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Research models of urinary bladder cancer for improved diagnostics and treatment

Raziskovalni modeli raka sečnega mehurja za izboljšanje diagnosticiranja in zdravljenja

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Abstract

Urinary bladder cancer is one of the leading malignancies in men with high recurrence rate, thus representing a huge economic and social burden. Many different research models are used for studying potential improvements in the diagnostics and treatment of bladder cancer. In this review, the most widely used models are described, since they represent the approximation to the molecular and biological processes occurring during human bladder carcinogenesis. Processes at the cellular level are investigated by *in vitro* models of cell cultures, while processes occurring in whole organisms are explored in different animal models. The results obtained by these two approaches should also be evaluated on human biopsy samples before any new diagnostic procedure or drug can be studied in clinical trials. We provide critical evaluation of advantages and disadvantages of specific models of the urinary bladder cancer and describe some of the studies using these models.

Izvleček

Rak sečnega mehurja je ena najpogostejših rakavih bolezni pri moških in ima visoko stopnjo ponovljivosti, zaradi česar predstavlja velik ekonomski in socialni problem. Za izboljšanje diagnosticiranja in zdravljenja raka sečnega mehurja se uporablja veliko različnih raziskovalnih modelov. V preglednem članku so predstavljeni najpogosteje uporabljeni modeli, saj predstavljajo približek dejanskemu molekularnobiološkemu dogajanju v človeku med boleznijo. Procesi na celični ravni se raziskujejo na *in vitro* modelih celičnih kultur, obnašanje celic v živem organizmu pa v različnih živalskih modelih. Izsledke raziskav na teh modelih je potrebno ovrednotiti tudi na človeških vzorcih, ki so nepogrešljivi, preden pride nov diagnostični postopek ali določena nova učinkovina v proces kliničnih študij. V članku so kritično ovrednotene prednosti in slabosti posameznega modela raka sečnega mehurja ter predstavljene nekatere raziskovalne študije.

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1 Introduction

1.1 Incidence and risk factors for the onset of urinary bladder cancer

Urinary bladder cancer is a disease of developed Western countries; however, it has also been spreading in developing countries in the past decade, becoming a global health and economic problem. On the global scale, it is ranked the sixth most frequently diagnosed malignancy in men (1). Another disconcerting fact is its very high recurrence rate, which is between 50% and 90% (1). Due to its frequency and ineffectiveness of the current therapy methods, which demand long-term monitoring of the already treated patients, urinary bladder cancer has a high social and financial burden on the society and individuals (2). The highest incidence is with men in Southern and Western Europe and North America, where it represents the fourth most common type of cancer (3). In Slovenia, bladder cancer is the eighth most frequent type of cancer among men (18.1 in 100,000), which places it fourth among Southern European countries (Table 1). For women, urinary bladder cancer incidence is currently significantly lower than for men (Table 1); however, it has been increasing since mid-90s, while for men it has been decreasing (1). The five-year survival rate of bladder cancer patients in Slovenia is 53.7%.

Table 1: Bladder cancer incidence and mortality in European Countries (age-standardised incidence and mortality per 100,000) (summarised from Antona et al.) (1).

	Incidence (male / female)	Mortality (male / female)
Central and Eastern Europe		
Poland (regional)	20.2 / 4.1	8.4 / 1.3
Czechia	19.8 / 5.4	6.2 / 1.6
Slovakia	16.1 / 3.6	5.4 / 1.2
Bulgaria	15.6 / 3.3	5.2 / 1.1
Northern Europe		
Denmark	27.4 / 8.4	6.7 / 2.3
Norway	21.9 / 6.4	4.9 / 1.6
Sweden	18.6 / 5.6	4.1 / 1.2
Ireland	11.6 / 4.2	3.6 / 1.4
Southern Europe		
Spain (regional)	36.7 / 5.0	8.2 / 1.1
Italy (regional)	33.2 / 6.1	5.8 / 1.0
Croatia	18.2 / 4.4	6.2 / 1.2
Slovenia	18.1 / 4.3	6.2 / 1.4
Western Europe		
Switzerland (regional)	26.2 / 6.3	4.0 / 1.3
Germany (regional)	21.8 / 5.5	4.2 / 1.3
Austria	20.3/ 5.2	4.2 / 1.1
The Netherlands	13.9 / 3.5	5.5 / 1.6

The main risk factor for bladder cancer remains tobacco smoking. Smokers have a 2.5-times higher probability for developing this type of cancer than non-smokers, with tobacco being responsible for approximately 50% of all cases and approximately 40% of all deaths from bladder cancer (4). With regard to this data, it is not surprising that the pattern of bladder cancer incidence and mortality rate mainly coincides with the pattern of smoking (5). The history of the disease must be taken into account, as the current recurrence pattern of bladder cancer matches with the smoking prevalence from 20–30 years ago (6). In the past decades, the prevalence of smoking has declined in numerous European countries, which is also reflected in the trend of lowering the incidence and fatality from bladder cancer for men in these countries. Quite the opposite, in some countries the prevalence of smoking has only begun declining recently or is even still growing, which pertains mainly to women (7). This means that the incidence and mortality from bladder cancer in these populations is still growing and

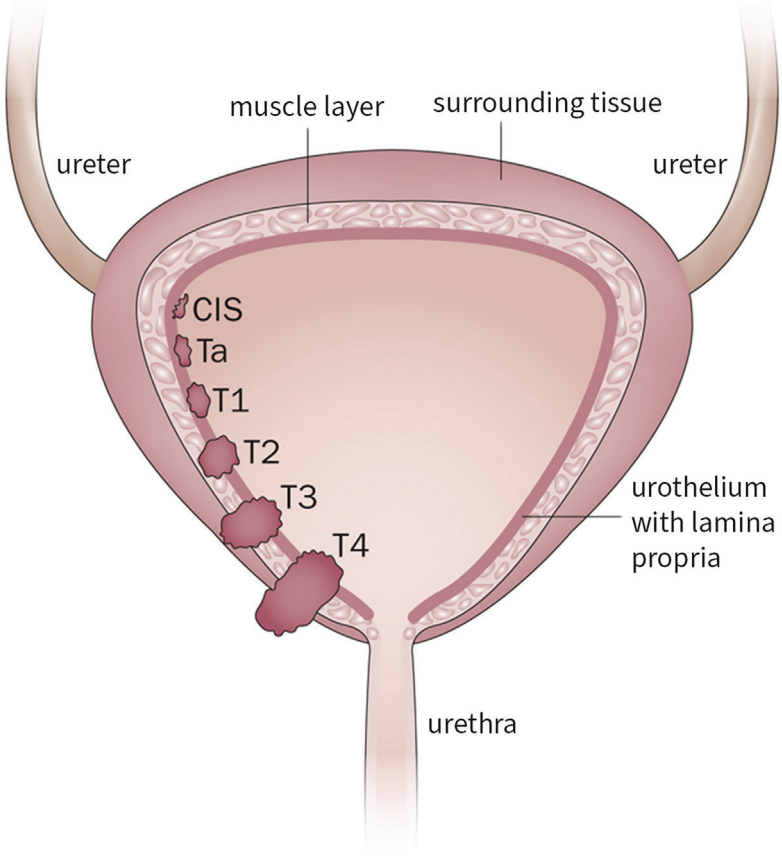

Figure 1: Classification of urinary bladder cancer by the depth of invasion (summarised from Mertens et al.) (13).

will only begin declining in a few decades.

Besides smoking, other known risk factors for bladder cancer include aromatic amines and other chemicals that are also present in colour, rubber and aluminium industries (8). These are followed by exposure to certain environmental pollutants, such as arsenic in drinking water (9). Studies show that inherited genetic factors may also play a potential role in carcinogenesis of the bladder (10). On the other hand, studies have not confirmed any role of diets in the development of bladder cancer (2).

1.2 Classification of urinary bladder cancer

Bladder cancer generally develops as a result of the transformation of epithelial cells that border on the bladder lumen and are called urothelial cells (11). Cancer transformation of urothelial cells in the urothelium leads to the onset of urothelial neoplasm. The first classification of urothelial neoplasms was made in 1973 and was used for nearly 30 years in spite of certain shortcomings (12). In 1998, the World Health Organisation (WHO) in cooperation with the International Society of Urological Pathology (ISUP) adopted a new WHO/ISUP classification that categorizes neoplasms by shape of changes to the urothelium and depth of the invasion of cancer urothelial cells (Figure 1 and Table 2). The WHO/ISUP classification for determining urothelial neoplasms is still in use today.

1.3 Detection and therapy of urinary bladder cancer

The most important factor for a favourable outcome of the disease is a quick diagnosis, as in the early stage, most urothelial neoplasms are non-invasive papillary carcinoma of the pTa type. Basic diagnostics of urothelial neoplasms is performed through anamnesis and clinical examination, which consists of cytological examinations of urine, and a cystoscopy and histopathology of the sample cells from the urothelial neoplasm, obtained during an operation. When setting a diagnosis, approximately 75% of patients have a non-muscle invasive urinary bladder cancer (pTa, CIS, T1), and approximately 25% have a muscle invasive urinary bladder cancer (stage ≥ T2) (15). For patients with a non-muscle invasive form of the disease, a five-year survival rate is relatively high (> 90%), however, the recurrence of the disease is exceptionally high (50–90%), which requires expensive long-term monitoring with invasive cystoscopy (1). The treatment for papillary forms of carcinoma is most often the transurethral resection of the bladder (TURB). The goal of this procedure is to remove all macroscopically visible parts of the carcinoma, which also provides us with biopsies of cancer

Table 2: WHO/ISUP classification of urothelial neoplasms and their histopathological characteristic (summarised from Ovčak, Epstein et al.) (12,14).

Classification	Histopathological characteristics
Normal urothelium	Up to 7 cell layers, in the superficia layer umbrella cells are characteristic, minimum cytological atypia and changes to cell construction.
Hyperplasia	
Simple hyperplasia	Urothelial thickening with no cytological atypia.
Papillary hyperplasia	The mucosa has a pebbly or papillary surface, growths in the shape of fingers, urothelium is unequally thick, and shows no cytological atypia.
Non-papillary (straight) changes with atypia	
Reactive (inflamed) atypia	Nuclei of urothelial cells are unequally enlarged, with the nucleoli clearly visible, and mitoses are present.
Atypia of unknown origin	A small distinction between a reactive atypia and a neoplasm makes diagnosis difficult. Because of intense inflammation process, the polymorphism of the nuclei is highly expressed.
Dysplasia	Severe changes which manifest as crumbling construction of cells, the cellular and nuclear polymorphism and increased mitotic activity.
Carcinoma *in situ* (CIS)	Presence of cells with large, unequally shaped nuclei, usually in the full width of the urothelium. Mitotic activity is also visible in the superficial and intermediate layer of the urothelium.
Papillary neoplasia	
Papilloma	Papillary benign tumour with narrow connective tissue, covered by urothelium, whose construction does not differ significantly from the normal.
Papillary urothelial neoplasm of low malignant potential (PUNLMP)	Papillary tumour with minimal irregularities in the construction of the cells of urothelium and minimum nuclei atypia. Urothelium is generally thickened, and the nuclei are larger than with papilloma, the basal layer has few mitoses.
Non-invasive low grade papillary urothelial carcinoma (pTa, low grade)	Irregularities in cell construction are more frequent, polymorphism of the nuclei is substantial. Changes in cell size, shape, and polarity. Mild mitotic activity is limited to the basal layer of the urothelium.
Non-invasive high grade papillary urothelial carcinoma (pTa, high grade)	Exceptional irregularities in the construction of cancer cells and what they look like. Irregular distribution of chromatin in the nuclei is noticeable, the nucleoli are clearly visible and irregularly shaped. Usual pathological mitoses are present in all layers of the urothelium.
Invasive carcinoma	
Invasion into the lamina propria (T1)	The presence of groups or individual cancer cells in lamina propria.
Invasion into propria muscularis (detrusor muscle) (T2)	The presence of cancer tissues between the thick layers of smooth muscles of the bladder wall is characteristic.
Invasion through detrusor muscle (T3)	The presence of cancer cells in tunica serosa.
Invasion into neighbouring organs (T4)	Infiltration of cancer tissue into neighbouring organs.

tissue for determining the stage, level and depth of the invasion (16). With T3 and T4 stages, the general type of therapy is a radical cystectomy with the removal of local lymph nodes, followed by systemic chemotherapy (17). It has been shown that cisplatin-based neoadjuvant chemotherapy before a radical cystectomy improves long-term survival rate by 6% (18). Approximately 50% of patients diagnosed with an invasive type of bladder cancer die within five years of diagnosis (19,20). Monitoring the statistics of the five-year survival rate shows that treating patients with this type of cancer has not improved significantly in the past 20 years (21). This makes bladder cancer, because of the high frequency and recurrence of urothelial neoplasms and poor survival rates of the invasive forms, a very serious problem for public healthcare. Future research should be focused on finding new methods and approaches in diagnostics and finding new indicators. The high recurrence rates of bladder cancer are most likely the consequence of the fact that in spite of the surgical removal of urothelial neoplasms visible in a cystoscopy, a few cancer urothelial cells remain in the urinary bladder, from which new neoplasms develop. Therefore, it is essential to find new methods of therapy, such as targeted drug delivery to cancer cells. Since 1990, when targeted therapy was first introduced in oncology, numerous therapies with targeted therapy have become established as part of cancer therapy, especially with leukaemia, gastrointestinal and colorectal tumours and kidney cancer (22). Unfortunately, none of the registered targeted medications have been proven to be effective in bladder cancer therapy (23). The main reasons for this most likely include: a) an exceptionally quickly obtained resistance of a tumour to therapy, which is the consequence of the molecular heterogeneity of cancer urothelial cells (24), b) the fact that urothelial cells act as a barrier, which can decrease administration of drugs to cancer cells at the cellular level, and c) numerous toxic side effects of therapy (23). The development of new types of therapies for bladder cancer requires representative, well-evaluated, replicable preclinical cancer research models, and the awareness of the advantages and shortcomings of each individual model.

2 Research models of urinary bladder cancer

The development of new methods of diagnosing and treating bladder cancer begins by studying fundamental principles of cell biology and by molecular studies on cell cultures (*in vitro* systems), followed by studies on more complex animal models (preclinical *in vivo* studies). Animal cancer models also enable the research of mechanisms behind currently used therapies (e.g., the Bacillus Calmette-Guerin vaccine therapy), and the options for their improvement (25). Of all the innovative therapy methods tested on model systems, only a few have developed to the level where a clinical study can be held on patients.

2.1 *In vitro* systems support fundamental studies of urothelial carcinogenesis

Cell cultures are an excellent tool for studying basic cellular-biological, molecular and genetic characteristics of cancer urothelial cells *in vitro*. The prevalent advantages of using *in vitro* systems of urothelial cells are the possibility to control the conditions and to directly observe cells in the culture. The growth of cancer urothelial cells, harvested from rodents, pigs or humans, presents the opportunity to study 'pure' cell populations without the effect of cells from other tissues. Cell cultures also enable first phase testing of new medical active substances, e.g., testing the mechanism of cell entry, transport within cells and mechanisms of action. In the literature, there are several published methods for growing cancer and normal

urothelial cells, from immortalized cell lines and various differentiated primary and secondary cell cultures to explant cultures (26,27). Cell lines of urothelial cells are harvested either from normal urothelial cells, which were exposed to chemical carcinogens, or from cancer cells of different tumours of the bladder. There are several cell lines of human origin (e.g., RT4, RT112, T24, UMUC3 and UMUC14), which represent research models of different types of bladder cancer, and express numerous genetic or morphological changes and changes in the expression of genes, detected in human urothelial neoplasms. For research purposes, mouse (e.g. MB49 and MBT-2) and rat cell lines (e.g. AY-27) are also used (29). All these *in vitro* systems are model systems for studying the effect of factors that monitor the growth and differentiation of normal and cancer urothelial cells (e.g., growth factors, signal molecules, mitogens, inhibitors). They also support changing the origin and the development of the cancer cell transformation after exposure to the carcinogen, and the first establishment of the effectiveness of anti-cancer active substances (30). Cell cultures also support studies of genetic changes related to the development of bladder cancer, and testing tumorigenesis and capability of cells to make metastasis. Even though *in vitro* systems provide an effective model system, as they are simple to use and provide a clear overview, they have their limitations, especially with the absence of a complex (tissue) environment. Metabolism (31) and responsiveness (32) of cells to signals from the environment in a cell line are for example notably different from what occurs in the cells of a normal or cancer tissue *in situ*. Cell lines grown in monolayers do not mimic the complexity of a three-dimensional order, characteristic for tissues in an organism. By using three-dimensional cell culture systems and components of extracellular matrix we can establish a more physiological model of the architecture and dynamics of a tumour (33); however, we cannot completely replace the complex environment of a tissue *in vivo*. The cells surrounding cancer cells *in situ*, e.g., immune system cells, mesenchyme cells, venal cells, the complexity of the connective tissue and other cell types have a major impact on the tumour. These limitations to cell cultures can be partly overcome with animal models, described in the next chapter.

In Slovenia, the centre for *in vitro* urothelial systems is at the Institute of Cell Biology of the Faculty of Medicine in Ljubljana. In the well-equipped cell laboratory, we grow primary and secondary cultures of urothelial cells, cell lines and also the culture of urothelial tissue used for studying urothelium differentiation and regeneration, for studying methods of internalizing active substances, cytotoxicity, etc. We also use *in vitro* urothelial cells to study methods of intercellular communication with membrane nanotubes (34), and with extracellular vesicles (35,36), which has come to the centre of research lately, and is believed to have a role in cancer. It is of key importance that besides cell lines of papillary and metastasizing urothelial cells we also have models of normal urothelial cell cultures. Namely, normal cell cultures can be differentiated to a degree similar to normal urothelial cells *in situ*, and therefore allow us to research similarities and differences in cellular processes. Moreover, combining normal cells and cancer cell lines in a co-culture, allow us to monitor relevant interaction between normal and cancer cells during carcinogenesis (e.g., how cancer cells attach to normal ones, how the intercellular communication between them occurs, how cancer cells migrate among normal ones).

2.2 Animal models of urinary bladder cancer are key for preclinical studies

Animal models are the central research connection between the simpler

in vitro systems and clinical studies with a final application of discoveries to benefit humans. The main advantage of animal models is their complexity, which provides a good simulation of cellular processes in humans, while there is still the option for controlling experimental conditions. The most frequently used types of animal models of bladder cancer are compiled in Table 3. They can be divided in several ways, e.g., by genetic background of cancer cells and host animals, to syngeneic (the same background) and xenogeneic (different backgrounds) models, and by the location of the tumour in a test animal, to heterotopic and orthotopic models. Heterotopic models of bladder cancer are the simplest to establish, as the implant of a suspension of cancer urothelial cells can be conducted by injection either subcutaneously (under the skin) or intraperitoneally (into the perineum). The tumour is therefore developed at the anatomical location in the organism that differs from the location of the originating cells, which begs the question whether the tumour develops the same in a different location of the body as it would in the organ from which the cancer cells have originated (37). Using orthotopic models is therefore more suitable, as the intravesical administration of cancer urothelial cells implants them directly into the urinary bladder (38).

The advantage of syngeneic animal models is the uniform genetic background, as the tumour develops from the tissue of the host animal. Syngeneic models include animals in which tumours occur spontaneously or they are induced. Tumours are induced with certain chemicals (e.g., 0.05-percent N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN)), with genetic modifications (e.g., by deleting the p53 gene for the suppressor protein for the P53 tumour), or a combination of both. Spontaneously occurring tumours are very rare in rodents and are, in more than 99% of cases, related to advanced age (39). The spontaneous model of urinary bladder cancer in dogs has been documented, as the spontaneous onset in dogs is similar to that in humans (40). With dogs, it is most often the invasive urothelial carcinoma of a high degree, with cellular biology and molecular characteristics, locations and frequencies of metastases, and responses to therapy similar to those of the invasive urothelial carcinoma in hu-

Table 3: Types of animal models used in preclinical studies of urinary bladder cancer (summary from van Kessel et al.) (29).

	Model type	Model
Genetic background	syngeneic model	spontaneously occurring cancer
		chemically induced cancer
		transgene models, genetically engineered mouse models
		cell transplant of the same animal species into a host animal
	xenogeneic model	cell transplant of a different animal species into a host animal
Position of the tumour	orthotopic xenogeneic model	foreign or own cells grown in an animal at the same anatomic location as the one from which the cells were isolated
	heterotopic xenogeneic model	foreign or own cells grown in an animal at the anatomically different location as the one from which the cells were isolated

mans. This makes the dog model a good supplement to the mouse and rat bladder cancer models (41). The biggest advantage of syngeneic models is the natural macro- and microenvironment of the tumour, including the active immune system of the test animal.

The xenogeneic models of bladder cancer are those where cancer cells of an animal are used and transplanted into a host animal of a different species. This makes the cancer cells more similar to the cancer of a donor organism (e.g., human), whose biology we are the most interested in. When transplanting human cancer cells into the bladder of test animals, the resulting neoplasms are generally more similar to bladder cancer in humans than if the (animal) syngeneic model of carcinogenesis is used. However, xenogeneic models have certain limitations. They can be used on animals with an immunodeficiency, which allows for the human cancer cells to survive in a foreign organism (42). In order to best preserve the heterogeneity of the tumour architecture of bladder cancer, the best option is to directly transplant a fresh xenograft from the patient into a test animal. The first study describing the method of preparing the xenograft from a patient was published in 2015 (43); however, its use is only slowly gaining attraction. In spite of the advancements in transplanting foreign or own urothelial cells, and the development of various genetically modified model animals, chemically induced bladder cancer models are still most often used for preclinical studies.

2.2.1 Chemically induced urinary bladder cancer

The most frequently used chemical carcinogen for inducing bladder cancer is BBN (44). BBN frequently crops up in the colour industry and is a metabolic product of different N-nitroso compounds, which are also present in cigarette smoke (45). Extensive histological, morphological, cellular biological, molecular and genetic analyses were conducted for the BBN-induced bladder cancer model, as well as comparisons with human bladder cancer (46,47). The biggest advantage of BBN is the absence of systemic toxicity and induction of exclusively urothelial neoplasms (48), which are equivalent to human urothelial neoplasms in terms of onset and timeline (46,47).

Pathological and genetic similarities between the BBN-induced bladder cancer model, and the human disease put this model among the most comparable systems for studying different stages of human urothelial neoplasms (Figure 2). As such it supports studies of the biology of cancer urothelial cells and the development of tumours using graphic, genetic, molecular, histological and electron-microscopic methods. The BBN-induced bladder cancer model is also very useful for evaluating new methods of treating bladder cancer and targeted delivery of active substances into cancer cells. This model is used for determining the efficacy of active ingredients administered intravesically into the organism (using a catheter into the urinal bladder) or in another manner, such as orally or intraperitoneally (27). Before the start of treating test animals, it is essential to confirm that the cancer urothelial cells are capable of accepting the active substance, which is usually tested on cell cultures.

BBN induces bladder cancer in mice, rats and dogs, while it has proven to be a weak carcinogen for hamsters and pigs (49,50). Studies most frequently use the BBN carcinogenesis model in mice and rats, which are fairly easy to handle and house, and their upkeep is not expensive. For ethical reasons, price and highly demanding conditions for housing, dogs are no longer used as model systems for carcinogenesis (51).

BBN is introduced into an animal in the form of a 0.05% solution in drinking water, with 0,005% or 0.5% solutions also used optionally, depending on the strain of mice or rats, and the level of the desired tumour (52). The ingested BBN

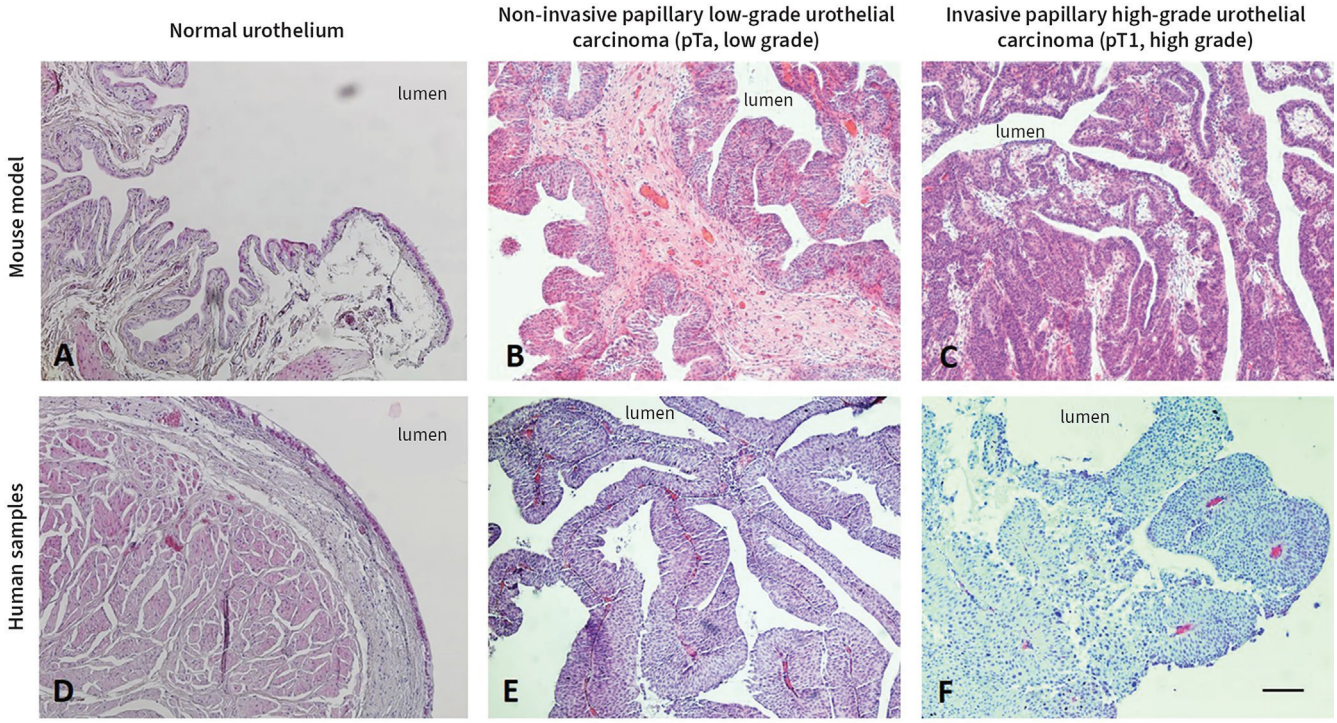

Figure 2: Paraffin sections, stained with haematoxylin and eosin. Normal mouse (A) and human (D) urothelium. The pathohistology of the mouse model of bladder cancer, induced by BBN (B - 0.05% BBN in drinking water 20 weeks; C - 0.05% BBN in drinking water 20 weeks, and then 15 weeks of drinking water without BBN) and human samples, with comparable degree of bladder cancer (E- pTa, low grade; F – pT1, high grade). Scale bar: 200 μm.

is metabolised in the liver, where the enzyme system of the alcohol/aldehyde dehydrogenase oxidises the BBN alcohol group into the carboxyl group, forming the N-Butyl-N-(3-carboxypropyl)-nitrosamine (BCPN). BCPN is excreted from the body through urine, which is stored in the bladder, and this is where it comes into contact with the urothelium. BCPN is a stable molecule, which forms covalent binds to the urothelial cells, and is directly responsible for the start of the process of the cancer transformation (53).

With regard to its mechanism of action, BBN is among genotoxic or DNA-reactive carcinogens (54). BBN causes damage to DNA molecules in urothelial cells and selectively induces bladder cancer in mice and rats (55). The intensity of the carcinogenesis and the stage to which an individual test animal develops bladder cancer (hyperplasia, dysplasia, CIS, low grade non-invasive papillary urothelial carcinoma, invasive carcinoma) depends on the concentration of BBN used, the duration of drinking water with BBN (2–24 weeks), the time from when they stop drinking the BBN water and until they are euthanised, the sampling of the urothelial tissue and the animal type and strain. With mice, pathogenesis generally first occurs as inflammation processes in lamina propria under the urothelium, followed by dysplasia with urothelial metaplasia of different stages, or without it, then followed by CIS and invasive carcinoma (56). Metastases are not typical for the mice model, as animals are euthanised because of possible obstructive uropathy before metastasis can occur. In the rat model, on the other hand, the result of BBN treatment is nearly exclusively non-invasive papillary carcinoma, low or high grade. During the development of the tumour, regular physical checks of the test animals are essential. With mice and rats, bladder palpation and

urine examination are used traditionally. Haematuria and palpable mass in the urinal bladder are signs that only develop in late and advancing stages of cancer. For monitoring a tumour, it is therefore recommended to use non-invasive *in vivo* imaging methods, such as ultra sound (US), endoscopy, cystoscopy and magnetic resonance imaging (MRI), as well as computer-assisted axial tomography (CAT), and bioluminescence. These imaging methods make it possible to monitor and measure tumours in detail, thereby decreasing the number of animals needed for the study. Besides the above, the *in vivo* imaging methods also make it possible to monitor the animals' general condition and establish the level of their suffering, which makes it possible to closely follow the established 3Rs of ethical rules: Replacement, Reduction, Refinement.

In Slovenia, the Institute of Cell Biology of the Faculty of Medicine in Ljubljana has used the mouse and the rat BBN model to analyse the changed expression of uroplakins (UP), which are characteristic for highly differentiated urothelial cells (57). We also used BBN models to study the selective binding of lectins (glycoproteins that specifically bind to certain sugar residue) to normal and cancer urothelial cells, which could provide improved diagnostics and targeted bladder cancer therapy (58).

2.2.2 Orthotopic models of urinary bladder cancer

Orthotopic models of bladder cancer enable a broad range of molecular and biological studies of cancer cells inside the host's healthy, normally differentiated urothelium, and new therapy methods. The xenogeneic orthotopic models are excellent at mimicking morphological characteristics of human tumours, while syngeneic orthotopic models are excellent at mimicking the natural systemic environment of an organism (immune and hormone system). From the perspective of implementation, orthotopic models are fairly demanding, and include two phases of establishing a tumour. In the first phase, cancer urothelial cells of human, mice or rat origin are grown in *in vitro* systems. The second phase requires successfully planting cancer cells in a prepared animal urinary bladder. The suspension of cancer urothelial cells must be introduced into the lumen of the bladder, which can be performed by injection with an open abdominal cavity, or by injecting the cellular suspension through a catheter through the urethra. Preparing the bladder means that a healthy, normal urothelium of the host animal is first chemically or mechanically damaged, otherwise the cancer urothelial cells cannot attach to it (59). After cancer cells are attached in the animal, its medical condition must be regularly monitored with a series of inspections of urine to check for haematuria and whether a pulpable tumour mass is growing in the urinary bladder. In order to monitor tumour growth, we can use non-invasive methods of *in vivo* imaging, already mentioned in chapter 2.2.1.

Orthotopic models of bladder cancer have certain limitations. For example, the induction of cancer development differs from the natural onset of the disease in humans. It is also not possible to conduct studies that would determine the specific immune response in mice with immunodeficiency, although these are needed for xenogeneic models. The time frame of metastases studies is also very limited with orthotopic models, as the test animals frequently die from obstruction of the urethra, caused by the primary tumour even before the onset of the invasive cancer and metastasis. When metastasis occurs, the locations of metastases in test animals often differ from those characteristic of human urothelial carcinoma (60).

With syngeneic orthotopic models of bladder cancer, cancer urothelial cells of the same species are implanted in the urinary bladder. The most widely used are the rat (61) and the mouse models, with whom cancer urothelial cells are trans-

planted from the same strain of the animal (38). The weakness of syngeneic orthotopic models is that these are cell lines of mouse or rat cancer urothelial cells with a chemically induced cancer transformation, which makes them differ from human cancer urothelial cells. Oncogene mutations, for which some human cell lines are known (for example T20 – *HRAS* and *TP53* mutations, RT4 cells – *RHOA* mutation, UMUC3 cells – mutations *PTEN* and *KRAS*), have not been discovered for the rat cell line AY-27, or for mouse cell lines MB49 and MBT-29 (29). The advantages of syngeneic orthotopic models for bladder cancer include the fact that test animals have flawless immune systems, which means that tumour growth and the effects of different therapies can be examined in the organ or in the organ system, where hormonal and immunological processes are uninterrupted. Even though the physical condition of the immune system and test animals is normal, and therefore similar to that of a human organism, it is important to mind the differences between the species and genders when interpreting results. At the Institute of Cell Biology of the Faculty of Medicine in Ljubljana we used the syngeneic orthotopic mouse model, with which we implanted mouse cancer urothelial cells MB49 directly into the bladder through a catheter. The success of the transplantation has increased after treating the urothelium with poly-L-lysine, which resulted in the desquamation of most of highly-differentiated superficial urothelial cells, so that the cancer cells were able to attach to non-differentiated urothelial cells and form a tumour (63). A histopathological analysis of these tumours showed that they are similar to human carcinoma *in situ* (CIS).

2.2.3 Transgene models – genetically engineered mouse models

Genetically engineered mouse models (GEM or GEMM) are frequently used for various studies of the biology of cancer cells, including the analysis of the tumour phenotype, modelling subtypes of diseases, studies of candidate genes and signalling pathways, and preclinical tests of potential active substances (64). The most important advantage of GEM models is that tumours develop anew in a micro-environment of the urothelium, similarly to chemically induced models, while they are also designed on exactly defined genetic changes. This allows us to study the impact of the changed, but still well-known gene expression on cancer development. On the other hand, an unfavourable trait is that there are relatively few described GEM models for bladder cancer, especially those with a muscle-invasive and/or metastatic phenotype. Most single mutations and also numerous combinations of mutations are mirrored as small changes to the phenotype (hyperplasia, CIS and non-invasive papillary urothelial carcinoma) (65). Due to the recent description of molecular changes characteristic for bladder cancer, which can be modelled in mice (66), GEM models are likely to play an increasingly significant role in the future.

The development of GEM models for bladder cancer has accelerated after the discovery of genes for uroplakins, which are integral transmembrane proteins characteristic for urothelial cells (67,68). The promoter for the mouse gene that encodes uroplakin II (UPII) can also be used for controlled introduction of oncogenic mutations into the urothelial cells of mice. At first the promoter for UPII was used in combination with oncoprotein SV40T, inactivating the tumour suppressor proteins p53 and pRb (retinoblastoma protein), for which it has been known that they are frequently mutated in human cancer urothelial cells (69). With transgene mice that were created this way, the most characteristic types are CIS and invasive urothelial carcinoma, some of which may also metastasize (69,70). The molecular profile of these mice is similar to humans (71).

Another advantage of GEM models is the high predictability of the development

of the tumour, which makes it easier to determine specific influences on the inhibition of the tumour's development. For GEM models, inbred strains of test animals are usually used, which significantly reduces the impact of different genetic backgrounds on the metabolism of active substances, on tumour's response and on the tumour's resistance to the active substance. These models allow us to research both innovative therapeutic approaches, as well as preventive strategies on different levels of the tumour's advancement (72).

Most of today's GEM models include a targeted insertion of genes into specific tissue. However, such bladder cancer models are increasingly being replaced by a system of the insertion of genes with Cre allele, which allow for an even better targeted insertion of genes, exclusively in the selected cellular type (65). It has been discovered that conditional activation of β-catenin in the bladder with Cre allele, tied to the expression of the *UPII* promoter gene (called UroII-Cre), causes hyperplasia, while along with activation of *Hras* and *Kras* genes, or the loss of the *Pten* gene function, it causes non-invasive papillary urothelial carcinoma (72).

The other method for studying the impact of the altered gene expression in the urothelium on the development and advancement of bladder cancer is by inserting the genes using adenoviruses. This requires a special catheterisation of the test animal and the insertion of adenoviruses with the code for the desired gene in the lumen of the urinal bladder (73). In practice, female mice are most often used; catheterising the males is more demanding because of the anatomy of the prostate and the length of the urethra. The advantages of inserting genes with adenoviruses is the possibility of entering the transgene (74) or a gene for Cre recombinase (such a system is called Adeno-Cre), which causes deletions of rodent genes, marked with flox/flox positions (73). This animal model is also relatively inexpensive. The biggest weakness of this system is an imperfect transfer of the viral DNA or RNA into the genome of the urothelial cells, and, depending on the success of the transfer, the long wait for the development of phenotype changes. However, a prior treatment of the bladder lumen with detergents, such as sodium dodecyl sulphate (SDS) and dodecyl-β-D-maltoside (DDM), improves the effectiveness of the insertion of the viral nucleotide acid (74); this is most likely because it causes small damages to the apical plasma membrane of urothelial cells. Using the Adeno-Cre system the genes for *Trp53* and *Pten* in urothelial cells were inactivated, causing the development of an invasive urinary bladder cancer, which frequently metastasized (73). Further studies of the Adeno-Cre models have confirmed their usability in preclinical studies of therapy in the bladder using rapamycin (75), and are the basis for clinical studies, aimed at assessing efficacy of intravesical testing with rapamycin in high-risk early stages of urinary bladder cancer (https://clinicaltrials.gov/ct2/show/NCT02009332). Similarly, preclinical studies on this model have shown the effectiveness of entering several different chemotherapeutics through the bladder, distributed along a special regime. These studies also led to clinical studies aimed at assessing the effectiveness of the treatment on humans (https://clinicaltrials.gov/ct2/show/NCT02202772). These cases show that preclinical studies on GEM models can be useful for testing the effectiveness of promising new active substances, as well as in the optimisation of target delivery of these substances.

2.3 Human samples, obtained from biopsies

The frequency and high recurrence of urothelial neoplasms call for future studies to focus on finding new diagnostic procedures and in finding new indicators, such as co-dependency of different diagnostic and prognostic markers. We still do not have any good markers for tumour

response that could be effectively used as a guideline when deciding on the most suitable method of therapy. Consequently, with bladder cancer, long-term postoperative monitoring of patients is essential. Discovering which markers are suitable for forecasting the survival rate of a bladder cancer patient and the advancement of the disease, or which patients will need to be actively monitored after the operation, and which will not, would significantly reduce the burden on the healthcare system. Human samples, obtained from biopsies, are most suitable for such studies. Along with *in vitro* and animal models it is also useful to verify if a certain new active substance can internalize into human cancer urothelial cells *in situ*. At our institute, we used transmission electronic microscope to analyse selective endocytosis of lectins, marked with colloidal gold, into normal and cancer urothelial cells, grown *in vitro*. We used the model of normal pig urothelial cells and two cell lines of cancer urothelial cells RT4 and T24. Normal urothelial cells did not result in endocytosis of lectins, while the cancer urothelial cells did (Figure 3). Next, we verified lectin endocytosis on human samples immediately after the biopsy (i.e., on *ex vivo* samples) and discovered that these cancer urothelial cells also result in lectin endocytosis (Figure 3 C, D). Because we confirmed lectin endocytosis not only in cell cultures, but also on human samples, these studies can conclude that lectins could be used for targeted delivery of active substances into cancer urothelial cells.

For long-term patient monitoring and retrospective studies, the most applicable are the archived human samples, obtained through biopsy, which are generally housed in paraffin or frozen at –80°C. Frozen samples can be used for performing immunohistochemical and other specific labelling, as well as various molecular, biochemical, and genetic analyses. In Slovenia, all paraffin blocks with patient samples are archived at the Institute of Pathology of the Faculty of Medicine in Ljubljana. When an appropriate ethical permission is granted, they can be used for different studies. The samples that had already been used for research purposes at the Institute of Cell Biology at the Faculty of Medicine in Ljubljana, are kept in our facilities. These include paraffin and epone (for transmission electronic microscopy) blocks, as well as frozen, cryo semithin and ultrathin slices and frozen samples for biochemical analyses and dried samples for scanning electronic microscopy.

3 Innovative approaches to diagnostics and therapy

The current methods of treating bladder cancer are usually only short-term solutions, as the tumours frequently recur. The most likely reason for high recurrence of bladder tumours is in survival of urothelial cancer stem cells after resection and cytostatic chemotherapy. Because conventional therapy methods using cytostatics only affect proliferative cells, the dormant cancer stem urothelial cells can survive. Understanding biological mechanisms that control the proliferation and differentiation of stem cells could lead to a development of innovative anti-cancer strategies and their combinations that would successfully remove the populations of all types of cancer urothelial cells, including cancer stem cells.

Besides protein markers that make it possible to monitor bladder cancer, such as uroplakins and purinergic receptors, new research is also focusing on protein sugar residue. Changes in the composition of sugar residue on the cell surface are a classic sign of a cancer transformation. Even though changes in glycosylation during cancer transformation of urothelium have not been studied in detail, it seems that they cause changes in interactions between cells and ligands (76). Such ligands are for example lectins, which have a specific affinity to binding sugar residues that allows them to mark target molecules. Due to their characteristics, lectins became highly

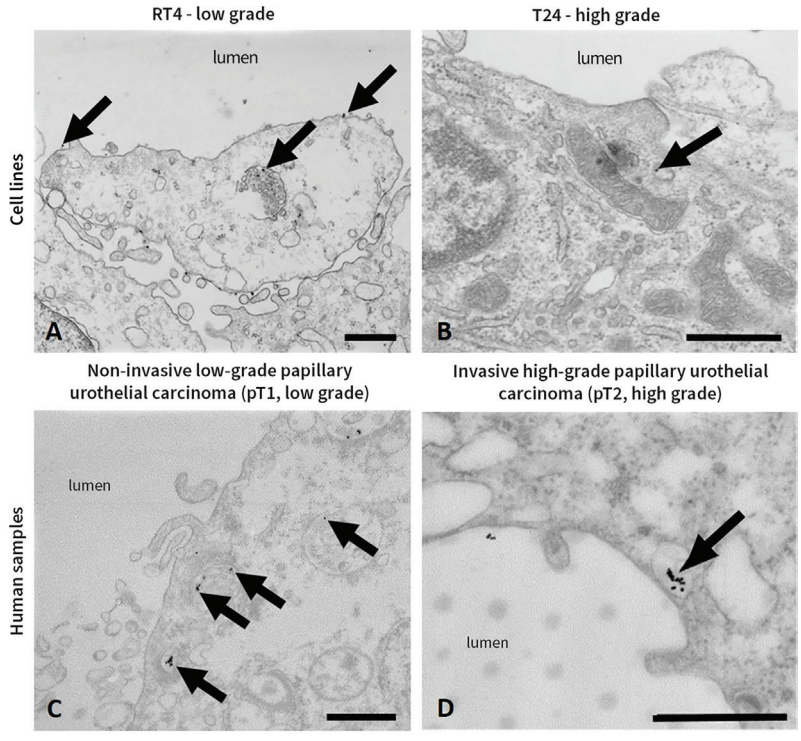

Figure 3: Transmission electronic microscopy of cancer urothelial cells with endocytosis-induced lectin jacalin, labelled with colloidal gold. Cells of cell lines RT4 (A) and T24 (B), grown in a growth medium *in vitro*. Colloidal gold, bound to lectin jacalin (arrows) is located on the surface of the cells and in the endocytic vesicles. Human sample cells were harvested *ex vivo* from biopsies of patients with non-invasive low grade papillary urothelial carcinoma (C) and non-invasive high grade papillary urothelial carcinoma (D). Colloidal gold, bound to lectin jacalin (arrows) is located in endosome of urothelial cell. Scale bar: 500 nm.

useful in research work, as they allow for characterisation and isolation of glycoproteins and studying sugar residues and their changes during cancer transformation (77). The method used for labelling sugar residue is called lectin histochemistry. It could represent an innovative approach to diagnosing and forecasting the course of the disease. It is possible to conduct a direct correlation between protein and sugar markers on the same tissue section with combined labelling of sugar residue with lectin histochemistry and proteins with immunohistochemistry (Figure 4). This combined method was introduced at the Institute of Cell Biology and we named it Combined Lectin- and Immuno-Histochemistry, (CLIH). We used cryo semithin sections of human normal urothelium and different urothelial neoplasms. The ACA (*Amaranthus caudatus aglutinin*) and JAC (jakalin) lectins were bound to the apical plasma membrane and apical cytoplasm of terminally differentiated superficial cells (umbrella cells), while DSA (*Datura stramonium aglutinin*) lectin was mainly bound to the intermediate cells of the normal urothelium (Figure 4). Antibodies against uroplakin labelled the apical plasma membrane of umbrella cells, where a co-localization of uroplakins with the ACA and JAC lectins was observed. The sample of the papillary urothelial neoplasm of low malignant potential (PUNLMP) had strong labelling of intermediate cells with all three lectins, i.e. the labellings were different than in the normal urothelium (Figure 4). The immunohistochemistry of uroplakins was negative (Figure 4). Since CLIH enable simultaneous detection of sugar residues and proteins, this could additionally contribute towards improved diagnostics. Lectins are also used in studies of targeted delivery of drugs into cancer cells (Figure 3), which could contribute towards more effective strategies for bladder cancer treatment. Besides lectins, our Institute has also tested the usability of nanodiamonds (78), the combination of UV radiation and nanoparticles from titanium oxide (79), as well as chitosan (80). All innovative methods of therapy for bladder cancer have been shown to be potentially useful.

4 Conclusion

Currently, no means for targeted delivery of active substances into cancer cells, which has a license for treating cancers, are in use for treating urinary bladder cancer. The completed clinical studies have had very limited success, often because of a lack of effectiveness and extensive side effects (29). On the other hand, new opportunities for targeted delivery of active substances and certain innovative

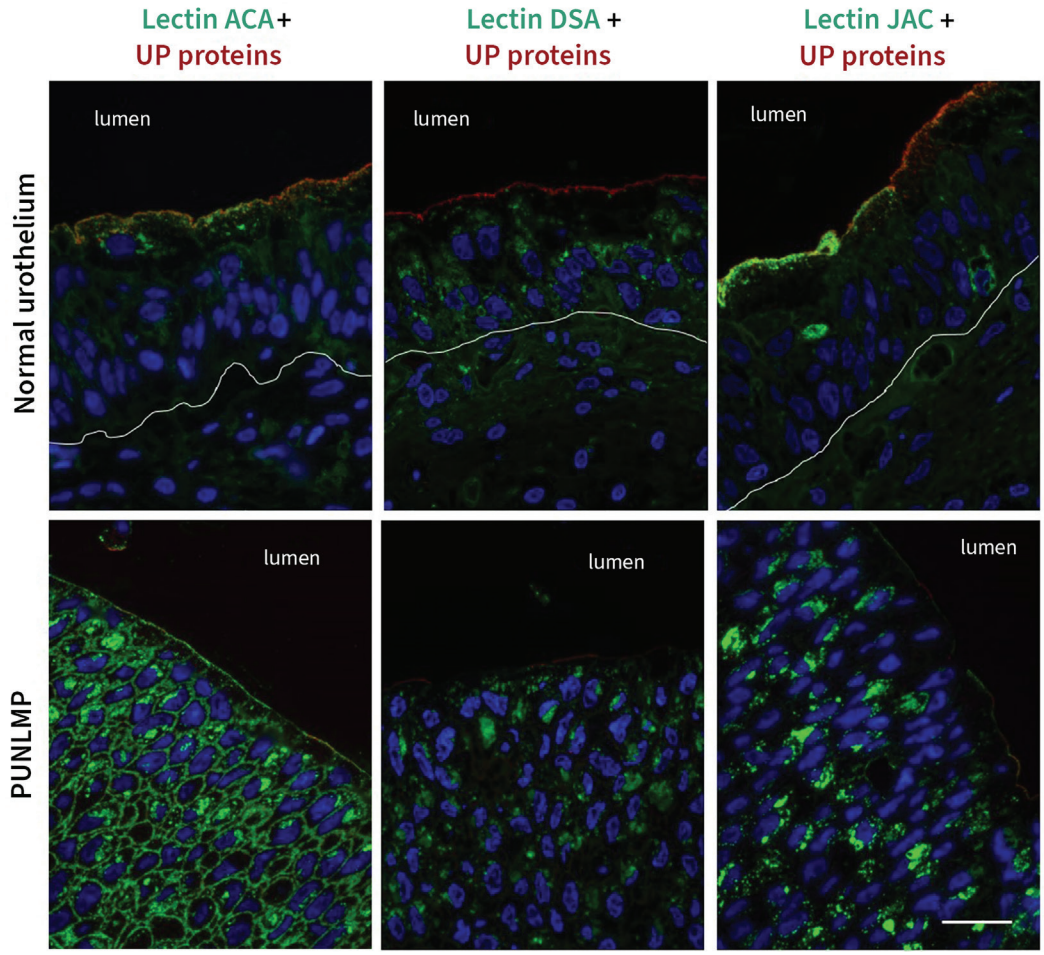

Figure 4: The method of combined lectin histochemistry and immuno-histochemistry (CLIH) on a normal human urothelium and a sample of the papillary urothelial neoplasm of low malignant potential (PUNLMP). The green fluorochrome labels lectins ACA – *Amaranthus caudatus* aglutinin, DSA – *Datura stramonium* aglutinin, JAC – jacalin. The red fluorochrome labels proteins uroplakins (UPs). The white line depicts the basal lamina. Scale bar: 20 µm.

technologies provide hope for a greater effectiveness of therapy in the future. The described research models are the foundation for preclinical and clinical studies that would bring higher success rates in detection, lower recurrence, and more effective treatment for urinary bladder cancer.

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