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Comprehensive analysis of 5-hydroxymethylcytosine in zw10 kinetochore protein as a promising biomarker for screening and diagnosis of early colorectal cancer

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Abstract

Background: As a new epigenetic biomarker, 5-hydroxymethylcytosine (5hmC) is broadly involved in various diseases including cancers. However, the function and diagnostic performance of 5hmC in colorectal cancer (CRC) remain unclear.

Results: High-throughput sequencing was used to profile 5hmC levels in adjacent normal colon, advanced adenomas, and CRC. The expression and 5hmC levels in zw10 kinetochore protein (ZW10) were significantly increased in the tissues and blood samples for patients with advanced adenoma and CRC, and were much higher in the early stages of CRC (I and II). The receiver operating characteristic analysis had potential diagnostic value for CRC. The area under the curve (AUC) of ZW10 5hmC levels in tissue samples of CRC was 0.901. In blood samples, the AUC was 0.748 for CRC. In addition, the ZW10 5hmC level had much higher diagnostic performance in early stages of CRC (AUC = 0.857) than it did in advanced stages (AUC = 0.594). Compared with FHC cell, ZW10 expression in HT29 cell was significantly increased. The ZW10 knockdown could inhibit cell proliferation and the ZW10 overexpression could promote cell proliferation in

ABBREVIATIONS: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; Adenoma, advanced adenoma; AUC, area under curves; BHLHA15, basic helix-loop-helix family member a15; CEP72, centrosomal protein 72; CRC, colorectal cancer; ctDNA, circulating tumor DNA; DEG, differentially expressed gene; DhMG, differentially hydroxymethylated gene; DhMS, differentially hydroxymethylated site; DPEP1, dipeptidase 1; ERAP2, endoplasmic reticulum aminopeptidase 2; FOXN3, fork-head box N3; GO, gene ontology; HC, healthy control; hMeDIP-qPCR, hydroxymethylated DNA immunoprecipitation-real time quantitative PCR; hMeDIP-seq, hydroxymethylated DNA immunoprecipitation sequencing; IHC, immunohistochemistry; KEGG, Kyoto encyclopedia of genes and genomes; LRIG1, leucine rich repeats and immunoglobulin like domains 1; mTOR, mechanistic target of rapamycin kinase; NID1, nidogen 1; Normal, adjacent normal colon; PI3K, PI3 kinase p85; qPCR, real time quantitative polymerase chain reaction; ROC, receiver operating characteristic; SDK1, Sidekick cell adhesion molecule 1; TCGA, the Cancer Genome Atlas; TMA, tissue microarray; ZW10, ZW10 kinetochore protein

HT-29 cell. Furthermore, ZW10 knockdown inhibited AKT and mTOR phosphorylation, and ZW10 overexpression promoted AKT and mTOR phosphorylation.

Conclusions: The ZW10 5hmC level may serve as an effective epigenetic biomarker for minimally invasive screening and diagnosis of CRC, and it has higher diagnostic performance in early stages of CRC than it does in advanced stages. In addition, ZW10 could regulate CRC progression through the AKT-mTOR signaling.

KEYWORDS
5-hydroxymethylcytosine, biomarker, colorectal cancer, zw10 kinetochore protein

1 | BACKGROUND

Colorectal cancer (CRC) is the third most common cancer and the second most common cancer-related cause of mortality in humans according to the latest global cancer statistics.[1] In China, the incidence and mortality of CRC have continued to increase.[2,3] Early diagnosis is critical for positive prognosis of CRC,[4,5] and consistently recommended by clinical practice guidelines. Hence, a variety of techniques have been utilized to implement this target.[6,7]

Colonoscopy is a crucial endoscopic strategy for CRC screening, but it has limitations such as its rate of missed lesions, incomplete coverage, invasiveness, and time and cost intensiveness.[8,9] These considerations have prevented the widespread use of colonoscopy. In urban China, the compliance rate for colonoscopy screening is only approximately 15% in the high-risk population for CRC.[10] Serum carcinoembryonic antigen quantification is a representative noninvasive method of CRC screening, while it has a low sensitivity of only 40 to 60%.[11,12] Similarly, the sensitivity of fecal immunochemical testing is also only 20 to 30%.[13-15] Therefore, the establishment of a simple new technique for CRC screening is urgently required.

The epigenetic modification 5-methylcytosine (5mC) is related to the development of various diseases including CRC.[16-19] Increasing evidence has indicated that 5mC can enable the diagnosis and prognosis for CRC.[20-22] Moreover, 5-hydroxymethylcytosine (5hmC), a stable derivative produced in DNA demethylation, is catalyzed by the ten-eleven translocation family.[23] As a new epigenetic biomarker, 5hmC modification is also associated with various diseases including cancer.[24-27] Although 5hmC has been reported to be involved in CRC progression,[16,28] the potential diagnostic value of 5hmC for early screening and diagnosis of CRC has seldom been investigated.

This study focused on evaluating the performance of 5hmC in screening and diagnosis for CRC. First, we used 5hmC sequencing to analyze the profiles of 5hmC in tissue samples from adjacent normal colon (normal), advanced adenomas, and CRC, and found differences in 5hmC levels among the groups. Second, blood 5hmC levels were verified, and the diagnostic value was performed through receiver operating characteristic (ROC) curve analysis. Finally, the underlying molecular mechanism of zw10 kinetochore protein (ZW10) was validated.

2 | METHODS

2.1 | Study design and participants

All participants were recruited from the departments of endoscopy and gastrointestinal surgery at Longhua Hospital (Shanghai, China). Informed consent was obtained from participants before sample collection. In order to maximally obtain precise information, we decided to include both precancerous lesion and CRC patients. The advanced adenomas were then chosen because they are considered as representative high-risk precancerous lesions for CRC.[29] The diagnostic criteria of advanced adenomas were defined as adenoma diameter > 10 mm with or without villous texture or high-grade neoplasia. Diagnoses of CRC were confirmed on the basis of pathological evidence. Healthy controls (HCs) without any colonoscopic evidence of advanced adenoma or CRC were also enrolled. A total of 31 HCs, 30 patients with advanced adenomas, and 30 patients with CRC were included (Table 1). All samples were frozen and stored at −80°C.

2.2 | Hydroxymethylated DNA immunoprecipitation sequencing

DNA was extracted from tissues by using the DNA Preparation Kit (Beyotime Biotechnology, shanghai, China), and DNA concentration and purity were measured using a NanoDrop instrument. Genomic DNA was sonicated to approximately 200–800 bp fragments that were immuno-

TABLE 1 Baseline patient characteristics

	Adenoma	CRC	HC
Total (n)	30	30	31
Age, mean (sd), yrs	60.37 (9.82)	61.09 (12.79)	60.73 (8.08)
Female, n(%)	10 (33.33)	10 (33.33)	18(58.06)
Male, n(%)	20 (66.67)	20 (66.67)	13 (41.94)
Localization n (%)			
Right (cecum, ascending, transverse)	6(20.00)	5 (16.67)	-
Left (descending, sigmoid)	22 (73.33)	11 (36.67)	-
Rectum	2 (6.67)	14 (46.67)	-
TNM stage, n (%)			
I	-	9 (30.00)	-
II	-	5 (16.67)	-
III	-	12(40.00)	-
IV	-	4 (13.33)	-

precipitated by anti-5-hmc antibody (39999, Active Motif, USA). By an Illumina HiSeq 4000, hydroxymethylated DNA immunoprecipitation (hMeDIP) sequencing (hMeDIP-seq) was performed according to the protocol, and then results were analyzed. Differentially hydroxymethylated sites (DhMSs) were identified by using a fold change of >2 and P value of < 0.05. Subsequently, overlapping differentially hydroxymethylated genes (DhMGs) were analyzed by using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The signal profiles of hMeDIP-seq data were generated using University of California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

2.3 | Real-time quantitative polymerase chain reaction analysis

The total RNA of tissue and blood samples was extracted using TRizol or TRizol LS reagent (Life Technologies, CA, USA), and purity and concentration were measured using a NanoDrop instrument. And then by using a reverse transcription assay kit (Invitrogen, Carlsbad, CA, USA), RNA was reverse-transcribed to cDNA. A real-time quantitative polymerase chain reaction (qPCR) was performed using SYBR Green kits (Invitrogen) according to a previous study.[30] Data were performed by the $2^{-\Delta\Delta Ct}$ method. The sequences of primers are showed in Supporting information Table S1.

2.4 | Immunohistochemical analysis

Tissue samples from the normal, adenoma, and CRC groups were fixed, and then cut into 4-μm sections for

immunohistochemistry (IHC). Tissue microarrays (TMAs) were obtained from Shanghai Outdo Biotech Co., Ltd (Shanghai, China). In brief, samples were incubated with the ZW10 antibody (ab21582, Abcam, USA), centrosomal protein 72 (CEP72) antibody (ab230333, Abcam), and dipeptidase 1 (DPEP1) antibody (ab230977, Abcam) overnight at 4°C. Subsequently, the secondary antibodies conjugated with horseradish-peroxidase were incubated for 1 h at 37°C. Finally, samples were stained, and then imaged.

2.5 | hMeDIP real-time qPCR

Modifications to 5hmC of DhMGs were determined through a hMeDIP real-time qPCR (hMeDIP-qPCR) assay. In brief, genomic DNA was extracted and sonicated, and these DNA fragments were further immunoprecipitated by the anti-5-hmc antibody (39999, Active Motif). The enriched DNA was amplified by qPCR, and the corresponding 5hmC enrichment was normalized using input. Primer sequences of hMeDIP-qPCR are showed in Supporting information Table S2.

2.6 | Cell culture and transfection

HT-29 CRC cells and normal colon cell (FHC) were obtained from the Type Culture Collection of the Chinese Academy of Sciences. And then cells were cultured in Dulbecco's Modified Eagle Medium including 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA), and incubated with 5% CO_2 at 37°C. HT-29 cells were seeded in a 6-well plate, cultivated for 24 h, and then transfected with ZW10 overexpression (ZW10-OE; Genechem, Shanghai, China)
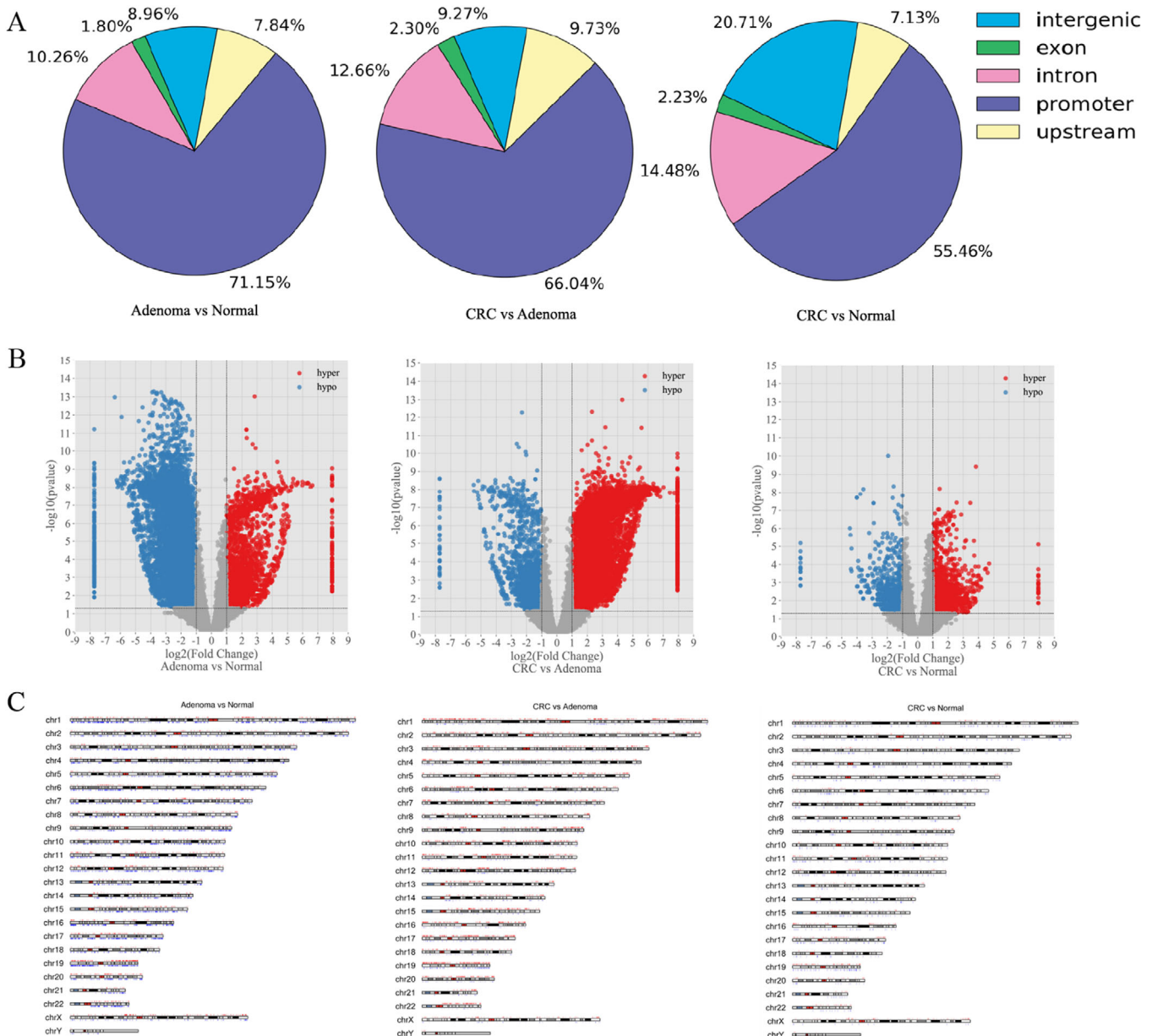
FIGURE 1 Distribution profiles of 5hmC in pairwise comparisons. (A) Percentage of 5hmC at different sites in pairwise comparisons; (B) DhMSs in the promoter visualized in volcano plots in pairwise comparisons; (C) distribution of DhMSs on the chromosome

plasmid and knockdown plasmid (shZW10; Genechem) by using Lipofectamine 2000 (Invitrogen).

2.7 | Western blot analysis

The total protein of transfected cells was extracted and quantified. Proteins were then transferred and blocked for 2 h in 5% fat-free milk at room temperature. Proteins were incubated with primary antibody overnight at 4°C, and incubated with secondary antibodies for 1 h. Images were captured and analyzed the density of band. ZW10 antibody (ab21582, Abcam) was obtained from

Abcam, and the antibodies of PI3 kinase p85 (PI3K) (4292), AKT serine/threonine kinase 1 (Akt) (2938), Phospho-Akt (Ser473) (4058), mechanistic target of rapamycin kinase (mTOR) (2983), and Phospho-mTOR (Ser2448) (5536) were obtained from Cell Signaling Technology. The β-actin antibody was obtained from HuaAn Biotechnology (JF53-10).

2.8 | Cell proliferation assay

Cell proliferation of transfected HT-29 was checked using the Cell Counting Kit-8 (Dojindo, Japan). In brief, HT-29 cells transfected with ZW10 were seeded in 96-well plates,
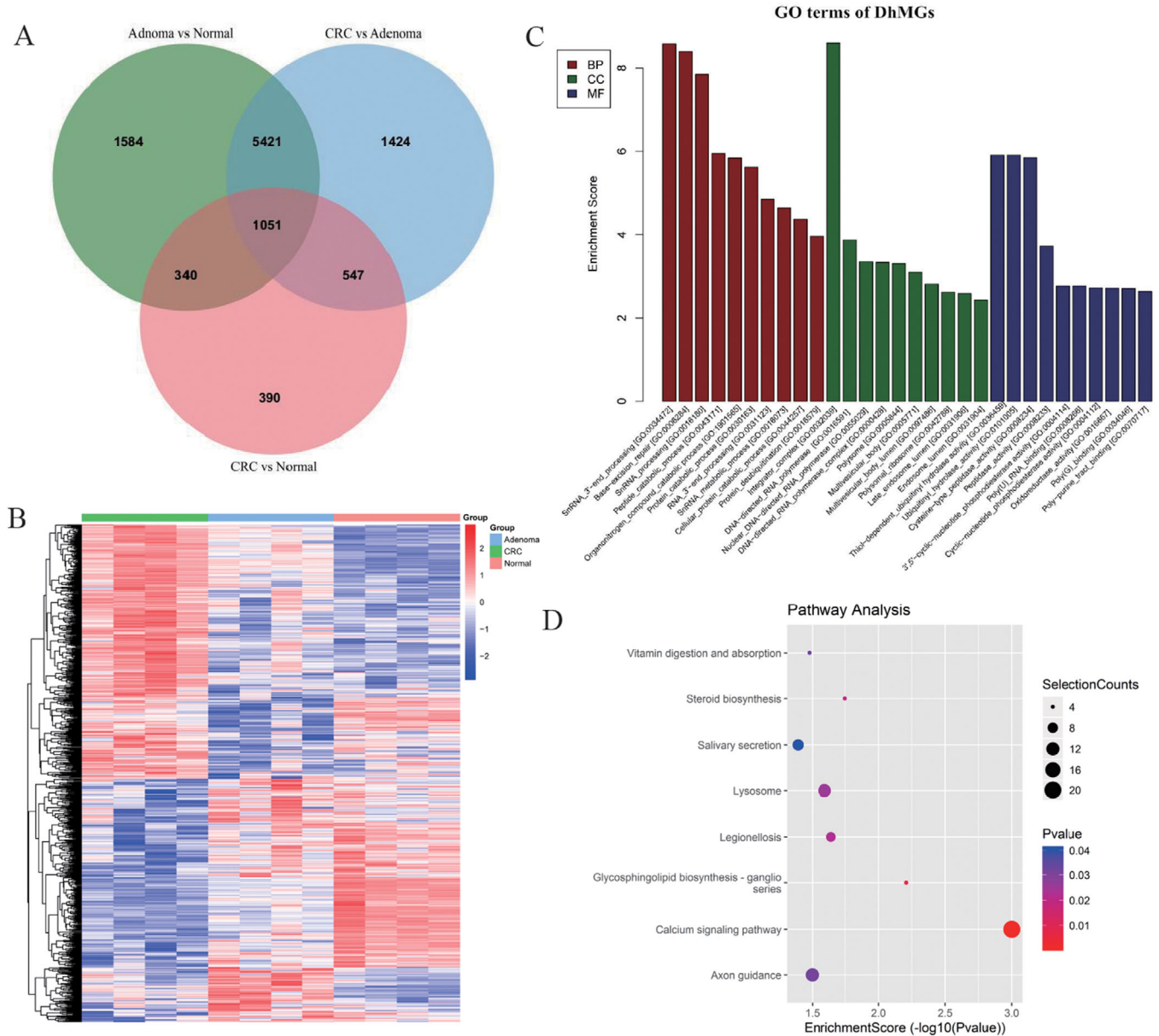
FIGURE 2 Functional analysis of DhMGs. (A) Venn diagram analysis of DhMGs in pairwise comparisons; (B) heat map of DhMGs; (C) GO analysis of DhMGs; (D) KEGG pathway analysis of DhMGs

including 1×10^4 cells per well, and incubated with Dulbecco's Modified Eagle Medium. After 0, 24, 48, and 72 h, CCK-8 was added to incubating cell for 1 h at 37°C. The absorbance was measured at 450 nm.

2.9 | Statistical analysis

Data are presented as mean ± standard error of the mean (SEM), and they were evaluated by two-tailed Student's t tests between two independent groups. $P < .05$ was considered statistically significant. Survival curves were estab-

lished through the Kaplan–Meier method. ROC curves were generated to assess the diagnostic performance of 5hmC in discriminating CNLs by using MedCalc software.

3 | RESULTS

3.1 | Profiling and identification of 5hmC in pairwise comparisons

To identify different profiles of 5hmC, high-throughput sequencing was performed in four samples of normal,
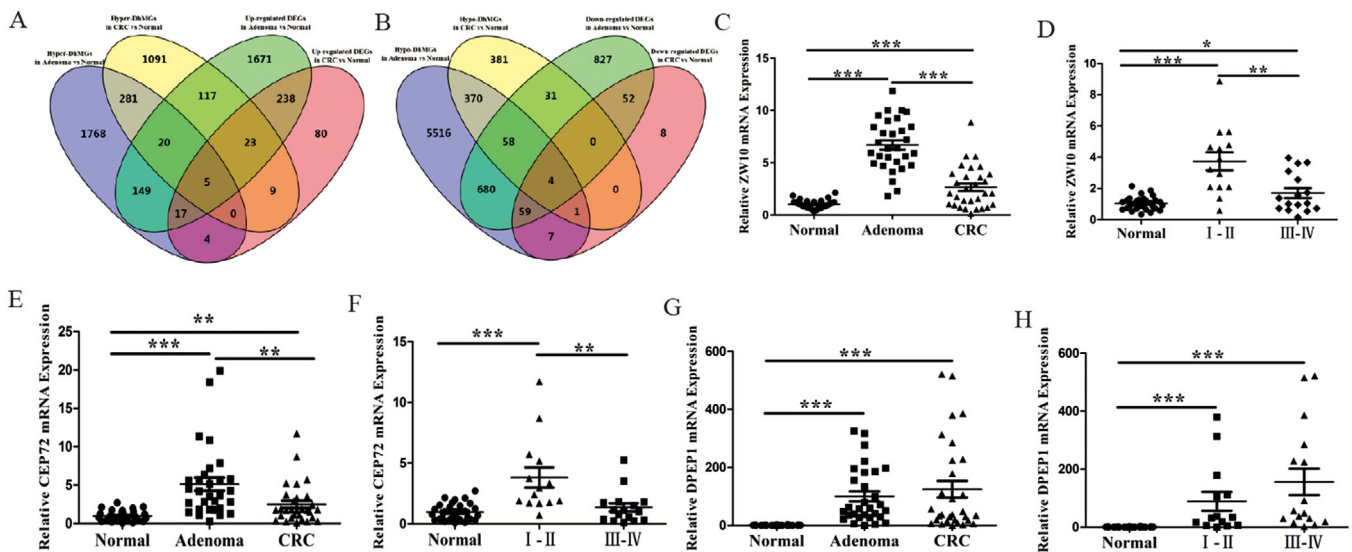
FIGURE 3 Expression analysis of DhMGs through qPCR. (A, B) Venn diagram verifying DhMGs and DEGs among the groups; (C, D) ZW10 expression among the groups; (E, F) CEP72 expression among the groups; (G, H) DPEP1 expression among the groups (normal = 30, adenoma = 30, CRC = 30). Data presented as means ± SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

adenoma, and CRC tissues. The distribution profiles of 5hmC included five transcript segments: promoter, exon, intron, intergenic, and upstream. The mainly modification sites of 5hmC located in the promoter domain, and the proportion was >50% (Figure 1A). The profile of 5hmC in the promoter domain was further analyzed with a threshold of a two-fold change and $P < .05$. Sets of 11 960, 12 321, and 2885 DhMSs were identified in the adenoma group compared with the normal group, in the CRC group compared with the adenoma group, and in the CRC group compared with the normal group, respectively (Figure 1B, Supporting information Figure S1A). Notably, 90% of DhMGs included only one 5hmC peak. In addition, the distribution of DhMSs was localized on chromosomes 1, 2, 3, 17, and 19 (Figure 1C, Supporting information Figure S1B).

3.2 | Functional analysis of DhMGs

The molecular functions of DhMGs were analyzed, and then sets of 8396, 8443, and 2328 DhMGs were obtained in the adenoma group compared with the normal group, the CRC compared with the adenoma group, and the CRC compared with the normal group, respectively. Overlapping DhMGs (1051) among the groups were filtered by a Venn diagram (Figure 2A), and a hierarchical cluster was produced (Figure 2B). Subsequently, the main biological functions of overlapping DhMGs were identified using GO and KEGG pathways, including the metabolic, steroid biosynthesis, lysosome, and calcium signaling pathways (Figure 2C and 2D).

3.3 | Expression analysis of DhMGs through real-time qPCR and immunohistochemistry

Differentially expressed genes (DEGs) were filtered out from the adenoma and CRC groups compared with the normal group according to previous mRNA sequencing data.[30] In the combined analysis with DhMGs, overlapping genes (five upregulated and four downregulated) were filtered out by a Venn diagram (Figure 3A and 3B, Supporting information, Table S3). RT-qPCR analysis indicated that only ZW10, CEP72, and DPEP1 showed significantly different expression levels in both the adenoma and CRC groups compared with the normal group (Figure 3C, 3E, and 3G, Supporting information, Figure S2D). The results of The Cancer Genome Atlas (TCGA) database also revealed that mRNA levels of ZW10, CEP72, and DPEP1 were markedly upregulated in the CRC group (Supporting information Figure S2A-C). Then, the expression levels of these three genes were investigated in early and advanced stages of CRC (Figure 3D, 3F, 3H). Compared with advanced-stages (III and IV) CRC, the expression levels of ZW10 and CEP72 were higher in early stage (I and II). However, the expression level of DPEP1 showed no significant difference between different stages of CRC.

To further verify the protein levels of ZW10, CEP72, and DPEP1, IHC analysis was performed. In accordance with the mRNA results, the protein levels of ZW10, CEP72, and DPEP1 were markedly upregulated in both the adenoma and CRC groups (Figure 4A). To further investigate the relationship between CRC prognosis and
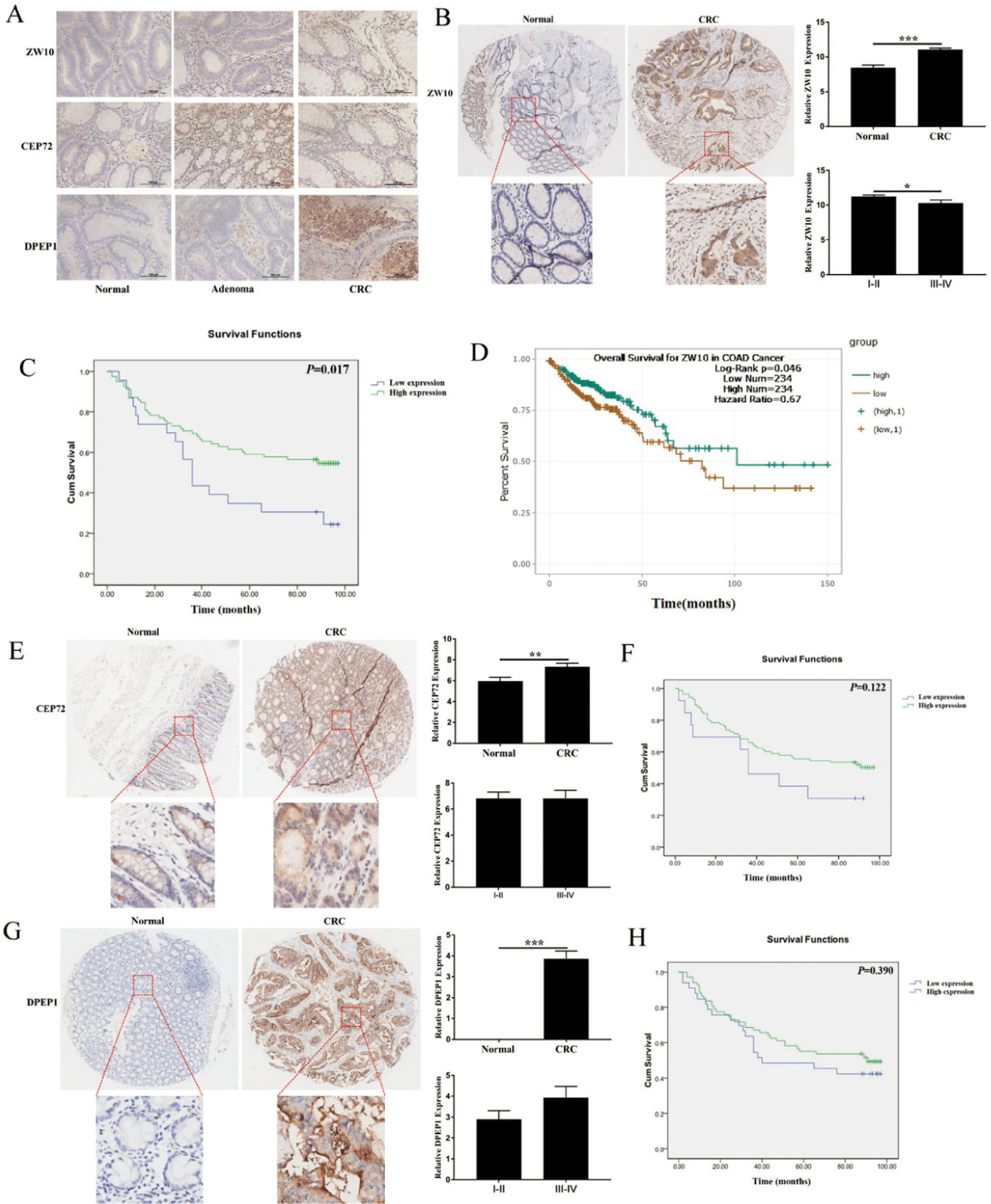
FIGURE 4 Expression analysis of DhMGs through IHC analysis. (A) IHC expression analysis of ZW10, CEP72, and DPEP1; (B) Expression analysis of ZW10 by using TMAs (normal = 79; CRC = 101 (I, II = 61; III, IV = 40)); (C, D) survival analysis of ZW10; (E) expression analysis of CEP72 by using TMAs (normal = 79; CRC = 101 (I, II = 61; III, IV = 40)); (F) survival analysis of CEP72; (G) expression analysis of DPEP1 by using TMAs (normal = 79, CRC = 101 (I, II = 61; III, IV = 40)); (H) survival analysis of DPEP1. IHC staining scores presented as means ± SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$
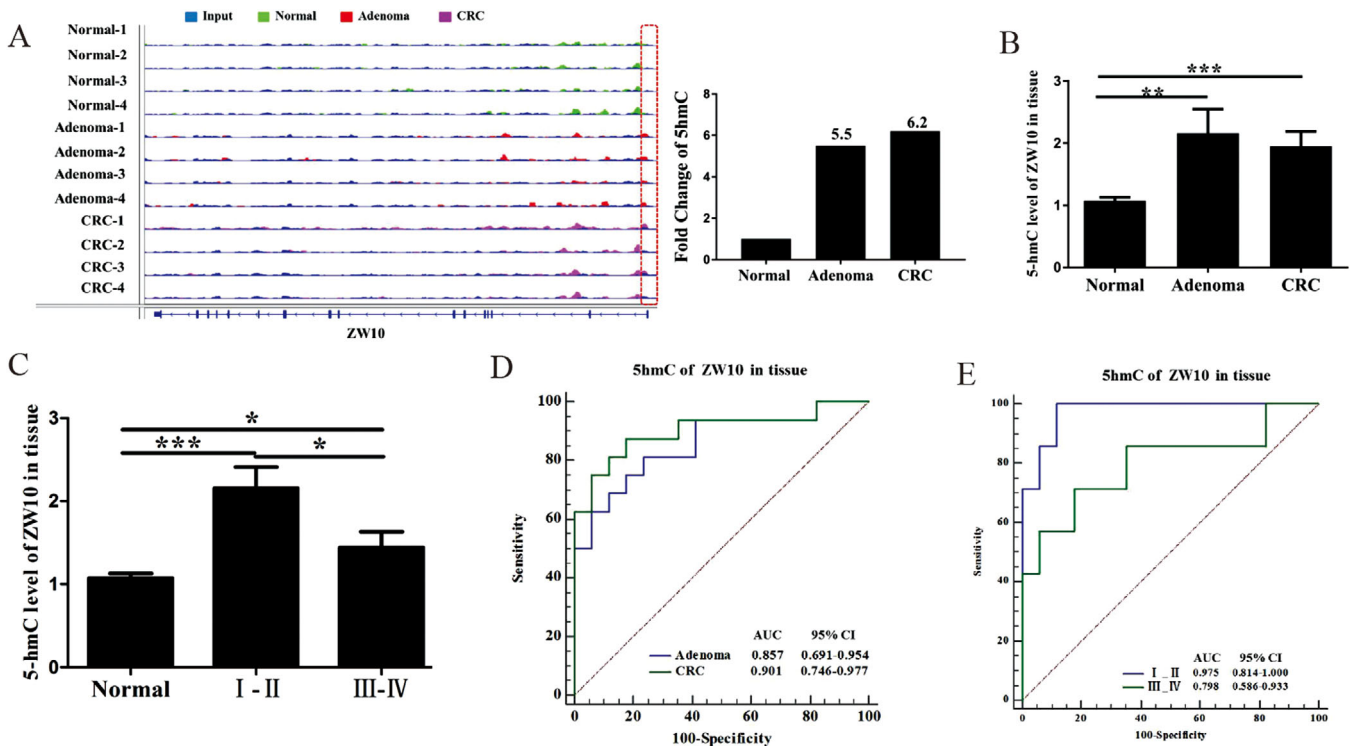
FIGURE 5 Analysis of 5hmC in ZW10 in tissue. (A) Abundances of 5hmC in the ZW10 promoter determined using UCSC Genome Browser; (B, C) 5hmC level in the ZW10 promoter determined through hMeDIP-qPCR (normal = 17, adenoma = 17, CRC = 17); (D, E) ROC analysis of 5hmC in the ZW10 promoter. Data presented as means ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

protein levels, TMA was performed through IHC staining. The IHC score of ZW10 was notably increased in the CRC group, and it was higher in patients with early-stage CRC (Figure 4B, Supporting information, Figure S3A). The patients with CRC and high ZW10 levels had higher overall survival, which was consistent with the TCGA result (Figure 4C and 4D). Although the IHC scores of CEP72 and DPEP1 were also increased, overall patient survival did not differ (Figure 4E to 4H, Supporting information, Figure S3B–E).

3.4 | ZW10 5hmC level as a potential biomarker for screening and diagnosing of early CRC

In a genome-wide analysis, the ZW10 5hmC level was notably increased in the adenoma (5.5 times) and CRC (6.2 times) groups, as presented in Figure 5A. The ZW10 5hmC level was further identified through hMeDIP-qPCR and was consistent with the genome-wide analysis (Figure 5B). Moreover, the ZW10 5hmC level was much higher in patients with early-stage CRC (Figure 5C). The area under the curve (AUC) was 0.901 (95% confidence of interval [CI]: 0.746-0.977) in CRC group (Figure 5D). And the AUC was 0.975 (95% CI: 0.814-1.000) in early-stage CRC and 0.798

(95% CI: 0.586-0.933) in advanced-stage CRC (Figure 5E). Although 5hmC levels of CEP72 and DPEP1 were notably increased in the genome-wide analysis, 5hmC levels did not differ significantly in the hMeDIP-qPCR (Supporting information Figure S4).

In the blood samples, ZW10 5hmC level was also notably increased in the adenoma and CRC groups compared with the HC group (Figure 6A), and ZW10 5hmC level was much higher in patients with early-stage CRC (Figure 6B). The AUC was 0.748 (95% CI: 0.618-0.852) in the CRC group (Figure 6C). Moreover, the AUC was 0.857 (95% CI: 0.721-0.943) in patients with early-stage CRC and 0.594 (95% CI: 0.438-0.738) in those with advanced-stage CRC (Figure 6D).

3.5 | Effect of ZW10 on proliferation and phosphorylation levels of Akt and mTOR

The expression and 5hmC levels in ZW10 were much higher in the early-stage CRC, so HT-29 was chosen to perform molecular mechanisms of ZW10 in vitro. As presented in Figure 7A, compared with FHC cell, the mRNA and protein level of ZW10 in HT29 cell was significantly increased. Then ZW10 knockdown and overexpression in HT-29 cell were performed (Figure 7B and 7C). The ZW10 knockdown inhibited cell proliferation, and the
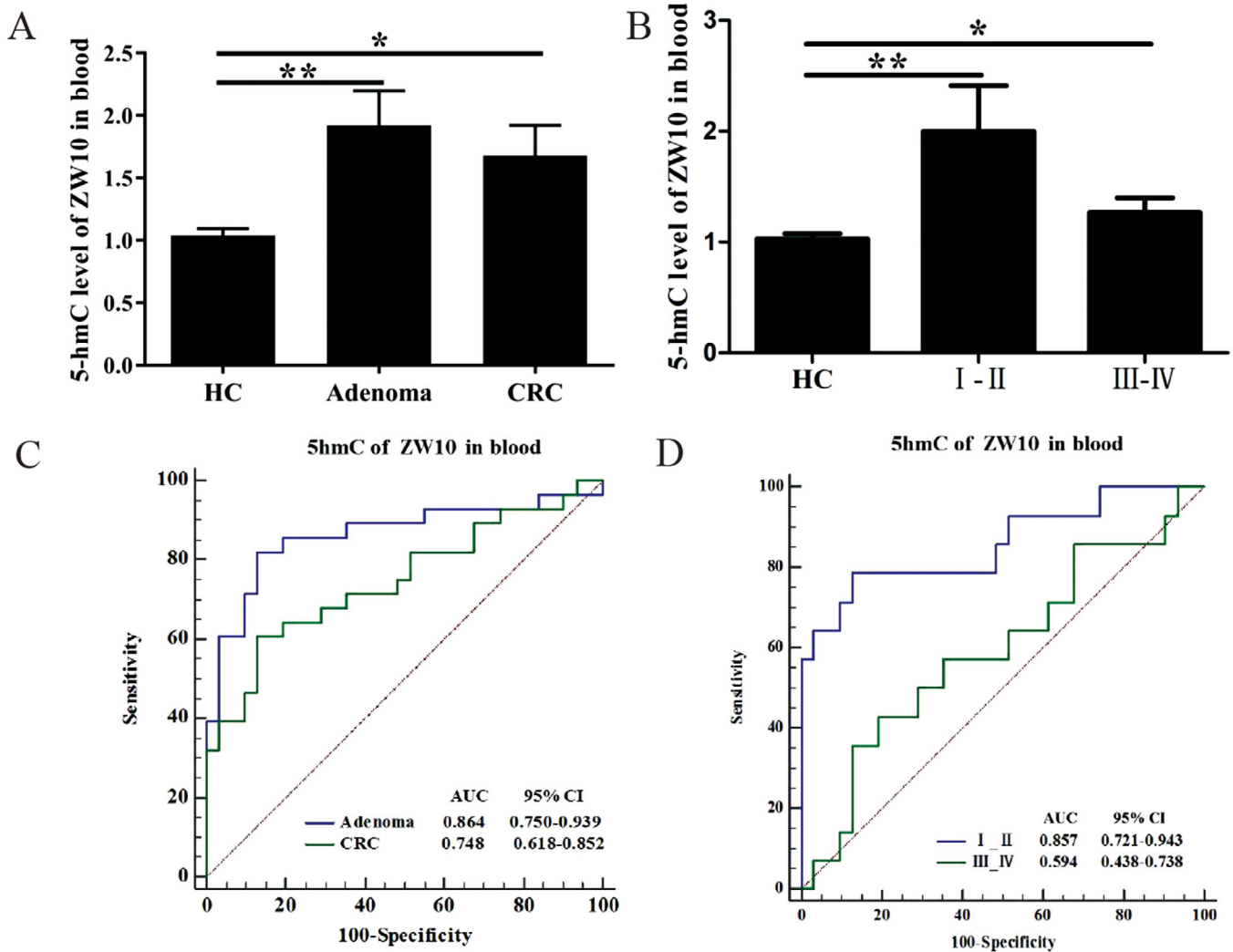
FIGURE 6 Analysis of 5hmC in ZW10 in blood. (A, B) Level of 5hmC in the ZW10 promoter determined through hMeDIP-qPCR (HC = 31, adenoma = 30, CRC = 30); (C, D) ROC analysis of 5hmC in the ZW10 promoter. Data presented as means ± SEM. [**]$P < .01$, [***]$P < .001$

ZW10 overexpression promoted cell proliferation at 48 and 72 h (Figure 7D). Furthermore, ZW10 knockdown inhibited AKT and mTOR phosphorylation, and ZW10 overexpression promoted AKT and mTOR phosphorylation (Figure 7E and 7F). These results indicated that ZW10 could regulate CRC progression through the Akt-mTOR signaling.

4 | DISCUSSION

As a novel epigenetic biomarker, 5hmC plays a critical role in the early screening and diagnosis of cancers.[24,31,32] We demonstrated that the ZW10 mRNA and protein level was markedly upregulated in CRC group, and more importantly, was much higher in the early stages than in the advanced stages of CRC. Survival outcomes indicated that the CRC patients with high ZW10 had more favorable

overall survival. The 5hmC level for ZW10 was notably upregulated in the tissue and blood samples of patients with CRC, and was much significantly higher in patients with early-stage CRC. The 5hmC level for ZW10 showed satisfactory diagnostic performance for early-stage CRC (AUC = 0.857). In addition, ZW10 could regulate CRC progression through the Akt-mTOR signaling.

ZW10, a mitotic kinetochore protein, is involved in mitotic kinetochore regulation, state of chromosome, and transmembrane trafficking.[33-36] Alterations in mitotic functions mediated by ZW10 can regulate CRC progression.[37] In this study, the expression of ZW10 was notably upregulated in CRC groups, and the patients with CRC and high ZW10 expression had more favorable overall survival, indicating that ZW10 is associated with the prognosis of patients with CRC. In addition, ZW10 is particularly crucial for centromeric SUMOylation and could promote the phosphorylation of mitotic arrest
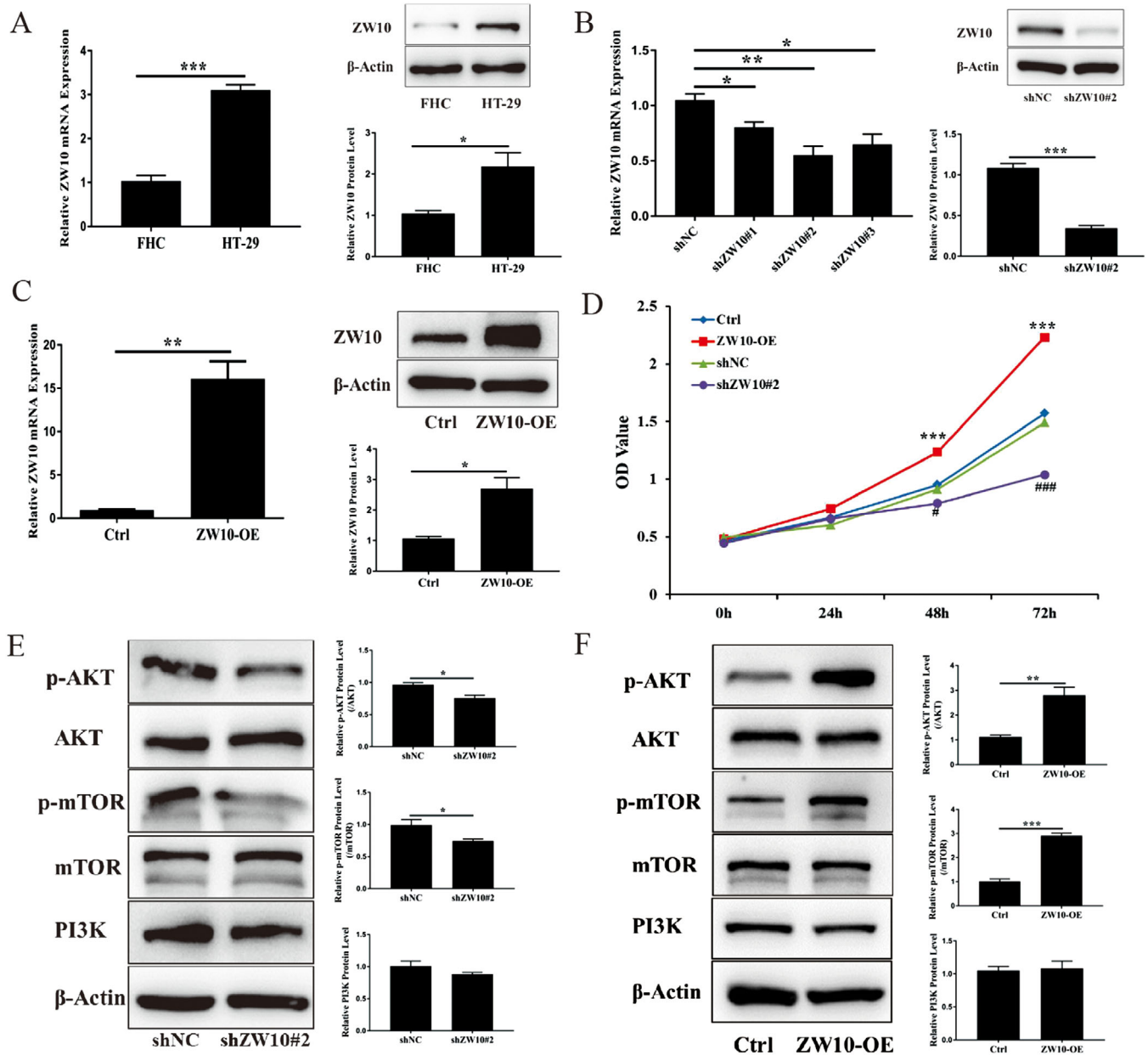
FIGURE 7 Effect of ZW10 on proliferation and phosphorylation levels of Akt and mTOR. (A) Expression of ZW10 in HT-29 cell; (B, C) Expression of ZW10 after overexpression or knockdown in HT-29 cell; (D) proliferation of HT-29 cell after ZW10 overexpression or knockdown; (E, F) Akt, mTOR, and PI3K protein expression after ZW10 overexpression or knockdown. Data presented as means ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

deficient 1.[38,39] These results indicate that ZW10 plays a central role through epigenetic modification. Moreover, patients with higher levels of DNA hydroxymethylation have a more favorable prognosis for CRC, and decreased 5-hmC is correlated with poor overall survival in cutaneous T-cell lymphoma and hepatoblastoma.[31,40,41] In this study, the 5hmC level for ZW10 was notably upregulated in tissue and blood samples of patients with CRC, and the ZW10 5hmC level was higher in patients with early-stage CRC compared with those with advanced-stage CRC; this is consistent with previously reported results. Our study

showed the gene expression and protein levels of ZW10 increased notably in the CRC group, and the AUC values of ZW10 mRNA and protein for discriminating CRC were 0.761 (95% CI: 0.634-0.862) and 0.720 (0.649-0.785) in tissue samples, respectively (Supporting information Figure S5A and B). Considering that the diagnostic value of tissue biomarker is much lower than that of blood biomarker, so ZW10 expression in blood samples was also verified. ZW10 mRNA level was notably upregulated in the blood samples of the CRC group, while the AUC for discriminating CRC was 0.681 (95% CI: 0.499-0.829), which was smaller

than that of the ZW10 5hmC in CRC (AUC = 0.748). Especially in early CRC, the AUC values of ZW10 5hmC was 0.857, while the ZW10 mRNA was only 0.707 (Supporting information, Figure S5C-F). Therefore, our results indicated that the ZW10 5hmC can serve as a promising biomarker for the early screening and diagnosis of CRC, and ZW10 could regulate CRC progression through the Akt-mTOR signaling. In addition, although the 5hmC level for CEP72 and DPEP1 was notably increased in adenoma and CRC groups in genome-wide analysis, the 5hmC level for CEP72 and DPEP1 showed no significant difference after increasing the sample size for verification by hMeDIP-qPCR. The difference in sample size (four in hMeDIP sequencing while in qPCR) and the presence of false positives may lead to differences of the results of CEP72 and DPEP1 in genome-wide and PCR analysis.

Patients with CRC diagnosed at early-stage have more favorable prognoses and survival than those with advanced-stage CRC. Because of its minimal invasiveness, rapid turnaround time, and high compliance rate, liquid biopsy is an expanding field for the screening and diagnosis of CRC.[42-44] A growing body of evidence suggests that microRNAs derived from extracellular vesicles or exosomes can enable the early diagnosis for CRC; these microRNAs include miR-21, miR-23a, miR-1246, miR-18a, miR-29a, and miR-92b.[45-48] In addition, circulating tumor DNA (ctDNA), which is fragmented DNA derived from a tumor, was found in blood, and increasing evidence indicates that ctDNA can serve as a promising biomarker in screening, early diagnosis, and prognosis for CRC.[49-53] Researches has also suggested that the methylation level of ctDNA can enable screening, early diagnosis, and prognosis prediction for CRC.[54-56] These findings indicated that both ctDNA and ctDNA methylation have application prospects as diagnostic biomarkers for CRC. Our study indicated that the 5hmC level for ZW10 could be served as a screening biomarker, not only for general CRC, but more importantly, for early-stage CRC. Nevertheless, this study had a few limitations. First, the sample size was insufficient. More patients with different stages of CRC should be included to verify the findings. Second, this study did not compare the diagnostic performance of 5hmC with previously reported diagnostic biomarkers for CRC. An exploration of the combined effect of 5hmC and biomarkers from other categories would be valuable in future studies.

5 | CONCLUSION

In summary, our results suggested that the 5hmC level for ZW10 derived from blood samples can serve as an effective epigenetic biomarker for minimally invasive screening and diagnosis of CRC, and can have much higher diagnostic performance for patients with earlier stages (I and II) compared with advanced stages (III and IV) of CRC. In addition, ZW10 could activate Akt-mTOR signaling to regulate CRC progression.

CONFLICT OF INTERESTS
The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This study was approved by the Ethics Committee of Longhua Hospital, and the informed consent was obtained from all participants.

AUTHORS' CONTRIBUTIONS
GJ and YD conceived, designed, and supervised the study. YD, ZY, YL, and YX collected samples. YD, DH, JX, CL, and YT performed the experiments, and YD and DH analyzed the data. YD and GJ wrote the paper. LZ, WZ, and HX edited and revised the article. All authors reviewed and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIAL
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONSENT FOR PUBLICATION
Not applicable

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.