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RESEARCH ARTICLE

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Impact of microRNA-375 and its target gene SMAD-7 polymorphism on susceptibility of colorectal cancer

Olfat Gamil Shaker[1] | Shereen Rashad Mohammed[2] | Asmaa Mohammed Mohammed[2] | Zeinab Mahmoud[3]

[1]Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt

[2]Departments of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Fayoum University, Al Fayoum, Egypt

[3]Departments of Tropical Medicine, Faculty of Medicine, Cairo University, Cairo, Egypt

Correspondence
Olfat Gamil Shaker, Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt.
Email: olfatshaker@yahoo.com

Background: Colorectal cancer (CRC) has a high morbidity and mortality. Many studies reported that mir-375 is frequently down-regulated in many cancers including esophageal cancer, hepatocellular carcinoma, breast cancer and leukemias.

Aim: Our aim was to study the expression of microRNA-375 and its target gene SMAD-7 polymorphisms (rs4939827) in CRC patients in comparison to control subjects and to correlate these results with clinical data of patients to elucidate their role in pathogenesis and early diagnosis of CRC.

Material and methods: The present study was conducted on 122 subjects divided into 86 patients with CRC and 36 age- and sex-matched controls. The followings were done to all subjects: full history taking, full clinical examination, complete blood picture, serum (ALT, AST), serum albumin, CEA, TLC, PLT, and creatinine. Gene expression of miRNA-375 from serum was done by real-time PCR. Gene polymorphism SNPs of SMAD7 (rs4939827) was also done in DNA extracted from blood by real-time PCR.

Results: As regards the polymorphism of SMAD7, we found that CC (wild) genotype has high percentage in controls compared to CRC cases (36.1% vs 15.1%). Meanwhile, the mutant and heterozygotes genotypes showed high percentage among cases compared to controls (33.7%, and 51.2% respectively) vs (22.2%, and 41.7% respectively) with no significant statistical analysis. There was a statistically significant high T-allelic frequency among cases and C-allelic frequency among controls. There was a statistically significant association between fold change in micro RNA (-375) and the susceptibility to CRC as there is down-regulation of the microRNA-375 in CRC group with fold change in 0.42±0.27.

Conclusion: Micro RNA-375 and rs4939827 SNP in SMAD7 could be considered as potential markers for detecting and early diagnosing CRC patients.

KEYWORDS
colorectal cancer, microRNA-375, SMAD-7gene polymorphisms

1 | INTRODUCTION

Colorectal cancer (CRC) is widespread worldwide, and despite improvement in treatment still leads to morbidity and mortality.[1] Many risk factors have a role in development of colon cancer. These risk factors may increase the rate of genetic mutations, and/or result in epigenetic modifications. These factors can be classified into: germline genetic mutations, associated diseases, personal or family history of CRC, environmental exposures, and demographic considerations.[2]

Small mother against decapentaplegic-7 (Smad-7) is a nuclear protein, and can transport between the cytoplasm and the nucleus. It negatively regulate TGF-β signaling by multiple mechanisms as by

blocking the phosphorylation of receptor-activated Smads or by competitive inhibition of complex formation of receptor activated Smads with common-mediated Smad4. Epithelial tissues show a very low expression level of Smad7, but it is upregulated in human pancreatic cancers.[3]

Genome-wide association studies (GWAS) showed that common alleles of Smad7 influence the risk of CRC.[4] Boulay et al.[5] showed that deletion of Smad7 brought better prognosis than patients with two copies of this gene, whereas increased copies of that gene is associated with a significantly bad prognosis.

The Smad7 gene encodes a protein that binds with the transforming growth factor (TGF)-β type I receptor, this causes its degradation and so inhibiting the phosphorylation of Smad2/Smad3 byTGF-β1.[6] Smad-7 can bind also with intracellular proteins and organize cell function by TGF-β1-independent pathways. Overexpression of Smad7 is accompanied by the development of many types of cancers as pancreatic, skin and lung cancers.[7]

MiR-375 is known as a pancreatic islet-specific miRNA as it regulates insulin secretion induced by glucose.[8] However, genome-wide miRNA expression profiling studies revealed that miR-375 is widely spread in tissues and organs and is significantly reduced in malignant cell, as hepatocellular carcinoma (HCC), gastric cancer (GC), esophageal carcinoma, head and neck cancer, it is considered an important cancer-related miRNA.[9]

1.1 | Aim of the work

Our aim was to study the expression of microRNA-375 and its target gene SMAD-7 polymorphisms in colorectal cancer patients in comparison to control subjects and to correlate these results with clinical data of patient group to elucidate their role in pathogenesis and early diagnosis of CRC.

2 | SUBJECTS AND METHODS

In this study, 86 Egyptian CRC patients diagnosed either by histopathologic, endoscopic or imaging evidence, were enrolled from Tropial Medicine, Al-Kasr Al-Eni Hospital, Faculty of Medicine, Cairo University. Also, 36 healthy individuals without any history of neoplasms or other diseases were recruited as controls. Characteristics of all enrolled subjects, including age, gender, family history, were collected. For CRC patients, complete blood picture, serum transaminases (ALT, AST), serum albumin, CEA and serum creatinine were done. BMI was calculated to all subjects. The study was performed with the approval of Faculty of Medicine, Cairo University local ethics committee and carried out in compliance with the Helsinki Declaration (2008). Informed consent was obtained from all the subjects enrolled in this study.

MiRNA-375 was detected by real-time PCR. RNA was extracted from serum then Real-time PCR was performed. Reverse transcription was carried out on 5 ng of total RNA in a final volume of 20 μL RT reactions (incubated for 60 minutes at 37°C, 5 minutes at 95°C, and then maintained at 4°C) using the miRNeasy serum/plasma Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For real-time PCR, 5 μL of diluted RT products (cDNA template) was mixed with SYBR Green Master Mix (Qiagen) in a final volume of 25 μL and universal up primer and miRNA forward specific primer (for -375). Real-time PCR reactions were performed with the following conditions: 95°C for 15 minutes, followed by 40 cycles at 94°C for 15 seconds, and 55°C for 30 seconds and 70°C for 34 seconds.

Genotyping was performed using real-time polymerase chain reaction (RT-PCR) with the Taq Man allelic discrimination assay using predesigned primer/probe sets SMAD7 r s4939827 (C_27913406_10) (Applied Biosystems, Foster City, CA, USA). DNA amplification was carried out in a 25 μL volume containing 12.5 μL Taqman master mix, 1.25 μL primer/probe, 1 μL DNA, and 10.25 μL H_2O. RT-PCR was performed using a Rotor gene QRT-PCR System (Qiagen) with the following conditions: after a denaturation time of 10 minutes at 95°C, 45 cycles at 60°C for 90 seconds and at 92°C for 15 seconds for annealing and extension were carried out, and fluorescence was measured at the end of every cycle and at the endpoint.

3 | STATISTICAL ANALYSIS

Data were collected and coded to facilitate data manipulation and double entered into Microsoft Access and data analysis was performed using SPSS software version 18 under windows 7 (SPSS Inc., Chicago, IL, USA). Simple descriptive analysis in the form of numbers and percentages for qualitative data, and arithmetic means as central tendency measurement, standard deviations as measure of dispersion for quantitative parametric data, and inferential statistic test.

3.1 | For quantitative parametric data

Independent student *t*-test used to compare measures of two independent groups of quantitative data. One-way ANOVA test was used in comparing more than two independent groups of quantitative data.

3.2 | For quantitative non parametric data

Kruskal–Wallis and Mann–Whitney tests were used in comparing more than two independent groups.

ROC curve "Receiver Operating Characteristic" was used to determine the Sensitivity and specificity for the microRNA-375.

4 | RESULTS

There were 122 subjects enrolled in our study, including 86 CRC patients and 36 controls. General characteristics of all subjects are listed in Table 1. It was shown that age, gender, and BMI were not significantly different between cases and controls. Description of site of tumor among cases was shown also in Table 1.

TABLE 1 Characteristics of the studied groups

Variables	CRC patients (n=86)	Control (n=36)	*P*-value	Sig.
Age, years (Mean±*SD*)	50.4±12.4	46.8±8.7	.1	NS
Male (n, %)	59 (68.6%)	19 (52.8%)	.1	NS
Female (n, %)	27 (31.4%)	17 (47.2%)		
BMI (≤25)	50 (58.1%)	17 (47.2%)	.3	NS
BMI (>25)	36 (41.9%)	19 (52.8%)		
Site of tumor				
Rectal	18 (20.9%)			
Colon	68 (79.1%)			

A statistically significant difference was found with *P*<.05 between study groups (cases, and controls) as regards CC (wild) genotyping with high percentage among controls 36.1% vs 15.1% among cases. There is no statistical significance difference with *P*>.05 as regard to mutant and heterozygotes genotypes although, there is high percentage of mutant and heterozygotes among cases (33.7%, and 51.2% respectively) vs (22.2%, and 41.7% respectively) for controls. There was a statistically significant difference with *P*<.05 between cases and controls as regards distribution of different alleles with high percentage of T-allele among cases and C-allele among controls (Table 2). There was a statistically significant difference with *P*<.05 between different cancer sites with high percentage of TT genotype among patients with rectal cancer, and high percentage of CT genotype among patients with colon cancer (Table 2).

Upon comparison of age and routine investigations with different genotyping we found a statistically significant difference between different genotyping as regards albumin level with high mean among patients with (wild CC) genotyping. On the other hand, there is no statistically significance difference with *P*>.05 between different genotyping as regards age, complete blood count, liver function parameters, creatinine, and CEA.

In the present work, there was statistically significant association between micro RNA (-375) and the susceptibility to CRC as there is down-regulation of the microRNA-375 in CRC group with fold change 0.42±0.27. At the same time, no statistically significant difference was found between cancer sites (colonic and rectal cancer) as regards to fold change in microRNA-375 levels. Meanwhile, there was no statistically significance correlation between microRNA-375 with all different laboratory investigations.

A highly significant correlation was found between miRNA-375 level and mutant and heterozygotes genotypes of SMAD-7 rs4939827 polymorphism in CRC (Table 3). Roc curve showed the sensitivity and specificity test for fold change in microRNA -375, they were 89.5%, 100%, respectively, with cut-off value of 0.6547.

TABLE 2 Comparison of different genotypes of SMAD-7 gene in different groups

Variables	Cases (n=86) No. (%)	Controls (n=36) No. (%)	*P*-value	Sig.
TT (mutant)	29 (33.7)	8 (22.2)	.3	NS
CT (heterozygote)	44 (51.2)	15 (41.7)	.4	NS
CC (wild)	13 (15.1)	13 (36.1)	.01	S
T allele	102 (59.3)	31 (43.1)	.02	S
C allele	70 (40.6)	41 (56.9)	.02	S
Variables	**Colon (n=68) No. (%)**	**Rectal (n=18) No. (%)**	***P*-value**	**Sig.**
TT (mutant)	19 (27.9)	10 (55.6)	.04	S
CT (heterozygotes)	39 (57.4)	5 (27.8)	.03	S
CC (wild)	10 (14.7)	3 (16.7)	.9	NS

Table shows that the wild genotype CC is common in controls. On the other hand the heterozygotes genotype is prominent in colon cancer while the mutant type is common in rectal cancer. S, significant; NS, non significant.

5 | DISCUSSION

Advances in diagnosis and treatment of CRC have improved patient outcome. But long-term survival and prognosis of patients rely on the stage of the tumor at the time of detection. The prognosis of patients diagnosed at advanced stage remains quite bad.[10] Current CRC screening tests are not convenient and the rate of screening is low. Most patients are diagnosed at late stages by colonoscopy, which is helpful in the detection of neoplastic lesions, but it is invasive and

TABLE 3 Comparisons of fold change in microRNA -375 in different genotyping

Variables	FC- microRNA -375 Mean	*SD*	*P*-value	Sig.
TT (mutant)	0.84938	0.901	.002	HS
CT (heterozygotes)	0.428692	0.3926	.005	HS
CC (wild)	0.59833	0.4364	.9	NS

Fold change in microRNA -375 was significantly associated with both mutant and heterozygots genotypes. NS, non significant; HS, highly significant.

causes abdominal pain.[11] Occult blood immunochemical test is convenient and inexpensive, but still has low sensitivity and specificity.[12] Therefore, there is an urgent need for noninvasive biomarkers to complement and improve diagnosis and prognosis of CRC.

Susceptibility of colorectal cancer is caused by genetic factors and majority of this seems to be due to multiple low-risk mutations. GWAS have identified multiple independent single nucleotide polymorphism (SNP) associated with colorectal cancer risk. The involvement of these SNPs in the occurrence of CRC may vary between populations, for example, due to allele frequencies or particular genetic and environmental factors that may modify the effect of the variants. Recognizing the effects in different populations may help in discovering disease mechanism.[13]

Several SNPs as rs4939827, rs4464148, rs12953717 on SMAD-7 gene are significantly correlated with the susceptibility to CRC. There is a controversy regarding the role of smad7 in tumor development depending on the type of the tumor. GWAS revealed that smad7 rs4939827 located in intron 3 is associated with altered susceptibility to cancer as CRC.[14]

MiR-375 was recognized as a pancreatic islet-specific miRNA regulating insulin secretion. Further study revealed that miR-375 is involved in glucose homeostasis, pancreatic islet development, mucosal immunity, lung surfactant secretion and tumorigenesis. MiR-375 has been found to be down-regulated in multiple types of cancer, and suppresses cancer by targeting several important oncogenes like AEG-1, YAP1, IGF1R, and PDK1.[15]

Meanwhile, we aimed to study the expression of microRNA-375 and its target gene SMAD-7 polymorphism (rs4939827) in colorectal cancer patients in comparison to control subjects and to correlate these results with clinical data of patient group to elucidate their role in pathogenesis and early diagnosis of the disease.

We studied the rs4939827 SNP of SMAD-7 in CRC patients and controls. We found that the wild type CC genotype has higher percentage in controls compared to cases (P=.01). The mutant genotype as well as the heterozygote genotype had higher percentage in patients than in controls. To some extent, this percentage is similar to the percentages of Kirac et al.[13] in which the proportion of each of the rs4939827 genotypes CC, CT and TT was 29%, 49%, and 22% in controls, respectively, and 21%, 47%, and 32% in cases, respectively. This results are also coincided in part with Abd Elfattah et al.,[16] who stated that SMAD7 rs4939827 TT genotype was associated with the rectal cancer but not the colon cancer as the frequency (13/30, 43%) in the rectal cancer patients than the colon cancer patients (9/47, 19%) at P=.042. No significant difference was found in the allele frequencies (P=.16) between the colon and the rectal cancer patients. The association with rectal more than colon may be explained by the involvement of the TGF-β signaling with malignant transformation of adenomas driven by chromosomal instability that occurs mainly in the rectum.[17]

In the present study, found higher frequency of allele T in CRC than in controls (59.3% vs 43.1% respectively, P=.02) and allele C among controls than patients (56.9% vs 40.6% respectively, P=.02). Kirac et al.,[13] coincided with us as they found that the T allele frequency in Croatian populations (AFT=49.6%). At the same time, T allele frequency in European populations was (AFT=52.9%).

Our results are coincided with Yan et al.,[18] who revealed that the T allele of rs4939827 was significantly associated with an increased risk of CRC under the allelic model in Caucasians. Our results are confirmed also by Zhang et al.,[19] who reported that SNPs rs7229639 and rs4939827 account for about 1% of the familial relative risk of CRC in East Asians and have an important role in the etiology of CRC.

In addition, Hong and Park[20] showed that rs4939827, rs4779584, and rs10795668 may contribute to the development of CRC in the Korean population and European populations.

On the other hand, our results are against the results of Yao et al.[21] who reported that there is no correlation between allele T frequencies and CRC in the Asian and African populations.

Our results showed that TT (mutant) genotype shows higher frequency in rectum cancer (55.6%) than in colon cancer (27.9%), (P=.04) while CT (heterozygous) genotype shows higher frequency in colon cancer (57.4%) than in rectal cancer (27.8%) (P=.03). Our results differ from result of Curtin et al.,[22] who observed that there is a significant association with distal colon tumors, but not in proximal or rectal cancers as regard SNP rs4939827 but coincided with Abd Elfattah et al.[16]

Studies revealed that miR-375 is profoundly aberrant (mostly down-regulated) in many human cancers, providing potential prognostic values[23] such as head and neck squamous cell carcinoma, non-small cell lung cancer, melanoma, glioblastoma multiforme, HCC, esophageal cancer, GC, breast cancer, or prostate cancer and it acts by inhibiting/repressing many well-known oncogenes.[24]

We found that there was a statistically significant association between micro RNA-375 and the susceptibility to CRC, that there is down-regulation of the microRNA-375 in CRC group with fold change 0.42±0.27. Our findings are coincided with Xu et al.,[25] who showed that miR-375 expression is frequently down-regulated in colorectal cancer tissue compared to the non-tumor counterparts and showed consistent correlations between tissue and plasma samples.

Dai et al.,[26] supported our results as they reported that miR-375 has been found to be significantly under-expressed, without any correlation with tumor size, histological grade, pT stage, pN stage and pTNM stage. In addition, Wang et al.,[27] showed that miR-375 was frequently down-regulated in human colorectal cancer cell lines and tissues when compared to normal human colon tissues. Phosphatidylinositol -4, 5- bisphosphate 3-kinase catalytic subunit alpha gene (PIK3CA) was identified as a potential miR-375 target by bioinformatics. In addition, miR-375 suppressed CRC cell proliferation and colony formation and led to cell cycle arrest.

Another study revealed that miR-375 reduced cell viability through the induction of apoptotic death by targeting YAP.[28]

To the best of our knowledge, our study is the first to detect significant correlation between microRNA (-375) and SMAD-7 polymorphism in which there is high mean of fold change in miRNA-375 among individuals with TT, and low mean among individual with CT. On the other hand, there is no statistically significance difference with P>0.05 between study groups as regards to CC genotype.

In conclusion, micro RNA-375 and rs4939827 SNP of SMAD-7gene could be considered as potential noninvasive markers for detecting and early diagnosing CRC.

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