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Research Article

Effects of capecitabine chemotherapy on the expression of CASC18, CASC19, and CASC20 lncRNAs in patients with colorectal cancer and the HCT-116 colorectal cancer cell line

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Abstract

Background: Colorectal cancer (CRC) is the third leading cause of cancer-related deaths. Capecitabine is a key chemotherapy drug for colorectal cancer (CRC). Long non-coding RNAs (lncRNAs) play a role in the pathogenic pathways associated with colorectal cancer. This investigation compared the expression patterns of CASC18, CASC19, and CASC20 in CRC patients before and after chemotherapy, as well as in healthy individuals. The effects of capecitabine on cell viability, apoptosis, cell cycle, and the expressions of CASC18, CASC19, and CASC20 were examined using HCT-116 cells.

Methods: CASC18, CASC19, and CASC20 expressions were assessed using qRT-PCR on patients' and healthy tissues. Furthermore, the receiver operating characteristic (ROC) curve analysis was used to evaluate the prognostic and diagnostic value of CASC18, CASC19, and CASC20 in CRC. The MTT test was used to assess capecitabine's cytotoxicity on the HCT-116 CRC cell line. Annexin-V/PI staining was used to examine apoptosis and cell cycle progression by flow cytometry. Gene expression differences between before and after capecitabine treatments were examined by qRT-PCR.

Results: Results showed that patients with colorectal cancer had lower levels of CASC18 but higher levels of CASC19 and CASC20 expression than the healthy group. Moreover, patients treated with capecitabine showed an increased CASC18 expression, while non-significant changes were observed in the expression of CASC19 and CASC20. The clinical factors were not associated with the expression of lncRNAs CASC18, CASC19, and CASC20. Furthermore, results indicated that CASC18, CASC19, and CASC20 are poor biomarkers for diagnosis of CRC. MTT analysis revealed a dose- and time-dependent cytotoxic effect for capecitabine on the HCT-116 cells. Capecitabine treatment led to the accumulation of the HCT-116 cells in the Sub-G1 phase, suggesting an apoptotic solid impact on the cells. Moreover, HCT-116 cells were arrested in the G2/M phases by capecitabine. Capecitabine also upregulated CASC18 expression in the HCT-116 cell line.

Conclusion: These results support the anticancer and cytotoxic effects of capecitabine and highlight its ability to increase CASC18 expression. Additionally, the findings indicate that CASC18, CASC19, and CASC20 are poor biomarkers for colorectal cancer (CRC) diagnosis.

Keywords

Colorectal cancer, CASC18, CASC19, CASC20, capecitabine, apoptosis, cell cycle

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Introduction

Around the world, colorectal cancer (CRC) is a major reason for death, accounting for 700,000 fatalities annually (Fitzmaurice et al. 2017; Moqadami et al. 2023). The primary cause of CRC death is post-operative metastasis and recurrence (Yang et al. 2020). At the time of first diagnosis, 20% of patients had metastasis, and nearly half of patients with primary disease may acquire metastatic disease (Silva-Fisher et al. 2020). Furthermore, patients with metastatic CRC had a five-year survival rate of only 14% (Siegel et al. 2017). There are currently several therapeutic options for patients with CRC, including radiation, surgery, immunotherapy, targeted therapy, cytotoxic chemotherapy, and combination techniques (Karimpur Zahmatkesh et al. 2023). The FDA approved capecitabine (Xeloda) as a commonly used oral chemotherapy drug for the treatment of multiple cancers, including colorectal cancer, breast cancer, pancreatic cancer, gastric cancer, and tumors known to be resistant to 5-fluorouracil (5-FU) (Xie et al. 2020; Ge et al. 2023). Although 5-FU-based chemotherapy is the primary treatment for CRC, it has drawbacks (Kadhum Kharmeet et al. 2024). Capecitabine is a prodrug form of 5-FU, which was created to solve the drawbacks of 5-FU, including systemic toxicity, lack of efficacy and selectivity, and resistance development (Alzahrani et al. 2023). Capecitabine is a fluoropyrimidine that is metabolized to 5-FU in cancer cells. It is converted into 5’-deoxy-5-fluorocytidine (5’DFCR), 5’-deoxy-5-fluorouridine (5’DFUR), and 5-FU by carboxylesterases (CES1 and 2), cytidine deaminase (CDD), and thymidine phosphorylase (TP), in both liver and tumor (Guichard et al. 2008; Zehra et al. 2024).

However, insufficient pre-treatment data exist to predict treatment response and resistance development (Silva-Fisher et al. 2020; Partin et al. 2023). While there are hopeful improvements in second-line treatment options for CRC patients employing cytotoxic or targeted medicines, the processes underlying metastatic progression are still poorly understood, preventing successful medication development (Garajová et al. 2017; Grothey et al. 2019). These statistics and inadequate treatment options underline the crucial need for new biomarker-driven medicines at diagnosis.

Prior studies have demonstrated that over 90% of DNA sequences are actively transcribed, and most of them are classified as non-coding RNAs, including long non-coding RNAs (lncRNAs) and microRNAs (Gu et al. 2020; Ghasemi et al. 2021b). LncRNAs are longer than 200 nucleotides and do not contain protein-coding sequences (Gu et al. 2020; Ahmadi et al. 2024). LncRNAs control gene expression at several levels and play an essential role in various biological processes (Kopp and Mendell 2018; Ostovarpour et al. 2021). It has been reported that mammalian cells exhibit aberrant expression of lncRNAs (Feng et al. 2020; Khajehdehi et al. 2023). Growing research suggests that lncRNAs may serve as potential biomarkers for the diagnosis and prognosis of various malignancies (Sha et al. 2018; Xu et al. 2020; Ghasemi et al. 2021a; Khajehdehi et al. 2022).

Recently, the human genome’s cancer susceptibility candidate 18 (CASC18) gene has been described as an lncRNA. Recent cancer research found that CASC18 knockdown blocked the epithelial-mesenchymal transition, which could be partially restored by inhibiting miR-20a-3p (Zhou et al. 2021). The CASC18 gene and the nearby genes NUAK1, APPL2, and OCC-1 are located in the 12q23.3 region, which has been identified as a potential cancer predisposition locus (Mehravar et al. 2016).

Chromosome 8q24.21 encodes cancer susceptibility candidate 19 (CASC19), a recently found lncRNA. CASC19 is significantly increased in malignancies of humans, including non-small cell lung carcinoma, cervical cancer, colorectal cancer, pancreatic cancer, gastric cancer, renal cell carcinoma, glioma, and nasopharyngeal carcinoma. Furthermore, clinicopathological markers and cancer progression were strongly associated with CASC19 dysregulation. CASC19 regulates a variety of cell phenotypes, including proliferation, epithelial-mesenchymal transition, migration, apoptosis, autophagy, cell cycle, invasion, and treatment resistance (Wang et al. 2023). CASC20 is a 1484-bp-long lncRNA identified on chromosome 20p12.3. Studies have proven that knocking down CASC20 reduces cancer cell proliferation, migration, and invasion. As demonstrated (Shan et al. 2022), CASC20 may also be an oncogene in some cancers.

Despite many reports on the dysregulation of lncRNAs CASC18, CASC19, and CASC20 in cancer, the impact of capecitabine treatment on the expression profile of these lncRNAs has not been considered yet. This study evaluated the expression profiles of the genes CASC18, CASC19, and CASC20 in colorectal cancer patients before and after chemotherapy and healthy controls. Further, the effects of capecitabine on apoptosis, cell cycle, and CASC18, CASC19, and CASC20 expression were investigated using HCT-116 human CRC cells.

Materials and methods

Tissue specimens

Ninety-eight tumor tissues from CRC patients before and after chemotherapy and 71 tissue samples from healthy individuals referring to Imam Reza Hospital in Tabriz, Iran, from 2021 to 2023 were included in this cohort study. Patients whose pathological reports were positive for CRC and who were diagnosed as CRC patients by the specialist were included in the case group. Patients who had a previous history of any cancer type, were diagnosed with familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer, or had already received any chemotherapy or radiotherapy were excluded from the patient group. Inclusion criteria for the control group were negative for any cancer types having any colon or intestine-related disorders. To avoid bias and confounding factors, the sex and age of the patients and controls were matched. Furthermore, during the capecitabine therapy, treatments with alternative therapeutics were avoided.

After being snap-frozen in liquid nitrogen, the samples were placed into RNase-free microtubes and preserved at -80 °C. Written informed consent was given by the participants. A qualified pathologist evaluated and described sample histopathologic features. The University of Tabriz's ethical committee approved this study (IR.Tabrizu.rec1403.113).

RNA extraction, cDNA synthesis, qRT-PCR

Trizol reagent was utilized to extract total RNA from tissues and cultured cells. Using the TaKaRa cDNA Synthesis Kit (Kusatsu, Japan), 500 ng of total RNA was reverse transcribed to produce cDNA. The Quantitative Real-Time PCR (qRT-PCR) System (Roche Molecular Systems, Inc., Pleasanton, CA, USA) was used to evaluate the expression of CASC18, CASC19, and CASC20. The housekeeping gene β-actin was employed as an internal control to ensure data normalization. The following conditions were used for all genes examined: 94 °C for 10 min, 96 °C for 15 s, 60 °C for 40 s, 72 °C for 40 s, and a final 5 min of 72 °C. Forward primer and reverse primer amounts were 0.3 µL and 0.3 µL, respectively, cDNA 1 µL (10 ng), SYBR Green Master Mix 10 µL, and dH2O 8.4 µL in a final volume of 20 µL. Table 1 provides a summary of the primer pairs used in this investigation. The $2^{-\Delta\Delta Ct}$ method was utilized to investigate the fold change in the mRNA expression level.

Table 1. Sequences of the primers used in the present study. The primers were designed using Gene Runner, and their specificity was checked with the BLAST program.

Gene Name	Primer Sequence
CASC18	Forward: 5'- CAGAGACTGCCACAAGCTCA-3'
	Reverse: 5'- TCCGGGGCTCCACATACATA-3'
CASC19	Forward: 5'- CCTGGGTTAGAACCCTGCTG-3'
	Reverse: 5'- TGGACAGCACCTTGAATGCT-3'
CASC20	Forward: 5'- GTGCTTGGGAGACAGTGAGT-3'
	Reverse: 5'- GAGGAGGGGAGGGTGGTTAT-3'
β-actin	Forward: 5'- AGAGCTACGAGCTGCCTGAC -3'
	Reverse: 5'- AGCACTGTCTTGGCGTACAG-3'

Cell culture

The HCT-116 cell line was purchased from the Pasteur Institute (Tehran, Iran). It was derived from human colon cancer, is known for its mutation in codon 13 of the Ras proto-oncogene, and is commonly used for studying colon cancer biology. In vitro studies have demonstrated its invasiveness and high motility characteristics (Rajput et al. 2008). HCT-116 cells were thawed from a stock vial containing 1 × 10^6 cells in a 37 °C water bath. The thawed cells were suspended in 5 ml of RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cell suspension was centrifuged at 1500 rpm for 5 minutes, and the supernatant was discarded. The cell pellet was resuspended in 5 ml of the same growth medium and transferred to a T25 flask. The cells were incubated in a 37 °C incubator (Memmert, Germany) with 5% CO_2. Cultured cells were checked under an inverted microscope until they achieved about 80% confluency (typically in 2–3 days). Then, the cells were briefly rinsed with phosphate buffer saline (PBS) and treated with 1 ml of 0.25% (w/v) trypsin + 0.53 mM EDTA solution to detach the cells (about 5 minutes). Afterward, 5 ml of complete growth medium was added to the flask, and the cells were collected by gentle pipetting. The cells were counted, and an appropriate number of the cells were used for each of the subsequent experiments. For long-term storage, cells were frozen in liquid nitrogen (1 × 10^6 cells/ml in FBS containing 10% DMSO).

Cell viability assay

The thiazolyl blue tetrazolium (MTT) test was applied to assess drug cellular viability and toxicity. Human HCT-116 cells were cultured in 96-well plates at a density of 1 × 10^4 cells per well. For 24, 48, and 72 hours at 37 °C, capecitabine (Xeloda) (Merck, Darmstadt, Germany) was administered at various concentrations (0-1000 µM). Each well contained a 100 µL aliquot of MTT 0.5%, which was incubated for three hours at 37 °C. After removing the medium, 150 µL of DMSO was added, and the plate was shaken for 20 minutes to dissolve the formazan crystals that formed inside the cells. Finally, absorbance at 570 nm was measured using an ELx 800 BioTek microplate reader (San Francisco, CA, USA). The logarithmic concentration-response curve was fitted using the GraphPad Prism 9 software, and the IC50 value was determined.

Assessing apoptosis

The method employed to detect cellular apoptosis was Annexin V-FITC/PI. 0.5 × 10^6 HCT-116 cells were seeded into each well of a 6-well plate. The cells were then incubated in a CO_2 incubator at 37 °C overnight. The cells were treated with capecitabine at 0 and 60.18 (IC50) µM concentrations. After two days, the cells were removed and washed with 500 µL of cold PBS. The cells were centrifuged for 5 minutes at 1300 rpm, then reconstituted in 500 µL of binding buffer and stained with 1 µL FITC Annexin V and 1 µL PI. The cells were kept at room temperature for fifteen minutes without exposure to light. After incubation, the samples were evaluated with a BD FACSCalibur cytometer (Becton Dickinson, USA). FlowJo v10.8.1 was used to express the results as a percentage of apoptotic cells.

Analysis of the cell cycle

After seeding 0.5 × 10^6 HCT-116 cells into each well of a 6-well plate, the plates were incubated with CO_2 at 37 °C overnight. For 48 hours, cells were exposed to 0 (control group) and 60.18 µM (IC50) dosages of capecitabine. After carefully rinsing the cells twice with PBS, they were collected and fixed for a whole night at 4 °C in cooled 70% ethanol. Following a PBS wash, the cells were stained for 45 minutes at 37 °C using a PBS solution containing 50 µg/mL of PI and 20 µg/mL of RNase A. A Becton Dickinson

(USA) BD FACSCalibur cytometer was used to analyze the samples. Using FlowJo v10.8.1, the arrangement of cells across the cell cycle phases was assessed.

Analytical statistics

GraphPad Prism version 9.0 and SPSS version 26 were employed to analyze data. The two groups were compared using a two-tailed t-test, and the mean ± SD was reported as the outcome. A one-way ANOVA test was used to analyze and compare three or more groups. To assess the flow cytometry test findings, FlowJo v10.8 was used. ROC curve analysis was done for the biomarker potency of the lncRNAs. P value < 0.05 was considered significant. Experiments were conducted in triplicate to ensure the reliability and reproducibility of the results. A data layout was created, and unbalanced data that could interfere with statistical analysis was removed.

Results

Patients' information

In this investigation, samples from 98 CRC patients with a mean age of 57 and 71 healthy controls with a mean age of 60 were included. Samplings from the patients were done before and after capecitabine treatment. Table 2 displays the demographic information for the participants. The age and sex factors did not differ statistically between CRC patients and the healthy group.

Table 2. The CASC18, CASC19, and CASC20 expression means and demographic information for the healthy group and patients before and after chemotherapy.

Variable	No.	mean (SD)			mean (SD)			mean (SD)			P Value			P Value		
		Before treatment			After treatment			Healthy group			(before treatment/after treatment)			(before treatment/control)		
		CASC 18	CASC 19	CASC 20	CASC 18	CASC 19	CASC 20	CASC 18	CASC 19	CASC 20	CASC 18	CASC 19	CASC 20	CASC 18	CASC 19	CASC 20
Age																
57≥	59	0.03 ± 0.03	0.05 ± 0.06	0.08 ± 0.08	0.03 ± 0.03	0.05 ± 0.05	0.07 ± 0.08	0.07 ± 0.01	0.04 ± 0.05	0.06 ± 0.10	0.09	0.53	0.89	0.48	0.34	0.35
57<	39	0.04 ± 0.07	0.05 ± 0.06	0.08 ± 0.09	0.04 ± 0.03	0.04 ± 0.04	0.06 ± 0.06	0.04 ± 0.05	0.03 ± 0.04	0.04 ± 0.05						
Sex																
Male	55	0.03 ± 0.06	0.06 ± 0.08	0.08 ± 0.9	0.03 ± 0.02	0.05 ± 0.05	0.06 ± 0.07	0.04 ± 0.05	0.03 ± 0.04	0.04 ± 0.05	0.55	0.30	0.35	0.23	0.75	0.55
Female	43	0.03 ± 0.04	0.05 ± 0.08	0.07 ± 0.06	0.04 ± 0.03	0.04 ± 0.04	0.07 ± 0.08	0.07 ± 0.08	0.04 ± 0.05	0.07 ± 0.11						
Tumor Site																
Rectum	50	0.04 ± 0.06	0.06 ± 0.08	0.07 ± 0.06	0.04 ± 0.03	0.05 ± 0.04	0.06 ± 0.08		_		0.40	0.39	0.69			
Colon	48	0.03 ± 0.03	0.05 ± 0.08	0.09 ± 0.09	0.03 ± 0.03	0.05 ± 0.06	0.07 ± 0.06		_							
Tumor histology																
Adenocarcinoma	61	0.02 ± 0.02	0.04 ± 0.07	0.07 ± 0.08	0.04 ± 0.03	0.05 ± 0.05	0.07 ± 0.08		_		0.05	0.48	0.77			
Mucinous adenocarcinoma	37	0.05 ± 0.07	0.06 ± 0.08	0.08 ± 0.08	0.03 ± 0.03	0.04 ± 0.04	0.05 ± 0.05		_							
Tumor differentiation																
Well/moderate	55	0.04 ± 0.06	0.06 ± 0.08	0.07 ± 0.07	0.04 ± 0.03	0.05 ± 0.05	0.07 ± 0.07		_		0.14	0.19	0.82			
Poor	43	0.03 ± 0.03	0.05 ± 0.08	0.08 ± 0.09	0.04 ± 0.03	0.04 ± 0.05	0.06 ± 0.07		_							
TNM																
I, II	52	0.02 ± 0.02	0.07 ± 0.09	0.07 ± 0.07	0.04 ± 0.03	0.04 ± 0.05	0.06 ± 0.07		_		0.80	0.62	0.46			
III, IV	46	0.04 ± 0.07	0.04 ± 0.05	0.08 ± 0.09	0.03 ± 0.03	0.05 ± 0.05	0.07 ± 0.07		_							
Lymph metastasis																
YES	40	0.04 ± 0.07	0.04 ± 0.05	0.08 ± 0.06	0.03 ± 0.03	0.04 ± 0.05	0.07 ± 0.09		_		0.16	0.76	0.80			
NO	58	0.03 ± 0.03	0.06 ± 0.09	0.08 ± 0.09	0.04 ± 0.03	0.05 ± 0.05	0.06 ± 0.06		_							
HPV infection																
YES	42	0.04 ± 0.07	0.04 ± 0.08	0.06 ± 0.06	0.04 ± 0.03	0.04 ± 0.04	0.07 ± 0.08		_		0.54	0.71	0.93			
NO	56	0.03 ± 0.04	0.06 ± 0.07	0.09 ± 0.09	0.04 ± 0.03	0.05 ± 0.05	0.06 ± 0.05		_							
Treatment responses																
Partial responses	51	0.03 ± 0.03	0.07 ± 0.10	0.06 ± 0.08	0.03 ± 0.02	0.05 ± 0.04	0.07 ± 0.07		_		0.64	0.08	0.47			
Complete responses	15	0.04 ± 0.05	0.0400 ± 0.04	0.09 ± 0.07	0.04 ± 0.04	0.05 ± 0.06	0.07 ± 0.07		_							
No change	32	0.03 ± 0.07	0.03 ± 0.02	0.10 ± 0.09	0.04 ± 0.04	0.05 ± 0.05	0.06 ± 0.07		_							

Expression levels of CASC18, CASC19, and CASC20 in patients and controls

The qRT-PCR results revealed that CRC patients before treatment with capecitabine had significant downregulation of CASC18 expression (0.036 ± 0.053) compared to the control group (0.057 ± 0.067, P value = 0.031). After treatment, the expression of CASC18 was significantly increased (0.039 ± 0.033, P value = 0.027), while it was lower than that of healthy controls (Fig. 1A). CASC19 expression was significantly upregulated in CRC patients before treatment (0.055 ± 0.078) compared to the control group (0.035 ± 0.049, P value = 0.006). However, there was no significant variation in CASC19 expression after and before treatments (P value = 0.740) (Fig. 1B). Moreover, CASC20 expression was considerably higher in CRC patients (0.080 ± 0.083) than in the control group (0.057 ± 0.082, P value = 0.015); however, no substantial change was seen in the expression of CASC20 before and after treatments (0.067 ± 0.073, P value = 0.282) (Fig. 1C). These findings demonstrated that capecitabine-based chemotherapy can alter CASC18 expression in CRC patients but not CASC19 or CASC20 expressions.

Relationship between clinical factors and the expression of lncRNAs CASC18, CASC19, and CASC20

There were no statistically significant differences in age, sex, tumor site, tumor histology, tumor differentiation, TNM classification, lymph node metastasis, HPV infection, or treatment responses between the CRC patients before and after capecitabine chemotherapy (Table 2).

Biomarker potency of CASC18, CASC19, and CASC20

The diagnostic and prognostic values of CASC18, CASC19, and CASC20 in colorectal cancer patients before and after chemotherapy and healthy controls were evaluated using receiver operating characteristic (ROC) curve analysis.

A comparison of the CASC18 expression in patients before chemotherapy and the healthy group showed an AUC of 0.597 (95% confidence interval (CI) 0.510–0.683; sensitivity = 59%, specificity = 50%), and before and after chemotherapy had an AUC of 0.588 (95% confidence interval (CI) 0.508–0.668; sensitivity = 62%, specificity = 53%). A comparison of the CASC19 expression in patients before chemotherapy

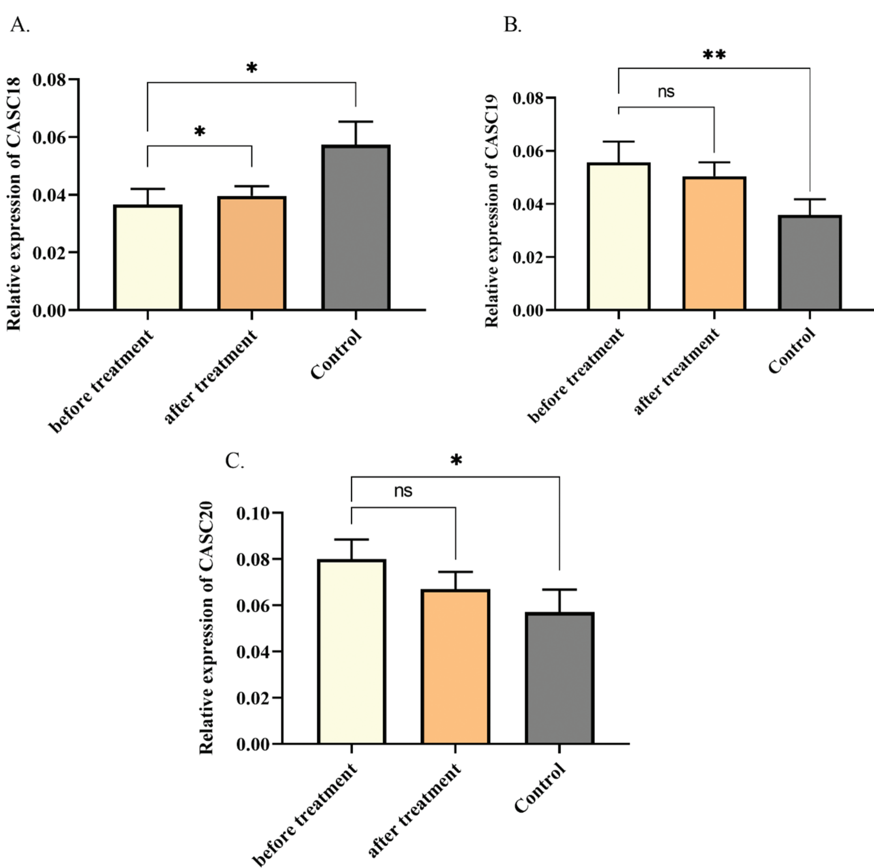

Figure 1. CASC18, CASC19, and CASC20 expression levels in CRC patients and healthy groups. **A.** CASC18 expression is significantly lower before treatment in CRC patients compared to the healthy controls with a P-value of 0.031 (*), and its expression is higher after treatment in CRC patients than before treatment with a significant difference (P-value = 0.027); **B.** CASC19 expression is considerably higher in CRC patients with a P value = 0.006 (**), but there are no significant differences in the expression of CASC19 between before and after treatment with a P value = 0.740; **C.** CASC20 expression is considerably higher in CRC patients with a P value = 0.015 (*), but there are no significant differences in the expression of CASC20 between before and after treatment with a P value = 0.282.

and the healthy group showed an AUC of 0.623 (95% confidence interval (CI) 0.536–0.710; sensitivity = 63%, specificity = 59%), and before and after chemotherapy had an AUC of 0.524 (95% confidence interval (CI) 0.442–0.606; sensitivity = 65%, specificity = 46%). A comparison of the CASC20 expression in patients and the healthy group showed an AUC of 0.609 (95% confidence interval (CI) 0.524–0.694; sensitivity = 65%, specificity = 52%), and before and after chemotherapy had an AUC of 0.550 (95% confidence interval (CI) 0.469–0.631; sensitivity = 63%, specificity = 48%). These results showed that CASC18, CASC19, and CASC20 are poor diagnostic biomarkers (Fig. 2).

Cytotoxic effect of capecitabine on the human HCT-116 colorectal cancer cells

The viability of the cells was investigated to determine the toxicity of capecitabine in HCT-116 cells. Cells were treated for 24, 48, and 72 hours at different drug doses. Capecitabine decreased the viability of HCT-116 cells in all 24-, 48-, and 72-hour treatment groups, as shown in Fig. 3. The IC50 values were 112.6 μM, 60.18 μM, and 50.34 μM for the 24 h, 48 h, and 72 h time points, respectively (Fig. 3). As a result, we treated the cells in the following tests with 60.18 μM for 48 hours.

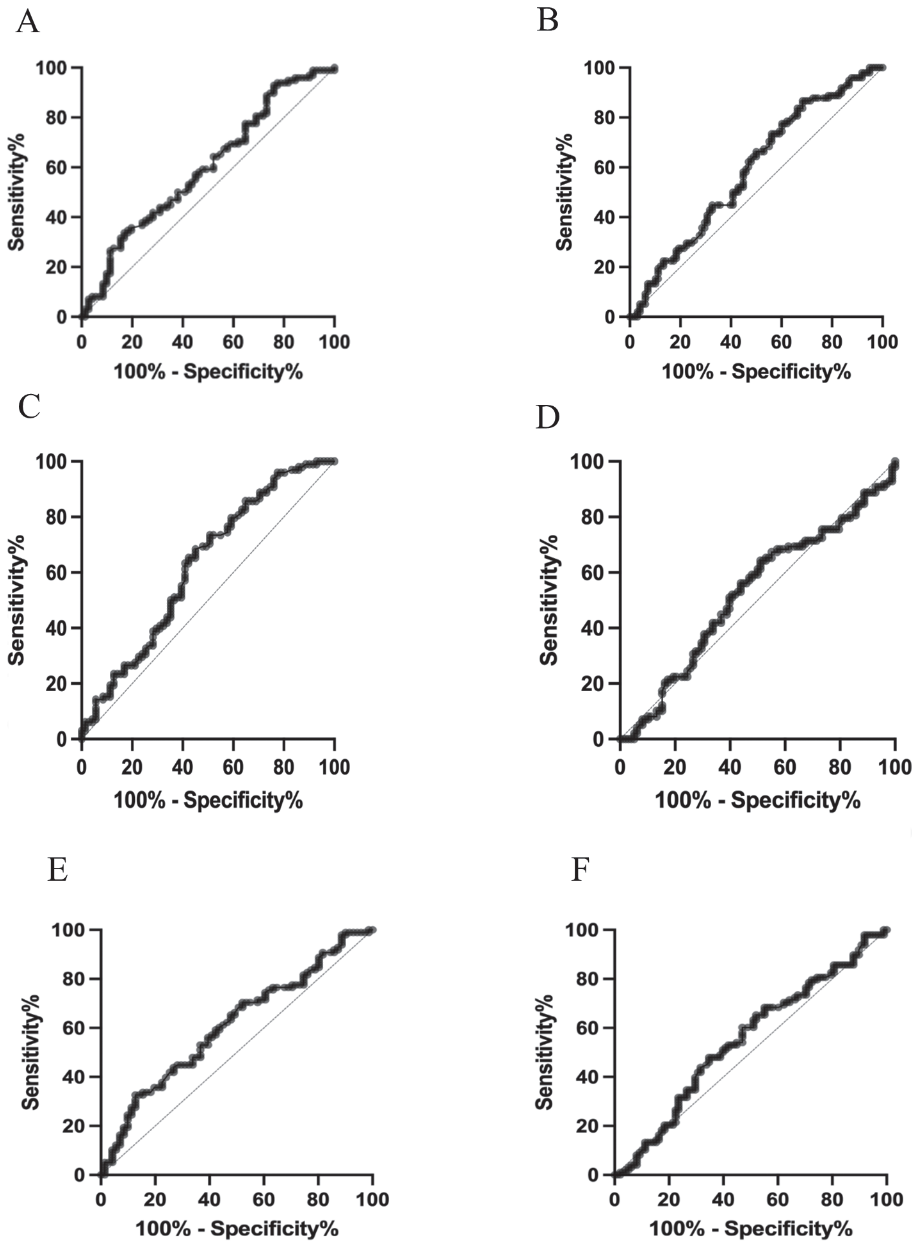

Figure 2. ROC curve analysis of **A.** CASC18 compared to before chemotherapy in CRC patients and the healthy group (AUc = 0.597 and P value = 0.031); **B.** CASC18 compared to before and after chemotherapy in CRC patients (AUc = 0.588 and P value = 0.032); **C.** CASC19 compared to before chemotherapy in CRC patients and the healthy group (AUc = 0.623 and P value = 0.006); **D.** CASC19 compared to before and after chemotherapy in CRC patients (AUc = 0.524 and P value = 0.555); **E.** CASC20 expression was compared between before chemotherapy and the healthy group (AUc = 0.609 and P value = 0.609) and **F.** CASC20 expression was compared in the patients before and after chemotherapy (AUc = 0.550 and P value = 0.224).

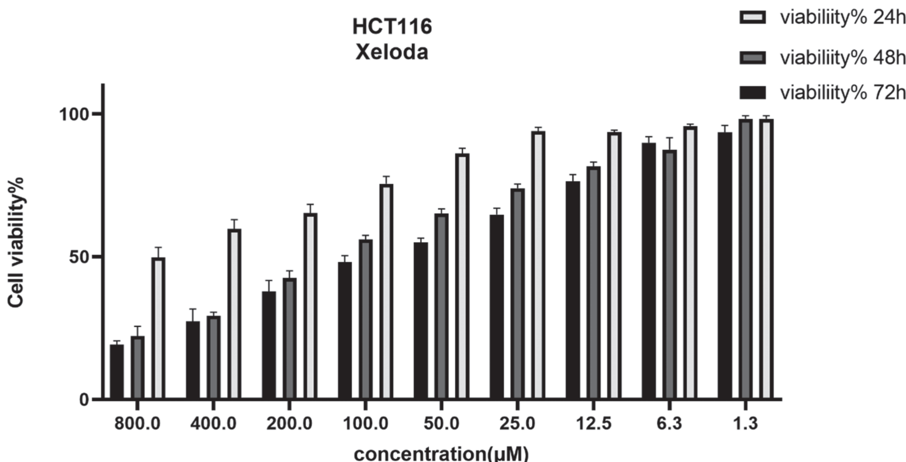

Figure 3. Assay for Viability Test: HCT-116 cells were treated with varying concentrations of capecitabine (0–800 μM) for 24, 48, and 72 hours, and their viabilities were evaluated. The results demonstrated concentration-dependent cytotoxic effects on the cells for each of the three time points.

Capecitabine effectively induced apoptosis in HCT-116 cells

Annexin V-FITC and PI staining validated capecitabine-induced apoptosis in HCT-116 cells. Apoptosis was detected by flow cytometry and compared between the capecitabine-treated and non-treated cells (Fig. 4). The findings showed a high percentage of apoptosis in the treated group in contrast to the untreated group (P value = 0.001). The apoptotic cell percent in the treated group was 72.46 ± 2.23.

Effect of capecitabine on the cell cycle progression of HCT-116 cells

The impact of capecitabine on the HCT-116 cell cycle progression was evaluated using flow cytometry. Capecitabine resulted in the accumulation of HCT-116 cells in the Sub-G1 phase (P value = 0.000), as Fig. 5 indicates. Capecitabine at 60.18 μM increased the Sub-G1 cell population by 27.68 ± 1.43% compared to the control. Thus, there was a significant decrease in the G2-M phase cell population by 4.44 ± 1.5% (P value = 0.002) but not in the G1 phase or S phase cell population by 61.73 ± 1.26% and 4.7 ± 2.03%, respectively (P values = 0.109 and 0.140, respectively) in the treated groups. These findings indicated that capecitabine substantially increased the induction of apoptosis in HCT-116 cells (Fig. 5).

Effects of capecitabine on the expression of CASC18, CASC19, and CASC20 in HCT-116 cells

QRT-PCR was used to measure CASC18, CASC19, and CASC20 expression levels in HCT-116 cells. Our findings demonstrated that the expression of CASC18 was significantly upregulated in contrast to the before treatment (P value = 0.03), while the expressions of CASC19 and CASC20 were downregulated compared to the before treatment, but the differences were not statistically significant (P value = 0.225 and P value = 0.244, respectively) (Fig. 6).

Discussion

Developing a more successful treatment for CRC patients may be obtained by a deeper understanding of the relationship between the tumor microenvironment and CRC cells. Treatments for CRC have not produced the most significant response rate or the fewest adverse effects for patients despite tremendous progress in our knowledge of the biology of CRC (Golshani and Zhang 2020). LncRNAs are essential in many human cancers, including CRC (Huang et al. 2017; Zeng et al. 2017; Lai et al. 2018). Disordered lncRNA expression is a common characteristic of human cancers (Haemmig et al. 2017; Jahangiri et al. 2023). Understanding the functions of critical lncRNAs is essential for diagnosing, prognosing, and treating many cancer types. Since research into lncRNAs as potential predictive biomarkers is still in its early stages, more lncRNAs should be examined, as only a few have been found in CRC. On the other hand, capecitabine treatment led to an increase in sub-G1 cells, highlighting a significant apoptotic effect on colorectal HT-29 cells. These results confirmed the anticancer properties of capecitabine (Alkharsan et al. 2024).

In the current study, we examined the expression of the lncRNAs CASC18, CASC19, and CASC20 in the tissues of CRC patients and healthy groups before and after treatment with capecitabine. Additionally, we investigated the effects of the capecitabine treatment on cell viability and apoptosis effects following the expression of these lncRNAs in HCT-116 colorectal cancer cells. In the tissue samples, we demonstrated that CASC18 expression was downregulated in CRC patients compared to the healthy group. Still, it was increased in the patients in response to the capecitabine treatment. We also demonstrated capecitabine's apoptotic effects through cell cycle and apoptosis assays, showing that the expression of CASC18 was upregulated in HCT-

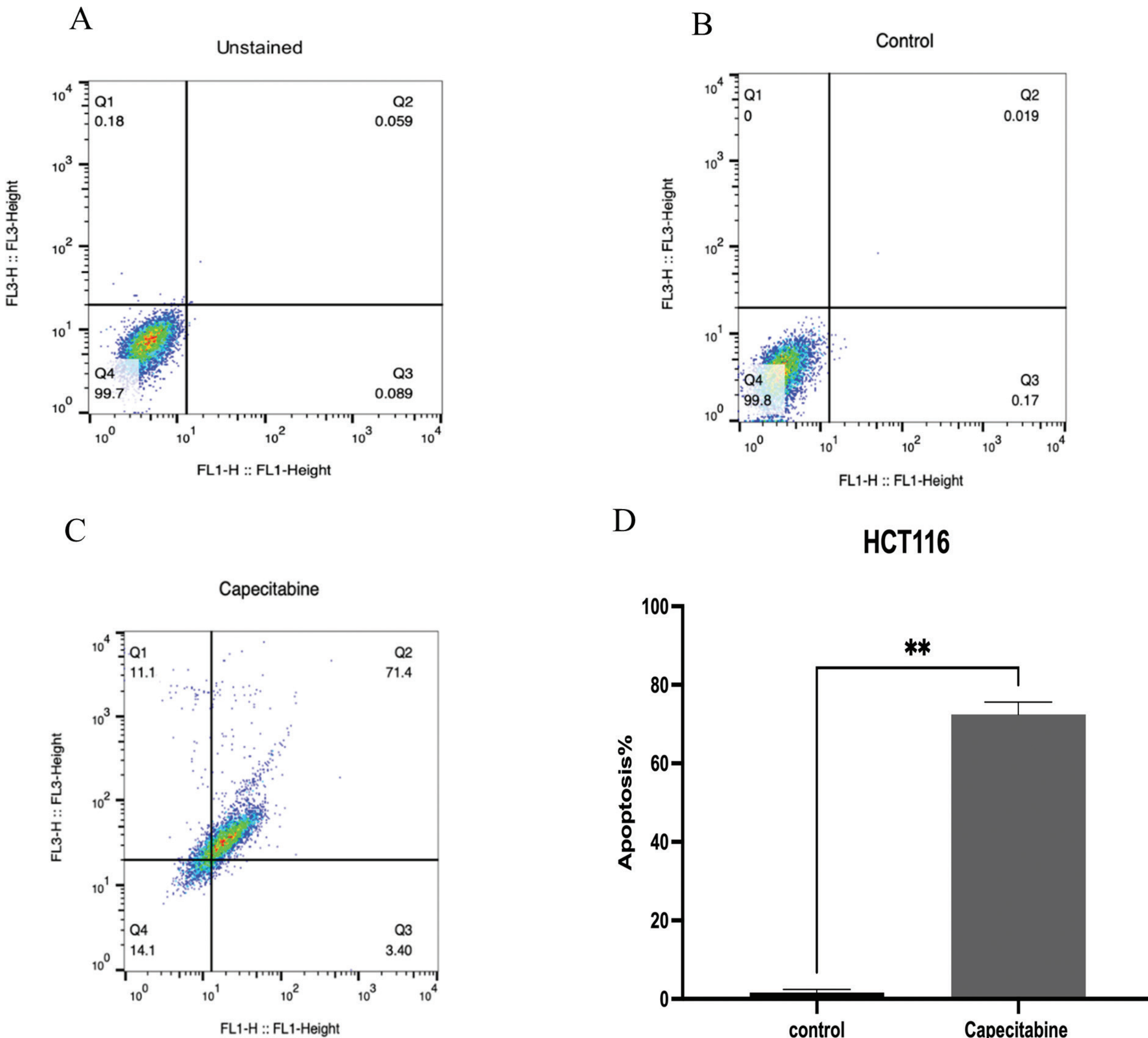

Figure 4. Apoptosis assay with flow cytometry: **A.** Unstained; **B.** 0, and **C.** 60.18 μM concentration of capecitabine for 48 hours was determined (%0.148, %0.189, and %74.8); **D.** This diagram showed that, compared to the control, the treatment with a 60.18 μM dose of capecitabine significantly caused apoptosis (P value = 0.001).

116 cells following capecitabine treatment. Also, compared to the healthy group, we showed that CASC19 expression was upregulated in CRC patients before treatment, but capecitabine treatment did not change its expression significantly. This finding was also approved by capecitabine treatment of the HCT-116 cells. Moreover, we showed that CASC20 expression was upregulated in CRC patients compared to the healthy group; however, the capecitabine treatment did not change its expression in the treated patients. Expression of CASC20 in HCT-116 cells also did not show a significant change in response to the capecitabine treatment, which was in line with the in vivo results. ROC curve analysis also demonstrated that CASC19 and CASC20 are poor diagnostic biomarkers in patients with CRC.

In a study, CASC18 was found to be upregulated in tumors from individuals with tongue squamous cell carcinoma (TSCC) who also had occult lymph node metastasis (OLNM). Importantly, they discovered that worse outcomes for TSCC patients and increased CASC18 expression were strongly linked with the existence of OLNM. Additionally, they showed that CASC18 knockdown prevented the epithelial-mesenchymal transition, which could be partially reversed by the miR-20a-3p inhibitor, repressing cell migration and invasion. They further established the molecular mechanism, showing that CASC18 was a ceRNA to scavenge miR-20a-3p and boost TGFB2 (transforming growth factor beta2) production and secretion (Zhou et al. 2021).

Wang et al. found that CASC19 expression was significantly upregulated in colorectal cancer metastases than in marginal tissues. Additionally, they showed that CRC's in vitro migratory ability was decreased by CASC19 expression knockdown (Wang et al. 2017). Wang et al. discovered an increase in CASC19 in clinical samples of gastric cancer. The rise in CASC19 in gastric cancer is related to lymph node metastases, poor overall survival, and TNM stage. Furthermore, compared to the human normal gastric GES-1, human gastric cancer cells (HGC-27, MGC-803, BGC-823, and AGS) had significantly higher expression of CASC19, according to a qRT-PCR analysis. Functionally, CASC19 knockdown inhibited the proliferation and migration of GC cells in vitro (Wang et al. 2019). CASC19 is noticeably overexpressed in non-small cell

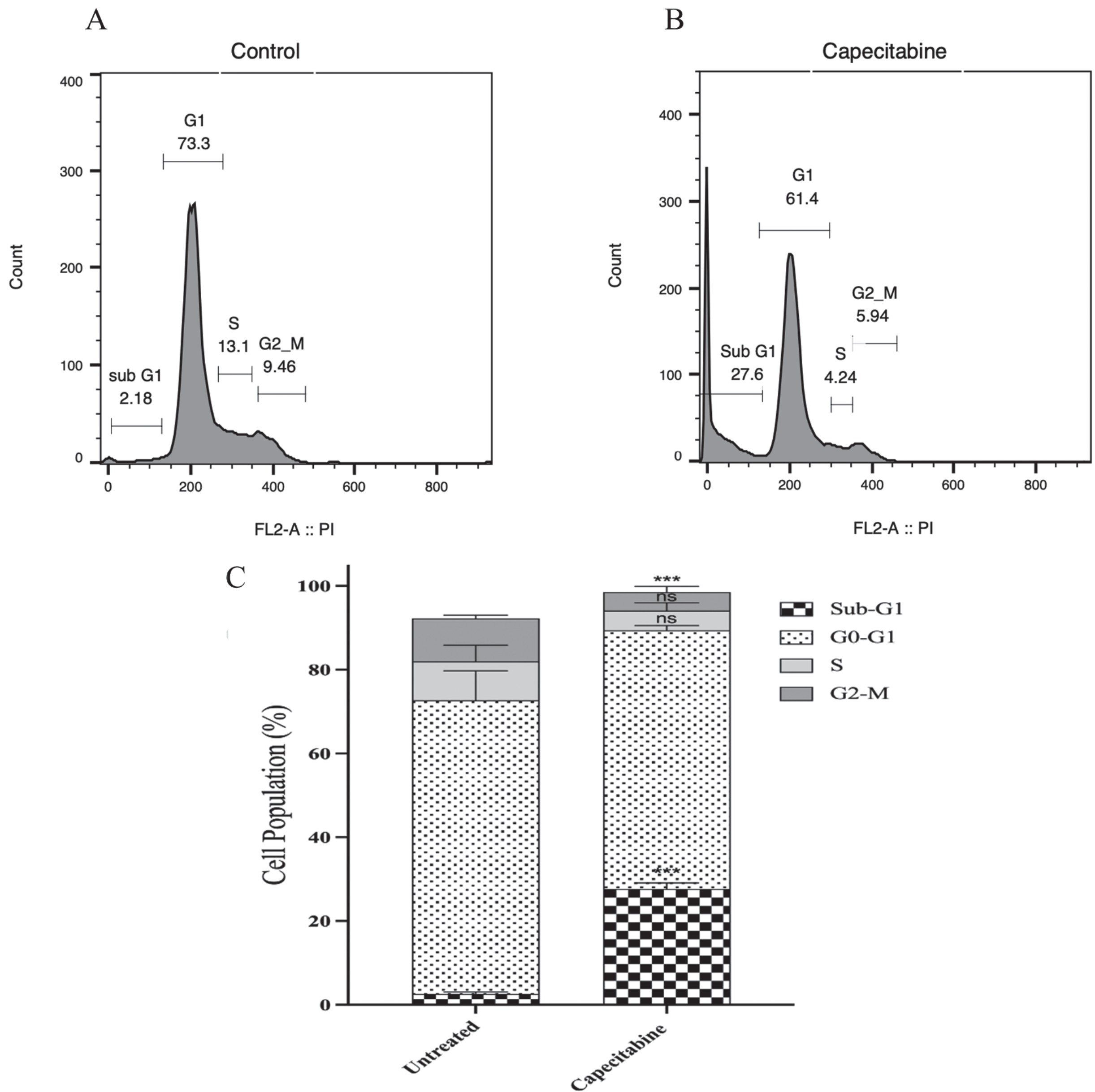

Figure 5. Cell cycle assay with flow cytometry: **A.** 0; **B.** 60.18 μM concentration of capecitabine for 48 hours was determined. Apoptosis was indicated by an increase in the Sub-G1 population in HCT-116 cells (27.68 ± 1.43%). Moreover, the G2/M phases of the cell cycle are arrested (4.44 ± 1.5%) in cells treated with 60.18 μM of the drug, but no substantial variations were shown in the G1 or S cell populations; **C.** The cell distribution across cell cycle phases is also shown in the bar graph.

lung cancer (NSCLC), and its lack inhibits metastasis and cell proliferation (Wang et al. 2020).

According to Shan et al., CASC20 expression was elevated in gastric cancer (GC) tumor tissues and several GC cell lines. In the GC patients, higher expression of CASC20 was associated with a poor prognosis and a significant likelihood of lymphatic metastasis. Silencing CASC20 in GC cells decreased migration and invasion, according to in vitro tests (Shan et al. 2022).

When taken orally, capecitabine functions as a prodrug of 5-FU that first transforms in the liver into active metabolites, which then release 5-FU in the gut to cause tumor suppression (Ge et al. 2023). Capecitabine may be an immunosuppressive drug with anti-cancer effects, as demonstrated by Zhang et al. They showed that capecitabine raised the number of anti-inflammatory cytokines in mice while selectively reducing some T cells and the concentration of associated pro-inflammatory cytokines. Moreover, they demonstrated that capecitabine can cause T-cell apoptosis both in vivo and in vitro (Zhang et al. 2021).

Based on our findings, capecitabine treatment effectively resulted in HCT-116 cells' apoptosis. We demonstrated that capecitabine caused HCT-116 cells to accumulate in the Sub-G1 phase, indicating that capecitabine significantly induced apoptosis in HCT-116 cells. Furthermore, we showed that 60.18 μM of capecitabine can arrest HCT-116 cells in the G2/M phases. Övey et al. discovered that the TRPV1 channels are directly responsible for the apoptotic effects of 5FU and capecitabine, commonly used to treat breast and colon cancer. These channels are crucial to all molecular pathways of apoptosis because they increase intracellular Ca^{2+} levels. It has been demonstrated that TRPV1 channels

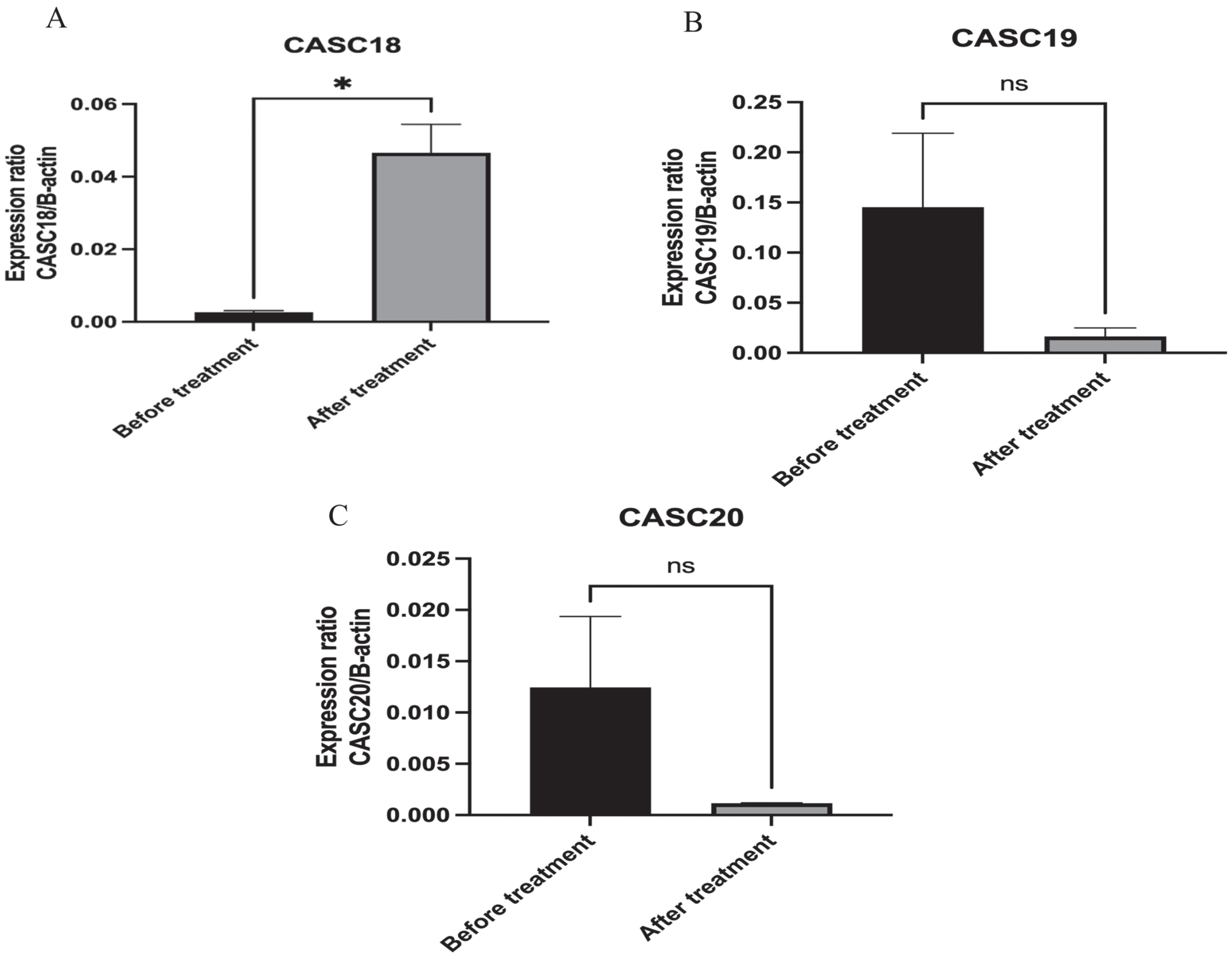

Figure 6. The qRT-PCR assay has shown that the HCT-116 cell treatment significantly increased CASC18 expression (P value = 0.03) compared to before treatment, but CASC19 and CASC20 expressions were not significantly different in the treated HCT-116 cells compared to before treatment (P value = 0.225 and P value = 0.244, respectively).

are essential for the growth and spread of tumors and for the oxidative stress and cell death that occur in tumor cells when they undergo chemotherapy (Suat Övey and Güler 2020).

Capecitabine has also been used in combination therapy protocols. According to research by Danesh et al., combinatorial treatment with capecitabine and pioglitazone hydrochloride-loaded triblock (TB) (PCL-PEG-PCL) nanoparticles offers an efficient method for targeted combinational colorectal cancer therapy. In CRC cell lines, they demonstrated a significant proportion of apoptotic cells in the TB nanoparticles loaded with capecitabine and pioglitazone hydrochloride (Pouya et al. 2023).

Conclusion

The study revealed that in colorectal cancer patients, the expression of CASC18 was lower than in healthy individuals. In contrast, expressions of CASC19 and CASC20 were elevated in the cancer patients. Interestingly, patients receiving treatment with capecitabine showed increased levels of CASC18 expression, which was approved by the treatment of the HCT-116 cells in vitro, highlighting the impact of this chemotherapy drug on tuning of the CASC18 gene expression. Additionally, capecitabine was shown to significantly induce apoptosis in these cells, leading to an accumulation in the Sub-G1 phase and causing a cell cycle arrest at the G2/M stages.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that experiments on humans or human tissues were performed for the present study.
Informed consent from the humans, donors or donors' representatives: University of Tabriz
The authors declared that no experiments on animals were performed for the present study.
Use of commercially available immortalised human and animal cell lines: Pasture Institute of Tehran.
The University of Tabriz's ethical committee approved this study (IR. Tabrizu.rec1403.113). All patients gave written informed consent. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (as revised in Edinburgh 2000), as reflected in its prior approval by the institution's human research committee

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Author contributions

NM wrote the main manuscript, and NM, MKK, and RS conceptualized it. MKK and MAHF wrote, reviewed, and edited it. RS, MN, and MKK performed formal analysis. MKK and MAHF did supervision and Visualization.

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Data availability

Availability of data and material: Data are available upon request.

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