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Role of c-Myc as a potent biomarker for diagnosis of colon cancer in Egyptians patients

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Immunohistochemical studies reported that colorectal cancer (CRC) is joined of high expression of c-Myc. Therefore, our study exhibited that c-Myc alone or combined with other markers can be a promising biomarker for CRC diagnosis. This study included colon cancer patients (n=80), benign growth patients (n=35) and normal individuals (n=30). c-Myc was recognized using western blotting and quantified by ELISA then Receiver-operating characteristic curve (ROC) was used for measuring its diagnostic performance. In colon cancer patients, c-Myc was identified at 62 KDa and detection rates of c-Myc increased in colon cancer patients (75.0%) than patients with benign growth (48.6%). Also, the mean c-Myc onco-protein level (OD) in colon cancer patients (1.6 ± 0.02 µg/mL) was significantly (p < 0.0001) higher than benign (1.1 ± 0.03 µg/mL). Additionally, both detection rate and level of c-Myc were significantly (p < 0.05) increased with late stages, lymph node invasion, distant organ metastasis and high grades. Based on ROC analysis, c-Myc yielded a good diagnostic performance for colon cancer detection, where it had an area under the curve (AUC) 0.87 (sensitivity75.0%, specificity 93.3%) to distinguish between colon cancer patients and normal individuals. Multivariate analysis yielded colon-score that had a valuable diagnostic power in colon cancer early diagnosis as it had AUC 0.89 in distinguishing between patients with benign growth and those with early stages (sensitivity 78.1%, specificity 81.8%). In conclusion, c-Myc can be used as an effective biomarker for colon cancer diagnosis particularly for differentiates early tumor stages from benign disorders.

Keywords: Colorectal cancer, Biomarkers, c-Myc, Expression, Early diagnosis.

INTRODUCTION

Colorectal cancer (CRC) is also known as colon cancer or bowel cancer (Jahanafrooz et al. 2020). Globally, CRC is the third most common diagnosed cancer and is the second reason of death (Zorzi et al. 2019). In Egypt, it ranks the 4th in females and the 6th in males; accounts for 4.2% of all cancer incidence and 3.6% of all mortality (GLOBOCAN, 2018). Development of CRC is resulting from environmental factors, epigenetic and genetic alterations (Thanikachalam and Khan, 2019). The time of diagnosis has a main role in survival rate as CRC is a disease that develops slowly and its symptoms appeared when reaches advanced stages. So the key of prognosis improvement and get the best treatment is early diagnosis (Sun et al. 2019). Although colonoscopy is a gold standard method for early diagnosis, it is uncomfortable and requires experience and skill of physicians (Zhang et al. 2019a). Therefore, there is a general focus and outstanding strategy on providing affordable and better method for

diagnosing and predicting CRC.

Additionally, prognosis of CRC at early stages performed by using biomarkers (Draht et al. 2018) which are biological molecules measured in blood, tissues, stool or other body fluids that are shown the biological state of body (Turano et al. 2019). CEA and CA19-9 are the most common biomarkers for CRC detection in spite of they had low sensitivity and specificity (Liu et al. 2018). So, to date, the search for novel biomarkers is a prior in CRC research due to there is no biomarker with a desirable specificity and sensitivity for clinical use (Álvarez-Chaver et al. 2018)

c-Myc oncoprotein is a transcription factor and one of the most common blood based biomarker for cancer diagnosis (Tansey, 2014); it acting as an oncogene and related to several processes such as metabolism, apoptosis, differentiation and proliferation (Venegas et al. 2018). Deregulation of c-Myc was found in most human cancers such as colorectal, lung, leukemia, lymphomas and pancreatic cancer (Jung et al. 2015). The relation between CRC and c-Myc is inconclusive and controversial as some studies like Lee et al. shown that expression of c-Myc in CRC is a poor prognostic factor (Lee et al. 2015). While some studies shown that c-myc overexpression can be a good prognostic factor for CRC patients and others shown that c-Myc did not predict prognosis (Toon et al. 2014; He et al. 2018). So the aim of this study is evaluation of c-Myc in colon cancer patients and how it can be valuable for diagnosis.

MATERIALS AND METHODS

Cases and clinical samples

After approval Ethics and Scientific Committees of the Mansoura University Hospitals, Mansoura, Egypt, 145 samples were collected and subdivided as follow: 80 patients with colon cancer (44 males and 36 females); mean age 50.7±11.8 years, 35 patients with benign growth (20 males and 15 females); mean age 48.8±11.4 and 30 normal individuals as negative control (17 males and 13 females); mean age 46.8±13.1 with no significant difference between three groups in mean age ($p > 0.05$). Additionally, colon cancer patients (n=80) were classified according to TNM system into 35 case with early stages (T1-T2) and 45 cases with late stages (T3-T4), also into 48 cases without lymph node invasion (N0) and 32 cases with lymph node invasion, and finally into 59 cases without distant organ metastasis (M0) and 21 cases with distant organ metastasis (M1). Also, they classified according tumor grade into 45 cases at low grade (G1-G2) and 35 cases at high grade (G3-G4).

Laboratory assays

By using automated biochemistry analyzer (Roche/Hitachi 917, Mannheim, Germany), all liver function tests including Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and albumin were measured on fresh serum. Also by using KX-21 Sysmex automated hematology analyzer (Sysmex Corporation, Japan), complete blood count especially platelets counting was measured on blood treated with EDTA-K3.

Identification of c-Myc onco-protein by SDS-PAGE and western blot

c-Myc onco-protein was recognized by subjecting serum samples from all participants to SDS-PAGE according to Laemmli, then by western blot according to Towbin (Laemmli, 1970; Towbin et al. 1979).

Detection of c-Myc onco-protein by ELISA

Concentration of c-Myc was detected by enzyme linked immunosorbent assay (ELISA) according to Attallah et al.'s protocol (Attallah et al. 2017) that previously explained the method for c-Myc by using ELISA reader (∑960 Metretech, Germany) where the intensity of color is proportional to the quantity of sample then to measure the concentration of each sample, standard curve was performed from series dilution.

Statistical analysis

By using statistical program for social science (SPSS 20 software for Windows 7), the data analysis was done. The qualitative variables were expressed in the form of percentages and frequency while the quantitative variables were expressed in the form of standard deviation and mean. Differences in continuous variables were appreciated using analysis of variance (ANOVA) or Student's *t*-test while for categorical variables were appreciated using Chai square (X^2) test. All tests with statistical significance and two-tailed were estimated and become significant when ($p < 0.05$). In our study, the correlation was detected by Pearson's correlation coefficient. Receiver-operating characteristic (ROC) curve analysis was used appreciate biomarker's specificity (true negatives percentage) and sensitivity (true positives percentage) performance. It was produced by plotting sensitivity (true positive rate)

vs. 1-specificity (false positive rate) for different cutoff points of a parameter. Additionally, the area under the curve (AUC) of ROC was used to evaluate the performance of the biomarker as the higher AUC value means that a best biomarker performance (English et al. 2016).

RESULTS

Clinical laboratory data

With respect to laboratory data, colon cancer patients had higher activities of liver enzymes (ALT and AST), WBCs count and neutrophils count but they had lower levels of serum albumin, Hb, RBCs counts, lymphocytes and platelets count in comparison with normal individuals and patients with benign growth with a statistically significant differences between three group of subjects in all comparisons ($p < 0.05$), Table 1. Moreover, by comparing colon cancer patients with benign group there was no significant difference ($p > 0.05$) in liver enzymes activities, serum albumin level and platelets count. While, there is significant differences between two groups of subjects in Hb levels, RBCs count, WBCs count, neutrophils and lymphocytes count ($p < 0.05$).

Identification of c-Myc onco-protein using western blotting

c-Myc was identified by SDS-PAGE followed by western blotting in all serum samples. At 62 KDa, exceptional sharp and light bands were seen in sera of colon cancer patients and benign patients, respectively while no response with sera of normal individuals, Figure 1A.

Detection of c-Myc in colon cancer group using ELISA

Mono-specific antibody for c-Myc was used as a probe in ELISA to quantified serum c-Myc onco-protein. The cutoff for ELISA technique is calculating as the mean optical density (OD) for 16 serum of normal people ± 3SD was equal 0.28, and serum samples from 20 colon cancer patients showed concentration above the cut off level.

The c-Myc onco-protein was detected in only 2 serum samples from 30 normal individuals with detection rate 6.7 % while, it increased with colon cancer progression where it was 84.4% (38/45) in patients with late stages (T3-T4) *vs*. 62.9% (22/35) in patients with early stages (T1-T2) stage, with a significant difference between two groups of subjects ($X^2 = 4.89$; $p = 0.027$). Also, c-Myc onco-protein was highly detected in colon cancer patients who had node invasion (N1) than those without lymph node invasion (N0) (93.8% *vs*. 62.5%, respectively). Moreover, 95.2% (20/21) of patients who had tumor metastasis (M1) were positive for c-Myc onco-protein compared to only 67.8% (40/59) of patients without tumor metastasis (M0). Additionally, the detection rate of c-Myc onco-protein was increase with tumor grade where it was 88.6% (31/35) in patients with high grade (G3-G4) *vs*. 64.4% (29/45) in patients with low grade (G1-G2).

Level of c-Myc onco-protein in colon cancer patients at different stages of cancer

Interestingly, the mean c-Myc onco-protein level (OD) in colon cancer patients (1.6 ± 0.02 µg/mL) were significantly ($p < 0.0001$) higher than benign (1.1 ± 0.03 µg/mL) and normal (0.4 ± 0.03µg/mL) groups, Figure 1B. Additionally, elevated c-Myc levels were significantly associated ($p < 0.05$) with some tumor severity features including, late tumor stages (1.7 ± 0.04 µg/mL), lymph node invasion (1.9 ± 0.01 µg/mL), and distant organ metastasis (1.8 ± 0.04 µg/mL) and high histological grades (1.9 ± 0.03 µg/mL), Figure 1C-F.

Diagnostic performance of c-Myc onco-protein for colon cancer detection

Based on ROC analysis this c-Myc yielded a good diagnostic performance for colon cancer detection where it had an AUC 0.87 with 75.0% sensitivity and 93.3% specificity at cutoff= 0.5 to distinguish between colon cancer patients and normal individual, Figure 2A. Also, c-Myc had a valuable power for identification of colon cancer patients from those with benign disorders with AUC 0.79, 75.0% sensitivity and 62.8% specificity at cutoff= 1.2, Figure 2B.

Correlation of c-Myc onco-protein with age and some biomarker in colon cancer patients

Pearson's correlation coefficient was then calculated to assess the relationship between age, some individual serum markers including neutrophils and lymphocytes count and c-Myc onco-protein in order to evaluate their roles in colon cancer diagnosis. There was a weak positive correlation between c-Myc onco-protein and age ($r = 0.323$; $p = 0.001$), neutrophils count ($r = 0.342$; $p = 0.001$) and a weak negative with lymphocytes count ($r = - 0.167$; $p = 0.055$), Figure 2C-E that means that there is no redundancy and they are exploring various biochemical abnormalities and using theses multiple markers

may increase colon cancer diagnostic accuracy.

Table 1: Clinical laboratory data of the study population

Variables	Control (n=30)	Benign disorders (n=35)	Colon cancer (n=80)	*P value*
Age (years)	46.8±13.1	48.8±11.4	50.7±11.8	> 0.05
Gender (male/female)	17/13	20/15	44/36	> 0.05
AST (U/L)	25.1±4.2	26.1±6.8	34.0±13.8	< 0.0001
ALT (U/L)	26.0±4.8	31.1±11.4	36.1±12.7	< 0.0001
Albumin (g/dL)	3.6±0.4	3.6±0.3	3.2±0.8	< 0.001
Hb (g/dL)	12.9±0.9	11.1±2.4	10.1±1.8	< 0.01
RBCs (x10^{12}/L)	5.6±0.3	4.1±0.5	3.4±0.6	< 0.01
WBCs (x10^{9}/L)	5.4±1.4	8.4±4.2	8.9±5.5	< 0.01
Neutrophils (x10^{9}/L)	4.7±1.9	5.2±2.9	9.4±5.8	< 0.0001
Lymphocytes (x10^{9}/L)	2.3±1.0	2.2±1.3	1.1±0.6	< 0.0001
Platelets count (x10^{9}/L)	307.9±92.0	252.3±84.1	213.6±72.6	0.039

Data were expressed as Mean± standard deviation. *P* value: $p < 0.05$ is significant.

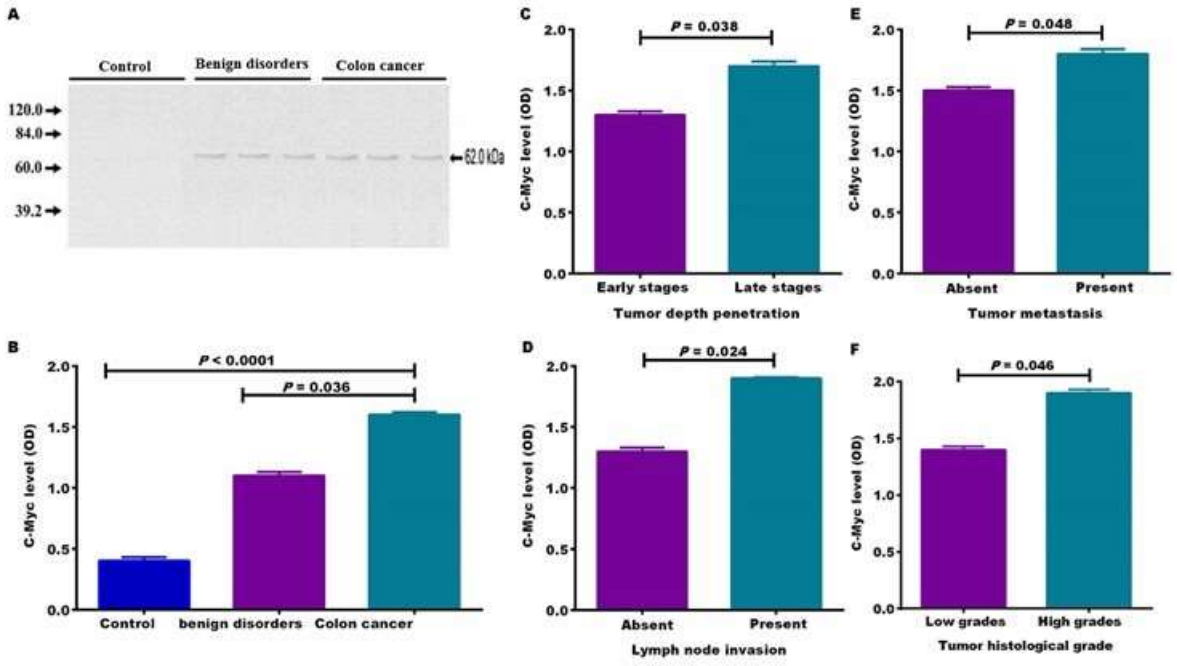

Figure 1: Identification of c-Myc onco-protein and its level with tumor progression. (A) Western blotting for c-Myc, (B) Level of c-Myc in three groups of subjects, (C) Level of c-Myc with tumor penetration, (D) Level of c-Myc with node invasion, (E) Level of c-Myc with metastasis and (F) Level of c-Myc with tumor grade.

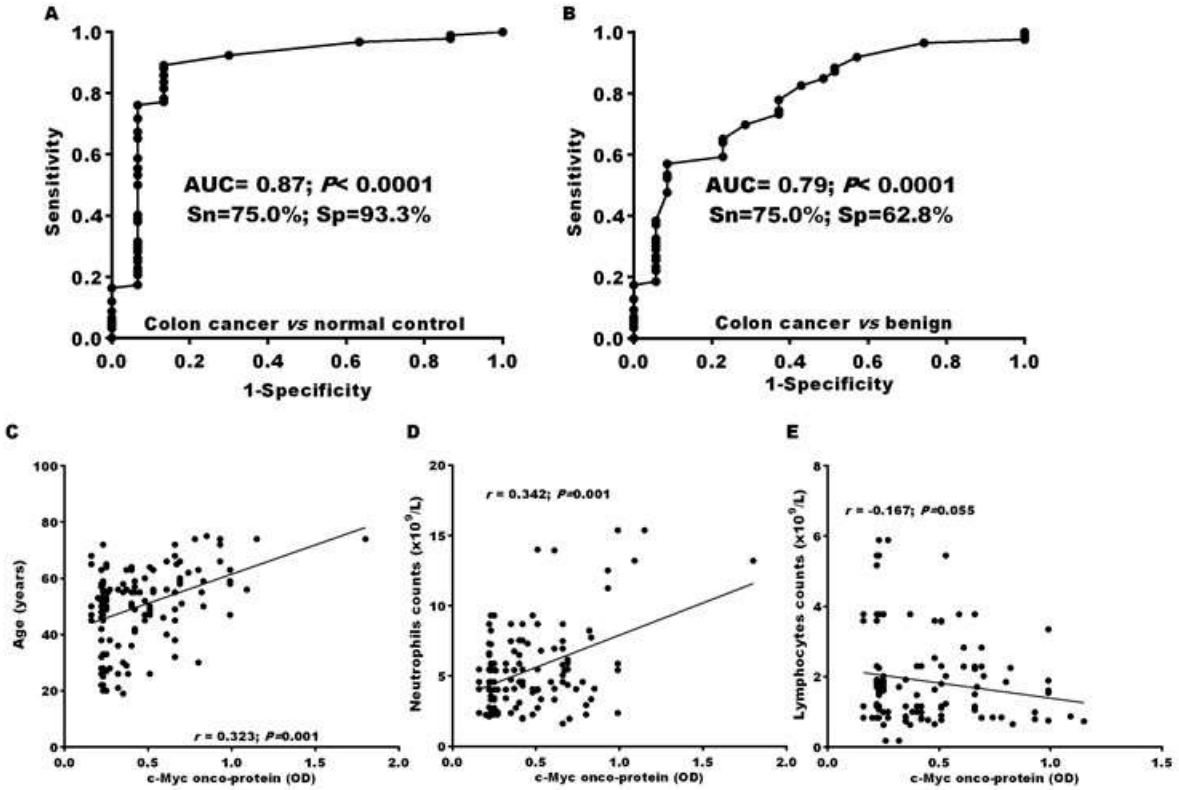

Figure 2: Diagnostic performance of c-Myc onco-protein for colon cancer detection and its correlation with age and some biomarker in colon cancer patients. (A) Diagnostic performance of c-Myc for colon cancer *vs*. normal, (B) Diagnostic performance of c-Myc for colon *vs*. benign, (C) Correlation between c-Myc and age, (D) Correlation between c-Myc and neutrophils and (E) Correlation between c-Myc and lymphocytes. AUC= area under curve, Sn= sensitivity, Sp= specificity, r= Pearson's correlation coefficient.

Consequently, multivariate discriminant analysis was used for calculating a function based on these variables (c-Myc onco-protein, age, neutrophils and lymphocytes count) for colon cancer detection. This obtained score was as the following (Colon-Score = 1.4 + 0.004 x age + 0.4 x c-Myc - 0.4 x lymphocytes + 0.04 x neutrophils).

Diagnostic performance of colon-score for colon cancer early detection

Based on the ROC analysis, it was found that the developed score could be effectively in colon cancer diagnosis where at cutoff= 2.0, colon-score yielded an AUC value of 0.95 when cancer patients were compared only to healthy individuals with a sensitivity 83.1% and specificity 97.3%, Figure 3A while when discriminating cancer patients from patients with benign growth yielded an AUC value of 0.94, with an optimal sensitivity 83.1% and specificity 94.6% at cutoff= 2.3, Figure 3B.

Interestingly, colon-score has a valuable diagnostic power in colon cancer early diagnosis. Colon-score at cutoff= 2.5 yielded an AUC 0.89 in distinguishing between patients with benign growth and those with early stages with sensitivity 78.1%, and specificity 81.8%, Figure 3C. In addition, colon-score has a promising diagnostic power in distinguishing between patients with benign growth and those without lymph node invasion or distant organs metastasis with AUC 0.88 and 0.89, sensitivity 75.0% and 77.9%, respectively at specificity 81.8%. Also, colon-score was 82.6% sensitive and 81.8% specific with AUC 0.92 in distinguishing between patients with benign growth and those with low grades, Figure 3D-F.

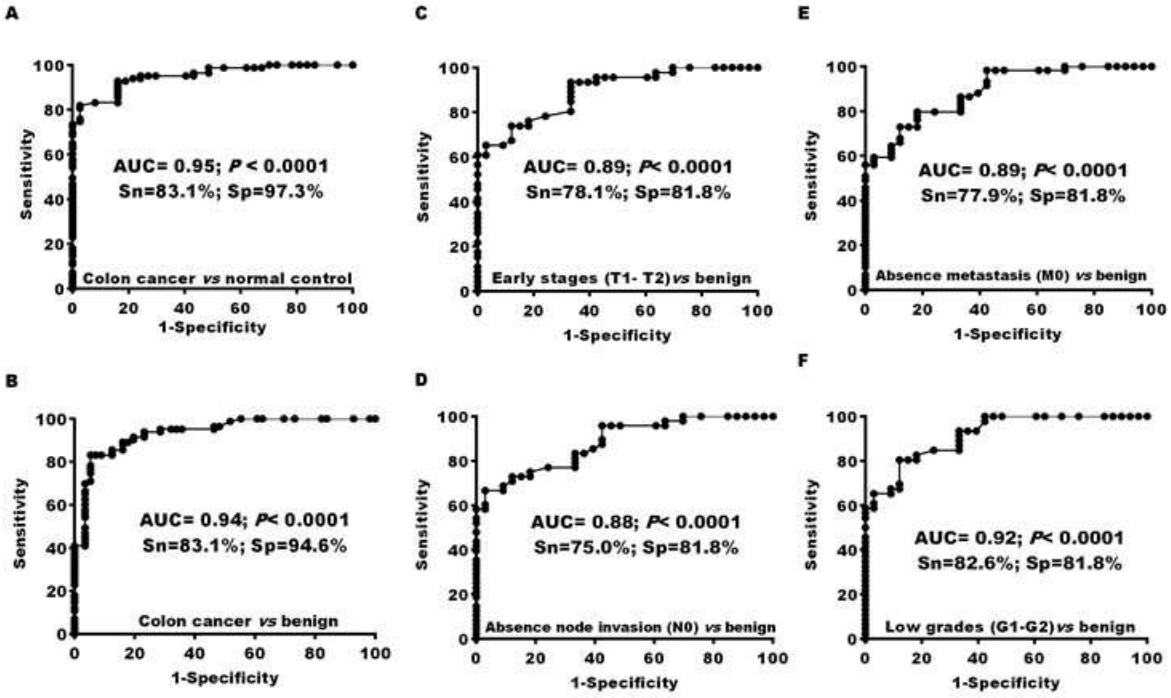

Figure 3: Diagnostic performance of colon-score for colon cancer early detection. (A) Colon cancer *vs.* normal, (B) Colon cancer *vs.* benign, (C) Early stages *vs.* benign (D) Absence node invasion *vs.* benign, (E) Absence metastasis *vs.* benign and (F) Low grades *vs.* benign.

DISCUSSION

A biomarker is a biological molecule measured in body fluids and it is a marker for physiological or pathological condition such as cancer. It may be a protein, antibodies, nucleic acids, peptide and lipids. It can be used for early diagnosis and prognosis of cancer (Turano et al. 2019). c-Myc is one of the most common blood biomarkers but there is an indecisive findings about its relation with CRC and its use as a biomarker for diagnosis (He et al. 2018). So this study aimed to evaluate c-Myc in colon cancer patients and identify its ability as a biomarker for diagnosis. At 62KDa, c-Myc was identified with a sharp band in colon cancer patients and a light band in benign growth patients like several authors who had also shown that c-Myc molecular weight range from 62-66 KDa (Litchfield et al. 2015; Attallah et al. 2017).

Generally, studies showed that c-Myc is overexpressed in ≥ 70% of CRC (He et al. 2018). Our results showed a significant increase of c-Myc in colon cancer patients compared to benign growth and normal individuals; also it was found increase of c-Myc with cancer progression. This overexpression was reported by previous studies including Wang et al. (2017) and Zhang et al. (2019b).

Several biomarkers affect positively on c-Myc expression where 90% of CRC occurs due to mutation in adenomatous polyposis (APC) that activate Wnt pathway and consequently affect β-catenin which is a transcription machine of c-Myc and results in overexpression of c-Myc (Elbadawy et al. 2019). Additionally, previous studies shown that Sphingosine Kinase-2 (SPHK-2) and Special-AT Rich Sequence binding protein-1 (SATB-1) results in overexpression of c-Myc (Mansour et al. 2016; Zhang et al. 2016).

Diagnostic performance of c-Myc was detected by using ROC curve and statistics shown that c-Myc had an AUC 0.87 with sensitivity 75.0% and specificity 93.3% in differentiation between colon cancer patients and normal individuals while it had an AUC 0.79 with sensitivity 75.0% and specificity 62.8% in differentiation between colon cancer patients and benign growth patients.

On the other hand, c-Myc has a good diagnostic performance for colon cancer detection and is superior compared to some biomarkers

including CEA and CA19-9 which are the most common biomarkers used and Tissue Inhibitor of metalloproteinases-1 (TIMP-1) which is a new biomarker used in colon cancer diagnosis. Interestingly, CEA and CA 19-9 have lower diagnostic performance (AUC 0.61, sensitivity 62.2%, specificity 60.0% and AUC 0.58, sensitivity 59.4%, specificity 55.6%, respectively) in differentiation between colon cancer patients and benign growth (Attallah et al. 2019). As for TIMP-1, it had (AUC 0.77, sensitivity 65%, specificity 87%) (Meng et al. 2018).

Due to the heterogeneity of disease, single biomarker is not ideal biomarker with high sensitivity and specificity; hence, the combination is required to obtain the high diagnostic performance (Fan et al. 2017). According to multivariate analysis and by using Pearson's correlation coefficient, a weak positive correlation between c-myc and age, neutrophils and a weak negative with lymphocytes count were found. This mean that, the combination between these 4 factors may increase colon cancer diagnostic accuracy rather than using each one alone and this combination yielded "colon score". This score could be effectively in colon cancer diagnosis as differentiate between colon cancer patients *vs*. benign growth patients and colon cancer patients *vs*. normal individuals with specificity 94.6% and 97.3%, respectively at sensitivity 83.1%. Interestingly, it was found that colon-score had a valuable diagnostic power in colon cancer diagnosis as it could differentiate between benign growth patients and early stages with sensitivity 78.1% and specificity 81.8%. Additionally, it can differentiate benign growth patients from those without lymph node invasion or metastasis with sensitivity 75.0% and 77.9%, respectively at specificity 81.8%. Also, it can distinguish between benign growth patients and those with low grades with sensitivity 82.6% and specificity 81.8%. From previous studies including Gao et al. who shown that when CEA and CA19-9 combined with CA72-4, CA125 and serum ferritin (SF), the sensitivity increased from 46.59 and 14.39%, respectively to 67.38% at specificity 73% (Gao et al. 2018). Also, combination between Neuron Specific Enolase (NSE), CEA, CA19-9, CA125 and CA242 yielded sensitivity 69.30% and specificity 84.60% (Luo et al. 2020). In addition to CEA and CA19-9, Hauptman and Glavač shown that combination between a panel of six miRNA (miR-335+ miR-29a+ miR-18a+ miR-15b+ miR-19a+ miR-19b) yielded sensitivity 78.6% and specificity 79.3% for colon cancer diagnosis (Hauptman and Glavač, 2017). All these findings shown that, colon score are superior to other established score for colon cancer diagnosis.

CONCLUSION

In conclusion, all these findings clarified the relation between c-Myc and CRC where c-Myc was overexpressed with cancer progression and TNM stages and is superior to some markers for colon cancer diagnosis due to its high sensitivity and specificity. Additionally, it can be used as an effective biomarker for colon cancer diagnosis at its different stages particularly for differentiates early tumor stages from benign disorders.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Attallah AM, El-Far M and Omran MM were chief investigators who conceptualized and designed the study. They equally participated in all parts of this final manuscript. El-Ghazi AS was investigator who collected data from the literature, collected samples and carried out the work. All authors read, reviewed and approved the final manuscript.

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