New diagnostic and prognostic markers of human urinary bladder tumours

Doctoral (Ph.D.) Theses

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Introduction

The incidence of tumourous cases, including bladder cancer, in Hungary is increasing year by year. Unfortunately, beside the visible tumours of the body surface and the well screenable breast cancer, early detection of cancer is not yet solved. There is no diagnostic tool at hand which could screen neoplasms early, fast, cheap and without pain. This is especially true in case of urinary tract tumours.

Yearly 2000 new bladder cancer cases are diagnosed in Hungary. The most common symptom raising the possibility of bladder neoplasms is painless haematuria. Smoking and contact with aromatic amines are the main predisposing factors.

Diagnosis and prognosis, further the planning of therapy of bladder cancer are mainly based on the histopathological analysis of transurethral resected specimens. Therapy is specified not only by histological classification, but by invasiveness of the tumour: muscle-invasive ones (30% of all cases) have a much worse prognosis and thereby need more radical therapy than the superficial, non muscle-invasive bladder cancers (70% of all cases). However, despite the fact that bladder cancer occurs as a superficial tumour, the majority (50-70%) of these entities will recur, independent of therapy, and 10-20% will progrediate into muscle-invasive stage. Another challenge is that some of the both macroscopically and microscopically uniform looking, either low or high risk tumours will recur or progress, while others will not. There is not a single marker at hand that could predict the potential biological behaviour of these tumours. Different studies reported many molecular changes in the carcinogenesis of urothelial cancer, however, the transformation of normal urothelia into cancerous tissue is not yet well characterized.

A helpful, non-invasive, non stressful aid for transurethral resection (TUR) is urine cytology, which is used since 1945. The procedure is of high sensitivity in case of poorly differentiated tumours, but has low sensitivity in well differentiated cases, which phenomenon restricts its usefulness. Furthermore, a negative cytology result does not exclude the possibility of a tumour. The specificity and sensitivity of urine cytology can be enhanced with FISH (fluorescence *in situ* hybridisation) technique. Currently, the UroVysion Bladder Cancer Recurrence Kit is available, also used by the 2nd Department

of Pathology of the Semmelweis University, which kit is approved as a diagnostic tool by the FDA (Food and Drug Administration). The kit comprises a mix of probes for the detection of 9p21 locus as well as the peri-centromeric region of chromosomes 3, 7 and 17. The specificity and sensitivity are appropriate for the expectations of diagnostics, however, its high cost and specific professional background needs restrict the spreading of this technique in routine diagnostics.

Within bladder cancers, according to the new WHO classification (2004), it is important to differentiate urothelial papillomas (UP), papillary urothelial neoplasms of low malignant potential (PUNLMP-k), low grade (LG) and high grade (HG, #208) tumours. Each tumour group has a different recurrence-free survival and progression rate, therefore these special cases need personalised, more aggressive treatment and better follow-up strategies. However, the descriptions in new histology classifications do not always give clear aid in differential diagnosis: almost 30% of tumours are unduly classified as HG, furthermore there are significant intra- and interobserver variabilities in the pathological findings. These data support the need for new markers that might assist the proper histologic/prognostic classification of bladder cancer, therefore administrating the choice of proper therapy, increasing life quality, and decreasing the expenses of treatment.

Several prognostic factors, including cell cycle regulators (p53, pRb), proliferation markers (Ki-67, survivin), oncogenes (EGFR, FGFR, VEGF) were investigated in recent years, however, there is no single marker which could overcome the prognostic and differential diagnostic value of common histopathological analysis.

Cell-to-cell connection structures have a very important role in urinary bladder. The physiological function of the urinary bladder, besides storage and transmission of urine, is to prevent the transportation of molecules from the urine to the space between the tissues or to the circulation. Claudins, the major proteins of tight junctions, are essential for the separation of extracellular compartments, especially in the uroepithelium, where separation of urine is essential to prevent tissue damage.

The prognostic and/or differencital diagnostic value of claudins is well known in many human tumours. There are only limited data available on claudin expression in the normal and tumourous urothelium, whereas its prognostic and differential diagnostic

value is not yet well characterized. In the upper urinary tract urothelium claudin-1 was detected in the basal/peribasal region, whereas claudins-3, -4 and -7 were observable in superficial localization. The expression of claudin-3 was strongly associated with growth pattern, stage and differentiation of upper urinary tract tumours, while claudins-1 and -4 were associated with stage. Boireau et al found no relevant difference in case of claudin-1 and -7 expressions in urinary baldder cancer in comparison to the tumour surrounding urothelium, whereas the change of claudin-4 expression proved to be significant in 26 of 39 cases. Claudin-4 expression of well differentiated carcinomas was found increased in comparison to the surrounding tumour free urothelium, while poorly differentiated tumours revealed decreased exppression in the same comparison. Decreased claudin-4 protein expression correlated well with one year survival. Since this study investigated claudin expression of urothelial carcinomas in comparison to surrounding urothelium this setup did not allow to make direct comparison between well and poorly differentitated UCCs.

II. Aims

According to the data summarized in the introduction, the following aims were developed:

- Our aim was to investigate the mRNA and protein expression of claudins-1, -2, -3, -4, -5, -7, -10 and Ki-67 protein expression in independent non tumourous samples, as well as in different stages and grades (low grade and high grade) of UCCs.

- Our aim was to characterize the claudin-1, -2, -3, -4, -5, -7, CK-5/6, CK-20, Ki-67 expression profiles and the localization of these proteins in UP, IUP, PUNLMP and non-invasive LG-UCC cases as well as in independent normal urothelium.

- Moreover, besides studying the potential differential diagnostic value of claudins, CKs and Ki-67, we investigated whether differences in claudin, CK, and Ki-67 expression bear with prognostic value regarding overall and recurrence-free survival.

- Our aim was to investigate the specificity and sensitivity of the FISH based *in situ* hybridisation technique in detecting UCCs in urine samples.

Materials and methods

Analysis of low grade, high grade and non-invasive bladder tumours:

Materials:

- 103 human, surgically resected, formalin-fixed, paraffin-embedded tissue blocks (86 UCCs /27 LG, 59 HG/, 17 non-tumourous urothelia) were studied
- 80 transurethral resection specimens were analysed: 15 IUPs, 20 UPs, 20 PUNLMPs, 20 LG-UCCs and 5 independent normal samples

Methods:

- Ventana ES (claudin-1, -2, -3, -4, -5, -7, -10, Ki-67) and Benchmark XT (tissue microarrays for claudin-1, -2, -3, -4, -5, -7, CK-5/6, CK-20 and Ki-67) automatic immunostainers were used for the analysis of protein expression (Ventana Medical System Inc., Tucson, AZ, USA)
- Fluorescence immunohistochemistry was administered for detection of co-localization and expression of claudin-4 and claudin-7
- Evaluation of immunohistochemistry was done with semi-quantitative (score x intensity) and quantitative (digital morphometry) methods
- Total RNA isolation followed by reverse transcription, then qRT-PCR reaction was carried out for the analysis of claudin-1, -2, -3, -4, -5, -7 and -10 mRNA expressions (reference gene: β-actin)

Detection of UCC from urine using FISH technique:

- Urine and histologic samples from 55 cases (43 histologically verified UCCs, 6 inflammations, 2 hyperplasias, 2 papillomas, 2 specific histological alterations of any type) were analysed
- Urine preparations (Vysis FISH Pretreatment Reagent Kit), FISH reaction (UroVysion Bladder Cancer Recurrence Kit (Vysis, Inc.) were carried out. Evaulation was done according to the published protocols.

Results

Immunohistochemistry and qRT-PCR analysis in non-tumourous epithelium: The epithelial expression pattern and intraepithelial distribution of individual claudins in the investigated cases were similar to previously published data of normal urothelium. Claudin-1 membrane positivity was mainly found in the basal layers. Perimembranous-cytoplasmic granular CLDN-2 expression was detected in the basal and parabasal epithelium. Claudin-4 and -7 membrane positivity was detected in the upper layers, declining towards basal layers of the epithelium, however, in some cases, claudin-7 membrane positivity was found in the whole thickness of the epithelium. CLDNs-3 and -5 were weakly detected in the upper layers in a few cases, mainly at the plasma membrane of umbrella cells. CLDN-10 was not detected. Scattered nuclear Ki-67 positivity was observed in both non-tumourous groups. Inflamed control samples revealed significantly higher claudin-2 and -4 protein levels and claudin-7 mRNA expression in comparison to non-inflamed control samples.

Immunohistochemistry and real time qRT-PCR analysis in tumours: Claudin-1, -2, -4 and -7 proteins were detectable in most cases. CLDNs-3 and -5 were only found in few, mostly well differentiated UCCs. CLDN-10 was not detected. The vertical distribution of CLDN-1, -2, -4 and -7 proteins within bladder tumours was similar to the control epithelium, while CLDN-4 positivity was mainly detected in the entire width of UCCs. In certain IUP samples, it was rather difficult to assess the basal or superficial orientation in H&E sections. It is noteworthy, therefore, that the standard intraepithelial differences in claudin distribution aided the orientation of the small sized tissue fragments.

Tumourous samples showed decreased claudin-1 and increased claudin-2, -4 and -7 and Ki-67 protein expressions while claudin-1, -2, -4, -7 mRNA levels were found increased in comparison to non-inflamed control samples.

HG tumours revealed significantly higher protein expression of claudin-4 and Ki-67 in parallel with significantly lower protein expression of claudin-7 in comparison to LG UCCs. Decreased claudin-1 and elevated claudin-2 and -4 mRNA levels were detected in HG in comparison to LG tumours. CLDN-4 protein expression characterized HG cases in

the entire width of the tumours contrary to LG cases showing vertical distribution similar to the normal pattern.

Claudin-4 and claudin-7 were mainly co-localized by immunofluorescence immunohistochemistry in both low and high grade UCC. Immunfluorescence also showed higher expression of claudin-4 and lower expression of claudin-7 in high grade UCC in comparison to low grade UCC.

In some cases of PUNLMPs and LG-UCCs only claudin-1 and claudin-4 were observed in the whole extent of the epithelium. On the other hand, however, in 73% of IUP cases (11/15) claudin-1 positivity appeared in the whole depth of the tumour tissue. Claudin-1 and -2 expressions were lower in LG-UCCs in comparison to UPs, IUPs and PUNLMPs. However, only IUPs revealed significantly higher claudin-1 expression when compared with UPs, PUNLMPs and LG-UCCs upon both morphometrical and scoring analyses. Claudin-3, -4 and -7 expressions did not show major differences between analyzed groups. None of the cases showed marked claudin-3 expression.

CK-5/6 expression was detected in the basal, while CK-20 expression in the superficial layers of the investigated entities in concordance with the literature. Dysregulated CK-20 expression was generally manifested in the increased number of positive cells situtated not only in the superficial, but also in the intermediate layers of LG-UCC. PUNLMPs were characterised by significantly lower CK-20 expression in comparison to normal urothelium and LG-UCCs. Otherwise, there were no measured differences between the groups.

LG-UCCs revealed significantly higher Ki-67 expression when compared with normal, UP and IUP cases.

Follow-up analysis: *Low grade and High grade UCCs:* The mean follow-up period was 45.96 (2-10) months. None of the LG patients (0/27), while 19 of the HG bladder cancer patients (19/59) died of UCC. Seventeen of these HG patients had T2 stage disease, while two patients had T1 disease.

Overall 64 Ta/T1 UCCs proved to be suitable for follow-up analysis (27 LG, 37 HG). Recurrence appeared in 13 (48 %) LG and 10 (17 %) HG cases. The mean recurrence-free survival (RFS) was 38 months in LG, whereas 47 months in HG cases.

Non-muscle invasive UCCs (Ta-T1) expressing CLDN-7 over the median revealed shorter RFS in comparison to UCCs expressing CLDN-7 under the median. Three patients from the HG Ta-T1 group (two died of UCC), while none from the LG Ta-T1 group progressed into muscle-invasive stage. HG tumours (including T2 UCCs) were associated with significantly shorter overall survival (OS) when compared to LG ones. In concordance with international data, T2 tumours (all of them were HG) of our study were associated with shorter OS in comparison to Ta/T1 tumours. Similarly, T2 patients treated with cystectomy had longer OS compared with those T2 patients not treated with radical surgical resection (14 versus 48 months).

Analysis of non invasive urinary bladder tumours: The mean follow-up period was 59.79 (3-126) months. One patient died of non-urological disease. All samples, except 2 PUNLMPs, were primary tumours. A total of 20 LG-UCC cases and 20 PUNLMPs, 18 UPs and 15 IUPs were suitable for follow-up analysis. Recurrence appeared in 16 cases (10/20 LG-UCCs; 6/20 PUNLMPs), the mean RFS was 21.43 (4-60) months in these cases. IUPs and UPs did not recur.

PUNLMPs showing decreased claudin-1 expression (under the median) revealed significantly shorter RFS in comparison to PUNLMPs highly expressing claudin-1 (over the median). Cases of LG-UCCs highly expressing CLDN-4 (over the median) showed significantly shorter RFS as compared with other non-invasive UCCs expressing CLDN-4 under the median. RFS showed no significant association with expressions of claudin-2 and -7, CK-5/6, CK-20 or with Ki-67.

Detection of UCC from urine using FISH technique: The cancer cell-specific chromosomal alterations detected in the urine samples by *in situ* fluorescent hybridization technique were compared with the histological findings of the transurethral resection specimens. Positivity criteria of UroVysion test were met in 34 cases; histologically verified bladder cancer in all 34 patients. Negative results were obtained in 16 cases from which 5 proved to be carcinomas of superficial stage Ta, based on the later histological findings. Altogether, based on the histological findings the specificity of the FISH test was rated as 100% and the sensitivity as 87%.

Of the 12 cases with duplicated FISH test (FISH was not only performed from the morning's second urination according to standard procedure, but from the first as well), in 3 of the 9 urothelial carcinomas both results were positive, in four cases one test was positive while the other could not be evaluated, in one case one test was positive and the other negative, whereas in one case one of the results was negative while the other could not be evaluated. Accordingly, 8 cases proved to be positive and 1 negative. All three cases without malignancy turned out to be negative with duplicated FISH test. There was no significant difference in the adequacy of the morning's first and second urination samples; the unevaluable FISH tests were evenly distributed among the two groups.

Of the 34 FISH-positive transitional carcinoma cases 2 stage T1 and 2 stage T2 cases displayed the complete loss of 9p21 to be the main genetic alteration (12%). In 30 cases (88%), including the 3 FISH positive cases of tumours with stage Ta, the gain of chromosomes 3, 7 and 17 was manifest. On the contrary, in 8 cases not only 3/7/17 multiplication was observed within one and the same cell, but complete 9p21 deletion was detected as well. Altogether 11 cases (32%) showed complete 9p21 loss.

The frequency of the cells showing cytogenetic abnormalities defined by the UroVysion test was significantly higher in T1 and T2 bladder carcinoma cases as compared with the superficial, noninvasive Ta stage, while no significant statistical relationship was found comparing the T1 and T2 stages. G3 tumours revealed significantly higher rate of cells showing genetical aberration in comparison to G1 tumours.

Conclusions

1. We proved that claudin and Ki-67 immunohistochemistry provides a useful aid in the differential diagnosis of different stages and grades of urinary bladder neoplasms, furthermore it might add additional prognostic information regarding recurrence-free survival. The used two evaluation methods (semi-quantitative and quantitative) confirmed each other, which might facilitate its application in routine work.

2. We demonstrated that HG tumours show significantly elevated claudin-4 and Ki-67, whereas significantly decreased claudin-7 protein expressions when compared with LG cases. Regarding claudin-4 mRNA expression, the similar change refers to regulation at mRNA level.

3. The elevated claudin-1 protein expression helps to differentiate inverted papillomas from urothelial papillomas, PUNLMPs and LG-UCCs. Furthermore, the standard intraepithelial localization of claudins might adjust the orientation of tissue specimens in doubtful cases.

4. Low claudin-1 expression in PUNLMPs, high claudin-4 expression in LG-UCC, whereas claudin-7 expression in Ta-T1 UCCs might provide a useful aid in selecting those patients with worse prognosis, who need closer follow-up.

5. The UroVysion technique produced excellent results regarding sensitivity and specificity. However, since the method is rather costly and time-consuming, it is unlikely to become widely used in routine diagnostic protocol. At present there is no other method that can be used instead of cystoscopy, but UroVysion could be an additional diagnostic tool providing help in doubtful cases.

5. New statements

- Claudin-4 and Ki-67 expressions were significantly higher, whereas claudin-7 expression was significantly lower in high grade UCCs in comparison to low grade cases.

- High expression of claudin-7 in Ta-T1 UCCs is associated with shorter recurrence-free survival.

- The significantly elevated expression of claudin-1 in inverted papillomas might help to differentiate these tumours from papillomas, PUNLMPs and non-invasive LG-UCCs.

- Low expression of claudin-1 is associated with shorter recurrence-free survival in PUNLMPs.

- High expression of claudin-4 is associated with shorter recurrence-free survival in LG-UCCs.

- The UroVysion test proved to be a reliable diagnostic tool with high sensitivity and specificity. Although for the time being UroVysion can not replace cystoscopy, it can be a helpful aid in the differential diagnosis of doubtful cases.

List of publications

In the topic of the doctoral thesis:

Törzsök P, Riesz P, Kenessey I, Székely E, Somorácz A, Nyirády P, Romics I, Schaff Z, Lotz G, Kiss A. Claudins and ki-67: potential markers to differentiate low- and high-grade transitional cell carcinomas of the urinary bladder. *JOURNAL OF HISTOCHEMISTRY & CYTOCHEMISTRY* 59:(11) pp. 1022-1030. (2011) **IF: 2.381**

Székely E, **Törzsök P**, Riesz P, Korompay A, Fintha A, Székely T, Lotz G, Nyírády P, Romics I, Tímár J, Schaff Zs, Kiss A. Expression of claudins and their prognostic significance in non-invasive urothelial neoplasms of the human urinary bladder. *JOURNAL OF HISTOCHEMISTRY & CYTOCHEMISTRY* 59: pp. 932-941. (2011) **IF: 2.381**

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Riesz P, Székely E, **Törzsök P**, Majoros A, Szendrői A, Dombóvári P, Romics I
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Zádori G, Gelley F, **Törzsök P**, Sárváry E, Doros A, Deák AP, Nagy P, Schaff Zs, Kiss A, Nemes B. Examination of claudin-1 expression in patients undergoing liver transplantation owing to hepatitis C virus cirrhosis. *TRANSPLANTATION PROCEEDINGS* 43: pp. 1267-1271. (2011) **IF: 0.993**

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