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FTHL17, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* are highly expressed in colon cancer patients and are regulated *in vitro* by epigenetic alterations

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ABSTRACT

Background: Colon cancer is a serious public health issue and a major cause of cancer-related mortality worldwide, including Saudi Arabia. Knowledge of genes associated with colon cancer development and progression is essential for identifying new cancer-specific biomarkers to improve the diagnosis of colon cancer.

Methods: The expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in 15 adjacent colon cancer and normal colon tissue samples from male patients were investigated using reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) assays. qRT-PCR analysis was also used to determine whether reducing DNA methyltransferase (via 5-aza-2′-deoxycytidine treatment) or histone deacetylation (via trichostatin treatment) increased the expression levels of the tested genes.

Results: The analysis of the 15 colon cancer and adjacent normal colon tissue samples revealed that all six genes were expressed in both groups, but their expression levels were significantly higher in the colon cancer group. Furthermore, the mRNA expression levels of the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, and *ADAM29* genes were considerably upregulated after treatment of HCT116 and Caco-2 cells with 5-aza-2′-deoxycytidine and trichostatin. However, the *CABS1* gene was activated only with trichostatin treatment.

Conclusions: The findings of this study suggest that *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* are suitable candidate biomarkers of colon cancer and their expressions are regulated by hypomethylation and hyperacetylation.

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1. Introduction

Colon cancer (CC) ranks as the third most significant contributor to cancer-related deaths among men and the fourth among women on a global scale [1]. As a cause of cancer-related mortality, CC ranks first among Saudi Arabian men and third among Saudi Arabian women [2]. CC is usually diagnosed at advanced stages in older patients [3]. However, Rasool et al. [4] reported that CC has become widespread among younger Saudi age groups. Moreover, approximately 73% of Saudi patients with CC from different age groups are diagnosed at advanced stages [5]. The situation has prompted a pressing requirement to discover new and promising diagnostic and prognostic markers, aiming to enhance the early detection of CC [6, 7].

Several studies have indicated that cancer-testis (CT) genes and CT antigens (CTAs) can be used as prognostic and therapeutic targets for the early detection and immunotherapy of different types of cancers [8-10]. The testis is the only normal tissue in which CT genes are generally expressed in the postnatal period. Many cancers, including ovarian cancer, melanoma [11], breast cancer [12], leukemia [13], and CC may aberrantly express these genes [10]. Most biological roles of CT genes are unknown; however, they appear to play a role in testicular spermatogenesis, and fertilization [7, 14]. CT genes may have abnormal expressions in malignant tissues owing to the shared properties between testicular and neoplastic cells, including the ability to divide and migrate [14]. Moreover, cancer cells may involve CTA-encoding genes in different roles such as tumor development, proliferation, and anti-apoptotic mechanisms [15]. Therefore, these genes represent potential and promising biomarkers for the early identification of several types of cancers, including CC, because of their distinctive and unique expressions in testicular and cancer cells [8, 10, 11].

The expressions of many CT genes are regulated by DNA methylation and/or histone acetylation [7, 16, 17]. DNA methyltransferase enzymes (DNMTs) are primarily accountable for the process of DNA methylation occurring within the gene promoter region [18]. Gene expression activation in different cancer types has been linked to DNA hypomethylation [7, 16, 19]. The expressions of some CT genes are stimulated in many malignancies because of carcinogenesis-related genome-wide hypomethylation [16, 17]. Furthermore, DNA methyltransferase inhibitor (DNMTi) drugs such as 5-aza-2'-deoxycytidine (5-aza-CdR) may be used to increase the expression levels of numerous CT genes in cancer cells [7, 16, 17]. Almutairi et al. (2022) reported that *CTAG1A*, *MAGE-A4*, *SCP2D1*, and *MAGE-B1* gene expression patterns in CC cell lines increased because of hypomethylation induced by 5-aza-CdR treatment [7].

For the histone acetylation mechanism, histone acetyltransferases can also stimulate CT gene expressions by adding acetyl groups in a process called hyperacetylation, which results in chromatin remodeling. On the other hand, histone deacetylases (HDACs) inhibit CT gene expressions by removing the acetyl group in a process called hypoacetylation [16, 20]. In fact, posttranscriptional alteration resulting from treatment of cancer cells with HDAC inhibitor (HDACi) drug like trichostatin A (TSA) has the potential to increase the expression levels of multiple CT genes [7]. Following treatment with a TSA compound, CT genes such as *CTAG1A*, *MAGE-A4*, *SCP2D1*, *MAGE-B1*, *TKTL2*, and *ACTRT1* were activated [7].

Novel CT gene biomarkers are usually identified as follows: CT genes that show (1) overexpression in cancer tissues compared with matched normal tissues and (2) exclusive expressions in cancer tissues can be considered cancer-specific biomarkers. Tarnowski et al. reported that six CT genes (*MAGEA3*, *OIP5*, *TTK*, *PLU1*, *DKKL1*, and *FBXO39*) could be used as biomarkers of CC because they were significantly overexpressed in CC tissues compared with normal colon (NC) tissues from the same patients [8]. On the other hand, Almutairi et al. reported that although many CT genes, including *SCP2D1*, *CTAG1A*, *TKTL2*, *SPZ1*, *LYZL6*, and *TEX33*, were expressed in various CC tissues, no such expressions were found in any of the NC tissue specimens from the same individuals [10].

CT-restricted genes show promise as potential therapeutic targets for cancer due to their specific expression limited to both the testis and malignant tumors. Early detection of CC can significantly decrease mortality rates. Consequently, it is crucial to pinpoint a novel biomarker specific to cancer, aiding in the early detection of the disease. In the present study, the first aim was to identify novel potential CT by evaluating the expressions of six CT genes (*ADAM29*, *CABS1*, *CABYR*, *CPXCR1*, *FTHL17*, and *PRM2*) selected randomly from the CTA database for Saudi Arabian CC patients. The second aim was to determine the epigenetic activation impacts of 5-aza-CdR or TSA on the selected gene expressions in CC cell lines.

Materials and methods

2.1. Ethical approval and sample collection

The research, identified by IRB No. IRB/1201/22 and Study No. SP22R/076/04, received approval from the ethics committee of King Abdullah International Medical Research Center. All individuals participating in the study were recruited from King Khalid University Hospital in Riyadh, Saudi Arabia. None of the patients had received chemotherapy or physical therapy. Eligibility for this experiment was determined by a group of surgeons and pathologists who monitored and diagnosed the patients using standard clinical, endoscopic, radiographic, and histological criteria for adenocarcinoma. Each patient signed an informed consent form after reading and understanding the privacy statement. Moreover, the patients were asked to complete a self-administered survey questionnaire regarding their demographics, health, and lifestyle habits, including cigarette smoking and alcohol use, as well as any relevant family history, personal medical history, and allergy symptoms or disorders. Tissue samples from 15 Saudi males with CC were taken and compared with 15 NC tissue specimens from the same individuals. Tissue samples from both patients diagnosed with CC and NC were placed in sterile tubes containing RNAlater solution (76106; Thermo Fisher Scientific, Warrington, USA). This solution helps maintain and stabilize the RNA's integrity during storage and transportation.

Table 1
Sequences of the designed sense and antisense primers for RT-PCR and the predicted product sizes of the resulting products for detecting the CT genes of interest

Gene symbol	Accession number	Chromosomal location	Category	Primer sequence (5'→3')	Ta*	Product size (bp)
ACTB	NM_001101.5	7	Sense	AGAAAATCTGGCACCACACC	58°C	553
			Antisense	AGGAAGGAAGGCTGGAAGAG		
ADAM29	NM_001130703.1	4	Sense	TTGGGCTCGCTTCAATGACA	58°C	716
			Antisense	AAGGCATCAACTGAGGTTGC		
CABS1	NM_033122.4	4	Sense	CCGAACATTTCATGCCAGTG	58°C	557
			Antisense	AAATACTGGCTCCCTCGGTA		
CABYR	NM_001308231.2	18	Sense	TGAGCAAACGGAAGCAGTTG	58°C	637
			Antisense	ACTAACTCGTGGTGACTGCT		
CPXCR1	NM_001184771.2	X	Sense	CTCCCAGGAAGATGTTGTTC	58°C	732
			Antisense	TTCCCAGAAGAGCTGCATGA		
FTHL17	NM_031894.3	X	Sense	AAGTACGACACCAACTGCGA	58°C	444
			Antisense	AATCTTGCGCAGGTTGCTCA		
PRM2	NM_001286356.2	16	Sense	AGGTGTACAGGCAGCAGTTG	60°C	199
			Antisense	GAGCGTCTTTTGCGCCTTCT		

Abbreviations: ACTB: actin beta; ADAM29: ADAM metallopeptidase domain 29; CABS1: calcium binding protein, spermatid associated 1; CABYR: calcium binding tyrosine phosphorylation regulated; CPXCR1: CPX chromosome region candidate 1; FTHL17: ferritin heavy chain like 17; PRM2: protamine 2; RT-PCR: reverse transcription polymerase chain reaction; Ta: annealing temperature; bp: base pair

2.2. Human CC cell line cultures and epigenetic drug treatments (5-aza-CdR or TSA)

In this study, two CC cell lines, namely HCT116 and Caco-2, were utilized. Dr. Bader Almutairi from King Saud University in Riyadh, Saudi Arabia, generously provided both cell lines. The cells were cultivated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at a temperature of 37°C in a 5% CO2 humidified incubator. For epigenetic drug treatments, the final concentrations of 5-aza-CdR (A3656; Sigma-Aldrich, Hilden, Germany) and TSA (T1952; Sigma-Aldrich, Hilden, Germany) were determined after dissolving the drugs in a dimethyl sulfoxide (DMSO) solvent (D8418; Sigma-Aldrich, Hilden, Germany). Each cell line was divided into four groups: one treated with 10 μM 5-aza-CdR for 72 hours, another treated with DMSO (serving as a negative control for 5-aza-CdR), one treated with 100 nM TSA for 48 hours, and another treated with DMSO (serving as a negative control for TSA) for 48 hours. The selection of the optimal times and dosages for 5-aza-CdR and TSA therapies was based on previous publications' results [2, 7, 19].

2.3. Extraction of RNA from CC, NC, and cultured cells

Around 30 mg of the CC and NC tissue samples were utilized for RNA isolation, while approximately 5 million cultured cells were used for total RNA extraction. The AllPrep DNA/RNA Mini Kit (80204; Qiagen, Foster City, USA) was employed to extract total RNA from the CC and NC tissue samples as well as the cultured cells, following the manufacturer's guidelines. The concentrations of the isolated RNAs were determined using methods detailed in previous publications [7, 10].

2.4. Synthesis of complementary DNA

The RNA was converted into complementary DNA (cDNA) at a concentration of 2000 ng/μL using a high-capacity cDNA reverse transcription kit (4368814; Applied Biosystems, Warrington, USA), following the manufacturer's protocols. Subsequently, the cDNA was diluted to a 1:10 ratio and stored at 20°C.

2.5. Reverse transcription polymerase chain reaction primers and setups and agarose gel electrophoresis

Each set of reverse transcription polymerase chain reaction (RT-PCR) primers was designed using a combination of the manual and automated methods described in earlier studies [7, 10]. Macrogen (Macrogen Inc., Seoul, South Korea) was used to synthesize all sense and antisense primers. The primer sequences and expected sizes of the resulting RT-PCR products of the selected genes are described in Table 1. To assess the cDNA quality from the NC, CC, treated, and untreated tissue samples, the *ACTB* gene was utilized. Moreover, the specificity of each gene primer was examined using cDNA derived from total RNA extracted from human testes (AM7972; Thermo Fisher Scientific, London, UK). The RT-PCR reaction mixture for each gene contained 8.4 μL of nuclease-free water, 0.8 μL of diluted cDNA (200 ng/μL), 0.8 μL of both sense and antisense primers, and 10 μL of BioMix Red (BIO-25006; BioLine, London, UK). The thermal cycling conditions for RT-PCR and subsequent agarose gel electrophoresis were carried out according to methodologies detailed in recent publications [2, 7, 10, 19].]. Specifically, 3 μL of a 100-bp DNA marker and 8 μL of each PCR product were loaded and separated on a 1% agarose gel.

Table 2
List of qRT-PCR primer sequences, their expected product sizes for detecting the human genes of interest, and the qRT-PCR cycling conditions

Gene symbol	Direction	Primer sequence (5'→3')	Ta*	Product size (bp)	qRT-PCR cycling conditions
GAPDH	Sense	GGGAAGCTTGTCATCAATGG	58°C	173	Pre-denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and primer annealing at 58°C for 1 min
	Antisense	GAGATGATGACCCTTTTGGC			
ADAM29	Sense	TTGGGCTCGCTTCAATGACA	58°C	138	
	Antisense	ATATGGACACAGTGCCTGTG			
CABS1	Sense	CCGAACATTTCATGCCAGTG	58°C	132	
	Antisense	TCTCTGCATCTCCTGTGTCA			
CABYR	Sense	TGAGCAAACGGAAGCAGTTG	58°C	149	
	Antisense	CTAACATCTGAGCAGCAAGC			
CPXCR1	Sense	CTCCCAGGAAGATGTTGTTC	58°C	148	
	Antisense	TAAGGGCTTGTGAGAGACCA			
FTHL17	Sense	AAGTACGACACCAACTGCGA	58°C	136	
	Antisense	GGAAGTAGCGGAAGAAGTTC			
PRM2	Sense	AGGTGTACAGGCAGCAGTTG	60°C	199	
	Antisense	GAGCGTCTTTTGCGCCTTCT			

Abbreviations: qRT-PCR: quantitative reverse transcription polymerase chain reaction; Ta: annealing temperature; bp: base pair

Table 3
Basic clinical information of the patients with CC and NC

Characteristic	CC (N%)	NC (N%)
No. of patients, n (%)	15 (100)	15 (100)
Age, mean (range), years	65 (35–96)	65 (35–96)
<65, n (%)	7 (47%)	7 (47%)
>65, n (%)	8 (53%)	8 (53%)

CC patients

Patient No.	Age, years	Cancer grade	TNM stage
1	49	2	T2N1M0
2	69	2	T2N2M0
3	65	2	T3N1M0
4	63	2	T3N1M0
5	38	1	T1N0M0
6	35	1	T1N0M0
7	69	2	T1N1M0
8	96	2	T1N0M0
9	71	2	T3N0M0
10	70	2	T3N2Mx
11	56	1	T3N1M0
12	65	2	T3N0Mx
13	80	2	T3N0Mx
14	67	2	T3N1Mx
15	83	2	T2N0Mx

Abbreviations: CC: colon cancer; NC: normal colon; TNM: tumor-node-metastasis

2.6. qRT-PCR primers and setups

Each set of quantitative RT-PCR (qRT-PCR) primers was meticulously and manually crafted following the optimal criteria outlined in prior studies [7, 10]. The sequences of the designed sense and antisense primers for qRT-PCR, along with their predicted sizes, are shown in Table 2. The qRT-PCR experiment was set up in a 96-well plate using iTaq Universal SYBR Green (1725120; Bio-Rad, Hercules, USA). Each well of the 96-well plate was filled with 10 μL of a mixture containing 2 μL of diluted cDNA (100 ng/μL), 5 μL of SYBR Green, 0.5 μL of sense and antisense primers, and 2.5 μL of nuclease-free water. Every sample underwent melt-curve analysis, and the qRT-PCR experiment was conducted in triplicate, repeated three times. The expression level of *GAPDH* was used as a reference for normalizing the qRT-PCR data.

2.7. Statistical analysis

The qRT-PCR analysis was conducted using the SPSS statistical package. In this analysis, statistical significance was attributed to the following *P* values: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$. These specific *P* values were considered as thresholds for determining different levels of statistical significance in the study.

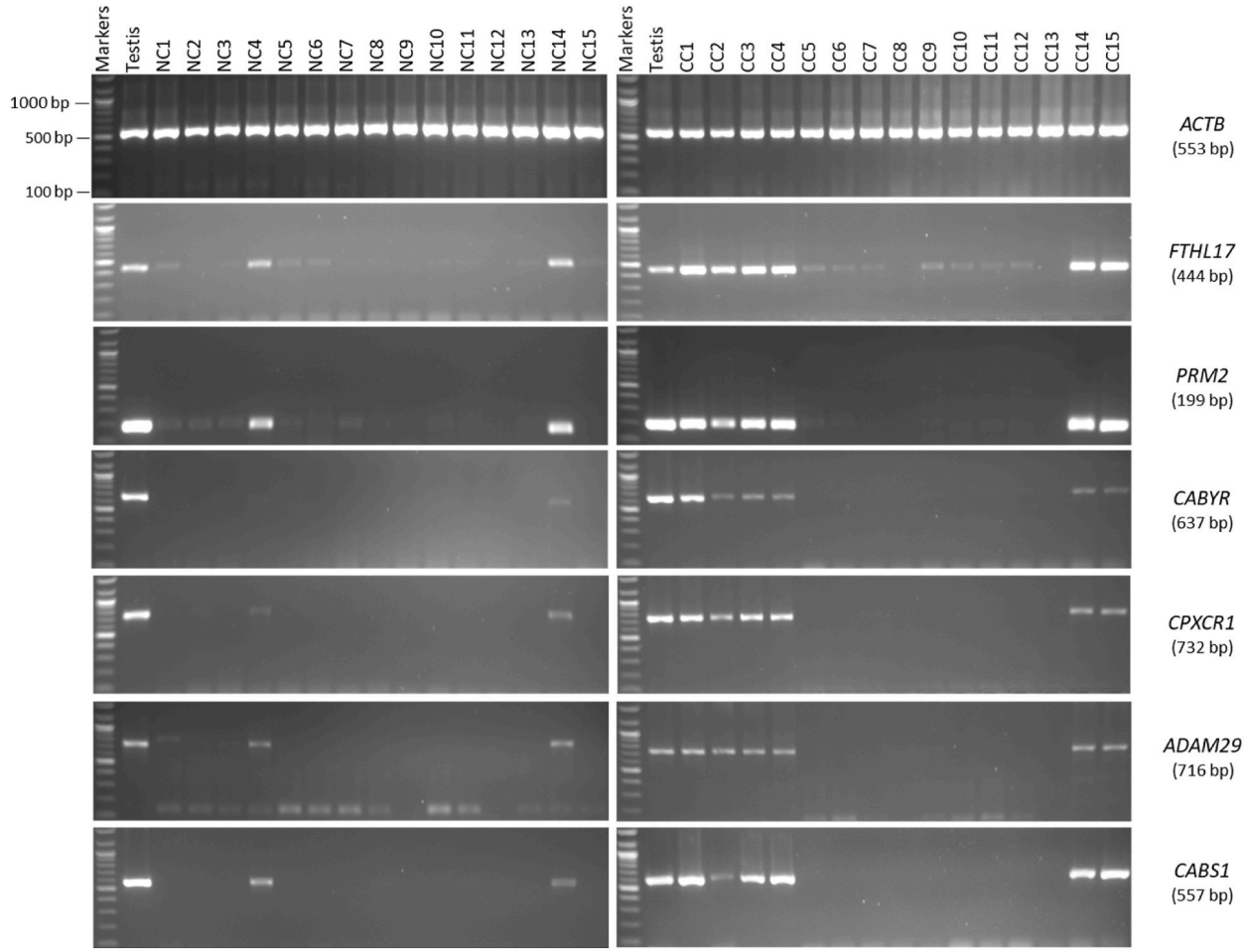

Figure 1. Analysis of the expressions of candidate CT genes in both normal colon (NC) and colon cancer tissue samples using a RT-PCR analysis. The agarose gel images show the RT-PCR results of the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes. Total RNA was isolated from 15 CC and adjacent NC tissue samples, and then all cDNA was generated from those samples. Each cDNA sample was evaluated on the basis of its ability to stimulate *ACTB* expression in humans. Human testis cDNA was used to evaluate the quality of the primers for each gene. On the right of the agarose gel images are the official symbol and predicted product size of each gene analyzed. Abbreviation: CT, cancer testis.

2.8. Bioinformatic RNA expressions

Data from more than 10,000 cancer patients in the Cancer Genome Atlas (TCGA) study, along with data from normal tissues in the Genomic Tissue Expression (GTEx) study were obtained via the online database OncoDB. Six genes (*FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1*) were selected for bioinformatics RNA expression analysis using OncoDB [21]. The expression data obtained from the database were analyzed statistically to identify any significant differences in gene expression between colon adenocarcinoma (COAD) and NC tissue samples. The Kaplan-Meier method was used to estimate the survival curves of the patients with altered expressions of the selected genes compared with those with unaltered expressions in the CC profile of the cBioPortal cancer genomics database. A total of 1269 patients were considered for the analysis, and their overall survival was recorded.

3. Results

3.1. Clinical data of the study subjects

Table 3 details some basic demographic and clinical data of the study participants. Fifteen participants who had both NC and CC were included in the research. The ages of the patients with CC ranged from 35 to 96 years (mean, 65 years). The patients with CC made up 47% of the population younger than 65 years, while 53% were older than 65 years. In most patients with CC (80%), the disease was classified as grade 2. The other clinical characteristics of the patients with CC, including their ages, cancer grades, and TNM staging, are detailed in Table 3.

Table 4
Summary of the positive expressions of the *FTHL17, PRM2, CABYR, CPXCR1, ADAM29,* and *CABS1* genes

Gene	No. of positive expressions in 15 CC tissue samples, n (%)	No. of positive expressions in 15 adjacent NC tissue samples, n (%)	*P* value
FTHL17	13 (87)	12 (80)	0.334
PRM2	12 (80)	11 (73)	0.334
CABYR	6 (40)	2 (13)	**0.040***
CPXCR1	6 (40)	2 (13)	**0.040***
ADAM29	6 (40)	3 (20)	0.082
CABS1	6 (40)	3 (20)	0.082

Abbreviations: NC: normal colon, CC: colon cancer. Values in bold indicate a significant result ($*P < 0.05$)

Table 5
Summary of the sequencing results for the *ADAM29*, *CPXCR1*, *CABYR*, and *CABS1* genes

Gene	Primer direction	Sequenced in NC tissue sample	Sequence identity (%)
ADAM29	Sense	14	99.21
	Antisense	14	99.40
CPXCR1	Sense	14	99.70
	Antisense	14	99.70
CABYR	Sense	14	98.28
	Antisense	14	98.47
CABS1	Sense	14	100
	Antisense	14	99.39

Abbreviation: NC: normal colon

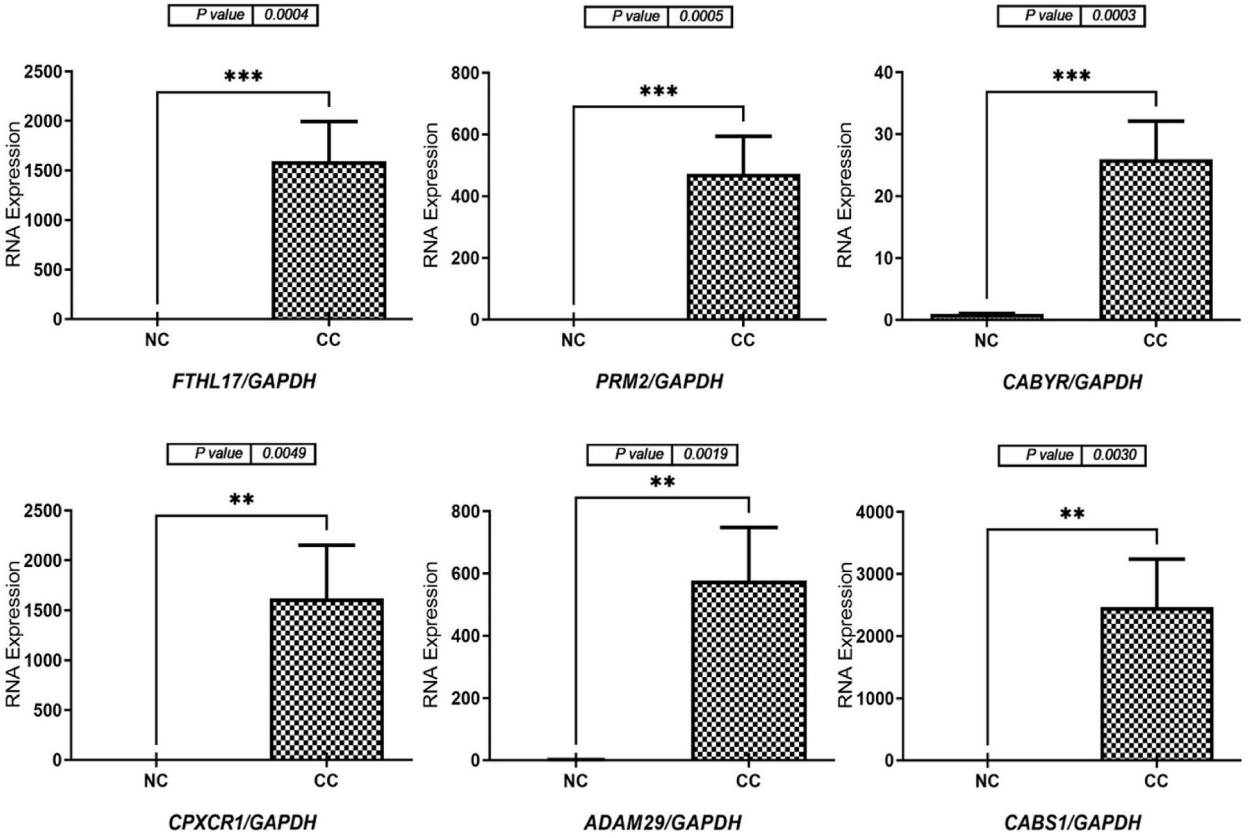

Figure 2. Analysis of the expressions of candidate CT genes in both normal colon (NC) and colon cancer tissue samples using a qRT-PCR analysis. The relative mRNA expressions of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in the CC and NC samples were determined using the qRT-PCR method. Normalization to *GAPDH* mRNA expression levels was performed for all genes. All genes were tested in three separate qRT-PCR assays, and the error bars show the standard error of the mean (SEM). Data were judged as statistically significant at the $**P \leq 0.01$ and $***P \leq 0.001$ levels.

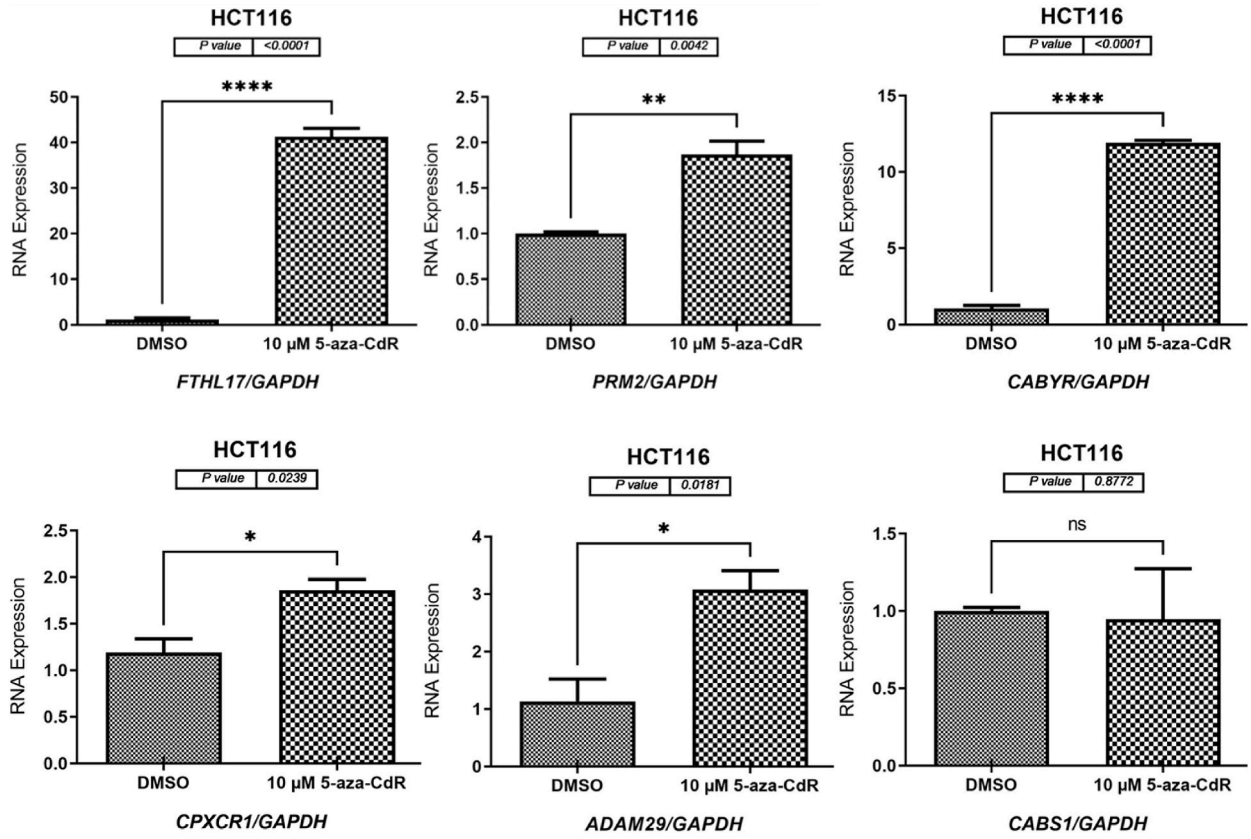

Figure 3. qRT-PCR analyses for the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes in the HCT116 cell line after treatment with 10 μM 5-aza-2′-deoxycytidine (5-aza-2-CdR) for 72 h. The gene expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in the HCT116 cell line before and after 5-aza-2-CdR treatment are shown in the bar chart. Since DMSO was utilized in the preparation of the 5-aza-2-CdR treatment solution, it was also used to treat the HCT116 cells in the control group. All gene expression levels were normalized to the mRNA expression level of the *GAPDH* housekeeping gene. The error bars represent the standard error of the mean (SEM) for three independent qRT-PCR experiments for each gene. All results with $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{****}P \leq 0.0001$ were considered statistically significant. Abbreviation: ns, not significant.

3.2. Expression profiles of the selected CT genes in the CC and matched NC tissue samples

The mRNA expressions of six CT genes (*ADAM29*, *CABS1*, *CABYR*, *CPXCR1*, *FTHL17*, and *PRM2*) were investigated in 15 CC and adjacent NC tissue samples to identify genes that could be used to detect CC in its early stages. The RT-PCR findings indicated that all CT genes were expressed in both the CC and NC tissue samples. However, the band intensity was stronger in the CC than in the NC tissue samples (Figure 1). The most frequently expressed CT gene in the CC tissue samples was *FTHL17* (87%), followed by *PRM2* (80%; Figure 1). The frequency of the expressions of the remaining CT genes, namely *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1*, in the CC tissue samples was 40% (Figure 1). Moreover, the positive expressions of the evaluated genes were compared between the CC and NC tissue samples (with faint or strong bands indicating a positive case). Table 4 shows that only the *CABYR* and *CPXCR1* genes were significantly positively expressed in the CC samples ($P = 0.040$; Table 4). To confirm the RT-PCR results through DNA sequence analysis, the RT-PCR products of *ADAM29*, *CABS1*, *CABYR*, and *CPXCR1* in the NC tissue samples were sequenced using both sense and antisense primers. The DNA sequencing analysis conducted on the RT-PCR products of *ADAM29*, *CABS1*, *CABYR*, and *CPXCR1* in the NC tissue samples revealed notable sequence resemblance to the *ADAM29*, *CABS1*, *CABYR*, and *CPXCR1* genes upon comparison with the reference sequences available in the NCBI database using the BLAST program (Table 5). Therefore, the outcomes of the sequencing procedure were in agreement with the results obtained from the RT-PCR analysis.

3.3. qRT-PCR analysis of gene expressions in the CC and NC tissue samples

In order to confirm with greater accuracy, the expression patterns of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in both CC and NC tissue samples were further scrutinized using qRT-PCR analysis. As depicted in Figure 2 based on the qRT-PCR data, these genes exhibited notably elevated expression levels in the CC tissue samples when compared to the corresponding NC tissue samples. Statistical analysis indicated significantly higher mRNA expression levels for *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and

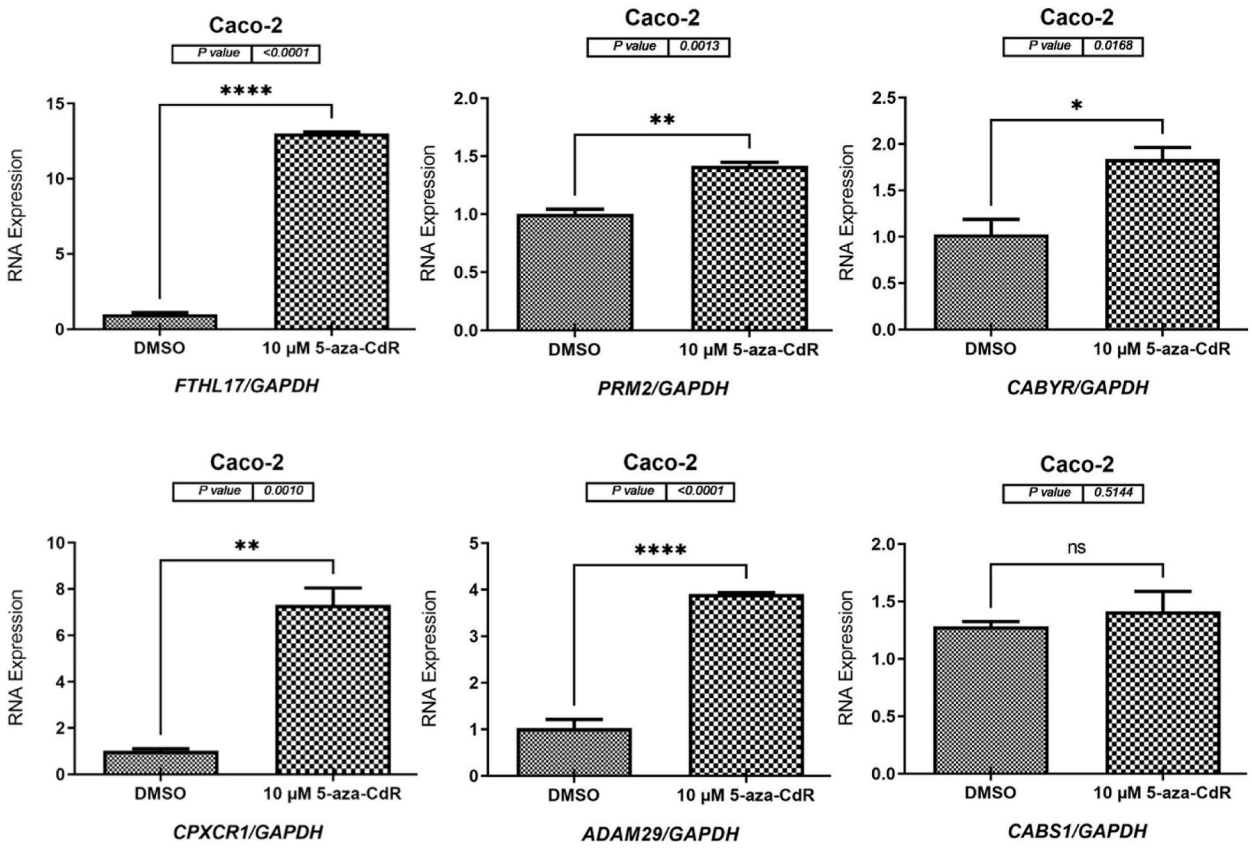

Figure 4. qRT-PCR analyses for the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes in the Caco-2 cell line after treatment with 10 μM 5-aza-2′-deoxycytidine (5-aza-2-CdR) for 72 h. The gene expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in the Caco-2 cell line before and after 5-aza-2-CdR treatment are shown in the bar chart. As DMSO was utilized in the preparation of the 5-aza-2-CdR treatment solution, it was also used to treat the Caco-2 cells in the control group. All gene expression levels were normalized to the mRNA expression level of the *GAPDH* housekeeping gene. The error bars represent the standard error of the mean (SEM) for three independent qRT-PCR experiments for each gene. All results with $*P \leq 0.05$, $**P \leq 0.01$, and $****P \leq 0.0001$ were considered statistically significant. Abbreviation: ns, not significant.

CABS1 in CC samples compared to NC tissue samples (P = 0.0004, P = 0.0005, P = 0.0003, P = 0.0049, P = 0.0019, and P = 0.0030, respectively; Figure 2). These findings affirm the consistency between the qRT-PCR and RT-PCR results (Figure 1). Based on the qRT-PCR outcomes, it is concluded that all six genes examined displayed overexpression in CC tissues in comparison to NC tissues. Hence, these genes hold promise as potential biomarkers for CC within the Saudi population.

3.4. Effects of 5-aza-CdR treatment on the expression patterns of the CT genes in CC cell lines

Two CC cell lines, HCT116 and Caco-2, were treated with 10 μM 5-aza-CdR as a DNMTi for 72 h. *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* gene expressions were analyzed using qRT-PCR. The 5-aza-2-CdR agent did not cause any noticeable changes in cell morphology. Figure 3 shows that when compared with the HCT116 cells treated with DMSO, those treated with 5-aza-CdR showed significantly higher expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, and *ADAM29* in the qRT-PCR analysis ($P \leq 0.0001$, $P = 0.0042$, $P \leq 0.0001$, $P = 0.0239$, and $P = 0.0181$, respectively). In addition, Figure 3 shows that neither 5-aza-CdR nor DMSO treatment resulted in a statistically significant change in the *CABS1* gene expression in the HCT116 cell line. Similar qRT-PCR results were found in the Caco-2 cell line. Compared with the Caco-2 cells treated with DMSO, those treated with 5-aza-CdR showed substantially upregulated expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, and *ADAM29* (Figure 4; $P \leq 0.0001$, $P = 0.0013$, $P = 0.0168$, $P = 0.0010$, and $P \leq 0.0001$, respectively). Figure 4 demonstrates that neither 5-aza-CdR nor DMSO treatment significantly altered the CABS1 gene expression in Caco-2 cells. Otherwise, no significant difference in *CABS1* gene expression was detected in the Caco-2 cells treated with 5-aza-CdR (Figure 4).

3.5. Effect of TSA treatment on the expression patterns of CT genes in CC cell lines

HCT116 and Caco-2 colon cancer cells were subjected to treatment with a hyperacetylation modulator known as TSA, functioning

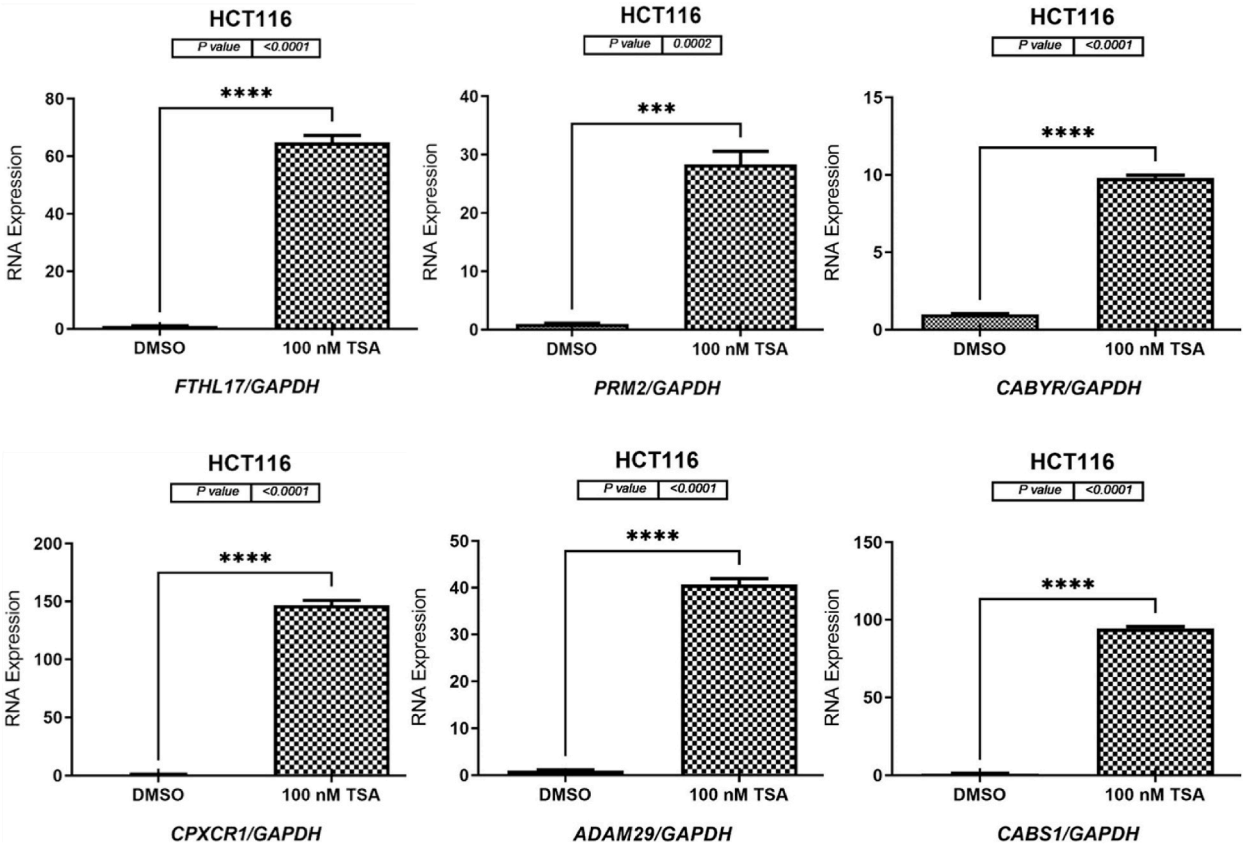

Figure 5. qRT-PCR analyses for the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes in the HCT116 cell line after treatment with 100 nM trichostatin (TSA) for 48 h. The gene expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in the HCT116 cell line before and after TSA treatment are shown in the bar chart. As DMSO was used in the preparation of the TSA treatment solution, it was also used to treat the HCT116 cells in the control group. All gene expression levels were normalized to the mRNA expression level of the *GAPDH* housekeeping gene. The error bars represent the standard error of the mean (SEM) for three independent qRT-PCR experiments for each gene. All results with ***$P \leq 0.001$ and ****$P \leq 0.0001$ were considered statistically significant.

as a HDAC inhibitor (HDACi). The dosage of TSA administered in this investigation was 100 nM for 48 h. In addition, no observable changes in cell morphology due to TSA treatment were detected in either the HCT116 or Caco-2 cell lines. However, the analysis revealed that compared to the control treatment with DMSO, the TSA treatment significantly stimulated the expression of all examined CT genes (*FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1*) in both the HCT116 and Caco-2 cells ($P \leq 0.0001$ and $P \leq 0.0001$, $P = 0.0002$ and $P \leq 0.0001$, $P \leq 0.0001$ and $P = 0.0003$, $P \leq 0.0001$ and $P = 0.0055$, $P \leq 0.0001$ and $P = 0.0004$, and $P \leq 0.0001$ and $P = 0.0260$, respectively), as shown in Figures 5 and 6, respectively.

3.6. RNA expressions of the selected genes in the COAD and NC samples based on data from publicly available databases

The expression levels of the six genes (*FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1*) were investigated in tissue samples from patients with COAD compared with NC tissue samples using data from TCGA databases. By using publicly available bioinformatics data and RNA expression analysis, all six genes were found to be overexpressed in the COAD tissue samples (Figure 7). This finding demonstrates that these genes were considerably overexpressed in the COAD tissue samples (n = 308) compared with the NC samples (n = 41). These results raise the possibility that these genes may contribute to the development or progression of CC.

4. Discussion

Currently, because it is more challenging to treat advanced cases of CC, late detection of CC is a major contributor to the increasing cancer-related mortality in Saudi Arabia. Consequently, validation of CT gene expressions in several patients with CC can be useful for detecting CC earlier and enhancing the likelihood of treatment. No previous study has documented *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, or *CABS1* gene expressions in the setting of CC.

Primers capable of amplifying certain CT gene family members were identified in this study. The expressions of the six genes in CC using the specific primers were validated using RT-PCR analysis of the fresh tissue samples from 15 patients with CC and matched

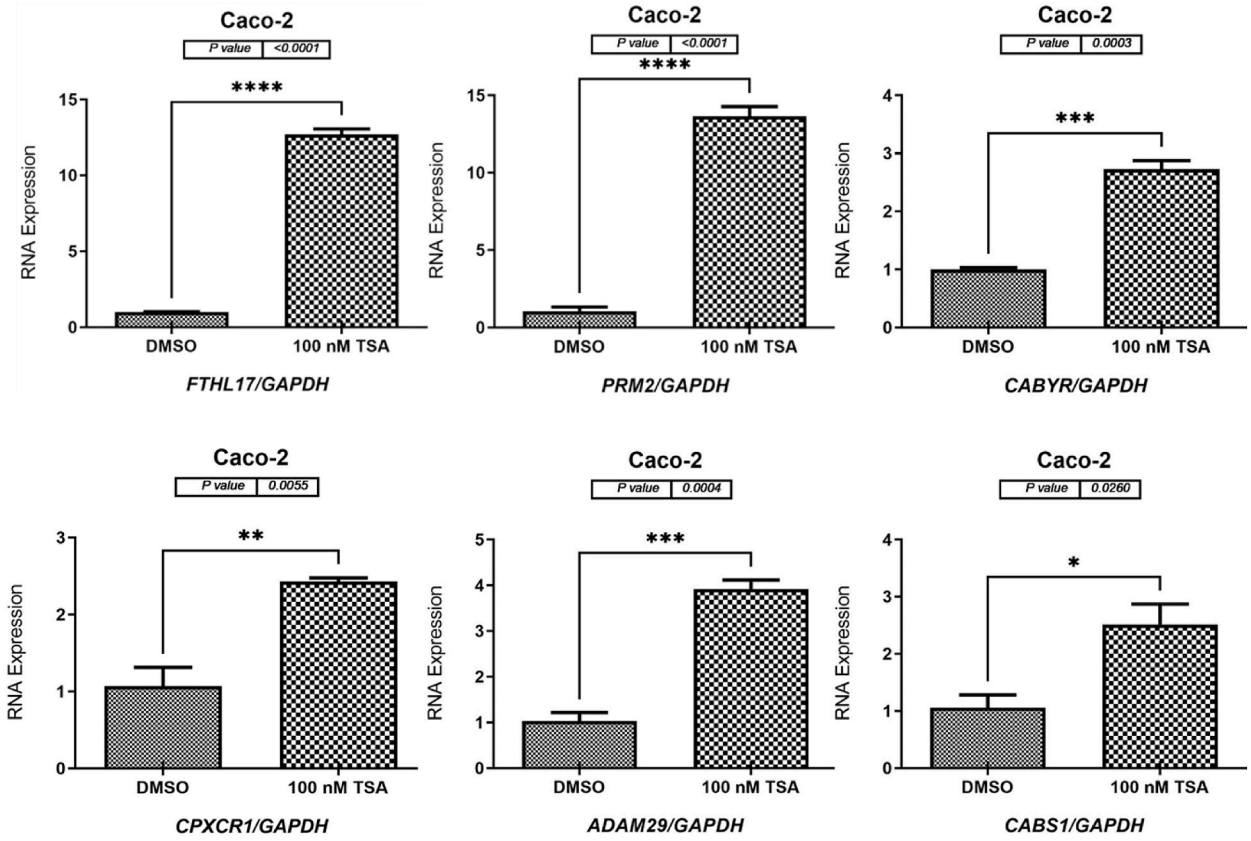

Figure 6. qRT-PCR analyses for the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes in the Caco-2 cell line after treatment with 100 nM trichostatin (TSA) for 48 h. The gene expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* in the Caco-2 cell line before and after TSA treatment are shown in the bar chart. As DMSO was used in the preparation of the TSA treatment solution, it was also used to treat the Caco-2 cells in the control group. All gene expression levels were normalized to the mRNA expression level of the *GAPDH* housekeeping gene. The error bars represent the standard error of the mean (SEM) for three independent qRT-PCR experiments for each examined gene. All results with $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$ were considered statistically significant.

participants with NCs. To prevent false-positive results due to contaminated genomic DNA, primers were chosen from different exons (Table 1). The primary objective of this study was to assess the expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes in CC tissue samples as compared with adjacent NC tissue samples to determine whether these genes hold potential as biomarkers for the early detection of CC. The findings revealed that all genes exhibited overexpression (upregulation) in the CC tissue samples when compared with the NC tissue samples, as validated by qRT-PCR analysis. Collectively, the results consistently indicated higher expression levels of these genes in CC tissue samples than in NC tissue samples. Consequently, their expression patterns categorized them as members of the CT gene group. This implies that these genes might serve as valuable biomarkers for the early detection of CC. Previous studies have identified the same findings for the *FTHL17, PRM2, CABYR, CPXCR1, ADAM29,* and *CABS1* genes in different types of cancers. For example, the *FTHL17* gene was expressed in bladder, breast, and lung cancer cells but was silent in normal somatic tissues [22]. The *PRM2* gene has not yet been studied in cancer; however, it has been identified as a CT gene in chronic leukemia and colorectal cancer [23]. *CABYR* mRNA expression was detected in 15 of 36 and 5 of 14 lung cancer tissue samples and cancer cell lines, respectively; however, it was also detected in adjacent noncancerous tissues or normal cell lines [24]. The *ADAM29* expression level was increased in breast and gastric cancer tissue samples relative to normal tissue samples. Consequently, it may be used as a predictive factor in human breast and gastric cancers and as a potential therapeutic target for these cancers [25, 26]. Little information is available on the human *CPXCR1* and *CABS1* genes in cancer. The TCGA database was also used to analyze the expression levels of the six genes in patients with COAD. The results demonstrated that all six genes were considerably overexpressed in the patients with COAD relative to the healthy controls with NCs. These findings emphasize the clinical importance of the overexpression of these genes in CC tissue.

The stronger band intensities of the *FTHL17, PRM2, CABYR, CPXCR1, ADAM29,* and *CABS1* genes in the CC tissue samples were associated with grade 2 cancer when compared with grade 1 cancer. While this study included CC tissue samples from patients with grade 1–2 cancer, it did not include any samples from patients with grade 3–4 cancer, which is a limitation of this study. To determine if the expressions of the genes correlate with disease grade, it is ideal to evaluate a sufficient number of specimens from each CC grade; grade 4 and 5 malignancies are of particular interest. In this study, whether *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, or *CABS1* gene

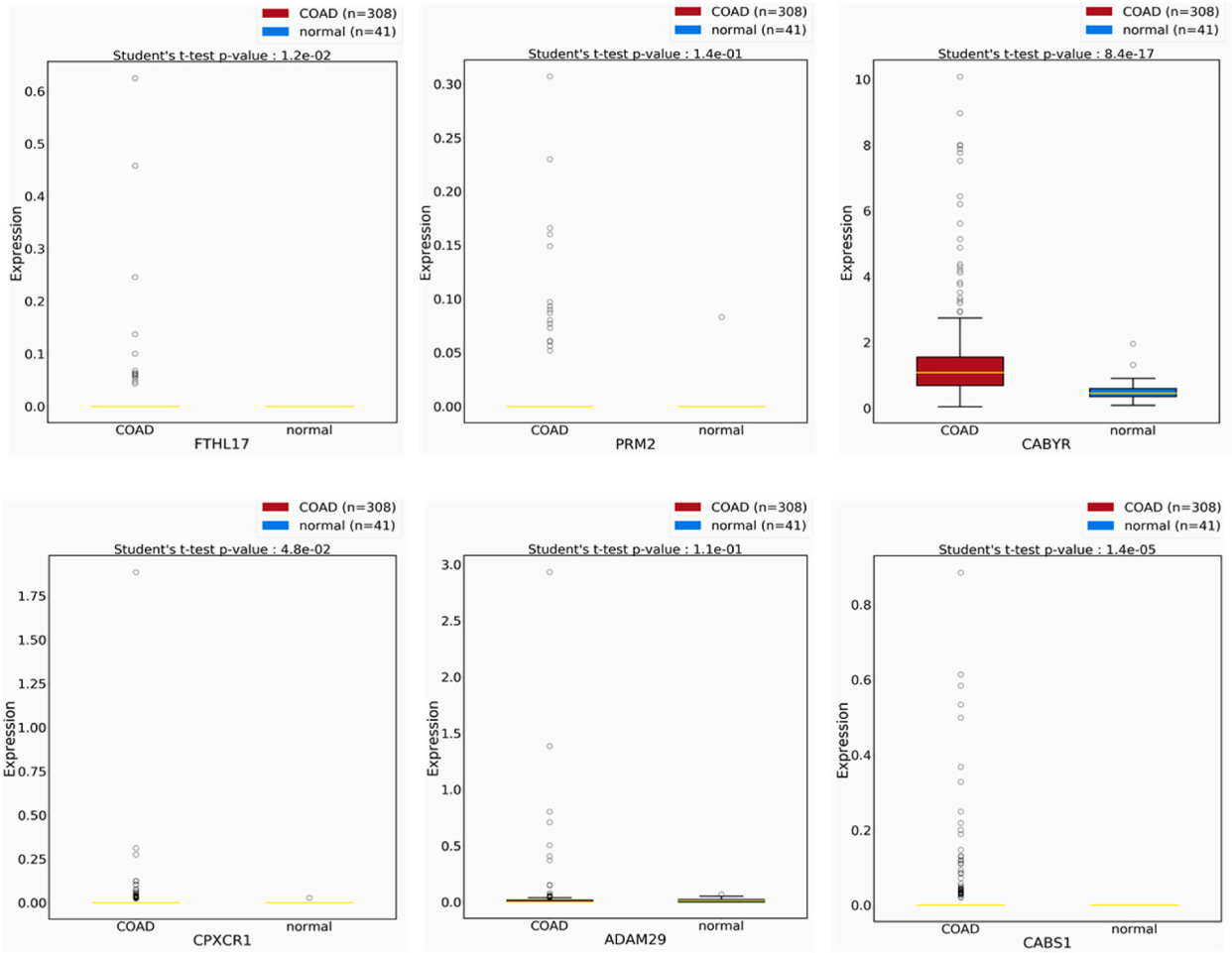

Figure 7. RNA expression patterns of the *FTHL17, PRM2, CABYR, CPXCR1, ADAM29,* and *CABS1* genes in colon adenocarcinoma (COAD) and normal colon (NC) tissue samples across multiple databases, including TCGA and GTEx. These analyses were performed using 308 COAD tissue samples and 41 NC tissue samples.

expression correlates with tumor grade could not be determined because of the small number of samples analyzed.

Moreover, the higher frequencies of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* gene expressions (overexpressed genes) in the CC tissue samples than in the adjacent NC tissue samples may be the result of other factors controlling their expressions in CC tissues. For instance, it is likely that epigenetic control of CT gene expressions in cancer, such as demethylation or histone deacetylation inhibition, can contribute to inducing the expression of several CT genes [7, 16, 27]. *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* gene expressions were examined to determine whether they increased through epigenetic regulation, specifically through decreased histone deacetylation or DNA methyltransferase. The HCT116 and Caco-2 cell lines, both freshly isolated from early-passage human CC tissues, were exposed to 10 μM 5-aza-2′-CdR for 72 h or 100 nM TSA for 48 h. The epigenetic results showed that the 5-aza-2′-CdR treatment activated the expressions of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, and *ADAM29* in the HCT116 and Caco-2 cells. However, no increased *CABS1* gene expression level was observed in either cell line at the same dose. The outcomes observed following treatment with 5-aza-2′-CdR indicate that the treatment can induce the expression of multiple genes, potentially indicating a certain degree of gene specificity in this response. These findings align with earlier research that identified differing expression levels among various CT genes in CC cell lines treated with 5-aza-2′-CdR [2, 7, 16].

In addition, the findings of this study prompt an important inquiry into why a substantial induction of gene expression was so significantly identified in the CC cell line treated with 5-aza-2′-CdR but not in the other cell lines treated with DMSO. Studies have indicated a decrease in the expression of *DNMT1*, a key enzyme involved in methylation repair, following the use of 5-aza-2'-CdR [28, 29]. In this study, it was observed that the expression level of the *DNMT1* gene decreased in the HCT116 cells treated with 5-aza-2'-CdR compared to those treated with DMSO (Figure 8). Further investigations are required to ascertain whether treatment with 5-aza-2'-CdR can also lead to reductions in the expression levels of other types of *DNMT* genes.

Inhibiting histone deacetylation with TSA treatment resulted in the most significant induction in gene expression. The expression levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* were significantly higher in the HCT116 and Caco-2 cells treated with

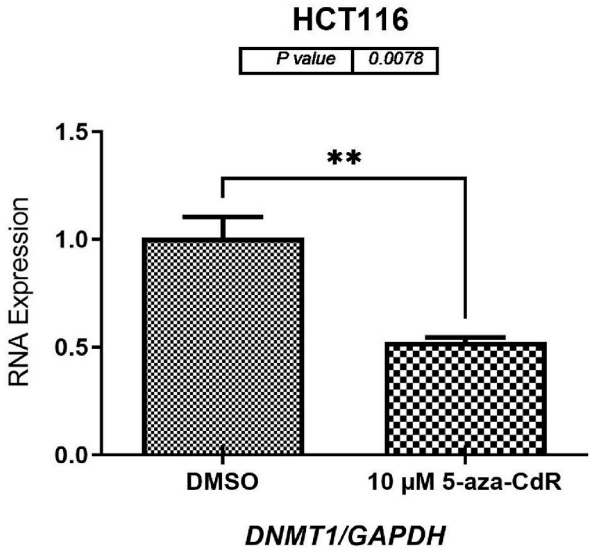

Figure 8. qRT-PCR analysis of *DNMT1* expression in HCT116 cells treated with 10 µM 5-aza-2-CdR for 72 h. The gene expression levels of *DNMT1* in HCT116 cells before and after 5-aza-2-CdR treatment are shown in the bar chart. As DMSO was used in the preparation of the 5-aza-2-CdR treatment solution, it was also used to treat the HCT116 cells in the control group. The *DNMT1* expression levels were normalized to the mRNA expression level of the *GAPDH* housekeeping gene. The error bars represent the standard error of the mean (SEM) for three independent qRT-PCR experiments for each examined gene. Result with $^{**}P \leq 0.01$ was considered statistically significant.

TSA than in those treated with 5-aza-2′-CdR. These results demonstrate that the inhibition of histone deacetylation is critical for controlling the expression of the genes investigated in this study. Our findings suggest that many mechanisms are involved in the regulation of these genes. Several CT genes have been identified as crucial for tumor development. Therefore, inactivating the expressions of these genes may be beneficial for lowering the proliferation-mediated burden of malignancies and maximizing the efficacy of other therapeutic modalities.

Lastly, owing to the selection of biopsy samples from individuals diagnosed with CC, the present study has several limitations. First, the insufficient clinical data and small tissue sample size prevented this investigation from using different experimental approaches to verify the reported results. In the present investigation, some specimens were obtained via biopsy; however, postsurgical specimens are deemed more reliable. Second, the present study examined the relationships between *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* gene expressions and the development of CC. However, future investigations are required to examine the relationships between the protein levels of these genes in adjacent NC and CC tissues. Third, the translational levels of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* gene expressions should be assessed in vitro studies. Fourth, more research is needed to determine whether 5-aza-2′-CdR treatment can also be used to decrease the expression levels of other DNMT gene types such as *DNMT3A* and *DNMT3B*. The present study was merely a retrospective investigation conducted in Saudi Arabia with a relatively small CC sample size. Therefore, future research should include assessments of various populations and larger samples.

5. Conclusions

The study focused on analyzing the expression patterns of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* genes within tissue samples obtained from patients diagnosed with CC and their corresponding adjacent NCs. The findings revealed significantly elevated gene expression levels in the CC tissue samples compared with the adjacent NC tissue samples. This suggests the potential utility of these genes as cancer-specific biomarkers for detecting CC at an early stage. However, additional investigations of their protein levels are needed to validate this finding. In addition, this work demonstrates that preventing methylation and histone deacetylation in CC cells can stimulate the expressions of the *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, and *ADAM29* genes in vitro. However, the *CABS1* gene expression was upregulated by TSA treatment. This study found that 5-aza-2-CdR treatment suppressed *DNMT1* expression, making it the primary regulator of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, and *ADAM29* gene expressions. Moreover, the transcriptions of *FTHL17*, *PRM2*, *CABYR*, *CPXCR1*, *ADAM29*, and *CABS1* were most strongly stimulated after the suppression of histone deacetylation with TSA treatment. Future cancer immunotherapies may include this epigenetic modulator owing to its important involvement in the transcriptional activation of the tested genes. More studies are needed to determine the outcomes of 5-aza-2-CdR therapy when administered at higher concentrations over extended durations or in combination with a TSA agent.

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Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the ethics committee of King Abdullah International Medical Research Center (IRB/1201/22, study No. SP22R/076/04, approved 27 June 2022).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

The data are available from the corresponding author when required

CRediT authorship contribution statement

Turki M. Alrubie: Investigation, Methodology, Software, Writing – original draft. **Jilani P. Shaik:** Formal analysis. **Abdullah M. Alamri:** Validation. **Mohammad Alanazi:** Formal analysis. **Alaa T. Alshareeda:** Data curation, Resources. **Ayyob alqarni:** Data curation, Resources. **Homoud G. Alawfi:** Data curation, Resources. **Sarah M. Almaiman:** Investigation. **Mikhlid H. Almutairi:** Funding acquisition, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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