Claudins as Prognostic Factors for Renal Cell Cancer

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Abstract. *Background: Claudins are tight junction proteins and their expression is often different in normal and corresponding tumor cells. In the present study, we determined how the expression of claudins 1-5 and 7 correlated to survival, grade and stage of patients with renal cell cancer (RCC). Patients and Methods: Primary tumor samples were collected retrospectively from 229 RCC patients. Claudins were detected by immunohistochemistry using commercial monoclonal antibodies against claudins 1-5 and 7. Median survival time was 6.5 years confidence interval (CI) (4.5-8.5, n=224). Kaplan-Meier survival estimated method was used in survival analyses. Results: Positive expression was detected in 62%, 67%, 45%, 55%, 7% and 35% of cases for claudins 1, 2, 3, 4, 5 and 7, respectively. High expression of claudin 2 was observed in 20% of cases while high expression of other claudins was less frequent. Claudins were compared to classical prognostic factors. On cross-tabulation, claudin 1 ($p<0.001$) and claudin 2 ($p=0.009$) were significantly associated with lower-grade and higher-grade tumors, respectively. None of the claudins was significantly associated with tumor stage or patient survival. Conclusion: Claudins 1 and 2 were associated with tumor grade. However, none of the claudins was a more powerful prognostic factor than tumor stage.*

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Clinical stage and histological grade are the most powerful prognostic factors in renal cell carcinoma (RCC), although new prognostic markers, including proliferation index (MIB-1), anti-apoptosis regulator (*BCL-2*), apoptosis regulator (*BAX*), Vascular endothelial growth factor (VEGF), and claudins have been promoted (1-4). Although MIB-1 is a proliferation marker generally associated with tumor size, nuclear grade and necrosis, it has not been found to be an independent prognostic factor of RCC (2, 3). The *BCL-2* gene has an inhibitory effect on apoptosis while *BAX* promotes it. Some studies have reported that they have no independent association with the prognosis of patients with RCC (2, 3). In one study, VEGF was an independent prognostic predictor of outcome (4), but the result was not corroborated by a subsequent study, which, however, suggested that VEGF was significantly correlated with tumor stage and grade (5).

Tight intercellular junctions lie adjacent to the apical end of the lateral cell membrane surface. They have two functions: barrier function and fence function. The barrier function regulates the passage of ions, water and macromolecules through paracellular spaces; this function also operates in cancer cells (6). The fence function maintains cell polarity (6, 7). Tight junctional proteins form a trafficking and signalling platform that regulates cell growth, proliferation, differentiation, and dedifferentiation (7). More than 40 different proteins have been located at the tight junctions of epithelial, endothelial and myelinated cells. Two main components of the tight junction filaments have been identified: occludin and claudin. The latter is a protein family with more than 20 members (6).

The expression of claudins is abnormally regulated in several human cancers. In particular, claudin 3 and claudin 4 are frequently overexpressed in several neoplastic conditions, including ovarian, breast, pancreatic, and prostate cancers (8, 9), while claudins 3, 4 and 7 are overexpressed in bladder, thyroid, fallopian tube, stomach, colon and uterus carcinomas

(9). Claudin 4 positivity was associated with a favorable prognosis in triple-negative breast cancer (defined as the absence of estrogen and progesterone receptors and negative HER2 expression). The same study reported that claudin 4 positivity was associated with poorer and claudin 3 positivity with better prognosis in luminal breast cancer (10). Claudins 3 and 4 have diagnostic value in Paget's disease and in differentiating diffuse mesothelioma from metastatic pleural adenocarcinoma (11, 12). Metastatic lower-lip squamous-cell carcinomas had higher claudin 1 expression than nonmetastatic tumors (13). Expression of claudins was lower in diffuse gastric carcinoma when compared to the intestinal type of gastric cancer (14). Immunostaining of claudin 4 and claudin 5 was less marked in one study that included RCC (15), suggesting that these substances might influence renal cancer; however only 9 cases of RCC were included in the study. The same study reported that immunostaining of claudin 3 was occasionally reduced. There are only few studies on the association between the claudins and, the prognosis, development and dissemination of RCC. Two studies have reported that claudin 7 can be used to differentiate between oncocytomas and the chromophobe type of renal cancer in difficult cases (16, 17). Claudin 3 and claudin 4 associated with overall survival based on univariate analyses, but they were not independent predictors of survival (17). Claudin 1 was reported to be an independent prognostic factor and a possible diagnostic marker for papillary renal cell carcinoma (18).

The aim of this study was to investigate expression of claudins 1-5 and 7 in a substantial set of renal cell carcinomas and to compare their expression with the histology and the other known prognostic factors of renal cell cancer.

Materials and Methods

Patients. The study population consisted of 229 cases collected retrospectively (demographics in Table I). A total of 224 patients underwent nephrectomy and there were 5 (2%) autopsy samples, which were excluded from the survival analysis. The operations had been performed between 1985 and 1995 at either the Tampere University Hospital or the Tampere Hospital, Finland. Follow-up was performed for all patients according to clinical practice. Clinical stage was assigned using the TNM 2002 Classification of Malignant tumors (19). Median follow-up was 4 years interquartile range (IQR), (1.27-7.24). Patient history was collected from the records of the two participating hospitals. The ethics committee at the Tampere University Hospital approved the research protocol and the National Authority for Mediolegal Affairs approved the use of tumor samples.

Histopathologic assessment. Archival formalin-fixed, paraffin-embedded RCC material was used. All tissue samples were re-evaluated and classified and graded by one of the authors (PK); a 1 mm core biopsy from the highest grade area of each tumor was transferred to a multi-tissue block for further immunohistochemical analysis. All tumors were graded according to the Fuhrman system and classified according to the Heidelberg classification (20, 21). Histology and grade of renal cell cancers are depicted in Table I.

Table I. *Baseline patients' characteristics*.

Patients (N=229)	135 men (59.0%)
	94 women (41.0 %)
Median age at the time of nephrectomy	65 (IQR 55.9-71.9)
TNM classification	
T1	107 (46.7%)
T2	29 (12.7%)
T3	39 (17.0%)
T4	4 (1.7%)
N+	13 (5.7%)
M1	37 (16.2%)
Stage	
1	104 (45.5%)
2	29 (12.7%)
3	40 (17.4%)
4	56 (24.4%)
Histology	
Clear cell renal cell carcinoma	207 (90.4%)
Papillary renal cell carcinoma	12 (5.2%)
Chromophobe renal cell carcinoma	5 (2.2%)
Sarcomatoid	2 (0.9%)
Unclassified	3 (1.3%)
Gradus	
1-2	23 (10.0%)
3	115 (50.2%)
4	91 (39.7%)

Immunohistochemistry. The primary antibodies used for immunostaining are designed from Zymed Laboratories Inc (South San Fransisco, CA, USA) and were designed for use in formalin-fixed, paraffin-embedded tissues. They were polyclonal rabbit anti-claudin 1 (clone JAY.8), monoclonal mouse anti-claudin 2 antibody (clone 12H12), polyclonal rabbit anti-claudin 3 (clone Z23.JM), monoclonal mouse anti-claudin 4 (clone 3E2C1), monoclonal mouse anti-claudin 5 (clone 4C3C2) and polyclonal rabbit anti-claudin 7 (clone ZMD.241). Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 10 minutes. After 60-minutes of incubation with the primary antibody (dilution 1:50 for anti-claudin 1, 2, 3, 4, 5 and 7), a biotinylated secondary anti-rabbit antibody and Histostain-SP kit (Zymed Laboratoris Inc) were used on the sample. In all cases, the colour for immunostaining was developed by diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were made by substituting non-immune rabbit or mouse serum and phosphate buffered saline for the primary antibodies. Immunostaining results were categorized as follows: 0, no immunostaining; 1, weak immunostaining (<50% membrane-bound positivity); 2, moderate immunostaining (50% to 90% membrane-bound positivity); or 3, strong immunostaining (>90% membrane-bound positivity), as shown in Figure 1. Expression of claudins was evaluated only in tumor cells.

Statistical analysis. Statistical analysis was performed using the IBM SPSS Statistic for Windows version 14.0.2. The differences between categorical variables were tested using the Pearson's X^2-test or Fisher's exact test. Survival was analysed by using the

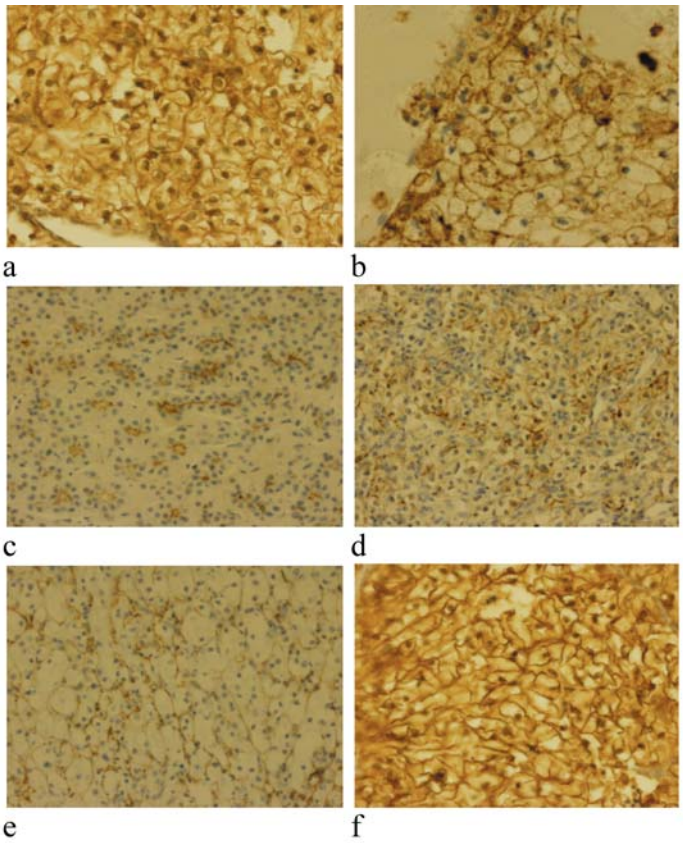

Figure 1. *Immunostaining of claudins 1 (a), 2 (b), 3 (c), 4 (d), 5 (e) and 7 (f) in RCC.*

Kaplan-Meier's survival estimated method. Univariate analysis adjusted for age and gender was performed using the Cox's proportional hazards model.

Results

Median age of the patients was 65 years (IQR 56-72) (Table I). Expression of claudins is described in Table II. Membrane-bound expression for claudins 1, 2, 3, 4, 5 and 7 was detected in 62.2%, 67.4%, 44.6%, 54.7%, 7.4% and 35.4 % of samples, respectively. Strong immunoreactivity was present in claudins 1, 2, 3, 4, 5, 7, 8.5%, 20.2%, 6.2%, 12.0%, 2.1% and 7.3 % of cases, respectively. Claudin 2 was most immunoreactive and was detected in 67.4 % of samples, while claudin 5 was negative in 92.6% of the samples. Many tumors had a rich vascular network and the vessels were strongly positive for claudin 5.

Claudin 2 was positive in all papillary and all chromophobe types of RCC. Papillary RCC was most strongly positive for claudin 2, 3 and 4, with weaker or no staining for other claudins. However, this study represents the most common RCC type, as 90.4% of the tumor samples included clear cell carcinomas.

Table II. *Expression of claudins 1, 2, 3, 4, 5 and 7 in renal cell carcinoma.*

	– n (%)	+ n (%)	++ n (%)	+++ n (%)
Claudin 1	71 (37.8)	54 (28.7)	47 (25.0)	16 (8.5)
Claudin 2	63 (32.6)	50 (26.0)	41 (21.2)	39 (20.2)
Claudin 3	107 (55.4)	46 (23.9)	28 (14.5)	12 (6.2)
Claudin 4	87 (45.3)	54 (28.1)	28 (14.6)	23 (12.0)
Claudin 5	176 (92.6)	8 (4.2)	2 (1.1)	4 (2.1)
Claudin 7	124 (64.6)	26 (13.5)	28 (14.6)	14 (7.3)

–, No immunostaining; +, weak immunoreactivy; ++, moderate immunoreactivy; +++, strong immunoreactivy.

The expression of studied claudins was compared to tumor stage and grade, both being prognostic factors of RCC (Tables III and IV). Cross-tabulation indicated that both claudin 1 ($p<0.001$) and claudin 2 ($p<0.009$) expression was significantly associated with tumor grade (Table IV). Claudin 1 expression was associated with lower-grade tumors and

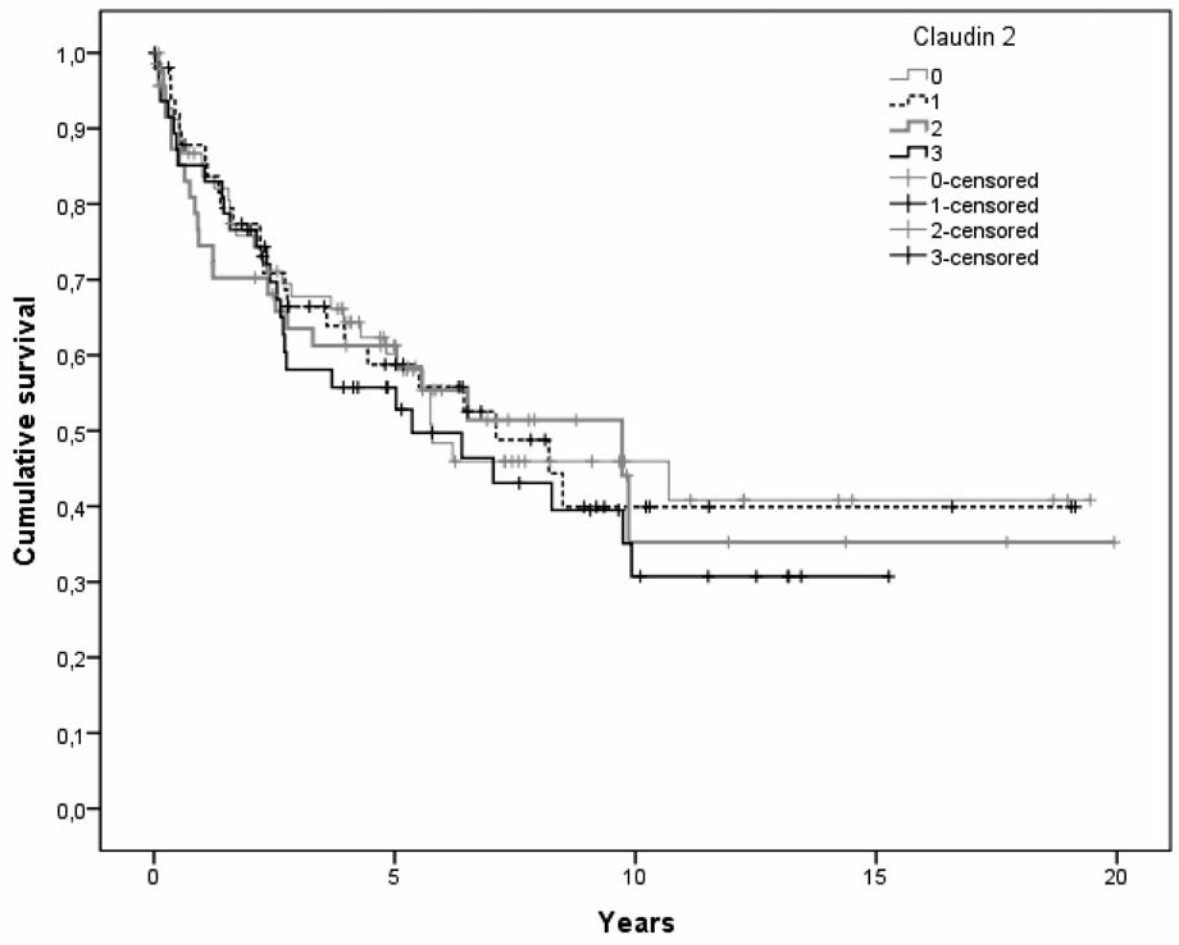

Figure 2. *Kaplan-Meier analysis of claudin 2 association with renal cell carcinoma survival.*

claudin 2 expression with higher-grade tumors. Claudin 4 was nearly statistitically significantly associated with RCC tumor grade (p=0.069). None of the claudins were significantly associated with tumor stage. The most significant results of the univariative analysis are summarized in Table V. Both classical prognostic factors (grade and stage) were statistically significantly associated with survival (Table V). Claudin expression was not independently associated with patient survival (Figure 2 and Table V).

Discussion

The present study was undertaken to evaluate expression of claudins 1-5 and 7 in RCC samples and to study the clinical significance of that expression. Over half of the samples showed positivity for claudins 1, 2 and 4, while claudins 3, 5 and 7 were more poorly expressed. In addition, we evaluated the association between claudin expression, tumor grade and stage, the latter of which reported to be associated with patients' survival in RCC (1-4). Expression of claudins has been studied in several different tumor types. Down-regulation of claudin 4 and overexpression of claudins 2, 3 and 5 have been reported in prostate adenocarcinomas compared with benign prostatic hyperplasia (22). In the same study overexpression of claudin 3 correlated with perineural invasion.

In another study, samples of prostatic adenocarcinoma were studied and decreased expression of claudin 1 and high expression of claudins 3 and 4 in prostatic adenocarcinoma samples correlated with poor prognosis (23). Overexpression of claudins 3 and 4 correlated with myometrial invasion in a study of endometrial tissue variants (24) while in another study overexpression of claudins 3 and 4 was correlated with poorer prognosis in clear cell RCC (25).

There are only few studies on claudin expression in RCC related to clinical data, including a limited study from our Hospital (15). One of those showed that claudin 1 expression was associated with poor survival in renal cell cancer (18).

Table III. *Associations of stage and claudin expression. Shown are Number of cases (n) and percentages are shown. Analysis was performed by Pearson Chi-square test or Fisher's exact test (marked with *).*

	Stage				
	1 n (%)	2 n (%)	3 n (%)	4 n (%)	*p*
Claudin 1					0.331
0	21 (28)	15 (38)	29 (48)	14 (42)	
1	25 (33)	15 (38)	13 (22)	9 (27)	
2	21 (28)	7 (17)	14 (23)	9 (27)	
3	9 (12)	3 (7)	4 (7)	1 (3)	
Claudin 2					0.097
0	31 (40)	6 (15)	25 (41)	10 (29)	
1	21 (27)	11 (27)	13 (21)	7 (21)	
2	14 (18)	10 (24)	12 (20)	11 (32)	
3	12 (15)	14 (34)	11 (18)	6 (18)	
Claudin 3					0.639*
0	42 (55)	19 (45)	36 (80)	21 (62)	
1	17 (22)	13 (31)	15 (25)	6 (18)	
2	13 (17)	8 (19)	6 (10)	3 (9)	
3	5 (6)	2 (5)	3 (5)	4 (12)	
Claudin 4					0.208
0	34 (44)	13 (31)	35 (57)	13 (39)	
1	22 (29)	14 (33)	14 (23)	12 (36)	
2	12 (16)	6 (14)	8 (13)	6 (18)	
3	9 (12)	9 (21)	4 (7)	2 (6)	
Claudin 5					0.533*
0	71 (93)	40 (98)	55 (90)	29 (88)	
1	3 (4)	0 (0)	4 (7)	2 (6)	
2	0 (0)	1 (2)	1 (2)	1 (3)	
3	2 (3)	0 (0)	1 (2)	1 (3)	
Claudin 7					0.416*
0	44 (57)	27 (64)	42 (70)	25 (76)	
1	12 (16)	6 (14)	7 (12)	4 (12)	
2	12 (16)	7 (17)	10 (17)	2 (6)	
3	9 (12)	2 (5)	1 (2)	2 (6)	

Table IV. *Associations of grade to claudins are expressed by number of cases (n) and percentages. Analysis was performed by Pearson Chi-square test or Fisher's exact test (marked with*).*

	Grade			
	1-2 n (%)	3 n (%)	4 n (%)	*p*
Claudin 1				<0.001
0	3 (13)	33 (30)	46 (54)	
1	7 (30)	32 (29)	26 (30)	
2	8 (35)	36 (33)	10 (12)	
3	5 (22)	9 (8)	4 (5)	
Claudin 2				0.009
0	14 (61)	33 (29)	29 (33)	
1	5 (22)	26 (23)	21 (24)	
2	3 (13)	33 (29)	13 (15)	
3	1 (4)	21 (17)	25 (28)	
Claudin 3				0.519
0	11 (48)	58 (51)	53 (62)	
1	8 (35)	29 (25)	18 (21)	
2	2 (9)	20 (18)	9 (10)	
3	2 (9)	7 (6)	6 (7)	
Claudin 4				0.069
0	14 (61)	38 (34)	47 (54)	
1	5 (22)	38 (34)	23 (26)	
2	2 (9)	22 (20)	10 (11)	
3	2 (9)	15 (13)	7 (8)	
Claudin 5				0.953*
0	22 (96)	102 (92)	80 (92)	
1	1 (4)	4 (4)	5 (6)	
2	0 (0)	2 (2)	1 (1)	
3	0 (0)	3 (3)	1 (1)	
Claudin 7				0.218*
0	11 (48)	70 (62)	59 (68)	
1	5 (22)	13 (12)	15 (17)	
2	4 (17)	19 (17)	10 (12)	
3	3 813)	10 (9)	3 (3)	

The present study showed that low expression of claudin 1 is associated with higher tumor grade and also that claudin 4 did not reach statistical significance in association higher tumor grade (p=0.069). It has been previously shown, that moderate-to-strong expression of this claudin is associated with decreased survival in patients with RCC (17).

We have here included 229 RCC samples and found that only 7 % were positive for claudin 5, which is a tight junctional protein. Claudin 4, however, was expressed in 55 % of cases, claudin 3 in 46% and claudin 7 in only 35% of cases. Claudins 1 and 2, again, were more highly expressed (Table II). Median survival time was poor, only 6.5 years but this, however, is in line with our previous larger study where we reported that median overall survival was 5.9 years, 3.4 years and 12 months between obese, normal or underweight patients with RCC, respectively (26). Our data included patients whose RCC has been diagnosed between 1985 and 1995. During this period, computerized tomography and ultrasound were not used as widely as nowadays and thus RCC might have been diagnosed later than nowadays. Our data are part of a larger study which demonstrated that prognosis and diagnosis of RCC has improved by using imaging procedures (27). In addition, targeted-therapies have also improved outcomes in advanced RCC (28).

Table V. *Age- and gender-adjusted univariate and multivariate associations of different staining patterns with other main prognostic parameters were tested using Cox regression models with results given as the hazard ratios (HR) and 95 % confidence intervals (CI) (n=224).*

		Adjusted	Variative
	n	HR	[95% CI]
Grade			
1-2	22	1.00	
3	114	2.84	[1.01-7.99]
4	88	4.76	[1.69-13.4]
Stage			
1	77	1.00	
2	43	2.33	[1.25-4.33]
3	61	2.78	[1.59-4.83]
4	34	9.03	[5.01-16.3]
Claudin 1			
0	79	1.00	
1	64	0.82	[0.51-1.32]
2	53	0.69	[0.42-1.15]
3	18	0.52	[0.22-1.22]
Claudin 2			
0	71	1.00	
1	52	0.90	[0.53-1.55]
2	49	1.00	[0.59-1.72]
3	47	1.09	[0.64-1.84]

There are some differences between claudin expression in RCC when compared to other epithelial cancers. Claudins 3 and 4 are usually strongly expressed in carcinomas of the genitourinary area, such as endometrial and ovarian epithelial tumors and prostate carcinomas. RCC seems to have a decreased claudin expression, at least regarding claudin 4 (15). Claudin 5 has been reported to be specific to endothelial cells, yet immunohistochemical expression of this protein has been found comparatively often in malignant tumors such as ovarian of gastric carcinomas (15). In our series of RCC, claudin 5 expression was low.

The pattern of claudin expression in RCC most likely reflects its expression in the corresponding non-neoplastic tissues of the kidney. Kidney adenocarcinomas originate from tubular epithelial cells. In rabbits, claudins 1, 2 and 4 are expressed in proximal tubule cells, Henle's loop and collecting segments, claudin 3 in the proximal and collecting tubules and claudin 7 in the proximal tubulus, while claudin 5 is absent from tubular cells (25). Interestingly, in our RCC samples, claudins 5 and 7 showed the lowest expression levels suggesting little to no expression of the proteins in tubular cells (25). Most tumors were ordinary kidney clear cell adenocarcinomas. The few papillary and chromophobe types of RCC were universally positive for claudin 2 immunostaining. Claudins 2, 3 and 4 were strongly expressed only in papillary RCC. The papillary-type RCC is less responsive to modern drugs developed in the last decade. Mesenchymal epithelial-transition inhibition alone and in combination with inhibition of epidermal growth factor receptor is a new target being explored for the treatment of papillary type RCC (29). Future experience will show, whether this result is useful for the clinical differential diagnosis between RCC subtypes. Some other tumors are known to show differences in claudin expression by phenotype or histological subtype. In gastric carcinomas, diffuse carcinoma exhibits reduced claudin expression compared to the intestinal subtype and mesotheliomas. In epithelioid mesotheliomas claudin express more strongly than sarcomatoid subtype (15).

Reduced expression of claudin 1 was associated with high-grade tumors. This is consistent with the concept that less differentiated tumors tend to lose their differentiation markers. Dysregulation of claudin expression has been associated with epitheliomesenchymal transition, which could influence the metastatic behaviour of tumors. Abrogated claudin expression could influence cell attachment, decrease cohesion of cancer cells and promote metastatic spread. Observations consistent with this hypothesis have been reported both for breast cancer associated with claudin 7 (30) and for esophageal cancer associated with claudin 3 (31). With respect to RCC, we did not find any association between expression of any claudins and either metastasis or stage. We found that claudins 1 and 2 may have additional prognostic value for patients with RCC. Both claudins were significantly associated with tumor grade.

Conclusion

The tight junctional proteins claudin 1 and 2 were significantly associated with tumor grade. None of the studied claudins were significantly associated with survival in RCC patients. The prognostic value of claudins for patients with RCC merits further investigation.

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