Chapter 2
Bladder Carcinogenesis and Molecular Pathways

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2.1 Introduction

Bladder cancer is primarily a "carcinogenesis driven" cancer. Exposure to carcinogens such as polycyclic aromatic hydrocarbons (PAH), aromatic amines (AA), and nitrosamines through cigarette smoking, occupational exposure, and hair dyes, among other substances, is strongly associated with increased risk for bladder cancer (DeMarini 2004; Murta-Nascimento et al. 2007). Many of these carcinogens form bulky DNA adducts, eventually causing mutations and chromosomal aberrations (Castaño-Vinyals et al. 2007; Veglia et al. 2008). Such molecular events take place when potential carcinogens are "activated" during the process of detoxification by metabolic enzymes that are involved in the metabolism of xenobiotic compounds. Genetic polymorphism in many of these genes is associated with risk for developing bladder cancer. In this first part of this review, we will discuss the relationship between chemical carcinogens and the development of bladder cancer. Furthermore, we will describe the role of the carcinogen-metabolic enzymes and the polymorphism in the genes encoding them in the development of bladder cancer. The exposure of the bladder urothelium to carcinogens that are excreted through urine has given rise to the theory of "field effect" and it is used to explain the multifocality and recurrent nature of bladder cancer. The heterogeneity of low- and high-grade bladder tumors to invade and metastasize has given rise to the concept of "divergent molecular pathways" for bladder cancer development. In the second part of this review, we will discuss the clonal origin of this multifocal disease and elaborate on the molecular pathways in the development of low- and high-grade bladder tumors.

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2.2 Bladder Carcinogenesis

2.2.1 Bladder Carcinogens and DNA Adduct Formation

The major risk factor for bladder cancer development is the exposure of the urothelium to chemical carcinogens. PAHs and aromatic amines (AA) are the most widely known and studied bladder carcinogens, as described in the literature. These carcinogens, when converted into active forms due to body's response to detoxify them, form DNA adducts in urothelial cells. DNA adducts are known to induce point mutations and such mutations in oncogenes (e.g., H-ras) or tumor suppressor genes (e.g., p53) cause cellular transformation. Additionally, point mutations in DNA repair enzymes can cause chromosomal instability leading to cellular transformation. Chromosomal instability is a major factor in the development of invasive bladder cancer (Florl and Schulz 2008).

2.2.2 Phase I and II Enzymes

The xenobiotic metabolism involves two different classes of enzymes. Phase I enzymes (e.g., cytochrome P450 dependent monooxygenases) are usually involved either in oxidation (to form *N*-hydroxy, phenol, or dihydrodiol intermediates) or reduction, which activates carcinogens into a reactive form (Talalay 1989). For example, AAs (e.g., 4-amino biphenyl) are converted into the *N*-hydroxy amine form by CYP-A2. The *N*-hydroxy arylamines can be transported through the blood, where it can form adducts with hemoglobin. These *N*-hydroxy AAs (electrophilic) pass through renal filtration into bladder lumen and can form adducts with DNA (nucleophilic) in urothelial cells (Kadlubar 1990; Kadlubar and Badawi 1995). In fact, urinary mutagenecity is correlated with the levels of 4-aminobiphenyl adducts in exfoliated urothelial cells from smokers (Talaska et al. 1991). Chemical fractionation of urine from smokers indicates that much of the mutagenic activity is due to PAH or AA (DeMarini 2004).

2.2.2.1 Cytochrome P450 (CYP) Monooxygenases

CYP450 enzymes are a superfamily of microsomal enzymes with more than 20 members. These enzymes are extremely polymorphic and the polymorphism in some of the enzymes is associated with higher risk for bladder cancer development. The CYP profile is altered in different types of cancers. For example, important CYPs in bladder urothelium that are reported are CYP1B1 and CYP4B1 (Roos and Bolt 2005). In a case–control study Grando et al. found that CYP1A1 (A2455 -> G) polymorphism significantly associated with risk for bladder cancer (Grando et al. 2009). However, Srivastava et al. found no association between the

CYP1A1*2A genotype and increased risk for bladder cancer among people from North India (Srivastava et al. 2008). Similarly, Fontana et al. found no association between CYP1A1 or CYP1B1 polymorphisms and bladder cancer risk (Fontana et al. 2009). Among the Japanese population, people carrying CYP4B1*1/*2 or *2/*2 genotypes were found to have increased risk for developing bladder cancer than individuals with the CYP4B1*1/*1 genotype (Sasaki et al. 2008). Extensive metabolizer genotype CYP2D6*1A was reported to be significantly associated with the development of transitional cell carcinoma of the bladder cancer, rather than squamous cell carcinoma of the bladder (Abdel-Rahman et al. 1997). Contrarily, carriers of at least one CYP2A6*4 allele were reported to have lower risk for developing bladder cancer than noncarriers (Song et al. 2009). However, Querhani reported no association between CYP2D6*4 allele and susceptibility to bladder cancer in Tunisian population (Ouerhani et al. 2008). Altayli et al. also found no association between polymorphism in CYP1A2 or CYP2D6 genes and risk for bladder cancer (Altayli et al. 2009).

Taken together, polymorphisms in CYP genes have different degree of association with the development of bladder cancer and this association may depend on ethnic origin, as well as, smoking history.

2.2.3 Phase II Enzymes

Phase II enzymes are mainly involved in detoxification of chemical carcinogens, such as AA and PAH. The inducers of phase II enzymes have an electrophilic olefin or related electron-deficient center that is susceptible to attack by nucleophiles. Thus, all inducers of phase II enzymes are "Michael reaction acceptors" characterized by olefinic or acetylenic linkages that become electrophilic by conjugation with electron withdrawing groups (Talalay 1989; Talalay et al. 1988). Examples of well-studied phase II enzymes in the context of bladder cancer are *N*-acetyl transferases (NAT) and glutathione-*S*-transferases (GSTs). Polymorphisms in these genes are associated with bladder cancer risk.

2.2.3.1 *N*-Acetyl Transferases (NAT)

NAT1 and NAT2 isoenzymes catalyze both N-acetylation (deactivation) and O-acetylation (activation) of aromatic and heterocyclic amines (Franekova et al. 2008). They catalyze the transfer of acetyl group from acetyl-CoA to AA and hydrazine substrates. AA and PAH are O-acetylated by both NATs. Although NAT1 and NAT2 share 87% nucleotide homology (only 55 out of 290 amino acids are different), NAT2 has three- to fourfold affinity to bladder urinary carcinogens such as 4-ABP than NAT1 (Hein 2006). The association between polymorphisms in NAT1 gene and bladder cancer risk is not well established. Fast acetylation status of NAT1 has been associated with risk for colorectal cancer, however, no such

correlation was found in metaanalysis of case–control and cohort studies (Sanderson et al. 2007). Certain alleles of NAT1 (NAT1*14, NAT1*15) do not have enzyme activity, however, their association with risk for bladder cancer has not been evaluated (Franekova et al. 2008).

A number of single nucleotide polymorphisms in the coding region result in the slow acetylator phenotype (i.e., NAT2) is unable to efficiently acetylate bladder carcionogens such as AA and PAH. The slow acetylator phenotype has been associated with risk for a variety of carcinomas, including bladder cancer. The proposed mechanism for the association between the slow acetylator phenotype and bladder cancer is that the slow acetylation of AA by NAT2 is competed out by the metabolic activation of these AAs by CYP enzymes (Hein 2006). Therefore, the slow acetylator phenotype combined with Fast CYP phenotype carries higher risk for developing bladder cancer than either phenotype alone or the reverse phenotype (i.e., fast NAT2 and slow CYP). NAT2 alleles that contain arginine64->glutamine; isoleucine114->threonine, arginine197->glutamine, or lysine268->arginine substitutions are associated with slow acetylator phenotype. The association between NAT2 polymorphism and the risk for bladder cancer has been summarized in a variety of excellent reviews (Hein 2006; Franekova et al. 2008; Sanderson et al. 2007). Recent studies show that the slow acetylator NAT2 phenotype increases risk for bladder cancer by about threefold in a variety of ethnic groups and the risk may be higher in females (Ouerhani et al. 2008; Fontana et al. 2009; Song et al. 2009). However, the slow acetylator NAT2 phenotype is not related to racial differences among blacks and whites regarding the risk for developing bladder cancer (Muscat et al. 2008). Smokers with slow acetylator phenotype may have up to 12-fold increase risk for developing bladder cancer (Rouissi et al. 2009) and this may explain the variation observed among smokers regarding bladder cancer development.

2.2.3.2 Glutathione-*S*-Transferase (GST)

In humans, eight distinct gene families encode different GSTs: alpha, mu, theta, pi, zeta, sigma, kappa and chi (or omega; Franekova et al. 2008). Among these, GSTM1, GSTT1, and to a lesser extent GSTP1 are the most well studied with respect to the risk for developing bladder cancer. As reviewed by Franekova et al., GSTT1 (theta group) detoxifies smaller reactive hydrocarbons, whereas, GSTM1 (mu group) detoxifies PAH (Franekova et al. 2008). Polymorphism in GSTM1, GSTT1, and GSTP1 has been well documented in bladder cancer. Earlier studies showed increased GSTP1 and GSTM1 activity in bladder tumor tissues when compared to normal bladder tissues. GSTT1–1 and GSTM1–1 are genetically deleted (nonfunctional alleles GSTT1*0 and GSTM1*0) in a high percentage of the human population, with major ethnic differences. For example, 20% of Caucasians are homozygous for a null allele GSTT1*0 (Franekova et al. 2008). Similarly, nearly 50% of the Caucasian population may have GSTM1 null phenotype. GSTM1 and GSTT1 null genotypes are associated with an increased risk of bladder cancer and

the risk increases further when GSTT1 null phenotype is combined with smoking or occupational exposure to AA (Salagovic et al. 1998; García-Closas et al. 2005; Moore et al. 2007; Yuan et al. 2008). Double negative individuals (GSTM1 and GST1 null) have even higher risk for developing bladder cancer (El Nouby et al. 2008; Song et al. 2009). Similarly, G/G genotype of the GSTP1 gene polymorphism may also be associated with risk for bladder cancer (Srivastava et al. 2005a, b). Interestingly, Rouissi et al. found that individuals with NAT2 slow acetylator and wild type GSTT1 and GSTM1 null phenotype have the highest risk for bladder cancer in Tunisian population (Rouissi et al. 2009).

The two electron reductase NADP(H) dehydrogenase quinine 1 (NQO1) can either activate or detoxify quinones from AA or PAH intermediates (Joseph et al. 1994). Similarly, sulfotransferases are a supergene family of enzymes that catalyze sulfonation of several xenobiotic compounds. Sulfonation of a nucleophilic group decreases its activity; however, may also generate electrophilic species, which can then form DNA adducts. There are three sulfotransferase families (SULT1, SULT2 and SULT3), each with more than ten members (Franekova et al. 2008). Polymorphisms in both NQO1 and SULT genes have been shown to be associated with risk for bladder cancer. For example, subjects carrying both the NQO1 C/T and T/T genotypes and the SULT1A1 G/G genotype have nearly fourfold increased risk of developing bladder cancer than noncarriers. The risk doubles if these individuals are either current or former smokers (Wang et al. 2008). C to T base change at position 609 of the human NQO1 cDNA (C609T) changes Proline 187 to Serine. Individuals with this change have trace amount of NQO1 protein and no NQO1 activity (Siegel et al. 1999). Variant allele carriers of the NQO1 (P187S) polymorphism may have a higher risk for high-stage bladder cancer than noncarriers at diagnosis. Furthermore, patients with NQO1 (R139W) variant allele carrier along with Ta/T1 high-grade bladder cancer may have shorter disease-free survival than noncarriers (Sanyal et al. 2007). Similar to the polymorphisms in NQO1 gene, the SULT1A1 gene possesses a G→A polymorphism that changes Arginine 213 to Histidine. The Histidine (213) allele has been shown to have low activity and low thermal stability. Zheng et al. found reduced risk for developing bladder cancer if the individuals are either homozygous or heterozygous for Histidine 213 (Zheng et al. 2003). Recently, Figuerora et al. in a case–control study involving over 2000 subjects reported that polymorphisms in an aldo-keto reductase gene AKR1C3 and in aryl hydrocarbon nuclear translocator gene significantly associate with risk for developing bladder cancer (Figueroa et al. 2008).

Taken together, the risk for bladder carcinogenesis is dependent on exposure to carcinogens, as well as, the relative enzyme activity of phase I and II enzymes. Individuals with fast acetylator phase I enzyme (i.e., faster conversion of PAH and AA into active electrophilic species), when combined with slow acetylator phenotype for NAT2 enzyme or the polymorphisms in GST enzymes, which render the phase II enzyme to have low activity (i.e., slower detoxification rate), have the highest risk for bladder cancer while individuals with opposite phenotype for phase I and II enzymes have the lowest risk.

2.3 Field Cancerization and Clonal Origin of Bladder Cancer

Synchronous and metachronous tumors, as well as frequent tumor recurrence, can be explained by the concept of "field cancerization," where the entire bladder urothelium is exposed to carcinogens and the entire urothelium is primed to undergo transformation (Braakhuis et al. 2003). This concept was initially introduced by Slaughter et al. (Slaughter et al. 1953) in 1953, when studying oral squamous cell carcinoma. It has now been applied to a variety of tumors, including bladder. When exposed to carcinogens, urothelial cells can accumulate independent point mutations, gene deletions, or duplications, some of which can cause cellular transformation. Thus, field cancerization should lead to independent transformation of many urothelial cells resulting in genetically unrelated tumors. With the discovery of cancer stem cells, the field cancerization concept has been modified. It is suggested that the resident urothelial stem cells within the urothelium, when exposed to carcinogens, undergo transformation into cancer stem cells, and the clonal expansion of different cancer stem cells results in multifocality and recurrent tumors. Additional accumulation of genetic alterations (mutations, loss of heterozygosity (LOH)) introduces more heterogeneity in bladder tumors (Habuchi 2005; Cheng et al. 2009).

The second concept is the clonal origin of multifocal and recurrent tumors. The clonogenic or single progenitor cell hypothesis states that genetic and phenotypic diversity, observed in multifocal bladder tumors, is the progressive accumulation of genetic alterations in clones of a single transformed cell. In addition to field cancerization and clonal expansion, epigenetic modifications and cellular microenvironment can further contribute to tumor heterogeneity (Duggan et al. 2004). Combining the concept of field cancerization, cancer stem cells, and clonal origin of bladder tumors Höglund expanded the concept of the field effect further, suggesting a new model "field first-tumor later" (Höglund 2007). In this model, aberrant cells (due to carcinogen exposure), with a stem cell or stem-cell like properties spread in the urothelium by cellular displacement, creating fields of premalignant cells. Tumor development at multiple sites then depends on the frequency and timing of critical genetic events in individual cells in such a field. Hence, recurring tumors originate from the field of same premalignant tumors, and not from previous overt tumors. Therefore, the "field" remains even after the removal of the primary tumor(s) (Braakhuis et al. 2003). This concept can also explain clinical observations that tumor recurrence is actually the occurrence of a new tumor in the bladder.

Genetic analyses such as evaluation of LOH using microstatellite markers, X-chromosome inactivation assays, comparative genomic hybridization (CGH), and fluorescence in situ hybridization (FISH) have been used in support of either the field cancerization effect or the clonal origin of bladder cancer. LOH is the most frequent alteration in bladder cancer. Among all genetic alterations, LOH has about 75% frequency followed by chromosome length alterations (25%; Berger et al. 2002; Fadl-Elmula 2005). LOH in chromosome 9 has been used in support of the clonal origin concept. Junker et al. reported that in their study, 80% of the cases of recurrent tumors from the same patient had the same LOH in chromosome 9

(Junker et al. 2005). Contrarily, Dahse et al. used p53 mutational analysis to conclude that recurrences may occur through genetically unrelated primary tumor sites. Furthermore, synchronous and metachronous tumors may have polyclonal origin due to field cancerization effect (Dahse et al. 2003). In addition of the chromosome 9 LOH, using CGH analyses, Prat et al. found gains of chromosome 1q, 2p, and 17q loci and loss of 4q locus in multicentric tumors. They concluded that accumulation of chromosomal alterations is a form of clonal evolution from a single progenitor cell. Furthermore, heterogeneity present in the same tumor is the result of genetic evolution of a clonally expanded progenitor cell, which also, probably, occurs in the synchronous tumors in a same patient (Prat et al. 2008).

The epithelial and mesenchymal components in sarcomatoid urothelial carcinoma may also arise from a single progenitor cell. Armstrong et al. reported that in sarcomatoid urothelial cell carcinoma, both epithelial and mesenchymal components have identical p53 mutations, and, therefore, have the same clonal origin (Armstrong et al. 2009). Similarly, Sung et al. found identical pattern of LOH with six polymorphic microsatellite markers and X-chromosome inactivation in both the carinomatous and sarcomatoid components (Sung et al. 2007). Small cell carcinoma of the bladder is often mixed with transitional cell carcinoma. Using microstatellite markers on chromosomes 3p25–26, 9p21, 9q32–33, and the TP53 locus and an X-chromosome inactivation assay, Cheng et al. reported that both the coexisting tumor components originate from the same cells in the urothelium (Cheng et al. 2005). Lymph node metastases and primary tumors can also be traced to a common clonal origin. Using LOH in chromosome 9 and 17p13 (p53 locus) and X-chromosome inactivation analysis, Jones et al. showed that metastasis often arises from only a single clonal population in the primary tumor. Additional gene variations then arise during clonal evolution of urothelial carcinoma (Jones et al. 1993, 2005a). Contrarily, using the same allelic markers, the same authors reported that each of the coexisting tumors in multifocal urothelial carcinoma has a unique clonal origin. These tumors arise from independently transformed urothelial progenitor cells as a result of the field cancerization effect (Jones et al. 2005b).

A review of the study cited above and the published literature suggest that multifocal and recurrent tumors are a result of both clonal origin and field cancerization effect. However, these two are not necessarily mutually exclusive events. Changes in the urothelial progenitor cells can trigger clonal expansion, but accumulation of different genetic alterations in different clones (due to field cancerization effect) results in tumor heterogeneity. Depending upon the microsatellite marker analyses, one may find evidence for one of these concepts.

2.4 Chromosomal Aberrations in Bladder Cancer

Rearrangements and/or chromosomal aberrations are most common in chromosome 9 and occur in more than 50% of all bladder tumors. These alterations are present in both low-grade nonmuscle-invasive tumors and in high-grade muscle-invasive tumors. Thus, the loss of chromosome 9 is considered an early event in bladder

cancer and the regions most often are the 9p21 locus and three or more regions in the long arm of chromosome 9 (pq22, 9q32–33, and 9q34) (Fadl-Elmula 2005). The candidate tumor suppressor genes in these loci are CDKN2A/ARF (p16/p14ARF; 9p21), CDKN2B (p15; 9p21), PTCH (Gorlin Syndrome gene; 9q22), DBC1 (deleted in bladder cancer 1 locus; 9q32–33), and TSC-1 (tuberous sclerosis syndrome gene; 9q34) (Knowles 2006, 2008). Gain of chromosome 7 (chromosome 7 trisomy) is a common finding in bladder cancer and it is one of the three chromosome gains that are examined to make an inference on the UroVysion test (a FISH test for bladder cancer). A known consequence of this chromosome gain is the increased number of alleles for epidermal growth factor receptor (Knowles 2006).

Other chromosomal aberrations that are detected at a higher frequency in bladder cancer include rearrangements in chromosome 1, 8, and 11. For example, amplification of the chromosome 1Q32 has been reported in bladder cancer tissues using CGH array on a whole genome BAC/PAC cosmid. Mouse double minute 4 (MDM4) homologue is the amplified gene in this locus and the amplification occurs in tissues that express wild type p53 (Veerakumarasivam et al. 2008). Allelic imbalance at chromosome 1q36 locus is associated with poor survival among patients who receive chemoradiation therapy following cystectomy for muscle-invasive bladder cancer (Matsumoto et al. 2004). Most frequently, chromosomal aberrations associated with bladder cancer are the deletion of the 8p locus and gain of 8q locus. CMYC is the candidate oncogene on 8q24 and alternations in CMYC gene, including copy number changes, are associated with bladder cancer (Zaharieva et al. 2005). Similarly, in a genome-wide SNP association study involving over 4000 bladder cancer casesand over 37,000 controls, polymorphism in the chromosome 8q24 locus (allele T of rs9642880) was found to confer susceptibility to nine smoking-related cancer cases, including bladder cancer (Park et al. 2008). Contrarily, tumor suppressor genes such as human beta defensin-1 and MTUS1 have been found on chromosome 8p23 and 8p22 loci, respectively, which may explain why the loss of chromosome 8q is associated with bladder cancer (Di Benedetto et al. 2006; Sun et al. 2006).

Aberrations in chromosome 11 (polysomy) may be found in up to 70% of bladder tumors (Watters et al. 2002; Panani et al. 2004). Amplification of the 11q13 locus has been reported in bladder cancer. Putative candidate oncogenes located in this region are CCND1 (cyclin D1: PRAD1, bcl-1), EMS1, FGF3 (Int-2), and FGF4 (hst1, hstf1). Zaharieva et al. evaluated the involvement of these genes in a FISH study involving over 2000 bladder specimens. The frequency of gains and amplification of all four genes was observed in about 70% of the specimens and correlated with tumor grade and stage. In addition, the amplification correlated with poor survival and the progression of T1 tumors (Zaharieva et al. 2003). Similarly, Shao et al. observed CCND1 translocation and amplification only in bladder cancer patients (Shao et al. 2004). Contrary to the 11q13 locus, loss of the 11p locus is observed frequently in patients with bladder cancer (Brunner et al. 2008).

In addition, chromosomal imbalances have been observed a variety of loci, including 1q, 2q, 4q, 10p, 10q, 11p, 11q, 12q, 13q, 15q, 17p, and 19q. By CGH array, common chromosomal alterations included gain of 1p, 1q, 12q, 16p, 17q, and

19p as well as loss of 4q and 9p, in most of the cases (Brunner et al. 2008; Chan et al. 2009). As reviewed recently by Knowles, low-grade and high-grade tumors show different allelic imbalance. For example, as discussed below, FGFR3 mutations are associated with low-grade tumors, whereas, the LOH of 10q is frequent in muscle-invasive tumors. This LOH site harbors the PTEN gene, which is a negative regulator of Akt signaling (Knowles 2008).

TP53 mutation is an event in bladder tumors and its relation with tumor progression and the molecular pathways of bladder cancer development has been extensively examined. The p53 gene is located in the chromosome 17p13 locus. The expression of mutated P53 is highly elevated particularly in invasive bladder cancer. The wild type P53 protein has a half-life of 15–30 min, however, mutated P53 has a longer half-life (Nishiyama et al. 2008). Therefore, nuclear accumulation of mutated P53 can be detected by immunohistochemistry, although in one study accumulation of wild type P53 protein has been reported (Abdel-Fattah et al. 1998). About 75% of P53 mutations are missense substitutions, while the remaining include frame shift and deletion mutations (Nishiyama et al. 2008). The missense mutations are often in the DNA binding domain and, hence, the mutated protein loses its transactivation activity (73). The rate of P53 mutations in infiltrating tumors is about 60% and P53 pathway is inactive in T1G3 tumor (López-Knowles 2006; Nishiyama et al. 2008). The progression-free survival is often significantly shorter in patients with tumors expressing mutant TP53 (Ecke et al. 2008). Mutated P53 is also an independent predictor of death among patients with muscle-invasive bladder cancer and of cancer specific mortality, following radical cystectomy (Salinas-Sánchez et al. 2008; Shariat et al. 2009). However, wild type P53 can also accumulate in the nucleus and have prognostic significance. Datar et al. reported that not only the presence of mutation but mutation site in the P53 gene is also associated with disease outcome (i.e., time to first recurrence). For example, they reported that the mutation in exon 5 have similar outcome as the wild type P53 gene. Mutations in exon 8 had intermediate outcome, and the mutated P53 gene with mutations in several exons is associated with the worst outcome (George et al. 2007). With over 500 studies conducted on P53 mutations and its relation to bladder cancer, alterations in P53 remain one of the heavily investigated areas in bladder cancer.

Inhibition or alteration in the retinoblastoma (Rb) pathway occurs commonly in high-grade invasive tumors. However, LOH at or around the Rb locus has not been well studied. Miyamoto reported that LOH at the Rb locus occurs in 80% of invasive tumors but only in 20% of the low-grade tumors (Miyamoto et al. 1996). Wada et al. have reported that LOH found around (at 13q11–12.1) or at the Rb locus (13q14.3) is found in about 20%–30% of cases. Furthermore, the LOH at the Rb locus significantly correlates with tumor grade and stage (Wada et al. 2000).

Recently, using whole-organ histologic and genetic mapping six chromosomal regions, critical for clonal expansion of in situ neoplasia were identified; these include 3q22–24, 5q22–31, 9q21–22, 10q26, 13q14, and 17p13 (Lee et al. 2007; Majewski et al. 2008). LOH at these sites was found to persist through the entire sequence of neoplasia, from morphologically normal regions to invasive carcinoma. Some of the target genes identified in these regions have been termed as “forerunner

genes" and these genes are thought to be relevant for the development of bladder cancer. The concept of forerunner genes proposes three waves of genetic hits. First wave encompasses clonal expansion of phenotypically normal-appearing urothelial cells over large portions of bladder mucosa. The second wave is associated with subregions of clonally expanded cells showing some features of dysplasia. The last wave is associated with full transformation. The two of the six chromosomal regions include the P53 locus (17p13) and the Rb locus (13q14). As an evidence for the forerunner gene concept two new genes ITM2B and P2RY5 were identified. These genes, when silenced by methylation, contribute to the development of neoplasia (Crawford 2008; Majewski et al. 2008). Additional candidate genes include GPR38, CAB39L, RCBTB1, and ARL11. The forerunner gene concept is highly attractive, however, the identity and the functional utility of many of the genes remains unknown. Furthermore, it remains unexplored whether and how the forerunner gene concept can explain the tumor heterogeneity, polymorphism in phase I and II enzymes, and divergent pathways of bladder cancer development.

2.5 Molecular Pathways for Bladder Cancer Development

Unlike prostate cancer, bladder cancer is rarely an incidental finding. Clinical and pathological evaluations identify three different phenotypes in bladder tumors. Low-grade tumors are hyperproliferative lesions. Neoplastic cells continue to proliferate, induce neovascularization and develop into nonmuscle-invasive tumors. These tumors can extend into the bladder lumen, but rarely invade the basement membrane and penetrate into bladder wall. Low-grade tumors account for nearly 75%–80% of bladder tumors. Low-grade tumors do not "progress" to become high-grade tumors, and therefore, must have a distinct molecular pathway of development. The second phenotype in bladder tumors is high-grade tumors. These tumors are made up of highly proliferative neoplastic cells, which also have the ability to invade lamina propria and beyond. In molecular terms, a Ta tumor is not necessarily similar to a T1 tumor because the latter already has established invasive activity (Lee and Droller 2000). The third pathway of development is carcinoma in situ (CIS). These are hyperproliferative, but highly invasive bladder cancer cells that spread horizontally, maintaining a flat appearance. In 2%–30% of cases, CIS can penetrate the basement membrane and lamina propria, with ultimate progression rate about 30%–50% (Lee and Droller 2000).

Based on these clinical observations, Droller was the first to suggest divergent pathways for the development of low-grade and high-grade tumors and CIS (Droller 1981). In 1993, Jones and Droller suggested that divergent, yet somewhat interconnected molecular pathways, may be involved in the development of the three distinct types of tumors (Jones and Droller 1993). Around the same, based on the molecular signatures available at that time, Spruck et al. proposed 2-pathway model for bladder cancer: one arm encompassing low-grade tumors, which display LOH in chromosome 9, and the other arm representing high-grade tumors characterized by p53 mutations (Spruck et al. 1994). They made an observation

that LOH in chromosome 9 is present at a higher frequency (34%) than in CIS and dysplasia. In contrast, they found that only 3% of the Ta tumors had mutated p53, while in CIS and dysplasia lesions, 65% of p53 was mutated. CIS and high-grade tumors from the patient had different mutations, supporting three divergent pathways of bladder cancer development (Spruck et al. 1994). As discussed above, the LOH in chromosome 9 occurs in >50% of all bladder tumors, regardless of tumor grade and stage. In addition, both synchronous and metachronous lesions show identical LOH at chromosome 9 loci, suggesting that the LOH in chromosome 9 is a very early event, prior to the molecular divergence of low-grade and high-grade tumors (Knowles 2006).

Mutations in FGFR3 and p53 have been suggested as the molecular signatures for the divergent pathways of low- and high-grade bladder tumor development. Mutations in fibroblast growth receptor (FGFR) 3 occur at high frequency (60%–80%) in noninvasive low-grade tumors, whereas, p53 mutations are common in high-grade invasive tumors and CIS. FGR3 is a member of the FGF-receptor family that binds more than one FGF family member (Eswarakumar et al. 2005). Binding of an FGF ligand to FGFR3 induces receptor dimerization, which then activates the tyrosine kinase activity of the receptor. FGFR3 mutations are associated with high expression of the mutated FGFR3 protein. For example, Tomlinson et al. reported that 85% of the tumors with mutated FGFR3 showed high expression of FGFR3 protein versus 42% of the tumors with high FGFR3 protein levels expressed wild type FGFR3 (Tomlinson et al. 2007). FGFR3 mutations constitutively activate receptor tyrosine kinase, which leads to downregulation of Akt, cell cycle-regulators, and activation of the MAP kinase pathway. Germline FGFR3 mutations that are identical to those found in bladder cancer induce achondroplasia, hypochodroplasia, and neonatal lethal forms of thanatophoric dysplasia (TD I and II) (Eswarakumar et al. 2005).

Using CGH analysis, Junker et al. recently reported a negative correlation between FGFR3 mutations to chromosomal aberrations (Junker et al. 2008). Furthermore, they observed a negative correlation between tumor stage and FGFR3 mutation frequency (Ta: 69%, T1, 38%, ≥ T2, 0%). Similar correlation was also observed between tumor grade and FGFR3 mutations (G1, 72%, G2, 56%, G3, 4%). Earlier studies also had made similar observations regarding the high frequency of FGFR3 mutations in low-grade, low-stage tumors (Bakkar et al. 2003; Rieger-Christ et al. 2003; van Rhijn et al. 2004). FGFR3 mutations are also found in benign urothelial papilloma and flat urothelial hyperplasia, but no mutations are found in the normal urothelium from either healthy controls or from patients with bladder cancer (Otto et al. 2009). Similarly, Lotto et al. reported that 45% of the inverted urothelial papillomas have FGFR3 mutations and the majority of the mutations are found in exon 7 (Lott et al. 2009). Kompier et al. in a recent study followed 118 patients for 8.8 years and reported that FGFR3 mutations are prevalent in both primary and recurrent tumors (63%). Patients were found to have different mutations in different tumors. However, in 81% of the recurrent tumors, the same mutation that was present in the primary tumor was found. Furthermore, patients with mutated FGFR3 had low-grade and low-stage tumors than patients with wild type FGFR3 (Kompier et al. 2009).

A direct comparison of FGFR3 and TP53 mutations in bladder tumors has revealed an inverse correlation between mutated FGFR3 and mutated TP53, i.e., TP53 was expressed at a higher frequency (>50%) in high-grade tumors, whereas, FGFR3 mutations were found in low-grade tumors (Bakkar et al. 2003; Rieger-Christ et al. 2003; van Rhijn et al. 2004). Furthermore, the occurrence of FGFR3 and TP53 mutations may be mutually exclusive (van Rhijn et al. 2004), suggesting that FGFR3 and TP53 mutations represent molecular signatures of the two divergent molecular pathways of bladder cancer development.

Consistent with the idea that FGFR3 and TP53 mutations represent two divergent pathways of bladder cancer development, Lamy et al. found a higher frequency of FGFR3 mutations in low-grade superficial tumors and a higher percentage of TP53 mutations in high-grade invasive tumors (Lamy et al. 2006). However, they observed higher frequency (85%) of FGFR3 mutations and a lower frequency (3%) of TP53 mutations in G2 tumors when compared to G1 tumors (FGFR3 mutations: 54%; TP53 mutations: 23%). The dichotomy of FGFR3 and P53 mutations in G1 and G3 tumors may not exist when comparing G1 and G2 tumors. In this regard, in a study of 119 patients with T1G3 disease, Hernandez et al. found neither a mutually exclusive occurrence of FGFR3 and TP53 mutations, nor any correlation between TP53/FGFR3 mutation status and tumor recurrence (Hernández et al. 2005). The authors suggested that since T1G3 tumors have undergone more molecular changes than low-grade tumors, the "good prognosis" associated with FGFR3 mutations is no longer observed in T1G3 tumors.

These studies suggest that although the "two divergent pathway" model for bladder cancer development and progression is attractive, there is overlap and/or crossover between these two pathways. Based on the current molecular understanding, the two divergent pathways for the development of low- and high-grade bladder tumors are summarized in Fig. 2.1. Since clinically, low-grade/low-stage tumors can progress to high-grade while the latter tumors do not regress to low-grade, it is most likely that the crossover occurs when low-grade tumors, with mutated FGFR3 representing good prognosis, become invasive by acquiring TP53 mutations and/or other alterations such as chromosome 9 LOH, including p16 loss, downregulation of pRb, p21, $p27^{kip-1}$, overexpression of MIB-1and TSC-1 LOH (Mhawech-Fauceglia et al. 2006). It is noteworthy that van Rhijn et al. found that a combination of FGFR3 and MIB-1, but not FGFR3 and TP53, is an independent predictor of prognosis (van Rhijn et al. 2004). Therefore, molecular signatures of low- and high-grade tumors may include MIB-1 or TSC-1 status, in addition to FGFR3 and P53 mutations (Lokeshwar, 2006).

2.6 Summary

The etiology of bladder cancer has been extensively investigated. Our current knowledge is that exposure of the bladder urothelium to carcinogens such as PAH and AAs, through smoking or occupational exposure, is the main cause of bladder cancer.

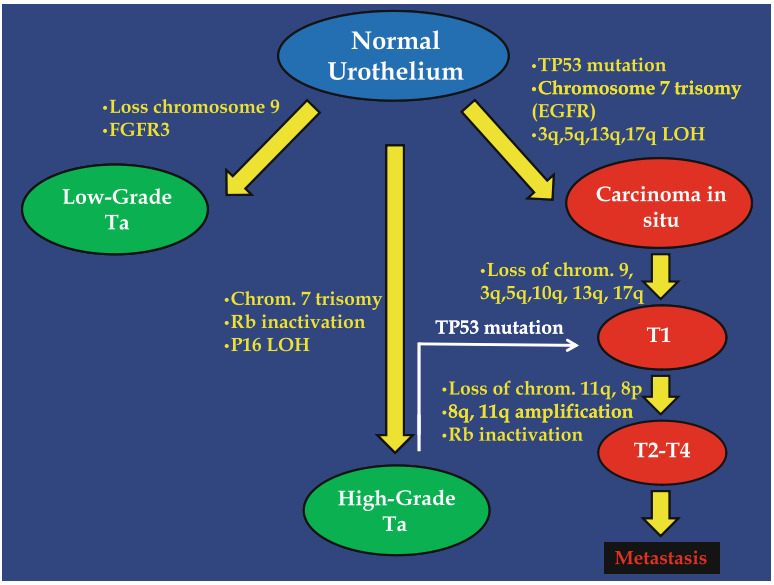

Fig. 2.1 Putative molecular signatures of bladder cancer development and progression

Low-grade tumors, accounting for 80% of all diagnosed cases, frequently recur even after the removal of the primary site. The prognostic for patients with metastatic high-grade tumors is often poor. Multifocal and frequent tumor recurrence has been suggested to be the result of both clonal origin and field cancerization effect. Additionally, ethinic and gender related polymorphism observed in genes encoding for phase I and phase II xenobiotic detoxifying enzymes have been associated with increased risk of developing bladder cancer for individuals of fast phase I acetylator and slow phase II acetylators phenotypes. Moreover, a number of chromosomal aberrations, such as loss of chromosome 9, amplification of chromosome 7 and 11q13, and genetic mutations (i.e., pRb, FGFR3, and TP53), have been associated with both risk for developing bladder cancer, as well as, tumor progression. Recently, MIB-1 and TSC-1 status have been suggested as possible molecular signature markers of low- and high-grade divergent pathways, in addition to FGFR3 and TP53 status.

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