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REVIEW ARTICLE

Molecular mechanisms in urinary bladder carcinogenesis

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Summary

Urinary bladder cancer accounts for approximately 5% of all newly diagnosed malignancies in the developed world. Smoking, occupational exposure and dietary factors constitute the most important exogenous risk factors for bladder carcinogenesis. Yet, individuals with seemingly equal exposure to environmental carcinogens develop bladder cancer in an unpredictable manner. This is probably attributed to the fact that DNA repair capacity varies in human populations, pointing the role of genetic susceptibility in human cancer. Numerous studies demonstrated that certain genetic and epigenetic alterations are fairly constant. Loss of heterozygosity (LOH) at chromosome 9 is an aberration found in urothelial cell carcinoma (UCC) of all stages and grades as well as in dysplastic urothelium, possibly representing an early event in urinary bladder carcinogenesis. On the contrary, gains of 3p can only be found in tumors demonstrating highly malignant behavior. Microsatellite instability (MSI) is another frequent finding in urinary bladder cancer. This has led many investigator groups to employ the analysis for MSI for early diagnosis of UCC with promising results. The silencing of certain genes such as $p16^{INK4A}$ and DAPK by aberrant methylation of their promoter region also represents an important mechanism in carcinogenesis. Similarly, alterations in certain tumor suppressor genes and proto-oncogenes result in uncontrolled cell proliferation, reduced apoptosis and have been associated with more aggressive UCC phenotypes. Undoubtedly, the application of these observations in clinical practice will make a breakthrough in the management of bladder cancer.

Key words: bladder cancer, carcinogenesis, DNA, markers, oncogenes, risk factors

Introduction

Cancer of the urinary bladder is the most common malignancy of the urinary tract. Approximately 70,000 new cases were diagnosed in the United States in 2010, whereas 15.000 patients died from bladder cancer related causes that year [1]. Moreover, it represents one of the most costly malignancies because of its frequent recurrences. According to epidemiological studies, the incidence in white men 2-fold higher compared to black men and the male-to-female ratio is 3-4:1, the latter though having lower 5-year survival rates, possibly due to poorly understood biological factors [2]. In men it is the 4th most common cancer representing 7% of all newly diagnosed cancers [1]. Generally, it is a disease of older individuals, with median age at diagnosis 69 years in males and 71 in females. Although more than 90% of the cases are diagnosed in patients older than 55 years of age, bladder cancer can occur at any age, even in young adults and children. During the last years urinary bladder cancer incidence rates remained fairly constant in men, whereas increased slightly in women [3]. Mortality rates have decreased in both men and women, reflecting the improvements in diagnosis and treatment, currently being the 7th cause of death from cancer in men and the 8th in women.

The most common histologic type is UCC, observed in more than 90% of the cases. Other rarer histologic types include squamous cell carcinomas, adenocarcinomas, and non-epithelial malignancies [4,5]. Bladder tumors which are predominantly urothelial in histology, may also exhibit foci of glandular or squamous differentiation. At presentation 75-85% of the patients are diagnosed with superficial disease, whereas the remaining

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15-25% are diagnosed with invasive disease and about one-third of them manifest distant metastasis. It is estimated that more than 60% of the superficial tumors will recur at least once and progress to less differentiated or invasive neoplasms in 10-15% of cases [6]. Growing evidence suggests that superficial and invasive tumors in the majority of cases arise via two distinct, but overlapping pathways [7]. This hypothesis is sustained by the fact that many muscle invasive tumors demonstrate no evidence of a superficial precursor lesion, but seem to arise from severe epithelial dysplasia or carcinoma *in situ* (CIS) [8]. Furthermore, UCC of the urinary bladder represents a mixture of heterogeneous cell populations, with distinct genotypic and phenotypic patterns characterising early and late stages of the disease [9].

Until now, and despite extensive research, the role of tumor markers in the diagnosis and prediction of the natural history [10-13] of bladder cancer remains to be clarified. Thus, in current clinical practice clinicians try to predict the behavior of bladder tumors in individual patients using scoring systems and risk tables, mostly based on morphologic criteria and history. Various tumor markers have been commonly employed. Nonetheless, molecular biology techniques and clinical experience have shown highly malignant potential even in newly diagnosed, low grade superficial tumors suggesting that with the current systems neither recurrence, nor progression rate can be predicted accurately. Therefore, a better understanding of the natural history of UCC is expected from molecular biology. Molecular biological research is focused on identifying high risk patients, on developing reliable early diagnostic "markers", on the search for non-invasive monitoring techniques and on developing more efficacious therapies. Understanding the biology of bladder cancer is the key to reduce morbidity and mortality.

Exogenous risk factors

Bladder carcinogenesis is thought to develop from the interaction of environmental exposures and genetic susceptibility [14]. Many environmental carcinogens, but primarily aromatic amines are implicated in the development of bladder cancer (Table 1). UCC follows the general concept of multistep carcinogenesis and it is likely that multiple lesions on the DNA of target cells are required for malignant transformation. Moreover, similar carcinogens may facilitate the development of different genetic alterations due to the microenvironment of transitional epithelium.

Smoking

Cigarette smoking is considered the main known exogenous risk factor for UCC of the urinary bladder [15]. Regarding other, less common histologic types, evidence is sparse but a trend towards an increased risk has also been demonstrated. Population studies in different parts of the world estimate that 50% of UCCs are directly attributable to cigarette smoking. More specifically, a linear relationship has been proven between duration and intensity of smoking and risk. On the contrary, results on patterns of inhalation are not clear. Cessation of smoking leads to a 40% reduction of bladder cancer risk within one year, however the risk remains increased for as many as 25 years afterwards.

Tobacco smoke is a heterogeneous mixture containing 4-amino-biphenyl, 2-naphthylamine and many other carcinogens which are metabolised by xenobiotic metabolising enzymes such as N-acetyltransferases (NAT) and glutathione S-transferases (GST). Carcinogens present in tobacco smoke are associated with DNA adduct formation [16] leading to the induction of specif-

Table 1. Carcinogenetic mechanisms and clinical significance of various exogenous risk factors for bladder cancer

Risk factor	*Carcinogen*	*Mechanism of carcinogenesis*	*Clinical significance*
Tobacco use	4-amino-biphenyl 2-naphylamine etc.	DNA adduct formation, base changes in oncogenes or tumor suppressor genes (e.g. TP53)	Approximately 50% of cases, higher cancer-specific mortality rates
Occupational exposure	Aniline dyes, chlorinated aliphatic hydrocarbons, aldehydes, etc.	Metabolites reacting with nucleic acids and cellular macromolecules, metabolic activation and toxic effects	Approximately one-third of cases, long latency period
Environmental exposure	Arsenic, nitrites/nitrates, ionizing radiation	Oxidative stress, genotoxic effects, DNA repair inhibition and methylation changes	Contaminated regions, food processing, high grade tumors
Chronic inflammation	Schistosomiasis, indwelling catheters, urinary stones	Increased proliferation, reduced apoptosis, downregulation of p27 and upregulation of bcl-2	Squamous cell carcinoma, 70% of cases in Egypt, aggressive behavior

ic base changes in oncogenes or tumor suppressor genes [17]. In particular, smoking has been associated with TP53 gene mutations, which in turn are associated with aggressive UCCs behavior [18]. Thus, not surprisingly, smokers present with higher grade and stage tumors, ultimately having higher cancer-specific mortality rates compared to non-smokers. Interestingly, tobacco smoke seems to exert minor or no effect on the incidence of FGFR3 gene mutations, found primarily in superficial tumors with favorable prognosis [19].

Occupational exposure

The relation between selected industries or occupations with bladder cancer was first described in 1895 by von Ludwig Rehn [20]. Currently it is estimated that 20-27% of bladder cancers are directly attributable to occupational exposures, representing the second most important exogenous risk factor [21]. Numerous industrial chemicals such as aniline dyes, chlorinated aliphatic hydrocarbons, benzidine, coal soot, several aldehydes as well as 2-napthylamine and 4-amino-biphenyl have been implicated in bladder carcinogenesis [22]. Occupations associated with high risk of developing bladder cancer include painters, metal workers, leather workers, hairdressers, dry cleaners, mechanists, plumbers and physicians. The latency period between exposure and clinical presentation is related to exposure intensity and workplace characteristics, but also on individual susceptibility. Regardless of individual's susceptibility, the mode of action of these carcinogens includes the reaction of metabolites with nucleic acids and cellular macromolecules, metabolic activation, as well as toxic effects. The dose-response relationship of irreversible DNA damage seems to be linear, while a no-effect level cannot be defined [23].

Environmental exposure

Several compounds, found naturally or as waste in the environment, are involved in urinary bladder carcinogenesis. In particular, chloride and arsenic in ground water, dietary nitrites and nitrates, ionizing radiation (IR) and chronic inflammation due to infectious microorganisms represent well recognized risk factors of bladder cancer [14].

Inorganic arsenic, the most abundant and toxic form, in drinking water induces lung, primary skin and urinary bladder cancers in humans. A recent metaanalysis demonstrated the relation between high, though not low, levels of arsenic in drinking water and the development of UCC of the urinary bladder [24]. The presumable molecular mechanisms for arsenic carcinogenesis are oxidative stress, genotoxic damage, DNA repair inhibition, epigenetic events such as DNA methylation changes, and activation of certain signal transduction pathways leading to aberrant gene expression [25-27].

Dietary nitrites and nitrates, related to cooking and processing of meat, but also found in soil as a result of the uncontrolled use of fertilisers and pesticides, have also been associated with the development of UCC of the urinary bladder. Approximately 70% of the orally ingested nitrate is excreted in the urine. Nitrosation, taking place in the bladder, seems to exert genotoxic and epigenetic aberrations in urothelial cells [28]. A recent study reported a significant positive relationship of dietary nitrates with bladder cancer [29], whereas Ferrucci et al. in a similar study presented only a weak association [30]. To complicate things even more, a preceding cohort study concluded that nitrate intake does not influence bladder cancer risk [31]. These findings suggest a rather controversial role of dietary nitrites and nitrates in urinary bladder carcinogenesis.

There is strong evidence relating exposure to IR with the development of bladder cancer. Most data are derived from Japanese atomic bomb survivors and from patients who had undergone external beam radiation therapy (EBRT) [32]. IR has been shown to promote carcinogenesis by oxidative stress leading to aberrant methylation of the promoter region of certain cancer related genes [33]. Recent studies performed in Ukraine population after the Chernobyl accident, demonstrated that long-term, low-dose of IR predisposes to bladder cancer possibly via an oxidative stress-p53 overexpression pathway [34,35]. Moreover, IR seems to stimulate significant activation of DNA repair enzymes, as well as alterations in p38-MAPK (mitogen-activated protein kinase) cascade and cytoplasmic retention of NF-κB subunit [34].

Chronic inflammation

Schistosomiasis is the second most common parasitic infection, with almost 200 million people being currently infected and with more than 700 million people at risk for infection [36]. Bladder schistosomiasis is considered a definitive cause of urinary bladder cancer, particularly of invasive squamous cell carcinoma (SCC), with an associated 5-fold risk. Carcinogenesis due to helminth infections is a rather complex process, involving several different mechanisms, with chronic inflammation being the key feature [37]. Recently, a group of investigators concentrated on the molecular pathways underlying the association between schistosomiasis and SCC [37]. According to their results, chronic infection with *S. Haematobium* seems to induce increased proliferation, decreased apoptosis, as well as downregulation

of the tumor suppressor p27 and upregulation of the antiapoptotic molecule Bcl-2. What is more, preliminary data support that genetic polymorphism of GST influences susceptibility of *S. Haematobium* infected patients for developing SCC of the urinary bladder [38].

Genetic susceptibility

Interestingly, only a small fraction of individuals exposed to the above mentioned risk factors actually develop bladder cancer. Furthermore, incidence rates vary considerably among races, with the highest met in Caucasians and the lowest in Asians. In recent years various genetic susceptibility factors have been studied in relation to bladder cancer. Currently, research has focused on the significance of genetic polymorphism in the GST supergene family. GSTs are a family of phase II enzymes that play an important role in the protection of cells from the oxidative stress products, as well as from several environmental carcinogenic compounds [39,40]. A review of the literature reveals many studies highlighting the relationship between GST polymorphisms and bladder cancer risk. More specifically, the results of 3 recent metaanalyses suggest that the GSTT1 and GSTM1 null phenotypes are associated with a modest increase in the risk of bladder cancer [41-43]. In these metaanalyses risk was also stratified by smoking and there was no statistical association with GST polymorphisms [42-44].

N-acetyltransferase2 (NAT2) gene encodes the NAT2 enzyme which activates and deactivates arylamine and hydrazine drugs and carcinogens. Polymorphism of the NAT2 gene divides individuals into slow, intermediate and rapid acetylators. Recent evidence indicates a relation between NAT2 slow acetylator phenotype and increased risk of bladder cancer, particularly among cigarette smokers [43,44]. Conversely, the lower frequency of slow acetylators in Asian populations may be responsible for the lower incidence of UCC in this ethnic group. Finally, polymorphisms of the X-ray repair cross-complementing 1 (XRCC1) and the human oxoguanine glycosylase 1 (hOGG1) genes, implicated in the base excision repair (BER) mechanism for DNA damage repair, are also considered risk factors of bladder cancer [45].

Monoclonal versus oligoclonal origin

UCC of the urinary bladder is characterised by the development of multiple synchronous and metachronous tumors at distant locations within the organ, often before the onset of clinical symptoms. The clonal origin of these tumors is of paramount importance as far as diagnosis and treatment are concerned [46]. Clonality, however, is not easily defined and it is highly dependent on the markers used to establish it. Three major theories have been proposed to explain the origin of multifocal and recurrent urothelial carcinomas: (1) intraluminal seeding; (2) intraepithelial migration; and (3) field-cancerization effect [47]. The first theory assumes that shredded cells from the primary tumor are reimplanted in the normal mucosa and initiate the development of a macroscopic new tumor. This theory is supported by the fact that urothelial carcinoma cells have been shown to be capable of adhering to the injured urothelium in animal models. Correspondingly, in the intraepithelial migration model spreading takes place by intraepithelial migration of cancer cells. The field-cancerization effect theory suggests that mutational stress induces independent genetic alterations within the urothelium, eventually leading to the development of multiple tumors. The field-cancerization effect hypothesis is sustained by the fact that the chronology of tumor appearance does not coincide with the genetic evolution of cancer cells [48] as well as by the observation that the application of a carcinogen into the bladders of chimeric mice results in the development of multiple tumors of oligoclonal origin.

The first two theories imply that tumors are clonally related, while the third theory supports non-clonal or oligoclonal origin via a pan-urothelial carcinogenic insult. Currently, there is no consensus on which theory is most predominant, even though it has been a topic for many investigators. Current evidence suggests that the origin of synchronous and metachronous tumors may be either oligoclonal or monoclonal, even in the same patient [49-51].

Genetic and epigenetic aberrations

Chromosomal aberrations

UCC of the urinary bladder comprises different subtypes, with specific cytological features and genetic characteristics. As a result, some UCCs appear as superficial papillary lesions with indolent clinical phenotype and a negligible risk for progression, whereas others demonstrate poor differentiation, invasive growth and ultimately highly malignant behavior. In recent years, comparative genomic hybridization (CGH) studies and single nucleotide polymorphism (SNP) arrays identified several numerical and structural chromosomal aberrations in newly diagnosed and recurrent bladder tumors of various grades and stages [52,53]. Some of these aberrations predominated in low grade, superficial tu-

mors, possibly representing initial steps in carcinogenesis, while others were related to progression (Table 2).

The most frequent chromosomal aberrations in bladder cancer involve loss of heterozygosity (LOH) at chromosome 9 [54,55], resulting in loss of function of several tumor suppressor genes. It is found in all grades and stages of papillary and solid tumors, but also in normal, hyperplastic and dysplastic urothelium [56], possibly representing an early, or even the initial event in urinary bladder carcinogenesis [57]. In the majority of the cases the chromosome's long arm is affected at loci 9q34, 9q22 and 9q32-33 where TSC1, PTCH1 and DBC1 are located respectively. Less frequently, the short arm is affected, particularly at 9p21 coding CDKN2A, a tumor suppressor usually inactivated by homozygous deletion [58]. Other chromosomal aberrations found in multiple, low grade superficial tumors include gains of 8q, 11q13-q14, 12q13-q15, 13q12, 20q and losses of 2q32, 11p, 11q21, 13q13 [52]. These aberrations correspond to early events, most likely playing a role during the genesis of the tumors.

Over a decade ago several CGH studies demonstrated that the progression of a superficial bladder tumor to muscle-invasive disease is associated with the occurrence of specific chromosomal aberrations [53,59,60]. According to these studies, gains of 3p22-24, 1q and 5p as well as losses of 4p11-15, 5q15-23, 6q22-23, 10q24-26, 18q12-23, 2q, 11q, 8p and 17p were strongly associated with higher risk of progression in pT1 patients. On the other hand, specific aberrations in chromosomes 3, 5, 7, 20 and 17 ploidy can be found only in aggressive tumors with no evidence of a superficial precursor lesion, suggesting a different carcinogenic pathway [61,62].

Table 2. Chromosomal aberrations in superficial vs. invasive urothelial cancer of the urinary bladder

Chromosomal aberrations	*Superficial tumors*	*Invasive tumors*
Deletions	9q22.3	4p11-15
	9q34	5q15-23
	9q32-33	6q22-23
	9p21	10q24-26
	2q32	18q12-23
	11q21	17p
	13q13	8p
Gains	11q13-q14	3p22-24
	12q13-q15	1q
	13q12	5p
	20q	17q

Microsatellite instability

Microsatellites, also known as simple sequence repeats (SSRs) are sections of DNA consisting of sequences of repeating units of 1-6 bp in length. Microsatellites at some cases may become unstable and can shorten or lengthen. MSI is a condition manifested by damaged DNA due to defects in the normal DNA repair process. Abnormalities of DNA-mismatch repair genes have been detected firstly in tumors of patients with hereditary non-polyposis colorectal carcinoma (HNPCC) or Lynch syndrome, where MSI arose from germ-line and somatic mutations in one of four DNA-mismatch repair genes. In the majority of the cases these genes are hMLH-1 and hMSH-2, which encode enzymes responsible for repairing nucleotide mispairs, insertions or deletions that are produced during DNA replication.

An interesting observation is that bladder cancer patients whose tumors presented with high MSI had longer survival than patients whose tumors lacked this feature [63]. Considering this, investigators have concluded that analysis of MSI could be used for the identification of patients subsets with better survival. According to the results of a different study, the detection of MSI demonstrated prognostic value, especially in patients younger than 71 years of age with T2-T3N0M0 tumors [64]. Thus, many research groups suggest the use of MSI analysis in the early diagnostics strategy and screening of bladder cancer in urine sediments or bladder washings. The sensitivity of this method appears to be around 83% and specificity close to 100%. Bartoletti et al. developed a rapid and cost-effective automatic multiplex polymerase chain reaction amplification. They demonstrated 80.8% sensitivity and 85.1% specificity and was proposed as an alternative to conventional urine cytology in the superficial UCC [65].

Aberrant DNA methylation

DNA methylation involves the addition of a methyl group to the number 5 carbon of the cytosine-pyrimidine ring resulting in gene silencing. The process is carried out by 3 enzymes: DNMT1, DNMT3a and DNMT3b. DNMT1 seems to be responsible for copying DNA methylation patterns to the daughter strands during DNA replication. In humans most cytosine methylation occurs in CpG-poor regions, as well as in repetitive regions. Methylation-induced silencing of genes is an epigenetic mechanism, functionally equivalent to mutations and deletions. It is probably due to acquired defects in the DNA methylation machinery and has been reported in many different neoplasms including UCC of the urinary bladder where it represents a common event [66].

In recent years the methylation status of numerous tumor suppressor genes, proto-oncogenes, cell cycle regulatory and cell adhesion genes (Table 3) has

Table 3. Epigenetically altered expression of genes in urothelial cancer of the urinary bladder

Gene	*Function*	*Significance*	*References*
$p16^{INK4A}$	Progression from G1 to S phase by inactivating cyclin-dependent kinase 4 and cyclin-dependent kinase 6	Related to progression and poor prognosis	Dhawan et al. 2006 [74]
$p14^{ARF}$	Progression from G1 to S phase by inhibition of mdm2	Related to recurrence and/or progression	Negaes et al. 2008 [70] Lin et al. 2010 [69] Toki et al. 2010 [71]
DAPK-1	Mediator of γ-interferon induced programmed cell death	Correlated with biological behavior	Brait et al. 2008 [67] Hoque et al. 2006 [68]
APAF-1	Central role in the apoptosis regulatory network	Correlated with grade and stage, marker of recurrence	Brait et al. 2008 [67] Phé et al. 2009 [75]
IGFBP-3	Encodes IGFBP-3 protein which alters the interaction of IGFs with cell surface receptors	Prognostic marker for recurrence/progression	Hoque et al. 2006 [68]
APC	Regulates transcription of several critical cell proliferation genes	Related to progression	Enokida et al. 2008 [66] Negaes et al. 2008 [70]
E-Cadherin	Regulates cell adhesion	Progression of CIS	Dhawan et al. 2006 [74] Lin et al. 2010 [69]
RASSF1a	DNA repair via an interaction with XPA protein	Related to progression and shortened survival	Dhawan et al. 2006 [74] Toki et al. 2010 [71]
RARβ	Regulates gene expression, cellular signaling and embryonal morphogenesis	Development of TCC, correlated with stage and grade	Toki et al. 2010 [71] Jarmalaite et al. 2008 [73]
CRBP1	Morphogenesis, cellular proliferation and differentiation	Development of TCC	Toki et al. 2010 [71]
EDNRβ	Activates a phosphatidylinositol-calcium second messenger system	Related to progression	Enokida et al. 2008 [66]
CDH1	Calcium-dependent cell-cell adhesion	Associated with shortened survival	Enokida et al. 2008 [66]
FHIT	Possible involvement in cell growth control and apoptosis	Associated with shortened survival	Jarmalaite et al. 2008 [73] Phé et al. 2009 [75]
LAMA3 LAMB3 LAMC2	Encode laminins implicated in cell differentiation, adhesion, migration and signaling	Related to poor prognosis	Enokida et al. 2008 [66]

CIS: carcinoma in situ, TCC: transitional cell carcinoma

been studied in patients with UCC of the urinary bladder [67,68]. According to the investigators' results, aberrant DNA methylation contributes mainly to tumor progression, but to some degree also to malignant transformation of normal urothelial cells. Therefore, hypermethylation of the promoter region of certain genes, such as p14, CRBP1, RARβ and RASSF1A, appears to be involved in early stages of bladder carcinogenesis, sometimes maintained also during tumor progression [69-71]. Interestingly, epigenetically aberrant regulated p14 in histologically normal urothelium is associated with shorter recurrence-free interval. In a similar fashion, global DNA hypomethylation induces genomic instability and has been associated with an increased risk of bladder cancer development [72].

What is more important, the hypermethylation rate has been reported to increase with tumor grade and stage and to yield prognostic information regarding tumor recurrence and/or progression [67,73]. Similarly, the methylation rate of certain genes such as E-cadherin, $p16^{INK4A}$ and RARβ shows a significant relationship with progression of CIS to muscle-invasive tumor, denoting another molecular pathway [74]. Taking the above into account, the methylation status of specific genes could be used to identify patients with either non-muscle invasive disease requiring radical treatment or a low progression risk suitable for less intensive follow up [75]. During the last years, there is much interest regarding the implication of mirtrons and microRNAs hypermethylation in bladder cancer [76]. According to the results of a recent study, hypermethylation of specific mirtrons and microRNAs is associated with the development and behavior of UCC, suggesting potential roles as diagnostic and prognostic biomarkers.

Apparently, aberrant methylation events in bladder cancer occur randomly and are individually selected during tumor progression. The development of novel therapeutic methods aiming at the reversal of hyper-

methylation is a compelling area of epigenetic research. Compounds that remove the methyl group can lead to re-expression of the affected genes and ultimately to the suppression of the malignant cells. Zebularine is an orally active demethylating agent, with minimal cytotoxicity *in vivo* and *in vitro* that has been tested successfully in mice [77]. The continuous administration of zebularine was shown to globally demethylate a substantial amount of hypermethylated regions [78]. Other agents that have been studied are the methyltransferase inhibitors which act by trapping DNMTs in a covalent complex on DNA, resulting in enzyme degradation. However, a major disadvantage of these agents is that they have to be incorporated into the genome in order to be active, which might cause mutations in the daughter cells.

Oncogenes and tumor suppressor genes

FGFR3

The fibroblast growth factor receptor 3 (FGFR3) gene encodes a protein which interacts with several fibroblast growth factors. This interaction results to the initiation of a cascade of downstream signals, influencing cell proliferation and differentiation. One of the most important discoveries in urinary bladder carcinogenesis is the importance of FGFR3 mutations. Activating mutations of the FGFR3 genes represent the most frequent and most specific genetic abnormality in low stage and grade UCCs of the urinary bladder [79]. In particular, FGFR3 mutations can be found in more than two-thirds of papillomas and non-invasive papillary carcinomas [80]. Moreover, FGFR3 positive tumors demonstrate lower recurrence rates compared to FGFR3 negatives ones [81]. The aforementioned findings, along with the observation that FGFR3 mutations occur rarely, if ever, in CIS and in high grade muscle-invasive tumors, suggest that FGFR3 activating mutations may be a key event in a molecular pathway leading to superficial, low grade UCCs. These mutations can be detected non invasively in urine specimens, offering a potential use in diagnosis, prognosis, and in the surveillance of patients [81].

H-ras

The Ras superfamily of oncogenes includes more than 100 members acting as molecular switches. They oscillate between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound states, involved in signal transduction from cell surface receptors to the nucleus [82].

The Ras superfamily includes 6 families, namely Ras, Rho, Arf, Rab, Ran and Rad. The Ras gene family represents one of the most frequently activated oncogenes family in human cancers. Until now, at least 3 cancer-related Ras genes have been identified in humans including K-ras, H-ras, and N-ras, the sequences of which are highly conserved. The possible mechanisms of the Ras pathway activation include gene mutation, gene amplification and functional activation through extracellular growth signals. Activated mutations of Ras proteins result in decreased intrinsic GTPase activity and resistance to inactivation by regulatory GTPase-activating proteins [83]. Given that Ras is involved in the transduction of mitotic and cell survival signals, Ras activation seems to promote cellular transformation. H-ras mutations are frequently found in bladder cancer, involved in both initiation and progression of tumors. Most of these mutations have been observed at codon 12 or 13 in exon 1 or at codon 59 or 61 in exon 2, but the reported frequency rates showed very high discrepancies from 6 to 84% [84-86].

PI3KCA

Phosphatidylinositol 3-kinases (PI3Ks) are a family of enzymes involved in many cellular functions including cell growth, differentiation, proliferation, motility, survival and intracellular trafficking [87]. A PI3K is composed of two subunits: a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. The former, also known as p110α, is encoded by the PI3KCA gene. Genomic alterations in PI3KCA as well as in other genes such as ATK1-2, PTEN and LKB1 involved in the PI3K pathway have been detected in many human cancers including UCC of the urinary bladder [88-90]. Recent studies demonstrated that PI3KCA mutations can be found in all stages and grades of bladder cancer, but mostly in superficial low grade tumors [91]. Another interesting observation is that Ras and PI3KCA have cooperative oncogenic action in many human cancers [92]. In UCC of the urinary bladder, PI3KCA and FGFR3 mutations seem to coexist in substantial percentage of superficial lesions [93]. These two observations indicate first that PI3KCA mutations represent an early event in bladder carcinogenesis. Moreover, FGFR3, Ras and PI3KCA seem to be a part of a pathway leading to superficial, low grade UCCs.

Retinoblastoma

Retinoblastoma (Rb) gene is located at chromosome 13q14.1-q14.2 and its product is a nuclear phosphoprotein, which plays a critical role in cellular prolif-

eration, differentiation and apoptosis. In non dividing cells the hypophosphorylated form of the Rb protein is bound to the transcription factor E2F, thereby blocking the transcription of genes involved in proliferation. In rapidly dividing cells Rb protein is hyperphosphorylated and releases E2F, which is then able to bind its target genes allowing cell cycle progression.

Recently, it was shown that the inactivation of the Rb pathway plays an important role in H-ras-induced bladder carcinogenesis, suggesting that oncogenic H-ras can stimulate compensatory activation of alternative tumor suppressor pathways [94]. Nevertheless, alterations in the Rb pathway have traditionally been implicated in the development of invasive urinary bladder cancer. It appears that Rb inactivation contributes to tumor invasion by EMT-changes and through Rho GTPase-mediated actin reorganization [95]. Numerous studies have demonstrated the significant association between Rb loss, tumor stage, and tumor grade [96,97]. In parallel, it has been shown that amplification and overexpression of E2f3 is also associated with increased tumor stage, grade and proliferation index in UCC of the urinary bladder [98]. Interestingly, a recent study related Rb inactivation and overexpression of E2f3 isoforms, E2f3a and E2f3b, to amplification of 6p22 in UCCs [99]. This genomic region contains 4 genes (prl, sox4, E2f3 and cdkal1) and appears to play a major role in tumor progression.

p53

The TP53 gene is located on the short arm of chromosome 17 (17p13.1) encoding p53, a protein with a central role in tumor suppression by initiating apoptosis or inducing cell arrest at the G1/S-phase in response to DNA damage through the induction of *p21waf1/cipi.* Abnormalities of TP53 have been described in many malignancies including colon, lung, breast, liver, esophagus and urinary bladder [100,101]. Also, germ-line mutations are implicated in the Li-Fraumeni syndrome [102]. In UCC of the urinary bladder, the most frequent alteration involves exons 5-11 of TP53, especially codons 280 and 285. Patients with tumors exhibiting TP53 mutations have higher grade and stage tumors, more frequent recurrences and shorter progression-free survival compared to patients with wild type tumors [103].

Another mechanism for TP53 gene inactivation is overexpression of the MDM2 oncogene. The MDM2 protein degradates p53, attending thus in a process of p53 protein inactivation, done independently of TP53 gene mutations. MDM2 actions are in turn inhibited by p14 which actually prevents p53 degradation by sequestering MDM2 [104]. Overexpression of MDM2 has been found in about 30% of UCCs, which, in consistence of the aforementioned data, were high stage and grade tumors [105].

Angiogenesis

As a general rule, neoplasms cannot grow more than 1-2 mm^3 unless there is adequate oxygen and nutrient supply. Moreover, growing tumors require a waste pathway for the removal of all biological end-products produced by the rapidly dividing cells. These requirements are fulfilled by angiogenesis, the process of blood vessel growth from the surrounding vasculature, considered fundamental in cancer biology [106].

Angiogenesis is stimulated by several factors, some deriving from the actual tumor cells, others from the host's immune cells that infiltrate the tumor and others from the extracellular matrix. Among all these stimulatory factors, the one most thoroughly studied is the vascular endothelial growth factor (VEGF). VEGF is produced by various cells in response to hypoxia and to certain cytokine or hormonal stimuli [107]. It promotes endothelial proliferation, migration and differentiation by binding to its type I (Flt-1) or type II (KDR) endothelial-specific tyrosine kinase transmembrane receptor. Numerous studies have demonstrated overexpression of VEGF or its type II receptor in patients with UCC of the urinary bladder [108,109]. Recently, there has been an association between the levels of VEGF expression in serum and urine, with higher risk of progression [110]. In another study performed on 58 patients having a mixture of superficial and invasive UCCs of the urinary bladder, the serum levels of VEGF were correlated with tumor stage, grade, presence of CIS and local invasion [111]. In recent years, much interest has been focused on the development of methods blocking the VEGF/VEGFR angiogenesis pathway [112,113]. Yet, the only agent that has been proven effective in phase III clinical trials is bevacizumab, a humanised monoclonal antibody that is tested only against renal cell carcinoma. Clinical trials evaluating the effectiveness of angiogenic inhibitors in patients with locally advanced UCC of the urinary bladder are in progress at this time [114].

Basic fibroblast growth factor (bFGF) is a member of the fibroblast growth factor family that induces endothelial cell proliferation and organization of into tube-like structures, promoting in this way angiogenesis. Increased expression of bFGF has been identified in biopsy specimens, but also in serum and urine samples of patients with UCC of the urinary bladder [115,116]. What is more, the concentration of bFGF in the serum and urine shows a relationship with tumor stage.

The increased synthesis of prostanoids, especially prostaglandin E_2, appears to have a central role in malignant cell proliferation, inhibition of apoptosis and stimulation of angiogenesis [117, 118]. Cyclooxygenase-2 (COX-2) is an enzyme involved in prostanoid synthesis that has been the focus of extensive research since its inhibition represents a potential therapeutic tool for cancer. COX-2 overexpression in UCC of the urinary bladder has been well documented in several studies. Moreover, enhanced expression of COX-2 has been associated with muscle-invasive and high-grade disease [119].

Lymphangiogenesis

For more than two centuries lymphatic spread has been identified as a major pathway for cancer dissemination. Until recently it was considered a passive process, entailing malignant cell migration through the regular routes of lymphatic drainage. During the last decade there has been increasing interest regarding the mechanisms of lymphatic spread, and today it has become apparent that it is an active process, depending on the pre-existing and on newly formed lymphatic vessels, both of which serve as conduits for cancer dissemination [120-121].

A relatively early event in the natural history of muscle-invasive UCC of the urinary bladder is its spread to the regional lymph nodes. The lymphatic microcirculation of the bladder is located from the adventitia through the lamina propria [121]. Invasion of a lymphatic capillary by adjacent malignant cells occurs through opened interendothelial gaps or by provoking the opening of closed gaps. Unfortunately, the mechanisms responsible for the growth of new lymph vessels (lymphangiogenesis) during bladder cancer progression are not very clear. One scenario implicates macrophages expressing the lymphatic endothelium-specific hyaluronan receptor (LYVE-1) [121-123]. These macrophages seem to migrate chemotactically from the circulation to integrate to the growing lymphatics. LYVE-1 is a cell surface receptor mainly expressed on lymphatic endothelial cells. Its physiological function has not yet been identified; nevertheless it is used as a lymphatic endothelial cell marker. On the other hand, growing evidence suggests that VEGF-C and VEGF-D, via VEGFR-3, possess a key regulatory role in the process of lymphangiogenesis in a variety of cancers including UCC of the urinary bladder [124]. Regardless however of which molecular pathways regulate lymphangiogenesis in UCC of the urinary bladder, it is a process that seems to contribute considerably to the lymphatic spread of muscle-invasive tumors [125].

During the last few years novel lymphatic endothelial cell markers have been developed. These markers provide a reliable discrimination between blood vessels and lymphatic vessels and can be used therefore to assess the significance of lymphangiogenesis in bladder cancer. The first study designed to investigate the prognostic value of lymphangiogenesis in bladder cancer established a relationship with tumor stage [126]. Two years later a study performed to evaluate the association of lymphangiogenesis with pathological parameters and survival in patients with muscle invasive UCC, demonstrated a correlation between peritumoral lymphatic vessel density (LVD) and metastases to the regional lymph nodes [127]. Such observations stimulated the use of LVD as a diagnostic tool for the detection of lymph node metastases and yielded promising results [128]. Given the importance of lymphangiogenesis in cancer dissemination, investigators are also making efforts to develop methods for its inhibition [129]. It remains however to find out whether these methods can alter favorably the management of UCC of the urinary bladder.

Conclusion

Various exogenous factors have been shown to induce oxidative stress and genotoxic damage on the DNA of urothelial cells. These bladder carcinogens act with a probable linear dose-response relationship and induce specific changes in oncogenes or tumor suppressor genes, influencing bladder cancer development, progression and prognosis. The latency period between exposure and clinical presentation depends on exposure intensity and duration, as well as on individual's susceptibility which is related to DNA repair capacity. Human DNA repair machinery comprises GSTs, NAT2, XRCC1, hOGG1 and many other genes involved in the detoxification of bladder carcinogens and DNA repair. Polymorphism in the relevant genes is associated with altered DNA repair capacity, thereby influencing susceptibility to bladder cancer.

In this review we presented distinct genotypic and phenotypic patterns in superficial low grade UCCs vs. high grade muscle-invasive UCCs. In the literature, there is solid evidence indicating that UCCs arise through two separate, but somewhat overlapping pathways. The pathway leading to superficial low grade tumors is primarily characterized by LOH at chromosome 9. This has been widely proven that results in the loss of function of several tumor suppressor genes including TSC1, PTCH1, DBC1, as well as CDKN2A and CDKN2B. In addition, a substantial percentage of

low grade and stage tumors is characterized by activating mutations of certain oncogenes such as FGFRe, H-Ras and PI3KCA. The first two are components of the Ras-MAPK signalling pathway, whereas PI3KCA has a central role in the PI3K pathway, both regulating cell growth, proliferation and survival. On the other hand, high grade muscle invasive UCCs and CIS are characterized by deletions or mutations of tumor suppressor genes, including p53 and Rb. These alterations are absent or very unusual in early UCCs, but have been frequently reported in severe urothelial dysplasia, signifying its relation with muscle-invasive UCCs.

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