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Association between DANCR and NRAS genes expression and colorectal cancer in Egyptians, Potential diagnostic markers

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Abstract

Colorectal cancer (CRC) is the third leading malignancy with high morbidity and mortality worldwide. Our study aimed to investigate the role of the Differentiation Antagonizing non-protein coding RNA (DANCR) and Neuroblastoma RAS viral oncogene homolog (NRAS) genes in the development and progression of CRC. The expression of DANCR and NRAS genes were assessed by quantitative real-time polymerase chain reaction (RT-qPCR) in serum samples of 40 patients diagnosed as CRC and 40 subjects free from CRC included as a control group. The demographic, laboratory and clinicopathological data of the subjects were reviewed to detect their association with the DANCR and NRAS genes. The level of gene expression of both DANCR and NRAS showed highly statistically significant upregulation in the cases group compared to the control group ($p < 0.001$ for both). The best cutoff point of DANCR gene expression to differentiate cases group from the control was > 1.693. This value has excellent sensitivity (87.5%) and specificity (92.5%). The area under the curve was 0.918 with high statistically significant value ($p < 0.001$). The best cutoff point of NRAS gene expression to differentiate cases group from the control was > 1.094. This value has excellent sensitivity (87.5 %) and specificity (90%). The area under the curve was 0.917 with high statistically significant value ($p < 0.001$). Our study showed the role of DANCR and NRAS genes as potential novel biomarker for early prediction of CRC in Egyptians.

Keywords: Colorectal Cancer, DANCR, NRAS, Gene expression, Biomarker.

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1. Introduction

The third most common cancer worldwide and the fourth most common cause of cancer death is colorectal cancer [1]. It is the seventh most prevalent cancer in Egypt, accounting for 3.47% of male and 3% of female cancer cases [2]. The intricate connections between environmental and genetic factors are linked in this multifactorial disease which is known as CRC. Inflammatory Bowel Disease (IBD) and DNA repair defects are among common causes [3]. Although remarkable progress in understanding of CRC has been made in recent years, the underlying exact molecular mechanisms of CRC remain unclear. Patients with CRC are usually diagnosed at aggressive stages with poor prognosis due to lack of effective predictive markers [4-5]. Conventional prognostic factors include clinical and laboratory criteria have limitations to predict patient outcome. Therefore, understanding molecular mechanisms of colorectal cancer to find effective targeting markers for early detection, prognosis and potential therapeutic modalities is necessary [6-7]. DANCR gene produces a long non-coding RNA which are a class of nonprotein- coding RNAs with more than 200 nucleotides in length [8]. Based on genomic location and function, lncRNAs are classified as various types, such as intron lncRNAs, intergenic lncRNAs and enhancer lncRNAs. Recently, lncRNAs have been widely studied due to their crucial role in genome packaging, chromatin dynamics, gene regulation, cellular pathways and biological processes. LncRNAs also play a pivotal role in the oncogenesis, infiltration and metastasis of CRC [9]. RAS genes encode proteins that have a pivotal role in cell signaling. Their mutations are seen around 52% of colorectal cancer [10]. So, these genes are an ideal target due to the important role of RAS genes family in development of several neoplasms [11-12]. RAS gene family involved in cell survival and colorectal cancer pathogenesis through interaction with mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) [10].

The detailed mechanisms of colorectal cancer pathogenesis remain to be exactly determined. Further studies are needed to clarify the mechanisms of lncRNA DANCR and NRAS in CRC. In our study, we evaluated expression of those genes in CRC patients.

2. Patients and methods

2.1. Patients, sample collection and preservation

The present case control study was carried out in Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt. The study included 40 CRC patients in addition to 40 apparently healthy subjects as a control group. All subjects were selected from Mansoura Gastrointestinal Surgery Center, Mansoura University, Mansoura, Egypt between April 2022 and May 2023. Informed consents were taken from all participants in the study. The study protocol was approved by Institutional Research Board (IRB) of Mansoura Faculty of Medicine (IRB code. MD.21.12.570). All patients were subjected to full history taking, and complete clinical examination. Patients included in this work were diagnosed with colon cancer based on the clinic-pathological parameters, including patient's age, tumor stage, were obtained from clinical records. Exclusion criteria included Patients who underwent chemotherapy, radiotherapy or adjuvant treatment before surgery, Patients with other malignancy and liver diseases. The TNM classification system for colon Cancer Staging designated by the 8th edition of the American Joint Committee on Cancer (AJCC) (2018) is based on three keys: The Size of the tumor (T): How far has the cancer grown into the layers of the colon wall or rectum. The spread to nearby lymph nodes (N). The spread (metastasis) to distant sites (M), According to the TNM classification system, patients were further subdivided into four groups: Group I (Stage I): (4 cases), Group II (Stage II): (9cases), Group III (Stage III): (16 cases), Group IV (Stage IVA): (1 case). 5ml of venous blood was collected from all subjects (patients and controls) and added to Ethylene Diamine Tetraacetic Acid (EDTA) containing vaccutainers for mRNA extraction, The blood samples were transferred from EDTA containing vaccutainers into a 15 ml falcon tube and 10 ml of the RBCs lysis buffer was added on the blood samples.

2.2. RNA extraction and gene expression

RNA extraction was done according to the protocol of [13] using QIAzol™ reagent kit purchased from Qiagen, Germany. The integrity of RNA was evaluated by loading RNA samples on agarose gel electrophoresis. The RNA concentration and purity of the samples were assessed using the NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). The purity of RNA was assessed in each sample via two optical density (OD) ratios (A260 / A280 and A260 / A230). The RNA samples with 1.8 to 2.0 A260/A280 ratios were used. The isolated RNA was then stored at −80°C for subsequent reverse transcription. Complementary DNA (cDNA) was synthesized using COSMO cDNA synthesis kit (COSMO cDNA synthesis kit, England), in accordance with manufacturer's instructions. The cDNA was synthesized using the thermal cycler (Applied Biosystem, USA) with the following program of 5 min at 25°C, 15 min at 45°C and 5 min at 85°C. The synthesized cDNA samples were stored at −20°C. The RT-qPCR assays were carried out on the 7500 Real Time PCR System, Applied Biosystem, USA, with HERAPLUS SYBR® Green qPCR Master Mix (2X), Birmingham Research and Development Park, Birmingham, WF10308001, and Gene-specific real time qPCR primers. It was performed according to the method described by Freeman et al., (1999) [14]. Each 20 µL reaction mix contained 10 µL of HERAPLUS SYBR® Green qPCR Master Mix (2X), 2 µL of the synthesized cDNA, 1 µL of forward primer, 1 µL of reverse primer and the remaining 6 µL was RNase free water. The primers for the DANCR and NRAS genes were chosen from NCBI databases [http://www.ncbi.nlm.nih.gov/tools/primer-blast]. The primer sequences were checked for the melting temperature, product length, GC ratio, 3' complementarity and self-complementarity using Primer3 v.4.1.0 software [http://primer3.ut.ee/]. The forward primer sequence of the DANCR gene was 5′-CCTTGAGCTCCAGGAGTTCGTCT -3′ and the reverse primer was 5′- GCTTGTGCCTGTAGTTGTCAACCT -3′. The forward primer sequence of the NRAS gene was 5′-ATGACTGAGTACAAACTGGTGGT -3′ and the reverse primer was 5′- CATGTATTGGTCTCTCATGGCAC -3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene, and its primers were selected based on published sequences [15]. The forward primer sequence of the GAPDH gene was 5′-GTCAAGGCTGAGAACGGGAA-3′ and the reverse primer was 5′-AAATGAGCCCCAGCCTTCTC-3′. The Real Time PCR reactions were performed with the following thermal cycling program of 2 min at 95°C, followed by forty cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. The analysis of melting curve of all the reactions was performed for evaluation of the specificity of the products.

2.3. Interpretation of results

Relative quantification (RQ) of mRNA expression was estimated using the comparative threshold method (ΔΔCt) [16]. The data were presented as RQ of the target mRNA, normalized as regards the mRNA of the reference gene GAPDH and in respect to the control samples. The fold change was calculated using the equation $RQ = 2^{-\Delta\Delta Ct}$.

2.4. Statistical analysis

The collected data were introduced to a PC using Statistical Package for Social Science (SPSS) Version 25. Qualitative data were expressed as count and percent. Quantitative data were initially tested by Shapiro-Wilk's, then expressed as mean ± standard deviation (SD) for parametric numerical data or median and interquartile range (IQR) for normally distributed numerical data. Qualitative data were compared via Chi-square test (or Fisher's exact test), whereas quantitative data were compared via independent samples t-test or nonparametric Mann–Whitney U test. Receiver operating characteristic (ROC) curve analysis was used to determine the discrimination accuracy of the diagnostic test to distinguish between CRC and control [17]. Comparisons of area under ROC curve (AUC) were performed. Logistic and ordinal regression analyses were used for prediction of risk factors, using generalized linear models. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated.

The results were considered statistically significant if *p* value ≤ 0.05 for any used test.

3. Results and Discussion

Nearly 98% of the human genome is non-coding RNAs (short ncRNAs <200 nucleotides and long ncRNAs >200 nucleotides), proposing their promising impacts on normal and pathological processes in various disorders [18-20]. Tumorigenesis and the development of cancer are linked to abnormal expression of non-coding and protein-coding mRNAs. Gene imprinting, histone modification, chromatin remodeling, transcriptional interference, nuclear transport, transcriptional activation, and cell cycle regulation are some of the mechanisms by which long noncoding RNAs (lncRNAs) regulate pathophysiological processes. RNA polymerase II mainly transcribes them [20]. The present study involved 40 colon cancer patients; they were higher prevalence of male gender in the two study groups (65%). Their mean age in colorectal cancer patients was 54.3, there were 15 cases (37.5%) with normal weight and 25 cases (62.5%) with overweight or obesity. Among the cases group, there were 18 smokers (45%). The right side was affected in 18 cases (45%) while the left side was affected in 22 cases (55%). Regarding the tumor stage, there were higher prevalence of stage III C (25%), stage III B (22.5%) and stage III A (17.5%). We found that there was no statistically significant difference in the expression of both DANCR and NRAS according to the gender or smoking. There was weak non-significant correlation between age and relative expression of both DANCR and NRAS genes. Expressions of DANCR and NRAS genes were assessed in different stages of CRC cases aiming to clarify their role in development and promotion of CRC. In the current study, statistically significant DANCR gene overexpression is found in CRC cases compared to the corresponding control cases ($p<0.001$).

This is consistent with Bahreini et al. (2021) who found that expression level of DANCR was significantly up-regulated in colorectal cancer tissues as DANCR could regulate NRAS expression by sponging miR-145-5 in colorectal cancer patients [20]. Similarly, Shen et al., (2020) found that DANCR level in the CRC tissue and serum was significantly increased, and serum DANCR expression was decreased in post-operative patients as compared with that in pre-treatment patients and recurrent patients [21]. Wang et al., (2018) concluded that DANCR was highly expressed and correlated with proliferation and metastasis in CRC. In addition, they demonstrated that DANCR and HSP27 were both targets of microRNA-577 (miR-577) and shared the same binding site. Furthermore, they revealed that DANCR promoted HSP27 expression and its mediation of proliferation/metastasis via miR-577 sponging. Finally, using an in vivo study, they confirmed that overexpression of DANCR promoted CRC tumor growth and liver metastasis [22]. The Ras genes, HRAS, KRAS, and NRAS, are members of the Ras family with GTPase activity. These genes function as molecular switches in the cell, where Ras GTP represents the active state, while Ras GDP represents the inactive state [23]. The function of Ras proteins in the regulation of cellular signal pathways has attracted the attention of numerous researchers in the last few decades [24]. Studies have shown that these proteins have significant roles in regulating cell motility, controlling cell apoptosis, and organizing cell proliferation [25-27]. The distinct role of each Ras member can be correlated with various posttranslational modifications (PTMs) at the C-termini, where each protein localizes a different subcellular membrane based on its modification and subsequently stimulates a specific signaling pathway [28].

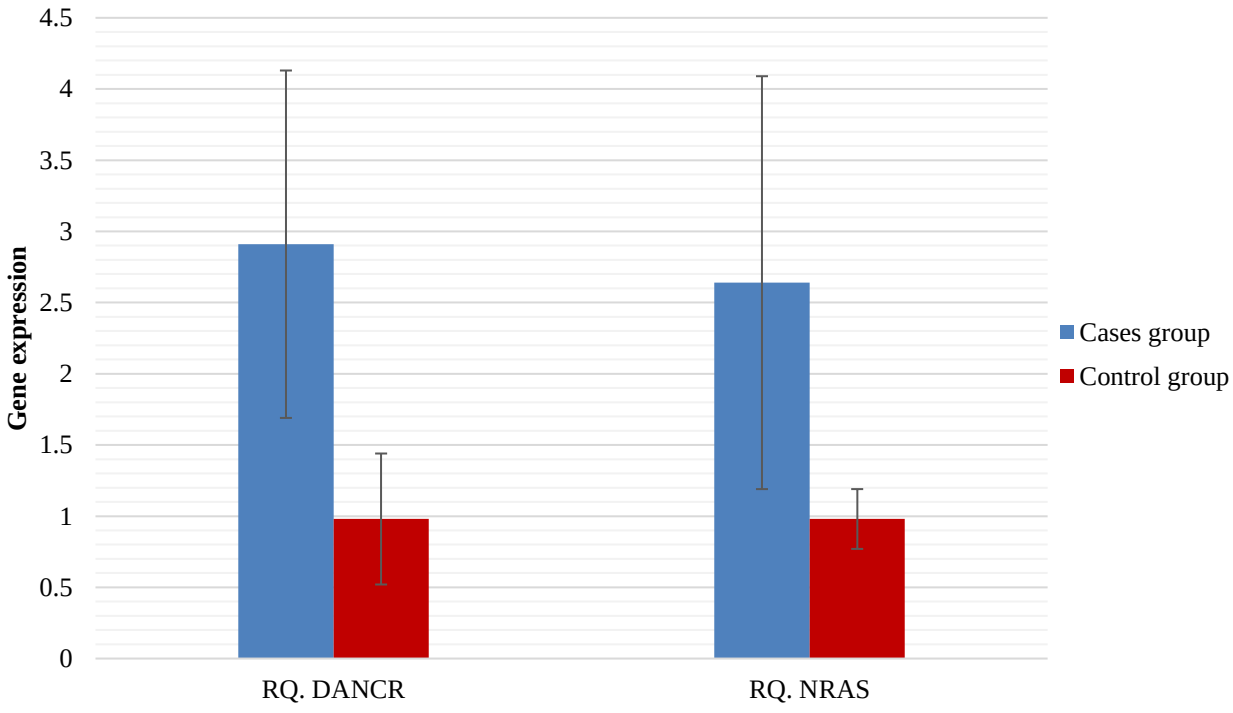

Figure 1: The relative gene expression in the two study groups.

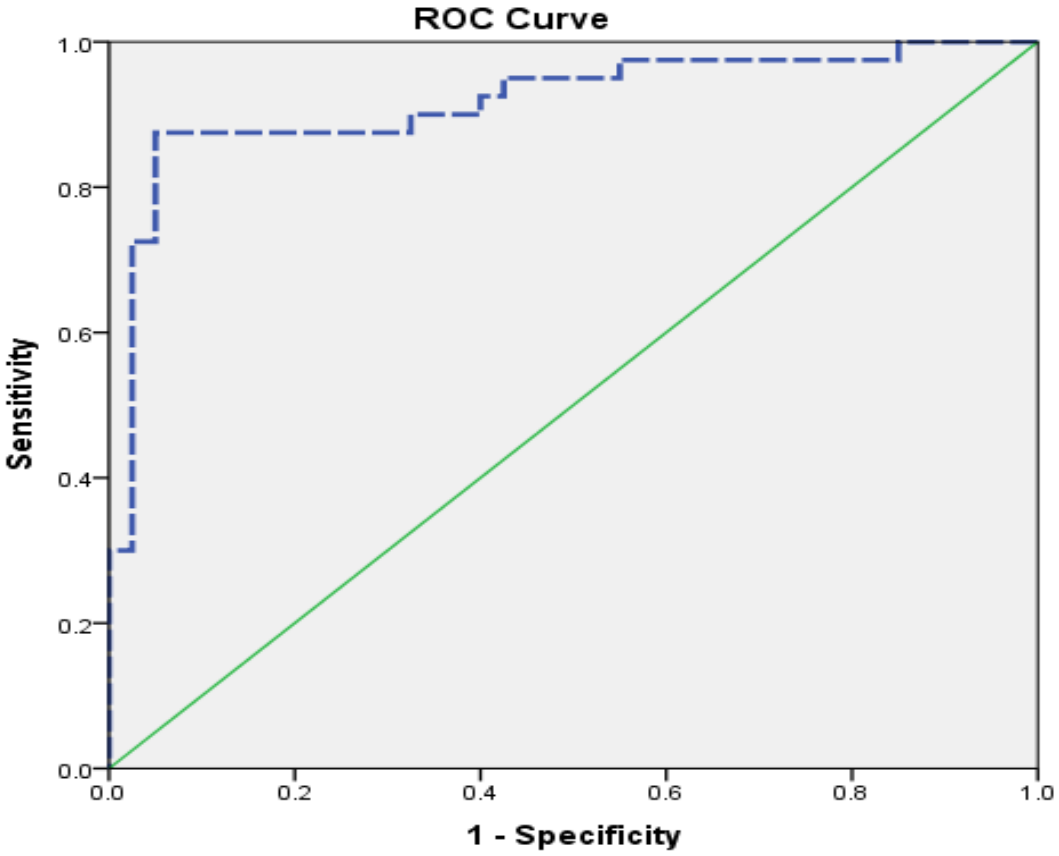

Figure 2: ROC curve of DANCR gene expression to differentiate cases group from control group.

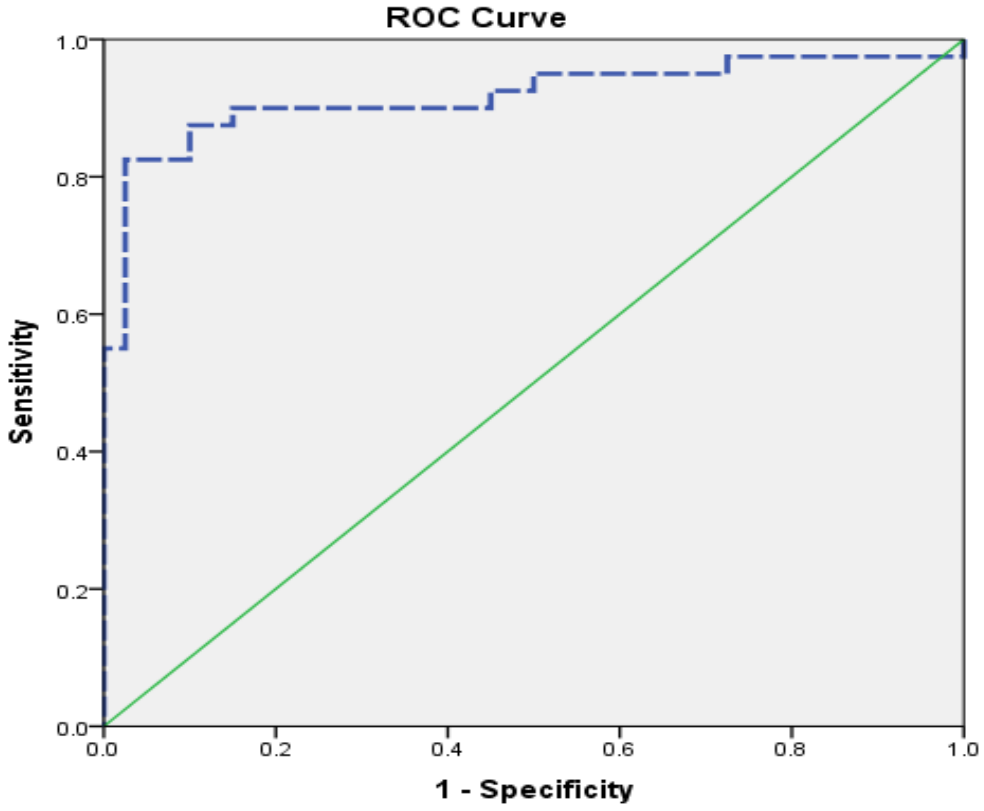

Figure 3: ROC curve of NRAS gene expression to differentiate cases group from control group.

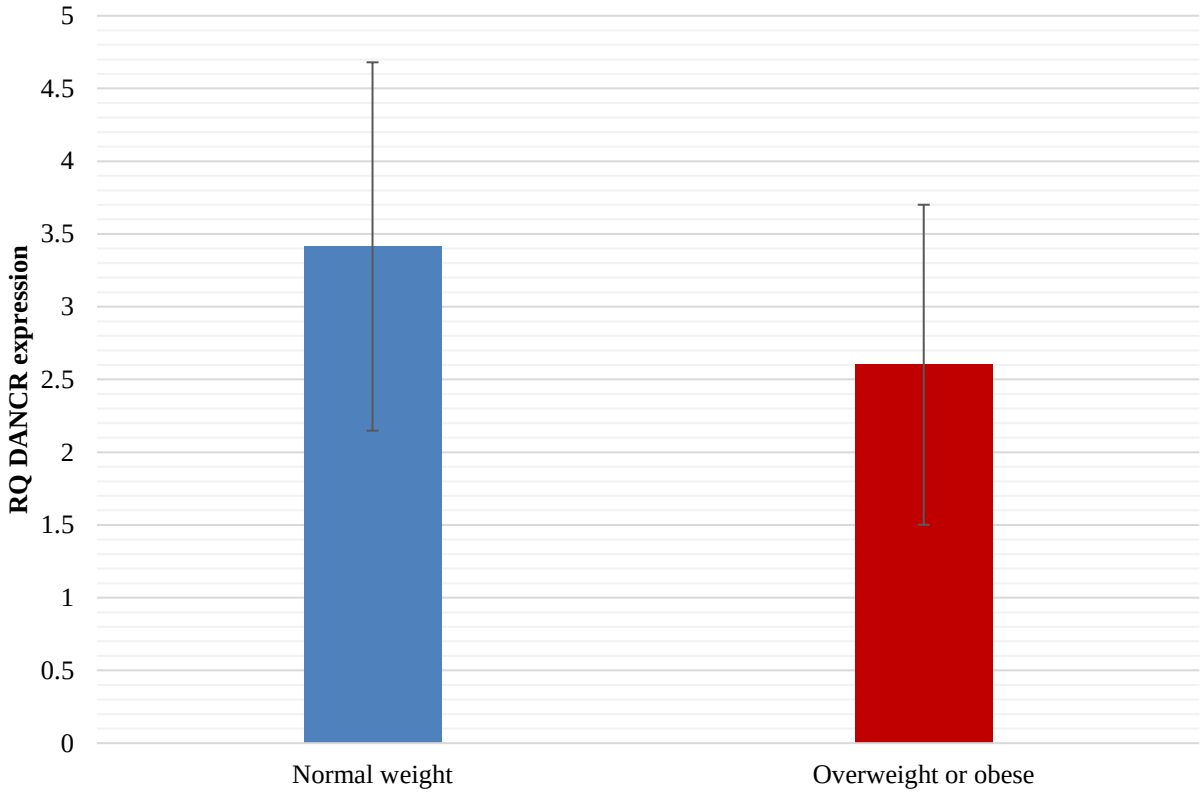

Figure 4: DANCR gene expression according to BMI.

Table 1: Body Mass Index (BMI) and smoking in the cases group Categorical data expressed as Number (%).

Variables	Cases group N = 40	
	Number	Percent
BMI		
Normal weight (20-25)	15	37.5 %
Obese (>30)	25	62.5 %
Smoking		
No	22	55 %
Yes	18	45 %

Table 1 shows that in the cases group, there were 15 cases (37.5%) with normal weight and 25 cases (62.5%) with obesity. Among the cases group, there were 18 smokers (45%).

Table 2: Site and stage of the tumors in the cases group Categorical data expressed as Number (%).

Variables	Cases group N = 40	
	Number	Percent
Site		
Right	18	45.0
left	22	55.0
Stage		
stage Ia	1	2.5
stage IB	1	2.5
Stage IC	2	5.0
Stage IIA	5	12.5
Stage IIB	3	7.5
Stage IIC	1	2.5
Stage III A	7	17.5
Stage III B	9	22.5
Stage III C	10	25.0
Stage IV A	1	2.5

Table 2 shows that the right side was affected in 18 cases (45%) while the left side was affected in 22 cases (55%). Regarding the tumor stage, there was higher prevalence of stage III C (25%), stage III B (22.5%) and stage III A (17.5%).

Table 3: Comparison of the relative gene expression in the two study groups P: probability.

	Groups		**Test of significance**	P value
	Cases group (n= 40)	**Control group (n= 40)**		
RQ. T DANCR				
Median	2.91	0.98	z = -6.438	< 0.001*
Range	0.658 – 5.983	0.459 – 2.985		
RQ. T NRAS				
Median	2.64	0.98	z = -6.428	< 0.001*
Range	0.655 – 5.984	0.748 – 2.057		

z: Mann-Whitney U-test, *: significant p value (< 0.05).

Table 3 shows that the level of gene expression of both DANCR and NRAS showed highly statistically significant up regulation in the cases group compared to the control group ($p < 0.001$ for both).

Table 4: Analysis of the diagnostic ability of DANCR gene expression to differentiate cases group from control group.

Diagnostic criteria	DANCR gene expression
AUC (95% CI)	0.918 (0.853 – 0.984)
Cut off point	> 1.639
Sensitivity	87.5%
Specificity	92.5%
NPV	82.5 %
PPV	92.5 %
Accuracy	90 %
P	< 0.001*

AUC: area under the curve. NPV: Negative predictive value
PPV: Positive predictive value P: probability.
*: significant p value (< 0.05).

Table 4 shows that the best cutoff point of DANCR gene expression to differentiate cases group from the control was > 1.693. This vale has excellent sensitivity (87.5 %) and specificity (92.5 %). The area under the curve was 0.918 with high statistically significant value ($p < 0.001$).

Table 5: Analysis of the diagnostic ability of NRAS gene expression to differentiate cases group from control group.

Diagnostic criteria	NRAS gene expression
AUC (95% CI)	0.917 (0.848 – 0.987)
Cut off point	> 1.094
Sensitivity	87.5%
Specificity	90%
NPV	80 %
PPV	82.5 %
Accuracy	87.5 %
P	< 0.001*

AUC: area under the curve, NPV: Negative predictive value, PPV: Positive predictive value, P: probability, *: significant p value (< 0.05).

Table 5 shows that the best cutoff point of NRAS gene expression to differentiate cases group from the control was > 1.094. This vale has excellent sensitivity (87.5 %) and specificity (90%). The area under the curve was 0.917 with high statistically significant value ($p < 0.001$).

Table 6: Correlation of NT by 2D (mm) with other variables.

	Age (Years)	
	r_s	P
RQ T. DANCR	0.037	0.821
RQ T. NRAS	0.144	0.375

r_s: Spearman's correlation, P: Probability.
Table 6 shows that there was weak non-significant correlation between age and relative expression of DANCR and NRAS.

Table 7: Analysis of DANCR gene expression according to the demographic data in the cases group.

Variables	DANCR gene expression	Test of significance	P value
Gender			
Males (n= 26)	2.914	z = - 0.057	0.955
Females (n= 14)	2.892		
BMI			
Normal weight (n= 15)	3.414	z = - 2.026	0.043*
Overweight or obese (n= 25)	2.601		
Smoking			
No (n= 22)	2.887	z = - 0.272	0.786
Yes (n=18)	2.929		

P: probability, Continuous data are expressed as median (Range), z: Mann-Whitney U-test, *: Statistically significant ($p< 0.05$).
Table 7 shows that in the cases group, there was no statistically significant difference in the expression of DANCR gene according to the gender or the smoking. However, the expression was statistically significantly higher in the normal weight cases compared to overweight and obese.

Table 8: Analysis of expression of NRAS according to the demographic data in the cases group.

Variables	NRAS gene expression	Test of significance	P value
Gender			
Males (n= 26)	2.710 ± 1.556	z = - 0.061	0.948
Females (n= 14)	2.521 ± 1.271		
BMI			
Normal weight (n= 15)	2.705 ± 1.928	z = - 0.545	0.586
Overweight or obese (n= 25)	2.608 ± 1.113		
Smoking			
No (n= 22)	2.522 ± 1.070	z = - 0.136	0.892
Yes (n= 18)	2.793 ± 1.833		

P: probability, Continuous data are expressed as median (Range), z: Mann-Whitney U-test, *: Statistically significant ($p< 0.05$).
Table 8 shows that in the cases group, there was no statistically significant difference in the expression of NRAS according to the gender, BMI or the smoking.

In the present study we found that statistically significant NRAS gene overexpression is found in CRC cases compared to the corresponding control cases ($p<0.001$). This is consistent with Bahreini et al. (2021) who found that expression of NRAS was significantly elevated in tumor samples compared to control group (15), the best cutoff point of NRAS expression to differentiate cases group from the control was > 1.094. This vale has excellent sensitivity (87.5%) and specificity (90%) and there was weak non-significant correlation between age and relative expression of NRAS. Yan et al., (2022) found that NRAS expression was significantly up regulated in lung adenocarcinoma tissue compared to normal tissue. Furthermore, NRAS expression was significantly correlated with more advanced stage and positive lymph nodes and associated with poor prognosis [29]. Tian li et al., (2020) reported that mutation of NRAS gene has no effect on the prognosis of acute myeloid leukemia patients [30].

4. Conclusions

Based on the DANCR and NRAS's values of specificity and sensitivity, these molecular biomarkers could be used in screening and differentiating colorectal cancer patients. It is still unclear whether specific pathways lead to the onset and spread of colorectal cancer. The application of DANCR and NRAS could be used as a probable promising strategy for early detection of this type of cancer. To pinpoint the unidentified components of the pathogenic pathways causing colorectal cancer, more research is necessary.

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Conflict of interest

The authors report no conflict of interest regarding this publication.

Author contributions

The idea and design of the study were contributed to by all authors. The preparation of materials, methods, and manuscript writing were handled by Maamon Essa and Sally Abdallah Mostafa and. Ahmed El-Ghawalby collected the samples and analyzed the pathology findings. Mohammed A. Zahran and Lamiaa F. Arafa oversaw the methodology, edited and revised the text. The final manuscript was read and approved by all writers.

Ethics approval

The study protocol was approved by Institutional Research Board (IRB) of Mansoura Faculty of Medicine (IRB code MD.21.12.570).

Informed consent

Informed consent was obtained from all individual participants included in the study.

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