The $N^\alpha$-methyladenosine (m$\alpha$A) erasers alkylation repair homologue 5 (ALKBH5) and fat mass and obesity-associated protein (FTO) are prognostic biomarkers in patients with clear cell renal carcinoma

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Objectives
To comprehensively investigate the role of the $N^\alpha$-methyladenosine (m$\alpha$A) erasers ALKBH5 and FTO in clear cell renal cell carcinoma (ccRCC), other RCC subtypes, and oncocytoma with respect to prognostic value and biomarker potential.

Patients and Methods
The collection of tissue samples was performed within the framework of the Biobank at the Centre for Integrated Oncology Cologne-Bonn. The gene expressions of alkylation repair homologue 5 (ALKBH5) and fat mass and obesity-associated protein (FTO) were determined using quantitative real-time polymerase chain reaction. ALKBH5 and FTO expressions were further investigated in ccRCC, papillary RCC, chromophobe RCC, sarcomatoid RCC, oncocytoma, and benign renal tissue using tissue microarrays.

Results
ALKBH5 mRNA, as well as ALKBH5 and FTO protein expressions, was significantly downregulated in ccRCC compared to normal tissue and most of the other studied tumour entities. Decreased mRNA levels of ALKBH5 and FTO correlated with a shortened overall and cancer-specific survival following nephrectomy.

Conclusions
Taken together, our present data indicate that the m$\alpha$A-demethylases ALKBH5 and FTO are dysregulated in ccRCC and could be used as prognostic biomarkers.

Keywords
m$\alpha$A, ccRCC, ALKBH5, FTO, biomarker

Introduction
Renal tumours are amongst the most common malignancies: 73 820 new cases and 14 770 deaths were estimated by the American Cancer Society in the USA for the year 2019 [1]. Thereof, RCC is the most common kidney cancer, of which ~80% is accounted for by the clear cell subtype (ccRCC) [2]. While patients with localised RCC are usually treated with curative intent, cure is mostly not attainable in patients with metastatic disease. By introducing targeted antiangiogenic and immune therapies significant therapeutic improvement has been made, but optimal sequencing of therapeutics is still unknown [2]. A more profound understanding of altered gene expression and RNA metabolism in ccRCC may lead to the discovery of a potential biomarker, which could help to provide an individualised therapy for each patient.

$N^\alpha$-methyladenosine (m$\alpha$A) is the most abundant RNA-modification in eukaryotic and human RNA, which was described decades ago [3]. In recent years, m$\alpha$A has gained increasing attention as scientists started to realise its extensive biological importance and vast prognostic and therapeutic potential. Similar to DNA and protein modifications, RNA methylation with m$\alpha$A is a reversible and continuous process, and is established by the interplay of methyltransferases (e.g. methyltransferase like 3 [METTL3]) and demethylases (e.g. alkylation repair homologue 5 [ALKBH5] and fat mass and obesity-associated protein [FTO]; also termed m$\alpha$A erasers), and several m$\alpha$A-binding proteins [4]. There are numerous studies suggesting a crucial role of m$\alpha$A and its modifiers for RNA metabolism at almost every cellular level including maturation, nuclear export, splicing, and decay [5–7]. In its broad biological function, it also impacts complex processes...
like meiosis, stem cell differentiation, and even circadian rhythm maintenance [8]. The m^6^A modification is involved in cancer initiation, tumour progression, and prognosis. Its dysregulation has been linked to diseases such as leukaemia, lung cancer, breast cancer, brain tumours, and pancreatic cancer [9–12]. Small-molecule targeting of m^6^A regulators revealed promising anticancer effects and are being proposed as a potential future treatment [13,14]. Recently, a retrospective study was published that showed for the first time that alterations of m^6^A modifiers impact the survival of patients with ccRCC [15]. But still little is known about m^6^A modifiers in ccRCC; therefore, in the present study, we investigated the expression of the RNA demethylases ALKBH5 and FTO in ccRCC.

**Patients and Methods**

**Patients**

The collection of tissue samples was performed within the framework of the Biobank at the Centre for Integrated Oncology (CIO) Cologne-Bonn. All patients underwent radical or partial nephrectomy at the Department of Urology at the University Hospital Bonn. Written informed consent for the collection of biomaterials was obtained from all patients. The study was approved by the Ethics Committee of the University of Bonn (vote: 127/17).

Fresh-frozen tissues from ccRCC and corresponding normal renal parenchyma were stored at −80 °C and used for mRNA expression studies. Archival formalin-fixed and paraffin-embedded tissues were used for immunohistochemistry. All tissues were re-evaluated by a pathologist and classified according the 2009 WHO classification. See Table 1 for clinicopathological parameters.

**Quantitative Real-time PCR**

RNA isolation was described in detail earlier [16]. In brief, total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) and treated with DNase (Ambion). The RNA quantity was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). The RNA integrity was determined by evaluation of the 28S and 18S ribosomal RNA bands in a gel electrophoresis. The gene expressions of ALKBH5 and FTO were determined by evaluation of the 28S and 18S ribosomal RNA bands in a gel electrophoresis.

The expression of ALKBH5 and FTO in ccRCC, papillary RCC (pRCC), chromophobe RCC (chRCC), sarcomatoid RCC, oncocytoma and benign renal tissue using a tissue microarray (TMA) as described previously [18]. In brief, paraffin sections were cut at 5-μm thickness, deparaffinised using xylene and rehydrated in graded ethanol. Slides were placed in citrate buffer (pH 6.0) and heated for 10 min at boiling temperature (microwave 600 W). After 30 min resting time and cooling for 15 min, the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The sections were washed with Tris-buffered saline and Tween 20 (Merck KGaA, Darmstadt, Germany). The slides were incubated with the primary antibodies (ALKBH5 1:200; FTO 1:1000) at 4 °C overnight. Signal detection was performed with Dako Envision + System-HRP labelled polymer (Dako, Hamburg, Germany) and the slides were finally counterstained using Meyer’s haematoxylin. The ALKBH5 and FTO staining intensities were evaluated semi-quantitatively.

**Immunohistochemistry**

ALKBH5 and FTO expressions were further investigated in ccRCC, papillary RCC (pRCC), chromophobe RCC (chRCC), sarcomatoid RCC, oncocytoma and benign renal tissue using a tissue microarray (TMA) as described previously [18]. In brief, paraffin sections were cut at 5-μm thickness, deparaffinised using xylene and rehydrated in graded ethanol. Slides were placed in citrate buffer (pH 6.0) and heated for 10 min at boiling temperature (microwave 600 W). After 30 min resting time and cooling for 15 min, the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The sections were washed with Tris-buffered saline and Tween 20 (Merck KGaA, Darmstadt, Germany). The slides were incubated with the primary antibodies (ALKBH5 1:200; FTO 1:1000) at 4 °C overnight. Signal detection was performed with Dako Envision + System-HRP labelled polymer (Dako, Hamburg, Germany) and the slides were finally counterstained using Meyer’s haematoxylin. The ALKBH5 and FTO staining intensities were evaluated semi-quantitatively.

**Table 1** Clinicopathological characteristics of the PCR study cohort.

| Characteristic                      | ccRCC 166 (61.9) | PCR cohort Normal 102 (38.1) |
|------------------------------------|------------------|-------------------------------|
| Sex, n (%)                         |                  |                               |
| Male                               | 108 (65.1)       | 71 (69.6)                     |
| Female                             | 58 (34.9)        | 31 (30.4)                     |
| Age, years, mean (range)           | 64.5 (27–89)     | 63.6 (36–89)                  |
| pT Stage, n (%)                    |                  |                               |
| pT1                                | 95 (57.2)        | –                             |
| pT2                                | 16 (9.6)         | –                             |
| pT3                                | 52 (31.3)        | –                             |
| pT4                                | 3 (1.8)          | –                             |
| Lymph node metastasis, n (%)       |                  |                               |
| pN0                                | 161 (97)         | –                             |
| pN1                                | 5 (3)            | –                             |
| Distant metastasis, n (%)          |                  |                               |
| M0                                 | 144 (86.7)       | –                             |
| M1                                 | 22 (13.3)        | –                             |
| Grade, n (%)                       |                  |                               |
| Grade 1                            | 21 (12.7)        | –                             |
| Grade 2                            | 108 (65.1)       | –                             |
| Grade 3                            | 28 (16.9)        | –                             |
| Grade 4                            | 9 (5.4)          | –                             |
quantitatively by two different investigators using QuPath software [19].

Statistical Analysis
Statistical analyses (t-test, Mann–Whitney U-test, Cox regression analyses, Kaplan–Meier estimates) were performed, as appropriate, with the Statistical Package for the Social Sciences (SPSS®, version 25 (SPSS Inc., IBM Corp., Armonk, NY, USA). Statistical significance was accepted at $P < 0.05$.

Results
ALKBH5 and FTO mRNA Expressions
Gene expressions of ALKBH5 and FTO were studied in 166 ccRCC and 106 normal renal tissues. ALKBH5 was significantly reduced in RCC compared to normal tissue ($P < 0.001$), while FTO expression levels were similar in both tissues ($P = 0.856$). The Spearman’s rank-order correlation test also showed a significant correlation between ALKBH5 and FTO levels ($P < 0.001$; Fig. 1).

Furthermore, both ALKBH5 ($P = 0.007$) and FTO ($P = 0.034$) were correlated with clinical M Stage in patients with ccRCC (Fig. 2). We did not observe associations with other clinicopathological parameters or grading (all $P > 0.05$). A prognostic potential for both genes was also indicated by Kaplan–Meier estimates: low expression levels of ALKBH5 and FTO were correlated with a shorter overall survival (OS: ALKBH5 log-rank $P = 0.007$; FTO log-rank $P = 0.033$) and cancer-specific survival (CSS: ALKBH5 log-rank $P = 0.018$; FTO log-rank $P = 0.029$; Fig. 3). Furthermore, univariate Cox regression analysis revealed ALKBH5 and FTO as predictors of survival.
Fig. 3 Low expression levels of ALKBH5 and FTO mRNA correlate with poor overall (A) and cancer-specific (B) survival.

ALKBH5 and FTO Protein Expressions

Immunohistochemical staining was performed using a TMA consisting of 147 ccRCC, 31 pRCC, 10 chRCC, 13 sarcomatoid RCC, 10 oncocytoma, and 30 normal renal tissues. The immunohistochemistry showed strong nuclear and weak to moderate cytoplasmic staining for both FTO and ALKBH5 see (Fig. S3). As expected, protein levels of both ALKBH5 ($P = 0.009$) and FTO ($P < 0.001$) were significantly decreased in ccRCC compared to benign tissue (Fig. 4). We did not observe associations with any clinicopathological parameters (all $P > 0.05$). Kaplan–Meier estimates indicated an insignificant trend towards shorter OS in patients with high FTO levels ($P = 0.079$). We could not find any prognostic information for ALKBH5.

ALKBH5 and FTO expressions were varied amongst RCC subtypes (Fig. 5). FTO protein levels in pRCC were elevated in tumours compared to benign tissue ($P = 0.009$). Compared
to ccRCC, both ALKBH5 (P = 0.003) and FTO (P < 0.001) protein levels were increased. FTO levels in chRCC were decreased compared to normal tissue (P < 0.001). ALKBH5 was increased compared to ccRCC (P = 0.012). In sarcomatoid RCC, both demethylases were reduced compared to normal tissue (ALKBH5 P > 0.001 and FTO P = 0.071). There was no difference to ccRCC.

ALKBH5 levels in oncocytoma were significantly elevated compared to normal tissue (P = 0.048), ccRCC (P < 0.001), and sarcomatoid RCC (P = 0.002). There was no significant difference to pRCC and chRCC. The amount of FTO was also increased in comparison with ccRCC (P < 0.001), sarcomatoid RCC (P = 0.001), and chRCC (P = 0.002).

The Spearman’s rank-order correlation test demonstrated a significant correlation between ALKBH5 and FTO for all RCC entities and for oncocytoma (ccRCC P < 0.001; pRCC P = 0.021; chRCC P = 0.001; sarcomatoid RCC P < 0.001; oncocytoma P < 0.001). We could not find any associations to other clinicopathological parameters for any entity.

**Discussion**

m^6^A plays a crucial role in tumorigenesis of different types of cancer. Several enzymes, including the erasers ALKBH5 and FTO, regulate m^6^A modification. They both belong to the AlkB homologue family and are classified as 2-oxoglutarate and iron-dependent nucleic acid oxygenases [20].

ALKBH5 catalyses the removal of the m^6^A modification on nuclear RNA (mostly mRNA). This demethylation activity subsequently affects nuclear RNA export and metabolism, gene expression, and even mouse fertility [6,21]. Furthermore, the AlkB homologues 1–8 and FTO have been shown to be able to repair several different DNA- and RNA-lesions [20]. Taken together this clearly indicates a broad biological function of this m^6^A regulator. ALKBH5 is highly expressed in glioblastoma stem cells and enhances cellular proliferation [21]. ALKBH5 knockdown in breast cancer also reduces the number of breast cancer stem cells [11]. ALKBH5 mRNA overexpression is a prognostic biomarker in pancreatic cancer [12]. This underlines that ALKBH5 is not only very likely to play a crucial role in the initiation and progress of cancer, but that it might even function as a future biomarker or even therapeutic target.

FTO (also known as alpha-ketoglutarate-dependent dioxygenase [ALKBH9]) also catalyses a wide range of biological oxidations [5,22]. FTO is known to affect the genesis of metabolic diseases and human obesity [23]. It was
the first m^6A-RNA demethylase discovered and shown to exhibit high demethylation activity toward m^6A. Further investigation revealed that FTO significantly impacts the amount of m^6A in a human cell [24]. FTO is overexpressed in acute leukaemia, cervical and breast cancer [9,25,26]. FTO inhibition in glioblastoma stem cells decreased cell growth and tumour progression [10]. Huang et al. [14] even performed small-molecule targeting of FTO, which also suppresses proliferation of acute myeloid leukaemia cell lines in vitro and indicates the potential for treatment.

In the present study, we found dysregulation of both demethylases in ccRCC: mRNA as well as protein expression of ALKBH5 and FTO were significantly reduced in ccRCC compared to normal tissue. This is contradictory to other cancer entities, where cancer cells were overexpressing the m^6A eraser genes [8,27].

Our present results are supported by the Kidney Renal Clear Cell Carcinoma (KIRC) The Cancer Genome Atlas (TCGA) dataset, in which we were able to find that low FTO expression significantly correlates with a poor OS (P = 0.014), while decreased ALKBH5 expression shows a strong tendency towards a shortened survival (P = 0.095). See Fig. S1 for details.

Downregulation of ALKBH5 and FTO may be explained by epigenetic and genetic alterations: the MEXPRESS software revealed that some CpGs sites within both genes are methylated and correlate with decreased gene expression levels [28] (see Fig. S2 for details). Copy number alterations also accompanied gene expression levels.

Furthermore, the mRNA expression indicated a prognostic role of ALKBH5 and FTO expression: low levels of both m^6A erasers significantly correlated with metastatic RCC and a poor OS and CSS.

Eventually, we found a dysregulation in the amount of protein of both demethylases in all other studied RCC subtypes including pRCC, chRCC, sarcomatoid RCC, and oncocytoma. Protein levels in ccRCC were lower than in all of them except for the sarcomatoid subtype, which is consistent with our previous findings, underlining a downregulation of both erasers in ccRCC in general.
Especially the increased expression of both ALKBH5 and FTO in oncocytoma as a benign renal lesion might be of further interest in future studies.

However, we were not able to show a significant prognostic relevance of FTO/ALKBH5 protein expression, although there was a non-significant trend towards poorer OS in patients with increased FTO expression.

Our present results also lack associations to several important clinicopathological parameters and we did not find that either ALKBH5 or FTO were independent predictors of patient survival.

To further and fully understand the biological role of the m^6^A erasers in ccRCC, a thorough investigation of the role of ALKBH5 and FTO will be needed.

**Acknowledgements**

The tissue samples were collected within the framework of the Biobank of the IOC Cologne-Bonn at the University Hospital Bonn.

**Conflicts of Interest**

None disclosed.

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**Abbreviations:** ALKBH5, alkylation repair homologue 5; cDNA, complementary DNA; ccRCC, clear cell RCC; chRCC, chromophobe RCC; CIO, Centre for Integrated Oncology;
FTO, fat mass and obesity-associated protein; m6A, N6-methyladenosine; pRCC, papillary RCC; sRCC, sarcomatoid RCC.

Supporting Information
Additional Supporting Information may be found in the online version of this article:

Fig. S1. Analysis of overall survival with respect to the gene expression level of ALKBH5 and FTO in the TCGA ccRCC cohort.

Fig. S2. The MEXPRESS software was used to determine ALKBH5 and FTO gene expression and promoter DNA methylation levels in the TCGA ccRCC cohort.

Fig. S3. The immunohistochemistry showed strong nuclear and weak-to-moderate cytoplasmic staining for both FTO and ALKBH5.