A systematic search strategy identifies cubilin as independent prognostic marker for renal cell carcinoma

Gabriela Gremel 1, Dijana Djureinovic 1, Marjut Niinivirta 2, Alexander Laird 3,4, Oscar Ljungqvist 5, Henrik Johannesson 5, Julia Bergman 1, Per-Henrik Edqvist 1, Sanjay Navani 6, Naila Khan 6, Tushar Patil 6, Åsa Sivertsson 7, Mathias Uhlén 7, David J. Harrison 8, Gustav J. Ullenham 2, Grant D. Stewart 4,10 and Fredrik Pontén 1,9*

Abstract

Background: There is an unmet clinical need for better prognostic and diagnostic tools for renal cell carcinoma (RCC).

Methods: Human Protein Atlas data resources, including the transcriptomes and proteomes of normal and malignant human tissues, were searched for RCC-specific proteins and cubilin (CUBN) identified as a candidate. Patient tissue representing various cancer types was constructed into a tissue microarray (n = 940) and immunohistochemistry used to investigate the specificity of CUBN expression in RCC as compared to other cancers. Two independent RCC cohorts (n = 181; n = 114) were analyzed to further establish the sensitivity of CUBN as RCC-specific marker and to explore if the fraction of RCCs lacking CUBN expression could predict differences in patient survival.

Results: CUBN was identified as highly RCC-specific protein with 58% of all primary RCCs staining positive for CUBN using immunohistochemistry. In venous tumor thrombi and metastatic lesions, the frequency of CUBN expression was increasingly lost. Clear cell RCC (ccRCC) patients with CUBN positive tumors had a significantly better prognosis compared to patients with CUBN negative tumors, independent of T-stage, Fuhrman grade and nodal status (HR 0.382, CI 0.203–0.719, P = 0.003).

Conclusions: CUBN expression is highly specific to RCC and loss of the protein is significantly and independently associated with poor prognosis. CUBN expression in ccRCC provides a promising positive prognostic indicator for patients with ccRCC. The high specificity of CUBN expression in RCC also suggests a role as a new diagnostic marker in clinical cancer differential diagnostics to confirm or rule out RCC.

Keywords: Cubilin, Renal cell carcinoma, Independent prognostic biomarker, Immunohistochemistry

Background

The Human Protein Atlas project has generated a comprehensive map of global gene expression patterns in normal tissues [1]. Through integration of antibody-based, spatial proteomics and quantitative transcriptomics, expression and localization of more than 90% of all human protein-coding genes have been analyzed. Whereas the majority of proteins show a widespread expression profile, subsets of tissue-enriched proteins have been defined [2], including proteins with enriched expression in the kidney [3]. To facilitate screening and discovery efforts for cancer-relevant proteins, the Human Protein Atlas also contains immunohistochemistry-based protein expression profiles for the 20 most common forms of cancer [4].

Renal cell carcinoma (RCC) is the most common type of cancer affecting the kidney. Several histological subtypes of RCC have been defined, the most frequent being clear cell RCC (ccRCC) [5]. Diagnosis and subtyping of RCC are achieved through the morphological analysis of tumor sections. The application of immunohistochemistry (IHC) can reveal important additional clues during the diagnostic work-up. A variety of antibodies have been described to guide pathologists during the diagnosis of
distant metastases from the kidney, to distinguish primary RCCs from benign mimics, and to differentiate RCC from malignancies derived from other retroperitoneal structures [6]. Most recently, PAX8 and PAX2 have shown improved RCC-specificity over the traditionally used RCC markers CD10 and RCC monoclonal antibody, although several female genital tract and thyroid tumors stain positive for both markers [7, 8].

The clinical risk stratification of RCC patients relies heavily on the assessment of histopathological parameters. Clear cell histology is significantly associated with a more aggressive disease progression and reduced overall survival [5]. For the prediction of recurrence in patients with localized ccRCC, algorithms were developed by teams at Memorial Sloan-Kettering Cancer Center (based on tumor stage, nuclear grade, tumor size, necrosis, vascular invasion and clinical presentation) [9] or the Mayo Clinic (based on tumor stage, tumor size, nuclear grade and histological tumor necrosis) [10]. More recently, gene expression signatures have been proposed to add prognostic value to conventional algorithms [11, 12].

The aim of this study was to utilize the vast data resources generated by the Human Protein Atlas project to identify novel biomarkers of clinical relevance for patients with RCC. Cubilin (CUBN) was identified and validated as a marker with the potential to classify RCC patients into low- and high-risk groups, as loss of CUBN expression was significantly and independently associated with less favorable patient outcome. In addition, CUBN expression appears highly specific for RCC compared to other types of cancer, rendering CUBN a possible clinical role in cancer differential diagnostics.

**Methods**

**Human Protein Atlas database searches**

Global mRNA expression data for 27 normal human tissues [1] was searched for genes specifically expressed in normal kidney and a maximum of six additional tissues. Genes with >5-fold higher fragments per kilobase of transcript per million mapped reads (FPKM) levels in normal human kidney compared to all other tissues and genes with 5-fold higher average FPKM level within a group of 2–7 tissues, including normal human kidney, were investigated further. Corresponding IHC-based expression data within the Human Protein Atlas database (www.proteinatlas.org and unpublished data) was evaluated manually.

Similarly, proteome-wide IHC-based expression data for 83 normal human cell types, corresponding to 44 normal tissues, was searched for proteins expressed in renal tubules or glomeruli and a maximum of nine additional cell types. Retention of protein expression in RCC was evaluated manually. IHC-based expression data for 216 cancer tissues, including up to 12 cases of RCC, were systematically queried for antibodies yielding positive IHC-staining primarily in RCC. Database searches were conducted using varying positive/negative definitions (e.g. negative or weak staining as cut-off) and various levels of specificity (e.g. staining in 50% or 75% of RCC cases and less than 10% or 25% of any other cancer type, respectively).

**Patient cohorts**

Initially, a tissue microarray (TMA) containing tumor material from 39 patients with available, corresponding transcriptomics data and protein lysates was used (Additional file 1: Table S1). In addition, three independent TMA cohorts were used. Cohort 1 was a multi-cancer cohort including 940 tumor samples, representing 22 different tumor sites (Additional file 2: Table S2, [13]). Formalin-fixed, paraffin-embedded (FFPE) tumor specimens were identified from the archives of Uppsala University Hospital, Falun Hospital and Lund University Hospital, where all cases were originally diagnosed between 1984 and 2011. A large fraction of samples (502 tumors) represented material from metastatic sites. For RCC, 20 primary tumors and 20 metastases were included. Cohort 2 included 167 primary, 103 venous tumor thrombi and 96 metastatic tumors from 183 RCC patients following radical nephrectomy at the Department of Urology, Edinburgh, between 1983 and 2010 (Additional file 3: Table S3, [14]). Written consent was obtained from study participants from cohort 2. Cohort 3 was assembled from 114 primary ccRCC samples (Additional file 3: Table S3) from patients diagnosed with metastatic RCC between 2006 and 2010 at one of seven Swedish medical centers (Uppsala, Göteborg, Örebro, Västerås, Gävle, Falun, Karlstad). All patients within this cohort had undergone a radical nephrectomy. Written consent was obtained from study participants from cohort 3.

**Tissue microarray construction, immunohistochemistry and annotation**

TMAs were constructed as described previously [14, 15]. Two antibodies targeting CUBN were tested (HPA043854 and HPA004133, Atlas Antibodies AB, Stockholm, Sweden). Automated IHC was performed as described previously [15]. IHC staining intensities and fractions of stained tumor cells were manually evaluated and each core annotated by two independent observers. Due to the large number of annotations this task was shared within a group of three observers (TP, NK, GG). Cases with divergent scores were reviewed by a third observer (DD) and consensus reached. Total cellular staining (including cytoplasm and cell membrane) was annotated. Cases were considered positive for CUBN if the fraction of stained cells was greater than 10% and the staining intensity showed at least moderate intensity.
RNA expression and Western blot analysis
RNA expression analyses were performed as described previously [2]. Western blot analysis was performed according to standard protocols.

Statistical analysis
For the calculation of sensitivity, specificity and positive predictive value (PPV) standard formulas were applied [16]. Kaplan–Meier survival curves were generated to evaluate the correlation between CUBN expression and patient survival. The log-rank test was used to compare patient survival in groups stratified according to CUBN expression. Cox proportional-hazards regression was applied to estimate hazard ratios in univariate and multivariate models. The \( \chi^2 \) test and Fisher's exact test were used to calculate the significance of associations between CUBN expression and clinicopathological parameters. Calculations were carried out using SPSS Statistics Version 22 (IBM, Armonk, NY).

Results
Target identification and antibody validation
The initial focus of this study was to identify kidney-specific proteins whose expression was partly or completely retained in RCC, a prerequisite for an RCC biomarker with prognostic and/or diagnostic value. Following searches within the Human Protein Atlas database, 15 proteins with preferential expression in RCC compared to all other included cancer types were identified (Additional file 4: Table S4). Following systematic antibody validation and immunohistochemical analysis of various TMA cohorts, CUBN was determined as the protein with the highest level of selective expression in RCC (Fig. 1).

Fig. 1 CUBN discovery pipeline and the standard Human Protein Atlas cancer test set. a The Human Protein Atlas database (www.proteinatlas.org and unpublished data) was systematically searched for cancer type-specific proteins using automated and manual searches. Staining patterns were reviewed and 15 proteins with RCC-enriched expression chosen for further antibody validation. Following extensive antibody validation and exclusion of antibodies with overlapping staining patterns, three antibodies were selected for validation of RCC-specific staining on multi-cancer TMA cohort 1. Two of these biomarkers were validated further on independent RCC-specific cohorts (cohort 2 and 3) and CUBN identified as highly RCC-specific protein. b CUBN staining on routine Human Protein Atlas cancer test set. Two antibodies, HPA004133 and HPA043854, targeting different epitopes on the same protein generated similar staining patterns. Red, orange and yellow coloring indicates cases with strong, moderate and weak staining, respectively. Grey corresponds to CUBN negative cases.
Two antibodies targeting CUBN underwent rigorous quality control measures. A comparison of mRNA and IHC-based expression levels in normal human tissues confirmed the specific expression of CUBN in kidney and small intestine (Additional file 5: Figure S1, [17]). Both antibodies specifically stained the proximal tubules of the kidney (Fig. 2a, [18]). Within the test TMA cohort IHC staining intensities correlated well with mRNA expression levels in the same tissues (Fig. 2a and b) and both antibodies produced a Western blot signal at approximately 460 kDa, the molecular weight of CUBN, which was only detected in IHC and RNA positive tissues (Fig. 2c). Additional signals at lower molecular weight were observed for both antibodies in samples with confirmed CUBN expression. These signals were regarded as products of protein degradation. Overall, both antibodies targeting CUBN showed high detection specificity and clone HPA043854 was used for further analyses.

**CUBN as RCC-specific protein**
A multi-cancer TMA cohort (cohort 1) was used to substantiate the RCC-specific expression of CUBN. CUBN staining was almost exclusively observed in RCC (Table 1) where 22 out of 39 cases (56%) were annotated as positive. Only one additional case of head and neck cancer (of 20 cases) stained positive for CUBN. This translated to a detection specificity of 100% and PPV of 96% for CUBN in RCC within this cohort. Approximately half of the included RCC samples in cohort 1 were of metastatic origin (20 out of 39 samples) and the expression of CUBN was well maintained in this setting (Additional file 6: Table S5). To further investigate the expression of CUBN during RCC progression, cohort 2 was analyzed. In primary tumors, a similar rate of CUBN positivity (58%) was observed, compared to cohort 1 (Additional file 6: Table S5). However, the number of CUBN positive cases significantly ($P < 0.001$) decreased from venous tumor thrombi with 39% CUBN positivity to metastatic samples with a positivity rate of 29%. Cohort 3 consisted of primary RCC material only with 60% of cases staining positive for CUBN (Additional file 6: Table S5).

**CUBN as marker for good prognosis in ccRCC**
Next, we investigated the prognostic relevance of CUBN in RCC. Patient survival information was available for two RCC cohorts (cohorts 2 and 3). Since all cases in cohort 3 and the majority of cases in cohort 2 were ccRCCs, we focused our analyses on this subtype. In cohort 2, stratification of patients according to CUBN positivity showed significant benefit for patients with CUBN positive tumors regarding both, overall survival ($P < 0.001$, Fig. 3a) and ccRCC-specific survival ($P < 0.001$, Fig. 3b). A similar effect was seen in cohort 3, where CUBN positive patient samples were linked to significantly longer overall survival ($P < 0.001$, Fig. 3c). For cohort 3, ccRCC-specific survival information was not available. Instead, the metastasis-free survival...
survival of patients initially presenting with localized disease was queried. There was no significant association of metastasis-free survival and CUBN expression overall ($P = 0.226$, Fig. 3d). However, CUBN positive ccRCC patients experienced a significant short-term metastasis-free survival benefit with $P = 0.01$ at 1-year follow-up and $P = 0.048$ at 5-years follow-up (Fig. 4).

Association of CUBN positivity with clinicopathological parameters and multivariate survival analysis in ccRCC

In cohort 3, positive CUBN staining was significantly associated with localized disease (Table 2, $P = 0.009$). A similar analysis in cohort 2 was not significant ($P = 0.317$). However, this may be due to the small number of patients that presented with distant metastases at diagnosis within this cohort. In cohort 2, the expression of CUBN was related to various other clinicopathological parameters (Table 2). A significant correlation was observed between positive CUBN expression and lower Fuhrman grade ($P = 0.006$) and negative nodal status ($P = 0.006$). No significant association between CUBN expression and T-stage was seen. For cohort 3 similar clinicopathological data were not available.

Univariate Cox regression analysis confirmed the relevance of CUBN as good prognostic marker for overall survival (Table 3, HR $0.411$, 95% CI $0.263$–$0.641$, $P < 0.001$), and ccRCC-specific survival (Additional file 7: Table S6, HR $0.334$, 95% CI $0.199$–$0.569$, $P < 0.001$). The association remained significant in multivariate analysis following adjustment for T-stage, Fuhrman grade and nodal status for both, overall survival (Table 3, HR $0.382$, 95% CI $0.203$–$0.719$, $P = 0.003$) and ccRCC-specific survival (Additional file 7: Table S6, HR $0.297$, 95% CI $0.142$–$0.620$, $P = 0.001$).

**Discussion**

We utilized the Human Protein Atlas resources to identify in an unbiased fashion, novel targets to improve and supplement currently used tools for the prognostication and differential diagnosis of RCC. Following state-of-the-art validation of antibodies targeting CUBN [19], we analyzed the expression of CUBN in normal human tissues, a large variety of cancers and two RCC-specific cohorts. We found that loss of CUBN expression in ccRCC patients was significantly associated with poor prognosis. Importantly, this observation was independent of T-stage, Fuhrman grade and nodal status, implying added clinical value of routine CUBN testing. In addition, we found the expression of CUBN to be highly specific to RCC, suggesting a potential use of CUBN in clinical cancer differential diagnostics as a complement to other diagnostic antibodies in cases where RCC needs to be confirmed.

CUBN is an endocytic receptor that is specifically expressed on epithelial cells in the proximal tubules of the kidney and in glandular cells of the small intestine [20]. In the kidney, CUBN mediates the reabsorption of filtered proteins such as albumin and transferrin [18], whereas in the small intestine, CUBN is primarily involved in the uptake of intrinsic factor-vitamin B$_{12}$ complex [21]. Even though the role of CUBN in normal kidney and small intestine has been well characterized and CUBN has been used as a marker for renal cell differentiation [22], the role of CUBN during RCC development and progression is largely unknown.

**Table 1** CUBN positivity rates on multi-cancer TMA cohort (Cohort 1)

| Cancer origin                  | N (912 total) | CUBN positive N (%) |
|-------------------------------|---------------|---------------------|
| Prostate                      | 57            | 0 (0)               |
| Colon                         | 59            | 0 (0)               |
| Breast                        | 60            | 0 (0)               |
| Stomach                       | 59            | 0 (0)               |
| Lung                          | 105           | 0 (0)               |
| Ovary                         | 60            | 0 (0)               |
| Endometrium                   | 59            | 0 (0)               |
| Cervix                        | 59            | 0 (0)               |
| Hepatocellular                | 28            | 0 (0)               |
| Neuroendocrine                | 30            | 0 (0)               |
| Sarcoma                       | 60            | 0 (0)               |
| Urothelial                    | 20            | 0 (0)               |
| Renal cell carc.              | 39            | 22 (56)             |
| - ccRCC                       | 30            | 18 (60)             |
| - other                       | 9             | 4 (44)              |
| Lymphoma                      | 20            | 0 (0)               |
| Melanoma                      | 20            | 0 (0)               |
| Testis                        | 18            | 0 (0)               |
| Oesophagus                    | 22            | 0 (0)               |
| Thyroid                       | 18            | 0 (0)               |
| Head and Neck                 | 20            | 1 (5)               |
| Pancreas                      | 51            | 0 (0)               |
| Cholangiocarc.                | 41            | 0 (0)               |
| Gall bladder carc.            | 7             | 0 (0)               |
| CUBN specificity$^{b}$        |               | 100%                |
| CUBN PPV$^{b}$                |               | 96%                 |

N number of patients, ccRCC clear cell renal cell carcinoma

*Percentage of positive cases within tumor type

$^{b}$For RCC compared to all other cases; PPV, positive predictive value
Fig. 3 Kaplan-Meier survival analysis of ccRCC patients, stratified according to CUBN expression. 

a Overall survival and b ccRCC-specific survival of patients in cohort 2. 

c Overall survival and d metastasis-free survival of patients in cohort 3

Fig. 4 Kaplan-Meier survival analysis of ccRCC patients, stratified according to CUBN expression. 

a One-year metastasis-free survival and b five-year metastasis-free survival of patients in cohort 3
exceedingly relevant in the clinical practice. The specificity and sensitivity of IHC staining for CUBN in cohorts of tumor tissue has provided an example of a novel diagnostic biomarker for RCC. Although extended studies regarding the expression pattern in additional tumors of relevance for differential diagnostics, e.g. adrenal gland tumors and other forms of clear cell cancer, are required to establish the usefulness of CUBN staining in clinical routine, the presented results indicate that this marker could be used for difficult cases where a diagnosis of RCC needs to be confirmed.

There is an unmet need for better tools for risk stratification of ccRCC patients. Several prognostic algorithms based on clinicopathological parameters have been proposed. For example, algorithms developed at Memorial Sloan-Kettering Cancer Center [9] or the Mayo Clinic [10] are used for the prediction of recurrence in patients with localized ccRCC. More recently, molecular phenotyping of RCC has shown promise in adding prognostic value to standard clinicopathological parameters. With ClearCode34, a 34-gene expression signature for the prognostic stratification of localized ccRCC patients was introduced and a combination of molecular and clinical parameters shown to provide better risk prediction than clinical variables alone [11]. Unlike mRNA-based assays, the immunohistochemical detection of CUBN can easily be implemented in routine pathology laboratories. An application of CUBN as marker for early disease spread and the added value of CUBN as a prognostic marker over clinical stage, grade and nodal status are promising and additional validation is highly desirable.

Functional studies to understand the mechanism linking the expression of a protein involved in re-absorption of proteins in proximal tubules and aggressiveness of RCC are needed. Previous studies showing that TGF beta reduces CUBN expression [23] and contributes to RCC aggressiveness [24] could provide one starting point to explore the biological background for the correlation between CUBN expression in RCC and prognosis. Extended functional studies regarding malignancy grade and also larger studies on well-defined cohorts with high quality clinical data from RCC patients will be needed to further explore the role of CUBN in RCC and to establish the clinical utility of this promising RCC biomarker.

Table 2 Association of CUBN positivity with clinicopathological parameters in ccRCC

| Variable               | Cohort 2 |                | Cohort 3 |                |
|------------------------|----------|----------------|----------|----------------|
|                        | N        | CUBN negative  |          | CUBN positive  |          |
|                        | N (%)    | N (%)          | P-value  | N (%)          | P-value  |
| Spread at diagnosis    |          |                |          |                |
| Local                  | 131      | 50 (96)        | 72 (91)  | 15 (33)        | 39 (57)  |
| Metastatic             |          | 2 (4)          | 7 (9)    | 31 (67)        | 29 (43)  |
| T-Stage                |          |                |          |                |
| T1-T2                  | 123      | 5 (10)         | 14 (19)  | n.a.           |          |
| T3-T4                  |          | 45 (90)        | 59 (81)  | 0.167a         |          |
| Fuhrman Grade          |          |                |          |                |
| 1–2                    | 95       | 8 (23)         | 31 (52)  | n.a.           |          |
| 3–4                    |          | 27 (77)        | 29 (48)  | 0.006a         |          |
| Nodal Status           |          |                |          |                |
| Negative               | 131      | 39 (75)        | 73 (92)  | n.a.           |          |
| Positive               |          | 13 (25)        | 6 (8)    | 0.006a         |          |

N: number of patients
a $\chi^2$ test
b Fisher’s exact test, n.a., not available

table

Table 3 Cox regression analysis of overall survival (Cohort 2)

| Prognostic factor       | Univariate |          |          |            |
|-------------------------|------------|----------|----------|------------|
|                         | HR (95% CI)| P-value  | HR (95% CI)| P-value  |
| CUBN (pos. vs. neg, ref)| 0.411      | 0.263–0.641| <0.001    | 0.382     | 0.203–0.719| 0.003 |
| T-Stage (T3-T4 vs. T1-T2, ref) | 1.897  | 1.002–3.593| 0.049    | 1.689     | 0.746–3.825| 0.209 |
| Fuhrman Grade (3–4 vs. 1–2, ref) | 1.822  | 1.059–3.136| 0.030    | 1.217     | 0.665–2.226| 0.524 |
| Nodal Status (pos. vs. neg, ref) | 4.208  | 2.397–7.386| <0.001   | 4.041     | 1.840–8.874| 0.001 |

HR: hazard ratio, CI: confidence interval
a Adjusted for all other variables; pos., positive; neg., negative; ref, referent group
Conclusions
In a quest to identify novel biomarkers for RCC, we have applied a systematic search strategy to exploit the extensive data resources of the Human Protein Atlas (www.proteinatlas.org). We identified CUBN as a marker for risk stratification of patients with RCC. Lack of CUBN expression was significantly associated with early disease progression and poor patient outcome, independent of T-stage, Fuhrman grade and nodal status. Owing to a highly RCC-specific expression profile, CUBN expression also has a potential role in clinical cancer differential diagnostics.

Additional files

Additional file 1: Table S1. Test TMA cohort composition. (DOC 34 kb)
Additional file 2: Table S2. Cohort 1 sample characteristics. (DOC 63 kb)
Additional file 3: Table S3. Available clinicopathological parameters of primary tumors in cohort 2 and cohort 3. (DOC 31 kb)
Additional file 4: Table S4. RCC-specific candidate biomarkers. (DOC 33 kb)
Additional file 5: Figure S1. Comparison of CUBN mRNA and IHC-derived protein expression in normal tissue. mRNA and protein expression levels were indicated as a percentage of the maximum. IHC-derived expression values were assigned numerical values; three for strong, two for moderate and one for weak staining. Staining intensities were averaged over the number of available tissue microarray cores (three cores per tissue type). (TIF 1128 kb)
Additional file 6: Table S5. CUBN positivity rates according to tumor site. (DOC 30 kb)
Additional file 7: Table S6. Cox regression analysis of ccRCC-specific survival (Cohort 2). (DOC 30 kb)

Abbreviations
cCRCC: Clear cell renal cell carcinoma; CUBN: Cubilin; DAB: 3,3'-Diaminobenzidine; FFPE: Formalin-fixed, paraffin-embedded; PPV: Positive predictive value; FPKM: Fragments per kilobase of transcript per million mapped reads; HR: Hazard ratio; IHC: Immunohistochemistry; PAQ: Paired box protein 2; PAX8: Paired box protein 8; RCC: Renal cell carcinoma; TGF: Transforming growth factor beta; TMA: Tissue microarray

Acknowledgements
The authors warmly acknowledge the staff of the Human Protein Atlas project in both Sweden and India for their efforts in generating the Human Protein Atlas. In particular, the authors would like to thank Sofie Gustafsson and IngMarie Olsson for constructing TMA cohorts 1 and 3, Dijana Cejan and Urban Rydberg for performing the IHC stainings and Ann-Sofi Strand and Cane Yaka for slide scanning. We are also grateful to Frances Rae and Craig Marshall (Health Sciences Scotland) for assistance with cohort 2 TMA construction.

Funding
This work was supported by the Swedish Cancer Society and the Knut and Alice Wallenberg Foundation. The work of DJH and GDS was funded by the Chief Scientist Office (grant number ETM37), Renal Cancer Research Fund and Kidney Cancer Scotland. The funding bodies provided basic financial support regarding salaries and materials and did not participate in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials
All primary data supporting our finding are confined within the manuscript, either as given data or in provided references. All IHC-based expression data will be made available on the Human Protein Atlas database (www.proteinatlas.org).

Authors’ contributions
FP conceived and designed the study and provided study supervision, technical and material support. GG contributed to study development and methodology, participated in acquisition, analysis and interpretation of data. DD participated in acquisition, analysis and interpretation of data. MN was partly responsible for design and acquisition of clinical data and biological material for cohort 3. AL was partly responsible for design and acquisition of clinical data and biological material for cohort 2. OL performed antibody validation and WB. HU performed antibody validation and WB. JB was partly responsible for design and acquisition of clinical data and biological material for cohort 1. PHE was partly responsible for design and acquisition of clinical data and biological material for cohort 1. SN was responsible for evaluation and scoring of immunohistochemically stained tissue microarrays. NK was responsible for primary annotation of immunohistochemistry. TP was responsible for primary annotation of immunohistochemistry. ÅS has taken part in analysis of RNAseq data. MU supervised antibody validation and RNAseq analyses. DJH was partly responsible for design and acquisition of clinical data and biological material for cohort 2. GU was partly responsible for design and acquisition of clinical data and biological material for cohort 3. GDS has taken part in study supervision and data analyses. All authors have read and approved the submitted manuscript, primary authors of manuscript text were GG, FP and GDS.

Competing interests
Two of the co-authors were employed at Atlas Antibodies AB and their contribution was technical and material support, essentially aiming to perform an extended validation of the cubilin antibodies. None of these co-authors have ownership in the Atlas Antibodies AB company. Three of the co-authors were pathologists and as such employed by Lab Surgpath. Their contribution to this study was to evaluate and annotate all the immunohistochemical staining patterns in TMAs representing cohort 1, 2 and 3. In performing this task they received salary from Lab Surgpath.

Consent for publication
Not applicable.

Ethics approval and consent to participate
This study was approved by the Research Ethics Committee at Uppsala University (2002–577, 2009/139 and 2011/473) and the Lothian Regional Ethics Committee (08/S1101/41 and 10/S1402/33). Written consent was required from study participants in TMA cohorts 2 and 3. All human tissue samples used in cohort 1 were anonymized in accordance with approval and advisory report from the Uppsala Ethical Review Board (2007–159), and consequently the need for informed consent was waived by the ethics committee. The use and analyses based on tissues in cohort 1 has previously been described (13).

Author details
1. Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden.  2. Department of Oncology, Radiology and Radiation Science, Uppsala University, Uppsala, Sweden.  3. MRC Human Genetics Unit, University of Edinburgh, Edinburgh, UK.  4. Edinburgh Urological Cancer Group, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK.  5. Atlas Antibodies AB, Stockholm, Sweden.  6. Lab Surgpath, Mumbai, India.  7. Science for Life Laboratory, Royal Institute of Technology, Stockholm, Sweden.  8. School of Medicine, University of St. Andrews, St. Andrews, UK.  9. Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, Hag Hammarskolds Väg 20, SE-751 85 Uppsala, Sweden.  10. Academic Urology Group, University of Cambridge, Box 43, Addenbrooke’s Hospital, Cambridge Biomedical Campus, Hill’s Road, CB2 0QQ Cambridge, UK.

Received: 14 October 2015 Accepted: 23 December 2016
Published online: 04 January 2017

References
1. Uhlen M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419. doi:10.1126/science.1260419.
2. Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djurinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide
integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics. 2014;13(2):397–406. doi:10.1074/mcp.M113.035600.

3. Habuka M, Fagerberg L, Hallstrom BM, Kampf C, Edlund K, Sivertsson A, et al. The kidney transcriptome and proteome defined by transcriptomics and antibody-based profiling. PLoS One. 2014;9(12):e116125. doi:10.1371/journal.pone.0116125.

4. Uhlen M, Bjorling E, Crispin PL, Roobijn SA, Thompson RH, Blute ML, et al. Histological subtype is an independent predictor of outcome for patients with renal cell carcinoma. J Urol. 2010;183(4):1309–15. doi:10.1016/j.juro.2009.12.035.

5. Tan PH, Cheng L, Rioux-Leclercq N, Merino MJ, Netto G, Reuter VE, et al. The human gastrointestinal tract-specific transcriptome and proteome as a diagnostic and prognostic biomarker. Am J Surg Pathol. 2011;35(9):816–26. doi:10.1097/PAS.0b013e1318216c112.

6. Laury AR, Perets R, Piao H, Krane JF, Barletta JA, French C, et al. A comprehensive analysis of PAX8 expression in human epithelial tumors. Am J Surg Pathol. 2011;35(9):816–26. doi:10.1097/PAS.0b013e1318216c112.

7. Ordonez NG. Value of PAX2 immunostaining in tumor diagnosis: a review and update. Adv Anat Pathol. 2012;19(6):301–9. doi:10.1097/PAP.0b013e3182713a32.

8. Sorbellini M, Kattan MW, Snyder ME, Reuter V, Motzer R, Goetzl M, et al. A postoperative prognostic nomogram predicting recurrence for patients with conventional clear cell renal cell carcinoma. J Urol. 2005;173(1):48–51. doi:10.1097/01.ju.0000035885.91935.d5.

9. Frank I, Blute ML, Cheville JC, Loehse CM, Weaver AL, Zincke H. An outcome prediction model for patients with clear cell renal cell carcinoma treated with radical nephrectomy based on tumor stage, size, grade and necrosis: the SSIGN score. J Urol. 2002;168(6):2395–400. doi:10.1097/01.ju.0000035885.91935.d5.

10. Brooks SA, Brannon AR, Parker JS, Fisher JC, Sen O, Kattan MW, et al. ClearCode34: A prognostic risk predictor for localized clear cell renal cell carcinoma. Eur Urol. 2014;66(1):77–84. doi:10.1016/j.euro.2014.02.035.

11. Choudhury Y, Wei X, Chu YH, Ng LG, Tan HS, Koh V, et al. A multigene assay to identify distinct prognostic subtypes of clear cell renal cell carcinoma with differential response to tyrosine kinase inhibition. Eur Urol. 2015;67(1):17–20. doi:10.1016/j.eururo.2014.06.041.

12. Gremel G, Bergman J, Djureinovic D, Edqvist PH, Maindad V, Bharambe BM, et al. A systematic analysis of commonly used antibodies in cancer diagnostics. Histopathology. 2014;64(4):396. doi:10.1111/his.12555.

13. Laird A, O'Mahony FC, Nanda J, Riddick AC, O'Donnell M, Harrison DJ, et al. Differential expression of prognostic proteomic markers in primary tumour, venous tumour thrombus and metastatic renal cell cancer tissue and correlation with patient outcome. PLoS One. 2013;8(4):e60483. doi:10.1371/journal.pone.0060483.

14. Kamp C, Olsson I, Ryberg U, Stjostedt E, Ponten F. Production of tissue microarrays, immunohistochemistry staining and digitalization within the human protein atlas. J Vis Exp. 2012(63). doi:10.3791/3620.

15. Spittalnic S. Test properties I: sensitivity, specificity, and predictive values. Hosp Physician. 2004;40(9):27–31.

16. Christensen EI, Venoust PJ, Nielsen R. Receptor-mediated endocytosis in renal proximal tubule. Pflugers Arch. 2009;458(6):1039–48. doi:10.1007/s00424-009-0685-8.

17. Christensen EI, Bilm H, Storm T, Weyer K, Nielsen R. Endocytic receptors in the renal proximal tubule. Physiology (Bethesda). 2012;27(4):223–36. doi:10.1152/physiol00022.2012.

18. O’Hurlery G, Stjostedt E, Rahman A, Li B, Kampf C, Ponten F, et al. Garbage in, garbage out: a critical evaluation of strategies used for validation of immunohistochemical biomarkers. Mol Oncol. 2014;8(4):783–98. doi:10.1016/j.molonc.2014.03.008.

19. Gremel G, Wanders A, Cedernaes J, Fagerberg L, Hallstrom B, Edlund K et al. The human gastrointestinal tract-specific transcriptome and proteome as defined by RNA sequencing and antibody-based profiling. Journal of gastroenterology. 2014. doi:10.1007/s00535-014-0958-7.

20. Bilm H, Venoust PJ, Nexo E, Hager H, Jacobsen C, Christensen EI, et al. Characterization of an epithelial approximately 460-kDa protein that facilitates endocytosis of intrinsic factor-vitamin B12 and binds receptor-associated protein. J Biol Chem. 1997;272(42):26497–504.