Apelin and apelin receptor expression in renal cell carcinoma

Vyr Tolkach, Yuri Tolkach1, Laura Esser1, Anika Kremer1, Laura Esser1, Stefan C. Müller1, Carsten Surphen3, Klaus Jung3, Mareta Ionka1, Glen Kristiansen1 and Stefan Hauser2

MATERIALS AND METHODS

RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue samples as described before.13 In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) from tissue bank at the CIO Cologne-Bornheim clinical characteristics in tissue bank at the Department of Urology at the University Hospital Bonn. Fresh-frozen tissues were stored at −80 °C prior to use. Total RNA was isolated from 166 clear-cell RCC and 102 normal renal tissue
The Cancer Genome Atlas analysis (TCGA cohort)
Clinical data and normalised mRNA expression data generated with Illumina HiSeq 2000 RNA sequencing platform, version 2 (data version 28.01.2016) were extracted from TCGA for patients with clear-cell RCC. After database construction with thorough control of data quality, 481 patient cases with complete mRNA expression and clinical information were available for analysis.

Immunohistochemistry analysis (Study cohort 2)
Three-hundred patients diagnosed with renal cell carcinoma after radical or partial nephrectomy at a single institution (Department of Urology, Charité—Universitätsmedizin Berlin, Germany; 1992–2004) were included in this study (Suppl. Table 1). The mean follow-up time was 117 months (total range: 1–267 months), allowing the calculation of overall survival as an endpoint. Formalin-fixed, paraffin-embedded archive tissue was used to construct a tissue microarray (TMA) with two tumours and two normal tissue spots (diameter 1 mm) from every patient. The tissue microarray was cut (3 µm thick) and mounted on superfrost slides (Menzel Gläser, Brunswick, Germany). After deparaffinisation with xylene and gradual rehydration, antigen retrieval was achieved by pressure-cooking in 0.01 mol/L citrate buffer for 5 min. Slides were incubated with primary antibody (APLNR rabbit polyclonal antibody, ThermoFisher Scientific, Catalogue number PA5-21285; Dilution 1:50). The slides were counterstained with haematoxylin and aqueously mounted. The immunohistochemical staining was evaluated blind to clinical outcome, clinical and pathological stage. Staining intensities were graded separately for cytoplasm and membrane of tumour cells and endothelial cells of tumour vessels. A 4-tier grading system (0: negative; 1: weakly positive; 2: moderately positive; 3: strongly positive) was used.

TMA Slides were also stained with CD34 antibody (monoclonal antibody, Dako/Agilent; Dilution 1:100, m7165) for microvessel density assessment and with PD-L1 antibody (monoclonal mouse anti-human antibody, Clone 22C3, Dako/Agilent; pharmDx kit). PD-L1 staining was evaluated by means of the percent of positive tumour cells in a TMA spot (membrane staining).

Cell lines
The following cell lines were used for western blot: Caki-1 (human ccRCC), 786-0 (human ccRCC), RC-124 (human kidney adult primary cell line, benign), as well as DU145 and PC-3 prostate cancer cell lines. All cells were cultured in their specific media (786-0, DU-145 and PC-3: RPMI-1640; Caki-1: McCoy’s 5A medium; RC-124: DMEM GlutaMAX medium; all media Thermo Fisher Scientific). The media were supplemented with 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin. The cells were grown in a humidified incubator at 37 °C with 5% CO2 and regularly correlated with histological grade (Pearson’s r = 0.0006; CSS: HR 4.1, 95% CI 1.7–9.7, p = 0.001) and presence of metastatic disease (Pearson’s r = 0.17, p = 0.009), while APLN mRNA expression showed no significant correlation.

In the survival analysis (n = 154, number of events: OS = 31, CSS = 21) APLN expression showed no prognostic association with either overall survival or cancer-specific survival. In contrast, APLNR was predictive for overall (OS) and cancer-specific survival (CSS) in Kaplan–Meier (Fig. 2a, b), univariate (OS: HR 3.5, 95% CI 1.7–7.0, p = 0.0006; CSS: HR 4.1, 95% CI 1.7–9.7, p = 0.001) and multivariate Cox analyses of mRNA expression, histological grade and pT-stage (OS: HR 2.9, 95% CI 1.4–5.8, p = 0.004; CSS: HR 3.5, 95% CI 1.5–8.4, p = 0.001), with lower expression predicting shorter survival times.

RESULTS
mRNA expression analyses (mRNA cohort)
APLN (p = 0.110) and APLNR (p = 0.105) mRNA expression levels were similar in normal (n = 102) and malignant tissues (ccRCC, n = 166) (Fig. 1). APLNR expression was inversely correlated with histological grade of the tumour (Pearson’s r = −0.17, p = 0.027), pT-stage (Pearson’s r = −0.20, p = 0.009) and presence of metastatic disease (Pearson’s r = 0.20, p = 0.009), while APLN mRNA expression showed no significant correlation.

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mRNA expression analyses (TCGA cohort)
In the TCGA ccRCC cohort, APLNR mRNA expression levels were negatively correlated with histological grade (Pearson’s r = −0.22, p = 5.2e−07) and pT-stage of the tumour (Pearson’s r = −0.23, p = 2.7e−07). APLN mRNA expression was only weakly inversely correlated with histological grade (Pearson’s r = −0.10, p = 0.036) and positively with APLNR mRNA expression (Pearson’s r = 0.10, p = 0.022).

Both low APLNR and low APLN mRNA expression levels were predictive of lower overall survival in patients with ccRCC in Kaplan–Meier (Fig. 2c, d) and in univariate Cox analysis with dichotomisation based on the optimised cut-off values (APLN: HR 2.1, 95% CI 1.6–2.9, p = 3.1e−06; APLN: HR 1.6, 95% CI 1.2–2.2, p = 0.004). However, independent prognostic significance in multivariate Cox analysis (gene mRNA expression, histologic grade, pT-stage, pN-status) was only observed for APLNR expression (HR 1.8, 95% CI 1.3–2.5, p = 0.0009; see also Suppl. Table 3).

Antibody validation
Results of western blot experiments are outlined in Fig. 3 (associated metrics in Supplementary Data 1) and Suppl. Figure 1.
A band with a molecular weight similar to that predicted for APLNR (~43 kDa) ±10% was detected in all cell lines used. Immunohistochemistry analysis (Immunohistochemistry cohort)

**Staining patterns.** Normal tissue demonstrated almost uniformly very high expression of the APLNR protein (Fig. 4). In tumour tissue, expression of APLNR was detectable in tumour cells (cytoplasm and membrane, highly correlated; Pearson’s $r = 0.46$, $p = 8.8e-15$) and on endothelia of tumour vessels (no significant correlation with staining of tumour cells), see Fig. 4. While cytoplasmic staining of tumour cells was negatively associated with overall patient survival, vascular staining associated positively with overall patient survival.

**Association with clinicopathological variables.** Papillary RCC (pRCC) and chromophobe RCC (chRCC) tissue samples demonstrated relatively low vascular and relatively high cytoplasmic APLNR expression in tumour cells, while clear-cell RCC (ccRCC) showed a wide spectrum of staining intensities in both cytoplasm of tumour cells and endothelium (Suppl. Table 4). Because of the small number of patients with pRCC or chRCC, we restricted further analyses to ccRCC ($n = 253$).

Endothelial expression in tumour vessels was significantly and inversely associated with histological grade of the tumour and pT-stage (Table 1), and not associated ($p > 0.05$) with pN-status, gender, age or status at the end of the follow-up (overall survival).

Cytoplasmic tumour cell expression was significantly and positively associated with tumour grade but was not with pT-stage, pN-status, gender or age (all $p > 0.05$).

**Microvessel density.** Median microvessel density (microvessels/mm²) for patients with ccRCC was 740, with pRCC 402 and with chRCC 263. Microvessel density was negatively correlated with ISUP grade of the tumour (Pearson’s $r = 0.22$, $p = 0.0003$) and was positively correlated with intensity of vascular APLNR expression (Pearson’s $r = 0.21$, $p = 0.0002$).

**Correlation with PD-L1 expression.** PD-L1 immunohistochemistry protein expression status was available for 72 patients with ccRCC with a range of 0-100% tumour cells positive for PD-L1 (median 15%). Of these patients 23 (31.9%) were completely negative, 15 other patients showed low levels of expression (<10%) in at least 1 tumour spot. A negative correlation was evident between APLNR expression and PD-L1 expression by tumour cells: for cytoplasmic APLNR expression: Pearson’s $r = -0.16$, $p < 0.001$, for vascular expression: Pearson’s $r = -0.19$, $p < 0.001$.

**Association with survival (ccRCC).** Endothelial expression in tumour vessels was significantly associated with overall survival
in Kaplan–Meier/log-rank (Fig. 5a) and univariate Cox-analysis (not shown), but completely lost its significance in multivariate Cox-analysis due to interaction with histological grade and pT-stage of the tumour.

Cytoplasmic tumour cell expression was significantly associated with overall survival in Kaplan–Meier analysis (Fig. 5b), univariate (Intensity “3” vs. Intensity “0–2” HR 2.12, 95% CI 1.33–3.40, \(p = 0.002\)) and multivariate (HR 1.68, 95% CI 1.02–2.78, \(p = 0.041\)) Cox-analysis.
of histological grade, pT-stage, R-status, and ECOG performance status to account for other mortality causes (Suppl. Table 5).

**DISCUSSION**

The apelin receptor is a G-protein-coupled receptor which binds a number of substances (apelin, APELA, ELABELA, Toddler) with many important functions in the cardio-vascular system, such as cardiac development, vasomotor tone, angiogenesis, myocardial inotropy, prevention of fibrosis and remodelling.\textsuperscript{6,7,14–17} Recently, it was identified as an essential gene for cancer immunotherapy, which can modulate interferon-γ responses in tumours and the effector function of CD8\(^+\) T cells.\textsuperscript{5} The loss of its function could reduce the efficacy of cancer immunotherapies.\textsuperscript{5}

To our knowledge, only one screening study has addressed the expression of APLN (but not APLNR) in ccRCC tumour tissue and adjacent normal tissue, with the only finding that APLN mRNA expression was higher in tumour tissue.\textsuperscript{12} In our study we have used three well-characterised cohorts of patients to perform a comprehensive evaluation of mRNA and protein expression of

| Table 1. Immunohistochemistry cohort: associations between APLNR protein expression and clinicopathological parameters in clear-cell RCC (n = 253) |
|---|---|---|---|---|---|---|---|
| | Tumour vascular expression, staining intensity | | Tumour cell cytoplasm expression, staining intensity | |
| | “0” | “1” | “2” | “3” | “0” | “1” | “2” | “3” |
| **Histological grade (WHO 2016), n (%)** | | | | | | | | |
| G1 | 1 (2.0%) | 17 (33.3%) | 17 (33.3%) | 16 (31.4%) | 7 (13.7%) | 34 (66.7%) | 8 (15.7%) | 2 (3.9%) |
| G2 | 5 (3.5%) | 45 (31.7%) | 56 (39.4%) | 36 (25.3%) | 15 (10.6%) | 73 (51.4%) | 38 (26.8%) | 16 (11.3%) |
| G3 | 3 (7.6%) | 14 (35.8%) | 14 (35.8%) | 8 (20.5%) | 1 (2.6%) | 11 (28.2%) | 17 (43.6%) | 10 (25.6%) |
| G4 | 9 (42.9%) | 9 (42.9%) | 1 (4.7%) | 2 (9.5%) | 3 (14.3%) | 8 (38.1%) | 7 (33.3%) | 3 (14.3%) |
| **p-level** | 3.6e–08 | | | | 0.002 | | | |
| **pT-stage, n (%)** | | | | | | | | |
| pT1 | 4 (2.7%) | 52 (34.9%) | 48 (32.2%) | 45 (30.2%) | 14 (9.4%) | 81 (54.4%) | 41 (27.5%) | 13 (8.7%) |
| pT2 | 3 (17.6%) | 2 (11.8%) | 10 (58.8%) | 2 (11.8%) | 0 (0%) | 7 (41.2%) | 8 (47.1%) | 2 (11.8%) |
| pT3 + pT4 | 11 (12.6%) | 31 (35.6%) | 30 (34.5%) | 15 (17.2%) | 12 (13.8%) | 38 (43.7%) | 21 (24.1%) | 16 (18.4%) |
| **p-level** | 0.002 | | | | 0.08 | | | |

\textsuperscript{a}Pearson’s Chi-squared test
APLN and APLNR in primary RCC tumours and to correlate tumour characteristics and patient survival.

At the mRNA level, we have demonstrated that APLNR expression is decreased in higher grade, higher stage and metastatic ccRCC tumours with independent prognostic significance for overall and cancer-specific survival. Immunohistochemical analysis of APLNR protein expression has provided important information concerning which compartments of the tumour tissue express the receptor. Both endothelial lining of the tumoral vessels and tumour cells express significant amounts of APLNR with relatively high variability between the cases. In support of our mRNA findings, we observe by immunohistology that APLNR expression in the vasculature and tumour cells associates inversely with tumour aggressiveness (pt-stage and grade as surrogates). Increasing aggressiveness is accompanied by higher APLNR expression in tumour cells and lower expression in vessels. Both expression parameters were associated with overall survival of patients, but only cytoplasmic expression in tumour cells associated independently in a multivariate model with common clinico-pathological variables. Interestingly, vascular APLNR expression was positively associated with microvessel density, which itself correlates negatively to tumour aggressiveness (ISUP grade). The western blots from ccRCC cell lines support this finding (Fig. 3).

APLN mRNA expression showed weak association with histological grade of the tumour only in the TCGA cohort, in contrast the influence on overall survival showed strong association but failed as an independent predictor.

Little is known about the role of APLNR/APLN axis during oncogenesis/tumour growth. In colon adenocarcinomas, it has been shown to be upregulated together with its ligand APLN, forming a putative autocrine loop, stimulating tumour growth. Apelin has been shown to promote lymphangiogenesis and lymph node metastasis in experimental models with melanoma cells. In a cholangiocarcinoma cell model and in in vitro/in vivo experiments with glioblastoma cells, inhibition of the APLN/APLN axis has resulted in decreased proliferation and angiogenesis. The role of the APLNR-axis for tumour neo-angiogenesis, especially under hypoxia, regulated by hypoxia-inducible factor (HIF)-1alpha, is well documented.

ccRCC is a highly vascularised tumour with high levels of intratumoural HIF, which accumulates due to inactivation of the von Hippel-Lindau gene. This could explain the generally high vascular expression of the APLNR and APLN in tumour tissues in our study. However, it is unclear why increasing aggressiveness of the tumour leads to decreased levels of APLNR expression. One possible explanation could be that APLN/APLN activation induces maturation of the tumour vasculature and improves the efficiency of immune therapy, while immature vessels could help the tumour to evade the immune response. Also, studies in glioblastoma suggest that release of APLN through endothelial cells triggers the response reactions from tumour cells expressing APLNR, so that an immuno-protective environment is created. However, the immunological effects of the APLN/APLN axis in ccRCC and other tumours are only incompletely understood and probably involve a complex interplay between vascular and cellular compartments of the tumour. Our analysis of microvessel density, which is associated both with aggressiveness of the tumour (ISUP grade) and vascular expression of APLN, once more supports this point. Importantly, while there is only one known isoform of APLNR, several different isoforms of APLN arise upon cleavage by endopeptidases and show different activity levels as well as different degrees of organ-specificity. These forms should be considered in further analyses.

Our study is limited. We have only used primary tumours for analysis. It would be interesting to compare the expression of APLNR/APLN in paired samples from primary tumours and metastases. Although our study provides the first thorough characterisation of APLN/APLN expression in renal cell carcinoma (especially in ccRCC as the dominant subtype) and its association with clinico-pathological variables and outcome, functional studies, especially those related to the associated immune processes, were not within the scope of our project and warrant further investigations.

Interestingly, in our study we were able to detect significant levels of negative correlation between APLNR expression in different tumour tissue compartments and PD-L1 expression by tumour cells in a subset of patients with ccRCC. This finding once more time underlines the potential relevance of APLNR for intratumoural immunological processes. The evaluation of treatment response to immune therapies (checkpoint inhibitors) as a function of APLN expression should be investigated in future studies.

CONCLUSIONS
In our study, we provide a comprehensive characterisation of APLNR and APLN expression in renal cell carcinoma. The main results are: (1) the significant correlation between ccRCC aggressiveness and APLN mRNA/protein expression, (2) characterisation of different APLN-expressing compartments in tumour tissue (tumoral vessels and tumour cells) with opposite correlations to tumour aggressiveness, (3) evidence of independent prognostic role of APLNR expression regarding patient survival, (4) correlation with PD-L1 expression by tumour cells in ccRCC.

Fig. 5 Survival analysis (Kaplan–Meier estimates) for APLNR protein expression in patients with clear-cell renal cell carcinoma (immunohistochemistry cohort), overall survival as endpoint. a APLNR expression on tumoural vessels; staining intensity: “0”—negative, “1”—weak expression, “2”—moderate expression, “3”—strong expression. b APLNR expression in cytoplasm of tumour cells; staining intensity (“0” negative, “1” weak expression, “2” moderate expression, “3” strong expression)
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AUTHORS CONTRIBUTIONS
Manuscript drafting: YT, JE, AK, GK, SH. Immunohistochemistry: YT, GK. mRNA experiments: JE, SH. Cell lines experiments: AK, LE, MT. TCGA analysis: YT. Data acquisition: JE, SH, OK, KJ, CS, SCM, MT. Data analysis: YT, JE, SH, GK.

ADDITIONAL INFORMATION
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Competing interests: The authors declare no competing interests.

Ethical approval: The study was approved by the ethic committee at the University of Bonn (vote: 317/17). The necessity of informed consent was waived by ethical committee. The study was performed in accordance with the Declaration of Helsinki.

Data availability: Study databases for mRNA cohort and immunohistochemistry cohort are available in electronic form as Supplementary data (Database IHC.xls, Database mRNA.xls). TCGA datasets used for in silico analyses were downloaded from the Broad Institute Firehose GDAC archive (https://gdac.broadinstitute.org/).

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