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ORIGINAL ARTICLE

Down-regulated claudin-7 immunoexpression in urothelial carcinoma of the urinary bladder

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KEYWORDS

Bladder cancer;
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Real-time PCR;
Tight junction proteins;
Urothelial carcinoma

ABBREVIATIONS

UC, urothelial carcinoma;
NU, normal urothelium;

Abstract ***Objectives:*** To analyse the gene-expression level of claudin-7 in urothelial carcinoma (UC) of the urinary bladder, and its relationship with clinicopathological variables.

Materials and methods: This study included 68 specimens of UC of the bladder, comprising 35 with non-muscle-invasive (NMI), stage Ta–T1, and 33 with muscle-invasive (MI) tumours, T2–T4, and 26 of normal urothelium (NU). Total RNA was extracted and 1 μg was reverse transcribed using a cDNA kit. RT-PCR was conducted using SYBR Green I dye to examine the expression levels of the target gene (claudin-7) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Using confocal-laser scanning light microscopy, immunohistochemistry (IHC) was used to validate the RT-PCR data. The correlation between claudin-7 and the clinicopathological variables was assessed.

Results: Claudin-7 was down-regulated in UC samples compared to NU samples ($P < 0.001$). NMI (Ta–T1) tumours had significantly higher claudin-7 expression

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(N)MI, (non-)muscle-invasive;
GAPDH, glyceraldehyde-3-phosphate dehydrogenase

than MI (⩾pT2) tumours ($P = 0.012$). There was no significant difference between patients with G1-2 tumours and those with G3 tumours ($P = 0.19$). There was no significant difference between patients with recurrent NMI UC and those with no recurrence ($P = 0.61$). IHC showed a lower expression of claudin-7 in the UC samples than NU samples, and in MI UC than in NMI UC.

Conclusions: These results indicate that a reduced expression of claudin-7 correlates with the invasiveness and progression of UC of the urinary bladder. Further studies are needed to validate claudin-7 as a marker for UC.

Introduction

The incidence of urothelial carcinoma (UC) is increasing, and unfortunately the methods used to detect UC, either initially or routinely over the follow-up, have remained unchanged for the last 60 years, and always involve invasive procedures such as cystoscopy and biopsy. For the initial diagnosis of tumour and for the follow-up of patients with UC several potential diagnostic and prognostic markers have been assessed, but none is in widespread use by clinicians, largely because the markers have limited sensitivity or specificity [1].

The claudins are a group of 24 distinct transmembrane proteins composed of four transmembrane domains and two extracellular loops, that latter being involved in the homophilic and heterophilic interactions with other adjacent claudins [2]. Claudins have individual patterns of expression that are specific to the tissue. Most cells express several claudin isoforms that interact homotypically and heterotypically to regulate junction permeability and confer the selectivity and strength of the tight junctions [3]. Claudin expression is either deregulated or lost in cancer [4], e.g. expression of claudin-7 is lost in both head and neck cancer, and invasive breast cancer [5,6]. The increase in the expression of claudins in cancer cells is also linked to increased invasiveness, through the recruitment of matrix metalloproteinases [7].

To date, little is known about the expression or biological roles of claudins in the normal urothelium (NU) or UC of the bladder. In the present study we examined the claudin-7 mRNA expression in the UC of the urinary bladder and its possible role in tumour invasiveness and progression.

Materials and methods

This study included 68 specimens of UC of the bladder, 35 with non-muscle-invasive (NMI) Ta–T1 tumours, 33 with muscle-invasive (MI) (⩾pT2) tumours, and 36 of NU (Table 1). Tissue samples were obtained from patients who underwent transurethral resection, partial cystectomy or radical cystectomy for bladder cancer at our institutions. Informed consent for this study was obtained from all patients, and the Research Ethics Committee of the Tokushima University, School of Medicine approved the study. No patients received chemotherapy, immunotherapy or radiotherapy. Specimens with carcinoma in situ were excluded. We used the International Union against Cancer and the criteria of the WHO classification system for tumour staging and grading, respectively [8].

Procedure

The procedure used was largely as described previously [9], except that we examined the expression levels of the target gene claudin-7, and compared them with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The selected sequences of sense and antisense primers used for the amplification of claudin-7 were: 5′-TGGGGAAAGGGTGGTGTTG-3′ (sense) and 5′-TTACCCAATTTTAACCACCAC-3′ (antisense). The immunostaining density and intensity results were obtained as reported previously [10].

The staining density was quantified as a percentage, i.e. (claudin-7 positive cells)/(all cells, i.e. propidium iodide-labelled cells), as follows: 0, no staining; 1, <25% cells; 2, 25–50% cells; 3, 50–75% cells; 4, >75% cells. Staining intensity was also indicated as 0, negative; 1–2, weak expression; 3–6, moderate expression, and 7–9, intense expression. These semiquantitative evaluations

Table 1 The patients' characteristics.

Variable	No. of patients
Total	68
Gender:	
Male	54
Female	14
Tumour stage:	
pTa	16
pT1	19
pT2	17
pT3	11
pT4	5
Tumour grade:	
1	11
2	27
3	30

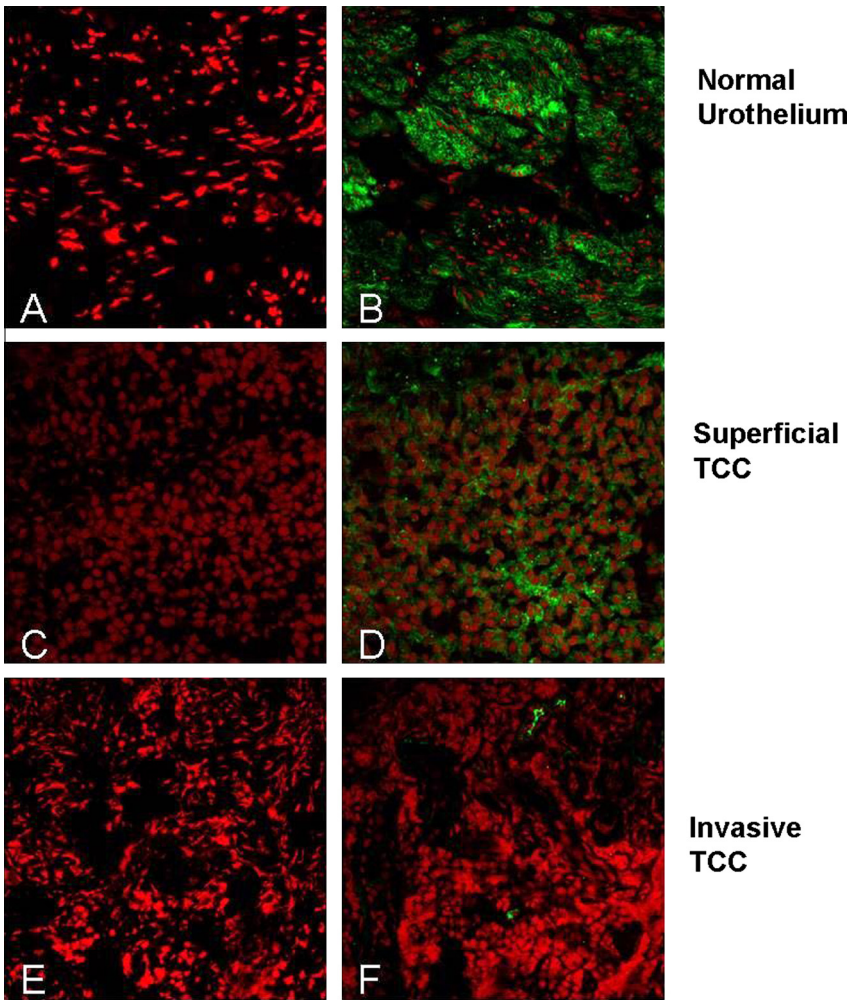

Figure 1 Immunostaining of claudin-7 in NU, NMI and MI bladder cancer specimens. Representative images of immunofluorescent (A–F) staining of bladder cancer epithelium with polyclonal anti-claudin-7 antibody are shown. (A) NU (negative control) with no primary antibody showed no staining. (B) Claudin-7 expression in the same sample (green). The nuclei were then counterstained with propidium iodide (red). (C) NMI UC (negative control) showed no staining. (D) Moderate green staining of the same sample indicated reduced claudin-7 expression. (E) MI UC (negative control) with no primary antibody showed no staining. (F) Minimal expression of claudin-7 in the same sample.

were carried out by two authors (M.G. and K.T.) independently, with 20 analysed areas of 10 sections from three patients in each tissue type.

The differences between claudin-7 expression and several clinicopathological variables, such as stage and grade, were assessed using Student's *t*-test. Values are presented as the mean (SEM), with statistical significance indicated at $P < 0.05$.

Results

Claudin-7 mRNA values in UC and NU tissues were normalised to GAPDH mRNA. The quantification of claudin-7 gene expression by real-time PCR showed a significantly greater mean ratio of claudin-7 to GAPDH expression in NU specimens than in UC, at 31185 (2071.7) vs. 17572.7 (3747.8) ($P < 0.001$). There was a greater mean ratio of claudin-7 to GAPDH expression in NMI (Ta-T1) than in MI ($\geqslant$pT2) tumours of the bladder, at 28942.8 (4121.8) vs. 5513.6 (3351), respectively ($P = 0.015$).

Patients with $\geqslant$pT2 tumours had significantly lower claudin-7 expression levels than patients with Ta–T1 tumours, at 5514 (3351) vs. 28942.8 (4121.8), respectively ($P = 0.012$). The difference between Ta and T1 tumours was not significant ($P = 0.683$). There was no significant difference in claudin-7 expression level in patients with G1-2 and G3 tumours ($P = 0.19$). There was no significant difference in claudin-7 expression level in patients with recurrent NMI UC and non-recurrent NMI UC ($P = 0.61$).

To validate the real-time PCR data, we stained representative samples of NU, NMI and MI UC with a specific antibody to claudin-7, using fluorescent immunohistochemistry for confocal laser scanning light microscopy. As seen in Fig. 1B NU samples had strong

homogenous staining for claudin-7, which was mainly expressed in the cytoplasm of the NU cells. By contrast, there was less pronounced immunostaining for claudin-7 in NMI UC tissue (Fig. 1D) and faint staining with MI UC (Fig. 1F).

Discussion

The loss of cell-to-cell adhesion is accepted to be an early event in the process of metastasis, thus permitting individual cancer cells to be liberated from the primary tumour. Hence claudins, a large group of essential tight-junction proteins, are frequently reported to be abnormally regulated in human carcinomas, play a role in tumorigenesis and identified as promising new targets for cancer detection, diagnosis and therapy [4].

Although claudin-7 has no established functional role in bladder cancer there is evidence supporting its role in cell-to-cell adhesion. We were the first to detect an up-regulation of claudin-7 in NMI UC and we suggested that claudin-7 might be an early marker of bladder cancer [11]. In the present study we further evaluated the correlation between claudin-7 expression and the invasiveness of the UC of the urinary bladder as a further step in validating it as a potential marker.

We showed that the level of expression of claudin-7 mRNA was significantly lower in UC of the urinary bladder than in the NU, and a significantly lower expression of claudin-7 mRNA in MI UC than in NMI UC. The greatest mean level of claudin-7 expression was in NMI Ta-T1 tumours, while there was lower expression in MI ($\geqslant$pT2) tumours. The results from confocal scanning microscopy supported the real-time RT-PCR findings, confirming the greater expression of claudin-7 in NMI than in MI UC of the bladder. From these results we show that the loss of claudin-7 correlates with tumour invasiveness and progression.

These results are similar to those reported by Kominsky et al. [6], who found that claudin-7 expression was lower in high-grade tumours of the breast than in adjacent normal epithelium. Using fine-needle aspirates from breast carcinoma, a similarly reduced claudin-7 expression correlated with high tumour grade, recurrence, and distant spread in a study by Sauer et al. [12]. Similarly, Sheehan et al. [13] reported that claudin-7 expression is down-regulated in prostate adenocarcinoma compared with benign glands. In addition, Usami et al. [14] stated that reduced expression of claudin-7 correlated with invasion and metastasis in squamous cell carcinoma of the oesophagus.

We investigated the clinical significance of claudin-7 expression for its usefulness as a prognostic variable in UC of the bladder, assessing, with other prognostic factors (tumour stage, tumour grade, age at surgery and gender) its effect on tumour recurrence. Interestingly, there were no significant differences between Ta and T1 tumours, or between G1-2 and G3 tumours, so there was no correlation between claudin-7 expression and tumour grade, and it could not be used to predict the recurrence of NMI UC.

Boireau et al. [15] stated that claudin-7 was down-regulated in bladder carcinoma, which coincides with our results. In addition, its localisation was largely restricted to the membrane in all carcinoma samples, with a fragmented appearance in high-grade tumours. They also suggested that gene-promoter methylation could be responsible for its down-regulation, similar to the situation in breast carcinoma.

Nakanishi et al. [16] examined the expression of claudin-7 in UC of the upper urinary tract, and stated that there was no evidence of a relationship between its expression and clinical variables or prognosis. These findings did not concur with our results for reasons that remain unclear. One possibility is that the function of claudin-7 might differ between the urinary bladder and the upper urinary tract.

The present study was done on 68 samples including all grades and stages of the UC of the urinary bladder and their matched NU. We obtained significant results for the expression of claudin-7 and its correlation with the clinicopathological variables.

However, there were some limitations, including deficiency in some data, e.g. patient age and smoking status. We did not evaluate the correlation between progression to metastasis and claudin-7 expression. We intend to include this in our ongoing research and will evaluate and analyse the recurrence-free, progression-free and overall survival in relation to claudin-7 expression. Finally, we encourage more research on the role of claudins in carcinogenesis and the progression of UC, and their prognostic value.

In conclusion, claudin-7 protein expression is down-regulated in UC of the urinary bladder compared with NU. Given the significant reported variability of claudins in numerous different cancers, further studies are needed to elucidate their functional role in tumorigenesis, and their increasing interest as novel drug targets in bladder cancer and other tumours.

Conflict of interest

No conflict of interest to declare.

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