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European Journal of Histochemistry 2020; volume 64:3141

# Combined lectin- and immuno-histochemistry (CLIH) for applications in cell biology and cancer diagnosis: Analysis of human urothelial carcinomas

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ABSTRACT

Lectin histochemistry (LHC) and immunohistochemistry (IHC), which demonstrate the composition and localisation of sugar residues and proteins in cell membranes, respectively, are generally used separately. Using these two methods, we previously demonstrated that malignant transformation of urothelial cells results in the alterations of protein glycosylation and reduced expression of urothelium-specific integral membrane proteins uroplakins (UPs). However, the correlation between these changes was not studied yet. To evaluate this correlation, we developed innovative method, which we named Combined Lectin- and Immuno-Histochemistry (CLIH). We used human biopsies of 6 normal urothelia and 9 papillary urothelial carcinomas, *i.e.* 3 papillary urothelial neoplasms of low malignant potential (PUNLMP), 3 non-invasive papillary urothelial carcinomas of low grade (pTa, l.g.), and 3 invasive papillary urothelial carcinomas of high grade (pT1, h.g.). We tested five different protocols (numbered 1-5) of CLIH on paraffin and cryo-semithin sections and compared them with LHC and IHC performed separately. Additionally, we carried out western and lectin blotting with antibodies against UPs and lectins *Amaranthus caudatus* agglutinin (ACA), *Datura stramonium* agglutinin (DSA), and jacalin, respectively. We showed that incubation with primary antibodies first, followed by the mixture of secondary antibodies and lectins is the most efficient CLIH method (protocol number 5). Additionally, 300 nm thick cryo-semithin sections enabled better resolution of co-localisation between sugar residues and proteins than 5 μm thick paraffin sections. In the normal urothelium, CLIH showed co-localisation of lectins ACA and jacalin with UPs in the apical plasma membrane (PM) of superficial umbrella cells. In papillary urothelial carcinomas, all three lectins (ACA, DSA and jacalin) labelled regions of apical PM, where they occasionally co-localised with UPs. Western and lectin blotting confirmed the differences between normal urothelium and papillary urothelial carcinomas. Our results show that CLIH, when used with various sets of lectins and antigens, is a useful, quick, and reliable method that could be applied for basic cell biology research as well as detailed subtyping of human urothelial carcinomas.

**Key words:** Lectin histochemistry; immunohistochemistry; combined lectin- and immuno-histochemistry (CLIH); paraffin sections; cryo-semithin sections; urothelium; papillary urothelial carcinoma.

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**Contributions:** All authors have made substantial contributions to the following areas of this manuscript: DZ, RR, conceived the project and designed the experiments, performed all the experiments and analysed the data; IS, collected human biopsy samples; DZ, wrote the manuscript. All authors edited, read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

**Conflict of interest:** None of the authors has any conflict of interest to declare.

**Availability of data and materials:** All data generated or analyzed during this study are included in this published article. /// The data used to support the findings of this study are available from the corresponding author upon request. /// The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate:** The study was conducted in accordance with the Helsinki Declaration and approved by the Slovenian National Medical Ethics Committee, No. 76/10/10.

**Informed consent:** obtained from all patients.

## Introduction

Urinary bladder cancer represents a substantial economic burden in developed countries, due to the highest lifetime treatment costs per patient of all cancers.[1,2] The majority of bladder cancer patients (75-80%) are first presented with papillary non-invasive (papillary urothelial neoplasm of low malignant potential (PUNLMP) ≈21% and non-invasive papillary urothelial carcinoma (pTa) ≈62%) or superficially invasive [invasive papillary urothelial carcinoma (pT1) ≈17%] urothelial carcinomas, whereas the remaining 20% to 25% of primary carcinomas are already muscle invasive [muscle invasive papillary urothelial carcinoma (pT2)].[3,4] PUNLMP, pTa and pT1 carcinomas can be removed by transurethral resection of the bladder (TURB). However, 70% of these patients will have at least one recurrent carcinomas, and up to 20-30% will eventually develop a more aggressive, muscle-invasive form.[4,5] At present, it is not possible to identify those PUNLMP, pTa and pT1 cases that will recur based on conventional histopathological assessment. Diverse immunohistochemical (*e.g.*, keratin and fibroblast growth factor receptor 3 (FGFR3) expression pattern) and molecular (*e.g.*, gene alterations, *FGFR3* mutations) markers have been suggested to predict recurrence, but conflicting results have been reported.[6-10] New diagnostic tools and personalized approaches are needed for more successful diagnosis and treatment of bladder urothelial carcinomas.

About 90% of bladder cancers arise from urothelium, a stratified epithelium that covers the luminal side of the urinary bladder.[4,11,12] The superficial terminally differentiated umbrella cells synthesize large amounts of transmembrane glycoproteins uroplakins (UPs).[13-15] In the apical surface of the normal urothelium, UPs are organized into urothelial plaques, which form one component of the blood-urine permeability barrier. N-linked glycans of UPIa, UPIb and UPIIIa are part of a glycocalyx, which form another component of the permeability barrier.[16-18] Previously, it was shown that urothelial carcinogenesis is accompanied by changes of UPs expression as well as sugar residues composition,[19-21] however, the correlation between these two attributes is not known.

The routine diagnosis of bladder cancer relies on histopathological evaluation of paraffin sections from biopsy samples. The immunohistochemistry (IHC) is sometimes additionally done for detection of keratins, while lectin histochemistry (LHC) is not accepted as a diagnostic tool, despite several studies demonstrated that it could improve diagnosis of bladder cancer.[22-25] Correlation between protein and sugar residues expression and localization would offer additional information about carcinoma subtypes.

In this respect, we introduce here the innovative Combined Lectin- and Immuno- Histochemistry (CLIH) method. We used lectins *Amaranthus caudatus* agglutinin (ACA), *Datura stramonium* agglutinin (DSA) and jacalin (lectin from *Artocarpus integrifolia*), since these lectins are promising for distinguishing between normal and cancer urothelium.[19] To develop the CLIH method for different microscopic modalities of fluorescence microscopy, we performed different protocols of CLIH on paraffin sections. Because the preparation of paraffin sections potentially alters antigen and sugar residues characteristics, we also tested the same protocols of CLIH on cryo-semithin sections. Moreover, cryo-semithin sections are 300 nm thick and therefore enable more precise co-localisation of protein and sugar residues expression. To sum up, CLIH is a versatile method, which could be applied to basic cell biology research of urothelial differentiation and analyses of urothelial carcinomas.

## Materials and Methods

Polyclonal rabbit antibodies developed against total bovine uroplakins - anti-UPs antibodies (pAb), which react strongly with UPIIIa, moderately with uroplakin Ia and Ib, and weakly with uroplakin II, were a kind gift from Prof. Tung-Tien Sun, Department of Cell Biology, New York University Medical School.[14] Fluorescein isothiocyanate (FITC) labelled lectins ACA, DSA and jacalin (JAC) were purchased from Vector Laboratories (Burlingame, USA). Lectin inhibitory sugars bovine submaxillary mucin for ACA, galactose for jacalin and chitin hydrolysate for DSA were also purchased from Vector Laboratories (Burlingame, USA). Secondary antibodies were goat anti-rabbit IgG conjugated with Alexa Fluor 555 (sAb-AF555; Thermo Fischer Scientific, USA) for IHC and CLIH and goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich, Taufkirchen, Germany) for Western blotting. We used Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). For protein concentration determination we used a bicinchoninic acid (BCA)™ protein assay kit (Pierce, Rockford, IL). For western and lectin blotting we used: enhanced chemiluminescence reagent (ECL; Amersham Biosciences, Buckinghamshire, UK ), Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), anti-FITC (Sigma-Aldrich), anti-actin (Sigma-Aldrich), and anti-α-tubulin (Sigma-Aldrich) antibodies. All other chemicals were obtained from Sigma-Aldrich, unless otherwise specified.

### Patients and sampling

The study was conducted in accordance with the Helsinki Declaration and approved by the Slovenian National Medical Ethics Committee, No. 76/10/10. The study population consisted of 9 patients with bladder cancer who underwent TURB. Informed consent was obtained from all patients. Two samples were acquired by cold-cup biopsies from each patient: i) the urothelial carcinoma, and ii) the normal urothelium 1 cm posterior from the interureteric ridge. Biopsies captured urothelium and part of lamina propria. Six (6) samples were considered as normal, since they had no signs of hyperplasia or dysplasia. For pathological staging and grading, the EAU Guidelines on Non-muscle-invasive Bladder Cancer were used.[4] Urothelial cancers were diagnosed as a papillary urothelial neoplasm of low malignant potential - PUNLMP (3 samples), non-invasive papillary urothelial carcinoma of low grade - pTa l. g. (3 samples) and invasive papillary urothelial carcinoma of high grade with lamina propria invasion - pT1 h. g. (3 samples). The samples were processed for paraffin and cryo-semithin sections, and western and lectin blotting.

### Sample preparations

For paraffin sections, samples were fixed with 4% formaldehyde (FA) in phosphate-buffered saline (PBS) overnight at 4°C and embedded in paraffin. Paraffin sections were 5 µm thick, cut from at least two different parts of each sample and stained with hematoxylin and eosin (HE).

For cryo-semithin sections, samples were fixed in 2% FA plus 0.05% glutaraldehyde in PHEM buffer for one h at RT and post-fixed in 0.5% FA in PHEM buffer overnight at 4°C. Samples were then washed in PBS and embedded in 12% gelatin. Then samples were incubated in 2.3 M sucrose at 4°C overnight. Lastly, samples were frozen with liquid nitrogen. Cryo-semithin sections, 300 nm thick, were cut in cryo-ultramicrotome (Leica, Microsystems, Germany). Then sections were collected on glass slides and air-dried. Cryo-semithin sections were washed in PBS and then in 0.1% glycine in PBS.

For lectin and western blotting, samples were homogenized in

ice-cold buffer (0.8M Tris-HCl, 7.5% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged and the protein concentration in the supernatant was determined by using a BCA™ protein assay kit.

### Immunohistochemistry

IHC was performed as previously described.[26,27] Briefly, all sections were incubated in 2.5% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT) to block nonspecific labelling. Then, sections were incubated overnight at 4°C with primary antibodies (pAb) anti-UPs antibodies (diluted 1:5000 for paraffin sections and 1:10000 for cryo-semithin sections). After washing in PBS, sections were incubated for 1 h at RT with secondary antibodies conjugated with Alexa Fluor 555 (sAb-AF555), diluted 1:400. A series of negative controls were performed, omitting the primary antibody or incubating sections with a non-relevant antibody. Sections were washed and immersed in Vectashield with DAPI.

### Lectin histochemistry

LHC was performed as previously described.[28] Briefly, non-specific labelling was blocked in 1% BSA, 0.005% Triton X-100 and 0.05% Tween 20 in PBS for 10 min at RT. After washing in PBS, sections were incubated with lectins, conjugated to FITC, for 30 min at RT. Sections were then washed and immersed in Vectashield with DAPI. Lectins were titrated and the optimal concentrations were used thereafter (ACA - 2 µg/mL, DSA – 20 µg/mL, jacalin – 2 µg/mL). Controls for LHC were performed using: i) substitution of lectin solution with the buffer alone; ii) incubation of each lectin in the presence of its hapten inhibitory sugar at the concentration of 0.75 M (bovine submaxillary mucin for ACA) and 1.11 M (galactose for JAC), while a stock solution of chitin hydrolysate for DSA was used.

### Protocols of CLIH

CLIH was performed on paraffin and cryo-semithin sections by the same concentrations of lectins and dilutions of antibodies as in IHC and LHC. Five different protocols were used for paraffin and cryo-semithin sections as shown in Table 1.

A series of negative controls were performed. Controls for IHC were performed by omitting the primary antibody or incubating sections with a non-relevant antibody. Controls for LHC were performed using: i) substitution of lectin solution with the buffer alone; ii) incubation of each lectin in the presence of its hapten inhibitory sugar at the concentration of 0.75 M (bovine submaxillary mucin for ACA) and 1.11 M (galactose for JAC), while a stock solution of chitin hydrolysate for DSA was used.

### Microscopy and image acquisition

Slides were analysed using a Plan-Apochromat objective (20×/NA 0.75) and an oil immersion objective (63×oil/NA 1.40) in a fluorescence microscope Axio Imager.Z1 (Zeiss, Jena, Germany). Filtersets used were from Zeiss: filter set 49 for blue fluorescence (excitation: G365, beamsplitter: FT 395, emission: BP 445/50); filter set 43 HE for red fluorescence (excitation: BP 550/25, beamsplitter: FT 570, emission: BP 605/70); filter set 10 for green fluorescence (excitation: BP 450-490, beamsplitter: FT 510, emission: BP 515-565). The images were taken at the same exposure time for uroplakins (red) (objective 20×: excitation time 206 ms; 63× oil: excitation time 60 ms), and for each lectin (green) (ACA - objective 20×: excitation time 667 ms; 63× oil: excitation time 381 ms; JAC - objective 20×: excitation time 667 ms, 63× oil: excitation time 401 ms; DSA - objective 20×: excitation time 118 ms, 63× oil: excitation time 118 ms), and for DAPI (blue) (objective 20×: excitation time 382 ms; 63× oil: excitation time 309 ms). Merged images were acquired in AxioVision Rel. 4.8 programme (Zeiss).

### Western and lectin blotting

From each patient, the protein sample (5 µg/lane) from the normal urothelium was loaded next to the protein sample (5 µg/lane) from different urothelial carcinomas. Proteins were size-fractionated on 7.5%, 12% and 15% SDS-polyacrylamide gels and then transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. For western blotting, membranes were blocked overnight at 4°C in 5% milk in PBS with 0.1% Tween (PBS-Tween) and incubated overnight at 4°C with pAb (1:10000). After washing, the membranes were incubated for 2 h at RT with sAb-peroxidase, diluted 1:1000. For lectin blotting, the membranes were blocked in RIPA buffer for 90 min at RT, washed in PBS-Tween and then incubated overnight at 4°C with lectins conjugated with FITC and diluted in PBS-Tween (ACA 1:2000, DSA 1:2000, jacalin 1:3000). After washing in PBS-Tween, membranes were incubated for 90 min with mouse anti-FITC antibodies (1:1000). After washing in PBS-Tween, membranes were incubated for 1 h with sAb-peroxidase, diluted 1:1000.

Membranes for western and lectin blotting were finally probed with ECL and exposed to X-ray films. To confirm equal protein loading, the blots were stripped and reprobed with anti-actin (1:2000) or anti-α-tubulin antibody (1:2000).

## Results

### ACA and jacalin bind to the apical surface of normal UPs positive human urothelium

On paraffin and cryo-semithin sections, the apical plasma membrane (PM) of normal urothelial cells was labelled with anti-UPs antibodies, while the labelling of fusiform vesicles was observed only in cryo-semithin sections (Figure 1 A,B). ACA and jacalin labelled apical PM of umbrella cells (Figure 1 C,D,G,H).

**Table 1. Five different protocols of CLIH.**

| Protocol number | Protocol summary | Protocol steps |
|---|---|---|
| Protocol 1 | LHC - IHC | lectin-FITC → washing → pAb → washing → sAb-AF555 → washing → DAPI → mounting → FM |
| Protocol 2 | LHC - FA - IHC | lectin-FITC → washing → FA → washing → pAb → washing → sAb-AF555 → washing → DAPI → mounting → FM |
| Protocol 3 | IHC - LHC | pAb → washing → sAb-AF555 → washing → lectin-FITC → washing → DAPI → mounting → FM |
| Protocol 4 | IHC - FA - LHC | pAb → washing → sAb-AF555 → washing → FA → washing → lectin-FITC → washing → DAPI → mounting → FM |
| Protocol 5 | pAb - lectin + sAb | pAb → washing → lectin-FITC + sAb-AF555 → washing → DAPI → mounting → FM |

FA, stabilization of bound lectins or primary antibodies with 4 % formaldehyde for 5 min at RT, followed by washing of sections with PBS; FM, examination with a fluorescence microscope Axio Imager.Z1 (Zeiss).

DSA labelled the cytoplasm of umbrella cells , while the apical PM was not labelled (Figure 1 E,F). To sum up, we showed that apical PM of UPs positive human umbrella cells is labelled by ACA and jacalin and not by DSA.

To test the specificity of lectin labelling we performed two types of negative controls. First, the negative controls using inhibitory sugars were performed. The results showed some lectin labelling especially with DSA, indicating that weak unspecific labelling could occur (Supplementary Figure 1). Second, the negative controls performed by omitting lectins were negative (*data not shown*).

### The comparison between different protocols of CLIH

For combined imaging of proteins and carbohydrates on the same tissue section, we first tested five different protocols on paraffin and cryo-semithin sections of normal urothelium with antibodies against UPs and lectin ACA. The results were similar regardless of which protocol (protocol 1 – protocol 5) was used (Figure 2). ACA and UPs were co-localized in the apical PM of superficial cells of the normal urothelium. Nevertheless, in the apical cytoplasm of these superficial cells strong ACA labelling was observed, with negative or weak uroplakin labelling (Figure 2). The difference between paraffin and cryo-semithin sections was observed in the intermediate and basal urothelial cells, in which ACA labelling was negative or weakly positive in paraffin sections

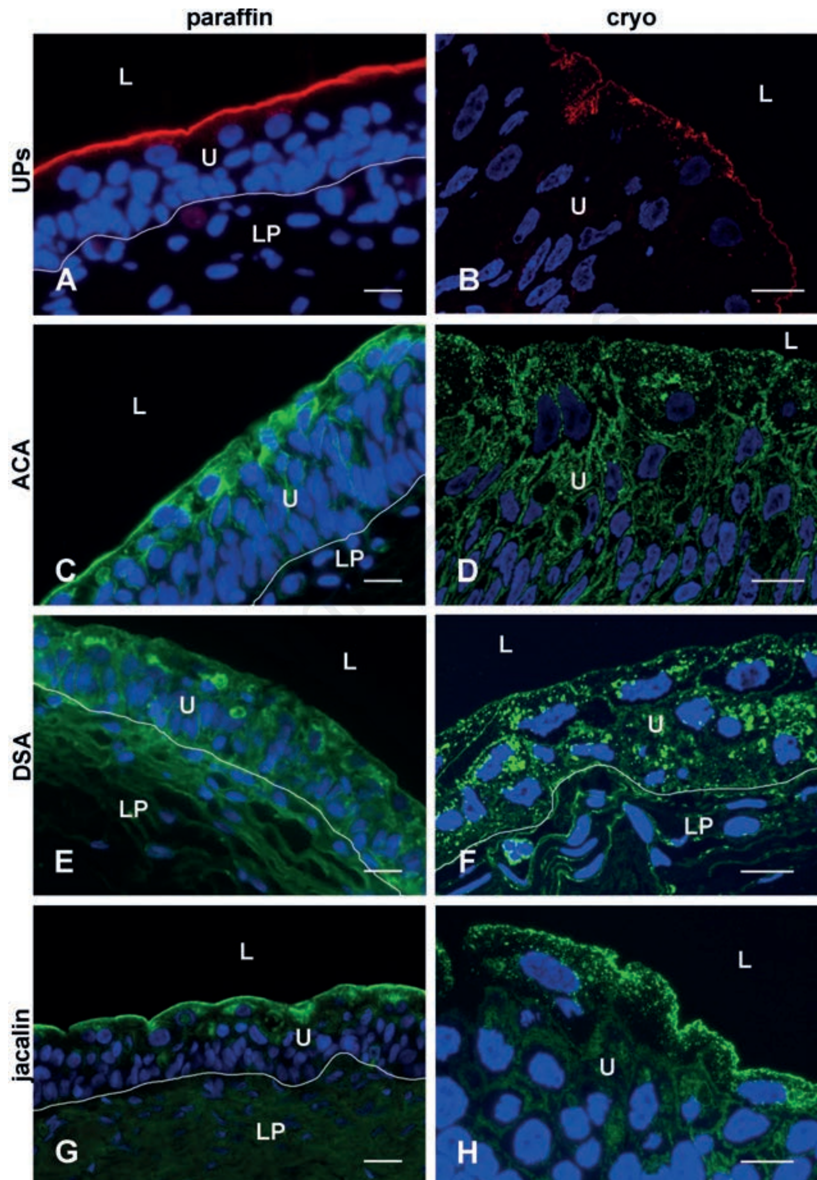


**Figure 1. Normal human urothelium with UPs positive apical PM (A,B) and sugar residues that bind ACA (C,D), DSA (E,F) and jacalin (G,H). Antibodies against UPs (red) label mainly apical PM of umbrella cells in paraffin (A) and cryo (B) sections. ACA (green) label mainly apical PM of umbrella cells in paraffin section (C), intermediate and basal cells in cryo-semithin section (D). DSA (green) label the cytoplasm of umbrella cells in paraffin section (E), cytoplasm of intermediate and basal cells in cryo-semithin section (F). Jacalin (green) label apical PM of umbrella cells in paraffin and cryo-semithin section (G,H). White line outline the basal lamina. L, lumen of the bladder; U, urothelium; LP, lamina propria. Scale bars: 10 µm.**

(Figure 2 A,C,E,G,I) and positive in cryo-semithin sections (Figure 2 B,D,F,H,J).

Second, we compared five different protocols on cryo-semithin sections of normal urothelium with antibodies against UPs and lectins DSA and jacalin. The results with these lectins were in general similar regardless of the protocol used (protocol 1 – protocol 5) (Supplementary Figure 2). The apical PM of superficial cells was uroplakin positive, while DSA did not bind to it. DSA labelling was observed in the cytoplasm of intermediate and basal urothelial cell, where no uroplakin labelling was detected (Supplementary Figure 1). Therefore, there was no co-localization between UPs and DSA labelling. On the other hand, lectin jacalin exhibited strong labelling of the apical PM of superficial cells (Supplementary Figure 1). Jacalin also weakly labelled the apical

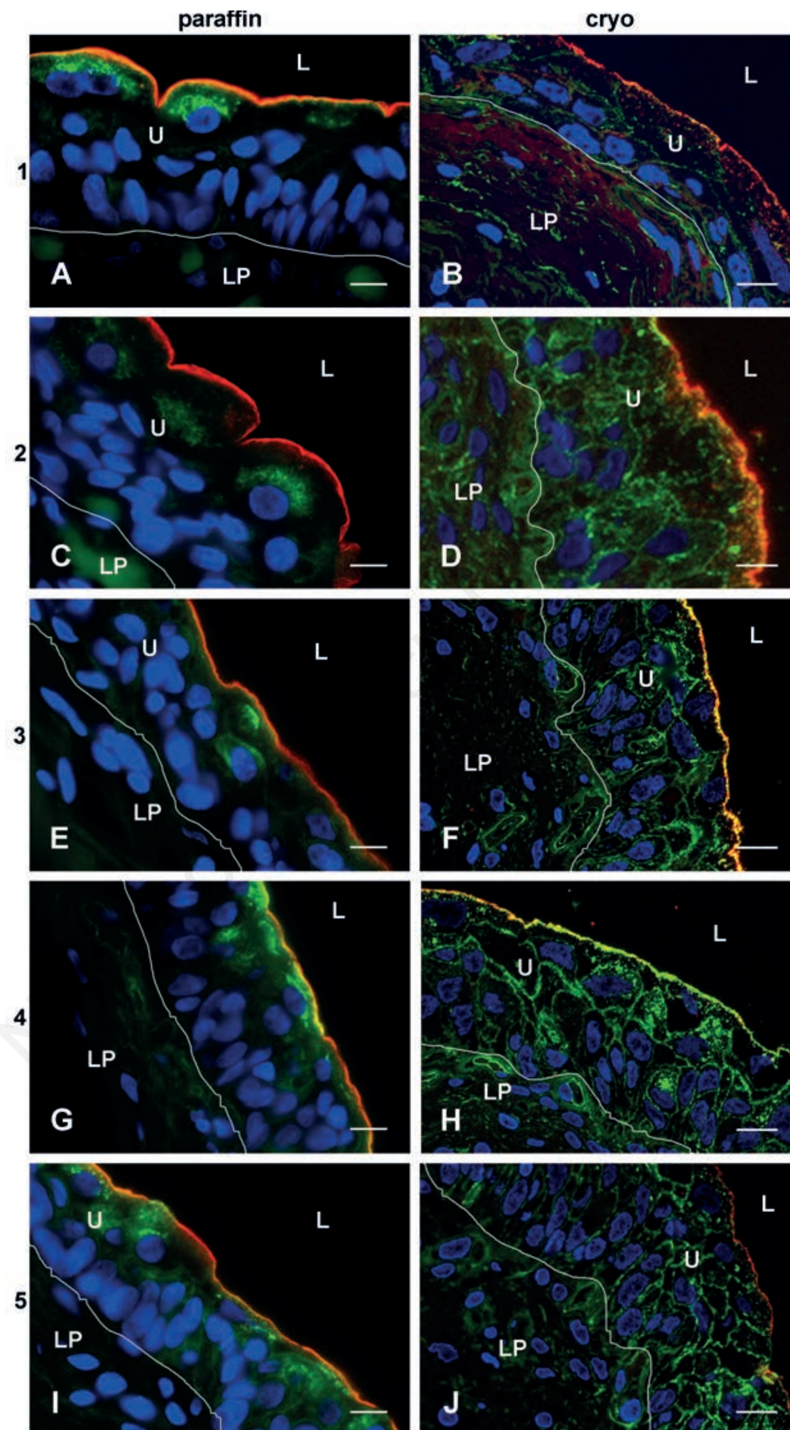


**Figure 2.** CLIH on paraffin (A,C,E,G,I) and cryo (B,D,F,H,J) sections of normal human urothelium with antibodies against UPs (red) and lectin ACA (green) following 5 protocols. Co-localisation between UPs and ACA labelling is present at the apical PM of umbrella cells. The cytoplasm of umbrella cells is labelled by ACA in paraffin and cryo-semithin sections, while the cytoplasm of intermediate and basal cells is labelled by ACA only in cryo-semithin sections. White line outline the basal lamina. L, lumen of the bladder; U, urothelium; LP, lamina propria. Scale bars: 10 µm.

cytoplasm of superficial cells and the cytoplasm of some intermediate cells, where uroplakin labelling was negative (Supplementary Figure 2).

To sum up, five different protocols of CLIH combining antibodies against UPs and lectins ACA, DSA or jacalin gave comparable results in normal urothelium. Since in the protocol 5, the primary antibodies were used first and the mixture of secondary antibodies and lectins was applied after washing, one or two incubations (with FA and separately with lectin and sAb) and washings were ommited (Table 1). Therefore, protocol 5 is the fastest one and should be considered in future research.

## Co-localization of ACA and jacalin with UPs in the apical PM of the normal human urothelium documented by CLIH

CLIH showed that lectins ACA and jacalin were co-localised with UPs in the apical PM of the umbrella cells, while there was no co-localisation between DSA and UPs (Figure 3). These results were in accordance with separate lectin histochemistry and immunohistochemistry (Figure 1) and demonstrated that co-localisation is observed in the apical PM and not in the cytoplasm of urothelial cells. The majority of apical PM of superficial cells was uroplakin positive and UPs co-localized with the ACA and jacalin labelling (Figure 2, Figure 3 D,L, Supplementary Figure 1 B,D,F,H,J). Interestingly, some portions of the apical PM were uroplakin negative, but still showed ACA and jacalin labelling (green apical PM in Figure 2H, Supplementary Figure 1 B,D,F). On the other hand, some portions were ACA and jacalin negative but showed positive uroplakin labelling (red apical PM in Figure 2 B,C,L,J, Supplementary Figure 1 F,H,J). These results indicated that lectins ACA and jacalin does not bind to UPs, but presumably to some other proteins of the apical PM. To check this notion, we performed lectin blotting with all three lectins and western blotting with antibodies against UPs. In the samples of normal urothelium, ACA and jacalin bounded predominantly to the glycoprotein of approximately 60 kDa, while molecular weights of UPs are 47 kDa (UPIIIa), 28 kDa (UPIb), 27 kDa (UPIa) and 15 kDa (UPII) (Supplementary Figure 2). On the other hand, DSA bounded predominantly to the glycoprotein of approximately 50 kDa, which is very close to the 47 kDa of the UPIIIa (Supplementary Figure 2), yet CLIH showed that there is no co-localisation between DSA and UPs (Figure 3H). We suggest that the results of CLIH, western blotting and lectin blotting should be compared when evaluating CLIH method.

The further analysis of co-localisation between UPs and lectins in the papillary carcinomas was focused on the apical PM, since biosynthesis of UPs and all other glycoproteins follows the same pathway from the endoplasmic reticulum to the Golgi apparatus and finally culminates in the apical PM.[29]

## ACA, jacalin and UPs are focally negative in most papillary carcinomas

All papillary urothelial carcinomas (PUNLMP, pTa, l.g. and pT1, h.g.) exhibited uroplakin positive and uroplakin negative regions of the apical PM (Figure 4 D-F). To quantify the expression of UPs, we performed western blotting. The expression of

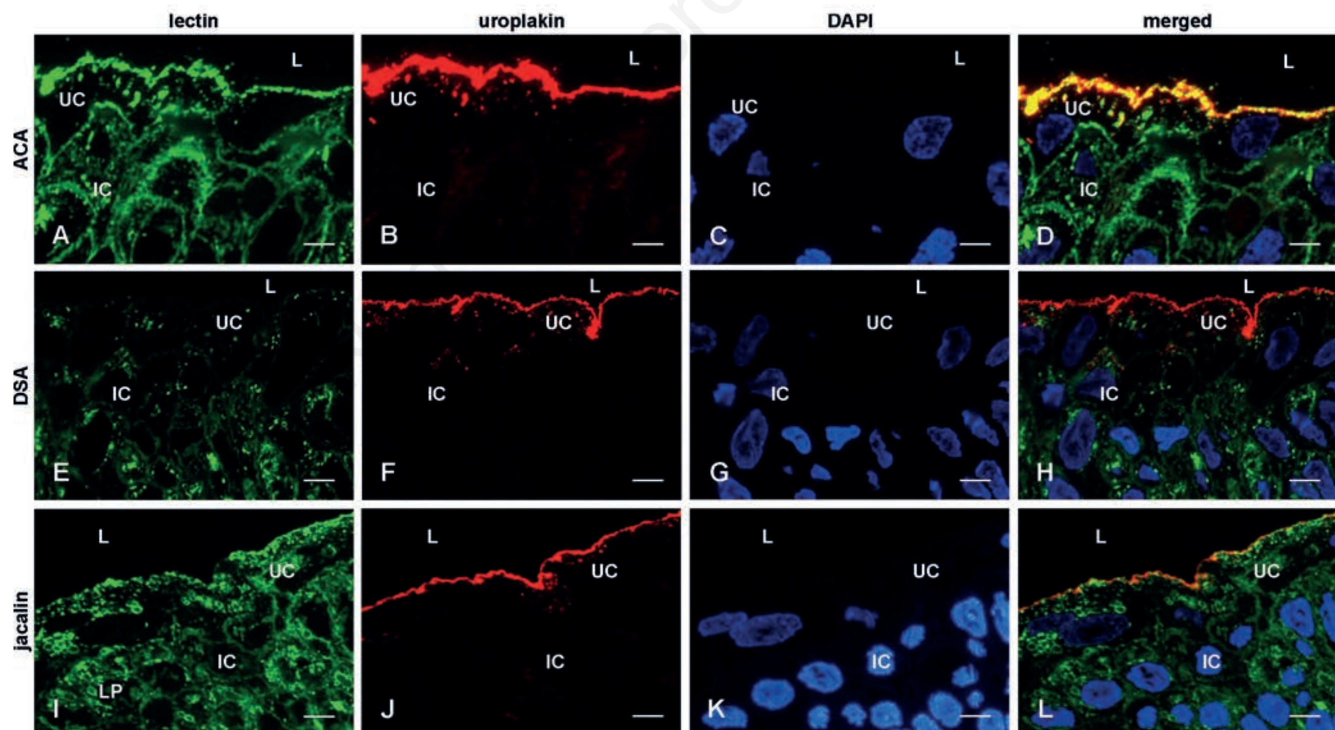


**Figure 3. CLIH following the protocol 5 on cryo-semithin sections of normal human urothelium with lectins ACA (green in A, D), DSA (green in E, H) and jacalin (green in I, L) and antibodies against UPs (red in B, D, F, H, J, L) (red). Co-localisation between UPs and ACA or jacalin labelling is present at the apical PM of umbrella cells (yellow in D and L), while there is no co-localisation between UPs and DSA. White line outline the basal lamina. L, lumen of the bladder; U, urothelium; LP, lamina propria. Scale bars: 10 µm.**

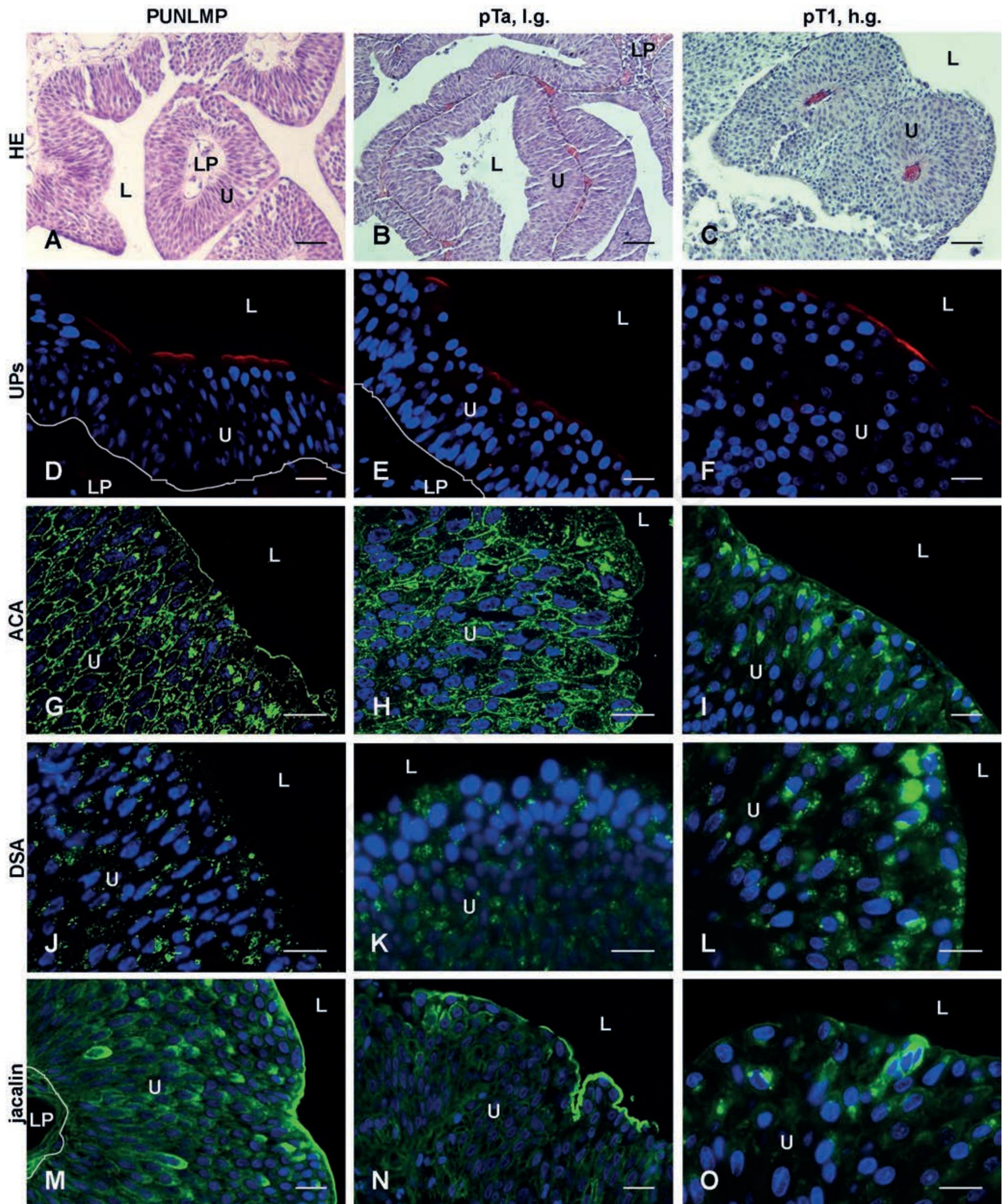


Figure 4. Urothelial papillary carcinomas (PUNLMP, pTa, l.g. and pT1, h.g.) stained with HE (A-C) and labelled with anti-UPs antibodies (D-F) and lectins ACA (G-I), DSA (J-L) and jacalin (M-O). UPs (red) are expressed in some regions of the apical PM (D-F). ACA and jacalin labelling (green) is focally negative, while DSA labelling (green) is entirely negative at the apical PM. All three lectins label some areas of the cytoplasm of superficial and intermediate urothelial cells. All the images are from paraffin sections except for G, H, J, which are from cryo-semithin sections. White line outline the basal lamina. L, lumen of the bladder; U, urothelium; LP, lamina propria. Scale bars: 20 µm.

UPs in all urothelial papillary carcinomas was positive (Supplementary Figure 3). In all samples, we detected the band at 47 kDa, which is the molecular weight of UPIIIa. In the sample of PUNLMP uroplakin expression was increased in comparison to normal urothelium, while in the samples of pTa, l.g. and pT1, h.g. it was decreased (Supplementary Figure 3).

Lectin histochemistry revealed that ACA and jacalin labelled some regions of the apical PM of the superficial cells and some regions of the cytoplasm of superficial and intermediate urothelial cells of all papillary carcinomas studied here (Figure 4 G-I,M-O). Similarly, as in normal urothelium, DSA did not labelled the apical PM of the papillary carcinomas, while some regions of the cytoplasm of superficial and intermediate urothelial cells were strongly labelled by DSA (Figure 4 J-L). When we compared the lectin histochemistry performed on paraffin and cryo-semithin sections, we observed that intracellular compartments labelled by lectins were visible in more details in cryo-semithin sections (Figure 4 G,H,J) than in paraffin sections (Figure 4 I,K-O). This was probably due to thickness of cryo-semithin sections (300 nm), which were more than 10 times thinner than paraffin sections (5000 nm). Our results show that lectin histochemistry on paraffin and cryo-semithin sections gives similar results with more detailed intracellular structures visible in cryo-semithin sections.

To analyse the correlation between uroplakin and lectin negative and positive regions of the apical PM, we performed CLIH on the paraffin sections of different papillary carcinomas.

## CLIH shows heterogeneity of superficial cells in papillary carcinomas

CLIH performed on all samples of papillary carcinomas (PUNLMP, pTa, l.g., pT1, h.g.) exhibited that similarly as in normal urothelium, lectin DSA did not labelled apical PM in papillary carcinomas and no co-localisation between UPs and DSA was observed (Figure 5). CLIH also revealed that in all samples of papillary carcinomas UPs and lectin ACA or UPs and lectin jacalin co-localized in some regions of the apical PM, while in other regions only UPs or only lectins were positive and even some regions of uroplakin and lectin negative labelling were observed (Figure 5). This results again suggest that ACA and jacalin did not bind to UPs and points toward great heterogeneity of the superficial cancer

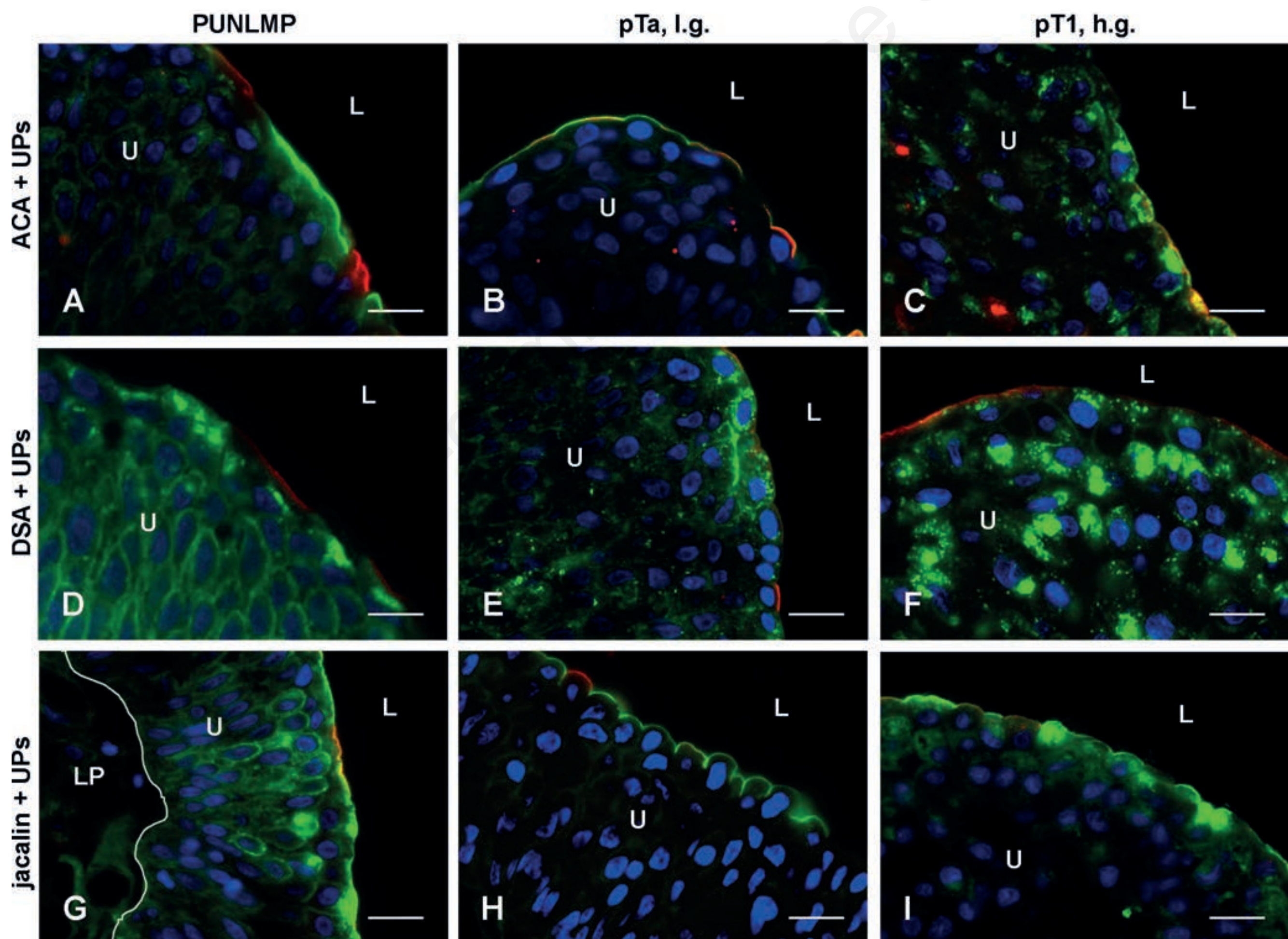


**Figure 5.** CLIH following the protocol 5 with lectins ACA (green in A-C), DSA (green in D-F) and jacalin (green in G-I) and antibodies against UPs (red) on the paraffin sections of PUNLMP, pTa, l.g. and pT1, h.g. UPs (red) are focally positive in the apical PM of all cancers. In some regions of the apical PM UPs co-localize with ACA and jacalin (yellow), while there is no co-localization with DSA. White line outline the basal lamina. L, lumen of the bladder; U, urothelium. Scale bars: 20 µm.

urothelial cells regardless of the type of carcinomas. To confirm this notion and to see whether the pattern of lectin binding to proteins separated by electrophoresis changes during bladder carcinogenesis, we performed lectin-blotting with the same lectins as lectin histochemistry and CLIH (Supplementary Figure 3). All three lectins bonded to sugar residues of proteins in all the samples (normal urothelium, PUNLMP, pTa and pT1), which is in accordance with the results of lectin histochemistry and CLIH. Additionally, lectin-blotting showed that all three lectins bonded to sugar residues of proteins with different molecular weights (Supplementary Figure 2). Some bands showed differences between normal and cancer urothelia, while others did not. The major differences between normal and papillary carcinomas were observed at approximate molecular weights as follows: for ACA at 80 kDa, 30 kDa and 17 kDa; for DSA at 245 kDa, 80 kDa and 17 kDa; and for JAC at 190 kDa, 30 kDa and 17 kDa (Supplementary Figure 3). In all papillary carcinomas glycoproteins with low molecular weight (approximately 17 kDa) were expressed, while in normal samples they were not expressed. On the other hand, the expression of some glycoproteins was diminished in carcinomas when compared to normal urothelium, especially in the samples of pTa, l.g.

To conclude, our results confirm that CLIH together with lectin blotting could be used to distinguish between normal urothelium and urothelial papillary carcinomas. Moreover, if different lectins and antibodies would be selected, CLIH could distinguish between different papillary carcinoma subtypes.

## Discussion

In our study, we tested five different protocols for the innovative method of CLIH for fluorescence microscopy. All the protocols gave comparable results and showed co-localisation between lectin and antibody binding. Since protocol number 5 (incubation in primary antibodies followed by incubation in mixture of secondary antibodies and lectins) is the fastest one, we recommend this protocol to be used in future studies. Moreover, we suggest that CLIH could be used as a new diagnostic tool for diseases where protein expression and glycosylation is altered during disease progression as well as for innovative basic cell biology research.

We performed LHC, IHC, and CLIH on paraffin and cryo-semithin sections of normal human urothelium and different urothelial papillary carcinomas. Paraffin sections are standard for histopathologic evaluations and paraffin blocks are stored for retrograde studies if needed. Therefore, paraffin sections are the best option for quick implementation of innovative methods into a routine diagnostic strategy. On the other hand, cryo-semithin sections offer better preservation of antigenic and carbohydrate residues as well as higher intracellular compartment visibility due to their more exact fixation, freezing and cutting, which is suitable also for transmission electron microscopy. Moreover, the thickness of cryo-semithin sections (300 nm) is approximately 17-times smaller than the thickness of paraffin sections (5000 nm) enabling more precise observation of co-localisation. This is due to the fact that in thinner cryo-semithin sections the fluorescence signal comes from one optical plane, which is similar to confocal microscopy.[30] In this study we demonstrated, by testing paraffin and cryo-semithin sections, that both types of sections are useful for CLIH and retrieve similar results. This is important, since the paraffin sections are widely used, cheaper and more permanent than cryo-semithin sections. Moreover, the majority of archived human samples are embedded in paraffin and could be used for CLIH in retrospective studies.

Our results showed that lectins ACA and jacalin bind to sugar residues in the apical PM of umbrella cells of normal urothelium, which is consistent with our previously published study.[19] Major sugar residues of the UPs are high mannose glycans, fucosylated complex glycans and N-linked complex glycans capped by sialic acid.[25] In normal urothelium specific glycoproteins like E-selectin, mucins 1, 2, 4, 6 and 7 and galectins are also expressed.[25] ACA and jacalin predominantly bind to galactosyl(β-1,3)*N*-acetilgalactosamine (Thomsen-Friedenreich antigen, Tn) and its sialylated form (sTn). Although Tn and sTn are thought to not be present in the normal urothelium,[31] in our study ACA and jacalin labelled apical PM of umbrella cells. Moreover, CLIH revealed that ACA and jacalin co-localise with UPs in the apical PM of umbrella cells. Labelling with ACA, DSA and jacalin was also observed in the cytoplasm of intermediate and basal cells in normal urothelium, but here no co-localisation with UPs was observed. We have previously shown changes of UPs in human bladder cancers by DAB immunohistochemistry.[32] Here we used immunofluorescence to confirm previous observations and because the immunofluorescence is more suitable for combination with second marker, *e.g.* lectin labelled with fluorochrome. In the samples of urothelial papillary carcinomas (PUNLMP, pTa, l.g. and pT1, h.g.) ACA and jacalin labelled some regions of the apical PM of superficial cells. The co-localisation with UPs was or was not present. These results indicate that ACA and jacalin do not bind to sugar residues of UPs and that superficial cancer urothelial cells are very heterogenic. Lectins ACA and jacalin bind to same sugar residue galactosyl (β-1,3)N-acetylgalactosamine and its sialated form, while DSA binds to galactosyl (β-1,4)N-acetylglucosamine oligomers. These bindings are thought to be preferential, yet lectin blotting revealed slightly different binding patterns of ACA and jacalin, especially to the proteins above 30 kDa (Supplementary Figure 3). Therefore, we assume that their affinities to sugars are not entirely specific, which was also supported by the results of negative controls performed with inhibitory sugars and reported elsewhere.[33-35] Moreover, DSA lectin blotting revealed similar band as ACA at approximately 80 kDa. Similarly, as CLIH, lectin blotting also showed differences between normal urothelium and papillary carcinomas. For example, lectins ACA and jacalin labelled band at the approximate molecular weight of 30 kDa in all samples of urothelial carcinomas, but not in normal urothelium. On the other hand, lectins ACA and DSA, which bind to different sugar residues, both labelled band of approximately 80 kDa in samples of normal urothelium, but not in the samples of urothelial carcinomas. Only in normal urothelium jacalin labelled bands at the approximate molecular weight of 190 kDa. There were also some alterations between different urothelial carcinomas. Interestingly, the sample of pTa, l.g. exhibited the lowest number of bands (Supplementary Figure 3). However, the band at approximately 17 kDa was present in blots of all three lectins in the samples of all carcinomas, but not in the sample of normal urothelium. Additionally, DSA blotting showed bands at around 47 kDa with very similar intensities as bands of UPIIIa (47 kDa), meaning strong bands in normal and PUNLMP samples and weaker bands in pTa, l.g. and pT1, h.g. samples. Yet CLIH showed no co-localization between DSA and UPs in normal and in papillary carcinoma samples. We, therefore, assume that DSA blotting showed bands of some other protein with similar molecular weight and similar expression pattern during urothelial carcinogenesis as UPIIIa. Our results show that blotting with different lectins can distinguish between normal urothelium and various urothelial carcinomas and therefore complement the results of lectin histochemistry and CLIH.

We conclude that CLIH method provides information about the correlation between protein expression (detected by IHC) and lectin labelling (detected by LHC) in the same tissue section. This

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is especially important for basic cell biology reaseach and when this information could improve diagnosis of a certain disease, especially cancers. For example, urothelial papillary carcinomas are accompanied by protein expression alterations and changes in carbohydrate composition, yet none of these facts alone do not benefit urologists and patients. We therefore analysed the results of CLIH to detect differences between carcinoma subtypes. To evaluate the diagnostic value of CLIH and its contribution to improved treatment and management of the urinary bladder cancer long-lasting studies including a sufficiently large number of patients with phenotypically different carcinomas are needed. Moreover, testing of various lectins and different protein markers should be performed in order to find the right combination.


## Acknowledgements

*We are grateful to Urša Kapš and Polona Glažar for their technical assistance. The authors acknowledge financial support from the Slovene Research Agency (research core funding No. P3-0108 and Infrastructure program MRIC UL IP-0510).*


## References


1. Svatek RS, Hollenbeck BK, Holmang S, Lee R, Kim SP, Stenzl A, et al. The economics of bladder cancer: costs and considerations of caring for this disease. Eur Urol 2014;66:m 253-62.
2. Sievert KD, Amend B, Nagele U, Schilling D, Bedke J, Horstmann M, et al. Economic aspects of bladder cancer: what are the benefits and costs? World J Urol 2009;27:295-300.
3. Schned AR, Andrew AS, Marsit CJ, Kelsey KT, Zens MS, Karagas MR. Histological classification and stage of newly diagnosed bladder cancer in a population-based study from the Northeastern United States. Scand J Urol Nephrol 2008;42: 237-42.
4. Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BW, Comperat E, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. Eur Urol 2013;64:639-53.
5. Schenk-Braat EA, Bangma CH. Immunotherapy for superficial bladder cancer. Cancer Immunol Immunother 2005;54: 414-23.
6. Alsheikh A, Mohamedali Z, Jones E, Masterson J, Gilks CB. Comparison of the WHO/ISUP classification and cytokeratin 20 expression in predicting the behavior of low-grade papillary urothelial tumors. World/Health Organization/Internattional Society of Urologic Pathology. Mod Pathol 2001;14:267-72.
7. Edwards J, Duncan P, Going JJ, Watters AD, Grigor KM, Bartlett JM. Identification of loci associated with putative recurrence genes in transitional cell carcinoma of the urinary bladder. J Pathol 2002;196:380-5.
8. Ramos D, Navarro S, Villamon R, Gil-Salom M, Llombart-Bosch A. Cytokeratin expression patterns in low-grade papillary urothelial neoplasms of the urinary bladder. Cancer 2003;97:1876-83.
9. Lopez-Knowles E, Hernandez S, Malats N, Kogevinas M, Lloreta J, Carrato A, et al. PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors. Cancer Res 2006;66:7401-4.
10. Barbisan F, Santinelli A, Mazzucchelli R, Lopez-Beltran A, Cheng L, Scarpelli M, et al. Strong immunohistochemical expression of fibroblast growth factor receptor 3, superficial staining pattern of cytokeratin 20, and low proliferative activity define those papillary urothelial neoplasms of low malignant potential that do not recur. Cancer 2008;112:636-44.
11. Nomura S, Suzuki Y, Takahashi R, Terasaki M, Kimata R, Hamasaki T, et al. Snail expression and outcome in T1 high-grade and T2 bladder cancer: a retrospective immunohistochemical analysis. BMC Urol 2013;13:73.
12. Witjes JA, Compérat E, Cowan NC, De Santis M, Gakis G, Lebret T, et al. EAU guidelines on muscle-invasive and metastatic bladder cancer: summary of the 2013 guidelines. Eur Urol 2014;65:778-92.
13. Koss LG. The asymmetric unit membranes of the epithelium of the urinary bladder of the rat. An electron microscopic study of a mechanism of epithelial maturation and function. Lab Invest. 1969;21:154-68.
14. Wu XR, Manabe M, Yu J, Sun TT. Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation. J Biol Chem 1990;265:19170-9.
15. Sun TT, Zhao H, Provet J, Aebi U, Wu XR. Formation of asymmetric unit membrane during urothelial differentiation. Mol Biol Rep 1996;23:3-11.
16. Yu J, Lin JH, Wu XR, Sun TT. Uroplakins Ia and Ib, two major differentiation products of bladder epithelium, belong to a family of four transmembrane domain (4TM) proteins. J Cell Biol 1994;125:171-82.
17. Lin JH, Wu XR, Kreibich G, Sun TT. Precursor sequence, processing, and urothelium-specific expression of a major 15-kDa protein subunit of asymmetric unit membrane. J Biol Chem 1994;269:1775-84.
18. Wu XR, Lin JH, Walz T, Haner M, Yu J, Aebi U, et al. Mammalian uroplakins. A group of highly conserved urothelial differentiation-related membrane proteins. J Biol Chem 1994;269:13716-24.
19. Zupancic D, Kreft ME, Romih R. Selective binding of lectins to normal and neoplastic urothelium in rat and mouse bladder carcinogenesis models. Protoplasma 2014;251:49-59.
20. Huang HY, Shariat SF, Sun TT, Lepor H, Shapiro E, Hsieh JT, et al. Persistent uroplakin expression in advanced urothelial carcinomas: implications in urothelial tumor progression and clinical outcome. Hum Pathol 2007;38:1703-13.
21. Ogawa K, St John M, Luiza de Oliveira M, Arnold L, Shirai T, Sun T, et al. Comparison of uroplakin expression during urothelial carcinogenesis induced by N-butyl-N-(4-hydroxybutyl)nitrosamine in rats and mice. Toxicol Pathol 1999;27: 645-51.
22. Neal DE, Charlton RG, Bennett MK. Histochemical study of lectin binding in neoplastic and non-neoplastic urothelium. Br J Urol.1987;60:399-404.
23. Langkilde NC, Wolf H, Orntoft TF. Lectinohistochemistry of human bladder cancer: loss of lectin binding structures in invasive carcinomas. Apmis 1989;97:367-73.
24. Okamura T, Ueda K, Ohtaguro K, Inoue K, Washida H, Mori M, et al. Lectin immunohistochemical evaluation of human bladder carcinomas. A comparison of Carnoy's and formalin fixation. Hinyokika Kiyo 1993;39:899-905.
25. Visnjar T, Romih R, Zupancic D. Lectins as possible tools for improved urinary bladder cancer management. Glycobiology 2019;29:355-65.
26. Zupancic D, Zakrajsek M, Zhou G, Romih R. Expression and localization of four uroplakins in urothelial preneoplastic lesions. Histochem Cell Biol 2011;136:491-500.
27. Sterle I, Zupancic D, Romih R. Correlation between urothelial differentiation and sensory proteins P2X3, P2X5, TRPV1, and TRPV4 in normal urothelium and papillary carcinoma of


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human bladder. Biomed Res Int 2014;2014:805236.
28. Wilson DB, Wyatt DP. Patterns of lectin binding during mammalian neurogenesis. J Anat 1995;186:209-16.
29. Višnjar T, Chesi G, Iacobacci S, Polishchuk E, Resnik N, Robenek H, et al. Uroplakin traffic through the Golgi apparatus induces its fragmentation: new insights from novel in vitro models. Sci Rep 2017;7:12842.
30. Takizawa T, Powell RD, Hainfeld JF, Robinson JM. FluoroNanogold: an important probe for correlative microscopy. J Chem Biol 2015;8:129-42.
31. Ferreira JA, Videira PA, Lima L, Pereira S, Silva M, Carrascal M, et al. Overexpression of tumour-associated carbohydrate antigen sialyl-Tn in advanced bladder tumours. Mol Oncol 2013;7:719-31.
32. Zupancic D. Heterogeneity of uroplakin localization in human normal urothelium, papilloma and papillary carcinoma. Radiol Oncol 2013;47:338-45.
33. Wu AM, Wu JH, Yang Z, Singh T, Goldstein IJ, Sharon N. Differential contributions of recognition factors of two plant lectins -Amaranthus caudatus lectin and Arachis hypogea agglutinin, reacting with Thomsen-Friedenreich disaccharide (Galbeta1-3GalNAcalpha1-Ser/Thr). Biochimie 2008;90: 1769-80.
34. Zschäbitz A, Krahn V, Gabius HJ, Weiser H, Khaw A, Biesalski HK, et al. Glycoconjugate expression of chondrocytes and perichondrium during hyaline cartilage development in the rat. J Anat 1995;187:67-83.
35. Cohen M, Varki NM, Jankowski MD, Gagneux P. Using unfixed, frozen tissues to study natural mucin distribution. J Vis Exp 2012:e3928.

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Received for publication: 30 April 2020. Accepted for publication: 10 June 2020.
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*European Journal of Histochemistry 2020; 64:3141*
*doi:10.4081/ejh.2020.3141*