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Association of lncRNA MEG3 Rs7158663 Polymorphism and Serum Expression with Colorectal Cancer in Egyptian Patients

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Abstract

Background: Colorectal cancer (CRC) is considered the third most common cancer around the world and second in terms of mortality. A significant aspect in its development is genetics. The risk of CRC and other clinicopathologic characteristics were investigated in this work in relation to the long non-coding RNA (lncRNA) MEG3 rs7158663 polymorphism, MEG3 expression, in an Egyptian population.

Methods: 160 CRC patients and 160 healthy controls were enrolled in this case-control study. The lncRNA MEG3 rs7158663 was examined using TaqMan Real-time PCR. RT-PCR was used to assess the levels of serum MEG3 expression.

Results: A significant higher expression of 'A' allele (risk allele) and A/A genotype in CRC cases vs. control subjects (P <0.001) Participants with A/A genotype had 4.8 times higher odds to exhibit CRC. Serum MEG3 gene expression was generally low in CRC patients, and it was considerably lower in those with the rs7158663 AA genotype than those with the GG genotype (P <0.001). It was found that CRC patients with the rs7158663 GA genotype had lower serum MEG3 expression levels than those with the GG genotype (P <0.001).

Conclusions: MEG3 low expression and MEG3 rs7158663 (AA) were associated with CRC risk in Egyptian patients and may serve as a diagnostic and prognostic marker for CRC patients.

Keywords: Colorectal cancer, MEG3, lncRNA, Polymorphism, Rs7158663.

Introduction

According to the International Agency for Research on Cancer (IARC) of the World Health Association (WHO) Colorectal cancer (CRC) ranks as the third most common cancer around the world and second in terms of mortality (1,2). Although more than 90% of CRC cases are diagnosed in individuals over age 55, CRC incidence is rising in younger populations at age of 40 in Egypt. Along with the high incidence rate of CRC in individuals under age 40 in Egypt, CRC is diagnosed at more advanced stages in these younger Egyptians (3-5).

Colorectal cancer (CRC) is one of gastrointestinal malignancy arising from either the colon or the rectum (6, 7). The colon might have it appear on either the right or left side. Position affects how CRCs behave in terms of how the illness develops and how long they live (8). The ascending colon, proximal two-thirds of the transverse colon, and the sigmoid colon are all parts of the right-sided CRC (RCRC) tumours, while the descending and sigmoid colon, as well as the distal one-third of the transverse colon and are parts of the left-sided CRC (LCRC) tumours (9, 10).

A chromatin-interacting long non-coding RNA (lncRNAs) maternally expressed gene 3 (MEG3) is an imprinted gene located at 14q32

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(NG_016853.2) that encodes a lncRNA associated with various human cancers (11). It has an important role in regulation of transforming growth factor-β (TGF-β) pathway (12) and inducesp53 tumor suppressor gene expression through different mechanisms. MEG3 can also suppress cell proliferation even in the absence of p53 (13).

MEG3 expression level is affected by another important tumor suppressor namely retinoblastoma protein (Rb) and it was documented that genetic deletion of Rb family members in mice has resulted in remarkable silencing of MEG3 expression. Conversely, activation of pRb enhanced the expression of MEG3 (14).Single nucleotide polymorphisms (SNPs) have been investigated as a potential biomarker of genetic background to determine the risk, course, and effectiveness of treatment for a variety of diseases. SNPs in MEG3 have been linked to cancer risk, chemotherapy toxicity, and cell phenotypes in different malignancies (15).

The lncRNA MEG3 has been discovered to be downregulated in several malignant tissues, including hepatocellular carcinoma, gastric cancer, and ovarian cancer (16). MEG3 has also been identified as a tumour suppressor in breast cancer. However, one pilot investigation found that plasma samples from CRC patients had higher levels of MEG3 than non-cancerous controls (17).

We therefore sought to learn more about the link between the MEG3 rs7158663 SNP and CRC susceptibility, as well as the correlation with its serum expression and clinicopathological data. Additionally, the serum MEG3 expression in CRC patients was examined and associated with clinicopathological information.

Materials and Methods

This case-control study was conducted at The Medical Biochemistry Department, Faculty of Medicine, Mansoura University, Egypt, Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Cairo University, Egypt, and The Medical Oncology Unit at the Oncology Center at Mansoura University, Egypt.

The study involved 320 participants selected regards to the following criteria, and divided into two groups:

Inclusion criteria: Patients diagnosed with colorectal cancer without receiving chemotherapy or radiotherapy, diagnosis and cancer staging was done by Mansoura Medical Oncology Center.

Exclusion criteria: All patients with inflammatory bowel disease, inflammatory polyps, any benign lesions, other types of cancers, and CRC patients who stared chemotherapy, radiotherapy, or surgical removal.

Group 1: 160 CRC patients (110 males and 50 females).

Group 2: Cancer free controls (160 healthy subjects) including 120 males and 40 females. All cancer free controls had no history of CRC, inflammatory bowel disease, colorectal polyps, or other cancers.

Isolation of peripheral blood mononuclear cells

Five ml of Whole blood was collected from each subject and divided into two portions. Two ml in EDTA collection tubes for DNA extraction. The remained was centrifuged in a plain tube to give serum that was used for RNA extraction and real-time PCR.

RNA extraction& cDNA synthesis

Total RNA including lncRNA was extracted by the TRIzol reagent from serum (Zymo Research, Irvine, CA). NanoDrop2000 (Thermo. Fischer Scientific, Waltham, MA) was used to quantitate RNA. Before use, the total RNA samples were kept at -80 °C. Using SensiFAST cDNA Synthesis Kit (Bioline, Memphis, TN) reverse transcription was performed on the RNA in a final volume of 20 µl reactions.

Real time PCR

Using MEG3 (Forward: 5’TTTTGTGCCCAAGGCTCCTGGA-3’, Reverse: 5’-AGGGACTCAAGGAGCCAGGTTA-3’) and

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GAPDH primers (Forward: 5′-CCCTTCATTGACCTCAACTA-3′ and Reverse: 5′-TGGAAGATGGTGATGGGATT-3′), MEG3 expression levels were evaluated using GAPDH as an internal control using the Hera plus SYBR Green qPCR kit (Willow fort, Birmingham, UK) according to the manufacturer's protocol. Fold change was calculated using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) for relative quantification normalized to an endogenous control (18).

DNA extraction

Using a Qia-amplification DNA extraction kit, DNA was isolated from whole blood according to manufacture instruction (Qiagen, USA).

Genotyping of SNP in MEG3 rs7158663

Using real-time polymerase chain reaction and the TaqMan allelic discrimination test, the MEG3 SNP rs7158663 was genotyped. predesigned unique primer/probe sets for lncRNA MEG3 rs71586 was used (Applied Biosystems, USA). DNA amplification was carried out in 25 µl total volume containing: 12.5 µl Taqman master mix. 1.25 primer/probe, 1 µl DNA (100 µg) and 10.25 H2O. Real-time PCR was performed using a Rotor gene Q Real Time PCR System (Qiagen, Valencia, CA, USA) with the following conditions: 10 min at 95 °C for denaturation then 45 cycles at 92 °C for 15 s then 60 °C for 90 s for annealing and extension were carried out and fluorescence was measured at the end of every cycle and at the endpoint.

Statistical analysis

Data were entered and analyzed using IBM-SPSS software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.) And SNP Stats web tool for SNP analysis (https://www.snpstats.net/start.htm).

Qualitative data was tested for by Chi-Square test of association and Fisher's exact test. Quantitative data were initially tested for normality using Shapiro-Wilk's test with data being normally distributed if P >0.050. Presence of significant outliers (extreme values) was tested for by inspecting boxplots. Quantitative data were expressed as mean ± SD or median (Q1-Q3) and compared between two groups using Independent-Samples t-test or Mann-Whitney U test. We also use the following: One-Sample Wilcoxon's signed ranks test, Point biserial correlation, Spearman's correlation, and Kruskal-Wallis H-. For any of the used tests, results were considered as statistically significant if p value ≤ 0.050.

Results

Association of MEG3 rs7158663 (G/A) with the risk of CRC

Our result shows a statistically significantly higher proportion of overweight and obesity in CRC cases compared to control (P = 0.007) but no statistically significant differences between the two groups as regards age or sex. In addition, there was a statistically significant difference in rs7158663 (G/A) distribution between the two groups, patients and control. Z-tests revealed a statistically significantly higher A/A genotype in CRC cases compared to control subjects, and a statistically significantly lower G/G genotype in CRC cases compared to control subjects (Table 1). We also revealed a statistically significantly higher 'A' allele compared to 'G' allele in group 1 compared to group 2, with moderate strength of association. A binary logistic regression analysis revealed that participants with 'A' allele (risk allele) had 1.9 times higher odds to exhibit CRC. In addition, there is a statistically significantly higher 'A/A' genotype compared to 'G/G-G/A' genotypes in group 1 compared to group 2, with moderate strength of association. A binary logistic regression analysis revealed that participants with 'A/A' genotype (risk genotype) had 4.8 times higher odds to exhibit CRC (Table 2).

SNP exact test for Hardy-Weinberg equilibrium (HWE) (n=320) shows that control subjects are in HWE (P = 1.000). And the best inheritance model is the recessive model. Participants with A/A genotype had 4.8 times higher odds to exhibit CRC (Table 3).

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Table 1. Comparisons between the two groups.

Characteristic	Group 1 CRC patients	Group 2 Cancer free controls	P value
Age (years)	**49.5 ± 11.7**	**49 ± 8.4**	**0.685**
Sex			
Male	110 (68.8%)	120 (75%)	0.214
Female	50 (41.3%)	40 (25%)	
Overweight / obesity	88 (55%)	64 (40%)	0.007
rs7158663 (G/A)			
G/G	78 (48.8%) a	96 (60%) b	<0.001
G/A	50 (31.3%) a	56 (35%) a	
A/A	32 (20%) a	8 (5%) b	

Data is mean ± SD for age (test of significance is Independent-Samples t-test), or N (%) for categorical data (test of significance is Chi-Square test). Z-tests with Bonferroni-adjusted p-values for comparisons of column proportions are presented as different letters if statistically significantly different. Group 1: 160 CRC patients, Group 2: 160 Cancer free controls, healthy subjects.

Table 2. rs7158663 (G/A) SNP analysis.

SNP	Group 1	Group 2	χ^2	φ	P value
Alleles					
'G' allele	206 (64.4%)	248 (77.5%)	13.369	0.145	<0.001
'A' allele	114 (35.6%)	72 (22.5%)			
Genotypes					
G/G-G/A	128 (80%)	152 (95%)	16.457	0.227	<0.001
A/A	32 (20%)	8 (5%)			

Data is N (%). Test of significance is Chi-Square test of association. Phi (φ) is a measure of the strength of association.

Table 3. SNP association with CRC (n=320, adjusted by Age, Sex, and Obesity).

Model	Genotype	Group 1 N (%)	Group 2 N (%)	AOR (95% CI)	P value	AIC	BIC
Codominant	G/G	39 (48.8%)	48 (60%)	r (1)	0.0002	428.9	451.5
	G/A	25 (31.2%)	28 (35%)	1.1 (0.52-2.2)			
	A/A	16 (20%)	4 (5%)	4.9 (1.5-16.1)			
Dominant	G/G	39 (48.8%)	48 (60%)	r (1)	0.048	439.8	458.7
	G/A-A/A	41 (51.2%)	32 (40%)	1.6 (0.83-3.03)			
Recessive	G/G-G/A	64 (80%)	76 (95%)	r (1)	<0.0001	427	445.8
	A/A	16 (20%)	4 (5%)	4.8 (1.5-15.3)			
Overdominant	G/G-A/A	55 (68.8%)	52 (65%)	r (1)	0.47	443.2	462
	G/A	25 (31.2%)	28 (35%)	0.83 (0.42-1.7)			
Log-additive	-	-	-	1.73 (1.08-2.77)	0.0011	433	451.9

AOR = odds ratio adjusted for age, sex, and obesity. AIC =Akaiki information criterion. BIC = Bayesian information criterion.

Down regulation of MEG3 serum gene expression levels in CRC patients

In the 160 CRC cases, the median MEG3 was 0.47, ranging from a minimum of 0.07 to a maximum of 1.13, with 25th and 75th percentiles of 0.245, and 0.788, respectively.

A One-Sample Wilcoxon Signed Rank Test was run to compare the median MEG3 in CRC cases compared to a hypothesized value (1.0) for control. The median MEG3 in CRC cases was statistically significantly lower than 1.0 (P <0.001).

Effect of rs7158663 genotype on serum MEG3 expression

We discovered that patients with CRC with the rs7158663 AA genotype had significantly lower serum MEG3 levels than those with the GG genotype (P< 0.001) (Table 4). CRC patients with rs7158663 GA genotype were found to have lower expression level of serum MEG3 than those with GG genotype (P <0.001) however, serum MEG3 levels were marginally significant between patients with GA and AA (Table 4 and Fig. 1).

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Table 4. Effect of rs7158663 genotypes on serum MEG3 expression.

Characteristic	G/G N=78	G/A N=50	A/A N=32	P value
MEG3 (Fold change)	0.78 (0.62-0.84)	0.33 (0.235-0.4575)	0.20 (0.14-0.25)	**<0.001**

Pairwise comparisons revealed that MEG3 (Fold change) was statistically significantly higher in G/G vs. A/A (Bonferroni adjusted P <0.001) and G/A (Bonferroni adjusted P <0.001), and significant between G/A and A/A (Bonferroni adjusted P = 0.003).

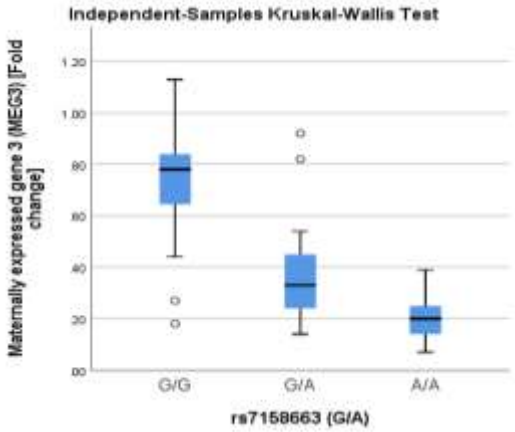

Fig. 1. Effect of rs7158663 genotypes on serum MEG3 expression. CRC patients with rs7158663 GA genotype have lower expression level of serum MEG3 than those with GG and AA genotype.

Diagnostic performance of serum MEG3

ROC curve shows that MEG3 at cutoff value ≤ 0.92 can significantly discriminate CRC from control (AUC = 0.975, P< 0.001) with 97.5% sensitivity and 100% specificity (Fig. 2).

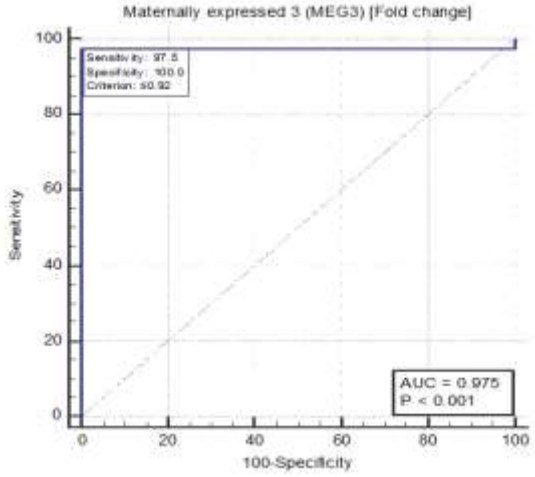

Fig. 2. ROC curve analysis to discriminate between case and control: cutoff value ≤ 0.92, AUC = 0.975, P < 0.001 with 97.5% sensitivity and 100% specificity.

Correlation of rs7158663genotypes, serum MEG3 level with clinicopathological data

The results in Table 5 show a statistically significant difference between the three genotypes as regards ALT, serum triglycerides, 2HPPS, PLT, and CEA. Serum triglycerides were statistically significantly higher in A/A-G/G and statistically insignificant difference between A/A-G/A and G/A-G/G. PPBG Pairwise comparisons revealed no difference between genotypes. Platelet count was statistically significantly higher in G/G-A/A and G/A-A/A and statistically insignificant difference between G/G and G/A. Pairwise comparisons show a statistically significantly higher CEA in A/A compared to G/A and G/A compared to G/G and statistically insignificant difference between G/G and A/A.

Table 6 shows a statistically significant negative correlation between MEG3 fold change and ESR level, WBCs count, and platelet count a statistically significant positive correlation between MEG3 fold change and occurrence of rectal bleeding. As MEG3 fold change increases, the proportion of patients with rectal bleeding increases.

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Table 5. Comparisons of the three genotypes.

Characteristic	G/G N=78	G/A N=50	A/A N=32	P value
Age (years)	50 (40-55)	50 (39.5-56.75)	53 (50-63)	0.172
BMI (kg/m^2)	28 (23-30)	24 (23-29.25)	26 (22.25-29.5)	0.773
Sex				
Male	50 (64.1%)	36 (72%)	24 (75%)	0.447
Female	28 (35.9%)	14 (28%)	8 (25%)	
Overweight / obesity	48 (61.5%)	24 (48%)	16 (50%)	0.264
MEG3 (Fold change)	0.78 (0.62-0.84)	0.33 (0.235-0.4575)	0.20 (0.14-0.25)	**<0.001**
ESR	42 (9-66)	26 (18-45)	41.5 (25-57.25)	0.118
Iron deficiency anemia	20 (25.6%)	14 (28%)	14 (43.8%)	0.159
ALT	24.6 (19.35-34.45)	27.25 (23.725-35.2)	21.05 (17.875-34.95)	0.19
AST	24 (16-29.75)	23 (15-30.25)	23.5 (13.5-26)	0.387
Serum albumin (g/dl)	4.9 (4.3-5.4)	5.1 (4.5-5.4)	4.8 (3.6-5.4)	0.223
GGT	84 (57.5-98.25)	87 (74-98.75)	88 (52-98.25)	0.380
Serum triglycerides (mg/dl)	99 (61.25-121)	85.5 (58.5-108.75)	63 (45-93.5)	**0.015**
Serum total cholesterol (mg/dl)	167.5 (148-252)	165 (149-234)	198 (148-255)	0.669
FBG (mg/dl)	130 (84-168)	121 (78-155)	112 (95-206)	0.897
PPBG (mg/dl)	85 (76-96)	94 (85-98)	95 (85-104)	**0.039**
Serum creatinine (mg/dl)	0.9 (0.75-1.125)	0.95 (0.6-1.1)	0.95 (0.65-1.1)	0.879
Hemoglobin level	13 (9.1-13.6)	11 (8.95-13.5)	10.95 (7.55-13.75)	0.256
WBC count	5.6 (4.6-7.6)	6.8 (4.8-9.125)	5.8 (4.85-8.9)	0.099
Platelet count	243 (163-341)	236 (189-265)	283 (237.25-407)	**0.004**
CEA	18 (4.4-46)	4.7 (2.9-18)	35.5 (7.95-55.25)	**<0.001**
CA19-9	29 (16-63)	26.5 (15-69)	45 (20.25-74.5)	0.471

Notes: Data is median (Q1-Q3) for quantitative characteristics (test of significance is Kruskal-Wallis H-test), or N (%) for categorical data (test of significance is *Chi-Square test or $^{\$}$Fisher's exact test for rs7158663 genotypes). MEG3 = Maternally expressed gene 3.

Table 6. Correlation between MEG3 (fold change) and clinical-laboratory parameters.

Parameter	Correlation coefficient	P value
Age (years)	-0.124	0.117
BMI (kg/m^2)	-0.034	0.673
Sex	-0.073	0.358
Overweight / obesity	0.040	0.616
ESR	-0.208	**0.008**
Iron deficiency anemia	-0.012	0.877
ALT	0.080	0.324
AST	0.124	0.125
Serum albumin (g/dl)	0.017	0.834
GGT	-0.114	0.158
Serum triglycerides (mg/dl)	0.124	0.126
Serum total cholesterol (mg/dl)	-0.058	0.487
FBG (mg/dl)	0.006	0.946
PPBG (mg/dl)	-0.117	0.162
Serum creatinine (mg/dl)	-0.145	0.073
Hemoglobin level	-0.115	0.149
WBC count	-0.168	**0.034**
Platelet count	-0.161	**0.042**
CRC site	0.055	0.491
CEA	-0.002	0.987
CA19-9	-0.203	0.052
Abdominal pain	0.056	0.493
Constipation	0.031	0.698
Weight loss	0.107	0.188
Rectal bleeding	0.226	**0.005**

Notes: Correlation coefficient is Point Bi-Serial (r_{pb}) or Spearman's (r_s) correlation coefficient.

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Discussion

Our findings demonstrated a link between the MEG3 rs7158663 polymorphism and CRC risk. In CRC cases compared to control subjects, an 'A' allele (risk allele) and A/A genotype were shown to be statistically significantly higher. Additionally, MGE3 gene expression was lower in CRC patients compared to control.

To increase the longevity of CRC patients, it is crucial to identify the molecular causes of CRC's onset and progression. Previous research demonstrated that MEG3 functions as a tumour suppressor by demonstrating low expression of the long non-coding RNA in numerous cancer cell lines (19, 20).

MEG3 rs7158663 has shown a role in MEG3 folding and might affect the transcription factor binding site (21-22, 23). Using this information, we looked at the association between the lncRNA MEG3 rs7158663 polymorphism and the risk of CRC and clinicopathologic features of CRC in an Egyptian population. We also evaluated the expression of the lncRNA MEG3, which was examined for a relationship with various genotypes and alleles.

A lot of focus has been placed on aberrantly expressed lncRNAs in tumour tissues as prospective biomarkers for the diagnosis and prognosis of cancer. Through their interactions with DNA, RNA, protein, and/or their combinations, lncRNAs are linked to several stages in the development of cancer (24). They function as an important regulator in cancer pathways. Based on this, numerous studies have looked at circulating lncRNAs as a biomarker for CRC (25,26). Here, we show that serum MEG3 was expressed differently in CRC patients compared to controls and distinguished CRC from a different group with high sensitivity and specificity, suggesting that serum MEG3 represents a unique potential noninvasive biomarker for CRC diagnosis.

Our findings demonstrate that serum MEG3 is downregulated in CRC, which is compatible with its tumour suppressor function (27,28). This downregulation was consistent with earlier research in several tumour tissues and cell lines (29-31), which may have been mirrored in the serum. In this study, we additionally look at MEG3 rs7158663's functional significance. We discovered that rs7158663 AA CRC patients had significantly lower serum MEG3 levels than GG CRC patients ($P < 0.001$). The expression level of serum MEG3 was shown to be lower in CRC patients with the rs7158663 GA genotype than those with the GG genotype ($P < 0.001$) however, serum MEG3 levels were marginally significant between patients with GA and AA.

Notably, MEG3 expression is regulated by several mechanisms, including promoter methylation, transcription factors, RNA destabilization, lncRNA-lncRNA interaction, and post-transcriptional regulation by miR-499-5p (32). Additionally, our results propose rs7158663SNP as a new mechanism regulating MEG3 expression in cancer.

This result was associated with a statistically significant difference between the three genotypes as regards ALT, serum triglycerides, 2HPPS, PLT, and CEA. There was a statistically significant negative correlation between MEG3 fold change and ESR level, WBCs count, and platelet count. There was a statistically significant positive correlation between MEG3 fold change and the occurrence of rectal bleeding. As MEG3 fold change increases, the proportion of patients with rectal bleeding increases.

Similarly, previous study examined SNPs of MEG3 associated with colorectal cancer in Chinese patients and found that carriers of the mutant AA genotype of MEG3 rs7158663 had a higher risk for colorectal cancer compared with controls (33, 34) found a significant association between a MEG3 polymorphism and oral squamous cell carcinoma by interring the binding of miRNA. Also, Ali et al (16) also demonstrated a role for MEG3 rs7158663 in breast cancer patients.

MEG3 rs7158663 GA/AA genotype and A allele levels were considerably greater in breast cancer patients compared to controls, according to shaker et al. (15). Additionally,

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MEG3 rs7158663 G/A mutant A allele correlation with lower serum MEG3 expression levels supports our findings. The MEG3 polymorphisms (rs11627993 and rs7158663) may not be associated with prostate cancer susceptibility, according to Xu et al., 2020 (35).

The ROC curve was developed to evaluate the diagnostic performance of MEG3, and it produced high diagnostic accuracy (AUC = 0.975%), a sensitivity of 97.5%, and a specificity of 100% in distinguishing CRC cases from control subjects. Due to its great ability to discriminate between cases and controls, it demonstrated a positive prognostic value in CRC and suggested serum MEG3 as a potential noninvasive biomarker for CRC diagnosis.

Ethical approval

All participants in this study signed an informed consent after being informed about all steps of the study including publication step. This study was approved by an ethics committee, Faculty of medicine, Mansoura University with the following code (R.22.12.1988).

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Conflict of Interest

No conflict of interest.

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