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The role of claudin-6 in chromophobe renal cell carcinoma

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Summary. Background. The prognostic value of Claudin-6 (CLDN6) in non clear cell renal cell carcinoma (RCC) is still unclear.

Aim. To evaluate the prognostic impact of CLDN6 expression in a large cohort of chromophobe RCC (chRCC).

Material and Methods. Patients who underwent renal surgery due to chRCC were recruited. Clinical data were retrospectively evaluated. Tumor specimens were analyzed for CLDN6 expression by immunohistochemistry.

Results. 81 chRCC patients were eligible for analysis, thereof 10 (12.3%) patients were positive for CLDN6. No significant associations were found for CLDN6 expression and clinical attributes in patients with chRCC. Kaplan-Meier analysis revealed no differences in overall survival (OS) for patients with $CLDN6^-$ compared to $CLDN6^+$ tumors (87.0% versus 62.5%; p=0.174).

Conclusion. In chRCC CLDN6 expression is not associated with parameters of aggressiveness or survival. Due to the rare incidence of chRCC further studies with larger cohorts are warranted.

Key words: Renal Cell Carcinoma, Claudin-6, Chromophobe histology, Survival

Introduction

Claudins (CLDN) are essential structural functional components of tight junctions (TJ) (Furuse et al., 1998). TJs are intercellular junctions between epithelial cells in which the outer layers of the cell membranes fuse. This reduces the ability of larger molecules and water to pass between the cells. CLDNs show an abnormal expression in several human cancers. Therefore, they can be used as promising targets for cancer detection, diagnosis, and treatment (Morin, 2005).

CLDNs are mainly markers of epithelial differentiation. They can be found in nearly all carcinomas with tissue type and cancer type specificity. CLDN 4, 7 and 8 are, for example, useful markers in the differentiation of sarcomas (Facchetti et al., 2007; Osunkoya et al., 2009; Schaefer et al., 2017). Besides the diagnostic value of CLDN, several studies demonstrated their prognostic utility in different types of cancer. Prat et al. demonstrated that a low expression of CLDN 3, 4 and 7 is correlated with poor prognosis in breast cancer (Prat et al., 2010). In con-trast, Lechpammer et al. showed that low CLDN 3 and 4 expression was correlated with longer overall survival (OS) than high CLDN 3 and 4 in clear cell renal cell carcinoma (RCC) (Lechpammer et al., 2008). In summary, the prognostic value of different CLDNs in tumor types remains to be elucidated.

Nevertheless, CLDN6 shows a specific expression pattern in the form of reactivation. Kojima et al. demonstrated a reactivation of CLDN6 in endometrial cancer (Kojima et al., 2020). Aberrant-ly activated CLDN6 expression in non-small cell lung cancer was detected by Micke et al. (2014). Furthermore, Kohomoto

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Abbreviations. AJCC, American Joint Committee on Cancer; CAR, Chimeric antigen receptor T cells; chRCC, Chromophobe renal cell carcinoma; CLDN, Claudin; CSS, Cancer specific survival; DAB, Diaminobenzidine; DFS, Disease free survival; HRP, Horseradish peroxidase; IHC, Immunohistochemistry; ISUP, International Society of Urological Pathology; OS, Overall survival; PFS, Progression free survival; RCC, Renal cell carcinoma; TM, Transmembrane domains; TMA, Tissue micro arrays; WHO, World Health Organization; TJ, Tight junctions

et al. suggested that CLDN6 is a single prognos-tic marker in a subgroup of intestinal type gastric cancer (Kohmoto et al., 2020). Reactivation of CLDN 6 was negatively correlated with patient OS in all three mentioned studies.

Chromophobe RCC (chRCC) is the third most common RCC subtype and constitutes 5-7% of all RCC cases (Moch et al., 2016). This subtype has a favorable prognosis compared to other RCC subtypes. The 5-year survival rate is around 78-100% (Moch et al., 2016). Nevertheless, some patients show unfavorable clinical courses with large tumors and metastasis. Because of distinct nuclear atypia, chRCC should not be graded according to the WHO/ISUP grading sys-tem. Ohashi et al. demonstrated in their multi-institutional evaluation of chRCC that tumor ne-crosis and sarcomatoid differentiation are reproducible components of a two-tiered chromo-phobe tumor grading system (Ohashi et al., 2020).

Moreover, up to now no other established prognostic grading systems or biomarkers exist.

Therefore, the aim of this study was to evaluate the prognostic impact of CLDN6 in chRCC. To the best of our knowledge, this is the first study which has analyzed this aspect in the third most common RCC subtype.

Materials and methods

Patients and tumor characteristics

Eighty-one patients who underwent renal surgery for chRCC between 1996 and 2014 were identified using the electronic pathology register. Relevant clinical attributes relating to each tissue sample were collected with regard to tumor stage and histological subtype according to the AJCC 2018 TNM tumor staging system. Suitable specimens were selected by a pathologist (FE) and tissue micro arrays (TMA) were prepared from the primary tumor as previously described. The histological subtype was confirmed by a second uropathologist (AH). Our cohort does not include tumors with sarcomatoid differentiation or tumor necrosis. Patient data were retrieved from electronic patient charts, with follow-up data regarding overall survival (OS), and death being ascertained from the Munich Cancer Registry of the Munich Tumor Centre. The study was carried out according to the latest version of the Declaration of Helsinki and approved by the institutional ethics committee.

Procedures

Expression of CLDN 6 was determined by immunohistochemistry (IHC). 2 µm TMA slides were stained for CLDN 6 (Clone #PA5-67557, invitrogen, dilution 1:50) (Mikuteit et al., 2022). The antibody was applied for 30 min after heat pretreatment at 120°C for 5 min with Tris-EDTA buffer pH 9 and peroxidase blocking (Dako, Hamburg, Germany). Incubation with a horseradish peroxidase (HRP)-labeled secondary antibody polymer (EnVision, Dako) was conducted for 30 min followed by adding a diaminobenzidine (DAB) substrate chromogen solution (Dako) for 10 min and counterstaining for 1 min with hematoxylin (Merck, Darmstadt, Germany). Incubation procedures were performed at room temperature. Positive controls as well as negative control slides without the addition of primary antibody were included for each staining experiment. Paraffin-embedded human colorectal cancer tissue was used as the positive control. All stained tissue samples were assessed in a blind way by a pathologist (FE). The evaluation was performed under a Leitz ARISTOPLAN light microscope (Leica Microsystems, Germany) with a x10 eyepiece, a 22-mm field of view and x40 objective lens (Plan FLUOTAR x40/0.70).

The staining reaction was classified according to a semi-quantitative IHC reference scale previously described (Kurokawa et al., 2014). CLDN6 was localized primarily on the membrane and partly in the cytoplasm of tumor cells. Paraffin-embedded human colorectal cancer tissue was used as the positive control.

The staining intensity was scored from 0 to 3 (0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining) according to the H-score as already described (Fig. 1) (Birks et al., 2010; Wang et al., 2015; Gao et al., 2019). The area of staining was evaluated in percent (0-100%), a staining intensity score was defined by multiplying the score with the stained area (Kurokawa et al., 2014; Phan et al., 2015). Given the absence of normative data on cell membrane or cell cytoplasm staining intensity in the literature, values in our patient collective were dichotomized using the median of observed distribution as the cut off. Because of the limited number of cases we used a binary cutoff.

A CLDN6 staining lower or equal to the median was defined as CLDN6 low, and a staining higher than the median was defined as CLDN6 high. The median was 0, so all values above 0 were counted as $CLDN6^{+}$.

Statistical analysis

The primary endpoint of the study was OS. In the absence of death, the endpoint was censored at the last date of follow-up. The duration of follow-up was calculated from the date of surgery to the date of death or last known follow-up. Dependent upon the nature of variable, chi-square, Fisher's exact tests, Mann-Withney U-Test, and independent t-test were used as appropriate, to compare between patient/tumor characteristics and the corresponding subgroup with or without CLDN6 expression. Kaplan-Meier survival times were estimated, with subgroups being compared using the log-rank test. SPSS 27.0 (USA) was used for statistical assessment. Two-sided p-values below 0.05 were considered statistically significant.

Results

Patients' characteristics and claudin 6 expression

The median age of the cohort was 59.8 (range: 31-

79) years. Of the patients, 60 (74.1%), 14 (17.3%) and 7 (8.6%) presented with pT1, pT2 and pT3 tumors, respectively. 86.4% of the patients had AJCC Stage I/II. Furthermore, 6 (7.4%) of all patients presented with lymph node metastasis and/or synchronous distant metastasis. CLDN6 expression was found in 10 (12.3%) of the chRCC TMA specimens, respectively (Fig. 1). No associations between CLDN6^{+} expression and patient or tumor characteristics were identified (Table 1). There were no CLDN6^{+} patients with metastases, in the CLDN6^{-} group there were 3 (4.2%) patients with metastases.

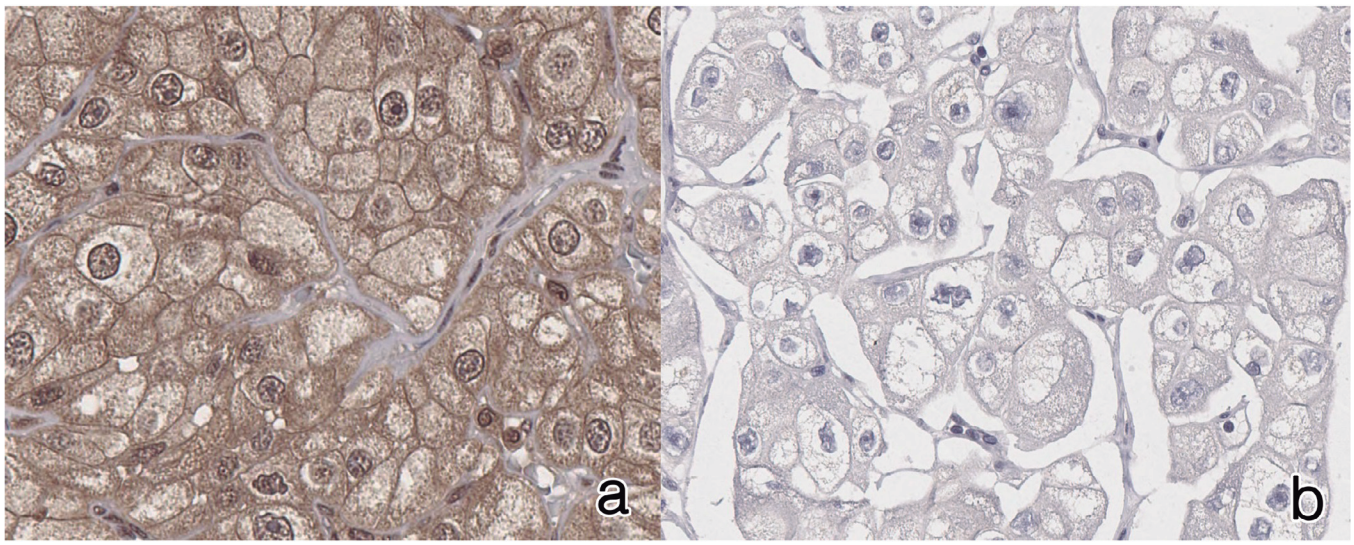

Fig. 1. Immunohistochemical staining of Claudin-6 in chromophobe renal cell carcinoma specimen. **a.** Positive. **b.** Negative. x 40.

Table 1. chRCC patient and tumor characteristics in dependence of Claudin-6 (CLDN6) expression.

Variable	All chRCC n=81 (100%)	CLDN6^{-} n=71 (87.7%)	CLDN6^{+} n=10 (12.3%)	p-value
Age, median (IQR) years	59.8 (52.9-69.1)	59.8 (51.3-68.3)	62.9 (55.4-72.6)	0.385[a]
Sex				0.458[b]
female	23 (28.4)	19 (26.8%)	4 (40.0%)	
male	58 (71.6)	52 (73.2%)	6 (60%)	
Stage (TNM 2010)				0.952[c]
pT1	60 (74.1)	53 (74.6%)	7 (70.0%)	
pT2	14 (17.3)	12 (16.9%)	2 (20.0%)	
pT3	7 (8.6)	6 (8.5%)	1 (10.0%)	
Cancer Stage (AJCC)				0.925[c]
Stage I	56 (69.1)	49 (69.0%)	7 (70.0%)	
Stage II	14 (17.3)	12 (16.9%)	2 (20.0%)	
Stage III	8 (9.9)	7 (9.9%)	1 (10.0%)	
Stage IV	3 (3.7)	3 (4.2%)	0 (0%)	
LN metastasis[#]				1.0[b]
N-	78 (96.3)	68 (95.8%)	10 (100.0%)	
N+	3 (3.7)	3 (4.2%)	0 (0.0%)	
Metastasis[#]				1.0[b]
M-	78 (96.3)	68 (95.8%)	10 (100.0%)	
M+	3 (3.7)	3 (4.2%)	0 (0.0%)	
Disease status				1.0[b]
Localized*	70 (86.4)	61 (85.9%)	9 (90.0%)	
Advanced[$]	11 (13.6)	10 (14.1%)	1 (10.0%)	

[#]: at time of renal surgery; *: localized disease= pT1/2 N0/M0; [$]: advanced disease= pT3/4 and/or N+ and/or M+. Legend: IQR: Interquartile range, NE: not evaluable; N- = lymph node status unknown or tumour cells absent from regional lymph nodes, N+ = regional lymph node metastasis present. [a]: Mann-Whitney-U test, [b]: Fisher exact test, [c]: chi square test.

Claudin 6 expression and clinical course

Median follow-up was 40.5 (IQR: 10.8-109.3) months. At the time of last follow-up, 46 (56.8%) patients were alive, 9 (11.1%) patients died and 26 (32.1%) patients were lost to follow up.

Kaplan-Meier analysis disclosed a 5 year- OS for CLDN6^{-} compared to CLDN6^{+} tumors of 87.0% compared to 62.5% (p=0.174, log rank) (Fig. 2).

Discussion

CLDNs are transmembrane components of TJs. They play a role of paracellular barrier and intracellular signaling, while regulating the proliferation, differentiation, and apoptosis of the epithelial cell. Zhang et al. detected CLDN 6 as a molecular biomarker in pan-cancer using multiple omics integrative analysis (Zhang et al., 2021). The database analysis results in an upregulated CLDN6 expression in 20 types of human cancer and CLDN6 downregulation in five cancer types. Furthermore, CLDN6 expression was closely correlated with both molecular subtype and immune subtype in several types of cancer, for example breast cancer or uterine corpus endometrial carcinoma. In addition, Zhang et al. reported on the prognostic role of CLDN6 in both of these tumors. CLDN6 expression significantly correlated with OS, disease specific survival (DSS), and progression-free interval (PFS) (Zhang et al., 2021).

Besides its prognostic relevance CLDN6 is an emerging target for therapeutic approaches. The treatment options include inter alia antibody drug-binding targets, radionuclide therapeutic tar-gets and new antibody therapeutic targets. Adra et al. proposed that a monoclonal antibody against CLDN6 might be useful against testicular germ cell tumor growth. CLDN6 as a target for monoclonal antibody is currently being investigated in a clinical trial (NCT03760081) (Adra et al., 2022).

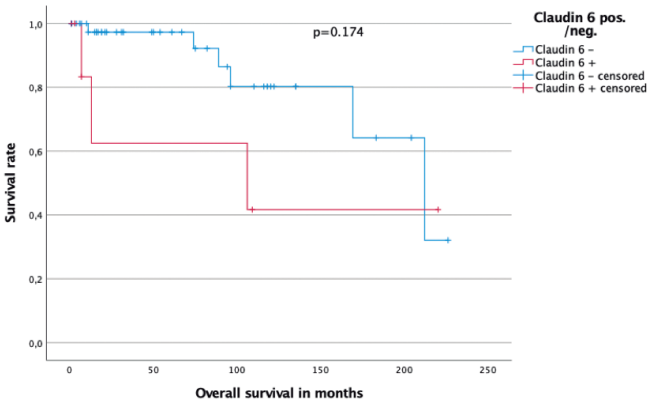

Fig. 2. 5-year overall survival for patients with chromophobe renal cell carcinoma in dependence of Claudin-6 expression. Kaplan-Meier analysis disclosed an OS for Claudin 6- compared to Claudin 6+ tumors of 87.0% compared to 62.5% (p=0.174, log rank).

Another therapeutic option currently being explored is chimeric antigen receptor (CAR)-T cell therapy targeting CLDN6. To date, this method is less effective in patients with solid tumors because of the limitations of tumor-specific targets. As we already know, CLDN6 is a surface antigen of carcinoembryonic cells and hence shows an ideal expression profile for CAR-T cells. Therefore, a CLDN 6 CAR-T cell approach is currently being investigated for multiple tumor entities (NCT04503278) (Li, 2021).

In summary, data regarding the prognostic impact of CLDN6 in several tumor types are still uncertain because of contradictory results. Therefore, the use of CLDN6 as prognostic marker is up to now not established in clinical or pathological routine. In RCC in general CLDN6 has not yet been adequately explored. Furthermore, the role for special subtypes of RCC, such as chRCC, remains to be elucidated.

The aim of our study was to evaluate the prognostic association of CLDN6 expression in chRCC with clinical parameters, tumor aggressiveness and OS. Our results showed no associa-tion between tumor stage and CLDN6 expression, neither in grading nor in stage. Furthermore, we detected no correlation between CLDN6 expression and OS. As already mentioned above, Ohashi et al. demonstrated in their study that tumor necrosis and sarcomatoid differentiation are reproducible components in chRCC (Ohashi et al., 2020). Unfortunately, we could not detect tumor necrosis or sarcomatoid differentiation in our cohort due to the use of TMA samples. Therefore, we could not find any correlations between an aggressive phenotype and CLDN6 expression. In a cohort of 66 patients with chRCC in the Cancer Genome Atlas (TCGA), the 5-year survival rate was 85.5%. There were no mutations in CLDNs found (clinical data obtained from https://portal.gdc.cancer.gov/).

Of course, our study shows several limitations, including the methodology of immunohistochemistry, the scoring system, the use of TMAs, the relatively low number of cases, as well as the retrospective analysis.

Hence, our study can give an initial indication that CLDN6 might not be a suitable prognostic marker in chRCC. Nevertheless, further multicenter studies are needed to evaluate the relevance of CLDN6 in RCC.

Conclusion

In summary, CLDN 6 expression is not associated with parameters of aggressiveness or survival. Therefore, it is not a predictive marker for chRCC. Future studies should focus in more detail on this entity.

Conflict of interest. All authors declare that they have no competing interests.

Author's contributions. Marie Mikuteit, Franziska Erlmeier and Sandra Steffens participated in the data interpretation and drafting of the manuscript. MM and SS performed the statistical analysis. Franziska

Erlmeier carried out the data acquisition. Stefanie Zschäbitz, Michael Authenrieth, Wilko Weichert and Arndt Hartmann revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethical standards. The study was approved by the Ethics Committee of the Technical University of Munich (384/13) in accordance with the German Human Research Act and with the Declaration of Hel-sinki. Informed consent was obtained from all individual participants included in the study. De-tails that disclose the identity of the subjects under study were omitted. The research involved no animals.

Informed consent. Informed written consent was obtained from all individual participants included in the study.

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Data availability statement. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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