# Serum Long-Noncoding RNA H19 and β-Catenin as Biomarkers for Early Diagnosis of Colorectal Cancer in Egyptian Patients: A Case Control Study

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## Abstract

Colorectal cancer (CRC) is the third most prevalent cancer and the second most common cause of cancer death; however, its early detection can improve the survival. Colonic polyps are considered one of the CRC's major risk factors. Throughout many biological processes and malignancies, the non-coding RNAs have essential functions. Certain long noncoding RNAs (lncRNAs), including H19, were supposed to be CRC possible biomarkers. Also, H19 has been reported to play a role in regulating the activity of β-catenin, a protein that regulates cell-to-cell adhesion, as well as gene transcription. The current work aimed to investigate the potential significance of LncRNA H19 relative serum expression level by quantitative polymerase chain reaction (q-PCR) and β-catenin by enzyme-linked immunosorbent assay (ELISA) as noninvasive biomarkers to discriminate between colorectal cancer and colonic polyps. The statistical analysis of the studied factors revealed that the serum expression of H19 and β-catenin in cancer cases were substantially greater than colonic polyp cases and normal control.

**Conclusion** The relative expressions of H19 and beta-catenin in the serum can significantly discriminate patients with CRC from those with polyp and normal controls, which could help when screening for CRC.

**Keywords**
- LncRNA
- H 19
- β-catenin
- CRC
- colonic polyp

## Introduction

Worldwide, colorectal cancer (CRC) is the third most common type of cancer, and it accounts for 10% of all cancer-related mortality.[1] Early detection of colon cancer can improve survival.[2] Among the essential risk factors, tubular and villous adenomatous colonic polyps are determined as CRC precursor lesions. From those adenomas, ~ 95% of sporadic CRC originates.[3]

Over the years, CRC begins to grow from adenomas even with no symptoms. It is believed that small lesions take ~ 10 years to develop to be invasive cancer. This protracted

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duration from adenoma to carcinoma seems to be of great benefit for early detection and diagnosis of colon cancer and also for its prevention.[2]

Screening by colonoscopy and sigmoidoscopy results in a reduction of both incidence and mortality of CRC.[4] Despite the benefit, they are invasive with high cost.[5] Fecal immunochemical test (FIT), although noninvasive, shows low sensitivity, which limits its use in practice.[6] Until now, it is still difficult to predict whether Cologuard (Exact Sciences Corporation, Madison, WI, USA), which is also a fecal test with a significantly higher cost, will have any better performance.[7]

Blood markers for CRC include carcinoembryonic antigen (CEA) and carbohydrate antigen CA 19–9. They are normally used to follow patients with known cancers, predict prognosis, assess response to treatment, and monitor for recurrence, but they are not sensitive enough for a diagnosis of CRC.[8]

A strategy for a higher screening rate and early diagnosis of CRC is urgently needed, and blood-based biomarkers are promising.

Non-coding ribonucleic acids (ncRNAs), which are RNA groups that do not code proteins, represent most of the transcripts of all RNAs inside the cell. It includes short ($\sim$ 22 nucleotides) microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (of $>$ 200 nucleotides). At the epigenetic levels, lncRNAs control gene expressions. Moreover, lncRNAs play important roles in the proliferation, apoptosis, and invasiveness of tumor cells as well as in the metastatic capacity of cancers.[9]

Importantly, lncRNAs were also found in human serum, plasma, urine, and other body fluids, where they are remarkably stable. These findings raised the possibility that circulating lncRNAs can be considered as a potential biomarker for the diagnosis of diseases, including cancer.[10]

Certain lncRNAs, one of which is H19, were recognized as possible biomarkers for CRC. The H19 gene encodes the covered, segmented, and polyadenylated 2.7 kb RNA, rather than a protein. The H19 lncRNA enhances the invasive and migratory potential of cancer cells by regulating target genes.[11] Also, H19 can regulate the activity of β-catenin,[12] which is a protein encoded via the catenin β1 (CTNNB1) gene. Beta-catenin is a target for miR-148b miR-200a. The H19 lncRNA can act as a competing endogenous RNA (ceRNA) of both miR-148b and miR-200a, leading to enhanced expression of β-catenin.[13,14] Also, H19 decreases cyclin-dependent protein kinase 8 (CDK8) expression by interacting with macroH2A, which is one of the core histones proteins that act as a regulator of CDK8. This reduction in CDK8 expression underlies changes in β-catenin activity by changing its phosphorylation.[15]

Beta-catenin acts as a regulator and coordinator for the adhesion between cells and is involved in the regulation of gene expression. In addition, β-catenin is a subdivision of the complex E-cadherin protein and could be considered as an intracellular transducer of signal through the wingless integrated (Wnt) signaling pathway that has a significant role in the control of the proliferation of intestinal cells.[16]

The measurements of H19 were performed in the plasma of patients with gastric and breast cancer, and its role as a potential non-invasive biomarker was shown in the two tumors.

However, the H19 level in the plasma of CRC patients is still unclear.[17] Also, many studies had focused on the detection of higher levels of β-catenin in cancer tissues but not in serum.[18] The present work aimed to examine the potential significance of lncRNA H19 relative serum expression level in addition to β-catenin as noninvasive biomarkers to discriminate between CRC and colonic polyps.

## Materials and Methods

To achieve the goal of the study, we enrolled 40 CRC (group I), 40 colorectal polyp patients diagnosed by colonoscopy (group II) who attended the department of gastroenterology of the Alexandria Main University Hospital, and 20 healthy controls (group III) of matched age and gender.

After a thorough clarification of all procedure steps, written informed consent was received from all participants. The ethics review board of the Faculty of Medicine at Alexandria University approved the study under the number 021023.

The exclusion criteria were patients with cardiac or hepatic disorders, renal diseases, autoimmune diseases, familial adenomatous polyposis (FAP), and malignancies other than CRC. Also, patients who had received systemic chemotherapy and radiotherapy, patients who were unwilling to participate, and patients who did not sign the informed consent form swere excluded from this work.

For the case-control study, the sampling size has been determined utilizing the epi info software, considering a power of 80% and a confidence level of 95%.

All patients of groups I, II, and III were evaluated clinically by taking a full history and complete clinical examination; then, 10 ml of venous blood was collected from them. Each blood sample was then divided into three aliquots: an ethylene diamine tetraacetic acid (EDTA) tube, a citrated tube, and a plain tube. In the latter, the blood was allowed to clot, then centrifuged at 1,200 xg for 10 minutes to separate serum samples, which were kept frozen at -80°C until use.

In groups I and II, the colon was evaluated by colonoscopy, and the obtained biopsy was referred for histopathological examination. Determination of H19 relative serum expression level using real-time quantitative polymerase chain reaction (q-PCR) and measurement of serum level of β-catenin using enzyme-linked immunosorbent assay (ELISA) were done for all groups

Total RNA separation from serum samples was performed using the Qiagen miRNeasy Mini Kit. (Cat. No. 217004). A nanodropper 2,000/2,000cc was used for measuring RNA condensation and purity; then, the High Capacity cDNA Reverse Transcription Kit was used for the synthesis of complementary deoxyribonucleic acids (cDNA) (Applied Biosystems, USA).[19] Each reaction contained 10 µg of RNA extract, 2 µl of reverse transcriptase buffer, 0.8 µl of deoxynucleotide triphosphate (dNTP), 1 µl of reverse transcriptase,

 

1µl of RNase inhibitor, 2 µl RT of random primers, and the total volume amounted to 20 µl, by adding nuclease-free water. The thermal cycle has been set for 10, 120, and 5-minute hold at 25°C, 37°C, and 85°C, respectively. Finally, at 4°C for 24 hours if not directly stored at – 20°C; then, the cycle was turned off. Quantitative PCR has been performed using the Applied Biosystems Step-one Real-time via Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, Cat. No. K0251) (Thermo Fisher Scientific, Waltham, MA, USA). Specific primers for H19 were as follows: forward primer 5-TGCTGCACTTTACAACCACTG-3, and a reverse primer 5-ATGGTGTCTTTGATGTTGGGC-3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control with a forward primer 5′-GAAGGTGAAGGTCGGAGTCAAC-3′ and a reverse primer 5′-CAGAGTTAAAAGCAGCCCTGGT-3′. Each reaction consisted of 12.5 µl of Maxima SYBR Green qPCR Master Mix (2X), 1 µl of forward primer (50 pmol), 1 µl of reverse primer (50 pmol), 0.1 µl of ROX Solution, 7.4 µl of nuclease-free water, and 3 µl of cDNA. In duplications, specimens have been analyzed. No template control was used in every assay. Quantitative PCR has been set as the following: 10 minutes premier cycle at 95°C, followed by 3-step cycling: (40 cycles) denaturation at 95°C, for 15 seconds; annealing at 56°C for H19 and 65°C for the GAPDH gene for 30 seconds, and the extension step at 72°C for 30 seconds. Melting curves were done to verify the specificity and identity of the product. A relative quantitative method (RQ = 2–ΔΔCT) has been used to calculate the fold modification between a sample and a normal control for H19.

**Determination of the serum level of human β-catenin** was performed by ELISA (NOVA, Beijing, China).

This type of ELISA kit used was the sandwich-ELISA. Beta-catenin antibodies were used for precoating the microelisa strip plate. Afterward, wells have then been used for standards or specimens; then, a horseradish peroxidase (HRP)-conjugated antibody specified for β-catenin was added to every well and incubated. Wells containing β-catenin and anti-HRP conjugated β-catenin turned blue, followed by yellow coloration after adding the stopping solution. Spectrophotometric measurement of the optical density (OD) was done at 450 nm wavelength.

### The Data Statistical Analysis[20]:

Using the IBM SPSS Statistics for Windows, Version 20.0 software package (IBM Corp., Armonk, NY, USA), the data have been analyzed and added to the computer. [21]

## Results

Statistical comparisons of the subjects' age showed significant differences between groups I and II. In group I, the age range was from 40 to 79 years, with a mean value of 55.6 ± 9.37. While the mean age in group II was 50.48 ± 7.29 years for a range from 40 to 68 years. In group III, the age range was from 32 to 67, with a mean value of 52.95 ± 8.68 years. Distribution of the studied cases in group I according to stage demonstrated in ►**Fig. 1**.

The hemoglobin level in group I ranged from 7.5 to 13.9 g/dl, in group II from 8.9 to 14.7 g/dl, while in group III it was

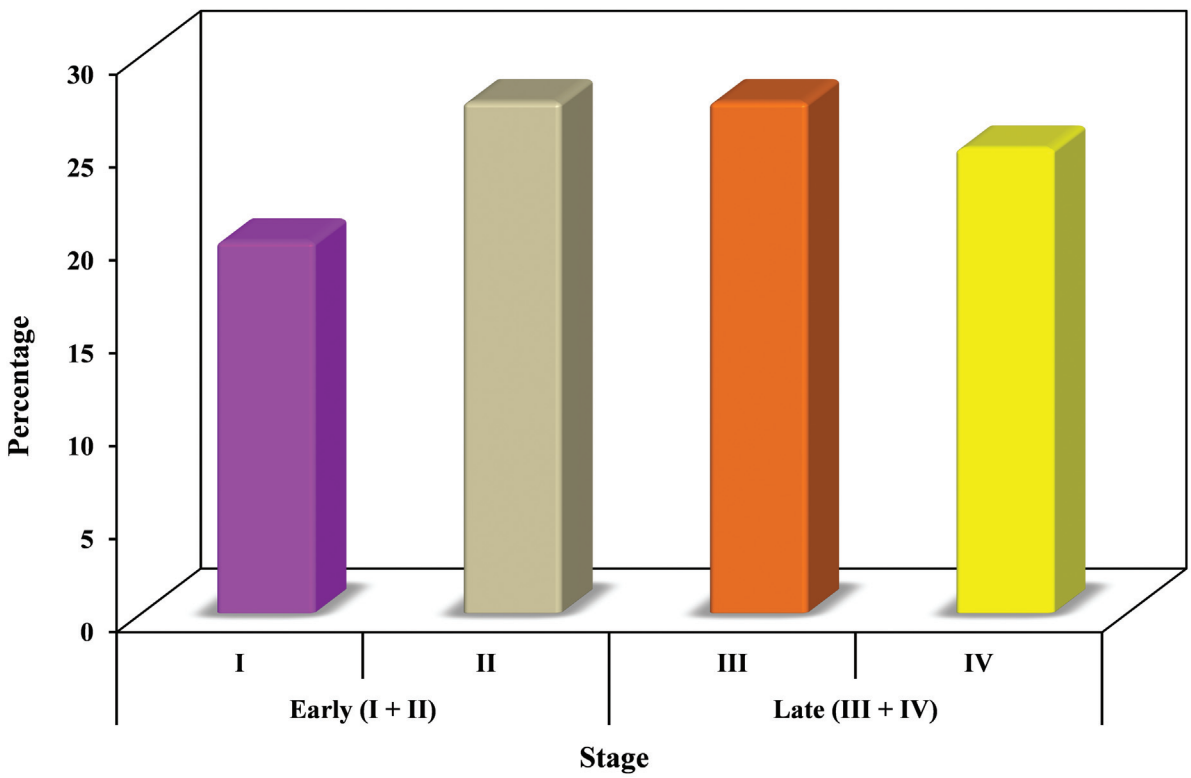


**Fig. 1** Distribution of the studied cases according to colorectal cancer stage in group I.

 

between 9.8 to 14.7 g/dl. In group I, the level of hemoglobin was significantly lower than that in groups II and III, but no significant difference has been found among groups II and III. The demographic and laboratory outcomes of all groups are demonstrated in ►**Table 1**.

Both CEA and CA19–9 levels in group I were significantly higher than those in groups II and III; however, the difference between the levels in groups II and III was not significant. The CEA and CA 19–9 levels were demonstrated in ►**Table (1)**.

The level of H19 in group I was significantly higher than that in groups II and III, while no significant difference between groups II and III was found. The level of β-catenin in group I was significantly greater than that in groups II and III, while the levels in groups II and III had no significant difference as shown on ►**Table (2)**.

There was no significant correlation between relative serum expression levels of H19 and different cancer stages (r: 0.112, p: 0.49), nor β-catenin and different cancer stages (r: 0.269, p: 0.09).

Receiver-operating characteristic (ROC) curve analysis of H19 relative serum expression, β-catenin, CEA serum level, and serum CA19,9 activity to predict group I from group II patients as in ►**Table 3**, ►**Fig. 2** and group I from group III in ►**Table 4**.

**Table 1** Demographic data and laboratory results of the studied groups

| | Group 1 ($n=40$) | | Group 2 ($n=40$) | | Group 3 ($n=20$) | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| **Sex** | | | | | | |
| **Male** | 17 | 42.5 | 21 | 52.5 | 5 | 25.0 |
| **Female** | 23 | 57.5 | 19 | 47.5 | 15 | 75.0 |
| **Age (years)** | | | | | | |
| **Min.–Max.** | 40.0–79.0 | | 40.0–68.0 | | 32.0–67.0 | |
| **Mean ± SD** | $55.60 \pm 9.37$ | | $50.48 \pm 7.29$ | | $52.95 \pm 8.68$ | |
| **Significance** | $p_1 = 0.021^*$, $p_2 = 0.489$, $p_3 = 0.535$ | | | | | |
| **HB (g/dl)** | | | | | | |
| **Mean ± SD** | $10.16 \pm 1.21$ | | $11.83 \pm 1.56$ | | $12.42 \pm 1.38$ | |
| **Significance** | $p_1 < 0.001^*$, $p_2 < 0.001^*$, $p_3 = 0.278$ | | | | | |
| **Erythrocytic sedimentation rate (ESR) (mm/h)** | | | | | | |
| **Median** | 67.50 (28.0–96.50) | | 29.50 (20.0–42.50) | | 14.50 (10.0–21.0) | |
| **Significance** | $p_1 = 0.007^*$, $p_2 < 0.001^*$, $p_3 = 0.006^*$ | | | | | |
| **Serum Albumin (g/dl)** | | | | | | |
| **Mean ± SD** | $28.95 \pm 7.33$ | | $35.74 \pm 5.46$ | | $40.14 \pm 3.29$ | |
| **Significance** | $p_1 < 0.001^*$, $p_2 < 0.001^*$, $p_3 = 0.023^*$ | | | | | |
| **C- reactive protein (CRP) (mg/l)** | | | | | | |
| **Median** | 7.30 (4.0–9.10) | | 3.05 (2.0–4.30) | | 1.20 (1.0–3.25) | |
| **Significance** | $p_1 < 0.001^*$, $p_2 < 0.001^*$, $p_3 = 0.049^*$ | | | | | |
| **CEA (ng/ml)** | | | | | | |
| **Median** | 8.11(6.3–11.7) | | 1.89 (1.24–3.2) | | 2.60(1.4–4.1) | |
| **Significance** | $p1 < 0.001^*$, $p2 < 0.001^*$, $p3 = 0.622$ | | | | | |
| **CA19–9 (U/ml)** | | | | | | |
| **Median** | 33.15(24.3–39.2) | | 7.71(2.4–15.2) | | 7.72(4.4–18.5) | |
| **Significance** | $p1 < 0.001^*$, $p2 < 0.001^*$, $p3 = 0.593$ | | | | | |

p1: p value for comparing between group I and group II.
p2: p value for comparing between group I and group III.
p3: p value for comparing between group II and group III.
*: Statistically significant at $p \leq 0.05$.
Group I: Colorectal adenocarcinoma.
Group II: Colorectal polyp diagnosed by colonoscopy.
Group III: Control.

 

**Table 2** Comparison between the studied groups according to H19 and Beta catenin

| | Group I ($n=40$) | Group II ($n=40$) | Group III ($n=20$) | *P* |
|---|---|---|---|---|
| **H19 (fold increase)** | | | | |
| **Min. – Max.** | 0.04–10.20 | 0.05–9.34 | 0.23–4.56 | 0.010* |
| **Median (IQR)** | 1.24 (0.54–2.17) | 0.70 (0.38–1.73) | 0.49 (0.29–1.01) | |
| **Significance** | p1 = 0.019*, p2 = 0.006*, p3 = 0.411 | | | |
| **β-catenin (pg/ml)** | | | | |
| **Min.–Max.** | 127.0–4,520.0 | 99.0–3,395.0 | 90.50–1,009.0 | 0.001* |
| **Median (IQR)** | 305 (199.7–509.5) | 211.25 (139–305) | 151.0 (119.5–233.3) | |
| **Significance** | p1 = 0.009*, p2 < 0.001*, p3 = 0.138 | | | |

p1: p value for comparing between group I and group II.
p2: p value for comparing between group I and group III.
p3: p value for comparing between group II and group III.
*: Statistically significant at $p \leq 0.05$.
Group I: Colorectal adenocarcinoma.
Group II: Colorectal polyp diagnosed by colonoscopy.
Group III: Control.

**Table 3** Receiver-operating characteristics curve analysis of H19 serum expression ($2^{-\Delta\Delta CT}$), β-catenin, carcinoembryonic antigen serum level, and serum CA19.9 activities for group I versus group II

| | AUC | *P* | 95% C.I | Cut off | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|---|---|---|
| **CEA** | 0.963* | < 0.001* | 0.914–1.011 | > **4.56** | 95.0 | 92.50 | 92.7 | 94.9 |
| **CA19-9** | 0.943* | < 0.001* | 0.896–0.991 | > **19.45**# | 95.0 | 87.50 | 88.4 | 94.6 |
| **H19** | 0.653* | 0.019* | 0.532–0.774 | > **0.85** | 67.50 | 62.50 | 64.3 | 65.8 |
| **Catenin** | 0.681* | 0.005* | 0.563–0.799 | > **215.5** | 67.50 | 57.50 | 61.4 | 63.9 |
| **H19 + Catenin** | 0.724* | 0.001* | 0.612–0.836 | | 35.0 | 87.50 | 73.68 | 57.38 |
| **CEA + H19 + Catenin** | 0.965* | < 0.001* | 0.917–1.013 | | 90.0 | 95.0 | 94.74 | 90.48 |
| **CA19-9 + H19 + Catenin** | 0.948* | < 0.001* | 0.905–0.992 | | 87.50 | 87.50 | 87.50 | 87.50 |

Abbreviations: AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; *p*-value, probability value; PPV, positive predictive value.
*: Statistically significant at $p \leq 0.05$
Group I: Colorectal adenocarcinoma.
Group II: Colorectal polyp.

## Discussion

The lncRNA H19 was overexpressed in many types of cancer, like esophageal, gastric, hepatic, bladder, and breast cancer.[22] The present work has been conducted for assessing the serum H19 expression significance and to discuss the probability of using it as a serum biomarker for early CRC diagnosis in Egyptian patients.

Our study revealed that the H19 expression levels in cancer cases were significantly higher than those of colonic polyp patients.

In accordance with our results, Gharib E et al.[23] found that among CRC cases, H19 expression in fecal colonocytes was higher than in colonic polyp patients. Also, Galamb et al. stated that the expression of H19 was elevated in the cancer tissue of CRC patients.[24]

Our study also showed that H19 expression level in cancer patients was significantly high when compared with controls, while polyp patients and controls did not show significant differences.

In agreement with our findings, the study by Ismail D et al. on Egyptian CRC patients stated that the expression level of H19 had shown an 11.38-fold increase in CRC cases in comparison with controls. However, H19 expression had not shown any significant difference among CRC cases who had and even those that did not have regional lymph node metastasis.[19]

Moreover, the study by Gharib et al. showed greater expression of H19 in fecal colonocytes in CRC cases than in controls.[23] Also, many studies were performed on CRC tissue in comparison to surrounding normal tissues, and they found that the level of H19 expression in malignant tissue was higher than in normal tissue, as demonstrated by Yang et al., Yang et al., Ding et al. and Liang et al.[13,22,25,26]

Acting as a competitive endogenous RNA (ceRNA) for miR-138 and miR-200a, lncRNA H19 enhances the tumor growth

 

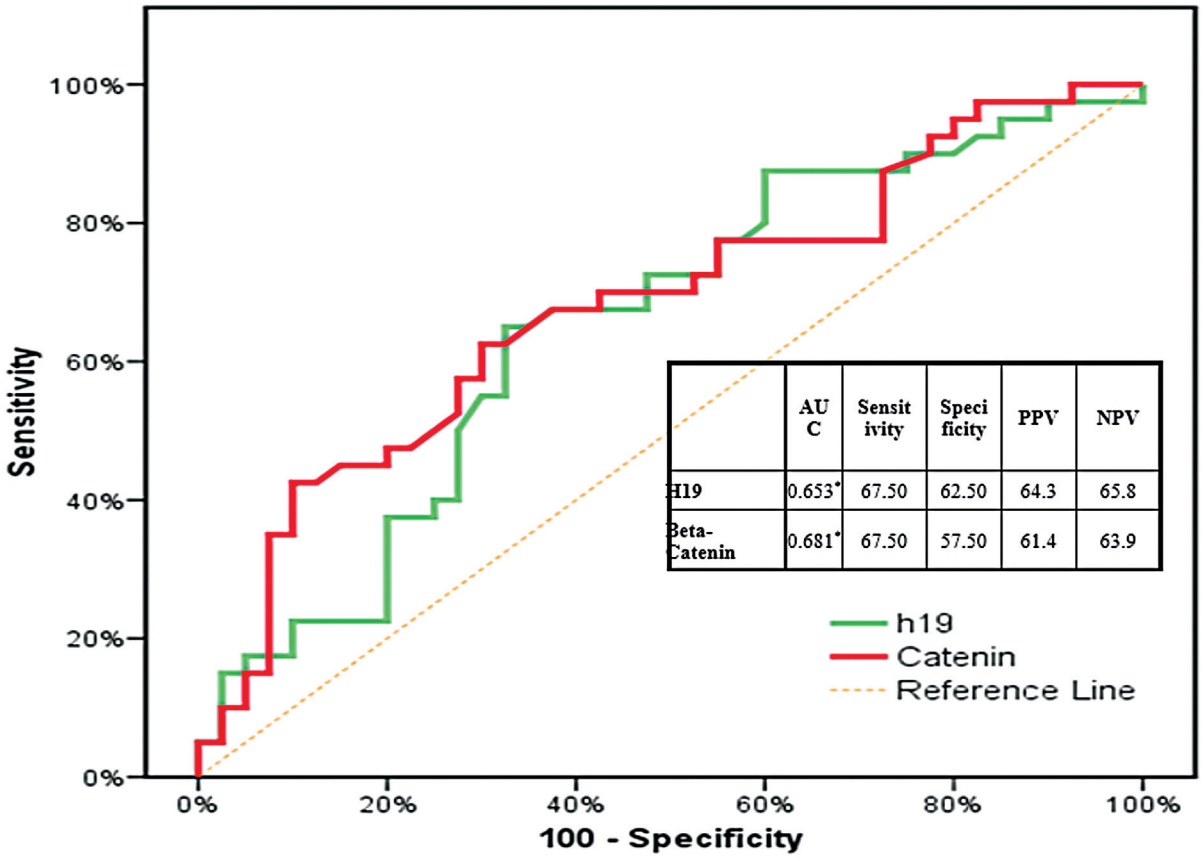


**Fig. 2** Receiver-operating characteristic curve to differentiate group I from group II.

**Table 4** Receiver operating characteristics curve for different parameters to differentiate group I from group III

| | AUC | *P* | 95% C.I | Cut off | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|---|---|---|
| CEA (ng/ml) | 0.976* | < 0.001* | 0.943–1.009 | > **4.6** | 95.0 | 90.0 | 95.0 | 90.0 |
| CA19-9 (U/ml) | 0.938* | < 0.001* | 0.875–1.002 | > **19.3** | 95.0 | 80.0 | 90.5 | 88.9 |
| H19 (fold increase) | 0.715* | 0.007* | 0.575–0.855 | > **0.54** | 72.50 | 50.0 | 74.4 | 47.6 |
| Catenin (pg/ml) | 0.763* | 0.001* | 0.634–0.891 | > **198.5** | 75.0 | 65.0 | 81.1 | 56.5 |

Abbreviations: AUC, area under a curve; CI, confidence interval; NPV, negative predictive value; *p*-value, probability value; PPV, positive predictive value.
*: Statistically significant at $p \leq 0.05$.
Group I: Colorectal adenocarcinoma.
Group III: Control.

by antagonizing their activities. It also causes the de-repression of their endogenous goals: vimentin, ZEB1, and ZEB2, which are markers for mesenchymal cells. Also, H19 sponged miRNA let-7 and modularized the expressions of let-7 targets, providing strong proof that H19 is a natural sponge for miRNAs causing endothelial-mesenchymal transition (EMT).[26]

Contrary to our study, Yoshimizu et al. suggested that H19 might also play a role in tumor suppression in mice by using in vivo murine models of carcinogenesis. They found that *H19* controlled the size of experimental carcinomas, the number of polyps in the *Apc* murine model of CRC, and delayed the appearance of metastasis.[27] The discrepancy between such results and our results could be explained by the difference in the species and/or type of samples used since human and mouse have different genetics; the H19 locus may play a more complex role in humans than in mice. While our study was carried on samples from patients with sporadic polyp.

Wingless-related integration site (Wnt)/β-catenin signaling pathway initiates the carcinogenesis. Many studies focused on the detection of higher levels of β-catenin in cancer tissues but not in the serum. Fortunately, some researchers have documented that β-catenin could be identified in human serum, and its level is associated with hepatocellular carcinoma (HCC) growth due to hepatitis C, hepatitis B-linked diseases, diabetes mellitus II, phosphatase and TENsin (PTEN) hamartoma tumor syndrome, and early-onset ulcerative colitis. Although there is a hyperactivated Wnt/β-catenin signal and a great quantity of β-catenin aggregation

 

in CRC, the diagnostic significance of serum β-catenin remains uncertain among CRC cases.[18]

Our research demonstrated that serum β-catenin levels were significantly higher in cases with cancer compared with those with colonic polyp and controls. In the same line with our research, Li et al. stated that the level of serum β-catenin in cases with CRC is significantly elevated in relation to those in colonic polyp and in the control group.[18] Also, our findings are supported by Kobayashi et al., who reported in their study that nuclear overexpression of β-catenin was observed in 35% of early-stage CRC patients and 42% of invasive cancer patients but was not observed in patients with polyps from either sporadic or FAP cases. Most CRC cases that were accompanied by polyps showed nuclear overexpression of β-catenin in the cancer area, but this was not observed in the polyps component.[28]

Also, Wong et al. mentioned that β-catenin was overexpressed in 8% of polyps and all CRC samples in comparison to normal tissues. Higher levels in CRC were significantly associated with lymph node metastasis.[29] Also, Bourroul et al. found low expression of β-catenin in colonic polyps, whereas its expression was increased in colorectal carcinoma.[30–32]

Our study also showed that the levels of β-catenin among cases with colonic polyp and controls did not differ significantly. In contrast to our study, the level of β-catenin in the colonic polyp group was significantly higher than in healthy controls in a study conducted by Li et al.[18]

Finally, the ROC curve analysis was performed for analyzing the cut-off levels for H19 serum expression and serum β-catenin level above which patients are expected to have CRC, and we found that H19 and β-catenin did not show a better diagnostic performance over CEA or CA19–9. However, the combination of both markers with CEA or CA19–9 improved their specificity in the differentiation of CRC patients from those with polyp and controls. Thus, serum H19 and β-catenin can both help in the differentiation of CRC patients from colonic polyp patients and controls but cannot discriminate polyp patients from normal subjects.

The present study is important due to the high mortality rate of CRC, and the possibility of decreasing its mortality by reaching an early diagnosis. Also, the novelty of the study as, until now, very little is known about the significance of relative serum expression of H19 and β-catenin as biomarkers used in the early diagnosis of CRC. On the other hand, some limitations of the current study need to be addressed. First, the study subjects were restricted to the city of Alexandria. Second, the sample size of this study is relatively small, which may not have enough statistical power to explore the true association. Therefore, large population-based prospective studies with an ethnically diverse population are warranted.

## Conclusion

Serum expressions of H19 and β-catenin can significantly discriminate patients with CRC from those with polyp and from the normal population, which could help with screening for CRC.

### Conflict of Interests

The authors declare no conflict of interests.

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