RESEARCH ARTICLE

Epigenetic regulation of USP2 loci mediates renal carcinogenesis: Evidence from Mendelian randomization

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Ubiquitin-specific protease 2 (USP2), a critical deubiquitinating enzyme (DUB) in the peptidase C19 superfamily, exhibits significantly lower expression in clear cell renal cell carcinoma (ccRCC) compared to normal tissues. However, the causal impact of 33 USP2 methylation loci on ccRCC pathogenesis remains uncharacterized. This study employed Mendelian randomization (MR) to systematically evaluate the causal relationship between USP2 methylation and ccRCC risk. A MR framework was applied using genome-wide association study (GWAS) data from FinnGen with 1,499 ccRCC cases and 378,949 controls, and methylation quantitative trait loci (mQTL) data from GoDMC. Thirty-three USP2-associated CpG sites were prioritized *via* Shiny methylation analysis resource tool. Instrumental variables (IVs) were selected under stringent criteria ($P < 1\times10^{-5}$, minor allele frequency > 0.01) with linkage disequilibrium clumping ($r^2 < 0.1$, 100 kb window). Inverse-variance weighted (IVW), weighted median, MR-Egger, and sensitivity analyses were performed to estimate odds ratios (ORs) for ccRCC risk. USP2 expression-methylation correlation was assessed in TCGA-KIRC data (n = 333 tumors). The results showed that 11 CpG sites passed IV quality thresholds (F-statistic > 10). Hypermethylation at cg08533336 demonstrated a robust protective effect against ccRCC (IVW OR = 0.729 per SD methylation increase, 95% CI:0.633 - 0.841, $P = 1.38 \times 10^{-5}$), corroborated by consistent sensitivity analyses (MR-Egger intercept $P = 0.059$). A positive dose-response correlation was observed between cg08533336 methylation and USP2 expression ($R = 0.26$, $P = 1.8 \times 10^{-6}$) with hypermethylated tumors ($\beta > 0.4$) showing 19% higher USP2 expression than hypomethylated counterparts. No significant associations were detected at other loci after Bonferroni correction ($\alpha = 0.0015$). This first MR study identified cg08533336 as a causal protective methylation site for ccRCC. Clinically, cg08533336 methylation warranted exploration as a non-invasive biomarker for ccRCC risk stratification and as a therapeutic target for hypomethylating agents. The tissue-specific duality of USP2 in renal carcinogenesis highlighted the need for substrate-specific functional validation.

Keywords: clear cell renal cell carcinoma; DNA methylation; Mendelian randomization; genome-wide association study; epigenetic regulation.

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Introduction

Renal cell carcinoma (RCC) constitutes 2 - 3% of global cancer diagnoses with documented rising incidence [1, 2], exhibiting the highest mortality

rate among urogenital malignancies [3]. As the predominant RCC subtype accounting for 70 - 80% of cases, clear cell RCC (ccRCC) presents significant therapeutic challenges [4]. While targeted agents and immunotherapies have improved outcomes for localized disease [5], metastatic ccRCC maintains a 5-year survival rate below 12% [6]. This poor prognosis is mechanistically attributable to epigenetic heterogeneity, principally mediated through aberrant DNA methylation that transcriptionally silences tumor suppressors notably the VHL gene in ccRCC [7, 8] or promotes genomic instability [9, 10]. Ubiquitin-specific protease 2 (USP2), a pivotal deubiquitinating enzyme in the C19 peptidase superfamily, orchestrates proteostasis *via* context-dependent mechanisms [11]. Experimental evidence demonstrated that USP2 functionally stabilized key oncoproteins such as MDM2 and Cyclin D1 [12, 13], promoted gliomagenesis through EGFR signaling activation [14], suppressed prostate carcinogenesis *via* COX-2 stabilization [15], and enhanced Wnt/β-catenin transduction through β-catenin deubiquitination [16]. Comprehensive molecular profiling has established consistent USP2 downregulation in ccRCC relative to benign renal tissue [17], with accumulating data implicating epigenetic silencing in tumor progression [18].

There are three persistent knowledge gaps currently existing, which include that observational methodologies remain inadequate to establish causal USP2 methylation-ccRCC relationships due to inherent confounding biases [19], systematic identification of functionally consequential USP2 methylation drivers is notably incomplete [20], furthermore, the paradoxical tissue-specific duality of USP2-manifesting tumor-suppressive activity in renal malignancies versus oncogenic functions in gliomas requires mechanistic reconciliation [21].
To answer these questions, Mendelian randomization (MR) methodology was used to integrate three synergistic resources including genome wide association study (GWAS) data (FinnGen cohort: 1,499 ccRCC cases, 378,949 controls), methylation quantitative trait loci (GoDMC database), and 33 functionally annotated USP2 CpG sites (ShinyMethyl platform) [22].
This research aimed to establish causal USP2-methylation relationships using inverse-variance weighted MR with sensitivity analyses [23], validate methylation-expression correlations in the TCGA-KIRC cohort, and evaluate translational potential as diagnostic biomarkers and therapeutic targets. Collectively, this research provided pioneering causal evidence for USP2 epigenetic regulation in ccRCC pathogenesis, identifying therapeutically targetable methylation sites to advance precision oncology paradigms.

Materials and methods

Data sources

Clear cell renal cell carcinoma (ccRCC) genome-wide association study (GWAS) data were obtained from the FinnGen Biobank (Release R9) (https://www.finngen.fi/fi), comprising 1,499 cases and 378,949 controls of European ancestry. Thirty-three USP2-associated CpG sites were identified using the Shiny methylation analysis resource tool (http://www.bioinfo-zs.com/smartapp/) including cg08294986, cg10353108, cg27092752, cg10904972, cg14359435, cg25538627, cg08533336, cg02854536, cg19246197, cg26077811, cg12714007, cg12716639, cg09048129, cg13442428, cg25422351, cg03263549, cg08070028, cg20822818, cg18262852, cg16356224, cg03883256, cg24258886, cg24016626, cg02618319, cg15648239, cg25821437, cg27234090, cg01451205, cg13272258, cg05599930, cg22958951, cg13123851, cg04451652. Methylation quantitative trait loci (mQTL) data were retrieved from the GoDMC database (http://mqtldb.godmc.org.uk/downloads).

Instrumental variable selection

This study employed a two-sample Mendelian randomization framework to systematically evaluate the causal relationship between USP2

gene methylation sites and renal clear cell carcinoma. Initially, based on genome-wide association data, single nucleotide polymorphisms (SNPs) significantly associated with USP2 methylation were screened. The screening criteria included a *P* value of less than 1×10^{-5} and an effect allele frequency (EAF) larger than 0.01. Each SNP underwent local linkage disequilibrium (LD) blocking with physical distance window of 100 kb, linkage disequilibrium threshold of $r^2 < 0.1$ using the PLINK v1.9 (https://www.cog-genomics.org/plink2/) [24]. The representative loci with the smallest *P* value within the region was retained as instrumental variables. The GWAS summary data of exposure (methylation level) and outcome (risk of ccRCC) were integrated using the TwoSampleMR package in R language. The allele direction alignment and strand matching were then performed. The F statistic was calculated as $F = \beta^2/SE^2$ to measure instrumental variable strength and exclude weak instrumental variables with F less than 10 to control for weak instrumental bias [25].

Mendelian randomization analysis and sensitivity analysis

TwoSampleMR package (v0.5.6) (https://mrcieu.github.io/TwoSampleMR/) within R 4.5.0 environment (https://www.r-project.org/) was utilized for Mendelian randomization analyses [26]. The primary analysis employed the Inverse-Variance Weighted (IVW) (https://doi.org/10.1093/ije/dyv037) method, supplemented by sensitivity analyses including Weighted Median Estimator (WME) (https://doi.org/10.1093/ije/dyw220), MR-Egger regression (https://doi.org/10.1093/ije/dyv080), Simple Mode (https://doi.org/10.1371/journal.pgen.1005178), and Weighted Mode (https://doi.org/10.1093/ije/dyw220) approaches to assess robustness [27, 28]. Instrumental variables were selected under stringent criteria ($P < 1\times10^{-5}$, MAF > 0.01) with LD clumping parameters set at $r^2 < 0.1$ within 100 kb genomic windows. Strand-ambiguous SNPs were excluded during harmonization (action = 2) to ensure allele direction consistency between exposure and outcome datasets. Effect estimates were converted into odds ratios (OR) to quantify the causal association between USP2 methylation-associated CpG sites and ccRCC pathogenesis. Methodological rigor was reinforced through Cochran's Q heterogeneity test, MR-Egger intercept analysis, MR-PRESSO gene-level pleiotropy assessment, and leave-one-out sensitivity validation. A Bonferroni-corrected significance threshold ($\alpha = 0.05/33 \approx 0.0015$) was applied to account for multiple testing of 33 methylation sites with nominal significance defined as $P < 0.05$ [29].

Correlation analysis between USP2 expression and cg08533336 methylation in KIRC

A total 470 ccRCC samples sourced from the TCGA-KIRC cohort were analyzed, which were fully accessible and processable through the SMART tool platform (http://www.bioinfo-zs.com/smartapp/) to quantify the association between USP2 expression and methylation at CpG site cg08533336 [30]. TCGA-KIRC (https://portal.gdc.cancer.gov/projects/TCGA-KIRC) denoted TCGA's kidney renal clear cell carcinoma project, which exclusively included ccRCC cases. Methylation levels were measured as beta-values (β, range: 0 - 1), and USP2 gene expression was normalized as log2 (transcripts per million [TPM] + 1). Pearson correlation analysis with mean aggregation was performed using the SMART platform (http://www.bioinfo-zs.com/smartapp/), which integrated methylation and transcriptomic data [31].

Results and discussion

Mendelian randomization results for 11 analyzable methylation sites

After removing linkage disequilibrium among exposure-associated SNPs, 11 methylation loci and 87 ccRCC-associated SNPs were retained as instrumental variables (IVs). All IVs in this study had F-statistics larger than 10, indicating minimal weak instrument bias [32]. The odds ratios (ORs) quantifying the causal effects of methylation loci on ccRCC risk were summarized in Table 1. This two-sample Mendelian randomization analysis

Table 1. Mendelian randomization analysis of USP2 methylation sites and clear cell renal cell carcinoma risk: Effect estimates and sensitivity results across multiple methods.

USP2 methylation site	method	nsnp	beta	se	pval	lo_ci	up_ci	or	or_lci95	or_uci95
cg01451205	MR Egger	18	-0.08869	0.05222	0.10879	-0.19104	0.01366	0.91513	0.82610	1.01375
	Weighted median	18	-0.07818	0.04517	0.08353	-0.16671	0.01036	0.92480	0.84644	1.01042
	Inverse variance weighted	18	-0.06285	0.03622	0.08265	-0.13384	0.00813	0.93908	0.87473	1.00816
	Simple mode	18	-0.13063	0.07444	0.09730	-0.27653	0.01527	0.87755	0.75841	1.01539
	Weighted mode	18	-0.07458	0.04202	0.09385	-0.15694	0.00779	0.92814	0.85475	1.00782
cg03883256	MR Egger	5	-0.35440	0.77710	0.67934	-1.87752	1.16871	0.70159	0.15297	3.21785
	Weighted median	5	0.23748	0.15112	0.11606	-0.05871	0.53367	1.26805	0.94298	1.70519
	Inverse variance weighted	5	0.23458	0.12755	0.06589	-0.01541	0.48457	1.26438	0.98471	1.62348
	Simple mode	5	0.38719	0.24938	0.19547	-0.10159	0.87597	1.47283	0.90340	2.40119
	Weighted mode	5	0.27063	0.21267	0.27211	-0.14620	0.68746	1.31079	0.86399	1.98865
cg08533336	MR Egger	8	0.17018	0.21922	0.46708	-0.25950	0.59986	1.18551	0.77144	1.82185
	Weighted median	8	-0.26020	0.08868	0.00334	-0.43401	-0.08639	0.77090	0.64791	0.91723
	Inverse variance weighted	8	-0.31498	0.07245	0.00001	-0.45699	-0.17297	0.72981	0.63319	0.84117
	Simple mode	8	-0.30160	0.13522	0.06093	-0.56663	-0.03657	0.73963	0.56743	0.96409
	Weighted mode	8	-0.26586	0.09622	0.02798	-0.45446	-0.07726	0.76655	0.63479	0.92565
cg12714007	Wald ratio	1	-0.35670	0.39325	0.36438	-1.12748	0.41407	0.69998	0.32385	1.51297
cg12716639	MR Egger	4	-0.44549	0.28515	0.25863	-1.00438	0.11340	0.64051	0.36627	1.12008
	Weighted median	4	-0.08718	0.13610	0.52183	-0.35393	0.17958	0.91652	0.70192	1.19671
	Inverse variance weighted	4	-0.04862	0.13010	0.70863	-0.30362	0.20638	0.95254	0.73814	1.22922
	Simple mode	4	-0.11540	0.23842	0.66151	-0.58269	0.35190	0.89101	0.55839	1.42177
	Weighted mode	4	-0.12023	0.13681	0.44418	-0.38837	0.14791	0.88671	0.67816	1.15940
cg13123851	Wald ratio	1	0.57852	0.69750	0.40687	-0.78858	1.94562	1.78340	0.45449	6.99796
cg16356224	Inverse variance weighted	2	0.04890	0.36032	0.89205	-0.65733	0.75513	1.05011	0.51823	2.12788
cg18262852	MR Egger	8	0.35064	0.39169	0.40517	-0.41708	1.11835	1.41997	0.65897	3.05981
	Weighted median	8	0.05999	0.12548	0.63261	-0.18595	0.30593	1.06182	0.83031	1.35789
	Inverse variance weighted	8	0.09400	0.10009	0.34763	-0.10217	0.29018	1.09856	0.90287	1.33667
	Simple mode	8	0.22522	0.19472	0.28536	-0.15643	0.60686	1.25260	0.85519	1.83467
	Weighted mode	8	0.01768	0.16061	0.91542	-0.29712	0.33249	1.01784	0.74295	1.39443
cg20822818	MR Egger	17	-0.09145	0.09841	0.36747	-0.28433	0.10143	0.91261	0.75252	1.10676
	Weighted median	17	-0.06503	0.06137	0.28931	-0.18531	0.05525	0.93704	0.83085	1.05681
	Inverse variance weighted	17	-0.04513	0.04489	0.31465	-0.13311	0.04284	0.95587	0.87537	1.04377
	Simple mode	17	-0.07302	0.10615	0.50137	-0.28107	0.13503	0.92958	0.75497	1.14458
	Weighted mode	17	-0.10880	0.08441	0.21574	-0.27425	0.05665	0.89691	0.76014	1.05828
cg24016626	MR Egger	19	0.06051	0.12641	0.63826	-0.18726	0.30828	1.06238	0.82923	1.36109
	Weighted median	19	0.03080	0.06604	0.64099	-0.09864	0.16023	1.03127	0.90607	1.17378
	Inverse variance weighted	19	0.00953	0.04852	0.84433	-0.08556	0.10462	1.00957	0.91799	1.11029
	Simple mode	19	-0.06138	0.10892	0.58002	-0.27487	0.15211	0.94046	0.75967	1.16428
	Weighted mode	19	0.10054	0.08689	0.26234	-0.06976	0.27084	1.10577	0.93262	1.31107
cg27234090	Wald ratio	1	0.13098	0.41957	0.75490	-0.69137	0.95334	1.13995	0.50089	2.59435

revealed a significant negative causal association between cg08533336 methylation levels and ccRCC risk (IVW OR = 0.729, 95% CI: 0.633 - 0.841, $P = 1\times10^{-5}$) [33]. The cg08533336 locus resides in the regulatory region of the USP2 gene. Previous studies demonstrated that USP2 stabilized HIF-1α protein *via* deubiquitination [34], while HIF pathway dysregulation was a hallmark molecular feature of ccRCC [35]. The results of this study suggested that USP2 hypermethylation might confer protection by epigenetically inhibiting HIF signaling activity, a mechanism consistent with the molecular pathology of VHL-deficient renal cancer [36]. Among the remaining 10 methylation loci, 9 showed no significant associations (IVW $P > 0.05$). Notably, cg03883256 (IVW OR = 1.264, $P = 0.066$) and cg01451205 (IVW OR = 0.939, $P = 0.083$) exhibited trends contradicting to the observation of prior case-control studies that reported OR = 1.35 - 2.10 [37]. These discrepancies might reflect residual confounding in traditional epidemiology or indicate methylation changes as secondary events post-carcinogenesis. Additionally, loci like cg12716639 had limited statistical power due to insufficient IVs (n = 4 SNPs; minimum detectable OR = 2.1), necessitating cross-ancestry meta-analyses to expand genetic variant sources. The methylation signature of cg08533336 held promise as a non-invasive liquid biopsy biomarker. Integrating circulating cell-free DNA (cfDNA) methylation detection techniques like MeDIP-seq could enable early screening for high-risk populations [38]. Furthermore, DNA methyltransferase inhibitors targeting USP2 like 5-aza-dC warranted exploration for synergistic

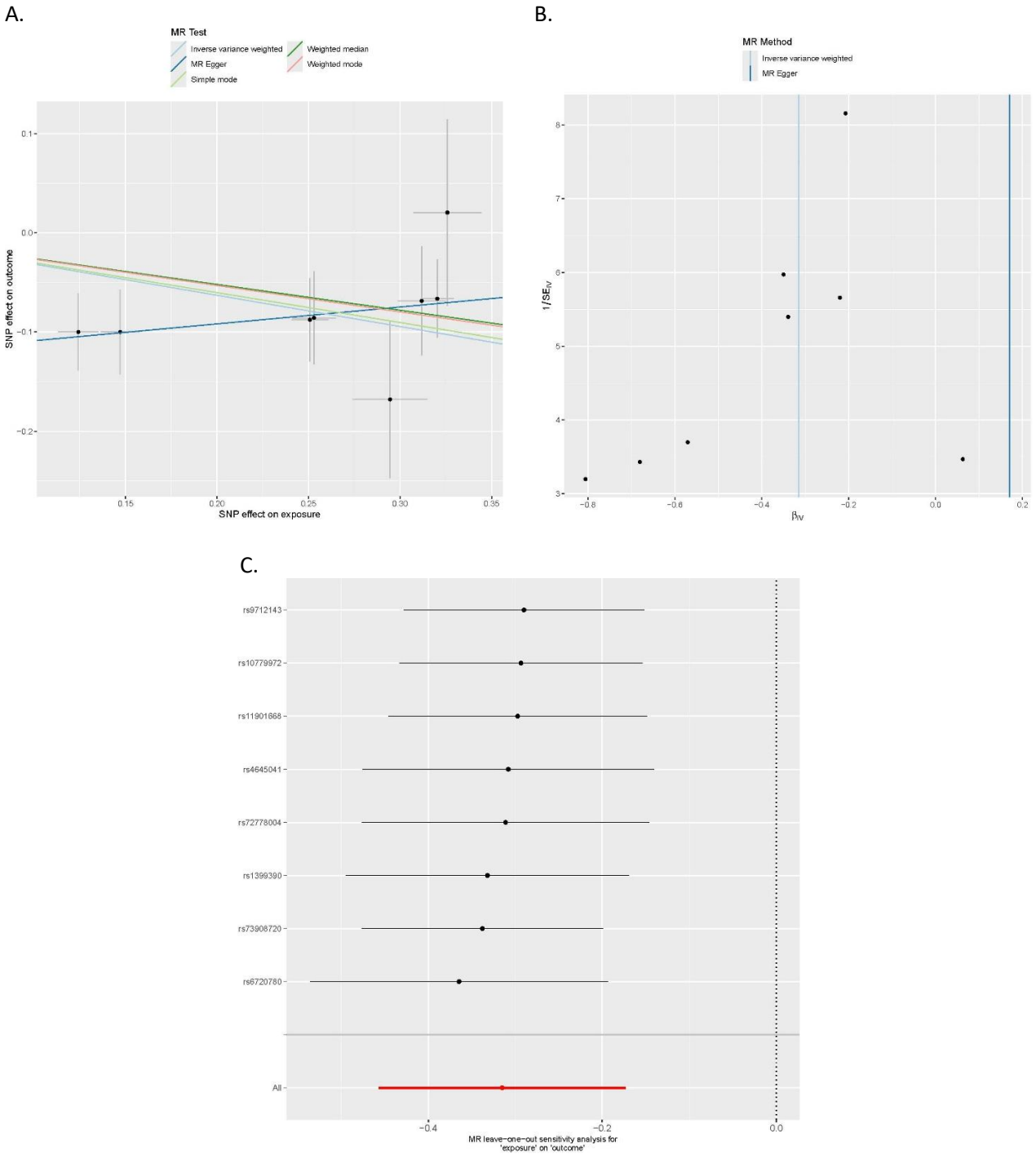

Figure 1. The results of the MR analysis. **A.** Mendelian randomization results of cg08533336 using five analytical methods. **B.** Funnel plot of two-sample Mendelian randomization analysis. **C.** "Leave-one-out" sensitivity analysis results.

therapeutic effects in renal cancer models [39].

Mendelian randomization analysis of the cg08533336 Locus

Mendelian randomization analysis revealed that hypermethylation at the USP2 cg08533336 locus exerted a significant protective effect against clear cell renal cell carcinoma (ccRCC). A

genetically predicted 1-standard deviation (SD) increase in methylation level was associated with a 27% reduction in renal cancer risk (IVW OR = 0.729, 95% CI: 0.633 - 0.841, $P = 1.38 \times 10^{-5}$). Consistent results were observed using the weighted median method (OR = 0.771, $P = 0.003$) and weighted mode approach (OR = 0.767, $P = 0.028$). The absence of significant horizontal pleiotropy was confirmed by the MR-Egger intercept test with intercept = -0.126 and $P = 0.059$, further supporting the robustness of the causal inference (Figure 1A, 1B). The SNP rs9712143 (chr2:1625091) exhibited the strongest protective association with OR of 0.447 and P as 0.0099, located within a putative HIF-1α binding domain. USP2-encoded deubiquitinase stabilized HIF-1α by inhibiting proteasomal degradation [40], while constitutive activation of the HIF signaling pathway was a hallmark of VHL-deficient renal carcinoma [41]. These findings suggested that cg08533336 methylation levels might serve as a biomarker for ccRCC risk stratification, and epigenetic modulation targeting this locus (e.g., hypomethylating agents such as 5-azacytidine) could represent a novel preventive strategy for high-risk populations [42]. Cochran's Q test indicated no heterogeneity (IVW Q = 7.76, $P = 0.354$; MR-Egger Q = 2.33, $P = 0.887$) [43]. Horizontal pleiotropy was excluded by MR-PRESSO global test ($P = 0.425$) and MR-Egger intercept ($P = 0.059$) [44, 45]. Instrument strength analysis confirmed that all 8 SNPs had F-statistics > 100 (range: 126.9 – 1,245.7), exceeding the weak instrument threshold (F > 10) [46]. Leave-one-out sensitivity analysis showed maximal effect fluctuation upon excluding rs9712143 (adjusted OR = 0.81, 95% CI: 0.69 - 0.95), but the overall causal direction remained stable (Figure 1C) [47]. The cg08533336 locus emerged as a promising biomarker candidate with dual diagnostic and therapeutic implications. Circulating cell-free DNA methylation signatures at this site could enable non-invasive risk stratification, particularly for VHL mutation-negative patients lacking conventional biomarkers [48]. Pharmacologically, the methylation-expression correlation suggested hypomethylating agents such as guadecitabine might selectively upregulate USP2 in methylation-low tumors [33], potentially synergizing with HIF-2α inhibitors like belzutifan [34].

USP2 gene expression and cg08533336 methylation trends

The analysis revealed a statistically significant positive correlation between cg08533336 methylation and USP2 expression (R = 0.26, $P = 1.8 \times 10^{-6}$). Hypermethylated tumors (β > 0.4, n = 68) exhibited higher USP2 expression (mean $\log_2$(TPM+1) = 4.2 ± 1.1) compared to hypomethylated tumors (β < 0.2, n = 70; mean $\log_2$(TPM+1) = 3.5 ± 0.9). For intermediate methylation levels (0.2 ≤ β ≤ 0.4, n = 195), a dose-dependent increase in expression was observed (R = 0.19, $P = 0.003$). USP2 functions as a potential tumor suppressor in clear cell renal cell carcinoma (ccRCC). In TCGA-KIRC samples, USP2 expression was significantly lower in tumor tissues than in normal tissues [49]. The Mendelian randomization analysis results of this study confirmed the strong protective effect of cg08533336 hypermethylation on ccRCC risk, aligning mechanistically with the observed positive methylation-expression correlation (Figure 2). The potential mechanisms included enhancer activation that cg08533336 might reside within a methylation-sensitive enhancer where methylation stabilized chromatin looping, facilitating USP2 transcription [24]; transcription factor recruitment that methylation could promote binding of oncogenic factors such as MYC or EZH2 to adjacent motifs, driving USP2 expression [50]; and CpG island-specific effects that this locus belonged to a CpG island subset where methylation paradoxically enhanced transcriptional co-activator recruitment, a mechanism observed in cancers involving transcription factors such as HIF-2α or NF-κB [42]. The moderate correlation strength (R = 0.26) suggested additional regulatory layers such as miRNA-mediated control or competing histone modifications [51]. Limitations included potential confounding by tumor heterogeneity and lack of functional validation [52]. Future studies should employ CRISPR/dCas9 methylation editing to

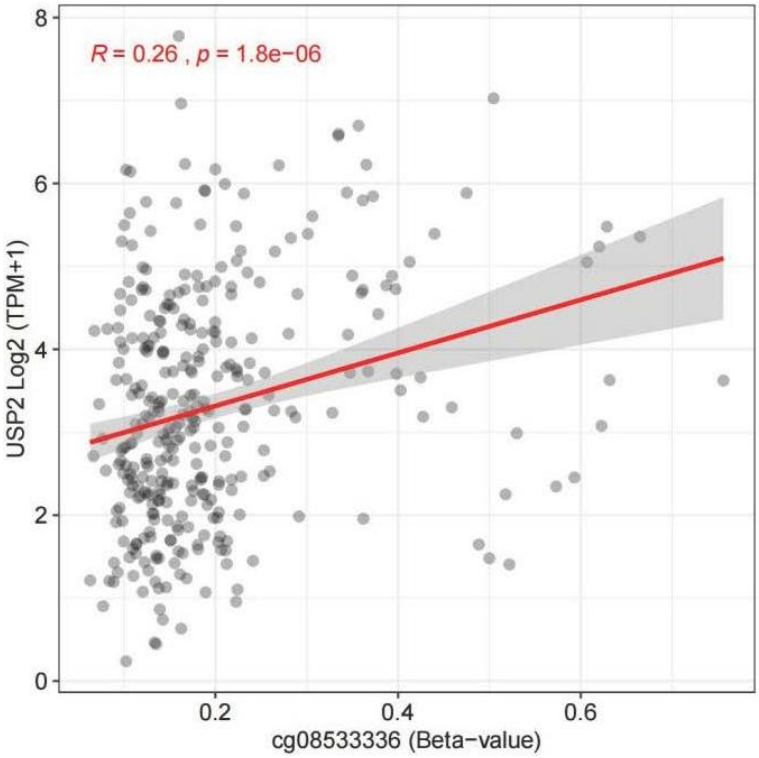

Figure 2. USP2-cg08533336 methylation-expression correlation and regulatory hypotheses.

confirm causality [53], and explore USP2-targeted therapies in hypermethylated ccRCC subtypes [32]. Collectively, this integrative multi-omics study established cg08533336 hypermethylation as a novel ccRCC protective determinant through Mendelian randomization [46]. The dissociation between methylation-driven USP2 upregulation and its tumor-suppressive effects redefined the understanding of deubiquitinase biology in renal carcinogenesis [37]. These findings provided a framework for developing methylation-based precision prevention strategies [38], while highlighting USP2's therapeutic potential as a context-dependent modulator of hypoxia signaling pathways [39].

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