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Analysis of the Relationship Between YWHAE and CDC6 Expression and Clinicopathological Characteristics of Colorectal Cancer

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ABSTRACT This study explores the expression of YWHAE and CDC6 in colorectal cancer (CRC) and their association with clinicopathological features and prognosis. mRNA expression profiles were obtained from The Cancer Genome Atlas (TCGA), and correlations with clinical data were analysed. Tissue microarrays from CRC patients were assessed using RNAscope and immunohistochemistry (IHC). Survival outcomes were evaluated by Kaplan-Meier and COX regression analyses. Both YWHAE and CDC6 mRNA were significantly upregulated in CRC tissues ($P < 0.05$) and showed a positive correlation ($P < 0.01$). IHC revealed high protein expression of YWHAE and CDC6 in CRC tissues, which was significantly associated with lymph node metastasis, pathological stage, and reduced survival ($P < 0.05$). Multivariate analysis confirmed both as independent prognostic factors. These findings suggest that YWHAE and CDC6 play a role in CRC progression and may serve as valuable biomarkers and potential therapeutic targets.

INTRODUCTION

According to global mortality statistics from 2020, cancer is one of the most common causes of death worldwide (Sung et al. 2021), and colorectal cancer (CRC) is the third leading cause of cancer-related deaths globally (Reilly et al. 2019), with its incidence rate steadily increasing. CRC is caused by multiple factors, with genetic alterations being one of the most important contributors to cancer development (Farooqi et al. 2019; Afrin et al. 2020). Identifying the key genes involved in cancer initiation and progression is crucial to improving the understanding of cancer mechanisms. Although advances in treatment options, such as radical surgical resection, have improved, the 5-year survival rate for CRC patients has not significantly increased (Yoshida et al. 2020; Kasindi et al. 2019). A major reason for treatment failure is tumour recurrence and metastasis, with nearly half of patients with advanced CRC dying from metastasis and recurrence after radical surgery (Renaud et al. 2019; Gagnière et al. 2020). Therefore, investigating the biological behaviour of CRC cells during cancer progression, identifying tumour-related biomarkers, and providing more clinical diagnostic options and treatment strategies to improve patient outcomes are of great importance.

The Cancer Genome Atlas (TCGA) is a publicly funded project that has systematically revealed a comprehensive molecular landscape of cancer biology (Tomczak et al. 2015). By integrating different types of tumours and using advanced bioinformatics tools, the project aims to identify similarities and differences among various cancers, with the goal of applying these insights to cancer research (Wang et al. 2016). The findings from the TCGA database have been validated in subsequent experimental studies in various cancers (Ganini et al. 2021; Chang et al. 2013).

YWHAE (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein), a gene located on chromosome 17p13.3, encodes a subtype of the 14-3-3 protein family. This protein is widely expressed in eukaryotic cells and has been detected in a variety of organisms, including wheat (Guo et al. 2018) and parasites (Tian et al. 2018) in goat blood cells. It interacts with various cellular proteins and serves as an adapter or scaffold protein for assembling multi-protein signalling complexes. Initially, YWHAE was studied in the context of neurological diseases, such as Par-

kinson's disease (Jiang et al. 2019) and excitotoxic brain damage (Smani et al. 2018), where it was found to regulate mitochondrial dysfunction and apoptosis (Li et al. 2021). Recent studies suggest that abnormal expression of YWHAE may also play an important role in tumorigenesis and cancer progression (Leal et al. 2016). YWHAE is upregulated in various cancers. For example, in gastric cancer, it promotes cell proliferation, invasion, and migration, while in breast cancer, its expression is associated with tumour size, lymph node metastasis, poor prognosis, and chemoresistance (Yang et al. 2019). In hepatocellular carcinoma, YWHAE promotes cell proliferation and metastasis (Ko et al. 2011). Although YWHAE is upregulated in many tumours, its specific mechanisms remain unclear. Recent work by Wang et al. (2023a) demonstrated that the novel norcantharidin derivative DCZ5417 can inhibit myeloma progression via the TRIP13-MAPK-YWHAE signalling pathway, suggesting YWHAE's active involvement in oncogenic signalling.

Cell division cycle 6 (CDC6), located on chromosome 17q21.3, encodes a member of the AAA+ ATPase family, which plays a crucial role in protein folding, degradation, intracellular transport, and DNA replication (Govatati et al. 2020). As a regulator of DNA replication initiation, CDC6 ensures precise cell cycle checkpoints. Abnormal CDC6 expression is associated with various cancers, where it may act as an oncogene (Mughal et al. 2019). It has been shown to promote cell proliferation, invasion, and metastasis in cancers such as glioma, gastric cancer, and pancreatic cancer (Zhang et al. 2020). Moreover, CDC6 is important for evaluating tumour grade and predicting prognosis (Zhang et al. 2020). Moreover, a pan-cancer analysis by Pu et al. (2024) revealed that CDC6 functions not only as a regulator of cell cycle but also as a potential immunomodulator, especially in pancreatic and colorectal cancers. Yang et al. (2022) reported that elevated CDC6 expression predicted poor prognosis in CRC, reinforcing its utility as a potential biomarker.

In previous studies by the research group, gene expression profiling revealed that silencing YWHAE in colon cancer cells led to significant changes in cell cycle-related genes, especially CDC6, which was notably downregulated. Although a few studies have reported on the expression of YWHAE and CDC6 in CRC, their correlation has not been confirmed. Additionally, both proteins are involved in pathways that influence cell division and tumour progression (Yang et al. 2019; Youn et al. 2020), particularly the ERK/MAPK signalling pathway, which plays a critical role in promoting cell proliferation and survival in cancers (Daldello et al. 2015; Wang et al. 2023b). The positive correlation observed between YWHAE and CDC6 expression in this study suggests that these proteins may work together to drive CRC progression. The ability of YWHAE to modulate various cellular proteins and the role of CDC6 in regulating cell cycle checkpoints may enhance tumour growth and metastasis when both are overexpressed.

Objectives

This study aimed to analyse the expression of YWHAE mRNA and CDC6 mRNA in CRC tissues using bioinformatics and to explore their relationship with clinicopathological features, prognosis, and potential molecular mechanisms. The researchers also collected paraffin-embedded CRC tissue samples to validate the bioinformatics findings using RNAscope in situ hybridisation and immunohistochemistry. The results will provide insight into the role of YWHAE and CDC6 in CRC.

METHODOLOGY

Bioinformatics Data Collection

YWHAE mRNA and CDC6 mRNA expression profiles and clinical datasets of colorectal cancer (CRC) were downloaded from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov). After removing cases with incomplete clinical information, a total of 400 CRC samples and 41 normal samples were included in the analysis. The CRC samples were divided into high and low expression groups based on the median expression levels of YWHAE and CDC6 mRNA with 200 cases in each group. A total of 400 CRC samples and 41 normal samples were retrieved from the TCGA database for transcriptomic and clinical correlation analyses.

Collection of Experimental Cases

Between January 2020 and November 2022, the researchers collected 100 radical resection CRC

surgical samples from the First Affiliated Hospital of Hainan Medical University to create tissue microarrays. Eligible patients had histologically confirmed CRC, complete clinicopathological data, and follow-up information, without prior chemotherapy or radiotherapy. Normal adjacent tissues were used for comparison. The researchers excluded patients with incomplete data, damaged samples, concurrent malignancies, serious comorbidities, or poor-quality microarray sections. After excluding samples with incomplete sections or tissue loss, 87 CRC and corresponding normal tissues were analysed. The researchers also purchased two colon cancer tissue arrays from Shanghai Outdo Biotech Co., Ltd. (HColA180Su13, batch XT17-012), comprising 90 cancer and normal tissue cases with 5 to 6 years of follow-up data. After removing failed samples, 77 cancer tissues and 62 normal tissues were analysed. All samples met the same inclusion criteria, and staging followed the 8th edition of AJCC CRC guidelines. No patients received preoperative radiotherapy or chemotherapy. The study was approved by the hospital's Ethics Committee, and informed consent was obtained from patients or their families. Combined with purchased arrays, the histological validation cohort consisted of 164 CRC tissues and 149 matched normal tissues. Among these, 77 cancer tissues had 5 to 6 years of follow-up data for survival analysis. Altogether, the study utilised 400 CRC and 41 normal TCGA samples for bioinformatics analysis, and 164 CRC and 149 normal tissues for histological validation, ensuring adequate power for statistical and prognostic evaluation.

RNAscope In Situ Hybridisation

Tissue microarrays were made from CRC tissues and adjacent normal tissues. A conventional HE-stained section was first made from each paraffin-embedded tissue, and the tissue was evaluated under a light microscope. The area of interest (without hemorrhage or necrosis) was marked, and the corresponding area on the paraffin block was identified. A 1.5mm hollow needle was used to extract the marked tissue, and the tissue was transferred into blank paraffin block molds. The tissue was arranged into arrays of 8x6 and 10x6 on the wax block. The tissue microarray slides were baked, deparaffinised, and treated with hydrogen peroxide at room temperature for 10 minutes. Target retrieval was performed using RNAscope® Target Retrieval Reagent at 95°C for 15 minutes, followed by cooling. RNAscope® Protease Plus solution was applied, and the slides were incubated at 40°C for 30 minutes. After two washes with distilled water, the YWHAE and CDC6 probes were applied, and the slides were incubated at 40°C for 2 hours. Signal amplification was performed using the RNAscope® 2.5 HD Detection Kit following the manufacturer's instructions. The slides were counterstained with hematoxylin and mounted. Staining was evaluated based on a semi-quantitative scoring system, where 0-10 signal dots per cell, positive cells less than 10 percent is considered as low expression, while more than signal dots per cell, positive cells more than 10 percent is considered as high expression.

Immunohistochemistry

The EnVision method was used for immunohistochemical staining. Paraffin sections were baked at 60°C overnight, deparaffinised in xylene, rehydrated, and treated with 3 percent hydrogen peroxide for 5 minutes. Antigen retrieval was performed in the Tris-EDTA buffer (pH 8.0). The slides were incubated with primary antibodies (YWHAE, diluted 1:500; CDC6, diluted 1:400) at 37°C for 1 hour. After washing with PBS, the slides were incubated with secondary antibodies for 20 minutes, followed by DAB chromogen and hematoxylin counterstaining. The results were evaluated by two independent pathologists, and the final expression levels were determined using a scoring system based on the product of staining intensity and the percentage of positive cells. Staining intensity was scored as 0, 1, 2, or 3 based on the depth of staining, while the percentage of positive cells was scored as 1 or 2, with 5 percent as the cutoff. A total score of less than 3 was considered low expression, while a score of 3 or more was considered high expression.

Statistical Analysis

Statistical analysis was performed using R (version 3.6.4) and IBM SPSS Statistics 26.0 software. Survival data and mRNA expression levels from the TCGA database were analysed using the Mann-Whitney U test for continuous data, and the chi-square test for categorical data. The correlation

between YWHAE and CDC6 expression was evaluated using Spearman correlation analysis. Kaplan-Meier curves were used for univariate survival analysis, and comparisons of survival rates were performed using the Log-rank test. Multivariate analysis was conducted using the COX proportional hazards regression model. A P-value of <0.05 was considered statistically significant.

RESULTS

Expression of YWHAE and CDC6 mRNA in Colorectal Cancer Tissues

Statistical analysis of TCGA data, including 400 CRC samples and 41 normal controls, revealed that YWHAE mRNA expression levels were significantly higher in cancer tissues (median: 423.78, IQR: 333.03–555.68) compared to normal tissues (median: 330.05, IQR: 296.76–375.78; $P < 0.001$). Similarly, CDC6 mRNA was markedly upregulated in CRC (median: 22.81, IQR: 15.57–33.20) compared to normal controls (median: 6.04, IQR: 4.74–8.41; $P < 0.001$), as shown in Table S1 and Figure 1. These findings suggest that both genes are transcriptionally activated in CRC.

Correlation Analysis Between YWHAE or CDC6 Expression and Clinical Pathological Characteristics in Colorectal Cancer

Correlation analysis between YWHAE or CDC6 mRNA expression and the clinicopathological characteristics of 400 colorectal cancer (CRC) samples from the TCGA database revealed no significant associations with gender, age, invasion depth, pathological stage, lymph node metastasis, or distant metastasis (Tables S2 and S3). To validate these results, 87 pairs of CRC and normal mucosal tissues were detected by RNAscope *in situ* hybridisation. Immunohistochemistry (IHC) was performed on 164 CRC and 149 normal tissues (Figs. 2 and 3).

In normal tissues, YWHAE mRNA was low in 78.16 percent of cases and high in 21.84 percent. CRC tissues, however, showed markedly higher YWHAE mRNA expression, with 73.56 percent of cases exhibiting high levels and 26.44 percent low (Table S4). IHC analysis indicated YWHAE protein was predominantly cytoplasmic, with high expression in 73.17 percent of CRC tissues versus 24.83 percent in normal tissues (Table S5).

For CDC6 mRNA in normal tissues, 79.31 percent of cases had low expression and 20.69 percent high. In CRC tissues, high expression was

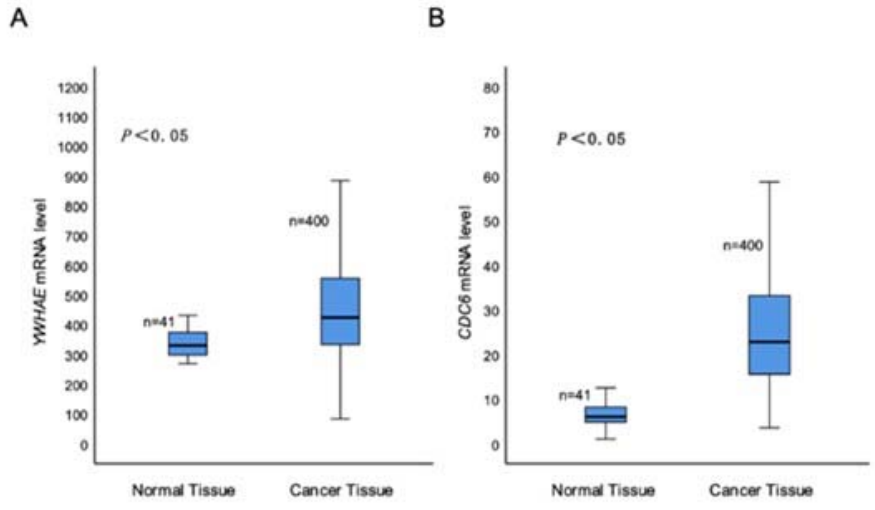

Fig. 1. YWHAE and CDC6 mRNA Expression in Colorectal Cancer Based on TCGA Data (A) YWHAE mRNA expression in CRC tissues. (B) CDC6 mRNA expression in CRC tissues. A P-value of <0.05 was considered statistically significant

Table 1: Correlation between YWHAE protein expression and clinicopathological characteristics in CRC

Clinicopathological characteristic	*Number of cases (n)*	*Low expression group*	*Expression high group*	*÷²-value*	*P-value*
Gender					
Male	100	26	74	0.09	0.764
Female	64	18	46		
Age					
≥60 years	103	24	79	1.756	0.185
<60 years	61	20	41		
Invasion Depth				3.244	0.072
T1 + T2	30	12	18		
T3 + T4	134	32	102		
Pathological Stage					
I + II	103	35	68	7.214	0.007
III + IV	61	9	52		
Lymph Node Metastasis					
No	109	35	74	4.617	0.032
Yes	55	9	46		
Tumor Grade					
I + II	135	37	98	0.13	0.718
III + IV	29	7	22		
Distant Metastasis					
Yes	8	1	7	0.88	0.348
No	156	43	113		

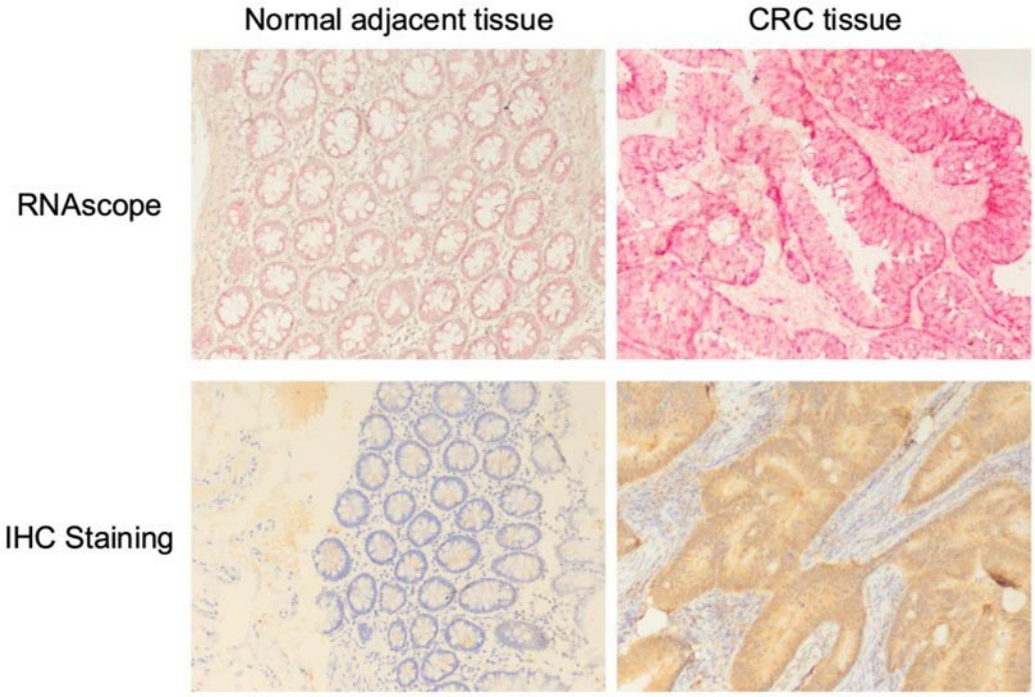

Fig. 2. Representative images for RNAscope and IHC analysis of YWHAE expression in normal adjacent tissue and CRC tissue (20×10)

Table 2: Correlation between CDC6 protein expression and clinicopathological characteristics in CRC

Clinicopathological characteristic	*Number of cases (n)*	*Low expression group*	*Expression high group*	*÷²-value*	*P-value*
Gender					
Male	100	36	64	0.698	0.404
Female	64	19	45		
Age					
≥60 years	103	33	70	0.279	0.598
<60 years	61	22	39		
Invasion Depth					
T1 + T2	30	11	19	0.161	0.688
T3 + T4	134	44	90		
Pathological Stage					
I + II	103	43	60	8.376	0.004
III + IV	61	12	49		
Lymph Node Metastasis					
No	109	43	66	5.098	0.024
Yes	55	12	43		
Tumor Grade					
I + II	135	43	92	0.972	0.324
III + IV	29	12	17		
Distant Metastasis					
Yes	8	1	7	1.670	0.196
No	156	54	102		

Table 3: Correlation analysis between YWHAE mRNA and CDC6 mrna expression in colorectal cancer

		YWHAE mRNA		*Number of cases (n)*	*r-val*	*P-val*
		High expression group	*Low expression group*			
CDC6 mRNA	High expression group	49	11	60	0.499	0.00
	Low expression group	15	12	27		
	Number of cases (n)	64	23	87		

Table 4 : Correlation analysis between YWHAE and CDC6 protein expression in CRC

		TWHAE mRNA		*Number of cases (n)*	*r-val*	*P-val*
		High expression group	*Low expression group*			
CDC6 Protein	High expression	98	11	109	0.454	0.000
	Low expression	22	33	55		
	Number of cases (n)	120	44	164		

Table 5: COX regression analysis of YWHAE protein expression in CRC patients

Clinicopathological factor	*Univariate analysis HR (95% CI)*	*P-value*	*Multivariate analysis HR (95% CI)*	*P-value*
Gender	0.749 (0.340-1.651)	0.473		
Age	0.929 (0.414-2.084)	0.858		
Tumor Grade	2.134 (0.803-5.670)	0.129		
Invasion Depth	4.495 (0.609-33.185)	0.141		
Lymph Node Metastasis	2.919 (1.347-6.328)	0.007	1.454 (0.128-16.460)	0.763
Pathological Stage	3.730 (1.717-8.103)	0.001	2.157 (0.167-27.880)	0.556
Distant Metastasis	8.272 (2.269-30.153)	0.001	4.063 (0.481-34.294)	0.198
YWHAE Expression	4.309 (1.291-14.375)	0.018	3.831 (1.136-12.922)	0.030

Table 6: COX regression analysis of CDC6 protein expression in CRC patients

Clinicopathological factor	*Univariate analysis CI)*	*HR (95% P-value*	*Multivariate analysis CI)*	*P-value*
Gender	0.749 0.340-1.651	0.473		
Age	0.929 0.414-2.084	0.858		
Tumor Grade	2.134 0.803-5.670	0.129		
Invasion Depth	4.495 0.609-33.185	0.141		
Lymph Node Metastasis	2.919 1.347-6.328	0.007	1.447 0.128-16.372)	0.765
Pathological Stage	3.730 1.717-8.103	0.001	2.096 0.162-27.075	0.571
Distant Metastasis	8.272 2.269-30.153	0.001	3.985 0.470-33.767	0.205
CDC6 Expression	3.323 1.251-8.827	0.016	2.805 1.038-7.576	0.042

Table S1: Expression of YWHAE and CDC6 in colorectal cancer

Group	*Normal tissue (n=41)*	*Cancer tissue (n=400)*	*Z-value*	*P-value*
YWHAE mRNA Expression	330.05 (296.76, 375.78)	423.78 (333.03, 555.68)	4.601	0.000
CDC6 mRNA Expression	6.04 (4.74, 8.41)	22.81 (15.57, 33.20)	9.789	0.000

Table S2: Relationship between YWHAE mRNA expression and clinicopathological characteristics in CRC (TCGA Data)

Clinicopathological characteristic	*Number of cases (n)*	*Low expression group*	*High expression group*	*χ^2-value*	*P-value*
Gender					
Male	216	116	100	2.576	0.108
Female	184	84	100		
Age					
e”60 years	289	144	145	0.012	0.911
<60 years	111	56	55		
Invasion Depth					
T1 + T2	81	38	43	0.387	0.534
T3 + T4	319	162	157		
Pathological Stage					
I + II	231	106	125	3.699	0.054
III + IV	169	94	75		
Lymph Node Metastasis					
No	239	110	129	3.753	0.053
Yes	161	90	71		
Distant Metastasis					
Yes	65	31	34	0.165	0.684
No	335	169	166		

Table S3: RNAscope detection of YWHAE mRNA expression in colorectal cancer and normal tissues

Group	*Number of cases (n)*	*Low expression (%)*	*High expression (%)*	*χ^2-value*	*P-value*
Normal Tissue	87	78.16 (68/87)	21.84 (19/87)	46.650	0.000
Cancer Tissue	87	26.44 (23/87)	73.56 (64/87)		

Table S1: Expression of YWHAE and CDC6 in colorectal cancer

Group	*Normal tissue (n=41)*	*Cancer tissue (n=400)*	*Z-value*	*P-value*
YWHAE mRNA Expression	330.05 (296.76, 375.78)	423.78 (333.03, 555.68)	4.601	0.000
CDC6 mRNA Expression	6.04 (4.74, 8.41)	22.81 (15.57, 33.20)	9.789	0.000

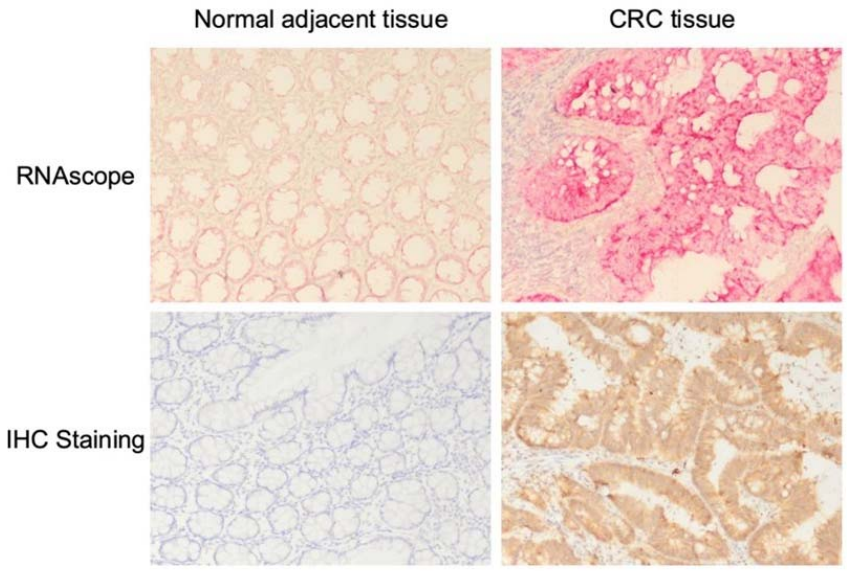

Fig. 3. Representative images for RNAscope and IHC analysis of CDC6 expression in normal adjacent tissue and CRC tissue (20×10)

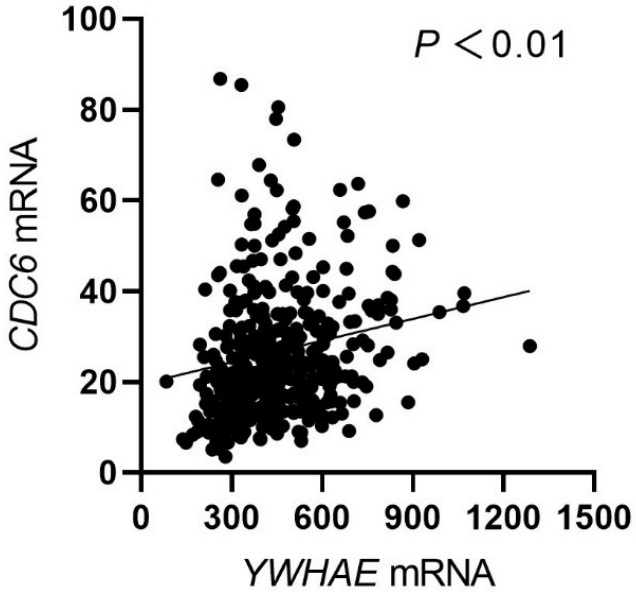

Fig. 4. Correlation Analysis of YWHAE mRNA and CDC6 mRNA Expression in CRC (TCGA Data). The correlation between YWHAE and CDC6 expression was evaluated using Spearman correlation analysis

Table S2: Relationship between YWHAE mRNA expression and clinicopathological characteristics in CRC (TCGA Data)

Clinicopathological characteristic	*Number of cases (n)*	*Low expression group*	*High expression group*	*χ^2-value*	*P-value*
Gender					
Male	216	116	100	2.576	0.108
Female	184	84	100		
Age					
e”60 years	289	144	145	0.012	0.911
<60 years	111	56	55		
Invasion Depth					
T1 + T2	81	38	43	0.387	0.534
T3 + T4	319	162	157		
Pathological Stage					
I + II	231	106	125	3.699	0.054
III + IV	169	94	75		
Lymph Node Metastasis					
No	239	110	129	3.753	0.053
Yes	161	90	71		
Distant Metastasis					
Yes	65	31	34	0.165	0.684
No	335	169	166		

Table S3: RNAscope detection of YWHAE mRNA expression in colorectal cancer and normal tissues

Group	*Number of cases (n)*	*Low expression (%)*	*High expression (%)*	*χ^2-value*	*P-value*
Normal Tissue	87	78.16 (68/87)	21.84 (19/87)	46.650	0.000
Cancer Tissue	87	26.44 (23/87)	73.56 (64/87)		

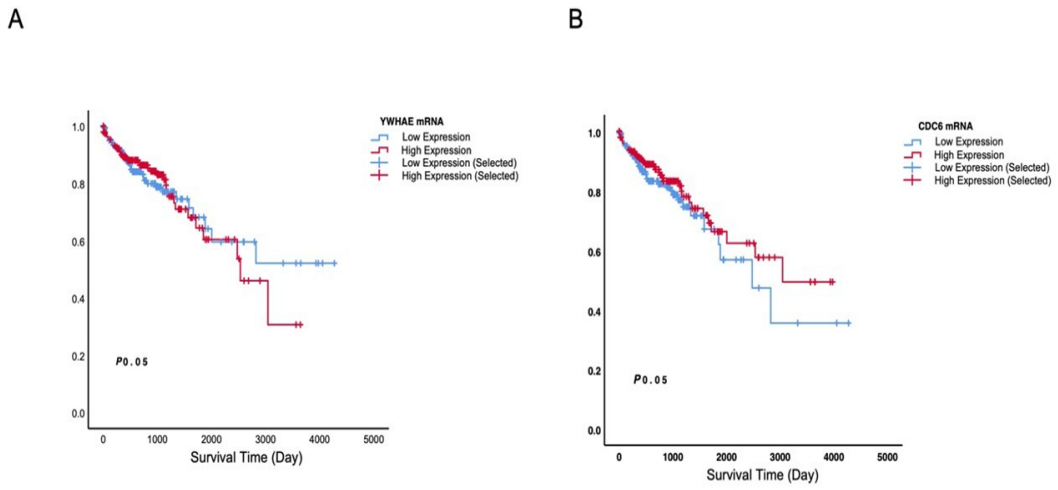

Fig. 5. Kaplan Meier Survival Curve of mRNA Expression in CRC (TCGA Data). (A) Kaplan Meier Survival Curve of YWHAE mRNA Expression in CRC. (B) Kaplan Meier Survival Curve of CDC6 mRNA Expression in CRC Kaplan-Meier curves were used for univariate survival analysis, and comparisons of survival rates were performed using the Log-rank test

Table S4: YWHAE protein expression in colorectal cancer and normal tissues

Group	*Number of cases (n)*	*Low expression (%)*	*High expression (%)*	*χ^2-value*	*P-value*
Normal Tissue	149	75.17 (112/149)	24.83 (37/149)	72.969	0.000
Cancer Tissue	164	26.83 (44/164)	73.17 (120/164)		

Table S5: Relationship between CDC6 mRNA expression and clinicopathological characteristics in CRC (TCGA Data)

Clinicopathological characteristic	*Number of cases (n)*	*Low expression group*	*High expression group*	*χ^2-value*	*P-value*
Gender					
Male	216	112	104	0.644	0.422
Female	184	88	96		
Age					
≥60 years	289	151	138	2.107	0.15
<60 years	111	49	62		
Invasion Depth					
T1 + T2	81	42	39	0.139	0.709
T3 + T4	319	158	161		
Pathological Stage					
I + II	231	108	123	2.305	0.129
III + IV	169	92	77		
Lymph Node Metastasis					
No	239	111	128	3.004	0.083
Yes	161	89	72		
Distant Metastasis					
Yes	65	38	27	2.223	0.136
No	335	162	173		

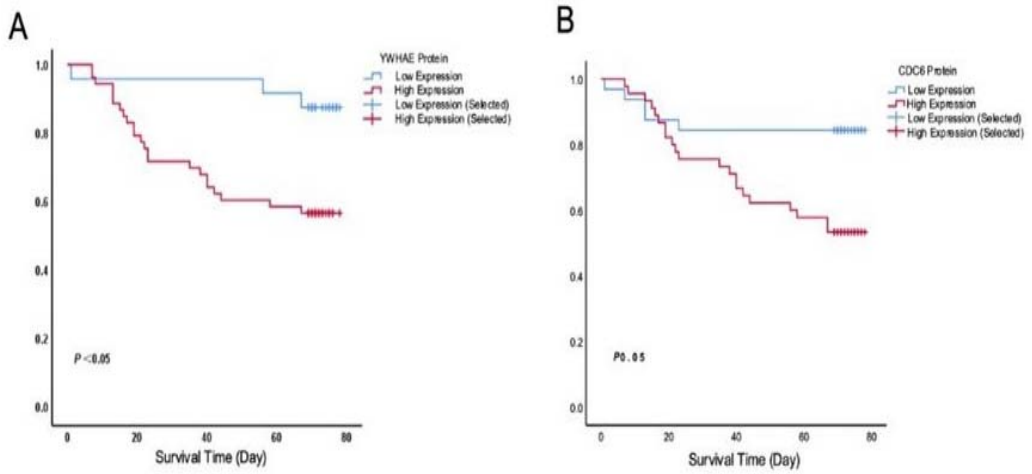

Fig. 6. Kaplan-Meier Survival Curve of Protein Expression in CRC Patients. (A) Kaplan-Meier Survival Curve of YWHAE Protein Expression in CRC Patients. (B) Kaplan-Meier Survival Curve of CDC6 Protein Expression in CRC Patients Kaplan-Meier curves were used for univariate survival analysis, and comparisons of survival rates were performed using the Log-rank test

observed in 68.97 percent of cases, with low expression in 31.03 percent, reflecting a significant difference between CRC and normal tissues (Table S6). IHC confirmed this trend, with high CDC6 protein expression in 66.46 percent of CRC tissues compared to 23.49 percent (Table S7).

Chi-square tests were used to analyse the correlation between YWHAE or CDC6 protein expression and clinicopathological characteristics in CRC patients. The results showed that both YWHAE and CDC6 protein expression were significantly correlated with lymph node metastasis and pathological stage ($P < 0.05$) (Tables 1 and 2).

Correlation Between YWHAE and CDC6 Expression in Colorectal Cancer

Spearman correlation analysis of TCGA data revealed a moderate but statistically significant positive correlation between YWHAE and CDC6 mRNA expression ($r = 0.291$, $P < 0.01$; Table S8, Fig. 4). This suggests these genes may be co-regulated or functionally related in CRC.

Validation in the RNAscope cohort further strengthened this observation, as in 87 tissue samples, 49 cases exhibited high expression of both YWHAE and CDC6, while only 11 had discordant expression (Table 3). The corresponding correlation coefficient ($r = 0.499$, $P < 0.001$) indicates a stronger association at the RNA level in real-world specimens.

Similarly, IHC analysis of protein expression showed consistent results, with a significant correlation ($r = 0.454$, $P < 0.001$) observed across 164 CRC samples (Table 4). These findings support the hypothesis that YWHAE and CDC6 may be involved in a shared signaling pathway.

Correlation Between YWHAE or CDC6 Expression with Prognosis in CRC Patients

Kaplan-Meier analysis was conducted to examine the overall survival (OS) of CRC patients based on the TCGA Data with high and low YWHAE mRNA or CDC6 mRNA expression. The results showed no significant difference in survival between the two separate groups (Fig. 5).

To further analyse the impact of YWHAE expression on prognosis, a Kaplan-Meier survival analysis was conducted on 77 CRC samples with 5 to 6 years of follow-up data. The results indicated that high YWHAE expression was associated with a shorter average survival time of 55.47 months, compared to 73.42 months for low expression, marking a significant difference (Table S9 and Fig. 6). Similarly, high CDC6 expression correlated with a reduced average survival time of 56.42 months versus 67.60 months for low expression, with a statistically significant difference (Table S10 and Fig. 6).

Univariate COX proportional hazards regression analysis indicated that lymph node metastasis, pathological stage, distant metastasis, YWHAE and CDC6 protein expression were significant predictors of poor prognosis. Multivariate COX regression analysis further confirmed that both YWHAE and CDC6 protein expression were independent risk factors for poor prognosis (Tables 5 and 6).

DISCUSSION

The findings demonstrated that both YWHAE and CDC6 mRNA and protein were significantly overexpressed in CRC tissues compared to normal tissues. High expression of YWHAE was strongly

Table S6: RNAscope detection of CDC6 mRNA expression in colorectal cancer and normal tissues

Group	*Number of cases (n)*	*Low expression (%)*	*High expression (%)*	*χ^2-value*	*P-value*
Normal tissue	87	79.31 (69/87)	20.69 (18/87)	40.990	0.000
Cancer tissue	87	31.03 (27/87)	68.97 (60/87)		

Table S7: CDC6 protein expression in colorectal cancer and normal tissues

Group	*Number of cases (n)*	*Low expression (%)*	*High expression (%)*	*χ^2-value*	*P-value*
Normal tissue	149	76.51 (114/149)	23.49 (35/149)	58.04	0.000
Cancer tissue	164	33.54 (55/164)	66.46 (109/164)		

Table S8: Correlation analysis of YWHAE mRNA and CDC6 mRNA expression in CRC (TCGA Data)

	YWHAE mRNA	*CDC6 mRNA*
YWHAE mRNA	1	0.291**
CDC6 mRNA	0.291**	1

Note: **P < 0.01, *P < 0.05.

associated with advanced pathological stage and lymph node metastasis, both markers of aggressive disease. Moreover, Kaplan-Meier survival analysis revealed that patients with elevated YWHAE expression had significantly shorter overall survival compared to those with lower expression levels, suggesting that YWHAE may serve as a key indicator of poor prognosis in CRC. This aligns with prior research indicating YWHAE's role in promoting tumour cell proliferation, migration, and resistance to apoptosis in several cancers, including breast, gastric, and renal cancers. These results are consistent with the growing recognition of YWHAE and CDC6 as key regulators of cancer progression (Cui et al. 2024; Yang et al. 2019). A recent study identified YWHAE as a downstream effector of the TRIP13-MAPK axis in multiple myeloma, where its inhibition suppressed tumor progression (Wang et al. 2023a). This finding supports the conclusion that elevated YWHAE expression contributes to aggressive tumour behavior in CRC, possibly via similar oncogenic pathways.

Similarly, CDC6, a key regulator of DNA replication and cell cycle progression, was found to be overexpressed in CRC tissues, with high levels correlating with lymph node metastasis and poor patient survival. CDC6's overexpression likely facilitates unchecked cell proliferation, contributing to tumour growth and metastasis, a finding consistent with its oncogenic role reported in cancers like lung, breast, and ovarian cancers. Univariate and multivariate analyses confirmed that CDC6 expression is an independent risk factor for poor prognosis, emphasising its potential as a therapeutic target. Additionally, the positive correlation between YWHAE and CDC6 expression suggests that these two proteins may act together in CRC progression, possibly through shared signalling pathways, such as the ERK/MAPK pathway, known to regulate cell proliferation and tumour development. This interaction highlights the need for further research into how YWHAE and CDC6 may cooperate to drive tumorigenesis, as dual targeting of these proteins could offer a new therapeutic approach for CRC treatment. CDC6 is a crucial initiator of DNA replication and has been recognised as a proto-oncogene in various solid tumors (Cui et al. 2024). A pan-cancer analysis by Pu et al. (2024) revealed that CDC6 not only promotes uncontrolled proliferation but also functions as an immunomodulatory molecule, influencing immune checkpoint expression and immune cell infiltration. These immunological roles may partly explain the association between CDC6 overexpression and poor prognosis in CRC, as observed in the study. Furthermore, Yang et al. (2022) demonstrated that CDC6 overexpression correlates with poor survival in CRC patients, which aligns with the Kaplan-Meier and COX regression results. Importantly, the analysis identified a positive correlation between YWHAE and CDC6 expression at both the mRNA and protein levels. This suggests a potential cooperative interaction in CRC pathogenesis. Previous reports have indicated that 14-3-3 family proteins, including YWHAE, can regulate the sta-

Table S9: Kaplan-Meier analysis of YWHAE protein expression and survival in CRC

YWHAE protein expression	*Number of cases (n)*	*Average survival time (months)*	*χ-value*	*P-value*
High expression	53	55.47	6.775	0.009
Low expression	24	73.42		

Table S10: Kaplan-Meier analysis of CDC6 protein expression and survival in CRC

CDC6 protein expression	*Number of cases (n)*	*Average survival time (months)*	*χ^2-value*	*P-value*
High expression	45	56.42	6.593	0.01
Low expression	32	67.60		

bility and localization of proteins involved in the cell cycle. It is plausible that YWHAE may stabilise CDC6 or facilitate its nuclear localisation, thereby amplifying its effects on DNA replication and cell proliferation. However, further functional experiments are needed to validate this hypothesis.

The findings on the elevated expression of YWHAE and CDC6 in colorectal cancer (CRC) align with previous research across various cancers (Yang et al. 2019; Lu et al. 2021; Lim and Townsend 2020), though it adds specific insights into their roles in CRC. For instance, prior studies have demonstrated that YWHAE is overexpressed in breast, gastric, and renal cancers, where it promotes tumour proliferation and metastasis (Yang et al. 2019). Similarly, CDC6 has been identified as an oncogene in cancers, where it facilitates uncontrolled cell cycle progression (Pu et al. 2024; Borlado and Méndez 2008). However, unlike many earlier studies that focused on these proteins individually (Kim et al. 2023; Yang et al. 2022), this study uniquely highlights the positive correlation between YWHAE and CDC6 expression in CRC, suggesting potential cooperative roles in tumour progression. This study also expands on previous research by demonstrating that both YWHAE and CDC6 are independently associated with poor prognosis in CRC, with high expression levels correlating with shorter survival times. While other studies have explored the individual roles of these proteins in different cancers, this research provides a comprehensive view of their combined significance in CRC and underscores the potential for dual-targeted therapies. Furthermore, by utilising both bioinformatics analysis and experimental validation through immunohistochemistry and RNAscope, this study offers a robust approach to understanding the molecular mechanisms underlying CRC progression, setting it apart from other studies that may rely solely on one method of analysis. Despite these insights, several limitations should be acknowledged. The study, while strengthened by both bioinformatics and histological validation, remains observational. Functional studies are required to elucidate the mechanistic interplay between YWHAE and CDC6. Additionally, while the study's cohort is one of the largest tissue-based validations for these markers in CRC, multicentre validation across diverse populations is necessary to generalise the findings.

CONCLUSION

In conclusion, the study identifies YWHAE and CDC6 as important biomarkers associated with CRC progression and poor prognosis. Their elevated expression levels in CRC tissues, coupled with their correlation to aggressive disease features, make them promising targets for therapeutic intervention. Future studies should focus on elucidating the molecular mechanisms through which these proteins interact and exploring their potential as dual targets in CRC therapy.

RECOMMENDATIONS

This study highlights the potential prognostic and therapeutic value of YWHAE and CDC6 in colorectal cancer. Based on the findings, the researchers recommend that future research further investigate the mechanistic interactions between these two proteins in tumour progression, particularly their roles in relevant signalling pathways such as ERK/MAPK. Clinical studies with larger, multicentre cohorts are warranted to validate their prognostic utility and assess their potential as dual biomarkers for personalised treatment strategies. Moreover, exploration of YWHAE and CDC6 as therapeutic targets may offer new avenues for the development of targeted therapies in colorectal cancer. Integrating transcriptomic profiling and histological validation should be encouraged in future biomarker studies to enhance diagnostic precision and clinical relevance.

DECLARATIONS

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study is approved by the Ethics Committee of The First Affiliated Hospital, Hainan Medical University, and approval number is 2022 (Research L) No. 53.

CONSENT FOR PUBLICATION

Informed consent was obtained from all individual participants included in the study.

DATA AVAILABILITY

The simulation experiment data used to support the findings of this study are available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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AUTHOR CONTRIBUTIONS

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