

Review

Molecular pathogenesis of urothelial bladder cancer

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Summary. Carcinoma of the urinary bladder is the second most common urologic malignancy. In addition, these tumors are one of the best understood genitourinary neoplasms with a well defined etiology, natural history, tumor biology, treatment options and outcome. 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. The intent of this review is to provide the reader an overview of our current understanding of this tumor from the standpoint of its molecular biology as related to tumor development and progression.

Key words: Bladder cancer, Molecular pathogenesis, Tumor Invasion

Molecular basis of 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 (Hunstein and Rehn, 1975). 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 figure 1 below (Droller, 1992). 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 (Hay, 1991; Loeb, 1991; Reznikoff et al., 1996; Weinstein, 2000; Goel et al.,

2001) necessary for carcinogenesis. Furthermore, chemical carcinogens may be genotoxic, non-genotoxic (Melnick et al., 1996) or induce epigenetic effects (MacLeod, 1996) with dose response relations being linear or non-linear (Swenberg et al., 1987; Lutz, 1990b). Endogenous mutagenic mechanisms such as DNA oxy-radical damage, de-purination and polymerase infidelity also contribute to carcinogenesis (Breimer, 1990; Lutz, 1990a; Loeb, 1991; Weinstein, 1991) 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 (Ames and Gold, 1990; Hay, 1991; Infante, 1991; Weinstein, 1991; Ames, 2001; McCormick, 2001). 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 19th 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 (Shopland et al., 1991). Several of the chemicals identified in tobacco smoke have been shown

to cause cancer in laboratory animals (Vineis and Caporaso, 1995). 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 (Talalay, 1989). 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 (Wormhoudt et al., 1999). 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

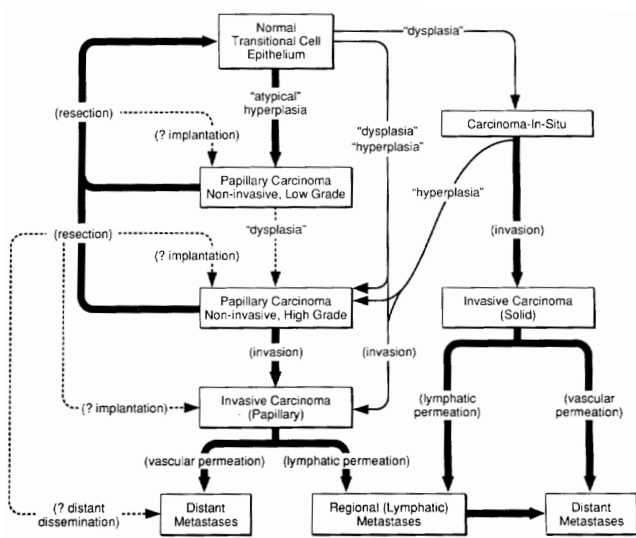


Fig. 1. Proposed pathway for bladder tumor development derived from clinical observation. Note that the superficial and invasive pathways are distinct, with divergence early in the process of tumorigenesis. (from Jones P.A. and Droller M.J. Pathways of development and progression in bladder cancer: new correlations between clinical observations and molecular mechanisms. *Semin Urol.* 11, 177-92, 1993).

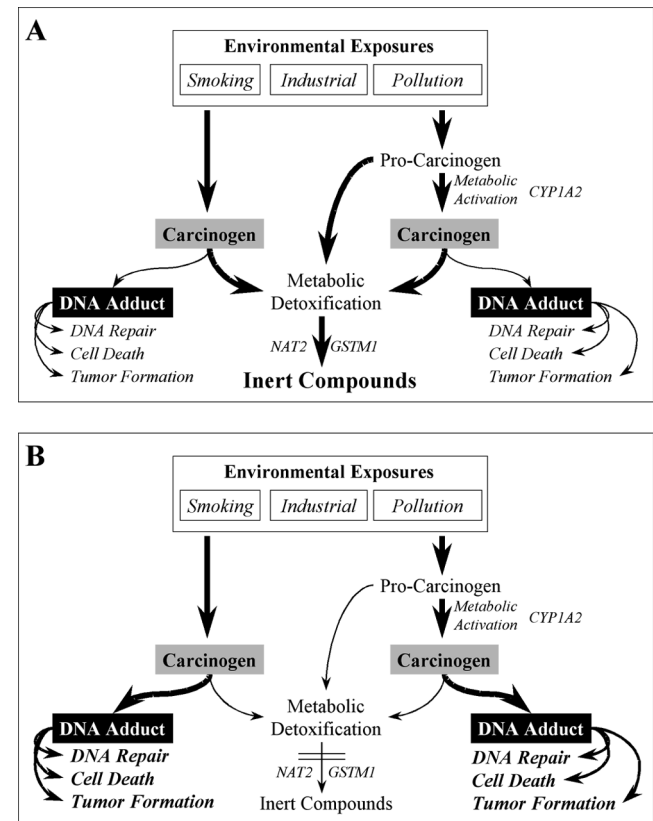


Fig. 2. Hypothetical model of carcinogen activation and detoxification and resulting cellular consequences in patients with normal detoxification mechanisms (A), and in individuals with abnormal detoxification mechanisms (B). CYP1A2: hepatic cytochrome P450 1A2; NAT 2: N-acetyltransferase 2; GST-M1: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

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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) (Butler et al., 1989). 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 (Kalow and Tang, 1991). 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 (Kaderlik and Kadlubar, 1995). 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 (Bryant et al., 1988). 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 (Wormhoudt et al., 1999). The NAT2 enzyme is encoded by a single polymorphic gene (Garte et al., 2001), with individuals having any two of several possible mutant alleles display a slow acetylator phenotype and hence exhibit impaired detoxification of carcinogenic arylamine (Bell et al., 1993a) (Fig. 2b). Several recent case control studies have investigated the relationship of NAT2 phenotype or genotype and bladder cancer risk (Hein, 1988; Branch et al., 1995; Risch et al., 1995) and have demonstrated that "slow acetylators", 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 (Hayes et al., 1993). 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 (Board et al., 1990) 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 (Board et al., 1990; Bell et al., 1993b). Thus, NAT2 and GST-M1 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 (Foster, 1979; Case et al., 1993). Both NAT2 and GST-M1 have shown racial/ethnic variations which may explain in part why

similar smoking habits result in different risks of bladder cancer (Bell et al., 1993b; Yu et al., 1994; Branch et al., 1995).

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 (Bos, 1998), which was the first proto oncogene found to be mutated in the T24 bladder cancer cell line (Parada et al., 1982). In addition, this molecule stimulates the activation of other downstream mediators which are associated with enhanced cell motility and invasion (Gildea et al., 2002). 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 (Fontana et al., 1996). In addition, an in vivo (Theodorescu et al., 1990) 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 differentiation. 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 (Fontana et al., 1996) or expression of a mutant protein (Parada et al., 1982) 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 (Christian et al., 1990; Pratt et al., 1992). Recent reports (Czerniak et al., 1990, 1992) 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. (Essigmann and Wood, 1993; Dipple, 1995). In addition, targeting of carcinogens to particular sites in DNA is determined by the nucleic acid sequence (Levy et al., 1992), 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 (Essigmann and Wood, 1993), 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→A:T base substitutions (Loechler et al., 1984). 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 (Marshall et al., 1984), and other chemical classes, such as tobacco-specific nitrosamines, have yet other spectra (Ronai et al., 1993). 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 (Greenblatt et al., 1994). 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 (Simoneau and Jones, 1994) and progression (Esrig et al., 1994). Mutations in the *P53* tumor suppressor gene are a frequent event in both transitional cell and squamous cell carcinomas of the bladder (Sidransky et al., 1991) 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 (Simoneau and Jones, 1994; Lu et al., 2002) and progression (Esrig et al., 1994; Lu et al., 2002; Rodriguez-Alonso et al., 2002). Recent immunohistochemical 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 non smoking patients showed an increased frequency of G:C→C:G transversions in both groups. While smokers did not have a different mutational spectrum than non smokers, they did exhibit a higher frequency of double mutation events (Habuchi et al., 1993b; Spruck et al., 1993). Interestingly, recent results suggest that *p53* might modulate the repair of DNA adducts generated from the human bladder carcinogen ABP in its target human uroepithelial cells. This implies that in *p53* null cells the unrepaired DNA damage could cause accumulation of mutation, which might contribute to increased genomic instability and neoplastic progression (Swaminathan et al., 2002). Mutations in *P53* are particularly detrimental due to this gene's multiple cellular regulatory and supervisory roles and Wouters, 1999).

An important question is whether genes that are crucial for the initial development of a specific tumor are required for maintaining the malignant phenotype of that

tumor. Recent studies (Weinstein, 2002) have shown that brief loss of oncogene (for example, *HRAS*) overexpression caused the tumor cells to differentiate into normal cells. Reintroducing a wild-type tumor suppressor gene (for example, *P53*) into human cancer cells where the respective endogenous gene is inactive usually promotes marked inhibition of growth, induction of apoptosis, and/or inhibition of tumorigenesis in mice (Weinstein, 2000). These results are unexpected because, if these cancer cells evolved simply through the stepwise acquisition of several mutations and altered gene expression, then correction of just one mutation should have only a modest inhibitory effect. Thus, some cancer cells seem to be "hypersensitive" to the growth-inhibitory effects of specific tumor suppressor genes (Weinstein, 2000). Taken together, these data are consistent with the notion that cancer cells are often "addicted to" (that is, physiologically dependent on) the continued activity of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype. These studies present an optimistic message with respect to new approaches for treating cancer. Clinical evidence that oncogene addiction exists is provided by specific antibodies against the Her-2/neu receptor (Vogel et al., 2002) that are being used to successfully treat breast cancer, and the striking therapeutic effects of the drug imatinib mesylate (STI571/Gleevec) that targets the *Bcr-Abl* oncogene in CML (Kantarjian et al., 2002). Likewise, the antitumor effects of viral vectors encoding the *p53* tumor suppressor gene (Kuball et al., 2002) may be due to tumor suppressor hypersensitivity.

Genetics 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 (Knowles et al., 1994). Studies of TCC revealed consistently high incidence of chromosomal abnormalities in chromosome 9 (Orlow et al., 1994) and 17p (Dalbagni et al., 1993).

Currently, it would appear that chromosome 9 (Simoneau et al., 1996) and *P53* (Reznikoff et al., 1996) changes may occur relatively early in the genesis of

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TCC while other changes such as EGFR (Theodorescu et al., 1998a,b) and E-Cadherin (Byrne et al., 2001; Shariat et al., 2001) 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 (Merlo et al., 1994), ovary (Schultz et al., 1995) and kidney (Cairns et al., 1995). A candidate tumor suppressor gene *CDKN2A:p16* was recently identified in the 9p21 region (Cairns et al., 1993), an area commonly altered in bladder cancer (Knowles et al., 1994). *CDKN2A* encodes a protein which is part of a new group of cell cycle inhibitory molecules known as cyclin dependent protein kinases (CDK) (Serrano et al., 1993). 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. Recently, this gene has been implicated in both tumor formation and progression when accompanied by changes in p53 (Friedrich et al., 2001; Korkolopoulou et al., 2001). The loss of p16, often accompanied by p15 loss is a very frequent occurrence in bladder cancer, occurring in up to 40% of cases (Gruis et al., 1995). 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 (Choisy-Rossi et al., 1999). 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 (Simoneau and Jones, 1994). 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 (Gonzalez-Zulueta et al., 1993). Microsatellites are sequences of polymorphic nucleotide repeats found throughout the human genome (Weber, 1990; Kwiatkowski et al., 1992), 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 (Peltomaki et al., 1993). 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 (Leach et al., 1993), which codes for an enzyme involved in DNA repair. Since microsatellite abnormalities found in TCC appear to be early changes (Gonzalez-Zulueta et al., 1993; Linnenbach et al., 1994; Orlow et al., 1994), 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. (1996) describes a patient who developed TCC of the 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 Kiemeny and Schoenberg (Kiemeny and Schoenberg, 1996) examined case reports and epidemiological studies on TCC and concludes that there is evidence for a

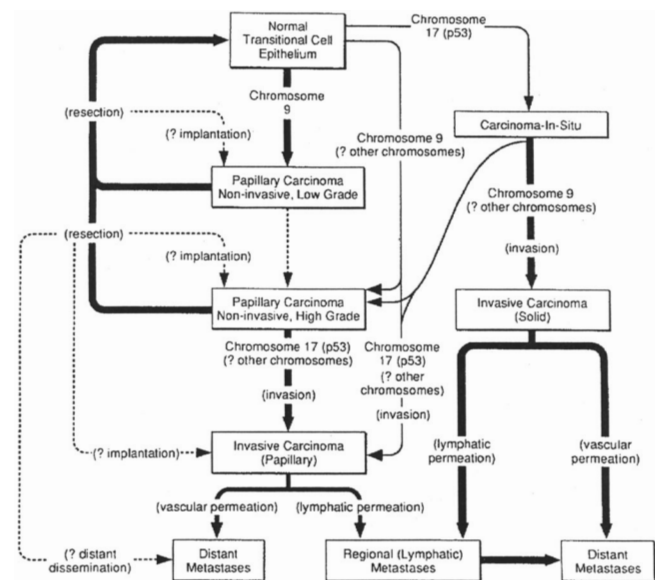


Fig. 3. Proposed pathway for bladder tumor development derived from molecular epidemiological data. The divergent, distinct pathways for superficial and invasive tumors parallels the model developed from observations of clinical biology (Fig. 1). From Jones P.A. and Droller M.J. Pathways of development and progression in bladder cancer: new correlations between clinical observations and molecular mechanisms. *Semin. Urol.* 11, 177-92, 1993.

familial bladder cancer gene, which is a distinct entity from the known cancer predisposition syndromes.

Importantly, microsatellite alterations are common in bladder cancer and analysis of genomic instabilities in urine samples has been recently evaluated as a method for bladder cancer screening with promising results in terms of sensitivity, specificity compared to classical techniques (Seripa et al., 2001; Zhang et al., 2001; Berger et al., 2002; Utting et al., 2002).

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 (Hunstein and Rehn, 1975). 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 (Soloway and Masters, 1980). 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.

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. The pendulum swung several times in the ensuing years. In the 1950's, Melicow, and Kaplan clearly demonstrated associated areas of dysplasia and pre-neoplasia in the urothelium intervening between sites of frank neoplasia. 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. These observations were later expanded on by Soloway (Soloway and Masters, 1980) and the specific mechanisms involved in tumor implantation delineated by See (See et al., 1989, 1990).

A definitive answer to the issue of the mechanism of bladder tumor recurrence was not provided until early in the 1990's. 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 (Sidransky et al., 1992). Subsequently, Habuchi demonstrated that heterotopic urothelial recurrence was associated with identical mutations in P53 at both the upper and lower track sites of occurrence (Habuchi et al., 1993a). Most recently this same group did microsatellite analysis on patients with multifocal metachronous tumor recurrence (Takahashi et al., 1998). 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 (Tsai et al., 1995). 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 (See and Chapman, 1987). 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 (See and Williams, 1992; See, 1993; See et

al., 1994). 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 (Turkeri et al., 1998). 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. (Crew et al., 1999). 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 (Mahadevan and Hart, 1990) 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 (Bernstein and Liotta, 1994). 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 (Rosin et al., 1995). 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 (Knowles et al., 1994; Rosin et al., 1995). In a recent series, Knowles (Knowles et al., 1994) 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 (Li et al., 1996), 4q (Polascik et al., 1995), 8p (Wagner et al., 1997), 18q (Brewster et al., 1994), 10 (Cappellen et al., 1997; Kagan et al., 1998), 15 (Wheless et al., 1994; Wick et al., 1996), and 17p (Reznikoff et al., 1996). Some of these changes have also been observed in a recently characterized highly tumorigenic variant of the T24 human bladder cell line (Gildea et al., 2000).

Previous studies on predominantly superficial bladder cancer specimens (Knowles et al., 1994) 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 (Cappellen et al., 1997), 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 (Kagan et al., 1998) 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 (Wick et al., 1996) 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 (Wheless et al., 1994).

Recently, our laboratory has developed a novel technique which combines information obtained from gene expression array analysis of human cancer cells with that of chromosomal position to allow expression mapping (Harding et al., 2002). Comparing the results obtained with this technique to those of comparative genomic hybridization (CGH), has allowed us to focus on areas of the genome which have both altered expression and chromosomal changes leading to the discovery of a new metastasis suppressor gene (see "Molecular and immunohistochemical changes associated with TCC progression" below).

Molecular and immunohistochemical changes associated with TCC progression

Studies utilizing immunohistochemical techniques (IHC) have suggested that overexpression of HRAS protein (discussed above) (Fontana et al., 1996), P53 (Lacombe et al., 1996) and the epidermal growth factor receptor (EGFR) (Lipponen and Eskelinen, 1994) in bladder tumors may be related to bladder tumor progression. Loss of RB (Reznikoff et al., 1996) and E-Cadherin (Schmitz-Drager et al., 1996) 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 (Mareel et al., 1997). This may explain the inverse relation between expression of E-cadherin and bladder tumor grade (Syrigos et al., 1995). 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 (Bringuier et al., 1993). These relationships to stage and grade were subsequently confirmed by other groups (Griffiths et al., 1996; Shimazui et al., 1996; Byrne et al., 2001; Shariat et al., 2001) while those to survival were sometimes (Shimazui et al., 1996) but not always (Lipponen and Eskelinen, 1995) shown, despite a correlation with progression (Byrne et al., 2001; Shariat et al., 2001) and distant metastasis (Mialhe et al., 1997). 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 (Gorgoulis et al., 1995) and higher rates of recurrence (Chow et al., 1997) and progression in superficial forms of the disease (Lipponen and Eskelinen, 1994). 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 (Nguyen et al., 1994), 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 (Sauter et al., 1994). 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 (Bos, 1998; Theodorescu et al., 1991).

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 (Chow et al., 1994), 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 (Messing and Reznikoff, 1987). Supporting the notion that EGFR overexpression is causally related to tumor progression and not merely an epiphenomenon are a number of in vitro (Theodorescu et al., 1991, 1998b) 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 (Cairns et al., 1991). RB alterations in bladder cancer as a function of stage was studied in 48 primary bladder tumors (Cordon-Cardo et al., 1992) 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. In addition, RB changes are associated with proliferative indices (Ioachim et al., 2000).

Two recent studies (Cordon-Cardo et al., 1997b; Cote et al., 1998) 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 (Grossman et al., 1998) however this is not found in all studies (Wada et al., 2000). These data indicate the some clinical utility of stratification of T1 bladder cancer patients based on *P53* and *RB* nuclear protein status. Thus 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 (Logothetis et al., 1992; Jahson and Karlsson, 1998), 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 (Cordon-Cardo et al., 1997a). 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 (Fujimoto et al., 1992). 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 (Soini et al., 1993), IHC detectable *P53* protein was studied in 42 bladder carcinomas. One out of 11 grade I (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 (Matsuyama et al., 1994) 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. (1993) 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 (Lipponen, 1993; Sarkis et al., 1993; Ioachim et al., 2000) 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) (Sarkis et al., 1993). 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 (Lipponen, 1993) reported an analysis of T1 tumors using immunohistochemistry and 20% positive nuclear staining as the cutoff value. The mean follow-up time was greater than 10 years. 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. The relationship of *P53* expression and BCG treatment response in patients with high risk superficial cancer has been evaluated but no clear concisus is yet apparent (Fontana et al., 1999; Pfister et al., 1999a; Zlotta et al., 1999; Peyromaure et al., 2002).

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 (Sarkis et al., 1995). 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 non-expressors 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 (Esrig et al., 1994). 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 (Furihata et al., 1993). 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 (Sauter et al., 1995; Pfister et al., 1999b). These data were consistent with a role of chromosome 8 alterations in bladder cancer progression (Wagner et al., 1997). However subsequent studies have not found statistical significant correlation between the methylation, expression of MYC gene and clinical-histopathological parameters (Sardi et al., 1998), between the MYC methylation pattern and clinical stage (Sardi et al., 1997). Furthermore, MYC overexpression did not correlate with tumor grade or tumor progression (Schmitz-Drager et al., 1997b). 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 (Novara et al., 1996; Ravery et al., 1997). However, other studies have failed to link ERBB2 expression levels as an independent variable predicting disease progression (Underwood et al., 1995). Other studies have indicated a high level of expression of this gene in malignant as compared to benign bladder epithelium (Underwood et al., 1995). 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 (Lianes et al., 1994). In addition, the simultaneous assessment of MDM2 and P53 was found to be independent factors for both disease progression and survival (Pfister et al., 1999b; Shiina et al., 1999). However, as with MYC and ERBB2 not all studies have shown assessment of this gene product to be independently related to tumor progression (Schmitz-Drager et al., 1997a).

Recently, our laboratory has used lineage related human bladder cancer cell lines, and DNA microarray technology to identify genes whose expression diminishes as a function of invasive and metastatic competence. The expression profile of such genes were

then evaluated in pathologically well characterized human tumors and one gene, RhoGDI2 was identified whose expression was suppressed as a function of tumor stage and grade in human cancer. When RhoGDI2 was transferred back into cells with metastatic ability that lacked its expression, it suppressed experimental lung metastasis while not affecting in vitro growth, colony formation or in vivo tumorigenicity. In addition, RhoGDI2 reconstitution in these cells blocked invasion in an organotypic assay and led to a reduction of in vitro motility (Gildea et al., 2002). These results indicate that RhoGDI2 is a metastasis suppressor gene, a marker of aggressive human cancer and a promising target for therapy.

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. The identification of heritable genetic mutations could possibly 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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