RESEARCH ARTICLE

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Association of *APC* Expression with Its Promoter Methylation Status and the Prognosis of Hepatocellular Carcinoma

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Abstract

Objective: The present study was aimed to investigate the *APC* expression, its promoter methylation status, the expression of *β-Catenin*, *c-Myc* and *Cyclin D1* and further explore their prognostic value in Hepatocellular carcinoma (HCC). **Patients And Methods:** Serum samples from 90 HCC patients and 27 healthy donors were collected in this study. The methylation-specific PCR (MSP) was performed to evaluate promoter methylation status of *APC* gene. RT-qPCR was used to detect the mRNA expression of *APC*, *β-Catenin*, *c-Myc* and *Cyclin D1*, meanwhile the protein expression were analyzed by Western blot. **Results:** The positive rate of *APC* gene methylation in HCC patients (46.67%) was higher than healthy donors (11.11%). *APC* gene exhibited marked hypermethylation in the patients of TNM III-IV stage when compared to the patients of TNM I-II stage , the methylation status of *APC* gene was correlated with tumor size and lymph node metastasis whereas the *APC* gene methylation showed no relationship with the patient's sex and age. *APC* methylation may be associated with *APC* expression level, *APC* expression in HCC cells is silenced by aberrant promoter hypermethylation. In HCC patients with methylated *APC*, the mRNA and protein expression of *β-Catenin*, *c-Myc* and *Cyclin D1* were higher than the unmethylated patient subgroup and healthy donors. **Conclutions:** The downregulation of *APC* in HCC samples was associated with promoter hypermethylation. *APC* methylation could be used as a novel diagnostic biomarker in HCC, which was associated with regulation of Wnt/*β-Catenin* signal pathway.

Keywords: *APC*- methylation- Wnt/*β-Catenin* signal pathway- Hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world and accounts for more than 80% of hepatic malignancy cases. HCC is a major health concern worldwide because of its high mortality rate, rising incidence and late diagnosis (Heimbach et al., 2018). Numerous evidence has confirmed that the carcinogenesis process of hepatocarcinoma not only depends on the genetic abnormality but also on epigenetic alterations (Bai et al., 2023). The Epigenetic modifications refers to the regulation of the expression of functional genes without gene sequence alterations, including DNA methylation, RNA methylation and histone modifications (Lu et al., 2023).

DNA methylation is one of most common epigenetic modifications and plays a role in cell differentiation. In DNA methylation, cytosine in CpG islands was catalyzed to 5-methylcytosine by methyltransferase, which affects DNA conformation and stability (Serdarevic et al., 2023). DNA methylation affects the function of oncogenes and tumor suppressor genes(TSG), as well as is considered diagnosis marker of HCC (Wang et al., 2023). The altered expression of TSG leads to accumulated physiological changes, contributing to tumorigenesis, the promoter hypermethylation of many TSGs has been confirmed to promote the neoplastic initiation and progression (Li et al., 2023).

Adenomatous polyposis coli gene (*APC*) located in 5q21 containing 21 exons, which has been reported to be a novel TSG. *APC* gene encoded a protein with 300 KD molecular weight consisting of 2843 amino acids (Hamada and Bienz, 2002). Increasing evidence suggests that *APC* protein plays an important role in regulating cell proliferation and apoptosis (Fang and Svitkina, 2022). *APC* was demonstrated to be a TSG in various tumors such as stomach cancer, endometrial cancer, breast cancer, lung cancer and prostate cancer (Aoki and Taketo, 2007). Kitchen-Goosen (2022) have revealed deletion of *APC* in Lysozyme M endometrial epithelial cells results in significantly more epithelial cells comparing with wild type mice, confirmed that loss of *APC* in endometrial

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epithelial cells can lead to endometrial hyperplasia and promote the pathogenesis of endometrial cancer. Low expression of *APC* was related to poor overall survival in endometrial cancer, indicated *APC* might be used as a novel diagnosis biomarker for endometrial cancer. In addition, hypermethylation of *APC* promoter is frequently observed in approximately 45% of human endometrial cancer. The level of *APC* mRNA expression in endometrial cancer patients was lower than that in healthy donors, *APC* expression was down-regulated by hypermethylation of *APC* promoter in endometrial cancer patients. Li et al revealed that 68.33% cases of colon cancer tissues were found with *APC* gene methylation, and *APC* gene was not methylated in tumor-adjacent normal tissues. *APC* gene methylation had a significant association with tumor size, differentiation degree and Dukes staging. Otherwise, the cell proliferation, invasion and migration of colon cancer cells was promoted by *APC* gene methylation, which indicate *APC* gene methylation is involved in the development and pathogenesis of colon cancer (Yang et al., 2023). *APC* was demonstrated to be an integral protein of the Wnt *β-Catenin* signaling pathway (Ge et al., 2023). However, the relationship with *APC* expression and the *APC* methylation in HCC remain unclear now, and the underlying association with Wnt/*β-Catenin* signal pathway needs further research.

In the present study, we examined *APC* expression level and methylation in HCC patients and healthy donors, and investigated the correlation between *APC* expression and its methylation status, We also examined the mRNA and protein expression of Wnt and *β-Catenin*, analyzed the correlations among *APC* methylation, *APC* expression and expression pattern of Wnt/*β-Catenin* signal pathway, and discussed its novel diagnosis value in HCC.

Materials and Methods

subjects

A total of 86 serum samples were collected randomly from HCC patients, and 27 serum samples were obtained from healthy donors at Yijishan Hospital of Wannan Medical College between 2019 and 2022. Fourty patients were classified as TNM I-II stage (UICC), Fourty-six patients as TNM III-IV stage. The average age of patients was 59 years (mean±SD: 43±12.8 years), ranging from 25 to 78 years. Written informed consent was provided by all patients, Ethics Committee of the first Affiliated Hospital of Wannan Medical College approved the protocol of the experiments.

Cell culture

Hepatocellular carcinoma HepG2 cell line was obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), and grown at 37 °C in a humidifed atmosphere in the presence of 5% CO_2.

DNA extraction

Genomic DNA Isolation was used by TIANamp Genomic DNA Kit (TIANGEN). The quality of extracted DNA visualized by Agarose gel electrophoresis, and NanoDrop microspectrophotometer (Thermo) was used to detect the purity of DNA.

Methylation of APC gene was detected by MSP method

MethPrimer 2.0 (http://www.urogene.org/methprimer/index1.html) was used to analyze the presence of CpG-islands in *APC* gene promoter and design the primers for MSP. Nucleotide sequences around the transcription sites (from −2000 to +500 bp) of *APC* gene promoter were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). The designed forward and reverse methyl-specific primers for *APC* was shown in follow: 5'-GAACCAAAACGCTCCCCAT-3', 5'-TTATATGTCGGTTACGTGCGTTTATAT-3'. The designed forward and reverse unmethyl-specific primers for *APC* was shown in follow: 5'-GTGTTTTATTGTGGAGTGTGGGTT-3', 5'-CCAATCAACAAACTCCCAACAA-3'. DNA was bisulfite-modified using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA, United States). MSP was performed to analyze the methylation status of *APC* gene promoter.

RNA Isolation

According to manufacturers' instructions, Trizol regeants(Invitrogen, United States) was used to obtain the total RNA from bone marrow samples. NanoDrop microspectrophotometer (Thermo) was used to measure the concentration and purity of the total RNA.

The mRNA expressions of APC, β-Catenin, c-Myc and Cyclin D1 were detected by RT-qPCR

A cDNA synthesis kit (TaKaRa, Dalian, China) was used to convert 500 ng of RNA into cDNA by reverse transcription, cDNAs was diluted with water treated by DEPC. SYBR premix Ex Taq (Qiagen, Hilden, Germany) was used to perform the real-time PCRs (RT-qPCR) on an ABI 7500 Real-time PCR system. The amplification parameters were set as the following: denaturation at 95 ˚C for 3 min, annealing at suiatable temperature(as shown in table 2) for 30 s, extending at 72 ˚C for 30 s. The primers for *APC*, *β-Catenin*, *c-Myc* and *Cyclin D1* were designed by the Primer 5 software, the primer sequences were shown in Table 1. Every examination was performed three times. Relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

The protein expression of APC, β-Catenin, c-Myc and Cyclin D1 were detected by western blot

Cell lysis buffer was used to lysate the cells to extract the proteins. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was then performed to separate the proteins. PVDF membranes was used to transfer the proteins. The membranes were incubated with primary antibodies at 4 ˚C overnight. Next, TBST was used to wash the membrane and was then incubated with secondary antibodies to resist the primary antibodies. β-actin was normalized for the quantitation of proteins and used as a control. The protein bands were analyzed using image J.

Statistical analysis

The data are listed as the means ± SD in the study. χ^2-test or Fisher's exact test was performed to assess the statistical significance in data between groups. All data were conducted by using SPSS software (SPSS Statistics 19).

Results

DNA methylation profile of APC in HCC cells, patients and healthy donors

MSP was performed to detect the methylation status of *APC* gene in 90 HCC patients and in 27 healthy donors. As shown in Figure 1 and Table 2, *APC* promoter methylation was found in the majority of HCC patients tested (46.67%, 42/90) and HepG2 cells, while the *APC* promoter methylation was examined in 3 of 27 healthy donors. The *APC* promoter methylation in HCC patients was signifcantly higer than healthy donors (P=0.001).

APC gene methylation profile in different TNM stage of HCC patients

As shown in Table 3, the methylation profile of *APC* gene in TNM stage patients was detected by MSP. *APC* gene exhibited marked hypermethylation in the TNM III-IV of HCC stage patients when compared to the patients of TNM I-II stage .

The relationship between the methylation status of APC gene and clinicopathological parameters

Results of the relationship between the methylation status of *APC* gene and clinicopathological parameters of HCC was shown in Table 3. Based on the methylation status of *APC* gene, we found that while *APC* gene methylation showed obvious correlation with tumor size and lymph node metastasis (P<0.05 and P<0.05), there was no correlation between *APC* gene methylation status and patients' age or sex, with a P-value of 0.215 and 0.892, respectively.

The mRNA and Protein expression of APC in HCC patients and control donors

APC mRNA and protein expression was investigated in 90 *APC* patients and control donors to study the role of the *APC* in HCC. Results of RT-qPCR showed that the average relative expression of *APC* mRNA in serum samples of HCC patients was significantly lower than that of control donors (Figure 2). Results of Western blot showed that the relative expression of *APC* protein in control donors was significantly higher than that of HCC patients (Figure 3).

The APC mRNA expression associated with APC hypermethylation

MSP results showed the positive rate of *APC* mRNA expression in HCC patients with *APC* methylation was significantly lower than that in control donors (Table 4) (P<0.05), suggesting that low or no *APC* gene expression in HCC patients may due to the *APC* gene promoter methylation.

The mRNAs and Proteins expression in HCC patients with and without APC hypermethylation

Through RT-qPCR and western blot analysis, we found that the mRNA and protein expression of *APC* in HCC patients with *APC* hypermethylation was lower than those without *APC* hypermethylation. Meanwhile, we examined

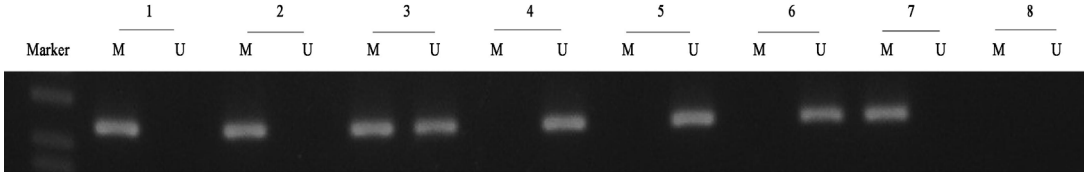

Figure 1. Methylation Status of *APC* Gene in HCC Cells, Patients and Healthy Donors. M: Methylation-specific PCR. U: Unmethylation-specific PCR. Line 1-2, Methylation status of APC gene in HCC patients. Line 3, Methylation status of APC gene in HepG2 cells. Line 4-5, Methylation status of APC gene in healthy donors. Line 6, Unmethylation positive control. Line 7, Methylation positive control. Line 8, Negative control.

Table 1. The Primers Used in RT-qPCR

Gene	Up-stream primer	Down-stream primer
APC	TTGTTTTTTTGTGTTGTAAAAATTATAGTA	ACACCTCCATTCTATCTCCAATAAC
β-Catenin	GATTACTGCTCTGGCTCCTAGC	GATTACTGCTCTGGCTCCTAGC
c-Myc	AGGAGCAGCAGAGAAAGGGAGAG	AGAGAGCCGCATGAATTAACTACGC
Cyclin D1	AGGCACGTCTTACATGACCA	CCCTAGTGCTGTTCTTCTGACA
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

Table 2. DNA Methylation Profile of *APC*

Group	n	Positive (n)	Methylation rate (%)	x^2	P
HCC	90	42	46.67	11.0933	0.001*
Control	27	3	11.11		

*P<0.05 compared with Control

Table 3. Relation of Clinicopathological Parameters to the Methylation Status of *APC* Promoter in HCC Patients

clinicopathological parameters	n	Positive (n)	Methylation rate (%)	x^2	*P*
TNM staging					
I-II	41	12	29.27	9.159	0.002*
III-IV	49	30	61.22		
tumor size (cm)					
≤5	42	14	33.33	5.625	0.018*
>5	48	28	58.33		
lymph node metastasis					
No	43	13	30.23	8.935	0.003*
Yes	47	29	61.7		
Age					
≤60	43	23	53.49	1.54	0.215
>60	47	19	40.43		
Sex					
Male	46	22	47.83	0.0185	0.892
Female	44	20	45.45		

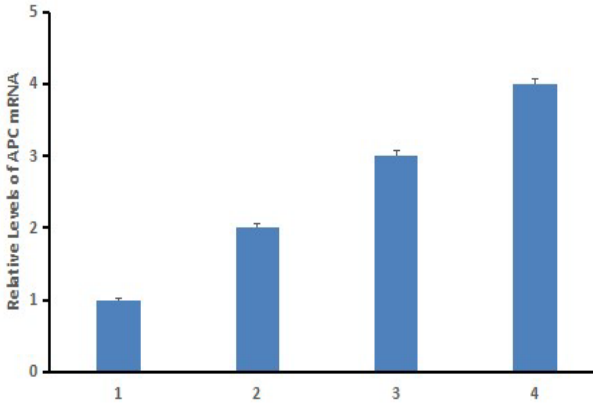

Figure 2. *APC* Gene Expression was Evaluated by RT-qPCR. 1-2: HCC patients; 3-4: control donors Compared with control *P<0.05

the mRNA and protein expressions of *β-Catenin*, *c-Myc* and *Cyclin D1*, the expression levels in HCC patients with were higher than their counterparts without *APC* hypermethylation (Figure 4). *APC* methylation may be associated with *APC* expression level, *APC* expression in HCC cells is silenced by aberrant promoter hypermethylation. In HCC patients with methylated *APC*, the mRNA and protein expression of Wnt and β-Catenin were higher than the unmethylated patient subgroup and healthy donors (Figure 5).

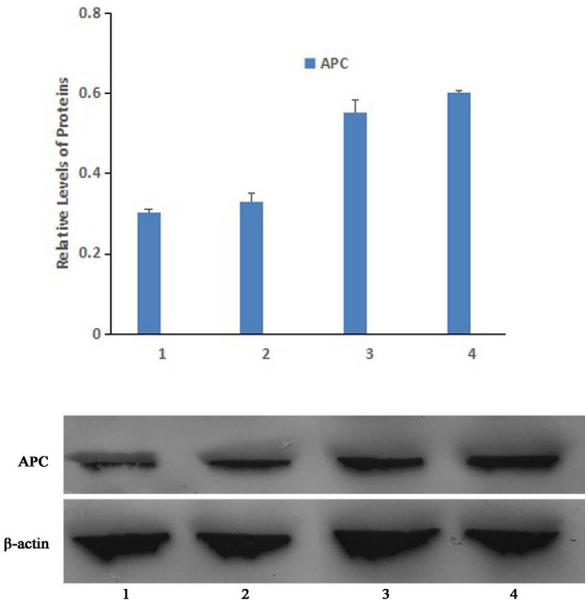

Figure 3. APC Protein Expression was Evaluated by Western Blot. 1-2: HCC patients; 3-4: control donors

Discussion

In this study, MSP assay result showed that the positive rate of *APC* gene methylation in HCC patients was higher than the matched healthy donors. *APC* gene exhibited marked hypermethylation in the patients of TNM III-IV stage compared to the patients in TNM I-II stage. Furthermore, we found that the *APC* gene methylation

Table 4. The Expression of *APC* mRNA in HCC and Control Donors Associated with Hypermethylation of the *APC* Promoter

Group	n	APC		The positive expression of APCmRNA (%)	x^2	P
		-	+			
Methylated APC	42	28	14	33.33	10.914	0.001*
Control	27	7	20	74.07		

*P<0.05, compared with control group

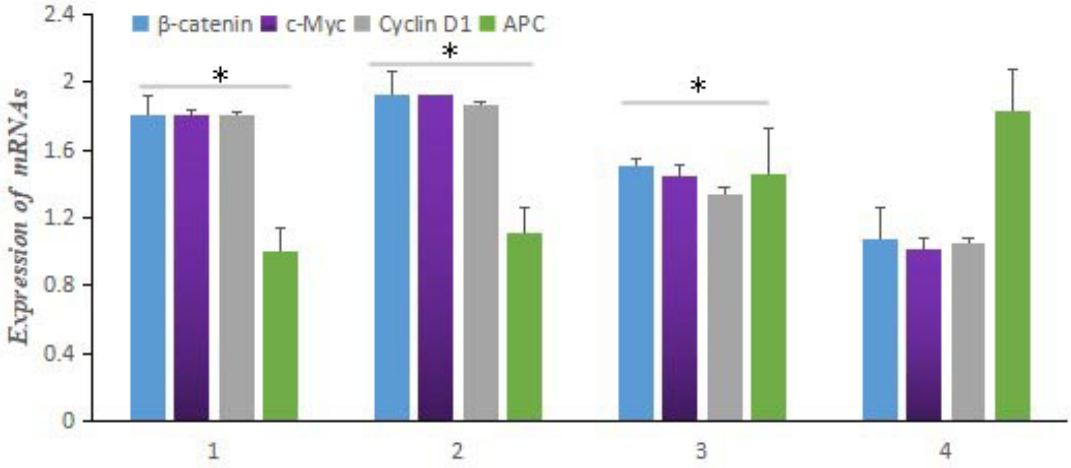

Figure 4. The mRNAs Expression in HCC Patients with and without *APC* Hypermethylation.1-2: HCC patients with *APC* hypermethylation; 3: HCC patients without *APC* hypermethylation; 4: control donors. Compared with control *P<0.05

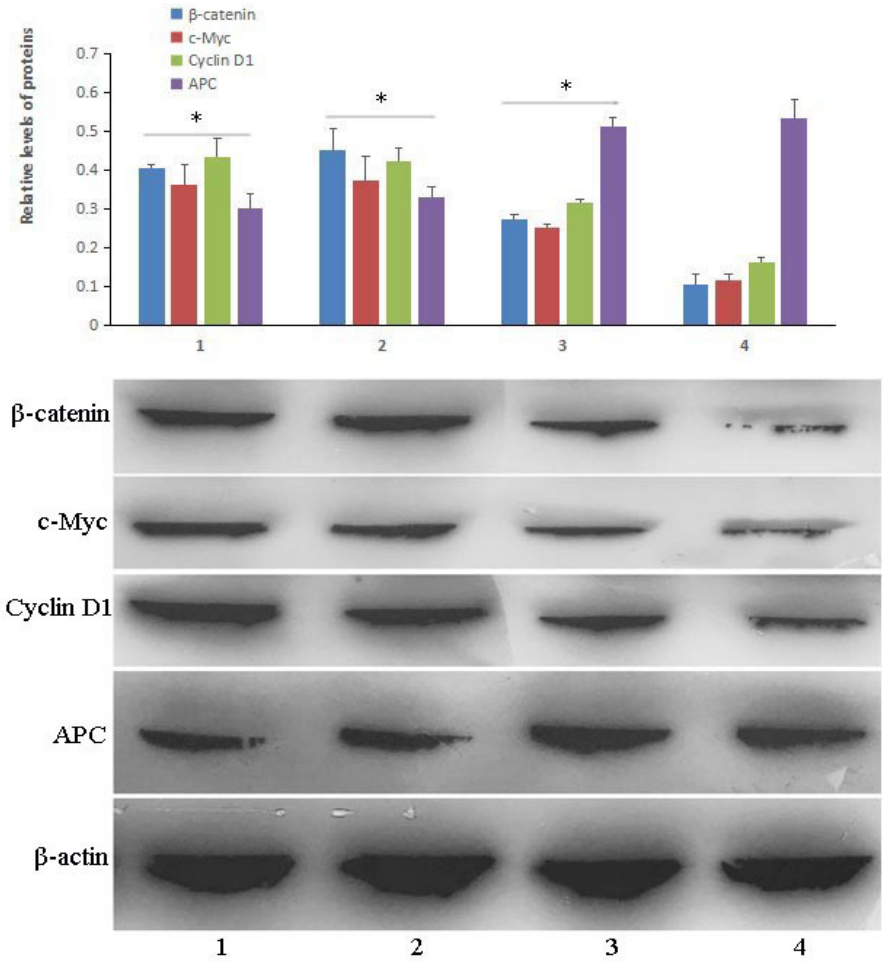

Figure 4. The Proteins Expression in HCC Patients with and without *APC* Hypermethylation. 1-2: HCC patients with *APC* hypermethylation; 3: HCC patients without *APC* hypermethylation; 4: control donors. Compared with control *P<0.05

was correlated with tumor size and lymph node metastasis whereas the *APC* gene methylation showed no relationship with the patient's sex and age. Second, *APC* expression in HCC patients was lower than healthy donors, and low *APC* expression level was associated with a poor prognosis of HCC. We found that *APC* gene promoter hypermethylation status may be a mechanism of its down-regulation. Finally, the mRNA and protein expression of Wnt and β-Catenin in HCC patients with methylated *APC* were higher than the unmethylated patient subgroup and healthy donors. Based on the research, we predicted *APC* methylation could be used as a novel diagnostic biomarker in HCC and was associated with regulation of Wnt/β-Catenin signal pathway.

Previous study revealed that APC expression was down-regulated in various tumors, including gastric cancer, colon cancer, lung cancer and prostate cancer (Lesko et al., 2014). Overexpression of APC decreased the cancer cell proliferation, migration and colony formation (Fang et al., 2022). Furthermore, it has also been reported that the expression levels of mRNA and protein in gastric cancer tissue were significantly lower than those in adjacent tissues (P<0.05) (Du et al., 2019). The 5-year cumulative survival rate of patients with downregulated *APC* expression in gastric cancer samples was significantly lower than that of patients with upregulated *APC* expression, indicating that dysregulated *APC* expression was associated with poor survival (Yang et al., 2018). Moreover, high *APC* expression was reported to be associated with a worse prognosis in patients with colorectal cancer (Liu et al., 2021).

In the current study, we found that the expression of *APC* in HCC patients was reduced compared with levels in healthy donors, and the patients with high *APC* expression exhibited better prognosis than patients with low *APC* expression. Then, we tried to investigate the mechanisms of *APC* dysregulation. DNA methylation has been demonstrated to play an important role in regulation of gene transcription as one of the most important epigenetic modifications, emerging evidence showed that aberrant DNA methylation patterns is involved in tumor metastasis and survival outcomes (Liu et al., 2017). Xian evaluated *CHD5* promoter methylation exited in seven gastric cancer cell lines and in primary gastric carcinoma tissues (73%, 11/15), *CHD5* promoter hypermethylation down-regulated the expression of *CHD5*, leading to the suppression of gastric cancer cell proliferation. *CHD5* functions as a TSG epigenetically silenced in gastric cancer(Wang et al., 2009). Feng (2016) revealed the rate of *APC* promoter methylation was 22.0% in non-small cell lung cancer (NSCLC) and 14.6% in corresponding non-tumor samples, patients with *APC* promoter methylation were found to be have shorter survival than patients without *APC* promoter methylation, *APC* methylation was reported as an independent prognostic factor in tumor samples. The study shows that *APC* methylation detected in lung tissue may be used as a predictive marker for diagnosis of NSCLC (Feng et al., 2016). Similarly, Trock (2012) reported that *APC* gene hypermethylation was found in the benign prostate and had high sensitivity and high predictive value for diagnosis of NSCLC patient. A previous meta-analysis have reported that hypermethylation of *APC* was significantly associated with the progression of bladder cancer and might be a promising biomarker for bladder cancer and other carcinomas (Bai et al., 2019). In this study, we explored the correlation between the methylation status of *APC* and its expression level. A negative correlation between *APC* expression level and its methylation was found in HCC, which suggested *APC* expression was down-regulated through the hypermethylation of *APC*.

APC functions as a tumor suppressor and is an integral protein of the Wnt β-Catenin signaling pathway, previous study explained APC could interact with β-Catenin and down regulate the level of β-Catenin in SW480 colon cancer cells (Van et al., 1999). β-Catenin binds to the Tcf/LEF family transcription factors and was demonstrated to be associated with cell proliferation. Inactivation of *APC* or mutated *APC* results in stabilization, translocation and accumulation of β-Catenin, and then the transcription of *c-Myc* and *Cyclin D1* was regulated and the cellular proliferation were increased, which indicated that Wnt/β-Catenin signaling pathway was involved in cell cycle regulation (Rubinfeld et al., 1997). However, the relationship with *APC* methylation and Wnt/β-Catenin signal pathway in HCC remain unclear. To further explore the potential association of *APC* methylation with Wnt/β-Catenin signaling pathway, we detected the mRNA and protein expression of β-Catenin in HCC patients with *APC* hypermethylation and those without *APC* hypermethylation. Result showed the mRNA and protein expression levels of β-Catenin, the expression in HCC patients with were higher than their counterparts without *APC* hypermethylation. In our fndings, *APC* methylation altered the expressions of β-Catenin, *c-Myc* and *Cyclin D1*, which might promote the cellular proliferation and involve in the pathological occurrence of HCC. The analysis showed that *APC* hypermethylation was positively associated with Wnt/β-Catenin signaling pathway which may suggest that *APC* hypermethylation is involved in HCC progression through these cancer-associated pathway.

In conclusion, Taken together, we found that the expression level of HCC was signifcantly downregulated in HCC and increased DNA methylation contributed to the aberrant *APC* expression. *APC* gene methylation was associated with different TNM staging tumor size and lymph node metastasis whereas it showed no relationship with the HCC patient's sex and age. Furthermore, the methylation status of *APC* was positively associated with Wnt/β-Catenin signaling pathway. In conclusion, our study showed that *APC* may serve as prognostic biomarkers for HCC patients in the future.

Author Contribution Statement

DW and WX: conceived and designed the research. YJL, JYC and QWH performed the experiments. PBL,RC and JLL analyzed the data. WX and YJL wrote the manuscript. DW and WX accomplished the project administration.

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Ethics statement

The studies involving human participants were in accordance with the ethical standards of the first affiliated hospital of Wannan Medical College institutional committee. Written informed consent for participation was not required for this study in accordance with the

institutional requirements.

Conflict of interest

No potential conflict of interest was reported by the authors.

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