RESEARCH ARTICLE

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Gene Expression Alterations of *TIMP3*, *ELASTIN*, *K-RAS*, and *BRAF* in Colorectal Cancer Patients with *H. pylori* Infection

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Abstract

Objective: Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related mortality worldwide, with over 1.9 million new cases and 0.9 million deaths reported in 2020. The role of Helicobacter pylori infection in CRC pathogenesis remains a significant area of research. This study aimed to investigate the association between *H. pylori* infection, and genetic alterations in CRC patients. **Methods:** A total of 110 blood and tissue biopsy samples were collected from CRC patients at Ghazi AL-Hariri Specialized Surgery Hospital in Baghdad, Iraq, between November 2023 and August 2024. An additional 36 samples from non-cancer patients were used as controls. Enzyme-linked immunosorbent assay (ELISA) was employed to detect *H. pylori*-specific immunoglobulins (IgG). Gene expression analysis of *ELASTIN*, *TIMP3*, *K-RAS*, and *BRAF* was performed using RT-qPCR. **Results:** The study found that *H. pylori* infection was present in 79.5% of CRC patients, with significant IgG seropositivity ($p < 0.05$). Gene expression analysis revealed a significant downregulation of *TIMP3* and alterations in *ELASTIN*, *K-RAS*, and *BRAF* ($p < 0.01$). **Conclusion:** In conclusion, chronic *H. pylori* infection may contribute to CRC pathogenesis through sustained inflammation and genetic dysregulation. The study highlights *TIMP3* suppression as a potential factor in CRC progression, warranting further investigation into its clinical implications.

Keywords: Colorectal Cancer- Helicobacter pylori- *TIMP3*- *K-RAS*- *BRAF*

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the second most common cause of cancer mortality worldwide. There are disparities in the epidemiology of CRC across different populations, most probably due to differences in exposure to lifestyle and environmental factors related to CRC [1]. The number of cases and deaths was higher in men than in women aged up to 80–84, with the highest rates observed in the oldest age group (≥95 years) for both sexes [2]. This increase will be the result of economic, social, environmental and generational changes in developed countries. The occurrence of colorectal cancer is associated with nonmodifiable risk factors, including age and hereditary factors, as well as modifiable factors related to the environment and lifestyle [3]. Helicobacter pylori that specifically colonizes the gastric epithelium causing the most common bacterial infection in the world [4]. Although most patients bearing an *H. pylori* infection are asymptomatic, all will sooner or later develop gastritis, the chronic inflammatory response and the presence of specific bacterial virulence factors, especially the cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), play a fundamental role in causing damage to host cell DNA and in the activation of specific pathways that sustain cell survival, with both processes often supporting each other [5]. Tissue inhibitor of metalloproteinases-3 (*TIMP3*) is vital in regulating several biological processes. *TIMP3* exerts antitumor effects via matrix metalloproteinase (MMP)-dependent and MMP-independent pathways. Due to promoter methylation and miRNA binding, *TIMP3* expression has been observed to decrease in various cancers. Consequently, the migration and invasion of cancer cells increases. Conflicting results have reported that expression levels of *TIMP3* in primary and advanced cancers are higher than those in healthy tissues [6]. Abnormal level of *ELASTIN* have been observed in many fibrotic diseases, including kidney, lung and liver fibrosis. Studies have shown that fibrosis is a contributing factor in cancer development, and is involved in early stages of CRC [7]. The assessment of RAS and *BRAF* mutational status is one of the main steps in the diagnostic and therapeutic algorithm of metastatic colorectal cancer (mCRC) [8]. This study aimed to study the link between *ELASTIN*, metalloproteinases 3 (*TIMP3*), *K-RAS*, *BRAF* and presence of *H. pylori* in colorectal cancer patients

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Materials and Methods

Patients and sampling

A Case-control study included total of 110 blood and tissue biopsies samples were collected from Ghazi AL-Hariri Specialized Surgery Hospital/ Medical City Hospital, Baghdad Iraq, in period extended from November 2023 to August 2024. In addition, 36 blood and tissue sample collected from patient free of colon cancer. Patients included in the present study were from different provinces in Iraq.

Classification of subjects and samples included in current study according to histopathological findings as patients was classified according to the site of tumor either in (colon, rectal, or rectosigmoid) and type of tumor either (poorly or moderate or well differentiated adenocarcinoma, or mucinous adenocarcinoma).

Patient cancer included both gender (male and female) aged from aged from 23 to 70 years old.

Control group included patient suffering from symptoms of digestive system problem especially on colon and rectal site with varies clinical cases including (celiac disease & autoimmune enteropathy, ulcerative colitis, congestion & edema, mild acute self-limited colitis, Hirschsprung disease [hypo ganglionic type], colonic polyps, or benign tumor), also blood and tissue samples were collected from both gender (male and female) aged from 6 to 69 years. Five ml of venous blood were collected in gel tube from patients, placed at room temperature for 30min and then, centrifuged at 4000 r.p.m for 5 min. to separate the serum, stored at -20°C.

Tissue biopsy from included subjects were kept at -20°C for molecular analysis. Data were collected from each patient and control group included (name, age, gender, previous treatment). Histopathology findings of each patient were obtained from laboratory reports.

Included criteria

Patients with colon cancer.

Excluded criteria

patients missing of histopathological reports (patients' data).

Ethical approval

This study was approved by ethical committee of the College of Medicine- AL-Iraqia University in Baghdad, (No. FM. SA/140 - Date 1/10/2023)

Screening for H.pylori infection

For more specific and sensitive diagnosis of H.pylori infection ELISA kit (BT-LAB, China) was used for detect specific antibodies including IgG and estimated according to company instructions. Average the reading from duplicate or triplicate samples. The results as follows

Cutoff Value = average Negative Control value + 0.15
While ODsample < Cutoff Value: Negative
While ODsample ≥Cutoff Value: Positive
Estimation the Gene expression alteration

Total RNA was extracted from colorectal tissue blocks using the TransZol Up Plus RNA Kit following the manufacturer's protocol, after deparaffinization with ethanol. Phase separation was achieved by chloroform addition, followed by centrifugation and ethanol-based RNA precipitation. The RNA pellet was purified through spin columns, eluted in RNase-free water, and stored at –20°C. RNA concentration and purity were assessed using a NanoDrop spectrophotometer, where samples with an A260/A280 ratio near 2.0 were considered suitable for downstream applications.

Complementary DNA (cDNA) was synthesized from total RNA using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix in a 20 µl reaction volume. The reaction was incubated at 25°C for 10 min, 42°C for 15 min, and 85°C for 5 sec, and the obtained cDNA was stored at –20°C. Gene expression of *ELASTIN*, *TIMP3*, *K-RAS*, and *BRAF*, in addition to the housekeeping genes GAPDH and β-actin (Table 1), was quantified using SYBR Green–based quantitative real-time PCR (qRT-PCR) on a QIAGEN Rotor-Gene Q system. Each 20 µl reaction contained TransStart® Top Green qPCR SuperMix, forward and reverse primers, and cDNA template. Cycling conditions consisted of enzyme activation at 94°C for 30 sec, followed by 40 cycles of 94°C for 5 sec (denaturation), 58°C for 15 sec (annealing), and 72°C for 20 sec (extension), with melt-curve analysis from 55–95°C. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method) [15].

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 21 is used to interpret the data. The information is given in the form of a mean, standard deviation, and ranges. ANOVA was used to compare between tested mean Data expressed as mean±SD. LSD test was used to calculate the significant differences between tested mean, the letters (A, B, C, D, E) represented the levels of significant, highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between tested mean. Values of $p>0.05$ were considered statically non-significant while $p\leq0.05$ considered significant results.

Results

Patients demography

In this study, general demographic criteria including gender showed there was no significance differences of the patients and control group in the gender percentage among male and female with ratio of patient 6:4 (26:18) male was more susceptible to get colon cancer than female, Table (2). The gender distribution between patients and controls is not significantly different, as $p > 0.05$, so, gender is not a confounding factor in this study. While there was a significance differences in age between patients and control group as age range of patients from (10-70) years, mean age 50.6±13. The p-value is 0.001, which is highly significant ($p < 0.01$). There is a significant difference in age distribution between patients and control groups. the patients group tends to be older, with a higher proportion

in the 42-62 and ≥63 years categories, Table (3).

Each subscript letter denotes a subset of Groups categories whose column proportions do not differ significantly from each other at the .05 level. Statistical analyses performed by the Chi-squared test; NS, no significant difference; Significant values are bolded.

Patients was grouped according to the site and type of colon cancer with highest site frequency was in colon 18 cases (40%), but highest colon cancer type was moderate differentiated adenocarcinoma with frequency 25 cases (56%), The most common tumors are those of the colon and rectal region. Additionally, mucinous tumors of the recto-sigmoid junction are rather prevalent. Specific sites such as the ileocecal valve, ascending colon, and appendix are uncommon (2.3% each).

The predominant tumor type is adenocarcinoma with moderate differentiation. Mucinous subtypes, such as LAMN and mucinous adenocarcinoma, make up a lesser percentage. Although they are rare, poorly differentiated tumors may be a sign of more severe illness. There appears to be some histological diversity in the sample, as evidenced by the 2.3% of well-differentiated neuroendocrine tumors, Table (4) and (5).

As control group included patients suffering from digestive system disorder but negative for colon cancer. Also control group was divided according to the site of samples and type of cases, as highest frequency site was colon with 17 cases (47%). but highest case type was colonic polyp with frequency 12 cases (33.3%). Given the prevalence of colon samples, it is likely that colonic diseases, not rectal or ileal problems, account for the majority of control group cases. The preponderance of biopsy samples (as opposed to surgical resections) suggests that the majority of control cases were probably diagnostic assessments rather than extensive surgical procedures, the most common result in the control group is colonic polyps, which is noteworthy.

Another frequent characteristic that indicates mild inflammation is congestion and edema. The prevalence of inflammatory and autoimmune diseases (such as celiac disease, UC, and IBD) is lower (around 16.8%). In limited cases, uncommon diseases such as benign tumors, adenomatous polyps, and Hirschsprung disease were detected, Supplementary Table (1) and (2).

Screening for Helicobacter pylori infection

The results showed a significance differences at p value 0.05 in the presence of IgG for both patients and control group but there were no significance differences in positive IgG between patients and control group. The number of patients have IgG against H.pylori was 34 (77%), while control 34 (94.4%) Figure 1. The controls' greater IgG positive may suggest: a- prior *H. pylori* exposure that did not cause the condition to worsen. b-a

Table 1. Primer Sequences Used in Current Study

Primers	Primer Sequences	References
BRAF	5′-AATGCTTGCTCTGATAGGAAAAT-3′	[9]
	5′-TAATCAGTGGAAAAATAGCCTC-3′	
K-RAS gene	5′-GTGGAGTATTTGATAGTGTATTAAC-3′	[10]
	5′-TGTATCAAAGAATGGTCCTGCA-3′	
Elastin	5'-CAGCTAAATACGGTGCTGCTG-3′	[11]
	5'-AATCGAAGCCAGGTCTTG-3′	
metalloproteinases 3 (TIMP3)	5′-AAGGTACTAGAAACAGACTCCTCCAG-	[12]
	5′-TTGATACAGGACAAGAACTTGAGTG-3′	
Beta- actin	5'-CGCGAGAAGATGACCCAGAT-3′	[13]
	5'-GCACTGTGTTGGCGTACAGG-3′	
GAPDH	5' -GAG TCA ACG GAT TTG GTC GT-3′	[14]
	5' -TTG ATT TTG GAG GGA TCT CG-3′	

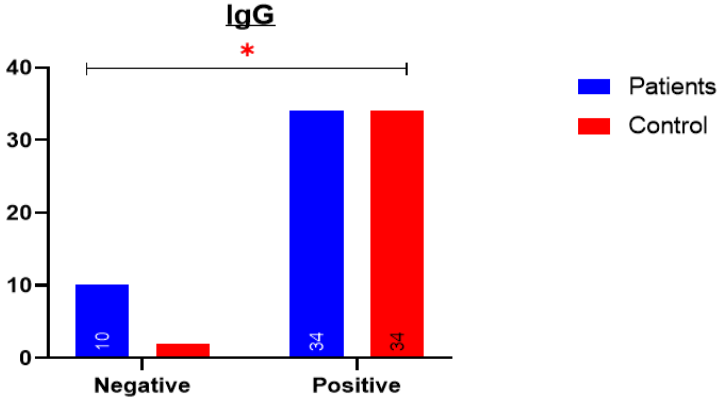

Figure 1. Comparison between Patients and Control Groups in IgG

Table 2. Comparison between Colon Cancer Patients and Control Groups in Gender

			Groups		Total	p-value
			Patients	Control		
Sex	Female	Count	18_a	21_a	39	0.12 NS
		%	40.90%	58.30%	48.80%	
	Male	Count	26a	15a	41	
		%	59.10%	41.70%	51.20%	
Total		Count	44	36	80	
		%	100.00%	100.00%	100.00%	

Table 3. Comparison between Colon Cancer Patients and Control Groups in Age

			Groups		Total
			Patients	Control	
Age groups	≤20	Count	0	6	6
		%	0.00%	16.70%	7.50%
	21-41	Count	12	12	24
		%	27.30%	33.30%	30.00%
	42-62	Count	22	14	36
		%	50.00%	38.90%	45.00%
	≥63	Count	10	4	14
		%	22.70%	11.10%	17.50%
Total		Count	44	36	80
		%	100.00%	100.00%	100.00%
Mean± SD			50.6136± 13.42198	38.6389± 17.32736	-
SE			2.02344	2.88789	-
p-value			0.001**		

Data were expressed as mean ± SD; Statistical analyses were performed by ANOVA. SD, Std. Deviation; SE, Std. Error of Mean; NS, no significant difference.

defensive immunological reaction that might have avoided serious consequences. while the patients' decreased IgG positive may indicate: a-a distinct pathogenic process in individuals in which *H. pylori* might not be the primary cause. b-IgG negative in certain people may be the result of a recent or temporary infection.

Figure 1, demonstrates the distribution of anti–Helicobacter pylori IgG antibodies among patients infected with *H. pylori* and the control group.

In the IgG-negative category, a higher number of negative cases was observed among patients (n = 10) compared with controls (n = 2). Conversely, in the IgG-positive category, both patients and controls showed an equal number of positive cases (n = 34 for each group) (Figure 2).

Table 4. Frequency of Site of Tumor in Colon Cancer Patients

Patients		Frequency	Percent %
Valid	appendiceal or Colonic	1	2.3
	appendix, tip	1	2.3
	Ascending Colon	1	2.3
	Colon	18	40.9
	Colon from liver metastasis	1	2.3
	Colonic mucosa	1	2.3
	Ileocecal valve	1	2.3
	Rectal	12	27.3
	Recto-sigmoid junction mucinous	2	4.5
	rectosigmoid	3	6.8
	Right Colon & appendix	1	2.3
	Sigmoid Colon	2	4.5
	Total	44	100

Changes in expression levels of tumorigenesis genes

The current study showed a significance decreased of expression level of *BRAF* gene with p value 0.01in comparing between patients and control group, Supplementary Table 3.

The current study showed a significance decreased of expression level of *ELASTIN* gene (fold change was reduced 10 times) with p value 0.001in comparing between patients and control (Supplementary Table 4). The current study showed a significance decreased of expression level of *TIMP3* gene (fold change was reduces in to half) with p value 0.001in comparing between patients and control group, Supplementary Table 5.

The current study showed a significance increased of expression level of *K-RAS* gene (fold change elevated to double) with p value 0.01in comparing between patients and control group, Supplementary Table 6.

Table 5. Frequency of Type of Tumor in Colon Cancer Patients

Patients		Frequency	Percent %
Valid	adenocarcinoma	7	15.9
	moderate differentiated glands & desmoplasia	2	4.5
	moderate to poorly differentiated	2	4.5
	moderate differentiated adenocarcinoma	25	56.8
	mucinous adenocarcinoma	3	6.8
	mucinous neoplasm (LAMN)	1	2.3
	Poorly differentiated adenocarcinoma	1	2.3
	Well differentiated adenocarcinoma	2	4.5
	well differentiated neuroendocrine	1	2.3
	Total	44	100

As comparison between presence of IgG and expression level of *BRAF*, *ELASTIN*, *TIMP3* and *K-RAS*, there was a significance relationship Ig G and IgA at p value 0.005 and 0.01 respectively, but no significance relationship with the presence of IgM, that may related to persistence of chronic infection of H.pylori in patients causes inflammation as a consequences reduces in the expression level of some genes such as *TIMP3*, thus aid in initiating of colon cancer, Supplementary Table 7, 8, 9.

Discussion

In the current study male was more susceptible to get colon cancer than female especially in age more than 50 years. This may relate to differences in life style or sex hormone or diet habit such as eating spacy food or smoking with aging human body defenses become weak with recurrent infection and accumulation of inflammatory products may causes mutations thus progress of cancer. Men are more likely to have a diet high in red and processed meat, be heavier consumers of alcohol, and more likely to smoke [16, 17]. Men also have a greater propensity to deposit visceral fat which is associated with increased risk of CRC [18].

An epidemiological study for colon cancer in Iraq included 73 patients from 2014-2016, they found that males are more prone to get colon cancer than females between 50 and 60 years, and site of cancer was Right

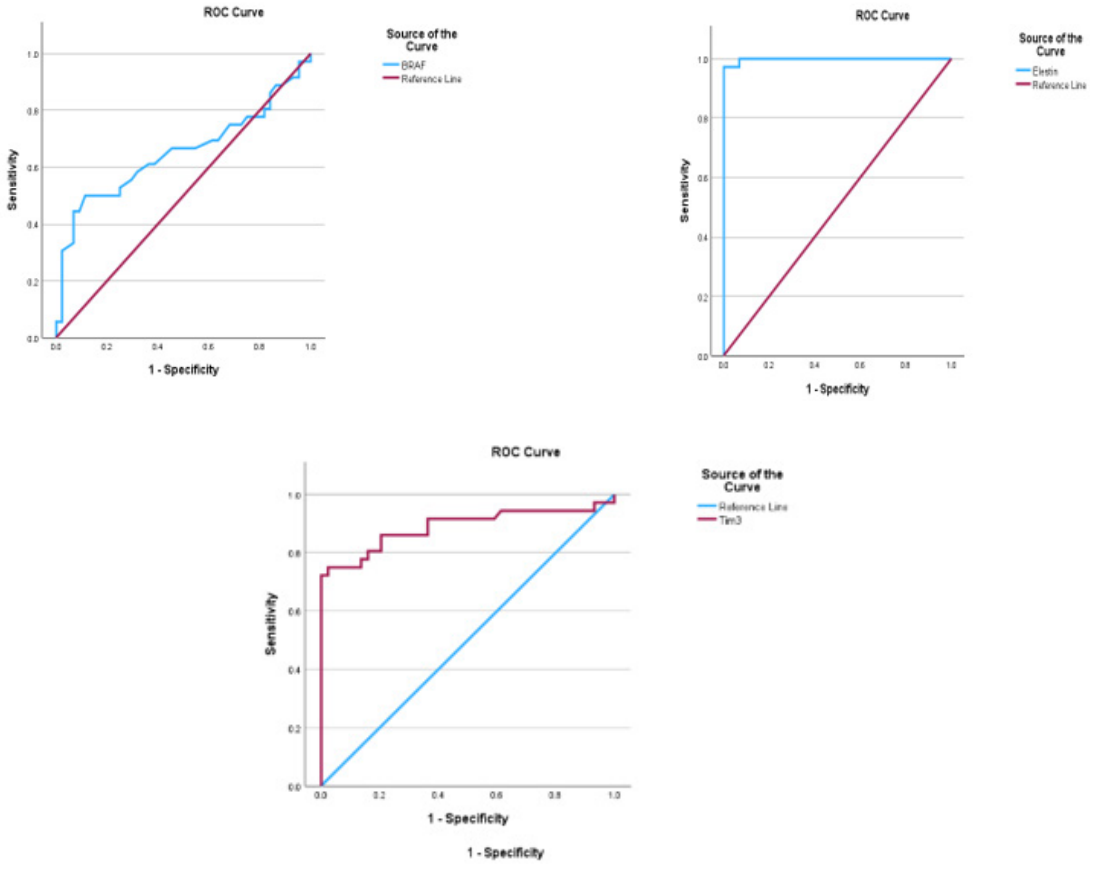

Figure 2. ROC Curve for Each Gene

colon cancer is more prevalent [19]. Another Iraqi study in 2023 described the main symptoms and main characteristics of CRC on 79 patients, they found in spite of older people are more likely to develop CRC, but young people can be affected and both younger and older individuals both had similar symptoms and clinicopathologic features which is bleeding per rectum [20]. In the current study, highest site frequency was in colon (40%), followed by rectal with 27.3% but highest colon cancer type was moderate differentiated adenocarcinoma with frequency (56%), despite the fact that colon cancer is categorized based on whether it is rectal or colon, yet It has been observed that there are differences between colon and rectal cancer with regard to etiology, genetics, anatomy, clinical manifestation, biological characteristic, responsiveness to treatment, and clinical outcomes. Diet, smoking, and physical activity are examples of lifestyle factors that have distinct effects on colon cancer than on rectal cancer. Treatments for colon and rectal cancer vary based on TNM stage. Colon and rectal cancers in stages I and IV are often treated as one single entity [21-23]. In a Chinese study, 145 patients with rectal cancer and 57 patients with right-sided colon cancer (RSCC), left-sided colon cancer (LSCC), and colon cancer were divided into three groups based on the location of the tumor. In order to assess the survival rates, they discovered that RSCC has a higher tumor size, poor differentiation, an advanced TNM stage, and a shorter survival time than LSCC and rectal cancer. Instead of a genetic difference, the advanced tumor stage in RSCC may be caused by the tumor's inherent proximal colon location feature. This could explain the shorter survival rate [24].

In the current study there was a significance presence of IgG antibodies against H.pylori. IgG for *H. pylori* is significantly higher in the control group (94.4%) than in patients (77.3%), with a p-value of 0.03. Patients are more likely to have negative IgG. The reason for this difference is unclear but could relate to differences in immune response, disease pathophysiology, or sampling bias. Several studies indicated that H.pylori induced colon cancer as a unique *H. pylori*-driven immune alteration signature characterized via a in regulatory T cells reduction and pro-inflammatory T cells. In addition, in the intestinal and colonic epithelium, *H. pylori* induced pro-carcinogenic STAT3 signaling and a loss of goblet cells, alters that have been shown to contribute—in combination with pro-inflammatory and mucus degrading microbial signatures to tumor development. Similar immune and epithelial alterations were found in human colon biopsies from *H. pylori*-infected patients [25]. Another suggested mechanism is chronic Inflammation with loss of cell cycle additionally the presence of CagA in *H. pylori* is associated with an increased risk of cancer. As infection causes the increasing of serum gastrin secretion, which can act as a growth hormone for the colonic mucosa cells. *H. pylori* can cause hypergastrinemia alone or together with changes in the normal gastrointestinal flora or modification of gut microbiota, suggesting an acceptable mechanism for the carcinogenicity of this organism [26].

An Iranian study in 2018 included 50 patients (33 adenomatous polyp and 17 colon cancer) investigated the association of *H. pylori* infection and the risk of adenomatous polyps and colon cancer and proposed that *H. pylori* infection can be considered as a risk factor for adenomatous polyps and colon cancer [27]. The current study showed a significance decreased of expression level of *BRAF* gene, as its role in cell the abnormal expression of *BRAF* genes effects in cell signaling activity, thus initiating of cancer. When comparing colon cancer patients to healthy controls, this study reveals notable changes in gene expression levels. Particularly with regard to *H. pylori* infection, the results point to a possible interaction between immune responses, genetic regulation, and chronic inflammation.

The current study showed a significance decreased of expression level of *ELASTIN* gene, a study in China investigate the role of ELA in initiating cancer, they found When CRC patient tumors were compared to nearby non-tumor tissues and healthy controls, there was an increase in the expression of the ELN gene. ELN protein was found to be higher in cancer cells than in healthy colon epithelial cells, indicating that ELN controls the growth of tumors and the surrounding environment in colorectal cancer [28]. Extracellular matrix (ECM) proteins regulate tumor growth and development in CRC. *ELASTIN* (ELN) is a component of ECM proteins involved in the tumor microenvironment [29]. The current study showed a significance decreased of expression level of *TIMP3* gene. As its role in inhibition cancer any reduction of gene expression level causes cancer. As a study in Taiwan in 2019 used MPT0B390 as inducer for restore expression of *TIMP3* genes and concluded the potential therapeutic applications of the *TIMP3* inducer, MPT0B390, for colorectal cancer treatment [30]. Tissue inhibitor of metalloproteinases-3 (*TIMP3*) is vital in regulating several biological processes. *TIMP3* exerts antitumour effects via matrix metalloproteinase (MMP)-dependent and MMP-independent pathways. Due to promoter methylation and miRNA binding, *TIMP3* expression has been observed to decrease in various cancers [6].

The current study showed a significance increased of expression level of *K-RAS* gene (fold change elevated to double). KRAS, a member of the RAS gene family, is one of the most studied oncogenes present in the short arm of chromosome 12. Among the three human RAS genes, namely, KRAS, NRAS, and HRAS, KRAS is reported to be the most frequently mutated gene. The KRAS gene encodes a 21kD KRAS protein involved in intracellular signal trans- duction processes. KRAS protein is activated upon binding with GTP, which is mediated by intracellular signals [31]. The significant reduction suggests that *TIMP3*-mediated immune suppression is compromised, possibly leading to a prolonged inflammatory response rather than immune escape. This supports the hypothesis that chronic *H. pylori* infection may drive inflammation, altering immune checkpoint expression. *K-RAS* mutations are one of the most common oncogenic drivers in colon cancer, leading to uncontrolled cell growth. A 2-fold increase in its expression suggests that *K-RAS* activation might be a dominant driver of tumorigenesis in these patients.

In conclusion, this study provides compelling evidence

of an association between Helicobacter pylori infection and colorectal cancer (CRC), highlighting its potential role in tumor progression. The high prevalence of *H. pylori* among CRC patients, particularly strains expressing CagA, suggests a link between bacterial infection, chronic inflammation, and genetic alterations contributing to colorectal carcinogenesis. Molecular analysis revealed significant downregulation of *TIMP3*, which may impair extracellular matrix regulation and promote tumor invasion.

Finally, *H. pylori* infection may serve as a significant risk factor for CRC by inducing chronic inflammation and driving oncogenic genetic mutations. These findings underscore the need for further studies to explore targeted prevention strategies, early diagnostic biomarkers, and potential therapeutic interventions aimed at mitigating the impact of *H. pylori*-related inflammation on colorectal cancer development.

Author Contribution Statement

All authors contributed equally in this study.

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