INTRODUCTION

Kidney cancer is one of 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. Kidney renal clear cell carcinoma (KIRC) is the most common subtype of renal cell carcinoma, accounting for 90% of all renal tumours. 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. 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 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
DNA methylation is one of the most well-studied epigenetic modifications in mammals and can contribute to renal tumourigenesis by silencing tumor-suppressor genes. Additionally, DNA methylation alterations have been demonstrated to be associated with clinicopathological features and patient survival. It has been reported that RCC DNA methylation represents a potential biomarker for early detection, prognosis and prediction of response to therapy because it is found early during carcinogenesis, including in precancerous lesions. Furthermore, as stable DNA marks that can be quantitatively measured, changes in DNA methylation are useful in detection strategies.

Cell polarity is a crucial phenomenon in many biological processes and is required for normal tissue integrity and tissue homeostasis. 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). Humans express two Lgl isoforms, Hugl-1 and Hugl-2; the latter is a 1020-amino acid protein containing 14 predicted WD40 repeats. 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. 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. In addition, other studies in our laboratory (unpublished) have shown that loss of Hugl-2 induces renal tumorigenesis and contributes to a poor prognosis in KIRC patients. In view of the potential role Hugl-2 plays in suppressing renal tumorigenesis, 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 tumorigenesis 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.

FIGURE 1 Hugl-2 expression and DNA methylation in kidney renal clear cell carcinoma (KIRC) samples. A, Hugl-2 mRNA expression. 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 = 2.632606e-02; Grade 1 vs Grade 3, P = 2.154100e-04; Grade 1 vs Grade 4, P = 2.425199999630891e-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.
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 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      | 7.1642e-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     | 26 964      | 5.43423e-01 |
| 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.

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) | Normal | Tumour | Correlation |
|---------------|-------------------------------------|--------|--------|-------------|
| 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 | Moderate    |
| 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 $-1.0$.

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. Hugl-1 and Hugl-2 also have tumour-suppressive effects in breast, gastric, colorectal cancer and lung adenocarcinoma. 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.

Recently, a substantial number of studies on the prognostic value of DNA methylation in RCC have been published. Peng 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. 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. Furthermore, methylation of PCDH17 in serum samples is frequent detected in RCC and is associated with poor outcomes.

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. 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. Comparisons between Hugl-2 DNA methylation and gene expression were obtained using MethHC.

| 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 |
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. 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. 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.
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. 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′- TTTGATCGAGTGTTTTGTGTTATTC-3′; Left U primer, 5′- TTGATTGAGTGTTTTGTGTTATTTGT-3′; and Right U primer, 5′- AATACCTCCTCTCTCAGTCTCGA-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)
4.8 Western blotting

Hugl-2 protein expression was detected by western blotting, as described previously.\textsuperscript{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.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

PEER REVIEW

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**SUPPORTING INFORMATION**

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

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