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CYP24A1, *AHR*, *CPEB4*, *TRIP13*, and *PIK3CA* genes expression in colorectal cancer patients: novel diagnostic biomarkers

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Abstract. – OBJECTIVE: This study aimed to investigate the *CYP24A1*, *AHR*, *CPEB4*, *TRIP13*, and *PIK3CA* mRNA expression in the blood of colorectal cancer patients in Egypt. This was performed to elucidate if there's a link between this gene expression and other clinicopathological characteristics of the tumor.

PATIENTS AND METHODS: A case-control study including 50 colorectal cancer patients and 50 healthy controls was conducted. Real-time polymerase chain reaction (rt-PCR) was utilized to assess the expression of *CYP24A1*, *AHR*, *CPEB4*, *TRIP13*, and *PIK3CA* mRNA in blood samples.

RESULTS: Patients with colorectal cancer had significantly higher levels of mRNA for the genes *CYP24A1*, *AHR*, *CPEB4*, *TRIP13*, and *PIK-3CA* ($p<0.001$, $p=0.021$, $p<0.001$, and $p<0.001$, respectively) compared to controls. Remarkedly, the gene expression of *AHR*, *TRIP13*, and *PIK-3CA* genes did not exhibit a significant correlation with the tumor stages ($p=0.379$, $p=0.095$, and $p=0.526$, respectively). However, there was a strong correlation between *CYP24A1* and *CPEB4* gene expression and tumor stages ($p<0.001$ and $p=0.002$, respectively).

CONCLUSIONS: Therefore, we can conclude that increased mRNA levels of *CYP24A1*, *AHR*, *CPEB4*, *TRIP13*, and *PIK3CA* in blood samples withdrawn from colorectal cancer patients could be a biomarker for the disease.

Key Words:
Biomarker, Carcinogenesis, Colorectal cancer, Gene expression profiling, Peripheral blood.

Introduction

Colorectal cancers start in the colon or rectum of the human body. Colorectal cancer is one of the most frequent reasons for cancer-related fatalities and the third most prevalent cancer type globally[1]. Early diagnosis of such cancer is connected to the discovery of gene biomarkers and the advancement of diagnosis techniques, all of which could improve the disease prognosis[2]. In research on carcinogenesis, gene expression profiling is used to discover specific changes in gene expression associated with the emergence of tumors and identify and categorize tumors according to their molecular characteristics[3].

There are startling differences between the gene expression patterns of adenoma and normal mucosa. According to numerous investiga-

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tions[4-10], it was reported that several gene groups have various expression patterns that help distinguish between cancerous and healthy tissues. The mitochondrial enzyme Cytochrome P450 Family 24 Subfamily A Member 1 (CYP24A1) reduces the antiproliferative effects of endogenously produced 1,25-2D3 while converting 1,25-2D3 (calcitriol) into a 24-hydroxylated (1,24,25 Trihydroxy vitamin3) product[11]. It is now categorized as an oncogene because of the altered expression observed in various cancers, including colorectal cancer (CRC)[12].

There are numerous ligands for the aryl hydrocarbon receptor (AHR). Most polycyclic hydrocarbons of tobacco smoke are aromatic hydrocarbons (dioxins). *AHR* gene overexpression is brought on by long-term environmental exposure, which has various detrimental effects, including immunological toxicity and human carcinogenicity[13]. Cytoplasmic Polyadenylation Element Binding Protein 4 (*CPEB*4) related mRNAs are substantially increased in several cellular processes crucial for carcinogenesis[14]. Thyroid hormone receptor interactor 13 (TRIP13) is essential for meiotic recombination, the spindle checkpoint, and chromosomal synapses[15].

According to different studies[16-18], numerous neoplasms have been shown to overexpress *TRIP*13. *In vitro*, TRIP13 may encourage the invasion, migration, and proliferation of colorectal cancer cells. In the Epidermal Growth Factor Receptor (EGFR) tyrosine-kinase domain, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) is a proto-oncogene that produces phosphatidylinositol-3-kinases (PI3K), which lead to the phosphorylation of AKT (cellular homolog of murine thymoma virus akt8 oncogene) and the AKT-mTOR (mammalian target of rapamycin) signal pathway. In human cancers, the phosphoinositol-3-kinase (PI3K) pathway was identified as an enzyme activity linked to a viral oncoprotein. The pleckstrin-homology (PH) domain of the serine/threonine kinase AKT is directly contacted by (phosphatidylinositol-3 kinase) PIP3 to bring AKT to the plasma membrane, where it is phosphorylated at Thr308 and Ser473. The 3-phosphoinositide-dependent protein kinase PDK1 phosphorylates Thr308, whereas Ser473 is phosphorylated by a molecularly undetermined kinase frequently referred to as PDK2[19].

Many biological processes, including proliferation, apoptosis, and growth, are regulated by AKT, which functions downstream of PI3K. However, other signaling pathways are also known to be regulated by PI3K activity and may be involved in PI3K-mediated carcinogenesis[19]. This pathway has received considerable attention in the studies[19] performed on human cancer since it is necessary for glucose metabolism, protein synthesis, the cell cycle, proliferation, growth, and survival. *CPEB*4, *CYP*24A1, *AHR*, *TRIP*13, and *PIK*3CA have been specifically named as a set of likely candidates for tumor development out of all the genes reported in the literature as having a potential impact on the development of tumors.

This research aimed to investigate these genes' expression in tumor cells in colorectal cancer patients' blood in Egypt in addition to correlating their expression and other tumor clinicopathological characteristics.

Patients and Methods

Subjects

Mansoura Faculty of Medicine's Ethical Committee approved this study with code: R.21.05.1339.R1.R2. Before participating in this study, all subjects signed a written consent form. We obtained clinical information and biochemical findings from patient medical records. The current case-control study, which had 100 participants (50 CRC patients and 50 controls), was conducted at the Faculty of Medicine, Mansoura University, Egypt, in the medical biochemistry, general surgery, and tropical medicine departments. Between June 2019 and May 2021, one hundred individuals were separated into /two groups: fifty CRC patients were recruited from Mansoura University Hospitals for the first group and fifty normal individuals for the second group.

CRC was diagnosed according to American College of Gastroenterology (ACG) guidelines[20]. This study excluded patients with malignancies other than colorectal cancer, patients with chronic inflammatory disorders, patients with septicemia, patients with liver cirrhosis, patients with pancreatitis, smokers, patients with inflammatory bowel diseases, and patients who received any chemotherapeutic agents. Every patient and member of the control group underwent a thorough physical examination as well as standard laboratory tests. These tests included a complete blood count, virology markers (hepatitis B virus antibody, hepatitis B surface antigen, and human immune deficiency virus antibody), liver function tests (serum

albumin, serum bilirubin, serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), and prothrombin time), serum creatinine, and tumor markers [carcinoembryonic antigen or (CEA) and carbohydrate antigen 19-9 (CA 19-9)]. Also, according to the tumor, node, and metastasis (TNM) staging, colorectal cancer staging was carried out[21].

RNA Extraction

RNA extraction was performed from blood samples in all the study groups. We gathered (5 mL blood samples in EDTA). In an adequately sized tube, one volume of whole human blood was mixed with five volumes of erythrocyte lysis (EL Buffer). The tube was swiftly vortexed twice while incubating on ice for 10 to 15 minutes. Following incubation, the tube was separated from the supernatant and centrifuged. A pellet of leukocytes will form after centrifugation, and RNA was extracted using the Mini Kit miRNeasy described by Qiagen manufacturer (Valencia, CA, USA). Total RNA from the nucleated cells was taken out.

RT-PCR

Supplementary Table I illustrates the *B-actin* and selected genes' primer sequences. Primer 3 software (https://primer3.ut.ee/) was used to create primer sets for the *CYP*24A1 gene, *AHR* gene, *CPEB*4 gene, *TRIP*13 gene, *PIK*3CA gene, and *B-actin* gene. Primers were designed to span exon-exon junction to preclude amplification of genomic DNA (Primers were chosen and designed so that one half hybridizes to the 3 ends of one exon and the other half to the 5 ends the adjacent exon of the target genes). *CYP24A1*, *AHR*, *CPEB4*, and *PIK3CA* genes were verified in The Cancer Genome Atlas Program (TCGA) data, while there was no data about *TRIP13* gene in TCGA data. Two Micrograms (ug) of RNA were added to reverse transcription with random primers using Gene-specific PCR primers purchased from Vivantis Technologies Sdn Bhd (Selangor, Malaysia) in proportion to the manufacturer's directions. As a control gene, we checked the integrity of cDNA using the amplification of *B-actin* gene simultaneously. The PCR amplifications were performed using a thermocycler (Applied Biosystem 7500, CA, USA) **Supplementary Table II**.

Statistical Analysis

The data was entered into and evaluated using Microsoft Excel. The Statistical Package for the Social Sciences (SPSS) 27.0 (IBM Corp., Armonk, NY, USA) was used to import and analyze the data. In categorical data, the baseline characteristics of the study population were presented as frequencies and percentages (%). On the other hand, the non-parametric quantitative data were exhibited as median and interquartile ranges, while mean values and standard deviations (SD) were utilized for the quantitative parametric data.

The Chi-Square and Fisher's exact tests were used to compare two or more distinct groups of qualitative data. Also, an independent *t*-test was performed to compare two quantitative parametric data sets. For continuous data, a Mann-Whitney test was performed to look for a significant difference between two groups that didn't have a normal distribution. The Registrars of Companies (ROC) curve assessed a test's diagnostic performance or precision in separating diseased and non-diseased cases. p-values < 0.05 and 0.01 were regarded as very significant and significant, respectively.

Results

Demographic Data of Both Groups and Tumor Characterization in the Colorectal Cancer Group

The two primary groups in this study were the 50 colorectal cancer patients (first group), who had an average age of 55.86±11.87 years and were made up of 22 men and 28 women, and the 50 healthy control subjects (second group), who had an average age of 54.04±15.45 years. In the colorectal group, 49 patients had the colon as their primary site of tumor growth, including 7 in the ascending colon, 5 in the caecum, 7 in the descending colon, 8 in the hepatic flexure, 12 in the sigmoid colon, 6 in the splenic flexure, and 4 in the transverse colon. There were 7, 16, 26, and 1 patients in stages I, II, III, and IV, respectively. Regarding the histopathology of colorectal carcinoma, 42 individuals had adenocarcinomas with moderate differentiation, 4 had adenocarcinomas with poor differentiation, 3 had villus carcinoma, and 1 had signet ring cell carcinoma (Figure 1).

Gene Expression Analysis

*CYP*24A1, *AHR*, *CPEB*4, *TRIP*13, and *PIK*-3CA had significantly higher levels of mRNA in the blood of colorectal cancer patients when com-

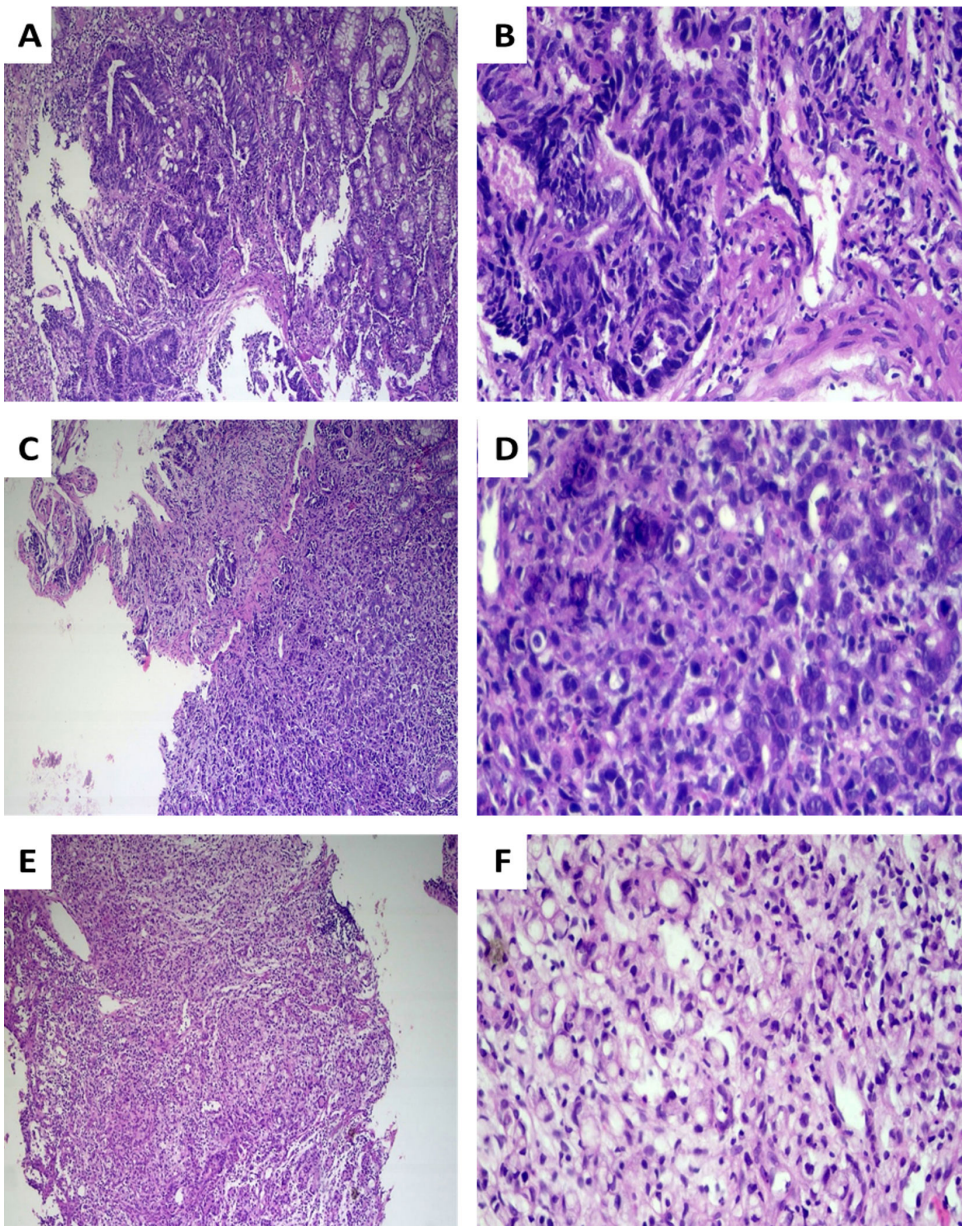

Figure 1. Moderately differentiated adenocarcinoma (**A**) at 200× and (**B**) at 400×, showing infiltration by malignant tumor tissue formed of small sheets and atypical glandular structures surrounded by desmoplastic stroma. The neoplastic epithelial cells show moderate atypia with hyperchromatic nuclei. Poorly differentiated adenocarcinoma (**C**) at 200× and (**D**) at 400×, showing tumor tissue formed of sheets and little acini lined by malignant epithelial cells and separated by fibrous tissue stroma. This exhibits a moderate to high degree of nuclear pleomorphism. Invasive signet ring cell carcinoma (**E**) at 200× and (**F**) at 400× shows tumor tissue formed of atypical cells with signet ring-like cell morphology. The cells show a high degree of nuclear pleomorphism.

pared to the controls ($p<0.001$, $p=0.021$, $p<0.001$, $p<0.001$, and $p=0.006$ respectively) (**Supplementary Table III** and Figure 2).

ROC Curve Analysis for Gene Expression to Identify Colorectal Cancer

The *CYP*24A1 gene's cut-off value was 0.89, with a sensitivity of 80%, a specificity of 6%, and an area under the curve (AUC) of 0.813. AUC was 0.634, sensitivity 82%, specificity 46%, and 0.795 was the cut-off value for the *AHR* gene. The *CPEB*4 gene's cut-off value was 0.875, with a sensitivity of 72%, a specificity of 58%, and an AUC of 0.718. The *TRIP*13 gene's cut-off value was 0.895, with a sensitivity of 74%, a specificity of 64%, and an AUC of 0.725. The *PIK*3CA

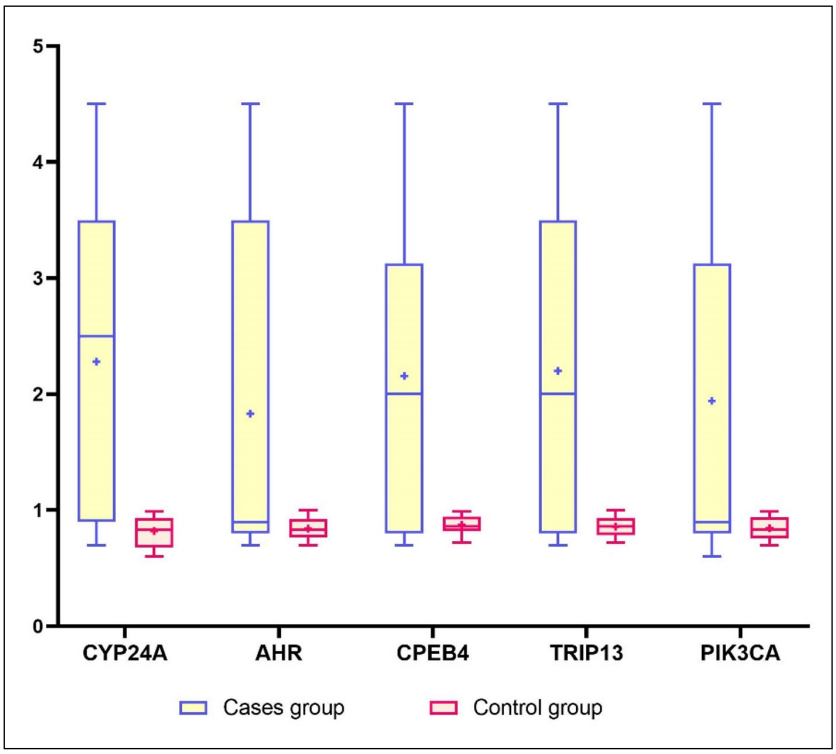

Figure 2. Gene expression analysis in both groups.

gene's cut-off value was 0.795, with a sensitivity of 86%, a specificity of 40%, and an AUC of 0.660 (**Supplementary Table IV** and **Supplementary Figure 1**).

Association of Gene Expression Levels and Tumor Staging

There was a significant association between *CYP*24A1 and *CPEB*4 gene expression and tumor

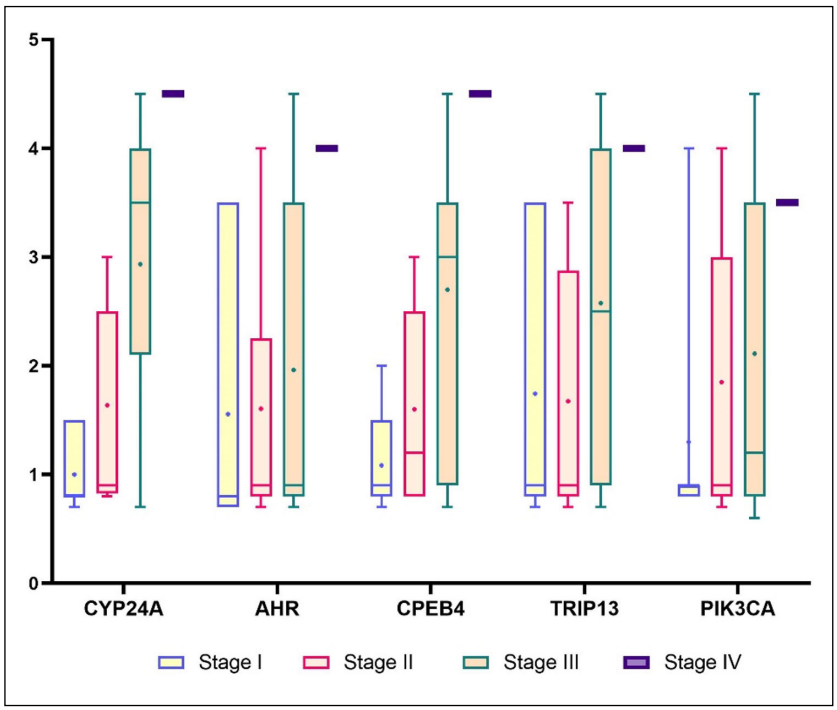

Figure 3. Association of gene expression levels and tumor staging.

Table I. Association of gene expression levels and tumor histopathology.

Stage	Number	CYP24A	AHR	CPEB4	TRIP13	PIK3CA
Moderately differentiated	42	2.75 (0.7-4.5)	0.9 (0.7-4.5)	2.25 (0.7-4.5)	2.5 (0.7-4.5)	0.9 (0.6-4.5)
Poorly differentiated	4	1.5 (0.8-4)	0.85 (0.7-1.5)	1.65 (0.8-4)	0.9 (0.7-4.5)	1.7 (0.9-3)
Signet ring cell carcinoma	1	3.5	0.9	3	2.5	0.8
Villous carcinoma	3	0.8 (0.7-0.9)	0.8 (0.7-1.5)	0.9 (0.8-0.9)	0.9 (0.9-2)	0.8 (0.8-0.9)
p-value		0.123	0.561	0.499	0.807	0.543

staging ($p<0.001$ and $p=0.002$, respectively). At the same time, we observed no significant association between *AHR*, *TRIP*13, and *PIK*3CA gene expression and tumor staging ($p=0.379$, $p=0.095$, and $p=0.526$, respectively) (**Supplementary Table V** and Figure 3).

Association of Gene Expression Levels and Tumor Histopathology

There was no significant association between *CYP*24A1, *AHR*, *CPEB*4, *TRIP*13, and *PIK*3CA gene expression levels and histopathological characters of the tumor ($p=0.123$, $p=0.561$, $p=0.499$, $p=0.807$, and $p=0.543$, respectively) (Table I).

Discussion

The different patterns of expression of the biomarkers could be used as a tool to support already-present histological elements in patient follow-up and treatment strategies in colorectal cancer.

Colon cancer is typically found at an advanced tumor stage, with a very low survival rate, despite the growing importance of screening procedures. Different gene expression patterns can be observed as colon cancer develops, as is well known. Based on all genome expression, previous research[22] aims to identify, create, and use therapeutically useful biomarkers for routine tumor classification diagnoses. To the best of our knowledge, this study is the first to elucidate the influence of different gene expressions in patients with colorectal cancer in Egypt. Our study outcomes could have a significant consequence for early diagnosis. In addition, it is planned to create and use useful biomarkers in routine tumor categorization diagnosis based on all genome expression investigations.

In this current study, *CYP*24A1 mRNA level was upregulated in colorectal cancer patients compared to controls. According to Androutsopoulos et al[23], active CYP1 enzymes were overexpressed in 35% of colon cancer cases. In contrast, *CYP*1A1 was overexpressed in 80% of colon cancer cases, whereas the mRNA of *CYP*1B1 was overexpressed in 60% of cases. Additionally, Androutsopoulos et al[23] discovered that bladder and colon cancers had increased mean *CYP*1B1 and *CYP*1A1 mRNA levels and mean CYP1 activity when compared to normal tissues ($p<0.05$).

This study showed a significant link between the expression of *CYP*24A1 and the tumor stage. This contrasts with Androutsopoulos et al[23], who found that CYP1 expression levels were independent of TNM status. Aldrghi et al[24] found a significant relationship between the gene expression of *CYP*24A1 and colorectal cancer. Still, it differs from our study that the samples were colon cancer tissue and control healthy tissue samples. These findings were in agreement with Sun et al[25], who found that the expression of *CYP*24A1 is strongly correlated with the development of CRC and may represent a unique predictive biomarker for CRC and can be used as a prognostic marker as it is correlated with tumor depth and tumor progression stages[25].

In the present investigation, colorectal cancer patients had higher *AHR* mRNA levels than healthy controls. Colon cancer cells exhibit moderate levels of *AHR* expression, according to the mRNA expression of *AHR* of 967 human cancer cell lines. However, it is unclear how these levels relate to those of normal intestinal epithelial cells[26]. Aldrghi et al[24] found a highly significant relationship between *AHR* gene expression and colorectal cancer. Still, it differs from our study that the samples were colon cancer tissue and control healthy tissue samples. The different expression rates of the *AHR* gene may be due to racial distribution, different sample sizes, and different technical methods for detection.

A link between aberrant *CPEB*4 expression and some cancer types suggests that *CPEB*4 may

considerably control cancer growth and metastasis[27]. It is particularly recommended that CPEB4 is a target for cancer treatment since it is thought to be crucial for cancer cell migration and invasion[28]. Furthermore, it is crucial to comprehend the biology of colorectal cancer, identify RNA-binding proteins associated with the disease, and perhaps establish new objectives for the prognostic biomarkers[29]. According to research[30], high mRNA levels have been associated with advanced tumor stages, metastasis, as well as reduced prognosis in individuals with colorectal cancer. *CPEB*4 is expressed at high levels in colorectal cancer tissues.

According to the current investigation, patients with colorectal cancer had higher levels of *CPEB*4 mRNA than healthy controls, which was consistent with Söylemez et al[31]. In contrast to our research, Xu and Liu[27] discovered that prostate cancer and the surrounding tissues had lower levels of *CPEB*4 mRNA than the control. According to the investigations, *CPEB*4 has been found in various types of tumor tissues. Given that *CPEB*4 has been identified as highly expressed in different types of tumors, it is believed to exert preoncogenic effects on tumor development, invasion, and vascularization. Another study[32] discovered that while pancreatic ductal cancer increased *CPEB*4 gene expression, hepatocellular carcinoma displayed a decrease. It is thought that *CPEB*4 is overexpressed in several malignancies, such as kidney, skin, and colorectal cancers, and that this overexpression may be advantageous for tumor development. High expression levels of *CPEB*4 are linked to a low prognosis in colorectal cancer. In addition, its potential involvement in tumor invasion and metastasis has been hypothesized[33]. Similar to this study, it has been noted that cases of colorectal cancer have significant levels of *CPEB*4 expression in their peripheral blood[34].

It has been established that *TRIP*13 is a protein localized in the kinetochore and facilitates proper cell division. Many kinetochore-localized proteins are abundantly generated in various malignancies[35]. *TRIP*13 has been associated with elevated expression or amplification in several types of human cancer[36]. In our study, the amount of *TRIP*13 mRNA significantly increased, in agreement with Söylemez et al[31]. Similar research was conducted by Kurita et al[37] on the variation in *TRIP*13 mRNA levels between cancer and normal tissues. They stated that *TRIP*13 is involved in developing and invading colorectal cancer cells and might be a marker for the illness. The advanced *TNM* stage was significantly linked with higher *TRIP*13 expression, according to Sheng et al[3]. It is important to emphasize that accurate chromosomal segregation in this scenario depends on the expression and function of the *TRIP*13 gene[38]. Thus, *TRIP*13 overexpression may be a general characteristic of colorectal cancer and a potential biomarker or signal for the disease's detection.

The proto-oncogene *PIK*3CA, which codes for phosphatidylinositol-3-kinases, is in the EGFR tyrosine-kinase domain (PI3K). As a result, the protein kinase B enzyme AKT is phosphorylated, activating the AKTmTOR signaling pathway. The phosphoinositol-3-kinase (PI3K) pathway has been recognized as an enzyme activity connected to a viral oncoprotein in human malignancies. According to Söylemez et al[31], it was shown in our investigation that peripheral blood *PIK*3CA mRNA levels rose in comparison to the control group. Numerous cancers, including colorectal cancer, have mutations in the catalytic subunit of PI3K, known as *PIK*3CA. Exon 9 and exon 20 at two hot locations, which account for 10% to 20% of colorectal cancers, have been reported to include about 80% of *PIK*3CA mutations[39-41]. Yan et al[42] explored the possible role of the *PIK*3CA mutation in colorectal cancer treatment. We looked at the relationship between *PIK*3CA mutation and first-line treatment response in 440 colorectal cancer patients' medical data.

*PIK*3CA gene mutations have been discovered to occur 9.55 percent of the time in colorectal cancer patients, and they have been linked to late TNM staging and low histological grade. Primary chemotherapy has been shown to have a worse response in colorectal cancer patients with the *PIK*-3CA mutation than in people without the mutation. Both *in vitro* and *in vivo* testing revealed low sensitivity of *PIK*3CA mutant tumor cells to first-line chemotherapy. The findings showed that the *PIK*3CA mutation activates PI3K/Akt, promoting chemotherapy-resistant colorectal cancer stem cells' survival and growth. Multiple meta-analyses[43-46] found that *PIK*3CA exon 20 mutations may be a sign of resistance to anti-EGFR therapy. Combining mRNA analysis with mutation analyses is necessary to accurately identify the *PIK*-3CA effect in patients with colorectal cancer.

Limitations

The current study had potential limitations as it is a small sample study, only one patient was in stage IV of the tumor. It is a single-center study, so a larger-scale validation study is required to prove these results.

Conclusions

In conclusion, according to the study's findings, a spike in the mRNA levels of *CYP*24A1, *AHR*, *CPEB*4, *TRIP*13, and *PIK*3CA in the blood of persons with colorectal cancer may be a potential biomarker for the diagnosis of this disease. The study's findings suggest that the increase in the mRNA levels of *CYP*24A1, *AHR*, *CPEB*4, *TRIP*13, and *PIK*3CA could contribute to the development of colorectal cancer and can be used as an indication of disease occurrence. Our data contain genetic information that might help accurately detect and diagnose colorectal cancer.

Authors' Contributions

Conceptualization, Ahmed Mohamed El Nakib, Mohamed Elsaeed, Ramy A. Abdelsalam, and Sally Abdallah Mostafa; Data curation, Ramy A. Abdelsalam, Khalil Wafi, and Engy Elekhnawy; Formal analysis, Mohamed Elsaeed, Ramy A. Abdelsalam, and Sally Abdallah Mostafa; Investigation, Ahmed Mohamed El Nakib, Mohamed Elsaeed, Khalil Wafi, Walaa A Negm, and Sally Abdallah Mostafa; Methodology, Ahmed Mohamed El Nakib, Mohamed Elsaeed, Ramy A. Abdelsalam, Khalil Wafi, and Sally Abdallah Mostafa; Resources, Mohammed Alrouji, Mansour Alsaleem; Software, Mohammed Alrouji, Mansour Alsaleem; Validation, Engy Elekhnawy, Gaber El-Saber Batiha, and Wala A Negm; Writing – original draft, Ahmed Mohamed El Nakib, Khalil Wafi, Mohamed Elsaeed, Ramy A. Abdelsalam, Engy Elekhnawy, Walaa A Negm and Sally Abdallah Mostafa; Writing – review and editing, Ahmed Mohamed El Nakib, Khalil Wafi, Mohamed Elsaeed, Ramy A. Abdelsalam, Engy Elekhnawy, Nada H. Aljarba, Walaa A Negm, and Sally Abdallah Mostafa. All authors have read and agreed to the published version of the manuscript.

Ethics Approval

Mansoura Faculty of Medicine's Ethical Committee approved this study with code: R.21.05.1339.R1.R2.

Informed Consent

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

Data Availability

Not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

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