Otoferlin is a prognostic biomarker in patients with clear cell renal cell carcinoma: A systematic expression analysis

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Abbreviations & Acronyms
ccRCC = clear cell renal cell carcinoma
CI = confidence interval
CSS = cancer-specific survival
Fer1L4 = Fer-1-like family member 4
HR = hazard ratio
IHC = immunohistochemistry
NA = not applicable
OS = overall survival
OTOF = otoferlin
PCR = polymerase chain reaction
PFS = progression-free survival
qPCR = quantitative polymerase chain reaction
qRT–PCR = quantitative real-time polymerase chain reaction
RCC = renal cell carcinoma
TCGA = The Cancer Genome Atlas
TMA = tissue microarrays

Objectives: To comprehensively investigate the role of otoferlin as a prognostic and diagnostic biomarker in clear cell renal cell carcinoma.

Methods: Three independent cohorts were used to study otoferlin in clear cell renal cell carcinoma: The Cancer Genome Atlas cohort (messenger ribonucleic acid expression; clear cell renal cell carcinoma $n = 514$, normal renal tissue $n = 81$); study validation cohort (messenger ribonucleic acid expression; clear cell renal cell carcinoma $n = 79$, normal renal tissue $n = 44$); and immunohistochemistry cohort (protein expression; clear cell renal cell carcinoma $n = 142$, normal renal tissue $n = 30$). Otoferlin gene expressions were extracted from The Cancer Genome Atlas database or determined using quantitative real-time polymerase chain reaction, respectively. Protein expression was assessed using immunohistochemistry staining against otoferlin on tissue microarrays. Correlations between otoferlin messenger ribonucleic acid/protein expression and clinicopathological data/patient survival were statistically tested.

Results: Otoferlin messenger ribonucleic acid expression was significantly upregulated in clear cell renal cell carcinoma compared with normal renal tissue. High expression levels correlated with advanced stage, higher grade and metastatic tumors, accompanied by independent prognostic significance for overall and cancer-specific survival. In contrast, otoferlin protein expression was downregulated in tumor tissue. Although, high otoferlin expression in clear cell renal cell carcinoma was positively correlated with histological grading and independently predictive of a shortened progression-free survival.

Conclusion: Our data suggest otoferlin as an indicator of tumor aggressiveness and as a prognostic biomarker for patients with clear cell renal cell carcinoma, leading to the conclusion that otoferlin could promote the malignancy of clear cell renal cell carcinoma.

Key words: biomarker, expression analysis, otoferlin, renal cell carcinoma, survival.

Introduction

Solid kidney tumors are among the most common malignancies worldwide. ccRCC is in the focus of therapy and translational research, as this subtype accounts for approximately 80% of renal tumors. As only surgical therapy of localized renal tumors can be carried out with curative intention, it is all the more important to optimize the therapeutic options of metastatic renal cell carcinoma. In recent years, the survival of patients with ccRCC has been improved by the introduction of immunotherapies, which in selected patients are also applied in combination with well-established angiogenic therapies. Nevertheless, the treatment response of patients is heterogeneous. The discovery of predictive biomarkers is indispensable for the ideal patient selection for targeted therapy. The focus is on the comprehensive investigation of altered gene and protein expression in ccRCC.

The ferlins are a group of six membrane-anchored proteins that are involved in various membrane processes. These include membrane fusion events, endo- and exocytosis, membrane repair and remodeling, and vesicle transport. As such membrane processes are essential for survival and proliferation, as well as signal transduction of benign and tumor cells, the ferlins gained interest as potential inducers or suppressors of tumor development. In recent years, altered expression patterns of some ferlin family members have thus been found in several tumor
entities. For instance, overexpression of myoferlin on mRNA and protein level with influence on carcinogenesis has been found in ccRCC, breast cancer, lung cancer and pancreatic adenocarcinoma.\textsuperscript{4–8} The expression level of the long non-coding RNA, Fer1L4, has been reported to be either increased or decreased depending on the tumor entity due to oncogenic or suppressive properties. In ccRCC, overexpression of Fer1L4 is associated with shortened survival, whereas lower expression levels were found in gastric and colon cancer leading to poor prognosis.\textsuperscript{9–11} The first member of the ferlin family, dysferlin, is also overexpressed in ccRCC and affects patient survival, as recently shown.\textsuperscript{12,13} The expression of the second ferlin family member, OTOF, has barely been investigated in cancer. Only a recent review analyzed the mRNA expression of the individual ferlins in various tumor tissues using the TCGA database, and showed a negative effect of OTOF on the survival of patients with ccRCC.\textsuperscript{14} As the name implies, OTOF is highly expressed in the inner hair cells and is essential for the proper functioning of the auditory system.\textsuperscript{15,16} Years ago, OTOF originally aroused interest, as mutations of its gene lead to a non-syndromic form of deafness.\textsuperscript{17,18} However, unlike the other ferlins, little is known about its relevance in oncological diseases. Given the paucity of data regarding the role of OTOF in ccRCC, we therefore carried out a comprehensive expression analysis in ccRCC, and investigated its association with clinicopathological parameters and survival.

**Methods**

**TCGA analysis**

OTOF mRNA expression data, as well as clinicopathological and survival data of patients with ccRCC, were obtained from TCGA Research Network (https://www.cancer.gov/tcga). After statistical exclusion of outliers using the z-method, where a z-score above ±3 or further from zero was considered an outlier, the cohort included a total of 595 tissue samples divided into 514 ccRCC and 81 normal renal tissue samples.

**qRT–PCR**

A total of 123 renal tissue samples (79 ccRCC and 44 normal renal tissues) were collected from patients who underwent radical or partial nephrectomy at the Department of Urology at the University Hospital Bonn (Bonn, Germany). The collection was carried out within the framework of the Biobank at the Centre for Integrated Oncology. Preoperative written informed consent was obtained from all individuals before enrolment. Table 1 shows the clinicopathological characteristics of the qPCR study cohort.

RNA isolation was described in detail earlier.\textsuperscript{19} In brief, fresh-frozen renal tissue samples were stored at −80°C until isolation of total RNA using the mirVana miRNA Isolation Kit, and then treated with DNase (both Ambion, Foster City, CA, USA). RNA quantity was measured by the NanoDrop spectrophotometer 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA integrity was determined by agarose gel electrophoresis. cDNA was prepared from 1 µg total RNA using the PrimeScript RT reagent Kit with genomic DNA Eraser; Takara Bio, Saint-Germain-en-Laye, France). qRT–PCR to determine the gene expression of OTOF was carried out on an ABIPrism 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qPCR was carried out with 5ng/µL cDNA template, 10 pmol/µL of each forward and reverse primer, and SYBR Premix Ex Taq II with ROX Plus (Takara Bio). The following primer sequence for OTOF was used: forward CAA-AGA-CGG-CAA-AGT-GGA-CG; reverse GGC-TTC-CTC-TGA-CCG-TTC-TC. Qbase+ software (Biogazelle, Ghent, Belgium) was used to calculate the relative mRNA expression levels that were normalized to β-actin and peptidylprolyl isomerase

### Table 1 Clinicopathological parameters of TCGA, qPCR study and IHC cohort

|                  | TCGA cohort | qPCR study cohort | IHC cohort |
|------------------|-------------|-------------------|-----------|
|                  | ccRCC       | Normal            | ccRCC     | Normal  | ccRCC   | Normal |
|                  | n = 514 (%) | n = 81 (%)        | n = 79 (%)| n = 44 (%) | n = 142 (%) | n = 30 (%) |
| Sex              |             |                   |           |         |         |         |
| Male             | 334 (65)    | 57 (70.4)         | 57 (72.2) | 32 (72.7) | 89 (62.7) | 21 (70.0) |
| Female           | 180 (35)    | 24 (29.6)         | 22 (27.8) | 12 (27.3) | 53 (37.3) | 9 (30.0)  |
| Age (years)      | 60.53       | 63.06             | 64.5      | 62.2     | 57.9     |
| Range            | 26–90       | 38–90             | 38–89     | 43–86    | 26–85    | 28–80    |
| Pathological stage |           |                   |           |         |         |         |
| pT1              | 266         | NA                | 41        | NA       | 59       | NA       |
| pT2              | 66          | NA                | 7         | NA       | 32       | NA       |
| pT3              | 172         | NA                | 30        | NA       | 49       | NA       |
| pT4              | 10          | NA                | 1         | NA       | 2        | NA       |
| Lymph node metastasis | 15      | NA                | 2         | NA       | 8        | NA       |
| Distant metastasis | 77       | NA                | 16        | NA       | 18       | NA       |
| Histological grade (WHO 2016) |      |                  |           |         |         |         |
| G1               | 14          | NA                | 11        | NA       | 44       | NA       |
| G2               | 224         | NA                | 47        | NA       | 94       | NA       |
| G3               | 203         | NA                | 17        | NA       | 3        | NA       |
| G4               | 73          | NA                | 4         | NA       | 0        | NA       |

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A as reference genes. The 2−ΔΔCT algorithm was used for the relative quantification of OTOF mRNA levels. Outliers were excluded using the z-method, as described previously.

**IHC**

OTOF protein expression was investigated in tissues (142 ccRCC and 30 normal renal tissues) archived in the Institute of Pathology at the University Hospital Bonn using a TMA. The construction of the TMA was described earlier.20 The sections (3-µm thickness) were cut from the TMA, mounted on slides, deparaffinized with xylene and rehydrated in graded ethanol. Antigen retrieval was achieved by pressure-cooking of the slides for 5 min in citrate buffer (pH 6.0). The Ventana Benchmark automated staining system (Ventana Medical System, Tuscon, AZ, USA) was used to carry out IHC. The slides were incubated with the primary OTOF antibody (dilution 1:200, catalog number HPA007502; Sigma Aldrich, St. Louis, MO, USA). Signal detection was carried out with an ultraView Universal DAB detection kit (Ventana Medical System). The slides were counterstained with hematoxylin. The staining intensities were evaluated independently by different investigators (AC, JS, YT). As a predominantly homogeneous expression pattern of OTOF was presented in immunostaining, a four-step grading system (0: negative, 1: weakly positive, 2: moderately positive, 3: strongly positive) was used. Normal renal tissue served as an ideal multistage negative and positive control, as strong staining of the distal tubules, weak-to-moderate staining of the proximal tubules and negative staining of the glomeruli were observed. This staining pattern was in line with the data from The Human Protein Atlas (http://www.proteinatlas.org). Tonsillar and liver tissues were used as complementary, appropriate negative control tissues, as they are known not to express OTOF (Fig. S1). As OTOF mainly localizes to intracellular membrane compartments, particularly to the trans-Golgi network, but also low levels have been detected at the plasma membrane, we did not distinguish between cytoplasmic and membranous staining in the evaluation of the immunostaining.21 In survival analyses, the cut-off to determine an overexpression was set at >1.

![Graphs showing OTOF mRNA expression analysis in TCGA cohort.](image)

**Fig. 1** OTOF mRNA expression analysis in TCGA cohort. (a) OTOF expression was significantly upregulated in ccRCC compared with normal renal tissue ($P < 0.001$). Significantly higher expression levels were assessed in (b) higher stage and (e) higher grade ccRCC, as well as in tumors with (c) lymphatic or (d) hematogenous metastasis (all $P < 0.001$). (f) Kaplan–Meier estimates for OTOF mRNA expression with OS as an end-point. Expression levels were dichotomized using the median as the cut-off. Overexpression was significantly associated with poor OS (log-rank $P < 0.001$).
Statistical analysis

Statistical analyses (Mann–Whitney U-test, Kruskal–Wallis test, Kaplan–Meier Estimator, Cox regression analyses) were made using SPSS Statistics version 24 (IBM, Ehningen, Germany). Using the survMisc package for R (The R Foundation for Statistical Computing, Vienna, Austria), optimal cut-off values of mRNA expression were determined for survival analyses. The determination was based on the evaluation of all relevant cut-offs in the univariate Cox regression analysis. Statistical significance was concluded at P < 0.05.

Results

OTOF mRNA expression analysis: TCGA cohort

OTOF gene expression was analyzed in the TCGA cohort (81 benign and 514 ccRCC samples). OTOF expression was significantly increased in tumor compared with normal renal tissue (P < 0.001; Fig. 1a). High expression levels of OTOF mRNA in tumor tissue were correlated with parameters of tumor progression and aggressiveness: high expression was associated with pT-stage, presence of lymph node and distant metastases, as well as histological grade (all P < 0.001; Fig. 1b–e). Interestingly, OTOF expression increased gradually with advanced pT-stage and higher tumor grade (Fig. 1b, e). Furthermore, overexpression of OTOF was predictive of OS in Kaplan–Meier (log-rank P < 0.001; Fig. 1f), univariate Cox regression analyses, overexpression of OTOF was significantly correlated with a shortened CSS (log-rank P = 0.022; univariate Cox regression: HR 5.316, 95% CI 1.072–26.369, P = 0.041; Fig. 2a), and showed a non-significant trend for shortened OS (log-rank P = 0.069; univariate Cox regression: HR 2.960, 95% CI 0.864–10.137, P = 0.084; Fig. 2b). Furthermore, multivariate Cox regression analysis showed OTOF as an independent predictor of CSS in patients with ccRCC (HR 7.901, 95% CI 1.368–45.632, P = 0.021; Table 3). A relevance of OTOF expression on progression-free survival could not be observed.

OTOF protein expression analysis: IHC

Next, we investigated OTOF protein expression in a TMA cohort including 142 ccRCC and 30 normal renal tissues. In contrast to the expression ratio on the mRNA level, we found a significantly increased OTOF protein expression in normal tissue (median staining score 2.0) compared with ccRCC tissue (median staining score 1.0, P < 0.001; Fig. 3a). A representative image of the OTOF IHC is shown in Figure 4. High expression, pT-stage, pN-stage, pM-stage and histological grade (HR 1.958, 95% CI 1.331–2.881, P < 0.001; Table 2).

OTOF mRNA expression analysis: study cohort

We further investigated the mRNA expression of OTOF in the present study cohort to validate the results obtained in the TCGA cohort. Similar to the TCGA cohort, significantly increased expression levels were observed in tumor tissue (P < 0.001; Fig. 2a). Unfortunately, we did not observe associations with clinicopathological parameters or grading (all P > 0.05). However, the prognostic potential of OTOF was confirmed in the study cohort. As shown by Kaplan–Meier estimates and univariate Cox regression analyses, overexpression of OTOF was significantly correlated with a shortened CSS (log-rank P = 0.022; univariate Cox regression: HR 5.316, 95% CI 1.072–26.369, P = 0.041; Fig. 2a), and showed a non-significant trend for shortened OS (log-rank P = 0.069; univariate Cox regression: HR 2.960, 95% CI 0.864–10.137, P = 0.084; Fig. 2b). Furthermore, multivariate Cox regression analysis showed OTOF as an independent predictor of CSS in patients with ccRCC (HR 7.901, 95% CI 1.368–45.632, P = 0.021; Table 3). A relevance of OTOF expression on progression-free survival could not be observed.

Table 2 OTOF mRNA expression in TCGA cohort: OS in patients with ccRCC

| OTOF expression | Univariate analysis | | Multivariate analysis |
|-----------------|---------------------|-----------------|
|                 | P-value | HR (95% CI) | P-value | HR (95% CI) |
| <Median         | 1.0      |               | 1.0      |               |
| >Median         | <0.001   | 2.717 (1.880–3.927) | 0.001   | 1.958 (1.331–2.881) |
| pT-stage        | 0.128    | 1.549 (0.881–2.724) | 0.788  | 1.083 (0.606–1.935) |
| pT1             | 1.0      |               | 1.0      |               |
| pT2             | <0.001   | 3.673 (2.534–5.324) | 0.003  | 1.946 (1.250–3.030) |
| pT3             | <0.001   | 11.743 (5.689–24.239) | 0.140  | 2.111 (0.783–5.690) |
| pT4             | 1.0      |               | 1.0      |               |
| pN-stage        | <0.001   | 3.359 (1.708–6.606) | 0.395  | 1.436 (0.624–3.301) |
| pN0             | 1.0      |               | 1.0      |               |
| pN1             | <0.001   | 4.428 (3.186–6.153) | <0.001  | 2.103 (1.385–3.193) |
| Histological grade (WHO 2016)† | | |
| G1 + G2         | 1.0      |               | 1.0      |               |
| G3              | <0.001   | 2.113 (1.408–3.171) | 0.041  | 1.558 (1.019–2.381) |
| G4              | <0.001   | 5.921 (3.831–9.152) | 0.003  | 2.212 (1.312–3.728) |

†G1 (n = 14) patients were combined with G2 patients for Cox analysis. G1 patients were few and had uniformly good outcomes (all patients alive at the end of the follow up, no cancer-related events). Therefore, their use as reference group in Cox analysis will distort all statistics, resulting in infinite HRs and CIs.
protein expression levels correlated with tumor grading ($P = 0.006$, G1 vs G2/G3; Fig. 3b). Similar to the mRNA expression levels, patients with OTOF overexpression in tumor tissue (staining score >1) had an unfavorable survival. OTOF overexpression was negatively predictive of OS, CSS and PFS, as shown by Kaplan–Meier estimates (OS: log-rank $P = 0.034$; CSS: log-rank $P = 0.012$; PFS: log-rank $P < 0.001$; Fig. 3c–e) and univariate Cox regression analyses (OS: HR 2.370, 95% CI 1.037–5.417, $P = 0.041$; CSS: HR 5.881, 95% CI 1.221–28.334, $P = 0.027$; PFS: HR 4.017, 95% CI 1.791–9.008, $P = 0.001$). In multivariate analyses (protein expression, pT-stage, pN-stage and histological grade), OTOF overexpression showed independent prognostic significance for PFS (HR 4.213, 95% CI 1.480–11.961, $P = 0.007$; Table 4).

The poor prognosis of patients with renal cell carcinoma with sarcomatoid features and the consequent urgency to find specific treatment options have prompted us to additionally investigate OTOF expression in a small cohort of patients with sarcomatoid renal cell carcinoma. Although we found a positive correlation of OTOF expression with histological grade in ccRCC, there was no significant difference in expression level in sarcomatoid renal cell carcinoma compared with ccRCC (median staining score 1.0).

**Discussion**

OTOF (fer1-like family member 2) is the second ferlin family member and indispensable for the functionality of the
auditory system. OTOF is predominantly expressed in intracellular membrane compartments, as it participates in the endocytic traffic from the trans-Golgi network to the plasma membrane and inversely. As intrinsic membrane proteins with multiple tandem C2 domains, ferlins are involved in the Ca\(^{2+}\)-regulated vesicle fusion. OTOF is primarily expressed in the inner hair cells of the mammalian cochlea, where it plays a key role in Ca\(^{2+}\)-dependent auditory neurotransmission. OTOF was shown to interact with t-SNARE proteins (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) in the process of synaptic vesicle cycle by serving as a calcium sensor for SNARE-mediated membrane fusion. In the inner hair cells of OTOF knockout mice, synaptic vesicle exocytosis is almost completely blocked, resulting in profound deafness of these mice. The proper function of OTOF is also important for the human auditory system. The only disease in humans associated with OTOF deficiency is DFNB9, a non-syndromic form of deafness, caused by mutations in the OTOF gene. Although it has been shown that OTOF mRNA is expressed in various tissues, to date only this disease phenotype could be detected. Unlike some other ferlin members, little is known about the relevance of OTOF in cancer. In the present study, we thus carried out a comprehensive evaluation of OTOF mRNA and protein expression in ccRCC and normal renal tissue in three independent cohorts to correlate its expression pattern with clinicopathological features and patient survival.

On the mRNA level, we showed that OTOF expression is increased in advanced stage, higher grade and metastatic ccRCC.

![Fig. 3](image-url) OTOF protein expression analysis in IHC cohort. (a) OTOF expression was downregulated in tumor tissue (median staining score 1; P < 0.001). (b) High protein expression was correlated with histological grade (P = 0.006). (c–e) Kaplan–Meier estimates for OTOF protein expression with OS, CSS or PFS as an end-point. The cut-off to determine an overexpression was set at staining intensity >1. OTOF overexpression was predictive of lower OS (log-rank P = 0.034), CSS (log-rank P = 0.012) and PFS (log-rank P < 0.001).
(lymphatic and hematogenous spread), which is accompanied by an independent prognostic significance for OS. Furthermore, the expression analysis in the present study cohort revealed OTOF as an independent prognostic indicator for shortened CSS. On the protein level, higher OTOF expression correlated with higher histological grade, and a shortened OS, CSS and PFS, which is in accordance with our mRNA findings. In a multivariate model, OTOF overexpression was independently predictive of progression-free survival. Interestingly, we observed an inverse expression pattern on the protein level; that is, lower OTOF expression levels in tumor compared with normal renal tissue. Possibly, this difference on the protein level can be attributed to post-transcriptional modifications of the mRNA molecule or interactions of, for example, microRNAs in the tumor cell that lead to a lower translation rate. However, the malignancy of the OTOF protein seems to be unaffected, as overexpression in ccRCC remained to be of prognostic relevance. Hence, the potential as a diagnostic biomarker on the protein level is currently not sufficient to replace established IHC markers and to complement pure microscopy in clinical routine. On the mRNA level, however, altered mRNA expression levels of OTOF might be useful to distinguish between renal cell carcinoma and normal renal tissue, and to determine high-grade and aggressive features.

To date, OTOF has only been functionally investigated in connection with auditory neurotransmission. Nothing is known about its biological role in carcinogenesis or tumor progression. However, expression analyses of other ferlin members in several tumor tissues revealed different interacting partners or signaling pathways that contribute to tumor development. For instance, an involvement of the long non-coding RNA Fer1L4 in the Pi3K/AKT pathway in osteosarcoma and lung cancer was found. In colon and gastric cancer, Fer1L4 was shown to regulate the expression of PTEN through associating with miR-106-5p or miR-18a-5p. For MYOF, a connection to the tyrosine kinase receptor pathway of the epidermal growth factor receptor has been described in ccRCC. Whether the oncogenic impact of OTOF in ccRCC can also be linked to these mentioned signaling pathways and molecules needs to be investigated in further functional studies. As OTOF is involved

![Fig. 4 OTOF protein expression analysis by IHC.](Image)

Table 4 OTOF protein expression in IHC cohort: PFS in patients with ccRCC

|                                      | Univariate analysis |                      | Multivariate analysis |
|--------------------------------------|---------------------|----------------------|-----------------------|
|                                      | P-value             | HR (95% CI)          | P-value               | HR (95% CI)          |
| OTOF expression > Median              | 0.001               | 4.017 (1.791–9.008)  | 0.007                 | 4.213 (1.480–11.961) |
| pT-stage T1                           | 1.0                 | –                    | –                     | –                    |
| pT-stage T2                           | 0.210               | 1.989 (0.680–5.822)  | 0.246                 | 3.589 (0.414–31.101) |
| pT-stage T3 + T4                      | 0.019               | 3.333 (1.218–9.117)  | 0.063                 | 7.376 (0.899–60.492) |
| pN-stage N0                           | 1.0                 | –                    | –                     | –                    |
| pN-stage N1                           | 0.007               | 4.631 (1.518–14.131) | 0.926                 | 1.092 (0.172–16.457) |
| pM-stage M0                           | 0.001               | 8.627 (3.725–19.982) | 0.222                 | 2.292 (0.521–63.504) |
| Histological grade (WHO 2016) G1     | 1.0                 | –                    | –                     | –                    |
| G2                                   | 0.037               | 3.087 (1.071–8.897)  | 0.370                 | 1.831 (0.487–6.855)  |
| G3                                   | 0.003               | 13.796 (2.403–79.192)| 0.021                 | 48.974 (1.788–1341.298)|
in membrane fusion events as a calcium-sensing protein, the link between calcium signaling and cancer might be an interesting approach for future investigations.

The results reported herein should be considered in light of some limitations. The small size of the study validation cohort, as well as the inhomogeneous distribution of clinicopathological data, should be mentioned here as a priority. Although the present study provides the first evaluation of OTOF expression in ccRCC and its link to clinicopathological parameters and survival, questions regarding the functionality of this protein in tumor development remain open. Functional studies considering interacting proteins and miRNA should follow to confirm the impact of OTOF on survival and to elucidate its involvement in the tumor biology of renal cell carcinoma for the response to future treatment options.

Our work is to be considered as the first study on the role of OTOF in ccRCC. We provided a comprehensive evaluation of OTOF expression on both mRNA and protein levels. In summary, the present study highlighted OTOF as an indicator of tumor aggressiveness and as a prognostic biomarker for patients with ccRCC leading to the conclusion that OTOF could promote the malignancy of ccRCC.

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Conflicts of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Positive and negative controls of OTOF IHC. (a) Normal renal tissue serves as multistage control of both positive and negative staining. Glomeruli: negative staining. Proximal tubules: weak staining. Distal tubules: strong staining. (b) Tonsillar and (c) liver tissue represent negative controls. Both show no staining. Object magnification: ×10. Scale bar: 50 μm.