The role of miRNA-4516 in regulating Bruton's tyrosine kinase expression and colorectal cancer progression in a sample of Iraqi population

Ahmed Sadoon Hassain,[1] Hiba Muneer Abdel Hassan Al-Khafaji,[2] Maryam Qasim Mohammed[3]

[1]College of Science, University of Misan, Maysan; [2]Department of Applied Sciences, Biotechnology branch, University of Technology, Baghdad; [3]Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

Abstract

Considering the second-highest global death rate, Colorectal Cancer (CRC) is the second most prevalent form of cancer in women and the third most frequent cancer type in men. Bruton's Tyrosine Kinase (BTK) is a soluble tyrosine kinase that plays essential functions in B cell maturation, development, and signaling. It has been discovered that BTK controls cell migration, survival, and proliferation in a variety of B-cell malignancies. The category of short non-coding RNAs known as microRNAs (miRNAs) is involved in several biological processes, including the development and propagation of tumors. The current study was designed to measure the gene expression level of the *BTK* gene and *miR-4516* in Iraqi CRC patients; 100 blood samples were collected, RNA extracted, converted into cDNA, and then expression levels were measured using quantitative real-time PCR. The results showed that there were statistically significant differences among the patients and the control with a P-value =0.005 in the expression level of *miR-4516*, while the results of the *BTK* gene showed that there were no significant differences between the CRC patients and control groups of the current study. This study reveals that non-detectable levels of *BTK* secretion may be attributed to *miR-4516* mediated suppression or due to *BTK* possessing a dual role in tumorigenesis, capable of either promoting tumor growth or inducing programmed cell death. Elevated levels of *miR-4516* are believed to contribute to the development of CRC by regulating the expression of specific genes, including *BTK*, making it a promising target for both monitoring and therapeutic of the disease.

Correspondence: Maryam Qasim Mohammed, Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq
E-mail: maryamqasim.ms.c.mic.2020@uomustansiriyah.edu.iq

Key words: Bruton's tyrosine kinase, *miR-4516*, colorectal cancer, gene expression.

Conflict of interest: the authors declare no conflict of interest.

Funding: no external funding was received for this work.

Ethics approval and consent to participate: the Ethics Committee of Mustansiriyah University approved the current study (approval no. 4267 on 29/9/2021). Before their involvement in the research, every participant signed an informed consent form.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

Acknowledgments: we express our sincere gratitude to the patients who participated in this study, despite facing significant health challenges. Their generous contribution was invaluable to this research.

Received: 29 August 2024.
Accepted: 29 December 2024.
Early view: 21 February 2025.

Publisher's note: all claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.

©Copyright: the Author(s), 2025
Licensee PAGEPress, Italy
Journal of Biological Research 2025; 98:12987
doi:10.4081/jbr.2025.12987

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial International License (CC BY-NC 4.0) which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

Introduction

The accumulation of genetic and epigenetic changes in colon epithelial cells causes Colorectal Cancer (CRC), a complicated disease. These alterations have the potential to interfere with a variety of biological activities, such as angiogenesis, DNA repair, apoptosis, and cell division.[1] CRC is a disease characterized by the abnormal proliferation of cells within the colon or rectum. In all disciplines of research and clinical practice, Rectal Cancer (RC) and Colon Cancer (CC) are considered a single disease entity, collectively referred to as CRC. This classification is predicated on the anatomical and functional unity of the large bowel, where both RC and CC originate.[2] CRC is the fourth type of cancer-related death globally and the third most common malignancy in terms of diagnoses.[3] According to the Iraqi Cancer Registry in 2020, CRC ranks third among the top ten most common cancers in Iraq with 2,210 patients and a 5.34% prevalence. By 2022, CRC had ascended to the second position in the top ten, with 2,871 cases and a 7.3% prevalence.[4]

Bruton's Tyrosine Kinase (*BTK*) gene is well-known for being extensively expressed in B cell malignancies like chronic lymphocytic leukemia, mantle cell lymphoma, and multiple myeloma in addition to its physiological roles.[5] Therefore, it was suggested that inhibiting *BTK* could have a therapeutic effect because it is a significant participant in the carcinogenic activity in these disorders.[6] *BTK* inhibitors have demonstrated encouraging anti-tumor effects, initially in B-cell malignancies and also in additional tumor types.[7] Immune and stromal scores had a positive correlation with *BTK* expression.[8] *BTK* is a predictive biomarker in a variety of cancers,

and it is intimately related to the remodeling of the tumor microenvironment.[9] Numerous investigations revealed that *BTK* stimulates tumor growth, angiogenesis, and immunosuppression and is strongly expressed in tumor-associated macrophages, the primary constituent of the tumor microenvironment (TME).[10,11] According to the National Center for Biotechnology Information (NCBI), the *BTK* gene codes for the production of the BTK protein; *BTK* gene is found on the Xq22.1 chromosome and is essential for several cellular functions, such as migration, survival, and proliferation; it is a protein-coding gene (molecular mass about 76,281 Da) with 21 exons and 659 amino acids. The size of the gene was 41,459 bases with minus strand orientation.[12] According to GeneCards, the Tec family is a group of non-receptor tyrosine kinases involved in various cellular signaling pathways; particularly in immune cells. They play a crucial role in regulating cell proliferation, differentiation, and activation. This includes *BTK*, which is essential for the development, maturation, and activation of B cells, mast cells, and myeloid cells. *BTK* activation is initially triggered by its membrane localization, which depends on the synthesis of phosphatidylinositol (3,4,5)-trisphosphate (PIP3).[13] Following its discovery, *BTK* has been thought to be a tissue-specific kinase that is only expressed in bone marrow-derived cells.[14] A complex post-transcriptional mechanism mediates the production of a novel isoform of BTK outside the hematopoietic compartment.[15] Furthermore, the findings that BTK is a potent oncoprotein acting downstream of the pathway and that its inhibition significantly impacts colon cancer cell proliferation and survival suggest that *p65BTK* acts as an oncogene and might be a novel, promising therapeutic target in this disease.[3] Deregulation of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (RAS/ERK) signaling pathway, a key intracellular cascade mediating cellular responses to extracellular stimuli, is a common occurrence in colon cancer. This pathway governs essential cellular processes, including growth, proliferation, differentiation, and survival.[15,16] Since *p65BTK* is an obligatory effector of RAS-mediated transformation and an entirely novel isoform of *BTK* according to previous investigation, it is overexpressed in colon malignancies, and its inhibition may have an impact on the clonogenicity of NSCLC cell lines by assessing colony growth in the presence of increasing *p65BTK* inhibitor doses.[17] Lung adenocarcinoma, skin melanoma, colon adenocarcinoma, uterine endometrioid adenocarcinoma, and diffuse large B-cell lymphoma, not otherwise defined, have the highest prevalence of mutations in *BTK*, which is altered in 1.27% of all malignancies.[18] The *BTK* gene encodes for the production of BTK, a protein that is required for B cell growth and maturation. Crucial chemical signals that instruct B cells to develop and generate antibodies are delivered by the BTK protein. Additionally, BTK plays a dual role in controlling apoptosis. The *BTK* gene promotes the activity of NF-kappa-B, which controls the expression of hundreds of genes.[19–21]

Endogenous, non-coding short RNAs known as microRNAs (*miRNAs*) are evolutionarily conserved and essential for regulating gene expression in essential physiological processes.[22,23] The *miRNAs* are important regulators during the development and tumorigenesis of a variety of biological mechanisms, including hematopoiesis, organogenesis, cell proliferation, differentiation, and apoptosis, as well as the occurrence, invasion, and metastasis of tumors, according to studies.[24] *MiRNAs* can function as either oncogenic or tumor suppressors by blocking the expression of tumor suppressor genes. They may achieve that by blocking the production of oncogenes. Because *miRNAs* are located inside fragile regions in the genome, they can be dysregulated by a wide range of genetic alterations, including deletions, amplifications, translocations, and point mutations.[25] *MiRNAs*, which include *miR-4516*, a short non-coding RNA (20–24 nt) that regulates the translation and stability of *miRNAs*, play a role in the post-transcriptional regulation of gene expression in multicellular organisms. *MiR-4516* gene possesses just one exon and is positioned on chromosome 16p13.3.[26] Previous research has indicated that overexpression of *miR-4516* has an impact on malignant tumors;[27,28] specifically, it has been shown to suppress pancreatic cancer cell proliferation, migration, and invasion while promoting cell apoptosis *in vitro*.[29] According to recent studies, *miRNAs* are not routinely expressed in the peripheral blood of CRC patients, but they are also linked to the development of CRC and may be employed as biomarkers.[30] As molecular biotechnology has developed in recent years, investigators have identified an assortment of *miRNAs* linked to CRC, each of which plays a role in the disease's sophisticated biological mechanisms.[31] Few findings on *miR-4516* being differentially expressed in colon cancer serum have been made, although it is present in the serum of patients with esophageal squamous cell carcinoma.[32] Nevertheless, little has been identified about *BTK*'s prognostic significance in human malignancies. The dual spectrum of action of *BTK* inhibitors on other binding enzymes (such as the ERBB family and the significance of *BTK* in TME) may be linked to the possible mechanisms of these inhibitors in solid tumors.[33] Oncogenes including *BTK* and *miRNAs* interplay in a complicated and constantly evolving mechanism. Understanding the precise processes through how *miR-4516* controls *BTK* expression in CRC may help to clarify the molecular pathophysiology of the condition and pinpoint potential therapies. As a result, the current study sought to assess *BTK* gene expression and the *miR-4516* that corresponds with it in Iraqi patients with CRC Patients.

Materials and Methods

Sample collection

The current study included 100 participants divided into two groups: in the first group (50 samples) there were patients diagnosed with CRC, and the second group (50 samples) was the control group. The mean ± SE of age was 54.8±10.21 and 55.6±9.48 years for CRC patients and control respectively. The men-to-women percentage of the patients group was 54.7% to 45.3% while in the control group was 53.4% and 46.6%, respectively. The samples were collected in the Oncology Hospital of the Medical City Department, Baghdad, Iraq, in the period from February 2022 to October 2022. The study design is shown in Figure 1.

Ethical approval

All procedures were performed following ethical principles of the International Ethical Guidelines for Health-related Research Involving Humans Prepared by the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO), National Commission for the Protection of Human Subjects of Biomedical, and Behavioral Research (NIH Publication No. 86–23, revised in 1996). The Ethics Committee of Mustansiriyah University approved the current study (approval no. 4267 on 29/9/2021). Before their involvement in the research, every participant signed an informed consent form.

RNA Extraction

Total RNA was directly extracted from the whole blood sample using the Easy Pure® Blood RNA Kit (cat. no. ER401) (TransGen Biotech company, Beijing, China) following the manufacturer's pro-

cedure. Total RNA was extracted according to the following steps.

i) The amount of 500 µL of Red Cell Lysis Buffer 2 (RCL2) was added to 200 µL of blood, and the mixture was thoroughly mixed by inverting it 4-6 times. The mixture was then incubated on ice for 10 minutes and mixed 2-3 times by inverting during the incubation period. The lysate was centrifuged at 400 × g for 10 minutes at 2-8°C, and the supernatant was discarded.

ii) The appropriate volume of Binding Buffer 7 (BB7) (300 µL) was added according to the sample requirement, and the cell pellet was thoroughly dispersed by vortexing. Then 150 µL of 96-100% ethanol was added. The mixture was thoroughly mixed by vortexing to disperse the precipitation that may have formed after the addition of ethanol. All subsequent centrifugation steps were performed at room temperature.

iii) The mixture was briefly centrifuged, and the entire content was added to a spin column. The mixture was then centrifuged at 12,000 × g for 1 minute, and the flow-through was discarded. Subsequently, 500 µL of Clean Buffer 7 (CB7) was added to the spin column, centrifuged at 12,000 × g for 30 seconds, and the flow-through was discarded.

Optional: When genomic DNA-free RNA was needed, 80 µL of DNase I working solution (prepared by mixing 10 µL of DNase I with 70 µL of DNase I Reaction Buffer) was added and incubated at room temperature for 5 minutes.

iv) An amount of 500 µL of Wash Buffer 7 (WB7) (ensuring ethanol was added to stock solution) was added to the spin column, centrifuged at 12,000 × g for 30 seconds, and the flow-through was discarded. This step was repeated twice.

v) The empty spin column was centrifuged at maximum speed (≥12,000 × g) to remove ethanol residue, and the column matrix was air-dried for several minutes. The spin column was placed into a clean 1.5 mL RNase-free tube, 50 µL of RNase-free water was added to the center of the column matrix, and the mixture was incubated at room temperature for 1 minute. The mixture was centrifuged at 12,000 × g for 2 minutes to elute the RNA. Finally, the eluted RNA was stored at -80°C.

Concentration and purity measurement

The concentration and purity of extracted RNA were evaluated using the NanoDrop One^{C} (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to determine the quality of samples for further evaluation in RT-qPCR.

Synthesis of complementary DNA

The EasyScript® (One-Step gDNA Removal and cDNA Synthesis Super Mix) Kits (TransGen Biotech Company, Beijing, China) with Cat number (AE311-02) were used to evaluate the cDNA synthesis. RNA was reverse transcribed into cDNA was performed in duplicate for two distinct purposes: i) the mRNA analysis: the first cDNA synthesis reaction was optimized for the amplification of target mRNA transcripts, specifically *BTK* and the housekeeping gene *GAPDH*; ii) the miRNA analysis: The second cDNA synthesis reaction was specifically designed for *miRNA-4516* and *U6* gene amplification. This was achieved by the sequential addition of 0.2 µL of 100 µM concentration of universal miRNA primers (CAGGTCCAGTTTTTTTTTTTTTTTVN)[34] and universal reverse

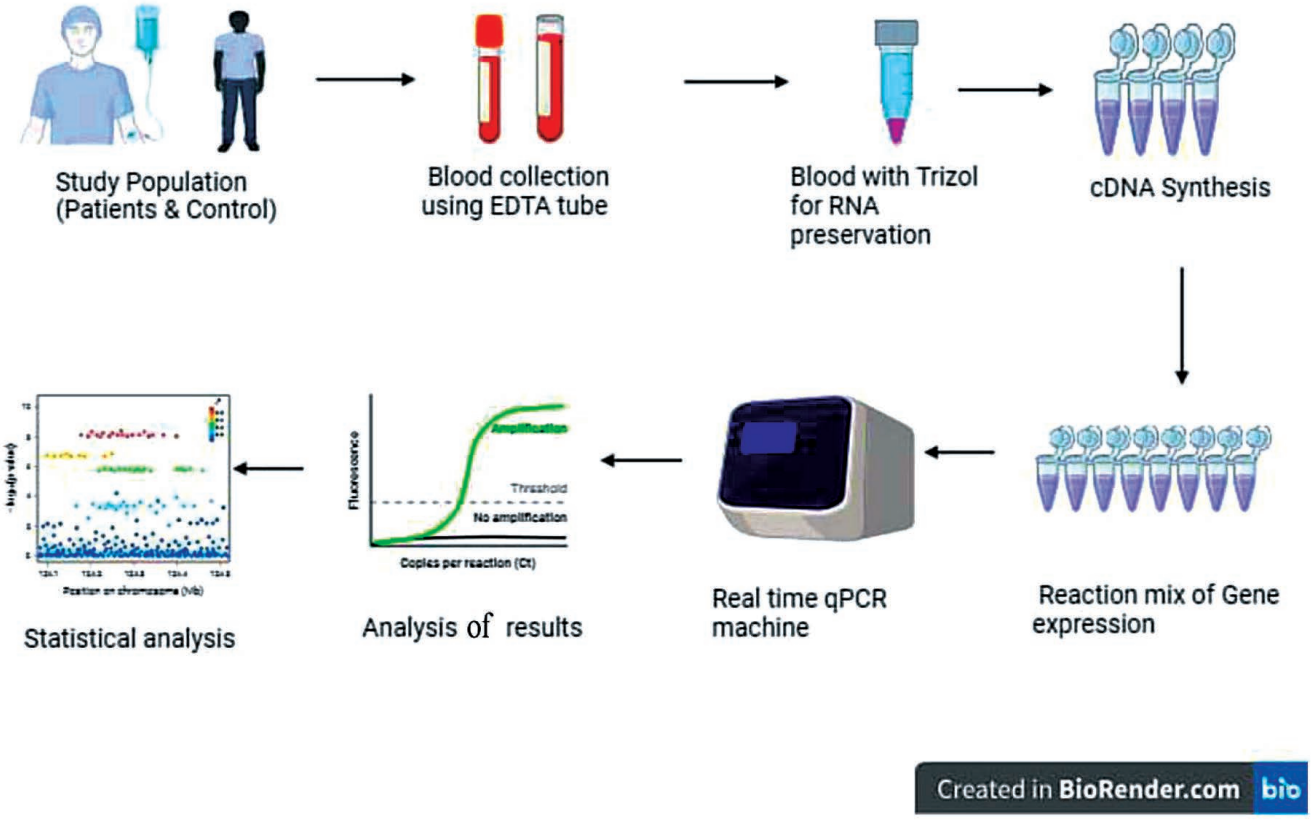

Figure 1. Study design of the current investigation.

miRNA primers (GCGAGCACAGAATTAATACGAC)[35] to the reaction mixture.

Reaction component of cDNA synthesis

Reaction components for each sample include the following components with final volume (22 µL): i) EasyScript® SuperMix (10 µL) contains essential reagents for reverse transcription, such as buffer, dNTPs, and enzymes; ii) Random Primers (1 µL) act as short, random sequences that bind to various RNA molecules, facilitating cDNA synthesis from a wide range of transcripts; iii) Anchored Oligo(dT) (1 µL) that represent Primers specifically designed to bind to the poly(A) tail of mRNA molecules, ensuring efficient cDNA synthesis from mRNA; iv) gDNA Remover (1 µL) action as an enzyme or chemical agent that specifically degrades gDNA, preventing its amplification in subsequent PCR reactions; v) RNase-free Water (3 µL) is used to adjust the final reaction volume; vi) E-mix Reverse Transcriptase (1 µL) characterizes the enzyme responsible for catalyzing the synthesis of cDNA from the RNA template using the provided primers; vii) Total RNA (5 µL) acts as the starting material containing the RNA molecules to be converted into cDNA.

Conditions of cDNA synthesis using thermocycler

The thermal profile steps for cDNA reverse transcription using thermo cycler (QIA amplifier 96, Qiagen, Hilden, Germany) involve three distinct stages. In the first step, the temperature is set at 25°C for 10 minutes, allowing random primers (N9) to bind to the RNA template. This facilitates the synthesis of cDNA from various RNA molecules. Subsequently, the temperature is increased to 42°C for 15 minutes in the second step, enabling the binding of anchored oligo(dT)18 primers to the poly(A) tail of mRNA molecules. This ensures efficient cDNA synthesis from mRNA specifically. Finally, in the third step, the temperature is rapidly increased to 85°C for 5 seconds, inactivating the reverse transcriptase enzyme and halting further cDNA synthesis. This process is carried out within the thermocycler and effectively converts RNA into cDNA, which can then be utilized for various downstream applications.

Gene expression

The primers were employed for gene expression reaction in the current study, and the specific sequences were provided by Alpha DNA Company, Montreal, Canada, whence they came in lyophilized form and were replaced with nuclease-free water until they reached a concentration of 10 µM. The primers used in the study and their reference sequence are shown in Table 1.

The reaction was performed with a final volume of reaction of 20 µL according to the manufacturer's instructions. The amount of TransStart® Top Green qPCR Super Mix (Cat no. AQ131-01, TransGen Biotech Company, Beijing, China) needed to prepare the necessary amount of reactions was calculated to be 10 µL. A quantity of 3 µL of cDNA as template, 1 µL each of the forward and reverse primers, and finally 5 µL of nuclease-free water was added to complete the final volume of the reaction. The stages and temperature of gene expression are shown in Table 2. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and *U6* are small nuclear RNA (snRNA) that act as a housekeeping gene. Gene expression levels were quantified for both patient and control groups using quantitative real-time PCR (qRT-PCR). Cycle threshold (Ct) values were determined for each target gene and a housekeeping gene. Data analysis was performed using two established methods: the ΔCt method and the ΔΔCt method for fold-change calculation.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 26 was used to calculate the mean ± SE (standard error) of the cycling threshold (Ct) of the target and references gene. *GAPDH* and *miRU6* were used as the reference genes for *BTK* and *miR-4516*, respective-

Table 1. Primers used in the current study.

Primer	Sequence (5′→3′ direction)	Temperature (°C)	Product size (bp)
Bruton's Tyrosine Kinase gene (*BTK*)[36]			126
Forward	TGCAAGGATGTCTGTGAAGC	60	
Reverse	GGACAGGCCGAAATCAGATA	60	
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene[37]			120
Forward	GAAATCCCATCACCATCTTCCAGG	62	
Reverse	GAGCCCCAGCCTTCTCCATG	60	
miR-4516[38]			72
Forward	ATGGGAGAAGGGTCGGGG	62	
Universal reverse	GCGAGCACAGAATTAATACGAC	62	
miR-U6[39]			73
miR-U6 forward primer	AGAGAAGATTAGCATGGCCCCT	60	
miR-universal reverse primer	GCGAGCACAGAATTAATACGAC	62	

Table 2. Stages and temperature of qRT PCR for *BTK*, *GAPDH*, *U6*, and *miR-4516* genes.

Stage		Temperature °C	Time/Sec.	Cycle
Stage 1	Denaturation	94	60	1
Stage 2	Denaturation	94	5	35
	Annealing	58	15	
	Extension	72	20	
Stage 3	Dissociation	65-95	1	1

ly. The ΔCT value, which was determined using the following formula: ΔCt = Ct (target gene) – Ct (internal control), represented the quantitative gene expression. Subsequently, the following formula was used to get the expression ratio: $2^{-\Delta Ct}$. The $2^{-\Delta\Delta Ct}$ method was used to compare the transcript levels across samples. The following equation was applied: ΔΔCt = ΔCt(test) - ΔCt(calibrator).[40,42] A P-value ≤0.05 means significant while if P-value ≤0.01 means highly significant.

Results

The concentration of the RNA samples ranged from 75 to 95 nanograms per microliter (ng/µL). This measurement indicates the amount of RNA present in a given volume of the sample. The absorbance (Optical Density, OD) of the sample was measured at 260 nm, which corresponds to the peak absorption of nucleic acids. The purity of the RNA was assessed using the A260/A280 ratio, which compares nucleic acid absorption at 260 nm to protein absorption at 280 nm. An A260/A280 ratio between 2.0 and 2.2 is generally indicative of high-purity RNA and the sample of this study had values of 2.1–2.2.

The *BTK* gene expression analysis revealed that the mean target gene expression in the patient group was 8.25(Ct), while the mean reference gene expression was 15.84(Ct), resulting in a ΔCt of -7.59 and a $2^{-\Delta Ct}$ of 192.67. In the control group, the mean target gene expression was 8.85, the mean reference gene expression was 15.77, the ΔCt was -6.92, and the $2^{-\Delta Ct}$ was 121.10. The results indicated no significant difference in mRNA expression of the *BTK* gene between the patient and control groups, with a fold-change of 1.58 and a P-value = 0.1.

For the *miR-4516* gene, the patient group exhibited a mean target gene expression of 7.73(Ct) and a mean reference gene expression of 17.39(Ct), leading to a ΔCt of -9.65 and a $2^{-\Delta Ct}$ of 804.81. In contrast, the control group had a mean target gene expression of 9.09, a mean reference gene expression of 17.26, ΔCt of -8.18, and a $2^{-\Delta Ct}$ of 289.35. The result demonstrated a significant upregulation of the *miR-4516* gene was observed in the CRC patient group, with a P-value = 0.005 and a fold-change of 2.78. Tables 3 and 4 summarize these findings.

The current study investigated the expression levels of *BTK* and *miR-4516* genes in a group of patients compared to a healthy control group. While no significant difference was observed in the expression of the *BTK* gene between the two groups, a significant upregulation of the *miR-4516* gene was detected in the patient group with a P-value of 0.005. These findings suggest a potential role of *miR-4516* in the pathogenesis of the disease under investigation.

Discussion

This study delves into the intricate interplay between the target gene (*BTK* gene) and the miRNAs (*miR-4516*) according to the MicroRNA Target Prediction Database (https://mirdb.org/). The *BTK* gene, a pivotal regulator of B-cell receptor signaling, has been strongly implicated in the pathogenesis of various malignancies, including CRC. Given its central role in governing cellular proliferation and survival, this gene presents a promising therapeutic target.

MiRNAs, such as *miR-4516*, offer a compelling approach to modulating gene expression. By targeting specific messenger RNAs, these small non-coding RNAs can regulate oncogene and tumor suppressor gene expression. Consequently, investigating the impact of *miR-4516* on *BTK* expression and downstream signaling may provide deeper insights into CRC pathogenesis and unveil novel targeted therapeutic strategies. Figure 2, adapted from the biorender platform (https://www.biorender.com/), illustrates the molecular mechanism by which microRNAs exert their influence on target genes, with a particular focus on post-transcriptional effects.

A growing amount of evidence indicates that deregulation of miRNAs plays a crucial role in the progression of CRC; however, the precise molecular mechanisms behind this condition remain not completely understood. MiRNAs are tiny, non-coding RNAs that

Table 3. Gene expression of *BTK* and *miR-4516* dependent on Δct methods.

Group	Mean of target gene	Mean of reference gene	Δct	$2^{-\Delta ct}$	Experimental group/ Control group	Fold	P-value
BTK Gene							
Patients	8.25	15.84	-7.59	192.67	192.67/121.10	1.58±0.32	0.1 NS
Control	8.85	15.77	-6.92	121.10	121.10/121.10	1.00±0.34	
miR-4516 Gene							
Patients	7.73	17.39	-9.65	804.81	804.81/289.35	2.78±0.31	0.005**
Control	9.09	17.26	-8.18	289.35	289.35/289.35	1.00±0.77	

(NS) means non-significant P-value. (**) means a highly significant difference between patients and control.

Table 4. Gene expression of *BTK* and *miR-4516* dependent on ΔΔCt methods.

Group	Mean ΔCt	Calibrator	ΔΔCt	$2^{-\Delta\Delta Ct}$	Experimental group/ Control Group	Fold	P-value
BTK Gene							
Patients	-7.59	-6.91	-0.67	2.05	2.05\1.58	1.58±0.32	0.1 NS
Control	-6.91	-6.91	3.70	1.58	1.58/1.58	1.00±0.34	
miR-4516 Gene							
Patients	-9.65	-8.17	-1.48	2.78	2.78/1.00	2.78±0.31	0.005**
Control	-8.17	-8.17	-0.01	1.00	1.00/1.00	1.00±0.77	

(NS) means non-significant P-value. (**) means a highly significant difference between patients and control.

control post-transcriptional levels of gene expression. *MiR-4516* has been linked in recent research to CRC as a putative tumor suppressor, indicating that it may target oncogenes including *BTK* to aid promote the onset and progression of the disease.[43,44]

According to the current investigation, the precise regulatory relationship between *miR-4516* and *BTK* in CRC remains to be fully elucidated; the available evidence suggests a potential inhibitory interaction. Given that *miR-4516* tumor-suppressive properties and *BTK*'s oncogenic role, their interplay warrants further investigation to unravel the underlying molecular mechanisms.[30,45] Tumors require a specific microenvironment to grow and metastasize. This microenvironment is characterized by a complex interplay of immune cells, blood vessels, extracellular matrix, signaling molecules, and nerve fibers.[43,47] Although the microenvironment's elements may vary significantly depending on where the tumor is located, each microenvironment contributes to the growth of tumors by using different signaling pathways to regulate gene expression and the microenvironment changes to varying degrees when under stress to keep the environment favorable for tumor growth. A large network of cell-to-cell signaling controls the modification of the tumor microenvironment.[48] While the outcomes of the *BTK* gene indicated that there were no statistically significant differences between the two groups of the current study, the results of the *miR-4516* test revealed that there were statistically significant differences between the patients and the control in the amount of *miR-4516* expression level.

This study shows that the high level of *miR-4516* in patients may be used as a biomarker to track the disease, while the low expression of the *BTK* gene may be attributed to its inhibition effect because it is targeted by the *miR-4516*, as *BTK* acts as either an inhibitor involved in the programmed death process or an inducing factor for tumor growth. Several malignancies, particularly CRC, have been connected to the onset and spread of aberrant *BTK* activation.[49]

BTK is an example of a pleiotropic gene with contradictory effects on cancer pathways. Recent research demonstrates that *BTK*, a crucial component of the p53 and p73 responses to damage, can operate as a tumor suppressor in other cells, despite long being thought of as being oncogenic in the setting of B cell malignancies.[6]

The inhibition of *BTK* has now been extended to the field of solid tumors, following findings on the role of *BTK* in various tumor microenvironment cells and non-hematological tumor cells when produced spontaneously.[50] A deeper understanding of the diverse roles that *BTK* plays in non-lymphocytic cells will be extremely beneficial for the development of innovative treatment plans for solid and hematopoietic malignancies. *BTK* inhibition exhibits promise as a therapeutic that targets important immune cells within the tumor microenvironment.[51] The development and progression of CRC have been associated with a diverse array of *miRNAs*, including *miR-4516*. While some *miRNAs* function as tumor suppressors by targeting oncogenes, others act as oncogenes by targeting tumor suppressor genes.[52]

In addition to cancer, *miRNAs* are important players in several molecular networks associated with autoimmune and inflammatory diseases. *MiRNAs* are essential for normal cellular processes and are also implicated in the processes that lead to the development of cancer, including angiogenesis, differentiation, apoptosis, proliferation of cells, and epithelial-mesenchymal transition. There is proof that chronic inflammation and cancer are related. While many *miRNAs* have been suggested as viable candidates for CRC diagnosis, conclusive findings remain difficult to obtain.[25]

Consistent with our findings, a previous study identified *miR-4516* upregulation as an independent determinant of primary hypertension, a novel finding in the literature. Further research

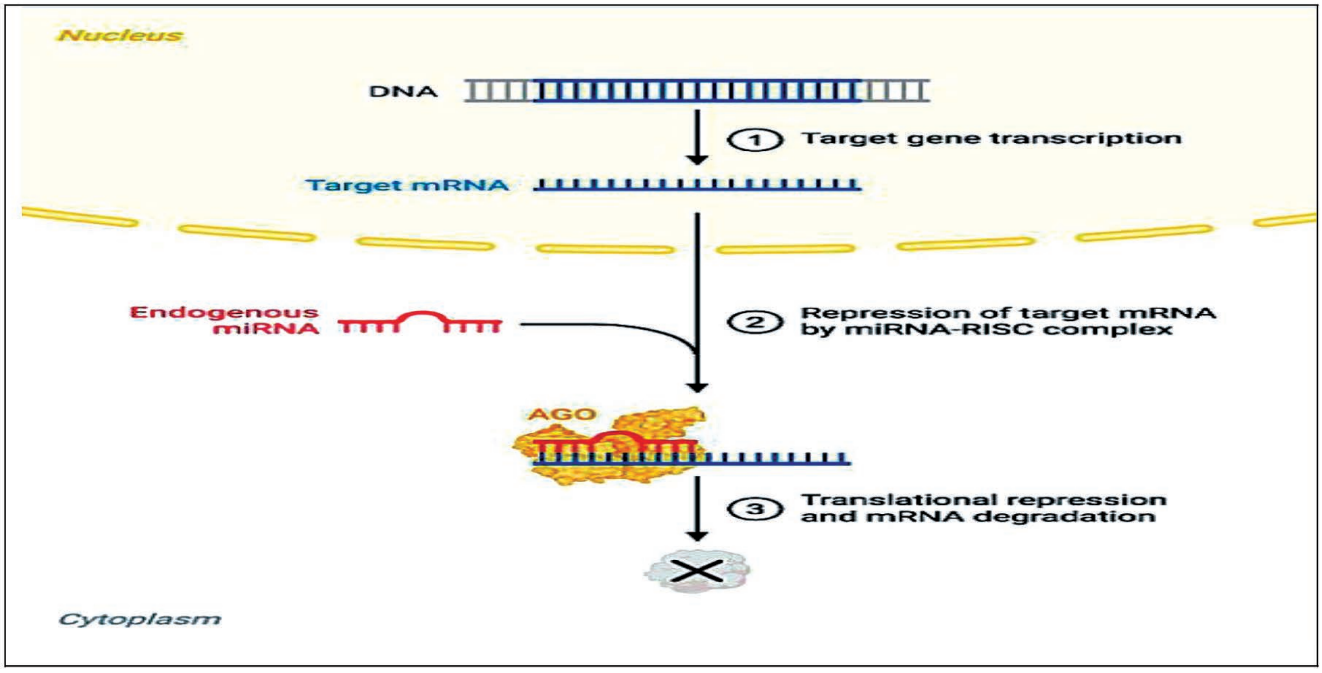

Figure 2. Molecular mechanisms that explain the association between *miR-4516* and *BTK* gene. Argonaute (AGO) proteins play a pivotal role in plant growth and development as the core components of RNA-induced silencing complex (RISC). Obtained from (https://www.biorender.com/).

building upon these results may deepen our understanding of the underlying mechanisms and potentially lead to novel therapeutic approaches.[53]

The pioneering study conducted by Yue *et al.* in 2017 was the first to establish a robust association between heightened *BTK* expression and adverse patient outcomes in approximately 50% of glioma cases. Their research demonstrated the efficacy of ibrutinib in significantly inhibiting glioma cell proliferation both *in vitro* and *in vivo*. This inhibitory effect is mediated through multiple mechanisms, including induction of G1 phase cell cycle arrest and modulation of cell cycle regulatory proteins. Furthermore, *BTK* inhibition effectively suppresses both constitutive and EGFR-stimulated activation of the NF-κB pathway, a critical signaling cascade implicated in glioma cell survival and proliferation. These compelling findings provide a strong foundation for future clinical trials evaluating the therapeutic potential of ibrutinib for glioma treatment.[31]

Jin *et al.* revealed that low *miR-4516* could be used as an independent risk factor for the prognosis of CRC.[30] Recent evidence suggests that *miRNAs* are important players in the development of tumors, acting as either tumor suppressors or oncogenes. Chen *et al*., for example, showed that overexpressing *miR-4516* reduced the proliferation, migration, and invasion of pancreatic cancer cells while increasing their apoptosis by adversely regulating *orthodenticle homeobox 1* (*OTX1*).[29]

The role of *BTK* in regulating innate immunity is a complex and expanding field of research. Targeting both the B-Cell Receptor (BCR) signaling pathway and *BTK* in Chronic Lymphocytic Leukemia (CLL) showed significant therapeutic promise; additionally, *BTK* inhibition demonstrated notable effects on B-cell chemotaxis and adhesion. Furthermore, the focus on *BTK* in myeloid cells opened up new avenues for modulating the tumor microenvironment.[54] Further research is necessary to fully elucidate the clinical potential of *BTK* inhibition across various disease states.

Conclusions

The precise functions of *BTK* in cancer remain elusive and necessitate further in-depth exploration. While some studies suggest its tumor-suppressive potential, others indicate its oncogenic properties. Notably, *BTK* exhibits context-dependent dual functionality, capable of both inducing apoptosis and promoting cell survival. The lack of significant alterations in *BTK* gene expression may be attributable to the inhibitory effects of *miR-4516*. The upregulation of *miR-4516* in CRC patients is a significant finding that could have profound implications for diagnosis and therapy. However, further research is imperative to unravel the precise mechanisms by which *miR-4516* contributes to CRC pathogenesis and to develop effective therapeutic strategies targeting this molecule.

The increased expression level of *miR-4516* in CRC patients presents a significant opportunity for advancing CRC research and clinical applications. Further investigation is needed to elucidate the precise mechanisms by which *miR-4516* contributes to CRC pathogenesis and to explore its potential as a diagnostic biomarker and therapeutic target. In-depth mechanistic studies, functional validation, and large-scale clinical trials are essential to translate these findings into effective clinical strategies. To confirm the results regarding BTK protein at the protein level, it is recommended to perform a Western blot analysis that allows for a more accurate determination of BTK protein expression levels and evaluation of any post-translational modifications.

References

1. Armaghany T, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. Gastrointest Cancer Res 2012;5:19–27.
2. Paschke S, Jafarov S, Staib L, et al. Are colon and rectal cancer two different tumor entities? A proposal to abandon the term colorectal cancer. Int J Mol Sci 2018;19:2577.
3. Basile D, Gerratana L, Buonadonna A, et al. Role of Bruton's Tyrosine Kinase in stage III colorectal cancer. Cancers (Basel) 2019;11:880.
4. Annual Report Iraqi Cancer Registry [Internet]. 2020. Available from: https://moh.gov.iq/upload/2991322580.pdf
5. Mouhssine S, Maher N, Matti BF, et al. Targeting BTK in B cell malignancies: From mode of action to resistance mechanisms. Int J Mol Sci 2024;25:3234
6. Rada M, Barlev N, Macip S. BTK: a two-faced effector in cancer and tumour suppression. Cell Death Dis 2018;9:1064.
7. Wang Y, Zhang LL, Champlin RE, Wang ML. Targeting Bruton's tyrosine kinase with ibrutinib in B-cell malignancies. Clin Pharmacol Ther 2015;97:455–68.
8. Qin Y, Lu F, Lyu K, et al. Emerging concepts regarding pro- and anti tumor properties of B cells in tumor immunity. Front Immunol 2022;13:881427.
9. Yang T, Hao L, Chen J, et al. BTK is a prognostic biomarker for multiple human tumors. 2022. Available from: https://www.researchgate.net/publication/365883496_BTK_is_a_Prognostic_Biomarker_for_Multiple_Human_Tumors
10. Weber ANR, Bittner Z, Liu X, et al. Bruton's Tyrosine Kinase: An emerging key player in innate immunity. Front Immunol 2017;8:1454.
11. Bi K-W, Wei X-G, Qin X-X, Li B. BTK has potential to be a prognostic factor for lung adenocarcinoma and an indicator for tumor microenvironment remodeling: A study based on TCGA data mining. Front Oncol 2020;10:424.
12. NCBI. BTK Bruton tyrosine kinase [Homo sapiens (human)] [Internet]. Available from: https://www.ncbi.nlm.nih.gov/gene/695
13. Gene cards [Internet]. Available from: https://www.genecards.org/cgi-bin/carddisp.pl?gene=BTK
14. Ponader S, Burger JA. Bruton's tyrosine kinase: from X-linked agammaglobulinemia toward targeted therapy for B-cell malignancies. J Clin Oncol Off J Am Soc Clin Oncol 2014;32:1830–9.
15. Grassilli E, Pisano F, Cialdella A, et al. A novel oncogenic BTK isoform is overexpressed in colon cancers and required for RAS-mediated transformation. Oncogene 2016;35:4368–78.
16. Omran MM, Fouda MS, Mekkawy SA, et al. Molecular biomarkers and signaling pathways of cancer stem cells in colorectal cancer. Technol Cancer Res Treat 2024;23:15330338241254060.
17. Giordano F, Vaira V, Cortinovis D, et al. p65BTK is a novel potential actionable target in KRAS-mutated/EGFR-wild type lung adenocarcinoma. J Exp Clin Cancer Res 2019;38:260.
18. AACR Project GENIE: Powering Precision Medicine through an International Consortium. Cancer Discov 2017;7:818–31.
19. The National Center for Biotechnology Information (NCBI) [Internet]. Available from: https://www.ncbi.nlm.nih.gov/
20. Mohamed AJ, Yu L, Bäckesjö C-M, et al. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. Immunol Rev 2009;228:58–73.
21. Uckun FM. Bruton's tyrosine kinase (BTK) as a dual-function

regulator of apoptosis. Biochem Pharmacol 1998;56:683–91.
22. Wang M, Yu F, Wu W, et al. Circular RNAs: A novel type of non-coding RNA and their potential implications in antiviral immunity. Int J Biol Sci 2017;13:1497–506.
23. Abdulhassn ML, Al-Khafaji HMAH, Mohammed MQ. Assessment of the TGF-β gene and its target miR-1908-5p gene expression and its role in the treatment of beta-thalassemia in Iraqi patients. Iraqi J Hematol 2024; 10.4103/ijh.ijh_82_24.
24. Khan AQ, Ahmed EI, Elareer NR, et al. Role of miRNA-regulated cancer stem cells in the pathogenesis of human malignancies. Cells 2019;8:840.
25. Kudelova E, Holubekova V, Grendar M, et al. Circulating miRNA expression over the course of colorectal cancer treatment. Oncol Lett 2022;23:18.
26. The National Center for Biotechnology Information (NCBI). 2024. Available from: https://www.ncbi.nlm.nih.gov/gene/100616258
27. Liu J, Chen D, Liu H, et al. A microRNA-4516 inhibitor sensitizes chemo-resistant gastric cancer cells to chemotherapy by upregulating ING4. RSC Adv 2018;8:37795–803.
28. Fuhua W, Chunyan W, Xiangyun G, et al. Expression of serum miRNA-4516 and its clinical significance and predicted target genes in hepatocellular carcinoma. Chin J Integr Tradit West Med Dig 2021;29:86–9.
29. Chen S, Xu M, Zhao J, et al. MicroRNA-4516 suppresses pancreatic cancer development via negatively regulating orthodenticle homeobox 1. Int J Biol Sci 2020;16:2159–69.
30. Jin X-H, Lu S, Wang A-F. Expression and clinical significance of miR-4516 and miR-21-5p in serum of patients with colorectal cancer. BMC Cancer 2020;20:241.
31. Yue C, Niu M, Shan QQ, et al. High expression of Bruton's tyrosine kinase (BTK) is required for EGFR-induced NF-κB activation and predicts poor prognosis in human glioma. J Exp Clin Cancer Res 2017;36:132.
32. Donati S, Ciuffi S, Brandi ML. Human circulating miRNAsreal-time qRT-PCR-based analysis: An overview of endogenous reference genes used for data normalization. Int J Mol Sci 2019;20:4353.
33. Metzler JM, Burla L, Fink D, Imesch P. Ibrutinib in gynecological malignancies and breast cancer: A systematic review. Int J Mol Sci 2020;21:4154.
34. Patel RK, West JD, Jiang Y, et al. Robust partitioning of microRNA targets from downstream regulatory changes. Nucleic Acids Res 2020;48:9724–46.
35. Ghareeb ZA, Al-Khafaji HMA-H, Al-Saedi MKA. Low-density lipoprotein receptor-related protein-1 and mir-205 expression for cardiovascular disease in familial hypercholesterolemia and non-familial hypercholesterolemia in Iraqi population. Iran J Ichthyol 2021;8:246–54.
36. Dai B, Chen A, Corkum C, et al. Hepatitis C virus upregulates B-cell receptor signaling: A novel mechanism for HCV-associated B-cell lymphoproliferative disorders. Oncogene 2016;35:2979–90.
37. Faeqali Jan M, Muneer Al-Khafaji H, Hasan Al-Saadi B, Aneed Al-Saedi MK. Assessment of interleukin-8 in bronchial asthma in Iraq. Arch Razi Inst 2021;76:913–23.
38. Umair Z, Baek M-O, Song J, et al. MicroRNA-4516 in urinary exosomes as a biomarker of premature ovarian insufficiency. Cells 2022;11:2797.
39. Chen Y, Guo Y, Li Y, et al. miR□300 regulates tumor proliferation and metastasis by targeting lymphoid enhancer□binding factor 1 in hepatocellular carcinoma. Int J Oncol 2019;54:1282–94.
40. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101–8.
41. Riedel G, Rüdrich U, Fekete-Drimusz N, et al. An extended ΔCT-method facilitating normalisation with multiple reference genes suited for quantitative RT-PCR analyses of human hepatocyte-like cells. PLoS One 2014;9:e93031.
42. SPSS. Statistical Package for the Social Sciences. 2019. p. Version 26.
43. Iorio M V, Croce CM. Causes and consequences of microRNA dysregulation. Cancer J 2012;18:215–22.
44. Lulli M, Napoli C, Landini I, et al. Role of non-coding RNAs in colorectal cancer: Focus on Long Non-Coding RNAs. Int J Mol Sci 2022;23:13431.
45. Ždralević M, Radović A, Raonić J, et al. Advances in microRNAs as emerging biomarkers for colorectal cancer early detection and diagnosis. Int J Mol Sci 2024;25:11060
46. Henke E, Nandigama R, Ergün S. Extracellular matrix in the tumor microenvironment and its impact on cancer therapy. Front Mol Biosci 2019;6:160.
47. Eble JA, Niland S. The extracellular matrix in tumor progression and metastasis. Clin Exp Metastasis 2019;36:171–98.
48. Messex JK, Liou G-Y. Targeting BTK signaling in the microenvironment of solid tumors as a feasible cancer therapy option. Cancers 2021;13:2198.
49. Cerrito MG, Grassilli E. Identifying novel actionable targets in colon cancer. Biomedicines. 2021;9:579.
50. McCay J, Gribben JG. The role of BTK inhibitors on the tumor microenvironment in CLL. Leuk Lymphoma 2022;63:2023–32.
51. Pal Singh S, Dammeijer F, Hendriks RW. Role of Bruton's tyrosine kinase in B cells and malignancies. Mol Cancer 2018;17:57.
52. Slattery ML, Herrick JS, Mullany LE, et al. The co-regulatory networks of tumor suppressor genes, oncogenes, and miRNAs in colorectal cancer. Genes Chromosomes Cancer 2017;56:769–87.
53. Özkan G, Ulusoy Ş, Geyik E, Erdem Y. Down-regulation of miRNA 145 and up-regulation of miRNA 4516 may be associated with primary hypertension. J Clin Hypertens (Greenwich) 2019;21:1724–31.
54. Good L, Benner B, Carson WE. Bruton's tyrosine kinase: an emerging targeted therapy in myeloid cells within the tumor microenvironment. Cancer Immunol Immunother 2021;70:2439–51.