REVIEW

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Occupational exposure to urinary bladder carcinogens – risk factors, molecular mechanisms and biomarkers

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Abstract

Bladder cancer (BC) is one of the most frequent forms of cancer, particularly in Caucasian population. Many environmental factors are recognized as carcinogenic in humans for this form of neoplasia and some of them are related to occupation. In order to illustrate these effects, we have selected several relevant cases with smoking and occupational exposure to carcinogens and their histopathological findings. We reviewed the most important research published in the field of environmental–genomic interaction in relation with the oncogenesis of BC. Three main directions have been identified and described in the article: the environmental factors involved in BC pathogenesis and evolution, the molecular mechanisms involved in cell mitosis control and xenobiotic metabolism related to the qualitative and quantitative exposure and, finally, the possible biomarkers of the tumor evolution. From the genomic and proteomic research, new biomarkers emerged that are in the validation process. Immunohistochemical methods open also new perspectives to the diagnostic algorithms and could serve as prognosis biomarkers.

Keywords: bladder cancer, oncogenesis, occupational risk factors, immunohistochemical biomarkers.

Introduction

Pathology of bladder cancer (BC) involves both external and genetic factors [1]; the 80% incidence of urinary bladder carcinomas in Caucasian population is a strong argument for its genetic determinism. Nevertheless, the current evidences favor the predominant role of environmental factors in the occurrence of sporadic carcinoma [2], and of their interaction with the genes. Bladder carcinoma is a relatively frequent form of cancer, occupying 7th place among all types of cancer in male population and 17th place in female population. In many European urology clinics, BC represents the highest percentage among oncological surgical interventions (56% from all cancer surgery) [3]. Early detection is essential due, as the survival is significantly diminished among patients who underwent surgery in any stage with regional lymph node invasion – N+ [4]. Furthermore, the necessity to acknowledge the etiological factors, methods of prevention and surveillance plays an important role. This review will present the most important occupational agents, the mechanism of environmental factors–gene interaction and the biomarkers for early detection.

Environmental factors

Occupational factors

Occupational agents are estimated to be responsible for 10% of bladder carcinomas [5]. In 2012, *The International Agency for Research on Cancer* (IARC) defined a list of 15 substances or technological processes as being carcinogens for urinary bladder; from a perspective of the number of carcinogens, only the lung and blood cells account with higher number of aggressors [1, 6].

The large number of substances carcinogenic for the urinary bladder could be related to several factors. Most toxic compounds reach bladder in an active form. Their

contact time with the bladder epithelium is longer than the contact with other tissues of the body. For example, arsenic is eliminated through the urinary system without any transformation inside the organism. Even with those glucurono-conjugated in the liver, as benzidine [5, 7], above a certain level, the metabolization possibilities of the liver are surpassed and benzidine appears unchanged in the urine. This level could be reached in occupational exposure.

The highest incidence of exposed workers is in the aluminum industry, in the colorants industry, in the pharmaceutical industry (for Chlornaphazine, Cyclophosphamide), in foundries, in the auto industry, as well as in any other technological process that uses auramine, aniline, aromatic amines based, magenta, toluidine, polyurethanes or mineral and cooling oils [6, 8–17]. Workplaces that use X rays or gamma rays are also part of the list [1].

A meta-analysis from Harling *et al.* [10] on 42 trials showed an increased risk (1.3–1.7 times higher) for hairstylists in the beauty salons, associated with exposure time, usually after 10 years of exposure.

In the pharmaceutical industry, the risk tends to zero in modern technological processes, which are strictly conceived from the perspective of employees' protection; the risk remains however elevated for the patients treated with antineoplastic drugs. In exchange, for herbalists from China, who prepare traditional medicines based on aristolochic acid, the risk remains high [18]; aristolochic acid was also supposed to be a risk factor for cancer associated with Balkan nephropathy [19].

Carcinogen agents enter the organism by the respiratory tract, digestive tract or by the dermal route; the latter is frequently ignored and therefore protection methods are less available for employees (gloves, respecting lunch spaces, etc.), especially in the industrial environment.

Exposure variations are very broad, depending on the technological process, the quantity of substance produced, the routes of ventilation and sealing, positioning of the worker from the source of pollution, route of absorption, etc. While malignancy latency period is high, considering the exposure at the previous workplaces is absolutely necessary. Golka *et al.* [9] identified nine cases of reported occupational BC, with a latency period from the starting time of exposure to the moment of diagnosis ranging between 17 and 45 years old; these cases were related to exposure to azo colorants which contained Solvent Red 19 – *N*-ethyl-1-{[4-(phenylazo)phenyl]azo}-2-naphthylamine (Sudan Red 7B) – or a mixture of *p*-phenylazoaniline-*N*-ethyl-2-naphthylamine and *p*-phenylazoaniline-*N*-ethyl-1-naphthylamine sprayed on metallic surfaces for identifying its' defects.

Non-occupational factors

When considering a diagnosis of occupational cancer, it is mandatory to exclude the non-occupational factors, because they might have synergistic action and the intensity of the non-occupational factor might be predominant. In terms of urinary BC, there are three main non-occupational factors that should be taken into account: (*i*) smoking; (*ii*) environment; and (*iii*) nutrition.

Smoking

There is enough evidence accumulated that smoking is by far the most important risk factor for BC [20]. The risk is related to the content in aromatic amines. Quantification through high-performance liquid chromatography (HPLC) of carcinogenic heterocyclic aromatic amines in tobacco smoke has identified the following aromatic compounds: 1-methyl-9*H*-pyrido[3,4-b]indole (Harman) with values between 250–2600 ng/cigarette, 9*H*-pyrido[3,4-b]indole (Norharman) with values three times higher, 2-amino-9*H*-pyrido[2,3-b]indole (A-alpha-C) at levels of 33–100 ng/cigarette and 2-amino-3-methyl-9*H*-pyrido[2,3-b]indole (Me-A-alpha-C) between 2–9.7 ng/cigarette [21, 22].

The risk of urinary BC is 2–4 times higher in smokers compared to non-smokers; on a cohort of 77 792 subjects, it was revealed that occupational risk is proportional to pack-year smoking, whatever the current smoking status is [23]. Smoking determines bladder carcinoma at younger ages and is, generally, more aggressive than in nonsmokers [24].

Environment

Second important non-occupational factor is related to the home microenvironment. Villanueva *et al.* showed that environmental exposure to chloride compounds, from disinfectant products in drinking water used for washing or swimming pool cleaning, are absorbed in multiple routes into the organism (digestive, respiratory, transcutaneous) and doubles the risk of bladder carcinoma [25]. This risk is due to trihalomethanes (chloroform, bromo-dichloro-methane, dibromo-chloro-methane and bromoform) and is correlated to a level of water chlorination above 50 μL/L (washing, swimming) independent from other risk factors.

Nutrition

Nutrition content and the cooking method, has also been related to carcinogenesis. Nitrate and nitrite content and heating meat at high temperature (*e.g.*, barbecue) releases heterocyclic aromatic amines, either through amino acids pyrolysis (glutamic acid, tryptophan), or by heating of the proteins at very high temperature [26–28]. In order to increase the risk, this consumption should be very high.

In terms of alcohol consumption, results are controversial and there is no current consensus regarding the risk associated with BC. A meta-analysis regarding several life style habits showed that there is convincing data regarding high risk of smoking in producing cancer, a slightly elevated risk regarding alcohol consumption, no association between consumption of coffee and tea and a moderately low risk in case of fruit consumption [20].

Examples of interactions between environmental factors

We have selected a series of some of the most representative cases diagnosed with bladder tumors, with traceable occupational history (the presence of work place carcinogens) correlated with the bladder tumor occurrence, who underwent surgery and received histopathological (HP) confirmation in the Department of Urology, "Prof. Dr. Theodor Burghele" Clinical Hospital, Bucharest, Romania, during 2015.

The first case of this series is the one of a 53-year-old male patient, with a body mass index (BMI) of 38 kg/m^2, ex-smoker (quitted five years ago with a history of over 10 pack-years), working as a radiology assistant, exposed to X rays, working with developing and fixing solutions, was diagnosed with a bladder tumor formation.

The HP report of the transurethral resection of bladder tumor (TURBT) in the Department of Urology showed thicker urothelium with no architectural abnormalities, with a branching arrangement but a monotonous appearance from cytological point of view. Nuclei were uniform, elongated, round-oval, slightly enlarged and more crowded relative to those in normal urothelium, with nuclear grooves and nucleoli either absent or inconspicuous (Figure 1, a and b). The morphological picture supported the diagnosis of papillary urothelial neoplasm of low malignant potential (PUNLMP), term introduced in the *World Health Organization* (WHO) 2003 Classification, defining those papillary proliferations with lower risk of recurrence than low-grade urothelial carcinomas and no progression in either stage or grade [29].

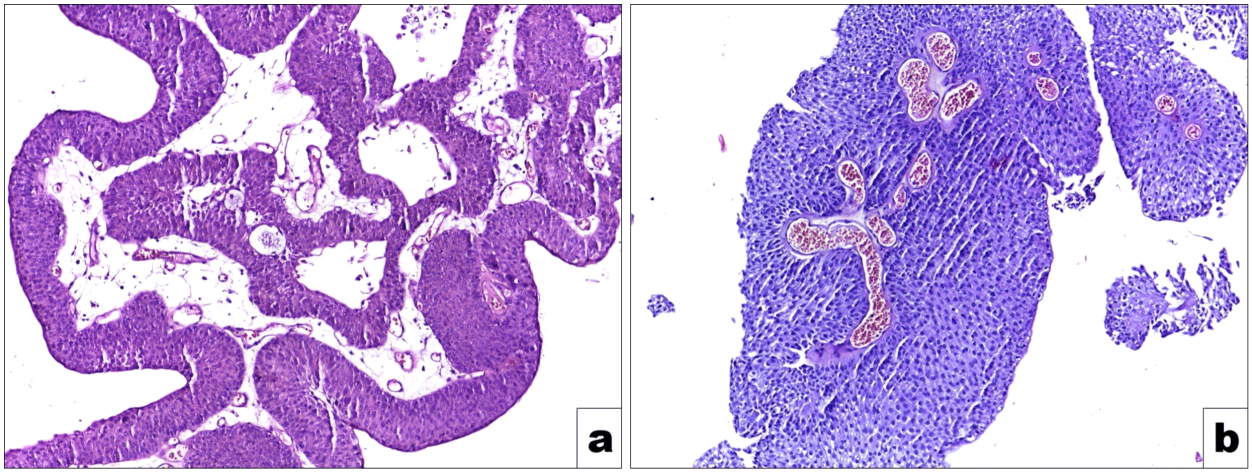

Figure 1 – ***Papillary urothelial neoplasm of low malignant potential (PUNLMP). Hematoxylin–Eosin (HE) staining: (a) ×40; (b) ×100.***

Another case was the one of a 52-year-old male patient, working as an auto mechanic for 23 years. He had a history of heavy smoking (more than 10 pack-years) and occupational exposure to diesel exhaust gases, asbestos, mineral oils. He discovered a bladder tumor in a general ultrasound medical check-up. He was referred to the Department of Urology, where he had been confirmed with the diagnosis. He underwent a bladder tumor endoscopic resection and he was diagnosed with a grade II papillary urothelial carcinoma (Figure 2a).

The third case, a medical female nurse, non-smoker, 58 years of age, has worked for 35 years in an oncology department, where she was administering cytostatic drugs, including Cyclophosphamide. She has undergone TURBT after the onset of gross hematuria. Histopathology report showed grade III papillary urothelial carcinoma with areas of glandular differentiation and infiltration in lamina propria (Figure 2b).

The fourth case is represented by a 58-year-old non-smoking male patient, who has worked as a dyer in a weaving manufactory for 37 years. At the work place, he was exposed to aniline dyes. He was suspected of a bladder tumor after the onset of hematuria. The HP diagnosis of the TURBT resected bladder tumor was poorly differentiated (grade III) urothelial carcinoma with invasion in the muscle layer of the bladder wall (pT2) (Figure 3a).

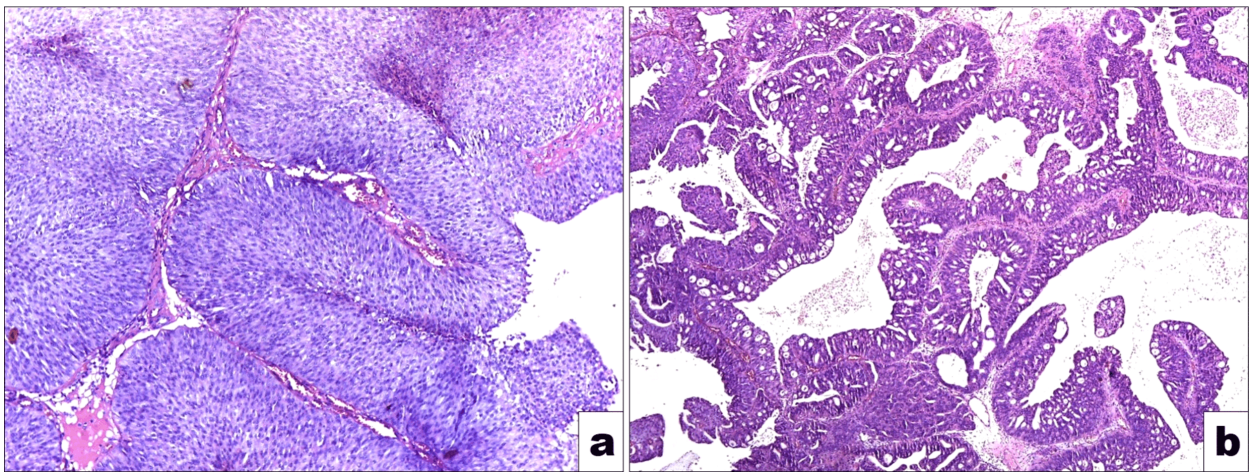

Figure 2 – ***(a) Grade II papillary urothelial carcinoma; (b) Grade III papillary urothelial carcinoma – areas of glandular differentiation. HE staining: (a) ×100; (b) ×40.***

Another rather interesting case was that of a 74-year-old male patient with a history of over 10 pack-years of smoking, who has worked as a foundry worker until nine years ago. He was exposed to foundry metal fumes (CO_2, sulfur compounds, fumes resulted from heating of the urea-formaldehyde resins), copper alloys, bronze and tin alloys, manganese steel, paraffin, SiO_2). He has been diagnosed with bladder carcinoma after an endoscopic resection and he received the indication of radical cystectomy, which he followed. His HP examination on the radical cystectomy specimen showed a grade III urothelial carcinoma infiltrating the entire bladder wall and extending further to the perivesical fat (Figure 3b).

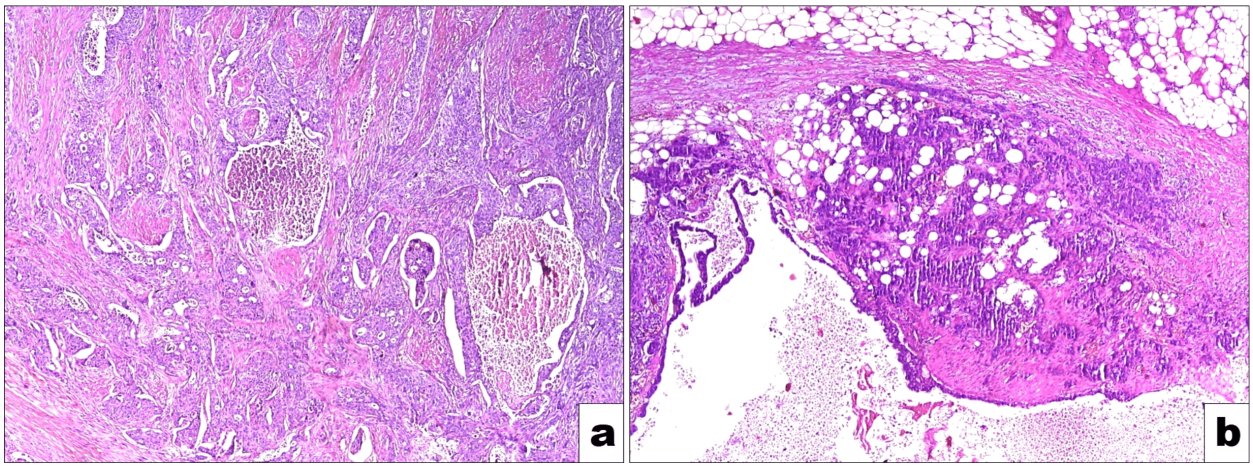

Figure 3 – ***(a) Grade III urothelial carcinoma – infiltration in the muscle layer of the urinary bladder; (b) Grade III urothelial carcinoma infiltrating the perivesical fatty tissue. HE staining: (a and b) ×40.***

The last cases were those of a 68-year-old male patient and a 44-year-old male patient. The former patient was a heavy smoker and has worked as a radiologist for over 38 years. The HP diagnosis after having resected a bladder tumor was urothelial carcinoma with squamous differentiation (Figure 4a). Cigarette smoking together with X-ray exposure are highly likely to be incriminated. The latter patient was also a heavy smoker (12 pack-years). He was defectoscopy worker in the same workplace – a clothing subassemblies enterprise – for 22 years, being exposed to metal cracks testing sprayers containing aromatic hydrocarbons, like aromatic amine 2-naphthylamine. His onset symptoms included gross hematuria and therefore he underwent a TURBT in the local urology departments. The HP examination revealed an infiltrative urothelial carcinoma with squamous differentiation (Figure 4b).

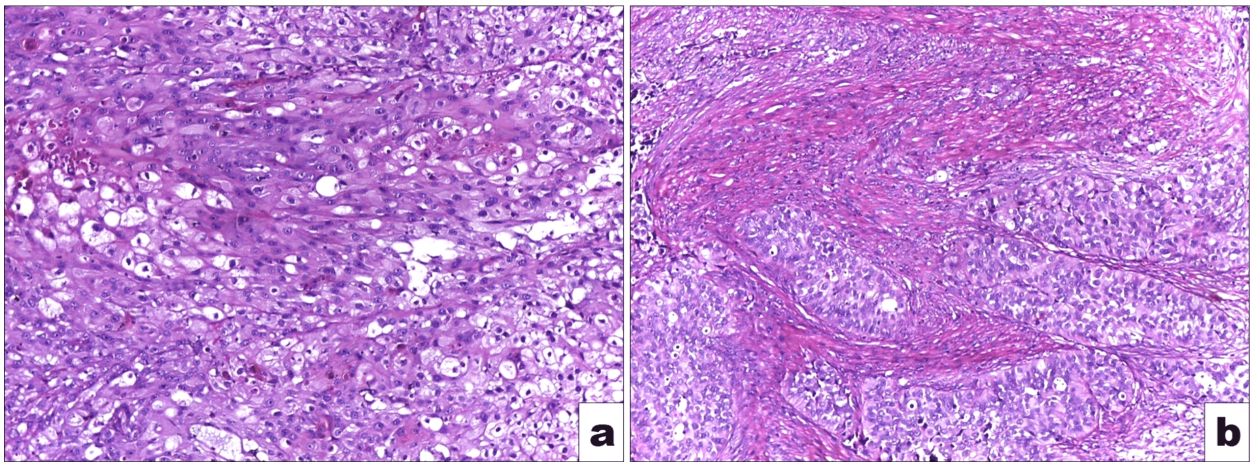

Figure 4 – ***(a) Urothelial carcinoma with squamous differentiation; (b) Urothelial carcinoma with squamous differentiation infiltrating the muscle layer of the urinary bladder. HE staining: (a and b) ×100.***

In all these cases, smoking significantly increased the risk and severity of the disease and decreased the occupational exposure time before diagnosis, compared to other reported data.

We have concluded there are several correlations, in terms of bladder tumors, with the presence of work place carcinogens.

Besides the work place exposure, we consider that smoking is a redoubtable co-carcinogen, which increases the risk and severity of the disease and decreases the occupational exposure time needed for the disease to occur.

Molecular mechanisms

IARC and *WHO* classify urothelial malignant tumors into two large categories: (A) non-invasive urothelial tumors (the majority of primary bladder neoplasms) and (B) infiltrating urothelial carcinoma. The former is further subdivided in two groups which can be seen separately or in combination, according to the gross aspect: (*i*) flat lesions, which include as malignant version only the urothelial carcinoma *in situ* (CIS) and (*ii*) papillary lesions, which include as malignant versions: (*a*) PUNLMP;

(*b*) low-grade papillary urothelial carcinoma; and (*c*) low-grade papillary urothelial carcinoma [29–31]. In terms of the degree of differentiation, is still valid the system proposed in 1973 by *WHO*, with three grades: grade I – well-differentiated (low grade); grade II – moderately differentiated (mild grade); and grade III – poorly differentiated (high grade) [31, 32]. Approximately 90% of the malignant tumors of the urinary bladder have an epithelial origin (carcinomas). Among these, 90% are represented by urothelial carcinomas [33]. Flat high-grade tumors confined to the mucosa and papillary tumors confined to the mucosa or invading the lamina propria are were gathered together, being labeled as non-muscle-invasive BCs but the clinical experience demonstrated that this term describes suboptimally the lesions [31].

The histological classification has developed progressively by profiling tumors with molecular markers. The profiling procedures will help to differentiate, from the time of diagnostic, the prognosis and will improve our knowledge on the specific oncogenesis mechanisms [5, 34]. The main research directions of the oncogenesis mechanisms are focusing on cellular mitosis regulation, xenobiotic metabolism and epigenetic modifications.

Cellular mitosis regulation

Cellular mitosis regulation is a complex process. When genotoxic compounds modify deoxyribonucleic acid (DNA) to form bulky adducts, disruptions of a single or of both chains, loss of bases or functional loss from alkylation, oxidation, hydrolysis or dimerization, the normal cellular controls identify these modifications and either dictate apoptosis, or allows repairing mechanisms to intervene. The normal capacity of DNA repair has an inter-individual variability, partially explained by the polymorphism in the frequency of the minor allele of the responsible genes.

Up to 35–40% of vesical cancers have loss of function modifications of the two main controllers of the G1 checkpoint, the *p53* and retinoblastoma (*RB*) genes [35, 36]. Mutations in *p53* gene influence the expression of serine/threonine kinase 15 (STK15)/breast tumor-amplified kinase (BTAK)/Aurora A kinase, a protein frequently amplified/overexpressed in carcinoma. This kinase stabilizes the spindle mitogen apparatus and assures the normal separation of the cells at the end of mitosis. Excess STK15 generates aneuploid cells. In vesical carcinoma, a positive association between the level of STK15 in the vesical tumor cells and the histological type (non-papillary, type 3 – undifferentiated, high invasiveness and aneuploidy has been found [37].

The more dysfunctional the tumor suppressor genes are, the higher is the malignancy potential of the tumor. For example, frequent mutations of *p53* gene are associated with high malignancy urothelial cancers and fibroblast growth factor receptor 3 (FGFR3) [38]. The genomic analysis of the tumoral tissue is therefore useful in prognosis evaluation [34].

Stern *et al.* reviewed the studies that have been published on repairing genes and concluded that there is evidence for an association between the risk of BC and the polymorphisms of three repairing DNA genes: excision repair cross-complementing (*ERCC*), nibrin (*NBN*) and *xeroderma pigmentosum*, complementation group C (*XPC*). *ERCC2* codes and DNA-helicase adenosine triphosphate (ATP)-dependent able to detach the modified part of the DNA strands and is involved also in DNA adducts repair [1].

NBN gene codes proteins that are involved in recruitment and activation of the phosphokinases in proximity of the damaged DNA, proteins maintaining the length of the telomeres by generating the substrate for the primer of telomerase, and takes part in the control of the cell checkpoints in G1 and G2.

Proteins coded by *XPC* gene play an important role in recognizing distortions of the double helix, monostrand abnormalities, including unpaired strands and initiating their repair. Many toxic compounds acting at bladder level generate DNA adducts; these include: smoking, 2-naphthylamine [39], acrolein [40], 4-aminobiphenyl [41]. Particular polymorphisms of *ERCC*, *NBN* and *XPC* genes become risk factors for cancer development.

Xenobiotic metabolism

Most studies dedicated to BC pathogenesis focused on three enzymatic systems involved in xenobiotic catabolism: (*i*) cytochrome P450 (mainly CYP1A1, CYP1A2, CYP1B1 and CYP2); (*ii*) *N*-acetyltransferase (NAT) and (*iii*) glutathione *S*-transferase (GST) system. Their products take part in the different stages of the aromatic amines metabolism.

Cytochrome P450 family

Exposure to aromatic amines, polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines or nitrosamines activate the aryl-hydrocarbon receptor and induces the transcription of CYP1A2 [42]. This positive feedback further increases activation. The phase I of the biotransformation starts with the cytochrome P450 (with CYP1A1 and CYPB1 as predominant isoforms) activation through *N*-oxidation of the amino groups and high electrophilic compounds formation [8, 43, 44]. At bladder cell level, particularly in an acid pH, these electrophilic compounds modify the genetic material of the urothelial cells.

Salinas-Sánchez *et al.* [43] studied the polymorphism of CYP1B1 and found an odds ratio (OR) of 4 for BC in occupations with high exposure for those with a combined genotype alanine/alanine (Ala/Ala) + leucine/valine (Leu/Val), demonstrating that this genetic profile becomes malignant in combination with occupational exposure.

On a large sample of 1040 subjects, Wang *et al.* [45] found a risk of BC 1.5–1.9 times higher; the magnitude of the risk correlated with the duration of occupational exposure to arsenic. The association of particular polymorphisms of cytochrome P450 family 2 subfamily E member 1 (*CYP2E1*) (insertion at the 96 base pairs), GST omega 1 (*GSTO1*) and GST omega 2 (*GSTO2*) (*G/G* genotype of *GSTO2–A-183G*) with smoking, occupational and environmental exposure to arsenic increased the risk nine times.

Transferases

Phase II reactions (esterification) imply several enzymes of which NATs and sulfur transferases are the most studied ones.

The metabolites resulting from these reactions are unstable; they release arylnitrenium ions that covalently

bind the DNA, forming adducts. This is one possible process that mutates genes controlling cell proliferation.

Arylamine NAT has two isomeric forms: NAT1 and NAT2 that catalyze the transfer of acetate to the nitrogen or oxygen atoms of the aromatic amines or of the hydrazine's substrates [44]. *N*-acetylation is a detoxifying reaction, while the *O*-acetylation is an activation one. Both isoforms of the enzyme are present in the bladder tissue [46].

NAT2 has multiple variants of the alleles; based on these, the population is divided in rapid and slow acetylators. In slow acetylators, metabolism of aromatic amines takes mostly the CYP1A2 *N*-oxidation pathway, the end products being activated at bladder cell levels, enzymatic or non-enzymatic [46].

In white population, the frequency of slow acetylators is 40–70%, which explains – at least in some extent – the higher incidence of BC to this population. The association with BC risk of the slow acetylators has been confirmed by several studies [47–49]. There are, however, some exceptions: benzidine, for example, can be acetylated either by NAT1 or by NAT2, and the result (*N*-acetyl-benzidine) is an active metabolite; after further acetylation, reaction catalyzed by NAT as well, to *N*,*N*'-diacetyl-benzidine the final excretion inactive product is formed [50]. Because the dominant enzyme pathway seems to be NAT1, the influence of rapid/slow acetylators NAT2 is minimal in benzidine exposure.

The family of GSTs from muscle (GSTM) includes four classes of enzymes that serve for the glutathione conjugation. They degrade to final end products excreted in urine many types of xenobiotics, including PAHs, benzo[a]pyrene, aniline dyes, 2-naphthylamine, 4-amino-biphenyl, 4-nitrobiphenyl, benzidine, 2-amine-1-naphthol, combustion gases and carbon soot, chlorinated aliphatic hydrocarbons, and some aldehydes, such as acrolein, ethylene oxide [51]. The genes related to these enzymes have various degrees of polymorphism; the most polymorphic are GST theta 1 (*GSTT1*) and *GSTM1* [52].

Absence of GSTs (genotype *GSTM1*-null) diminishes the capacity to detoxify xenobiotics and is associated with an increased risk for many types of cancers (pulmonary, ovarian, colonic, skin, and prostatic). For *GSTM1*-null genotype, the risk for BC increases by up to 50%; genotype *GSTT1* and *GSTM1*-null have a cumulative risk [53]. The elevated risk for BC has a direct relation to occupational exposure; for heavy smokers, the risk significantly increases [24, 54].

Epigenetic modifications

The third oncogenic mechanism includes the epigenetic modifications. Global hypomethylation [55] and regional hypermethylation (mainly in the CpG islands) are very frequent in malignant tumors are the most frequent ones [56, 57].

After hypermethylation, CpG islands are able to act as promoters. Invasive tumors have high hypermethylation and overexpression of DNA methyltransferase 1 (DMT1) [55]; this is related to abnormal silencing of some of the DNA repairing genes: MutL homolog 1 (*MLH1*) and O^6-methylguanine-DNA methyltransferase (*MGMT*) [58].

Nishiyama *et al.* [55] found an excessive expression of *DMT1* in early process of oncogenesis of the urothelial tumors and proposed this as a marker for monitoring exposed persons. Kawamoto *et al.* [59] described methylation of $p16^{INK4A}$ and $p14^{ARF}$ as markers of poor prognosis for bladder tumors, detectable in urine cytology.

Biomarkers

Blood, urine and bladder cells are commonly used as samples for identification of the markers for the early detection of bladder neoplasia and especially the malignant one. Exposure and biological indicators are used to monitor the exposed subjects.

Blood

The hemoglobin (Hb) adduct induced by different aromatic compounds represents o modified Hb due to binding to the *N*-hydroxylamine formed by aromatic amines oxidation. Smokers have higher concentrations. Recent studies showed that an increased level of Hb adduct, even in non-smokers or in never exposed to occupational oncogenic hazards, is associated with higher ORs for BC [60]. For example, exposure to 4-nitrobiphenyl (4-NB) could form 4-aminobiphenyl Hb adducts. 4-NB exposure is either environmental or endogenous: 4-NB is a product of diesel exhaust or of kerosene but can be generated also by the intestinal flora after exposure to food color additives or by fumes from heated cooking oil [61]. Aniline–Hb and *o*-toluidine–Hb adducts were used to monitor the exposed persons from rubber industry [62]. Aryl amines induce methemoglobinemia, and met-hemoglobinemia level has been also used as monitoring element.

Urine

Rapid/slow acetylators identification is estimated by their phenotype expression; after caffeine ingestion, the urine metabolites 5-acetylamino-6-amino-3-methyluracil (AAMU), 1-methylxanthine (1-MX), 1-methyluric acid (1-MU) and 1,7-dimethylxanthine (17X), are measured using HPLC [63]. The method is effective to identify subpopulation at risk, but it cannot be used for screening due to its high cost.

A general indicator of exposure to PAHs is 1-hydroxy-pyrene in urine, as pyrene is ubiquitous in PAH mixtures. Smokers have significantly higher levels; *CYP1A1* polymorphism influences these levels. In occupational non-exposed persons and nonsmokers, the recommended level is 0.24 μmol/mol creatinine; for smokers, the level is 0.76 μmol/mol creatinine. The maximum accepted level for the occupational exposed subjects is 1.476 μmol/mol creatinine [64]. In order to reach this value, the occupational exposure limits in the workplace environment should be reduced in many European countries.

For several substances, specific exposure indicators are available, *e.g.*, for benzidine, the acetylated metabolites can be measured [50]; for 4,4'-methylene-*bis*-(2-chloro-aniline) the conjugates compounds [11]; for arsenic, its level in blood or urine. The amount of tissue aromatic amine has been correlated by itself with higher incidence of associated diagnosis and could also be considered to influence prognosis in other forms of cancer [65].

The urinary level of proteins involved in angiogenesis regulation – vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) expression – help to differentiate BC from other chronic bladder diseases [66].

Cells morphology

Cytology

Morphology of cells from exfoliated bladder lavage is the golden standard diagnostic and the best detection method for BC. However, new tests increase the positive predictive value of cytology. Such tests are the ones that reveal mutations of DNA. *N*'-(3'-monophospho-deoxyguanosine-8-yl)-*N*-acetylbenzidine, the main alteration of the DNA chain induced by the benzidine metabolites is a specific biomarker of benzidine effect.

The micronucleus test is used to monitor many potential oncogenic exposures; the test identifies an abnormal fragment of nuclear material in cytoplasm, reflecting the damage produced on the chromosome(s) by the exposure to the external factor [67]. Even if this test is well known for a long time, it did not become a general screening test and it is not included in the urological guidelines. The actual indication of microsatellites test (particularly for chromosomes 8p and 9p) is limited for monitoring already diagnosed patients with BC for early detection of relapse [68].

Histology

HP examination is still a crucial tool in the assessment of bladder malignancies.

Immunohistochemistry (IHC) is one of its methods that may have a limited but distinct role in staging of BC. Thus, a possible useful role of IHC in the diagnosis of urothelial malignancies is the detection of early or obscured tumor invasion. This could be achieved by using broad-spectrum cytokeratins (CKs) like OSCAR or AE1/AE3 or CK 34βE12/high-molecular-weight cytokeratin (HMWCK). Another useful help is the distinction of muscle cells from desmoplasia and the highlighting of muscle contours for subclassification [69].

Besides these, another important role of IHC is to support urothelial lineage of urothelial carcinoma variant morphologies. The antibody panel used for this includes CK7, CK20, HMWCK, p63 (Figure 5, a and b), GATA3 and S-100 protein (S-100P). Thus, the next variants could be defined:

- *urothelial carcinomas* with or without divergent differentiation: HMWCK – 89%, p63 – 87%, S-100P – 86%, and CK7 – 80%;
- *urothelial carcinoma variants* (micropapillary, plasmacytoid, nested, clear cell, and microcystic): HMWCK – 96%, CK7 – 95%, S-100P – 96%, GATA3 – 88%, p63 – 69%, and CK20 – 61%;
- *undifferentiated carcinomas* (lymphoepithelioma-like carcinoma, small cell carcinoma, sarcomatoid carcinoma and carcinoma with rhabdoid and giant cells): p63 – 50%, and HMWCK – 49% [70].

The method is also useful in the differentiation of various spindle cell lesions, which have unique clinicopathological, prognostic, and therapeutic ramifications but significant immunohistochemical overlap between them. The antibody panel is complex including, on one hand, broad spectrum anti-CK antibodies (OSCAR or AE1/AE3) with either of CK5/6 or CK 34βE12/HMWCK and, on the other hand, p63, alpha-smooth muscle actin (α-SMA), desmin and anaplastic lymphoma kinase (ALK) 1. Thus, the next conditions could be defined:

- *pseudosarcomatous myofibroblastic proliferations* (PMPs), with positivity for pan-CKs, α-SMA and ALK1;
- *sarcomatoid carcinoma*, with positivity for several CKs and especially CK 34βE12 or CK 5/6 and p63;
- *leiomyosarcoma*, with positivity for α-SMA and overall absence of other markers [69, 71].

Another supportive confirmatory role is in the diagnosis of flat urothelial lesions. Some authors are stating that IHC has no role in the distinction of dysplasia *versus* carcinoma *in situ* [69]. Arias-Stella *et al.* suggested that staining results of CK20 and p53 immunomarking should be interpreted with caution in *de novo* atypia as long as, on one hand, very few patients without a prior diagnosis of BC progressed to diagnostic cancer (1 of 22) and, on the other hand, patients with a known history of BC had a substantial rate of recurrence, independent of staining pattern [72].

However, the use of a cocktail including CK20 and p53 (Figure 6, a and b) and human epidermal growth factor receptor 2 (Her2)/neu (Figure 7, a and b) could help the differentiation of reactive atypia from CIS in difficult cases, in the sense that the positivity of at least two of the three antibodies is strongly associated with CIS [73].

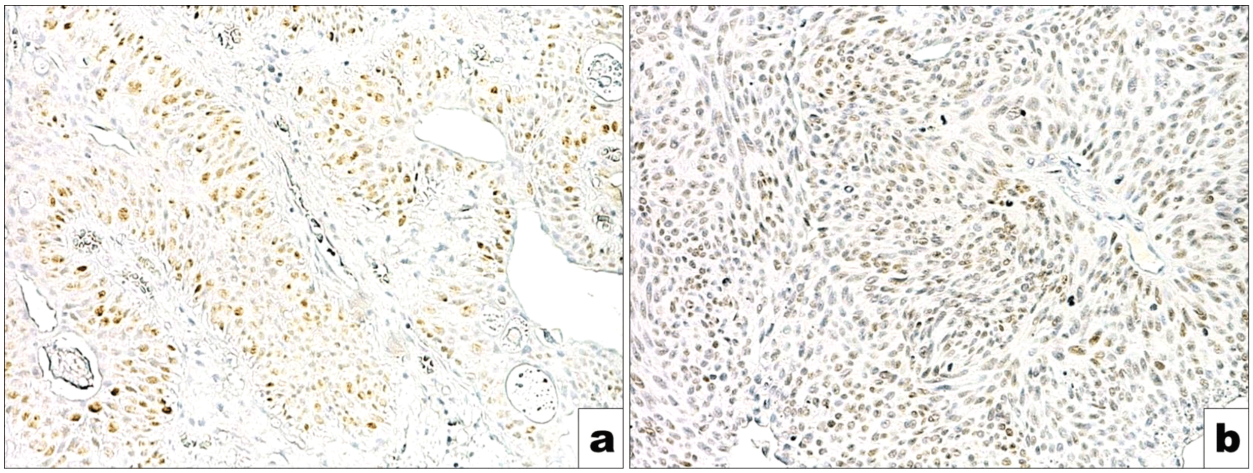

Figure 5 – ***p63 expression in urothelial carcinoma: (a) Low grade tumor; (b) High grade tumor. Anti-p63 antibody immunomarking: (a and b) ×100.***

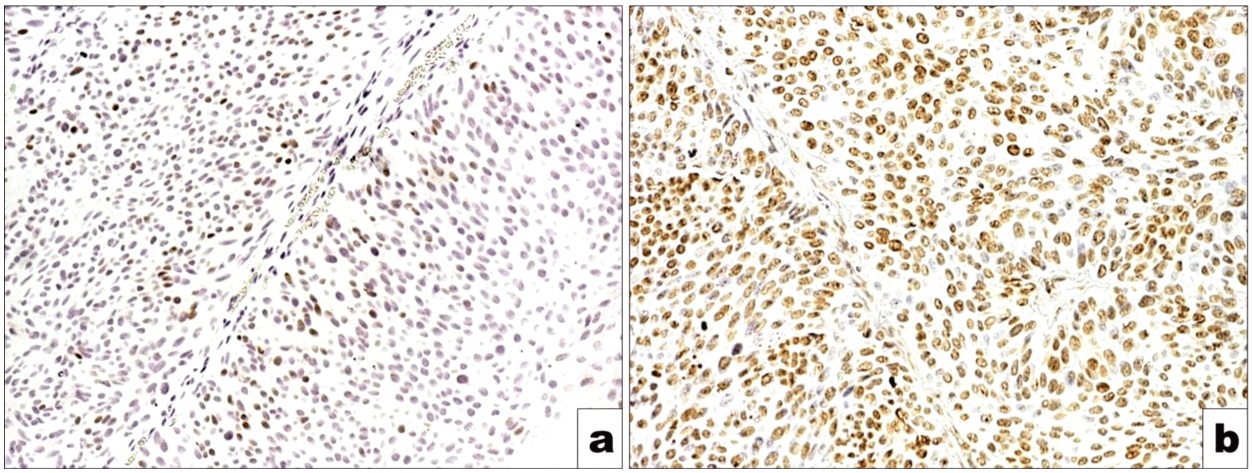

Figure 6 – ***p53 expression in urothelial carcinoma: (a) Low grade tumor; (b) High grade tumor. Anti-p53 antibody immunomarking: (a and b) ×100.***

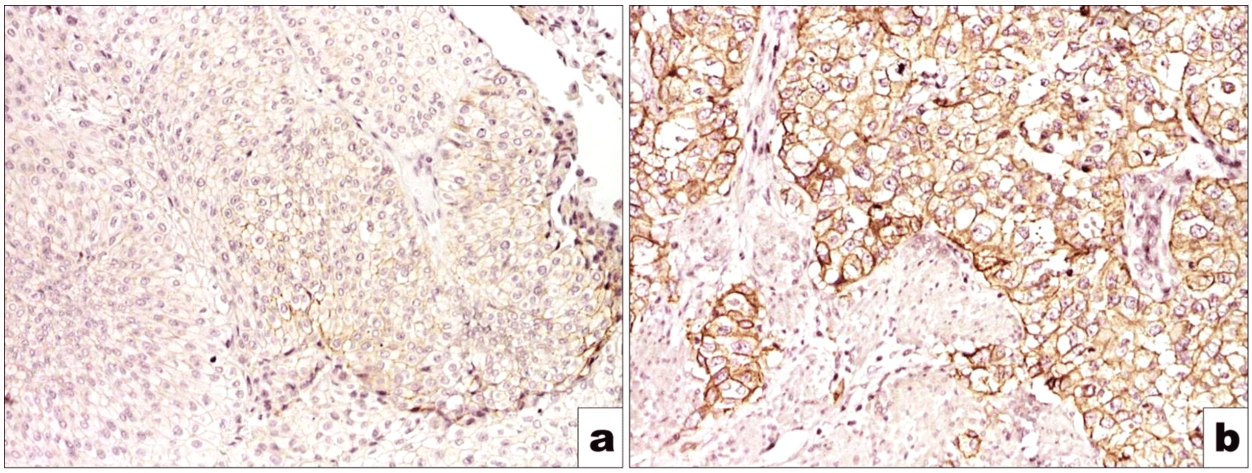

Figure 7 – ***Her2/neu expression in urothelial carcinoma: (a) Low grade tumor; (b) High grade tumor. Anti-Her2/neu antibody immunomarking: (a and b) ×100. Her2: Human epidermal growth factor receptor 2.***

Finally, many efforts have focused on defining algorithms of morphological investigation with prognostic value for bladder malignancies but, unfortunately, until now, there are no prognostic immunohistochemical or molecular studies that are recommended to be routinely performed on biopsy or resection specimens [69].

The role of the p53 in cellular division has been used as differentiator between malignant and non-malignant tumors in the diagnostic workup of multiple types of carcinomas. Still, there is no consensus on the validity of this biomarker in bladder tumors, as different studies have analyzed its value from different perspectives and found divergent results depending on the stage and on the presence of other comorbidities. Thus, while a large study of 3421 patients from 25 centers has found a significant correlation between staining percentages of p53 and the prognosis of the invasive forms [74], another study found that p53 expression did not associate significantly with tumor recurrence [75].

Adding the p63 marker in the panel, gave also contradictory results. Thus, while in one study, p53 and p63 did not solve the identification of prognosis for the non-invasive forms [74], in another study, p63 was able to differentiate between the low malignant potential and non-invasive papillary urothelial carcinoma low grade [76]. Relationship between infections and previous treatments, particularly bacillus Calmette–Guérin (BCG) local treatment, has also to be taken into consideration, as they might interfere with the conclusions [77–79]. However, El-Gendi & Abu-Sheasha reported an increased recurrence incidence and a shortened disease-free survival (DFS) time as the p63 decreased, concluding that p63 could represent a surrogate biomarker to predict urothelial BC recurrence [75].

Another marker taken into consideration for prognosis assessment was Ki67 (Figure 8, a and b) but its expression did not associate significantly with tumor recurrence [75].

A possible prognostic marker is the expression level of E-cadherin (Figure 9, a and b) at both messenger ribonucleic acid (mRNA) and protein levels. Studies demonstrated that of E-cadherin was significantly lower in recurrent tumors than in non-recurrent tumors regardless the degree of differentiation and stage [80, 81].

Genomics and proteomics

Genomics and proteomics [82] contribute to progress in early diagnostic of bladder tumors: there are ongoing preliminary studies to evaluate the efficacy of tests that measure enzymes involved in different stages of neoplastic protein synthesis [58, 83].

Proteomic profiles are able now to capture simultaneous expression markers for differentiation, growth,

apoptosis, angiogenesis, metastasis, coagulation, inflammation and immune response [84], as used in other forms of occupational related diseases [85–87]. For example, preliminary studies showed that a combination of 23 biomarkers in multivariate algorithms raised sensitivity to 91% and specificity to 80%: the algorithms included tumor markers (nuclear matrix protein 22 – NMP22 – a protein from the mitotic spindle apparatus released from bladder cells in large quantities in neoplasia), bladder tumor antigens, carcinoembryonic antigen, EGF and thrombomodulins [84].

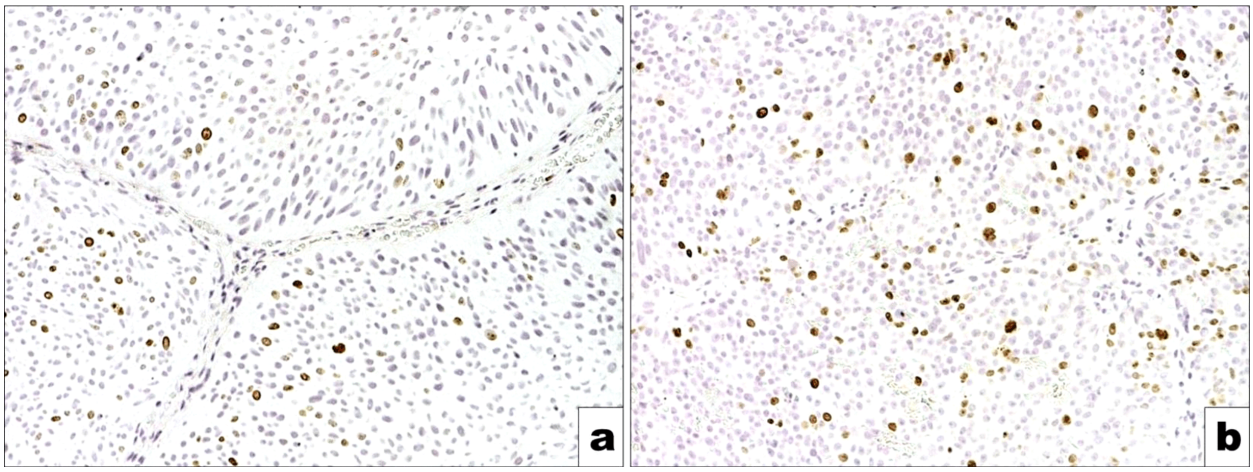

Figure 8 – ***Ki67 expression in urothelial carcinoma: (a) Low grade tumor; (b) High grade tumor. Anti-Ki67 antibody immunomarking: (a and b) ×100.***

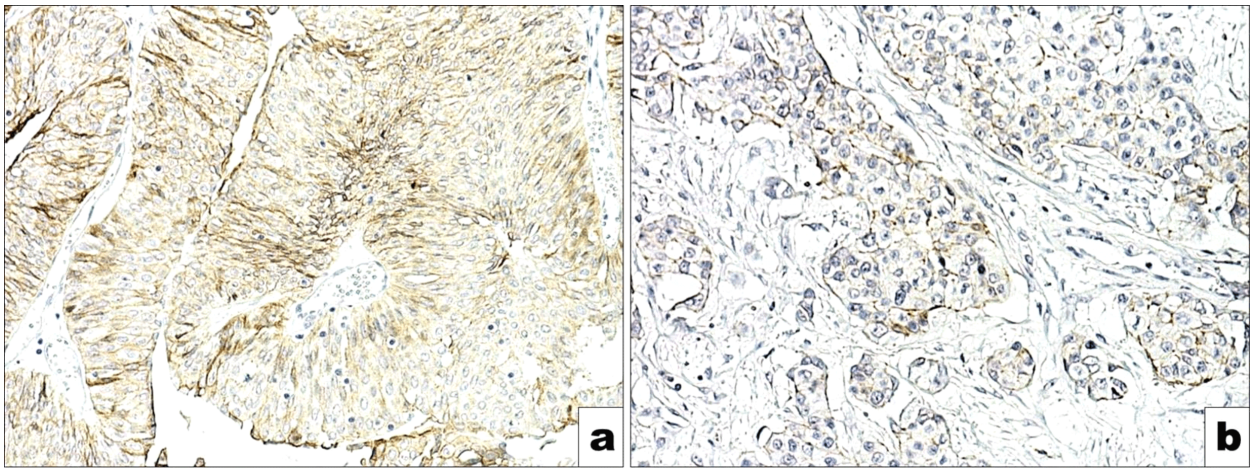

Figure 9 – ***E-cadherin expression in urothelial carcinoma: (a) Low grade tumor; (b) High grade tumor. Anti-E-cadherin antibody immunomarking: (a and b) ×100.***

As a concluding remark, one could say that screening for BC is not a current recommendation, except for the high-risk population, currently defined as heavy smokers (>40 pack-years) and/or occupationally exposed to carcinogens.

Microhematuria has been initially used, although its positive predictive value is low. Testing NMP22 has higher sensitivity (57.1–55%) compared to cytology and a similar specificity (89.8–85.7%) [83, 88].

Tumor antigens have variable sensitivities, between 29% and 91% and specificity ranging from 56% to 86% [89, 90] so that currently, there are no prognostic immunohistochemical or molecular studies that are recommended to be routinely performed on biopsy or resection specimens [69].

Conclusions

The review of the molecular mechanisms involved in the BC pathogenesis, the current biomarkers and the perspective of the possibility to translate the new -omics research in diagnostic tests is important in order to understand how these new methodologies are expected to change the diagnostic process of BC and to influence the occupational medicine screening tests. The role of IHC investigation is a remarkable field of research that can add significant value to the pathological investigation in the differential diagnosis, severity and prognosis of the urinary bladder carcinomas. Genomic and epigenomic research proposes new biological markers of biological effect but there is no consensus on their utilization when coming to monitoring the exposed workers. The lack of consensus and the limitations or their utilization are due to cost-effective reasons and to unavailable prospective studies. From ethical perspective, industrial interventions should focus on the primary prevention and limitation or avoidance of exposure.

Conflict of interests

The authors declare that they have no conflict of interests.

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