utilizing immunohistochemical techniques (IHC) have suggested that overexpression of *HRAS* protein (discussed above) *(42)*, *P53*

(112) and the epidermal growth factor receptor (*EGFR*) *(113)* in bladder tumors may be related to bladder tumor progression. Loss of *RB (64)* and *E-Cadherin (114)* expression has also been related to this transition. Below, we will discuss the evidence suggesting roles for these genes in bladder cancer progression.

E-CADHERIN (CDH1)

The disruption of intercellular contacts, which accompanies cell dissociation and acquisition of motility, is correlated with a redistribution of *E-cadherin* over the entire cell surface and within the cytoplasm. Normal urothelium expresses E-cadherin, a Ca^{2+} dependent cell adhesion molecule, located on chromosome 16q22.1 and shown to behave like an invasion suppressor gene in vitro and in vivo in experimental systems *(115)*. This may explain the inverse relation between expression of *E-cadherin* and bladder tumor grade *(116)*. Several investigators further examined *E-cadherin* expression in bladder cancer samples and sought a correlation with tumor behavior. In an early study on 49 patient specimens (24 superficial and 25 invasive tumors), decreased *E-cadherin* expression correlated with both increased grade and stage of bladder cancer. More importantly, abnormal E-cadherin expression correlated with shorter patient survival *(117)*. These relationships to stage and grade were subsequently confirmed by other groups *(118,119)* while those to survival were sometimes *(119)* but not always *(120)* shown, despite a correlation with distant metastasis *(121)*. This latter apparent inconsistency may be due to a lack of statistical power in the various analyses to demonstrate an effect.

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

Similar to *HRAS*, *EGFR* expression levels in bladder cancer have been associated with increasing pathologic grade, stage *(122)* and higher rates of recurrence *(123)* and progression in superficial forms of the disease *(113)*. As such they may be causally related to the transition from superficial to invasive disease. Most importantly, patients with increased *EGFR* expression on their tumor cells did not survive as long as patients with normal *EGFR* expression. However, when the comparison of survival was limited to patients with invasive bladder cancer, no significant difference was found between patients with high levels of *EGFR* expression and those with low *EGFR* values *(124)*, suggesting that EGFR overexpression might be associated with the phenotypic transition from superficial to invasive forms of disease. Interestingly, gene amplification and gene rearrangement does not appear to be a common mechanism for *EGFR* overexpression in bladder cancer *(125)*. However, superficial human bladder cancer cells which were engineered to overexpress either

mutated or normal *HRAS* also begin overexpressing *EGFR* at both the mRNA and protein levels, therefore HRAS might also play a role in transcriptional regulation of *EGFR* besides its role in *EGFR* signal transduction *(40,126)*.

Taken together, these data suggest that regulation of *EGFR* is altered in bladder cancer. In addition, since *EGF* is present in large quantities in urine *(127)*, with concentrations up to 10-fold greater per milliliter than those found in blood, this situation is likely to potentiate the consequences of EGFR overexpression since *EGFR's* in bladder cancer are functional *(128)*. Supporting the notion that *EGFR* overexpression is causally related to tumor progression and not merely an epiphenomenon are a number of in vitro *(126,129)* studies that have implicated this molecule in several of the steps involved in tumor invasion, such as cell motility.

Retinoblastoma (*RB*)

Deletions of the long arm of chromosome 13, including the *RB* locus on 13q14, were found 28 of 94 cases, with 26 of these 28 lesions being present in muscle-invasive tumors *(130)*. *RB* alterations in bladder cancer as a function of stage was studied in 48 primary bladder tumors *(131)* where a spectrum of altered patterns of expression, from undetectable *RB* levels to heterogeneous expression of *RB*, was observed in 14 patients. Of the 38 patients diagnosed with muscle invasive tumors, 13 were categorized as *RB* altered, while only 1 of the 10 superficial carcinomas had the altered *RB* phenotype. Patient survival was decreased in *RB* altered patients compared with those with normal *RB* expression.

Two recent studies *(132,133)* have also shown that *RB* and *P53* alterations can further deregulate cell cycle control at the G1 checkpoint and produce tumor cells with reduced response to programmed cell death. The imbalance produced by an enhanced proliferative activity and a decreased apoptotic rate may further enhance the aggressive clinical course of the bladder tumors harboring both *P53* and *RB* alterations. A study focusing on the clinical progression of T1 tumors has demonstrated that patients with normal expression of both proteins have an excellent outcome, with no patient showing disease progression. Patients with abnormal expression of either or both proteins had a significant increase in progression *(134)*. These data indicate the clinical utility of stratification of T1 bladder cancer patients based on *P53* and *RB* nuclear protein status. They suggest that patients with normal protein expression for both genes may be managed conservatively, whereas patients with alterations in one and particularly both genes may require more aggressive treatment. Conversely, conflicting results have been obtained when *RB* status has been examined in patients with invasive tumors *(135,136)*,

indicating perhaps that this gene may have its primary role in progression from superficial to muscle invasive disease rather than further downstream in the metastatic cascade.

P53

Genetic alterations of the *P53* gene, such as intragenic mutations, homozygous deletions, and structural rearrangements, are frequent events in bladder cancer *(137)*. Structural alterations of the *P53* gene were investigated using single strand conformation polymorphism (SSCP) in 25 bladder tumors and mutations in 6 of 12 invasive carcinomas were found, while only 1 of 13 superficial bladder tumors had such mutations *(138)*. Moreover, mutations were not identified in any of the 10 grade 1 and 2 lesions, while 8 of 15 grade 3 bladder carcinomas were found to have intragenic mutations. In another study *(139)*, IHC detectable *P53* protein was studied in 42 bladder carcinomas. One out of 11 grade 1 (9%), 12/22 grade 2 (55%) and 8/9 grade 3 (89%) tumors showed positivity for *P53*. There were significantly more *P53* positive cases in grade 2–3 tumors than in grade 1 tumors. There were significantly more *P53* positive cases in stage T2-T4 tumors than in stage T1 tumors. Another study *(140)* analyzed 42 specimens of transitional cell carcinoma by interphase cytogenetics with a fluorescence in situ hybridization technique (FISH) and found that *P53* deletion was significantly correlated with grade, stage, S-phase fraction, and DNA ploidy, while *P53* overexpression correlated only with grade. Moch et al. *(141)* studied the overexpression of *P53* by IHC in 179 patients and found that *P53* immunostaining to strongly correlate with tumor stage. In addition, this was driven by a marked difference in *P53* expression between pTa (37% positive) and pT1 (71%) tumors, while there was no difference between pT1 and pT2–4 tumors. Similarly, a strong overall association between *P53* expression and grade was driven by a marked difference between grade 1 (28%) and grade 2 tumors (71%), and there was no significant difference between grade 2 and grade 3 tumors.

Several groups *(142,143)* have investigated the possibility that altered patterns of *P53* expression correlated with tumor progression in patients with T1 bladder cancer. Patients with T1 tumors were retrospectively stratified into two groups with either <20% tumor cells (group A) with positive nuclear staining or >20% of cells with nuclear immunoreactivity for *P53* (group B) *(142)*. Disease progression rates were 20.5% per year for group B and 2.5% for Group A, with patients in group 2 having significantly shorter progression free intervals. Disease specific survival was also associated with altered patterns of *P53* expression. Another study *(143)* reported an analysis of T1 tumors using immunohistochem-

istry and 20% positive nuclear staining as the cutoff value. The mean follow-up time was greater than 10 yr. Progression and tumor grade were both significantly related to *P53* nuclear overexpression. However in this last study, *P53* expression was not an independent predictor of disease progression.

Other studies have attempted to clarify the role of *P53* as a prognostic marker in muscle-invasive tumors. In one study, *P53* was evaluated in 90 bladder tumors from 111 patients treated with neoadjuvant MVAC *(144)*. Patients with *P53* overexpression had a significantly higher proportion of cancer deaths. The long term survival in the *P53* overexpressors was 41% vs 77% in the nonexpressors independent of stage and grade. In another study, histologic specimens of transitional-cell carcinoma of the bladder, stages pTa to pT4 from 243 patients who were treated by radical cystectomy were examined for the IHC detection of *P53* protein *(8)*. Nuclear *P53* reactivity was then analyzed in relation to time to recurrence and overall survival. In patients with transitional-cell carcinoma confined to the bladder, an accumulation of *P53* in the tumor-cell nuclei predicted a significantly increased risk of recurrence and death, independently of tumor grade, stage, and lymph-node status. In a third study, IHC *P53* protein expression analysis was performed in 90 patients with transitional cell carcinoma of the urinary bladder *(145)*. Positive nuclear staining of tumor cells by the antibody to *P53* protein was detected in 32 cases, most of which were invasive and nonpapillary tumors and in high grade tumors. In addition, patients with tumors positive for *P53* staining had a significantly worse survival rate.

Other Genes

Early studies in bladder cancer have indicated a strong association of low level *MYC* (8q.24) gains with tumor grade, stage, chromosome polysomy, p53 protein expression, p53 deletion and tumor cell proliferation as assessed by Ki67 labeling index *(146)*. These data were consistent with a role of chromosome 8 alterations in bladder cancer progression *(105)*. However, subsequent studies have not found statistical significant correlation between the methylation, expression of *MYC* gene and clinical-histopathological parameters *(147)*, between the *MYC* methylation pattern and clinical stage *(148)*. Furthermore, *MYC* overexpression did not correlate with tumor grade or tumor progression *(149)*. Thus the role of this gene in bladder cancer development or progression is at present unclear.

Amplification and protein overexpression of the *ERBB2* gene located on 17q11.2-q12, has been suggested as a prognostic markers for patients with recurrent progressive bladder tumors *(150,151)*. However, other

studies have failed to link *ERBB2* expression levels as an independent variable predicting disease progression *(152)*. Other studies have indicated a high level of expression of this gene in malignant as compared to benign bladder epithelium *(152)*. From these studies it would appear that the role of *ERBB2* as a diagnostic marker may outweigh its usefulness as a prognostic indicator.

The *MDM2* (mouse double minute 2, human homolog of p53-binding protein) gene is located at 12q13-14 and codes for a 90 Kd nuclear protein which is a negative regulator of *P53*. In urinary bladder, a strong statistical association between *MDM2* and *P53* overexpression was found in addition to an association between *MDM2* overexpression and low-stage, low-grade bladder tumors *(153)*. In addition, the simultaneous assessment of *MDM2* and *P53* was found to be independent factors for both disease progression and survival *(154)*. However, as with *MYC* and *ERBB2* not all studies have shown assessment of this gene product to be independently related to tumor progression *(155)*.

CONCLUSION

We have attempted to review the current understanding of both the molecular pathogenesis, and the molecular basis for the biology of transitional cell bladder neoplasms. This status results from an amalgam of scientific disciplines, ranging from molecular epidemiology to urologic oncology, combining "forces" to exact an exponential growth in our knowledge of bladder tumor biology. We may stand poised on the brink of a true revolution in cancer management. The ongoing molecular dissection of all phases of tumor biology promises unprecedented change in the way we assess and manage neoplastic processes.

The identification of heritable genetic mutations could possibly already allow the early, specific, identification of individuals at risk for certain tumors. The recognition that both the individual genotype and the environment may combine for the ultimate determination of risk may allow patients to adapt their lifestyles for risk modification. For those patients with neoplastic disease, therapy can be tailored to the specific biology of the individual tumor. The ongoing integration of molecular biology with clinical science promises to bring all of this within our reach.

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