ORIGINAL ARTICLE

Expression Levels of *EIF4EBP1* in Colorectal Cancer: PBMCs versus Tissues

[1]Esraa H. Abd El-Ghani, [1]Mohamed A. El-Feky, [1]Salwa S. Eldin, [2]Mohamed A.S. Mahmoud, [1]Aliaa M.A.A. Ghandour*

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Egypt
[2]Department of Surgical Oncology, South Egypt Cancer Institute, Assiut University, Egypt

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Corresponding Author:
Aliaa Mahmoud Ali Ahmed Ghandour
Assistant professor of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Egypt
Tel.: 00201006199196
aliaaghandour@aun.edu.eg
aliaaghandour@yahoo.com

ABSTRACT

Background: *The autophagy is a physiological mechanism by which the cells break down and rapidly rebuild the cell components. The autophagy is crucial for the onset, the progression, the management, and the prognosis of colorectal cancer (CRC).* ***Objectives:*** *This study aimed to evaluate the diagnostic and the prognostic roles of the autophagy-related gene (ARG) eukaryotic translation factor 4E binding protein 1 (EIF4EBP1) in the CRC patients.* ***Methodology:*** *This study included 30 CRC patients and 30 healthy controls. The gene expression of EIF4EBP1 in the peripheral blood mononuclear cells (PBMCs) and the tissue using the real-time PCR (RT-PCR) was done.* ***Results and Conclusion:*** *The PBMCs and tissue expression levels of EIF4EBP1 were upregulated in the CRC cases with higher tissue expression than that of PBMCs. Receiver Operating Characteristic Curve (ROC) analysis showed the ability of EIF4EBP1 tissue and PBMCs expression levels to diagnose CRC and differentiate between grade II and III CRC.*

INTRODUCTION

Colorectal cancer (CRC) is considered the second deadliest and the third most prevalent malignancy globally [1]. Incidence rates especially through elderly decreased by approximately 1% year between 2011 and 2019 [2]. In industrialized nations, the prevalence of CRC is comparatively high and is still rising [3, 4].

Autophagy is a physiological process usually used by cells to break- down their components and swiftly rebuild them. In eukaryotes, autophagy is an intracellular mechanism that aids in the vacuole or lysosome's mass breakdown of cytoplasmic components. Coordination of many autophagy-related genes (ARGs) results in this conserved process [5]. Accumulating evidence suggests that autophagy enhances tumorigenesis by facilitating the acquirement of cancer hallmarks, and thus, its manipulation has been indicated as a promising approach to treating cancer [6].

Autophagy is crucial for CRC's onset, progression, management, and prognosis. It can prevent the growth and formation of tumors during the early stages of CRC. However, autophagy might increase tumor metabolism, mediate tumor resistance, and activate additional mechanisms that would encourage tumor growth when CRC worsens. Thus, there are numerous clinical applications for timing one's intervention in autophagy [7].

Eukaryotic translation factor 4E binding protein 1(*EIF4EBP1*) is a member of a family of translational repressor proteins that directly interact with eIF4E. Many ARGs and proteins can promote CRC metastasis. The high expression and activation of several mammalian targets of rapamycin (mTOR) signaling mediators, including mTOR, p70-S6 Kinase 1 (*S6K*), and *4E-BP1* in CRC, was reported. mTOR phosphorylates *4E-BP1* at variable sites, which facilitates the dissociation of *eIF4E* from *4E-BP1*, relieving the *4E-BP1* inhibitory effect on *eIF4E*-dependent translation initiation [8].Poor survival in diffuse large B-cell lymphoma, hepatocellular carcinoma, CRC, and all cancer entities has been linked to elevated *EIF4EBP1* levels. Nevertheless, there is insufficient evidence to determine the significance of *EIF4EBP1* expression as a prognostic tool in other distinct tumor types [9].

Currently, the most common clinical treatments for CRC are radiation, chemotherapy, and surgery [10].

Limited targeted therapy and a 5-year survival rate are available for advanced CRC cases. Drug resistance and severe side effects impair the effectiveness of chemotherapy, and the overall treatment outcome is still insufficient [11, 12].

Autophagy inducers may boost immunogenic cell death (ICD), increasing the effectiveness of some chemotherapy drugs [13]. Moreover, CRC is among the numerous cancer forms that benefit from immunotherapy, particularly in advanced stages of the

disease where conventional treatment is ineffective at preventing recurrence or produces a low survival rate [14]. Therefore, this study was carried out to evaluate the possible diagnostic and prognostic roles of *EIF4EBP1* in CRC.

METHODOLOGY

Ethical considerations

The study protocol was accepted by the local Ethical Committee of the Faculty of Medicine, Assiut University (IRB No.: 17200584) and was registered on Clinical Trials.gov (ID: NCT04729855). Informed consent was obtained from each participant in this study.

Study design

This case-control study was conducted in the Medical Microbiology and Immunology Department - Faculty of Medicine, South Egypt Cancer Institute and Medical Research Center at Assiut University.

The study included 30 CRC patients admitted to the Surgical Oncology Department, South Egypt Cancer Institute, from July 2021 to October 2023, and 30 healthy persons matched with the cases of CRC were included in the study as controls.

Clinical assessment of the included patients

Newly diagnosed CRC patients aged 25-65 were confirmed by histopathological examination and did not undergo any lines of treatment.

Samples collection and processing

Five milliliter fresh blood was withdrawn from each participant in EDTA blood collection tubes using a 5 ml sterile disposable plastic syringe (total number of blood samples: 30 from CRC cases + 30 from healthy controls = 60 blood samples). The separation of peripheral blood mononuclear cells (PBMCs) was done within 2 hours for best results [15].

Small-sized paired tissue samples were taken: one from the cancer tissue and another from the safety margin (at least 3 cm from the edge of the tumor) as a normal control tissue sample. The tissue sample was collected in a 1ml TRIzol™ reagent containing Eppendorf tube and stored at -80 °C [16].

Separation of PBMCs using Ficoll Histopaque-1077 (Biowest, France) (LOT: MS008Q1017) and RNA extraction from the whole blood using Invitrogen™ TRIzol™ reagent USA (Catalog Number: 15596026) was done.

Conversion of RNA into cDNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, USA) (Catalog. number #K1622) was done.

Gene expression by quantitative real-time PCR (q RT-PCR)

- Primers used for *EIF4EBP1g*ene [17] , and *GAPDH* (as housekeeping gene) [18] (Invitrogen, USA):

EIF4EBP1: Forward:5′-CCCGCTTATCTTCTGGGCTA-3′,
Reverse:5′-CTATGACCGGAAATTCCTGATGG-3′

GAPDH: Forward: 5′-CCCTTCATTGACCTCAACTA-3′
Reverse: 5′-TGGAAGATGGTGATGGGATT-3′

Components of RT-PCR

Maxima SYBR green master mix (2X) (Applied Biosystems, USA) (LOT: 00634195) (10 µL), Primer (forward) (1 µL), Primer (reverse) (1 µL) and DNase/RNase free water (3 µL) with a final volume equal to 25µl.The amplification process was done in Applied Biosystems 7500 Fast Real-Time PCR (serial no.275014287).

The PCR condition for all genes

Denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and finally annealing and extension at 60°C for 60 seconds. The relative gene expression was normalized to the level of *GAPDH* transcript, and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method [19].

Statistical analysis:

All statistical calculations were done using SPSS version 22. Data was statistically described in terms of mean ± standard deviation (±SD), or median and range when not normally distributed, frequencies (number of cases), and relative frequencies (percentages) when appropriate. Comparison of quantitative variables was done using Student t-test for normally distributed data and Mann Whitney U test as data was not normally distributed. Wilcoxon sign rank test was used to compare the paired quantitative data. For comparing categorical data, Chi-square ($\chi 2$) was used or Fisher Exact test instead of Chi-square ($\chi 2$) when expected frequency was less than 5. A receiver operating characteristic curve (ROC) analysis was used to find the best cut-off values to validate the prediction of CRC and its grade using studied biomarker. *P-value* is always a 2-tailed set significant at 0.05 level.

RESULTS

Demographic data of studied groups

Studied groups are matched in age and gender, with no statistically significant difference as shown in table (1).

Table 1: Demographic data of studied groups

Demographic data	Cases (n=30)		Controls (n=30)		*p*-value
Age (years)					0.917
• Mean ± SD	48.87 ± 6.94		49.03 ± 5.28		
• Median (range)	50 (32 – 64)		49 (38 – 61)		
Gender					0.766
• Male	23	(76.7%)	22	(73.3%)	
• Female	7	(23.3%)	8	(26.7%)	
Family history					
• Negative	10	(33.3%)			
• Positive	20	(66.7%)			

Data is presented as mean ± SD and median (range) or number (percentage). Significance is defined by *p-value* < 0.05.

Clinical and pathological data of the studied CRC cases

Bleeding per rectum was the most common clinical presentation among the studied CRC cases (83.3%); 66.7% presented with constipation, and 50% presented with loss of weight.

Regarding tumor site, recto-sigmoid cancer was the most common tumor site in 80%, 10% had ascending, and another 10% had descending colon tumors. 60% had a tumor size ≥ 3 cm, 56.7% had tumor grade III and (93.3%) had adenocarcinoma.

PBMCs and tissue expression levels of *EIF4EBP1* among studied groups

Table (2) shows that *EIF4EBP1*, both PBMCs and tissue levels, have statistically significant upregulation among the CRC cases compared to matched controls ($p < 0.001$).

By comparing PBMCs versus tissue expression of *EIF4EBP1* among CRC cases, tissue expression of both genes was significantly higher than PBMCs expression ($p < 0.001$).

PBMCs and tissue expression levels of *EIF4EBP1* among studied CRC cases according to different patients' characteristics, as shown in table (3)

Tissue expression was significantly higher than PBMCs expression in patients with either < 50 or ≥ 50 years old (p = 0.002 and 0.036), respectively, and among male patients (p <0.001). While PBMCs and tissue expression were comparable among female patients with no significant difference. Regarding family history of CRC, *EIF4EBP1* tissue expression among patients with a positive family history of CRC was significantly higher than PBMCs expression ($p < 0.001$). While PBMCs and tissue expression were comparable among those with a negative family history of CRC, with no significant difference between them. However, the *EIF4EBP1* PBMCs and tissue expression levels showed no significant difference according to age, sex, and family history of the studied CRC cases.

PBMCs and tissue expression levels of *EIF4EBP1* among studied CRC cases according to the patient's clinical presentation and tumor characteristics, as shown in table (4)

No significant difference was observed in *EIF4EBP1* expression levels either in PBMCs or in tissue and patients' clinical presentation or pathological type. Tissue expression levels were significantly higher than those of PBMCs in patients with tumor size < 3 cm or ≥ 3 cm (p = 0.050 and 0.002), respectively, in patients with tumor grade III (p = 0.001), in patients with recto-sigmoid tumor compared to those with colon cancer ($p < 0.001$) and in those with adenocarcinoma compared to mucinous type (p = 0.001). Furthermore, the *EIF4EBP1* tissue expression was significantly higher among patients with tumor size ≥ 3 cm compared to patients with tumor size < 3 cm (p = 0.004), and also *EIF4EBP1* tissue expression was significantly higher among patients with tumor grade III compared to patients with tumor grade II ($p < 0.001$). While *EIF4EBP1* PBMCs expression showed no significant difference according to tumor size and tumor grade of the studied CRC cases.

Table 2: Expression levels of *EIF4EBP1* in PBMCs versus tissue among the studied groups

Variable name	Cases (n=30)	Controls (n=30)	*p* value[1]
***EIF4EBP1* in PBMCs**			
• Mean ± SD	1.06 ± 0.54	0.48 ± 0.24	
• Median (range)	1.19 (0.04 – 1.78)	0.46 (0.09 – 0.85)	**<0.001**
***EIF4EBP1* in Tissue**			
• Mean ± SD	4.04 ± 6.40	0.56 ± 0.26	
• Median (range)	1.55 (0.45 – 33.61)	0.69 (0.10 – 0.97)	**<0.001**
***p* value[2]**	**<0.001**	0.165	

Data is presented as mean ± SD and median (range). Significance is defined by ***p-value*** < 0.05.
p value[1]: comparing both studied groups.
p value[2]: comparing PBMCs versus tissue samples in the same group separately.

Table 3: Relationship between expression levels of *EIF4EBP1* as regards different patients' characteristics

Demographic data	*EIF4EBP1* in PBMCs	*EIF4EBP1* in tissue	*p* value[1]
Age (years)			
< 50 years (n=15)	1.19 (0.04 – 1.78)	1.59 (0.45 – 33.61)	**0.002**
≥ 50 years (n=15)	1.19 (0.24 – 1.78)	1.51 (0.21 – 10.75)	**0.036**
***p* value[2]**	0.412	0.870	
Gender			
Male (n=23)	1.19 (0.04 – 1.78)	1.59 (0.45 – 33.61)	**<0.001**
Female (n=7)	1.19 (0.13 – 1.78)	1.42 (1.27 – 1.83)	0.310
***p* value[2]**	0.962	0.207	
Family history			
Negative (n=10)	1.50 (0.30 – 1.78)	1.43 (1.21 – 33.61)	0.333
Positive (n=20)	1.04 (0.04 – 1.78)	1.59 (0.45 – 12.19)	**<0.001**
***p* value[2]**	0.143	0.397	

Data is presented as median (range). Significance is defined by *p-value* < 0.05.
p value[1]: comparing PBMCs versus tissue samples. *p* value[2]: comparing the expression of the studied genes according to different patients' characteristics.

Table 4: Relationship between expression levels of *EIF4EBP1* as regards patients' tumor characteristics

Tumor characteristics	*EIF4EBP1* In PBMCs	*EIF4EBP1* in tissue	*p value*[1]
Tumor site			
Colon	1.01 (0.13 – 1.51)	1.41 (1.21 – 1.83)	0.173
Recto-sigmoid	1.19 (0.04 – 1.78)	1.59 (0.45 – 33.61)	**<0.001**
***p value*[2]**	0.561	0.129	
Pathological type			
Adenocarcinoma	1.19 (0.04 – 1.78)	1.55 (0.45 – 33.61)	**<0.001**
Mucinous	1.11 (0.73 – 1.49)	1.40 (1.21 – 1.59)	0.665
***p value*[2]**			
Tumor size			
< 3 cm (n=12)	0.88 (0.13 – 1.78)	1.45 (0.45 – 1.83)	**0.050**
≥ 3 cm (n=18)	1.26 (0.04 – 1.78)	2.12 (1.21 – 33.61)	**0.002**
***p value*[2]**	0.268	**0.004**	
Tumor grade			
GradeII (n=13)	1.04 (0.13 – 1.57)	1.39 (0.45 – 1.83)	0.064
GradeIII (n=17)	1.19 (0.04 – 1.78)	2.41 (1.27 – 33.61)	**0.001**
***p value*[2]**	0.363	**<0.001**	

Data is presented as median (range). Significance is defined by ***p-value*** < 0.05. *p-value*: comparing the expression of the studied genes according to different tumor characteristics.

The diagnostic and predictive ability of *EIF4EBP1* to detect CRC cases and differentiate CRC grades, as shown in table (5)

The diagnostic ability of EIF4EBP1 to detect CRC cases

The PBMCs expression levels were able to diagnose CRC at a cut-off value of ≥ 0.87; the AUC was 80.1% (95% CI: 0.680 – 0.922, $p < 0.001$) with a sensitivity of 66.7%, a specificity of 100.0% and accuracy of 83.3%. While *EIF4EBP1* tissue expression levels showed much more diagnostic ability to detect CRC at a cut-off value of ≥ 1.1, the AUC was 96.7% (95% CI: 0.931 – 1.0, $p < 0.001$) with a sensitivity of 96.7%, a specificity of 100.0%, and accuracy of 97.7% as shown in figure (1-a, b).

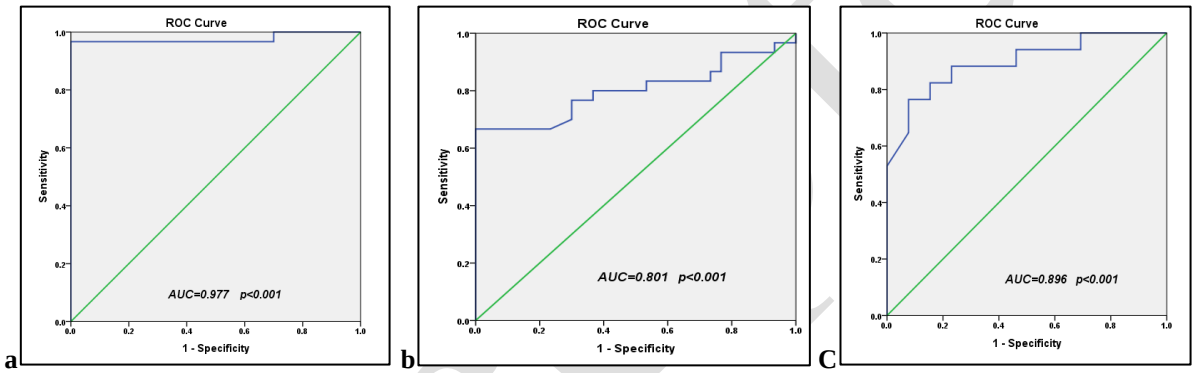
The predictive ability of EIF4EBP1 to differentiate between CRC grades

The PBMCs expression levels failed to differentiate between CRC grades at a cut-off value of ≥ 1.1; the area under the ROC curve was 60.2% (95% CI: 0.394 – 0.809, $p = 0.346$) with a sensitivity of 58.8%, a specificity of 53.8%, and accuracy of 56.7%. While tissue expression levels were able to differentiate between CRC grades at a cut-off value of ≥ 1.5, the area under the ROC curve was 89.6% (95%CI: 0.784 – 1.0, $p < 0.001$) with a sensitivity of 82.4%, a specificity of 84.6%, and accuracy of 83.3% as shown in figure (1).

Table 5: The best cut-off, sensitivity, and specificity for prediction of CRC and differentiating CRC grades by *EIF4EBP1* in PBMCs and tissue

Markers	Cut off	95%CI	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	*p-value*
EIF4EBP1 in PBMCs	≥ 0.87	0.680–0.922	66.7%	100.0%	100.0%	75.0%	83.3%	0.801	**<0.001**
EIF4EBP1 in tissue	≥ 1.1	0.931–1.0	96.7%	100.0%	100.0%	96.8%	98.3%	0.977	**<0.001**
EIF4EBP1 in PBMCs Grade III vs Grade II	≥ 1.1	0.394–0.809	58.8%	53.8%	62.5%	50.0%	56.7%	0.602	0.346
EIF4EBP1 in tissue Grade III vs Grade II	≥ 1.5	0.784–1.0	82.4%	84.6%	87.5%	78.6%	83.3%	0.896	**<0.001**

PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve; CI: confidence interval. *Significance is defined by *p-value* < 0.05.



a b C

Fig. 1: ROC curve for :(a) CRC detection in studied participants regarding expression levels of *EIF4EBP1* in PBMCs. (b) CRC detection in studied participants regarding expression levels of *EIF4EBP1* in tissues. (c) Prediction of CRC grades in studied cases regarding expression levels of *EIF4EBP1* in tissues. *EIF4EBP1* in PBMCs, tissue (blue), and reference line (green).

DISCUSSION

The present study investigated possible diagnostic and prognostic role of the ARG *EIF4EBP1* in CRC. This was done by comparing expression levels of the studied gene in PBMCs and tissue in CRC cases and healthy matched controls.

The mean age of CRC cases was 48.87 ± 6.94 which was consistent with Ali et al.[19] CRC was found to be more common in males (76.7%) than in females (23.3%), which was consistent with *Ali* et al.[19] and Ali Akbar-Esfahani et al. [20].

A higher incidence of CRC is found in males than females, with a 50% higher cumulative risk of developing CRC than in women [21]. Increased incidence of CRC in male patients older than 50 years was reported by several studies [22, 23]. Men's increased susceptibility to developing CRC may be attributed to several biological and behavioral factors [24]. Men tend to eat a diet rich in red and processed meat [25], are more liable to consume alcohol, smoke, and also deposit visceral fat [26, 27].

The present results showed that 66.7% of CRC cases had positive family history of CRC. This is consistent with findings of Roos et al.[28] and Samadder et al.[29]. Association between the risk of developing CRC and family history has been attributed to genetic and environmental factors [30].

Most patients suffered from bleeding per rectum (83.3%), constipation (66.7%), and loss of weight (50%). This is in harmony with many studies [19]. For CRC cases, recto-sigmoid cancer represented the commonest tumor site in 80 % of CRC cases; 10 % had ascended, and another 10 % had descending colon tumors. Adenocarcinoma represented 93.3%, and only 6.7% were mucinous. 60 % of CRC lesions were ≥ 3 cm and 40% were < 3 cm in size. Grade III represented 56.7%, and 43.3% were grade II with no lymph node, liver, or distant metastasis. This was consistent with Ali et al.[19].

To the best of our knowledge, this is the first study to estimate the expression level of *EIF4EBP1* gene in PBMCs versus tissue in CRC cases compared to healthy matched controls.

In this study, expression levels of *EIF4EBP1* in both tissue and PBMCs showed statistically significant upregulation among CRC cases compared to matched controls (p <0.001). For CRC cases, tissue expression level of *EIF4EBP1* was significantly elevated than that of PBMCs (p <0.001). It is consistent with results of Chao et al.[31] and inconsistent with Chen et al.[32].

The high expression levels of *EIF4EBP1* in cancerous tissue reflect that protein's important role in the carcinogenesis process. Phosphorylation of *EIF4EBP1* causes the release of *EIF4E*, loss of translational repression, and enhances protein synthesis by cancer cells [32]. Also, high levels of *4EBP1* were reported to induce hypoxia-mediated inhibition of cap-dependent mRNA translation, leading to hypoxia resistance, angiogenesis, and promotion of cancer cell survival and resistance [31].

In a trial to correlate PBMCs or tissue expression levels of *EIF4EBP1* among studied CRC cases to patients' characters, clinical presentation, and tumor characteristics, no significant difference was observed between *EIF4EBP1* expression levels either in PBMCs or in tissues and patients' clinical presentation, tumor site or pathological type. However, the *EIF4EBP1* tissue expression level was significantly higher among patients with tumor size ≥ 3 cm compared to those with tumor size < 3 cm (p = 0.004) and among patients with tumor grade Ⅲ compared to those with tumor grade Ⅱ (p < 0.001). This was consistent with Chao et al. [31] and with Diab-Assaf et al.[31].

Upon comparing tissue and PBMCs expression levels of *EIF4EBP1*, tissue expression level was significantly higher than that of PBMCs in patients with recto-sigmoid tumor compared to those with colon cancer (p < 0.001), adenocarcinoma compared to mucinous type (p = 0.001), in those with tumor size ≥ 3 cm and those with tumor size < 3 cm (p = 0.002 and 0.05, respectively) and in patients with tumor grade III (p = 0.001).

Furthermore, the *EIF4EBP1* tissue expression level was significantly higher among patients with tumor size ≥ 3 cm compared to those with tumor size < 3 cm (p = 0.004) and also with tumor grade III compared to those with tumor grade II (p < 0.001). All of the data mentioned above are consistent with studies by *Chao et al.* [31] who conveyed a significant statistical correlation between *EIF4EBP1* expression level and patients diagnosed with late-stage CRC (stages III and IV) and concluded a direct correlation between the expression levels of *4EBP1* and CRC progression, adverse prognosis and could be a prognostic indicator, a novel biomarker to predict the clinical outcome of patients with CRC and potential therapeutic target.

As regards the diagnostic ability of *EIF4EBP1* in PBMCs, ROC curve analysis showed that the expression level of *EIF4EBP1* in PBMCs was able to differentiate CRC cases from controls with a sensitivity of 66.7%, a specificity of 100 % and accuracy of 83.3% at a cut-off value of ≥ 0.87; AUC = 80.1% (95% CI: 0.680 – 0.922, p < 0.001). Tissue expression level of *EIF4EBP1* was much more sensitive and specific in differentiating CRC cases from healthy ones at a cut-off value of ≥ 1.1; AUC was 96.7% (95% CI: 0.931 – 1.0, p < 0.001) with a sensitivity of 96.7%, a specificity of 100 %, and accuracy of 97.7%.

Furthermore, these findings revealed that *EIF4EBP1* expression level in tissue could have a prognostic ability to predict grade III CRC and differentiate it from grade II, as evidenced by the ROC curve analysis. At a cut-off value of ≥ 1.5, the AUC was 89.6% (95% CI: 0.784 – 1.0, p < 0.001), and *EIF4EBP1* was able to predict grade III CRC and differentiate it from grade II with a sensitivity of 82.4%, a specificity of 84.6%, and accuracy of 83.3%. These results were consistent with Chen et al.[32] who reported that patients with low expression levels of *EIF4E* tended to have significantly longer overall survival (p = 0.039), and high expression levels of *EIF4E* were associated with more advanced stage and poorer prognosis.

All of the previously mentioned findings suggest the diagnostic and prognostic role of *EIF4EBP1* in CRC, which needs further evaluation in larger-scale studies.

CONCLUSION

EIF4EBP1 expression levels in both PBMCs and tissue showed significant upregulation among the CRC cases compared to healthy matched controls. Tissue expression levels were able to differentiate CRC cases from healthy controls and predict higher tumor grade. This reflects the possible use of this gene as a novel diagnostic and prognostic tool in CRC.

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Statements and Declarations

Competing Interests

The authors declare no competing interests.

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Ethical Considerations

Ethics approval

The study protocol was accepted by the local Ethical Committee of the Faculty of Medicine, Assiut University (**IRB No.: 17200584**), registered on Clinical

Trials.gov **(ID: NCT04729855)**, and was conducted following the principles embodied in the Declaration of Helsinki.

Consent to participate

All patients provided written informed consent.

Contributions

Dr. Esraa Hassan: Practical part, formal analysis, investigation, writing original draft, visualization, and funding acquisition.

Dr. Mohamed Ali Mohamed Elfeky and Dr. Salwa Sayed Ahmed Hassan Seif Eldin: Supervision, conceptualization, research administration, and funding acquisition.

Dr. Mohamed Abouelmagd Salem Mahmoud: Obtaining specimens, supervision, research administration, and funding acquisition.

Dr. Aliaa Mahmoud Ali Ahmed Ghandour: Editing, supervision, research administration, and funding acquisition.

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