ORIGINAL ARTICLE

DNA methylation of Hugl-2 is a prognostic biomarker in kidney renal clear cell carcinoma

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Abstract

It has been reported that loss of Hugl-2 contributes to tumour formation and progression *in vitro* and *in vivo*. However, whether Hugl-2 levels decrease during kidney renal clear cell carcinoma (KIRC) and the mechanism involved remain unknown. This study aimed to investigate whether DNA methylation of Hugl-2 reduces its expression, leading to the progression and poor prognosis of KIRC. Hugl-2 methylation and mRNA expression and KIRC clinicopathological data were extracted from The Cancer Genome Atlas (TCGA), and relationships among these factors were analyzed using UALCAN, MethHC, Wanderer and LinkedOmics web tools. We found that Hugl-2 mRNA and protein levels were reduced in KIRC tissues. Moreover, Hugl-2 mRNA levels were related to tumour grade and overall survival, and Hugl-2 methylation was increased in KIRC. According to the results of methylation-specific PCR, KIRC cells had higher Hugl-2 DNA methylation levels than HKC cells. Moreover, Hugl-2 DNA methylation correlated negatively with Hugl-2 mRNA and was also related to the pathology and T stage of KIRC patients. KIRC patients with high Hugl-2 DNA methylation also had shorter overall survival. Additionally, methylation of cg08827674, a Hugl-2 probe, was related to pathologic stage, T stage, neoplasm histologic grade, serum calcium level without laterality, M stage, N stage, and ethnicity. Furthermore, treatment with the DNA methylation inhibitor decitabine resulted in upregulation of Hugl-2 mRNA and protein levels in KIRC cell lines. These results indicate that Hugl-2 DNA methylation may be both a prognostic marker and a therapeutic target in KIRC.

KEYWORDS
cell polarity protein Hugl-2, DNA methylation, kidney renal clear cell carcinoma, prognosis

1 | INTRODUCTION

Kidney cancer is one the most frequent solid tumours worldwide, with approximately 403 300 new cases and 175 100 deaths from renal cell carcinoma (RCC) estimated to have occurred in 2018.[1] Kidney renal clear cell carcinoma (KIRC) is the most common subtype of renal cell carcinoma, accounting for 90% of all renal tumours.[2] Currently, curative therapy with surgery is an option only for patients with early-stage localized tumours. Patients with metastasis have high rates of morbidity and mortality.[3,4] Hence, there is a clinical need to identify tumour markers for preliminary screening and early detection of metastasis and to develop guidelines for drug development and use for KIRC.

DNA methylation is one of the most well-studied epigenetic modifications in mammals[5] and can contribute to renal tumourigenesis by silencing tumor-suppressor genes.[6] Additionally, DNA methylation alterations have been demonstrated to be associated with clinicopathological features and patient survival.[7,8] It has been reported that RCC DNA methylation represents a potential biomarker for early detection, prognosis and prediction of response to therapy[9-11] because it is found early during carcinogenesis,[12] including in precancerous lesions.[13] Furthermore, as stable DNA marks that can be quantitatively measured, changes in DNA methylation are useful in detection strategies.[14]

Cell polarity is a crucial phenomenon in many biological processes and is required for normal tissue integrity and tissue homeostasis.[15,16] As important members of the scribble complex, lethal (2) giant larvae (Lgl) proteins define the basolateral plasma domain and play a key role in regulating cell polarity with two other members: scribble homolog and disc-large homolog (DLG).[15] Humans express two Lgl isoforms, Hugl-1 and Hugl-2; the latter is a 1020-amino acid protein containing 14 predicted WD40 repeats.[17] Accumulating evidence suggests that loss of Lgl function results in disruption of polarized epithelial organization and affects signalling pathways that regulate cell growth, which are linked to human cancers.[18,19] Indeed, downregulation of Hugl-2 expression has been observed in breast cancer, colorectal cancer, gastric cancer and lung adenocarcinoma and has been associated with cancer progression.[20-23] In addition, other studies in our laboratory (unpublished) have shown that loss of Hugl-2 induces renal tumourigenesis and contributes to a poor prognosis in KIRC patients. In view of the potential role Hugl-2 plays in suppressing renal tumourigenesis, we postulated that Hugl-2 DNA methylation downregulates Hugl-2 protein expression and serves as a prognostic marker for KIRC. In this study, the relationship among Hugl-2 DNA methylation and expression levels and clinicopathological parameters in KIRC was investigated utilizing datasets from The Cancer Genome Atlas (TCGA).

2 | RESULTS

2.1 | Hugl-2 mRNA and protein expression in KIRC

Hugl-2 mRNA and protein expression were analyzed in normal and KIRC tumour specimens using UALCAN. We found that Hugl-2 mRNA ($P < 1e-12$, normal n = 72, tumour n = 533) and protein ($P = 1.93124477876003e-72$, normal n = 84, tumour n = 110) levels were significantly reduced in KIRC tissues compared to normal tissues (Figure 1A,B).

2.2 | Association among Hugl-2 mRNA, tumour grade and prognosis in KIRC

We next examined associations among Hugl-2 mRNA level, tumour grade and prognosis in KIRC using UALCAN and found that Hugl-2 mRNA levels correlated with neoplasm histologic grade (Figure 1C). Moreover, overall survival was significantly shorter for KIRC patients with low Hugl-2 mRNA expression compared to those with high Hugl-2 mRNA expression ($P = 1.4e-02$, Figure 1D). These data suggest that loss of Hugl-2 induces renal tumourigenesis and contributes to a poor prognosis in KIRC patients.
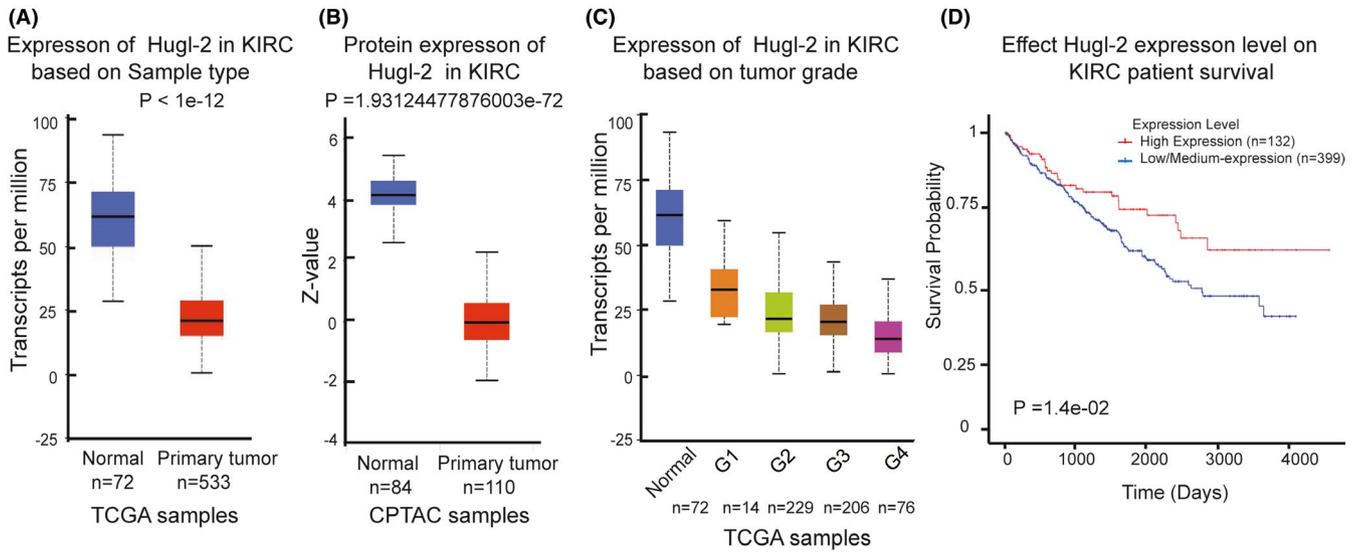
2.3 | Hugl-2 methylation in KIRC

First, we analyzed Hugl-2 methylation in normal and KIRC tumour specimens using the MethHC web tool. Based on the results, DNA methylation levels of the three Hugl-2 isoforms (a, b and c) were all elevated in the KIRC group compared to the normal group ($P < 5e-03$, Figure 1E). As shown in Figure 1F, data from the Wanderer web tool were similar ($P < 5e-02$, normal n = 160, tumour n = 324), with most of the Hugl-2 probes in the 450 methylation array exhibiting significant differences between KIRC and normal specimens. The DNA methylation of the Hugl-2 probes is provided in Table 1. Next, we assessed the methylation level of Hugl-2 in HKC, 786O, Caki-1 and Caki-2 cells using methylation-specific PCR (MS-PCR) analysis and found that the level was higher in 786O, Caki-1 and Caki-2 cells (Figure 1G).

Correlation between DNA methylation and Hugl-2 mRNA expression in KIRC was further analyzed using the MethHC web tool. The specific P-values are shown in Table 2. As indicated in Table 3, Hugl-2 DNA methylation correlated negatively with Hugl-2 mRNA expression.
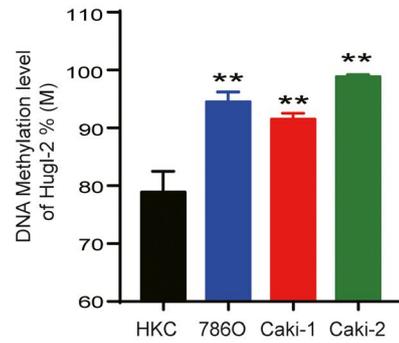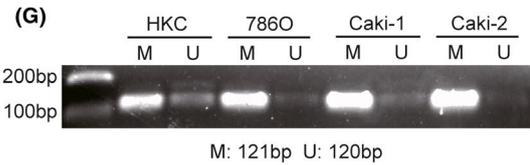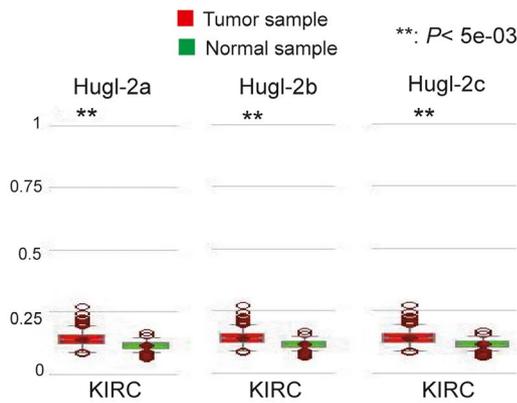
2.4 | Association of Hugl-2 DNA methylation and pathological features in KIRC

We used the LinkedOmics web tool to evaluate the association of Hugl-2 DNA methylation (all probes) and pathological features in KIRC. Hugl-2 DNA methylation (all probes) correlated with
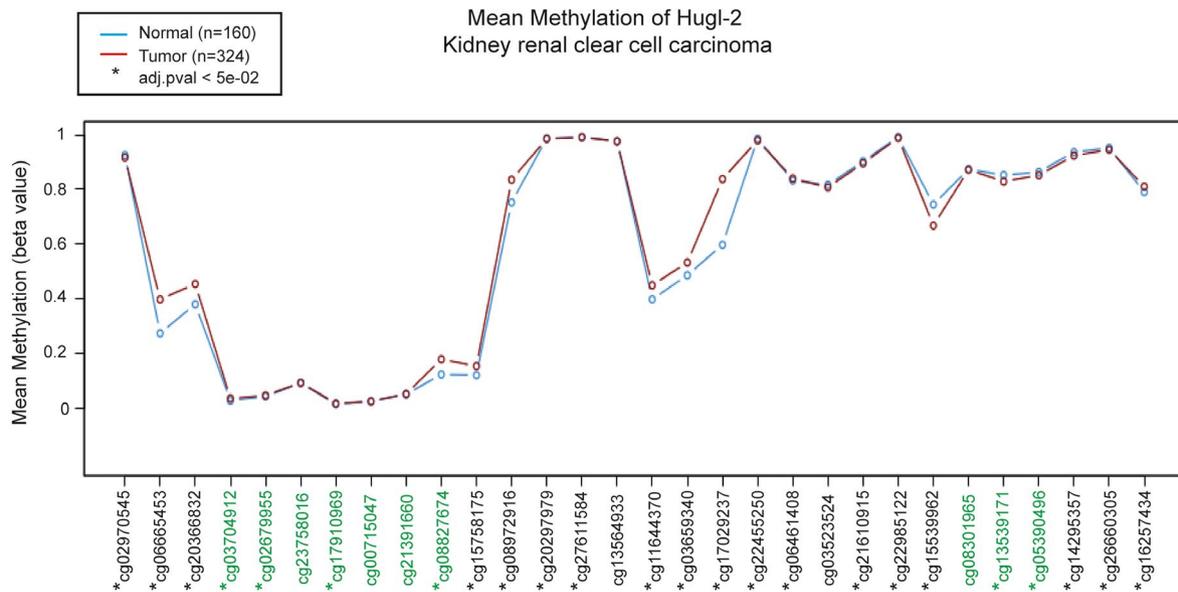
FIGURE 1 Hugl-2 expression and DNA methylation in kidney renal clear cell carcinoma (KIRC) samples. A, Hugl-2 mRNA expression. $P < 1e-12$. B, Hugl-2 protein expression. Z-values represent standard deviations from the median across samples for the given cancer type. $P = 1.93124477876003e-72$. C, Hugl-2 mRNA and tumour grade. Normal vs Grade 1, $P = 8.47759999977882e-08$; Normal vs Grade 2, $P = 1.62447832963153e-12$; Normal vs Grade 3, $P = 1.62447832963153e-12$; Normal vs Grade 4, $P < 1e-12$; Grade 1 vs Grade 2, $P = 1.620360e-02$; Grade 1 vs Grade 3, $P = 2.154100e-04$; Grade 1 vs Grade 4, $P = 2.42519999638091e-08$; Grade 2 vs Grade 3, $P = 2.680100e-02$; Grade 2 vs Grade 4, $P = 5.01219954429644e-10$; Grade 3 vs Grade 4, $P = 2.28719999995075e-06$. D, Hugl-2 mRNA and overall survival. $P = 1.4e-2$. Box plots and P-values in (A-D) were produced using UALCAN (http://ualcan.path.uab.edu/index.html). E, DNA methylation of three Hugl-2 isoforms in KIRC. Box plots and P-values were obtained using MethHC (http://methhc.mbc.nctu.edu.tw/php/index.php); **$P < 5e-03$. F, Mean Hugl-2 DNA methylation in KIRC samples. The green-colored font represents CpG islands; adj.pval represents the adjusted P-value and *$P < 5e-02$. The plot and P-values were produced in Wanderer (http://maplab.imppc.org/wanderer/). G, Methylation level of Hugl-2 promoter regions in HKC, 786O, Caki-1 and Caki-2 cells detected by MS-PCR. M, methylated; U, unmethylated; methylated and unmethylated levels were quantified as M/(M + U) × 100% and U/(M + U) × 100%, respectively. **$P < 5e-03$

(A)

Expresson of Hugl-2 in KIRC based on Sample type

P < 1e-12

(B)

Protein expression of Hugl-2 in KIRC

P =1.93124477876003e-72

(C)

Expresson of Hugl-2 in KIRC based on tumor grade

(D)

Effect Hugl-2 expresson level on KIRC patient survival

(E)

Comparison of average Hugl-2 beta value in tumor samples and matched normal samples

(G)

M: 121bp U: 120bp

(F)

Mean Methylation of Hugl-2
Kidney renal clear cell carcinoma

TABLE 1 Hugl-2 DNA methylation in normal tissues vs kidney renal clear cell carcinoma (KIRC) tumour specimens

Probe	Gene name	Wolcox_ stat	Adj.pval
cg02970545	TSEN54	33 368.5	5.34e-07
cg06665453	TSEN54	9893.5	1.29e-27
cg20366832	TSEN54	15 570	2.60e-12
cg03704912	LLGL2	13 766	1.97e-16
cg02679955	LLGL2	20 849	7.26e-04
cg23758016	LLGL2	25 300	7.16424e-01
cg17910969	LLGL2	15 028	1.99e-13
cg00715047	LLGL2	26 964	5.43423e-01
cg21391660	LLGL2	25 592.5	8.21272e-01
cg08827674	LLGL2	8775	2.30e-31
cg15758175	LLGL2	13 439.5	3.29e-17
cg08972916	LLGL2	5052	6.12e-46
cg20297979	LLGL2	17 902.5	6.53e-08
cg27611584	LLGL2	31 989.5	5.17e-05
cg13564933	LLGL2	26 568	7.16424e-01
cg11644370	LLGL2	16 203	5.21e-11
cg03659340	LLGL2	21 414	2.683e-03
cg17029237	LLGL2	3991	2.29e-50
cg22455250	LLGL2	29 244.5	2.7066e-02
cg06461408	LLGL2	15 343.5	9.14e-13
cg03523524	LLGL2	28 837	5.2701e-02
cg21610915	LLGL2	29 925	7.726e-03
cg22985122	LLGL2	34 557.5	5.58e-09
cg15539962	LLGL2	41 188	3.13e-25
cg08301965	LLGL2	26 309	8.15579e-01
cg13539171	LLGL2	31 277	3.59e-04
cg05390496	LLGL2	30 420.5	2.683e-03
cg14295357	LLGL2	31 841.5	7.59e-05
cg26660305	LLGL2	29 352	2.316e-02
cg16257434	LLGL2	16 375.5	1.07e-10

Note: The 'adj.pval' represents the adjusted *P*-value.

pathologic stage ($P = 8.22e-03$, n = 218) and T stage ($P = 3.88e-02$, n = 219; Figure 2A,B). These results indicate that Hugl-2 DNA methylation correlates with KIRC progression.

2.5 | Association of Hugl-2 DNA methylation and KIRC prognosis

The LinkedOmics web tool was also employed to examine the association of Hugl-2 DNA methylation and KIRC prognosis (Figure 2C),

TABLE 2 Correlation of Hugl-2 probe DNA methylation with Hugl-2 mRNA expression

Probe	*P*-value
cg02970545	−2.2204460492503e-16
cg06665453	2.2204460492503e-16
cg20366832	−2.2204460492503e-16
cg03704912	−2.2204460492503e-16
cg02679955	−4.4408920985006e-16
cg23758016	0
cg17910969	−4.4408920985006e-16
cg00715047	−2.2204460492503e-16
cg21391660	−2.2204460492503e-16
cg08827674	1.1102230246252e-16
cg15758175	0
cg08972916	−4.4408920985006e-16
cg20297979	0
cg27611584	0
cg13564933	−8.8817841970013e-16
cg11644370	−2.2204460492503e-16
cg03659340	−4.4408920985006e-16
cg17029237	−2.2204460492503e-16
cg22455250	−2.2204460492503e-16
cg06461408	−2.2204460492503e-16
cg03523524	−2.2204460492503e-16
cg21610915	0
cg22985122	1.1102230246252e-16
cg15539962	0
cg08301965	3.3306690738755e-16
cg13539171	2.2204460492503e-16
cg05390496	−2.2204460492503e-16
cg14295357	−4.4408920985006e-16
cg26660305	−2.2204460492503e-16

Note: *P*-values were generated using linear regression.

and Hugl-2 DNA methylation significantly ($P < 5e-02$) was observed to affect the overall survival of KIRC patients. In fact, overall survival was significantly shorter for KIRC patients with high Hugl-2 DNA methylation levels than those with low Hugl-2 DNA methylation levels (log-rank $P = 2.11e-02$, n = 216). These results indicate that high levels of Hugl-2 DNA methylation indicate a poor prognosis in KIRC.

2.6 | Correlation between individual probe methylation and Hugl-2 mRNA

All of the Hugl-2 probes in the 450 methylation array were further analyzed for correlations with Hugl-2 mRNA expression (Table 3), revealing a moderate or weak correlation between

TABLE 3 Correlation of Hugl-2 DNA methylation with Hugl-2 mRNA expression

| Probe | Spearman coefficient (Meth vs mRNA) | | Correlation |
	Normal	Tumour	
cg02970545	0.055	−0.149	Weak
cg06665453	−0.288	−0.235	Weak
cg20366832	−0.167	−0.321	Moderate
cg03704912	−0.247	−0.134	Weak
cg02679955	−0.314	−0.089	
cg23758016	−0.112	−0.079	
cg17910969	−0.172	−0.053	
cg00715047	0.057	−0.03	
cg21391660	−0.317	−0.213	Weak
cg08827674	−0.493	−0.407	Moderate
cg15758175	−0.339	−0.359	Moderate
cg08972916	0.054	0.067	
cg20297979	0.394	0.057	
cg27611584	−0.025	0.071	
cg13564933	−0.092	0.032	
cg11644370	−0.203	−0.448	Moderate
cg03659340	0.084	−0.323	Moderate
cg17029237	−0.088	−0.228	Weak
cg22455250	0.133	0.098	
cg06461408	0.303	0.096	
cg03523524	0.245	0.001	
cg21610915	0.171	−0.164	Weak
cg22985122	0.387	0.131	Weak
cg15539962	0.281	0.357	
cg08301965	0.211	−0.101	Weak
cg13539171	0.514	−0.029	
cg05390496	0.384	−0.024	
cg14295357	0.339	−0.015	
cg26660305	−0.075	0.166	
cg16257434	0.146	0.044	

Note: 'Meth' represents methylation. 'Weak' represents $r = 0.1$ to 0.3 or $−0.1$ to $−0.3$; 'moderate' represents $r = 0.3$ to 0.5 or $−0.3$ to $−0.5$; 'strong' represents $r = 0.5$ to 1.0 or $−0.5$ to $−1.0$.

every methylation probe and Hugl-2 mRNA expression in KIRC samples. The highest correlation in both the normal and tumour groups was between Hugl-2 mRNA expression and probe cg08827674 (Table 3), in the Hugl-2 CpG island (Figure 1F and Table 4). The target of probe cg08827674 (chr17: 73522539-73522588) is shown in Table 4. Based on data in the UCSC website (http://genome.ucsc.edu/), cg08827674 is present in the binding domains for transcription factors EZH2 (chr17: 73521793-73523024), MAX (chr17:73522087-73522636), and SIN3AK20 (chr17:73522230-73522685).

2.7 | Association of cg08827674 methylation and pathological features in KIRC

The association of cg08827674 methylation and pathological features of KIRC using TCGA data integrated from the Wanderer web tool was further assessed (Table S1). The results showed that cg08827674 methylation correlated with pathologic stage ($P = 2.03e-02$), T stage ($P = 2.20e-02$), neoplasm histologic grade ($P = 5e-04$) and serum calcium level ($P = 4.2e-02$) but not with laterality, N stage, M stage, or ethnicity (Figure 3).

2.8 | Restoration of Hugl-2 mRNA and protein levels by a DNA methylation inhibitor in KIRC cell lines
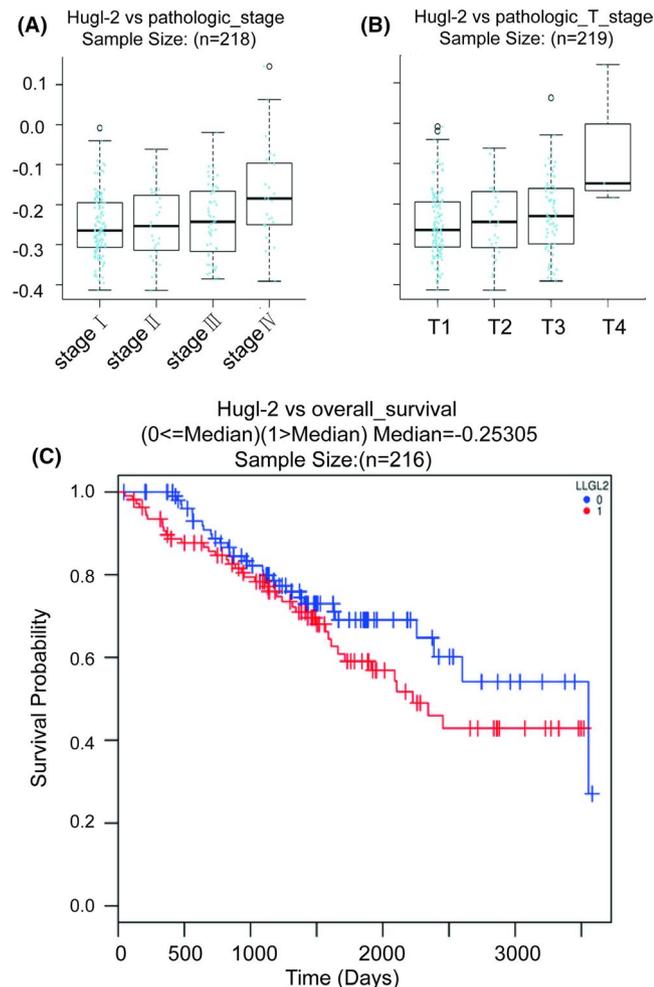
To verify the association of methylation with Hugl-2 mRNA levels, we evaluated the effect of decitabine (DAC), a methyltransferase inhibitor, on the expression levels of Hugl-2 in 786O, Caki-1 and Caki-2 cells and found that the mRNA level was higher in the DAC-treated cells than in the control cells (Figure 4A-C). Additionally, the mRNA levels of Hugl-2 increased as the concentration of DAC increased. Furthermore, treatment with 10 µmol/L of DAC for 72 hours enhanced the protein levels of Hugl-2 (Figure 4D), suggesting that elevated Hugl-2 DNA methylation may contribute to loss of Hugl-2 in KIRC.

3 | DISCUSSION

Increasing evidence has shown that loss of Hugl-2 contributes to tumourigenesis and progression *in vitro* and *in vivo*.[21-24] However, there is little information about whether and how Hugl-2 expression decreases during KIRC. Here, we show that Hugl-2 DNA methylation downregulates Hugl-2 mRNA and protein expression, promotes KIRC progression, and reduces the overall survival of KIRC patients. These conclusions are strongly supported by the following: (a) Hugl-2 mRNA expression is decreased in KIRC; (b) increased Hugl-2 DNA methylation correlates negatively with Hugl-2 mRNA expression; (c) Hugl-2 DNA methylation correlates with pathologic stage, T stage and poor prognosis in KIRC patients; and (d) methylation of the Hugl-2 probe cg08827674 correlates with pathologic stage, T stage, neoplasm histologic grade, and serum calcium level. Our findings represent an important contribution to the understanding of Hugl-2-mediated tumour suppression.

Renal cell carcinoma is one of the most common cancers with high mortality rates worldwide. Although many studies have been performed on KIRC, the clinical prognosis of these patients remains very poor, and the survival time of 90% of patients with metastatic KIRC is <5 years.[25] As with all cancers, researchers are striving to rapidly promote an understanding of the molecular biology of tumour formation and progression, which will provide the opportunity for developing new therapeutics and facilitating early diagnoses. Notably, loss of cell polarity is considered both a hallmark and precondition for human cancer.

It has been well documented that polarity proteins, including scribble, DLG5, and CRB3, among others, play suppressive roles in various types of cancers.[21,26-28] Hugl-1 and Hugl-2 also have tumour-suppressive effects in breast, gastric, colorectal cancer and lung adenocarcinoma.[20-23,29-31] Therefore, it is conceivable that Hugl-1 and Hugl-2 may also inhibit tumourigenesis in KIRC. However, using the LinkedOmics web tool, we only found that Hugl-2 DNA methylation is related to clinicopathological features in KIRC (Hugl-1 DNA methylation data are shown in Table S2). Based on this, we focused on the DNA methylation of Hugl-2 rather than of Hugl-1.

FIGURE 2 Association of Hugl-2 DNA methylation and clinical features in kidney renal clear cell carcinoma (KIRC) samples. Hugl-2 DNA methylation association with: A, pathologic stage ($P = 8.22$e-03, n = 218); B, T stage ($P = 3.88$e-02, n = 219); and C, overall survival ($P = 2.11$e-02, n = 216). Box plots and Kaplan–Meier plots were produced using LinkedOmics (http://www.linkedomics.org/login.php). A and B were statistically tested using the Kruskal–Wallis test, and C was analyzed using the Cox regression test

Recently, a substantial number of studies on the prognostic value of DNA methylation in RCC have been published.[6] For example, Peng D et al reported that prognostic models using 19 CpG sites in KIRC, which were identified using TCGA and gene expression omnibus databases, could be used to distinguish high- and low-risk patients and improve the predictive ability of the tumour node metastasis staging system.[32] Some potential prognostic methylation markers for RCC, such as SCUBE3, BNC1, GATA5, SFRP1, GREM1, RASSF1A, PCDH8, LAD1, NEFH and neural EGFL-like 1, have been validated.[6,33] Furthermore, methylation of PCDH17 in serum samples is frequent detected in RCC and is associated with poor outcomes.[34]

As mentioned above, aberrant DNA methylation is an early event in the process of carcinogenesis and increases gradually as the tumour progresses. Hence, the DNA methylation levels of precancerous lesions and early tumor detection are among the most promising methods for early diagnosis of cancer. In this study, we found that high Hugl-2 DNA methylation levels reduced Hugl-2 mRNA expression and further promoted the malignancy, invasion, and metastasis of renal tumours and decreased the survival time of KIRC patients. Our results suggest that Hugl-2 DNA methylation contributes to KIRC progression. This finding may provide a novel clinical marker for the early diagnosis, prognosis and treatment of KIRC.
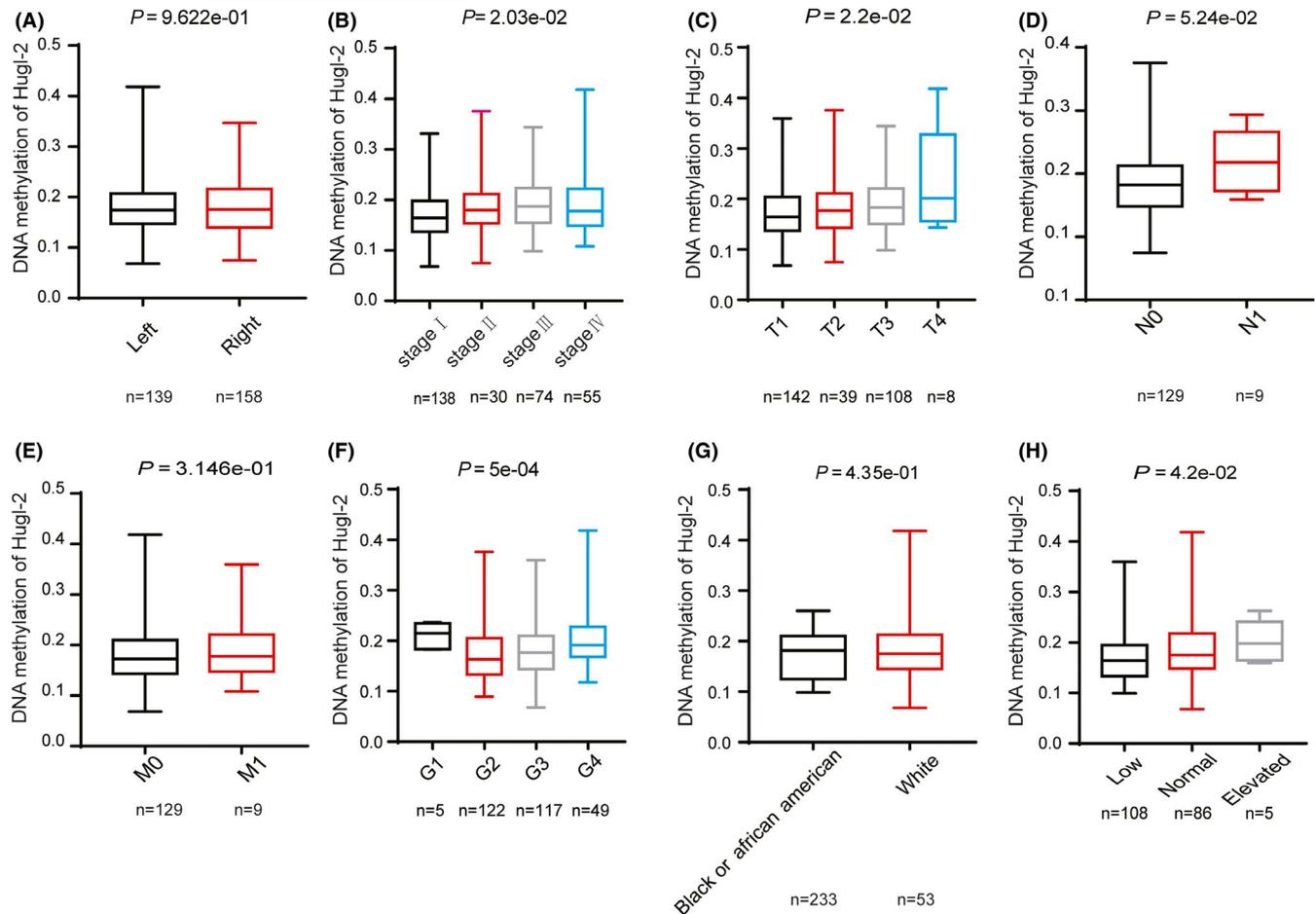
4 | MATERIALS AND METHODS

4.1 | mRNA and protein expression of Hugl-2, association between Hugl-2 mRNA and tumour grade

UALCAN (http://ualcan.path.uab.edu/index.html) is a web tool for analyzing tumour transcriptome data. The web tool provides publicly accessible cancer transcriptome data (TCGA mRNA sequencing), published gene expression data with graphs and plots, and patient survival information.[35] Hugl-2 mRNA and protein expression in normal and tumour specimens and the association of Hugl-2 mRNA and tumour grade in KIRC patients were comparatively analyzed using this tool.

4.2 | Hugl-2 methylation analysis

The human pancancer methylation database MethHC is a web-based resource focusing on DNA methylation in human diseases (http://methhc.mbc.nctu. edu.tw/php/index. php). MethHC integrates data covering gene expression, DNA methylation, microRNA expression, microRNA methylation, and the correlation of methylation and gene expression from TCGA.[36] Comparisons between Hugl-2 DNA methylation and gene expression were obtained using MethHC.

TABLE 4 Specific information for cg08827674

Probe	Chr	Cg_start	Cg_end	probe_start	probe_end	gene_start	gene_end
cg08827674	chr17	73 522 539	73 522 540	73 522 539	73 522 588	73 521 161	73 571 289

FIGURE 3 Association of cg08827674 methylation and pathological features in KIRC samples. A, Laterality (*P* = 9.622e-01); B, pathologic stage (*P* = 2.03e-02); C, T stage (2.2e-02); D, N stage (*P* = 5.24e-02); E, M stage (*P* = 3.146e-01); F, neoplasm histologic grade (*P* = 5e-04); G, race (*P* = 4.35e-01); and H, serum calcium level (*P* = 4.2e-02). *P*-values were generated using the Mann–Whitney test (A, D, E and G) and Kruskal–Wallis test (B, C, F and H)

Wanderer (http://maplab.imppc.org/wanderer/) is an intuitive web tool that can be employed to analyze gene expression and DNA methylation profiles from TCGA. This web tool provides the DNA methylation levels of Illumina Human Methylation 450 Bead Chip loci inside or in the vicinity of the queried gene.[37] Correlations between methylation and Hugl-2 gene expression were tested using the Spearman (*r*) correlation method.

Correlations were further examined between individual probes with methylation changes and mRNA expression using MethHC. A correlation was considered either weak (*r* = 0.1 to 0.3 or −0.1 to −0.3), moderate (*r* = 0.3 to 0.5 or −0.3 to −0.5), or strong (*r* = 0.5 to 1.0 or −0.5 to −1.0).

4.3 | Association of Hugl-2 DNA methylation with pathological features and overall survival in KIRC patients

We used the LinkedOmics web tool to analyze multiomics data for all 32 TCGA cancer types (http://www.linkedomics.org/login.

php). Using three analytical LinkedOmics modules, we can identify and analyze information about mRNA or protein expression signatures, biomarkers of clinical attributes, and putative target genes of transcriptional factors, microRNAs, or protein kinases; the analysis results are depicted as plots.[38] The association of Hugl-2 DNA methylation with pathological features and overall survival in KIRC patients was analyzed using this tool with Illumina Human Methylation 27K arrays and clinical data via nonparametric analysis.

4.4 | Cell culture

Human clear cell renal cell carcinoma cell lines 786O, Caki-1 and Caki-2 were obtained from the National Infrastructure of Cell Line Resource. 786O cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% FBS (HyClone). Caki-1 and Caki-2 cells were cultured in McCoy's 5A medium (HyClone) supplemented with 10% FBS (HyClone). All cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

FIGURE 4 Restoration of Hugl-2 mRNA and protein expression by DAC in KIRC cell lines. 786O (A), Caki-1 (B) and Caki-2 (C) cells were treated with 1, 5, or 10 μmol/L DAC for 48 h or 72 h, and mRNA levels of Hugl-2 were detected by qPCR. All data are presented as the mean ± SEM (n = 3, *P < 5e-02, **P < 1e-02). D, KIRC cell lines were incubated with 1, 5, or 10 μmol/L DAC for 72 h, and protein levels of Hugl-2 were analyzed by western blotting (n = 3, **P < 1e-02)
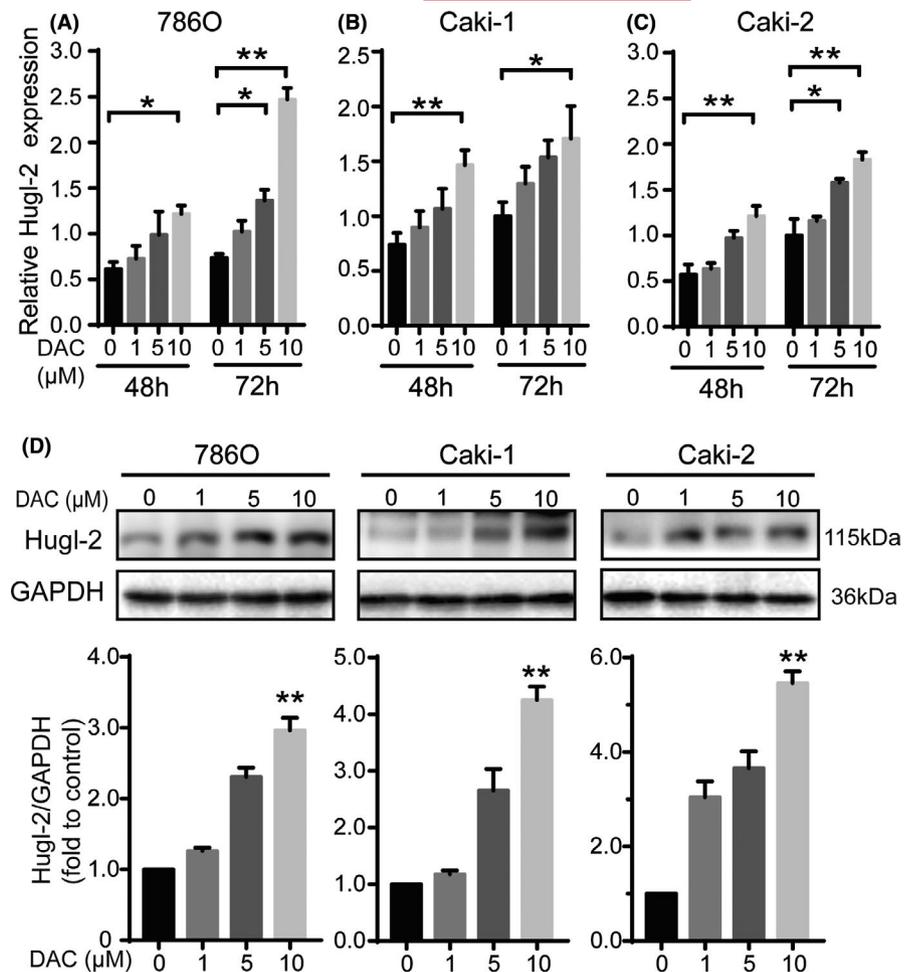
4.5 | DNA extraction, bisulfite modification and MS-PCR

Genomic DNA was isolated from HKC, 786O, Caki-1 and Caki-2 cells using a SteadyPure Universal Genomic DNA Extraction Kit (Accurate Biotechnology) according to the manufacturer's instructions. DNA modification was performed as previously described.[39] A total of 500 ng of DNA was bisulfite-modified with the EZ DNA Methylation-Gold kit (Zymo Research). Modified DNA templates were utilized for MS-PCR with Zymo Taq PreMix (E2003; Zymo Research) following the instructions of the manufacturer. The online software METHPRIMER(http://www.urogene.org/methprimer/) was applied for profiling of CpG islands in the region from −2000 to −200 bp upstream of ATG in the Hugl-2 promoters. The primer pairs used for MS-PCR are as follows: Left M primer, 5′-TTTGATCGAGTGTTTTGTGTTATTC-3′; Right M primer, 5′- AATACTTCCTCCTTCTAACCTCGA-3′; Left U primer, 5′- TTGATTGAGTG TTTTGTGTTATTTGT -3′; and Right U primer, 5′- AATACTTCCTCCTT CTAACCTCAAA -3′. PCR was performed using the following protocol: denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, annealing at 58.3°C for the methylated primer set and at 56.3°C for the unmethylated primer set for 30 seconds, and 72°C for 30 seconds, with a final elongation step of 72°C for 7 minutes. The MS-PCR product was visualized on a 2% agarose gel under ultraviolet (UV) light using a Gel Doc machine (Bio-Rad). The methylation level was calculated by the ratio of methylated and unmethylated levels, as follows: methylation (M) = M/(M + U); unmethylation = U/(M + U). The gray value of each band represents its relative expression, as measured using GELPRO32 software. Each reaction was performed in triplicate.

4.6 | DNA methylation inhibitor treatment

The same numbers of cells were seeded in each well of six-well plates and cultured in medium containing 1, 5, or 10 μmol/L DAC (Selleck) or vehicle (0.1% DMSO). The medium was refreshed every 24 hours over a 72-hour period. Cells were then harvested for quantification of Hugl-2 mRNA and protein levels.

4.7 | Quantitative real-time RT- PCR (RT-qPCR)

Total RNA was isolated from cells using RNA Fast 200 (#220010, Fastagen Biotech) and reverse transcribed into cDNA using PrimeScript RT Master Mix (TaKaRa Biotechnology)

according to the manufacturer's directions. qPCR was performed using SYBR Premix Ex Taq II (TaKaRa) with a Bio-Rad CFX96 system. The primers used were as follows: Hugl-2 forward, 5′-TTTAACAAGACGGTGGAGCA-3′, and reverse, 5′-GAGCTTGATGGCTCCAGAAC-3′; GAPDH forward, 5′-GTGGACCTG ACCTGC CGTCT-3′, and reverse, 5′-GGAGGAGTGGGTGTCGCT-3′. The experiment was performed in triplicate, and the level of Hugl-2 mRNA was normalized to that of GAPDH using the $2^{-\Delta Ct}$ method.

4.8 | Western blotting

Hugl-2 protein expression was detected by western blotting, as described previously.[40] After treatment with DAC, lysates were further centrifuged at 14 500 g for 15 minutes at 4°C, and the supernatants were collected and stored at −80°C. Total protein concentrations were determined using a BCA protein assay kit (Fdbio). Equal amounts of protein (100 μg) with loading buffer were separated on SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes (Roche). The following antibodies were used: anti-GAPDH (Proteintech) and anti-Hugl-2 (Abnova). Chemiluminescent signals were detected using Fdbio-Dura ECL (Fdbio).

4.9 | Statistical analysis

Statistical analyses were performed using GRAPHPAD PRISM Version 7.0 (GRAPHPAD Software). The statistical significance of differences between two groups was tested by the Mann–Whitney U test; the significance of differences among three or four groups was determined by the Kruskal–Wallis test. When assessing the DNA methylation inhibitor, the significance of differences among the four groups of Hugl-2 mRNA and protein expression was compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Correlation between overall survival and Hugl-2 DNA methylation was assessed using the Cox regression test. All statistical tests were two-sided, and all results are expressed as the mean ± SEM. All in vitro data were obtained from three experimental replicates with similar results.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.