Integrating HECW1 expression into the clinical indicators exhibits high accuracy in assessing the prognosis of patients with clear cell renal cell carcinoma

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Background: Although many intratumoral biomarkers have been reported to predict clear cell renal cell carcinoma (ccRCC) patient prognosis, combining intratumoral and clinical indicators could predict ccRCC prognosis more accurately than any of these markers alone. This study mainly examined the prognostic value of HECT, C2 and WW domain-containing E3 ubiquitin protein ligase 1 (HECW1) expression in ccRCC patients in combination with established clinical indicators.

Methods: The expression level of HECW1 was screened out by data-independent acquisition mass spectrometry (DIA-MS) and analyzed in ccRCC patients from the The Cancer Genome Atlas (TCGA) database and our cohort. A total of 300 ccRCC patients were stochastically divided into a training cohort and a validation cohort, and real-time PCR, immunohistochemistry (IHC) and statistical analyses were employed to examine the prognostic value of HECW1 in ccRCC patients.
Results: The expression level of HECW1 usually decreased in human ccRCC specimens relative to control specimens in TCGA ($p < 0.001$). DIA-MS, Real-time PCR, and IHC analyses also showed that the majority of ccRCCs harbored decreased HECW1 expression compared with that in normal adjacent tissues ($p < 0.001$). Additionally, HECW1 expression was reduced in ccRCC cell lines compared with the normal renal cell line HK-2 ($p < 0.001$). Moreover, lower HECW1 expression was found in ccRCC patients with a higher tumor node metastasis (TNM) stage, bone metastasis, or first-line targeted drug resistance ($p < 0.001$). Low HECW1 expression indicated higher TNM stage, SSIGN (Stage, Size, Grade, and Necrosis) score and WHO/ISUP grade and poor prognosis in ccRCC patients ($p < 0.05$). Even after multivariable adjustment, HECW1, TNM stage, and SSIGN score served as independent risk factors. The c-index analysis showed that integrating intratumoral HECW1 expression into TNM stage or SSIGN score resulted in a higher c-index value than these indicators alone for predicting ccRCC patient prognosis.

Conclusion: HECW1 is a novel prognostic biomarker and therapeutic target in ccRCC, and integrating intratumoral HECW1 expression with established clinical indicators yields higher accuracy in assessing the postoperative prognosis of ccRCC patients.

Keywords: Clear cell renal cell carcinoma, HECW1, Prognostic marker, Disease progression

Background
Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer, which ranks as the 6th and 9th most frequent cancers in men and women, respectively, in the United States [1]. Although radical nephrectomy, targeted therapies and immunotherapy have been developed for ccRCC, recurrence and metastasis still exist, which results in a poor prognosis for ccRCC patients [2]. Therefore, it is important to search for reliable prognostic biomarkers to monitor postoperative disease progression and recurrence, which is critical for clinical decision making [3].

In the clinic, tumor node metastasis (TNM) staging has been applied to evaluate the outcome of ccRCC patients [4]. The stage, size, grade and necrosis (SSIGN) scoring system and the University of California Los Angeles Integrated Staging System (UISS) stratify ccRCC patients into low-risk, intermediate-risk, and high-risk prognostic groups [4]. In addition, many molecular prognostic indicators for ccRCC have been reported, such as BAP1, PBRM1, PDZK1, and CBX4 [4–6]. However, there are still some limitations to these indicators, which cannot completely and accurately evaluate the prognosis of ccRCC patients.

Recently, our research group has employed data-independent acquisition mass spectrometry (DIA-MS) on 230 ccRCC patients and screen out the proteins related to ccRCC patients’ prognosis. Among the proteins, we found that HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1 (HECW1) was significantly down-regulated in ccRCC. HECW1, also named NEDL1, belongs to the E3 ligase HECT family [7]. HECW1 expression was initially identified in neuronal tissues, including the spinal cord [8]. HECW1 binds to the COOH-terminal region of p53, which promotes its transcriptional activation and proapoptotic function [9]. Furthermore, HECW1 has been described in some studies on malignant tumors.

Exome sequencing studies have revealed somatic mutations in HECW1, along with other novel driver genes, in non-small cell lung cancer (NSCLC) [10]. In addition, the novel mutant gene HECW1 was identified in muscle-invasive transitional cell carcinoma [11]. Moreover, HECW1 has been shown to degrade thyroid transcription factor 1 in follicular thyroid carcinoma cells [12]. However, the expression and prognostic significance of HECW1 in ccRCC is unknown.

In the present research, The Cancer Genome Atlas (TCGA) database and two cohorts of ccRCC patients were employed to detect HECW1 expression in ccRCC and to determine whether HECW1 expression is associated with disease progression and postoperative prognosis in ccRCC patients. Furthermore, the prognostic accuracy of HECW1, TNM stage, and SSIGN score were compared and integrated to achieve a reliable prognostic model.

Methods
Collection and analysis of public databases
Datasets from The Cancer Genome Atlas (TCGA) were downloaded from National Cancer Institute GDC Data Portal (https://portal.gdc.cancer.gov) using FirebrowseR package. The KIRC dataset from all TCGA cohorts were selected and 508 samples from ccRCC patients were filtered out by barcodes and gene mRNA expression profiles. Then, the gene expression profiles were normalized by using DESeq2 package. The expression of HECW1 was compared between 508 tumor samples and 72 normal adjacent tissues, and then also compared in 69 paired samples. Wilcoxon test was used to compare the expression of HECW1 between the ccRCC and normal adjacent tissues.

Data-independent acquisition mass spectrometry (DIA-MS)
Formalin-fixed paraffin embedding (FFPE) tissue blocks of 230 ccRCC patients were organized by seasoned biopsy
pathology doctors. A perforated sampler was used to drill into the FFPE tissues blocks and extract tissue cores (1 mm in diameter and about 1–1.5 mg in weight). Three tissue cores were made for each case as biological repeat samples. The DIA MS acquisition of peptides was performed on Dionex Ultimate 3000 RSLC Nano System.

**Patients and specimens**

300 ccRCC patients, who were pathologically diagnosed between 2012 and 2014 from Eastern Hepatobiliary Surgery Hospital (Shanghai, China), were recruited in this study to determine the prognostic significance of HECW1. The type of ccRCC tissues used in our study are postoperative ccRCC specimens. 58% (174/300) patients received partial nephrectomy while others (126/300) underwent radical nephrectomy. Another 60 paired ccRCC specimens were used for Real-time PCR analysis. This study followed the recommendations for prognostic studies of tumor biomarkers (REMARK) [13]. All experiments were approved by the institutional ethical review boards from all hospitals, and all written informed consents were obtained from the ccRCC patients. The clinical features of the ccRCC patients are listed in Table 1. Pathologic specimens were evaluated by two surgical pathologists, with stage and grade determined according to the 2017 American Joint Committee on Cancer guidelines and WHO/ISUP grade, respectively.

**Real-time polymerase chain reaction (real-time PCR)**

The Real-time PCR assay was described as we reported previously [14]. Briefly, total RNAs were extracted with RNAiso Plus (9108, Takara, Kusatsu, Japan) and their corresponding cDNAs were synthesized using a PrimeScript One Step RT reagent Kit (RR037A, Takara, Kusatsu, Japan). Real-time PCR was taken with SYBR Green Real-Time PCR Master Mix (QP201, Toyobo, Osaka, Japan) on an ABI PRISM 7300HT Sequence Detection System (Thermo Fisher Scientific, Waltham, USA). The primer sequences are shown: HECW1 (forward primer, 5’- ACTGCTGCTGGAAGACGTTGAAG-3’, and reverse primer, 5’- TCCTCCTCCTGCTCATCCTCCTCCTCCT-3’), GAPDH (forward primer, 5’-GGAAGGTGAAGTGGAGT-3’, and reverse primer, 5’-CCTGGAAGATGATGGG-3’). All results were normalized to the expression of GAPDH and fold change relative to the mean value was determined by $2^{-△△Ct}$.

**Immunohistochemistry (IHC)**

The ccRCC pathological sections were dewaxed, hydrated, and repaired with citric acid buffer (1:100) in a pressure cooker (3 min) before cooling to room temperature. The pathological sections were then incubated with the reagents of the hypersensitive immunohistochemical kit (Fuzhou Maixin Biological Company, Fuzhou, China). The endogenous peroxidase blocker (within kit) was incubated for 30 min, and the animal non-immune serum blocker (within kit) was incubated for 20 min. Rabbit anti-HECW1 antibody (ab121264, abcam, Cambridge, USA) or IgG antibody (ab37415, abcam, Cambridge, USA) was incubated at 4 °C overnight. The next day, the antibody was recovered and then incubated for 30 min with biotin labeled secondary antibody.

**Table 1** Demographics and characteristics of patients with clear cell renal cell carcinoma (ccRCC)

| Characteristics | NO. of cases(%) | Sum(n = 300) |
|-----------------|-----------------|--------------|
| Age(16y ~ 84y)  |                 |              |
| < 60            | 110(61.1)       | 182(60.7)    |
| ≥ 60            | 70(38.9)        | 18(39.3)     |
| Gender          |                 |              |
| Male            | 127(70.6)       | 215(71.7)    |
| Female          | 53(29.4)        | 85(28.3)     |
| WHO/ISUP Grading|                 |              |
| I-II            | 137(76.1)       | 228(76)      |
| III-IV          | 43(23.9)        | 72(24)       |
| TNM stage       |                 |              |
| I-II            | 161(89.4)       | 266(88.7)    |
| III-IV          | 19(10.6)        | 34(11.3)     |
| SSIGN           |                 |              |
| 1–4             | 167(92.8)       | 281(93.7)    |
| ≥ 5             | 13(7.2)         | 19(6.3)      |

SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis
reagent (within kit), and then incubated with Streptomy-
cetes antibiotic protein peroxidase reagent (within kit) for
30 min. Before and after each step of the above experiment,
wash with PBS buffer for 3 times (5 min/time). Then, DAB
color development kit (Fuzhou Maixin Biological Company,
Fuzhou, China) was used for staining (terminating accord-
ing to different antibody reaction time). The nucleus was
stained with hematoxylan for 5 min, followed by differenti-
sation solution for 2 s, blue return solution for 3 min, and
finally transparent and sealed slices were performed. The
IgG, positive, and negative controls for HECW1 have been
presented in supplementary Fig. S1F-G.

The presence of IHC staining for HECW1 was scored
semiquantitatively as negative (0), weakly positive (1+),
moderately positive (2+), or strongly positive (3+), and
the percentages of positive cells were also determined.
For each observed tissue component, a summary value
referred to as component H-Score was calculated by the
multiplication of the intensity score, which ranged from
0 to 3, by the percentage of positive cells, which ranged
from 0 to 100, and the total H-Score for a tissue section
was derived as the sum of the component H-Scores
weighted by the fraction of each component observed in
the tissue section (Supplementary Table S1).

Cell culture
The ccRCC cell lines were bought from the Cell Bank of
the Type Culture Collection of the Chinese Academy of
Sciences (Shanghai, China) in 2019. HK-2 cells were cul-
tured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM)
(12,100,046, Gibco, Waltham, USA). 786-O and 769-P cells were cultured in RPMI-1640
medium (C11875500BT, Gibco, Waltham, USA). Caki-1
cells were culture in McCoy’s 5A Medium (16600–082,
Gibco, Waltham, USA). L-glutamine is contained in the
culture medium. The culture medium of all cell lines
were supplemented with fetal bovine serum (FBS, 10%,
16,140,071, Gibco, Waltham, USA) and 1% penicillin/
streptomycin (15140–122, Gibco,Waltham, USA), and
the FBS was heat-inactivated (56 ºC, 30 min) before
using. The cell lines were cultured at 37 ºC in 5% CO₂.
Sunitinib and pazopanib-resistant 786-O cell lines (786-
O-SR and 786-O-PR) were established as described in
our previous research [14, 15]. The cell lines in this
study were authenticated by short tandem repeat (STR)
profiling and detected for mycoplasma contamination
using a Mycoplasma Detection Kit (Biotool, Neuhof,
Switzerland), and the most recent tests were conducted
in October 2020. All cell lines used in the study were
cultured within 40 passages.

Statistical analysis
Numerical data were expressed as the mean ± S.D. Two-
tailed Student’s t-test or Wilcoxon test was conducted
for continuous variables. Chi-square test or fisher’s exact
test was conducted for categorical variables. Time-
dependent receiver operating characteristic (ROC)
analysis was performed using ‘survivalROC’ package to
determine the optimal cut-off values of the H-scores of
HECW1. Survival curves were plotted using Kaplan-
Meier analysis and compared via log-rank test. Variables
with p values < 0.05 in univariate Cox proportional
hazards analysis were included in multivariate analysis.
Difference was considered significant at p < 0.05. Prognos-	ic accuracy of the HECW1 classifier and other prognostic
indicators was indicated by Harrell’s concordance index
using ‘rms’ package(c-index). All the statistical analyses
were performed using R-software (version 3.5.2).

Results
HECW1 expression is decreased in clear cell renal cell
carcinoma
The protein expression of HECW1 in ccRCC samples
was first determined by data-independent acquisition
mass spectrometry (DIA-MS) assay, which showed that
HECW1 expression was reduced in ccRCC (Fig. 1a). The
expression of HECW1 was then analyzed in unpaired or
paired ccRCC and normal adjacent tissues from the
TCGA. Lower HECW1 expression was found in ccRCC
in contrast to normal adjacent samples (Fig. 1b-c). To
validate this initial finding, matched postoperative
ccRCC specimens and their normal adjacent tissues were
employed. As expected, the expression level of HECW1
was down-regulated in ccRCC in contrast to the
adjacent renal tissues (Fig. 1d). Moreover, immunohisto-
chemistry (IHC) assays were performed with postopera-
tive ccRCC specimens and demonstrated that the
majority of ccRCCs harbored decreased HECW1 expres-
sion compared with that in paired normal adjacent tissues
(Fig. 1e). These findings indicate that the expression of
HECW1 is commonly reduced in ccRCC.

Low HECW1 expression indicates the progression of ccRCC
As the expression level of HECW1 is usually decreased
in ccRCC, we postulated that HECW1 expression might
be negatively associated with malignant characteristics of
ccRCC. First, a real-time PCR assay was employed to
show that the expression of HECW1 was reduced in the
ccRCC cell lines 786-O, 769-P, and Caki-1 compared
with that in the normal renal cell line HK-2 (Fig. 2a).
Additionally, IHC assays presented that down-regulated
HECW1 expression was observed in ccRCC specimens
with a high TNM stage in contrast to that with a low
TNM stage (Fig. 2b). Moreover, HECW1 expression was
reduced in ccRCC specimens with bone metastasis
compared with those without metastasis (Fig. 2c).
Furthermore, the mRNA expression of HECW1 was
down-regulated in sunitinib- or pazopanib-resistant 786-
O cells (786-O-SR or 786-O-PR, as we described previously [14, 15] in contrast to that in naïve 786-O cells (Fig. 2d-e). Additionally, IHC assays demonstrated that downregulation of HECW1 was observed in sunitinib- or pazopanib-resistant orthotopic ccRCC specimens (which were also described in our previous studies [14, 15] compared with naïve orthotopic ccRCC specimens (Fig. 2f-g). These results exhibit that low HECW1 expression indicates higher tumor stage, bone metastasis, and targeted drug resistance of ccRCC.
Low HECW1 expression is predictive of unfavorable prognosis in ccRCC patients

To examine whether low HECW1 expression indicates the post-operative prognosis of ccRCC patients, ccRCC specimens from postoperative ccRCC patients (n = 300) were employed. These samples were randomly divided into a training and a validation cohort at a 3:2 ratio (Fig. 3a; Table 1). First, IHC assays were applied to examine the expression level of HECW1, and then a time-dependent receiver operating characteristic (ROC) analysis was used to show that the optimal cut-off value for dividing ccRCC
patients from the training cohort into HECW1\textsuperscript{low} and HECW1\textsuperscript{high} groups was 105 (Fig. 3b-c; Table 2). As shown in Table 2, the HECW1\textsuperscript{low} group presented higher TNM stage, SSIGN score, and WHO/International Society of Urological Pathology (ISUP) grade. In addition, Kaplan-Meier survival analysis demonstrated that the HECW1\textsuperscript{low} group exhibited worse overall survival (OS) and progression-free survival (PFS) than the HECW1\textsuperscript{high}
group (Fig. 3d–e). The validation cohort of ccRCC patients was employed to corroborate the above results using the cut-off value derived from the training cohort and showed that low expression of HECW1 indicated unfavorable clinicopathological features and short survival in ccRCC patients (Fig. 3f–g; Table 3). To confirm the above results, the ccRCC patients were also stochastically divided into a training cohort and a validation cohort at a 1:1 ratio (Fig. 3a; Table 1). The analysis of these cohorts demonstrated that the HECW1\textsuperscript{low} group showed advanced TNM stage, SSIGN score, and WHO/ISUP grade and shorter OS and PFS than the HECW1\textsuperscript{high} group both in the training cohort and in the validation cohort (Fig. S1a–f; Tables 4 and 5). These findings present that low HECW1 expression indicates unfavorable clinicopathological features and short survival in ccRCC patients.

Combining intratumoral HECW1 expression and the clinical indicators exhibits higher prognostic accuracy in evaluating the postoperative prognosis of ccRCC patients

To further determine the prognostic value of HECW1 in ccRCC patients, univariate and multivariate Cox regression analyses were performed to examine whether HECW1 serves as an independent risk factor for predicting the OS and PFS of ccRCC patients. Even after multivariable adjustment of the clinical characteristics, HECW1 expression, TNM stage, and SSIGN score served as independent risk factors in both the training cohort (Tables 6 and 7) and the validation cohort (Tables 8 and 9) regardless of the ratio (3:2 or 1:1). Therefore, HECW1 expression in specimens serves as an independent risk factor for ccRCC patients’ prognosis.

Furthermore, we examined the prognostic accuracy of combining HECW1 expression with a current prognostic indicator, TNM stage or SSIGN score, in predicting ccRCC patient prognosis. Then, time-dependent concordance index (c-index) analysis was used in the training cohort (at a 3:2 ratio), which demonstrated that combining HECW1 and TNM stage or SSIGN score exhibited a higher c-index value than any of these indicators alone in predicting ccRCC patients’ prognosis (Table 10). The above results were also confirmed in the validation cohort (at a 3:2 ratio) and the training cohort and validation cohort (at a 1:1 ratio) (Tables 10 and 11). Taken together, these findings indicate that improved prognostic accuracy in predicting the postoperative prognosis of ccRCC patients can be accomplished by combining intratumoral HECW1 expression and existing clinical indicators.

**Discussion**

The identification of reliable and helpful indicators for evaluating ccRCC patient disease progression and prognosis is crucial for improving clinical therapies.
and patient survival [16]. Although numerous studies have reported that tumor biomarkers predict ccRCC patient prognosis, combining intratumoral markers and clinical indicators could predict ccRCC patient prognosis more accurately than any of these markers alone [17, 18]. Our present study integrates HECW1 expression into a prognostic model with the TNM stage or SSIGN score, which results in better accuracy in evaluating ccRCC patient prognosis than that achieved with only one of these indicators.

Given its role in DNA damage responses and p53-mediated apoptotic cell death, it is possible that the

Table 4 The correlation between HECW1 expression and clinicopathologic characteristics of patients with ccRCC in the training cohort (n = 150) (1:1 ratio)

| Characteristics  | HECW1                          | Sum (150) | P value |
|------------------|--------------------------------|-----------|---------|
|                  | High expression (90) | Low expression (60) |         |
| Age              |                                |           |         |
| < 60             | 47                             | 34        | 0.619   |
| ≥ 60             | 43                             | 26        |         |
| Gender           |                                |           |         |
| Male             | 61                             | 49        | 0.063   |
| Female           | 29                             | 11        |         |
| WHO/ISUP Grading |                                |           | < 0.001 |
| I–II             | 77                             | 31        | 108     |
| III–IV           | 13                             | 29        | 42      |
| TNM stage        |                                |           | 0.003   |
| I–II             | 87                             | 49        | 136     |
| III–IV           | 3                              | 11        | 14      |
| SSIGN            |                                |           | < 0.001 |
| 1–4              | 90                             | 49        | 139     |
| ≥ 5              | 0                              | 11        | 11      |

SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis

Table 5 The correlation between HECW1 expression and clinicopathologic characteristics of patients with ccRCC in the validation cohort (n = 150) (1:1 ratio)

| Characteristics  | HECW1                          | Sum (150) | P value |
|------------------|--------------------------------|-----------|---------|
|                  | High expression (94) | Low expression (56) |         |
| Age              |                                |           |         |
| < 60             | 64                             | 37        | 0.858   |
| ≥ 60             | 30                             | 19        |         |
| Gender           |                                |           |         |
| Male             | 64                             | 41        | 0.582   |
| Female           | 30                             | 15        |         |
| WHO/ISUP Grading |                                |           | 0.006   |
| I–II             | 82                             | 38        | 120     |
| III–IV           | 12                             | 18        | 30      |
| TNM stage        |                                |           | < 0.001 |
| I–II             | 93                             | 37        | 130     |
| III–IV           | 1                              | 19        | 20      |
| SSIGN            |                                |           | < 0.001 |
| 1–4              | 93                             | 49        | 142     |
| ≥ 5              | 1                              | 7         | 8       |

SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis
| Characteristics                      | Overall survival |          | Progression-free survival |          |
|-------------------------------------|-----------------|----------|---------------------------|----------|
|                                     | Univariate      | Multivariate | Univariate               | Multivariate |
|                                     | HR(95% CI)      | P value  | HR(95% CI)               | P value  |
|                                     | HR(95% CI)      | P value  | HR(95% CI)               | P value  |
| Age (≥60y vs < 60y)                 | 1.061 (0.498–2.288) | 0.879   | 0.664 (0.324–1.350)      | 0.664   |
| Gender (Male vs Female)             | 0.558 (0.211–1.476) | 0.24   | 1.154 (0.577–2.310)     | 0.686   |
| WHO/ISUP Grading (III-IV vs I-II)   | 3.901 (1.813–8.394) | <0.001  | 2.095 (0.889–4.938)     | 0.091   |
|                                     |                 |          | 2.345 (1.305–4.561)     | 0.012   |
|                                     |                 |          | 2.0999 (5.004–19.982)   | <0.001  |
|                                     | 11.791 (5.126–27.121) | <0.001  | 3.797 (1.633–8.829)     | 0.002   |
|                                     |                 |          | 9.9999 (5.004–19.982)   | <0.001  |
| SSIGN (≥5 vs 1–4)                   | 10030 (4199–23.961) | <0.001  | 3.872 (1.477–10.150)    | 0.006   |
|                                     |                 |          | 19.698 (9.141–42.449)   | <0.001  |
|                                     | 69 (9.253–514551) | <0.001  | 33.384 (4.218–264.224)  | <0.001  |
|                                     |                 |          | 15.432 (5.919–40.233)   | <0.001  |
|                                     |                 |          | 7.554 (2.602–21.930)    | <0.001  |

*SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis*
Table 7: Univariate and multivariate Cox regression analysis of HECW1 expression classifier and clinical characteristics with overall survival and progression-free survival in the validation cohort (n = 120) (3:2 ratio)

| Characteristics          | Overall survival | Progression-free survival |
|--------------------------|------------------|---------------------------|
|                          | Univariate       | Multivariate              | Univariate       | Multivariate |
|                          | HR(95% CI)       | P value                   | HR(95% CI)       | P value |
| Age (≥60y vs < 60y)      | 1.535 (0.690–3.417) | 0.294                     | 2.417 (1.052–5.554) | 0.038 |
| Gender (Male vs Female)  | 1.274 (0.542–2.994) | 0.579                     | 0.807 (0.316–2.061) | 0.654 |
| WHO/ISUP Grading (III-IV vs I-II) | 3.064 (1.396–6.723) | 0.005 | 1.029 (0.418–2.536) | 0.95 |
|                          | 3.636 (1.347–9.569) | 0.029 | 1.633 (0.587–4.456) | 0.34 |
| TNM stage (III-IV vs I-II) | 5.160 (2.272–11.719) | < 0.001 | 3.248 (1.171–9.011) | 0.024 |
|                          | 5.287 (2.292–12.195) | < 0.001 | 1.180 (0.477–2.923) | 0.72 |
| SSIGN (≥5 vs 1–4)        | 5.492 (2.135–14.130) | < 0.001 | 3.425 (1.025–11.443) | 0.045 |
|                          | 10.988 (3.921–30.795) | < 0.001 | 1.180 (0.477–2.923) | 0.72 |
| HECW1 (High vs Low)      | 13.743 (4.100–46.065) | < 0.001 | 7.480 (1.978–28.295) | 0.003 |
|                          | 21.144 (4.947–90.383) | < 0.001 | 10.613 (2.226–50.600) | 0.003 |

SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis
Table 8: Univariate and multivariate Cox regression analysis of HECW1 expression classifier and clinical characteristics with overall survival and progression-free survival in the training cohort (n = 150) (1:1 ratio)

| Characteristics                      | Overall survival |                       | Progression-free survival |                       |
|--------------------------------------|------------------|------------------------|---------------------------|------------------------|
|                                      | Univariate       | Multivariate           | Univariate                | Multivariate           |
|                                      | HR(95% CI)       | P value                | HR(95% CI)                | P value                |
|                                      |                  |                        |                           |                        |
| Age (≥60y vs < 60y)                  | 1.550 (0.681–3.525) | 0.296                  | 1.251 (0.599–2.615)       | 0.351                  |
| Gender (Male vs Female)              | 0.995 (0.409–2.424) | 0.992                  | 0.958 (0.435–2.110)       | 0.915                  |
| WHO/ISUP Grading (III-IV vs I-II)    | 3.804 (1.645–8.795) | 0.002                  | 1.412 (0.595–3.352)       | 0.434                  |
|                                      |                  |                        | 2.148 (1.032–4.469)       | 0.041                  |
|                                      |                  |                        | 10.054 (4.183–26.373)     | 0.001                  |
| TNM stage (III-IV vs I-II)           | 5.843 (2.222–15.363) | < 0.001                | 3.028 (1.115–8.222)       | 0.03                   |
|                                      |                  |                        | 9.042 (4.157–19.667)      | < 0.001                |
|                                      |                  |                        | 5.441 (2.329–12.712)      | < 0.001                |
| SSIGN (≥5 vs 1–4)                    | 9.235 (3.881–21.975) | < 0.001                | 2.635 (1.058–6.562)       | 0.037                  |
|                                      |                  |                        | 17.907 (8.057–39.800)     | < 0.001                |
|                                      |                  |                        | 7.604 (3.169–18.243)      | < 0.001                |
| HECW1 (High vs Low)                  | 51.099 (6.841–381.667) | < 0.001                | 29.907 (3.750–238.530)    | 0.001                  |
|                                      |                  |                        | 10.054 (4.183–26.373)     | < 0.001                |
|                                      |                  |                        | 5.072 (1.728–14.891)      | 0.003                  |

SSIGN: Stage, Size, Grade, and Necrosis. TNM: Tumor Node Metastasis
Table 9: Univariate and multivariate Cox regression analysis of HECW1 expression classifier and clinical characteristics with overall survival and progression-free survival in the validation cohort (n = 150) (1:1 ratio)

| Characteristics                      | Overall survival | Progression-free survival |
|--------------------------------------|------------------|---------------------------|
|                                      | Univariate       | Multivariate              | Univariate       | Multivariate |
|                                      | HR(95% CI)       | P value                   | HR(95% CI)       | P value       |
|                                      |                   |                           |                   |               |
| Age (≥60y vs < 60y)                  | 1.037 (0.480–2.244) | 0.926                    | 0.978 (0.455–2.101) | 0.955         |
| Gender (Male vs Female)              | 0.729 (0.296–1.796) | 0.492                    | 1.117 (0.510–2.450) | 0.782         |
| WHO/ISUP Grading (III-IV vs I-II)    | 3.954 (1.873–8.345) | < 0.001                  | 1.568 (0.657–3.745) | 0.311         |
| TNM stage (III-IV vs I-II)           | 9.069 (4.307–19.096) | < 0.001                  | 3.832 (1.437–10.223) | 0.007         |
| SSIGN (≥5 vs 1–4)                    | 7.223 (2.727–19.136) | < 0.001                  | 5.953 (1.773–19.982) | 0.004         |
| HECW1 (High vs Low)                  | 19.480 (5.883–64.500) | < 0.001                  | 7.483 (1.897–29.514) | 0.004         |

SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis
The prognostic value of HECW1 has not been reported in other types of tumors. For the first time, we demonstrated that low HECW1 expression indicated higher TNM stage, SSIGN score, WHO/ISUP grade, and poor prognosis in ccRCC patients. Although many prognostic biomarkers such as oncogenes, tumor suppressive genes, and tumor-infiltrating immune cells have been reported in evaluations of the postoperative prognosis of ccRCC patients [16], they have not been applicable to clinical practice. One of the reasons may be that these indicators have not been compared and integrated into established clinical prognostic models, such as the TNM staging system. Our present study not only demonstrated that HECW1 is a potential biomarker for ccRCC patient disease progression and survival rate but also compared the accuracy of combining HECW1 expression with current prognostic indicators, namely, the TNM stage or SSIGN score, with the accuracy of these indicators alone for predicting ccRCC patient prognosis. We found that combining HECW1 and TNM stage or SSIGN score presented a higher c-index value than any of these indicators alone for predicting ccRCC patient prognosis. Therefore, improved prognostic accuracy can be achieved by combining intratumoral HECW1 expression and clinical indicators when evaluating ccRCC patients' postoperative prognosis. However, ccRCC patients from only one clinical center were employed in this study.

The unpredictable expression of HECW1 may disrupt cell homeostasis and cause tumorigenesis [19, 20]. Recently, mutations of HECW1 have been identified in non-small cell lung cancer and muscle-invasive transitional cell carcinoma [10, 11]. Additionally, HECW1 has been found to negatively regulate ErbB4 protein expression via ubiquitin-mediated degradation in breast cancer [9]. In our present study, the expression of HECW1 in ccRCC was analyzed in TCGA and compared between matched postoperative ccRCC specimens and normal adjacent tissues in our clinical center. Altogether, these findings showed that the expression of HECW1 is commonly downregulated in ccRCC. Although the results of immunohistochemical studies showed that HECW1 expression was higher in 10 muscle-invasive transitional cell carcinomas than in normal adjacent samples [11], its expression may be based on the tumor type. The results of the present study also revealed that a low expression of HECW1 was associated with a high TNM stage, bone metastasis, and targeted drug resistance in ccRCC. In addition, the biological function of HECW1 was reported only recently in a paper showing that HECW1 promoted the proliferation, migration and invasion of non-small cell lung cancer cells. However, to elucidate the function of HECW1 in other malignant tumors, including ccRCC, additional studies are needed in the future.

### Table 10: C-index analysis of the prognostic accuracy of HECW1 and other variables for overall survival and progression-free survival in the training cohort (n = 180) and validation cohort (n = 120) (3:2 ratio)

| Characteristics | Overall survival (training cohort (n = 180)) | Validation cohort (n = 120) | Progression-free survival (training cohort (n = 180)) | Validation cohort (n = 120) |
|-----------------|---------------------------------------------|----------------------------|---------------------------------------------|----------------------------|
| TNM stage       | 0.7000(0.601–0.799) 0.664(0.549–0.745) | 0.699(0.616–0.782) 0.684(0.511–0.705) | 0.692(0.749–0.833) 0.780(0.76–0.887) | 0.680(0.469–0.607) 0.624(0.036–0.728) |
| SSIGN           | 0.686(0.51–0.705) 0.807(0.755–0.859) | 0.828(0.786–0.847) | 0.836(0.761–0.863) | 0.857(0.817–0.911) |
| HECW1 + TNM     | 0.870(0.825–0.915) 0.830(0.771–0.889) | 0.845(0.803–0.905) | 0.855(0.793–0.915) | 0.861(0.816–0.906) |

### Table 11: C-index analysis of the prognostic accuracy of HECW1 and other variables for overall survival and progression-free survival in the training cohort (n = 150) and validation cohort (n = 150) (1:1 ratio)

| Characteristics | Overall survival (training cohort (n = 150)) | Validation cohort (n = 150) | Progression-free survival (training cohort (n = 150)) | Validation cohort (n = 150) |
|-----------------|---------------------------------------------|----------------------------|---------------------------------------------|----------------------------|
| TNM stage       | 0.699(0.588–0.750) 0.686(0.588–0.784) | 0.659(0.566–0.752) 0.664(0.572–0.756) | 0.734(0.637–0.831) 0.607(0.521–0.692) | 0.781(0.731–0.831) 0.830(0.789–0.871) |
| SSIGN           | 0.684(0.598–0.770) 0.605(0.513–0.697) | 0.729(0.616–0.782) 0.624(0.536–0.728) | 0.829(0.775–0.883) 0.845(0.797–0.893) | 0.854(0.793–0.915) 0.861(0.816–0.906) |
| HECW1 + TNM     | 0.830(0.788–0.880) 0.847(0.799–0.895) | 0.854(0.793–0.915) | 0.861(0.816–0.906) | 0.854(0.793–0.915) 0.861(0.816–0.906) |

### SSIGN Stage, Size, Grade, and Necrosis, TNM Tumor Node Metastasis
Our future studies will include more ccRCC patients from other clinical centers.

Supplementary Information

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Additional file 1.

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Authors’ contributions

(I) Conception and design: C Wang, P Shen, and XG Cui; (II) Administrative support: C Wang, P Shen, and XG Cui; (III) Provision of study materials or patients: C Wang, KQ Dong, YN Wang, G Peng, X Song, and YW Yu; (IV) Collection and assembly of data: C Wang, KQ Dong, YN Wang, G Peng, X Song, and YW Yu; (V) Data analysis and interpretation: All authors; (VI) Manuskript writing: C Wang; (VII) Final approval of manuscript: All authors.

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Availability of data and materials

Datasets from The Cancer Genome Atlas (TCGA) were downloaded from National Cancer Institute GDC Data Portal (https://portal.gdc.cancer.gov) using FirebrowseR package. The data used and analyzed in this study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

All experiments were approved by the institutional ethical review boards from Eastern Hepatobiliary Surgery Hospital and in accordance with the Declaration of Helsinki and the Guidelines for Good Clinical Practice. Written informed consent was obtained from all ccRCC patients or their legal guardians.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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