QUANTITATIVE DETECTION OF MICRORNA-20A AMONG IRAQI COLORECTAL CANCER PATIENTS

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ABSTRACT

This study aimed to investigate the expression level of microRNA-20a in plasma samples collected from 20 healthy males and 20 healthy females' as non-cancerous controls, 34 males and 26 females who diagnosed with CRC, the age range for both poles was at age range of 23 to 66 years old, those patients didn't receive any chemical, radiological or surgical treatment and subjected to relative quantitative detection using RT-PCR technique. Specific primers designed for this purpose, the sequence of miR-20a retrieved from NCBI and miRBASE, the primers designed using stem-loop structure method, RNU-43 reference gene used for results normalization. The resulted data analyzed using the Livak method, the folding mean of CRC patients (12.78) was higher than the folding level of non-cancerous controls (1.6909), 28 patients were had higher folding levels than the mean of the controls while 32 patients were below the non-cancerous controls folding mean. Among 60 patients, 22 samples were had expression levels less than 1 folding level, 11 samples had expression levels less than 2 folding levels, 12 samples quantified folding less than 5 folding levels, 8 samples were less than 20 folding levels, and 7 samples with higher 20 folding level, such results indicated that the expression level of microRNA-20a is changed and dose not remain consistent in colorectal cancer patients, with varied quantified results due to different factors. No considerable statistical relevance to age and gender was revealed since P values were greater than 0.05 level in Students-t-test and ANOVA test.

Keywords: CRC, RT-PCR, Expression Level, Biomarker.

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الكشف الكمي للحمض النووي الرايبوزي المايكروي- 20a في المرضى العراقيين المصابين بسرطان القولون والمستقيم

هبة عبد الأمير محمد نها جوزيف قندلا نور صابر
مدرس مساعد استاذ مدرس مساعد

المستخلص

تهدف هذه الدراسة إلى التحقيق في مستوى التعبير لـ microRNA-20a في عينات البلازما التي تم جمعها من 20 ذكر سليم و 20 انثى سليمة كعناصر تحكم غير مصابين بالسرطان ، 34 ذكر و 26 انثى مصابين بسرطان القولون والمستقيم، مدى العمر لكلا قطبي الدراسة تراوح من 23 الى 66 سنة، هؤلاء المرضى لم يتلقوا أي علاج كيميائي أو إشعاعي أو جراحي وخضعوا للكشف الكمي النسبي باستخدام تقنيةالـ RT-PCR وعن طريق بادئات محددة مصممة لهذا الغرض، تم تصميم البادئات باستخدام طريقة بنية الحلقة الجذعية. تم استخدام الجين المرجعي RNU-43 لتطبيع النتائج و تم تحليل البيانات الناتجة باستخدام طريقة ليفاك ، كان متوسط التعبير الجيني لدى مرضى سرطان القولون هو 12.78 اي بمستوى اعلى من أعلى من مستوى التعبير الجيني لدى الأفراد الاصحاء (1.6909) ،وكان لدى 28 مريضًا مستويات تعبير أعلى من متوسط التعبير لدى الاشخاص الاصحاء بينما كان 32 مريضًا أقل من مستوى التعبير بالمقارنة مع الافراد الاصحاء. من بين 60 مريضًا ، كان لدى 22 عينة مستويات تعبير أقل من مستوى واحد ، و 11 عينة بمستويات تعبير أقل من مستويين للتعبير الجيني، و 12 عينة أقل من 5 اضعاف التعبير الجيني ، و 8 عينات كانت أقل من 20 ضعف التعبير الجيني ، و 7 عينات كانت اعلى من 20 ضعف التعبير الجيني بالمقارنة مع الاشخاص الاصحاء ، أشارت هذه النتائج إلى أن مستوى التعبير عن microRNA-20a قد تغير ولم يكن ثابتًا في مرضى سرطان القولون والمستقيم مع وجود نتائج كمية متباينة بسبب عوامل مختلفة. لم يتم الكشف عن أي صلة إحصائية كبيرة مرتبطة بالعمر والجنس لأن قيم P كانت أكبر من مستوى 0.05 في اختبار Student-t واختبار ANOVA.

الكلمات المفتاحية: سرطان القولون والمستقيم،تفاعل البلمرة المتعددي اللحظي، مستولى التعبير، مؤشر حيوي.

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INTRODUCTION

MicroRNAs (miRs) defined as single stranded, endogenous, non-coding RNAs molecules with nucleotide length of 21–24 nucleotides that play important roles in post-transcriptional mRNA regulation (23, 26). The human genome possesses over 2500 miRNAs molecules with the potential ability to target about 60% of mammalian genes (16). The cellular regulation mediated by miRs performed through inhibition and degradation of mRNA which resulted in controlling biological development, cell cycle, differentiation and stress response, scientific findings report that about 60-90% of mRNA transcripts are targeted by miRs (14, 18). The miRs involved in involved in human cancer through negatively influencing on the protein-coding oncogenes, other types inhibit the tumor suppressors so it can be either onco-miRNAs or tumor-suppressor miRNAs (24), the varied expression of miRs effect carcinogenesis, development of metastases, tumor progression and relation to tumor prognosis and miRNAs effects in resistance to chemotherapy (2, 11). MicroRNAs are linked to all types of cancers so that it surfacing as promising biomarkers can be implemented in the early detection and classification of human cancers (29). MiRNAs are linked to a vast range of human cancers including breast cancer (33) liver cancer (20), ovarian cancer, and colorectal cancer (12, 17). The fact that miRs mediate the regulation of cellular pathways involved in tumor growth, and since that miRs released from the human cells to the body fluids, it is possible to detect such molecules in blood plasma and serum and use it as potential marker and as a predicting tool in tumors diagnosis, although until now the miRNAs not enrolled yet in clinical routine diagnostic tests because there is no miRNA proved yet to be robust enough for diagnosis (30, 31, 35). Colorectal cancer (CRC) like any other types of cancers, the pathogenesis of the colorectal cancer is highly complicated and characterized by significant number of dysregulations of signaling networks and multiple gene mutations (10). Human MiR 20a located at chromosome 13 region q31.3 and one of important miRs that involve in various biological processes and progression of cancer, high expression levels of miR-20a have been detected in plasma, serum, tissues, and fecal samples with high sensitivity to CRC, miR20a exhibit useful potential clinical and diagnostic applications in CRC patients (8, 28, 32). In Iraq, genetic assessments of different genes and their roles in cancer prognosis and their impact investigated frequently (1, 3, 4, 15), however specified roles of miRNAs didn't receive enough attention. Therefore, the current study aimed to conduct an assessment to the level of miR20a in CRC Iraqi patients and possibilities to be used as diagnostic biomarker which could serve as predicting tool for CRC diagnosis instated of invasive diagnostic methods.

MATERIALS AND METHODS

Subject: The selected patients' samples collected from Gastroenterology and Hepatology hospital in Baghdad along with other samples which referred by a private physicians to private labs at Al-Karadha region and Al-Kindi Street, these samples collected through the period from June 2019 to December 2020.

Poles of Study: The study workflow procedure started with collecting 5ml of venous blood from CRC patients and 40 samples from non-cancerous controls, 0.4ml of all extracted plasma samples added to 0.6 ml Trizol reagent for extraction purposes (34), hybrid extraction method for miRs considered by further spin column purification step (7). All extracted samples checked by Qunatus fluorometer (Promega, USA) using specific QuantiFluor kit (Promega, USA). All extracted samples were in good concentration and purity.

Complementary DNA synthesis: Reverse transcriptase reaction carried by M-MLV Reverse Transcriptase (M1701, Promega, USA), M-MLV Reverse Transcriptase enzyme is an RNA-dependent double stranded deoxyribonucleic acid polymerase (DNA polymerase) used in cDNA synthesis procedure with RNAse H activity much weaker than Avian Myeloblastosis Virus (AMV) reverse transcriptase and reported to have greater sensitivity for gene expression (12, 11), the resulted concentration of synthesized cDNA ranged from 12-15 ng/µl for all constructed poles of this study (Patients

and Control). Quantitative PCR (qPCR) reactions were performed using the GoTag qPCR Master Mix (A6010, Promega, USA) with Bryt™ Green dye as an alternative to SYBR® Green I dye since Bryt™ Green dye less inhibitory on the amplification reaction and had brighter fluorescence during qualitative PCR run than master mixes contained- SYBR® Green I dye.

Amplification primers and qPCR assay

Amplification primer designed with primer premier 3 software, the sequence of miR20a retrieved from NCBI and miRBASE. In order to increase the melting temperature (Tm), 5' extension of 5-7 nucleotides considered to be added along with the forward primer sequence at the 5' end. the primers supplied by Macrogen (South Korea), the primers that used for the detection of miR20a-5p include miR-20a-5p-RT 5`-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CTACCT-3`and the forward primer include 5`-GTTGGTAAAGTGCTTATAGTGC-3`, RNU-43- RT quantified using 5`-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAATCAG-3` and 5`-GTGAACTTATTGACGGGCG-3`, the annealing temperature were 55°C for the RT primers and 55°C for the F primers, 5`-GTGCAGGGTCCGAGGT-3` used as universal primer. MIC RT-PCR instrument (Australia) used to for relative quantification purposes. Reaction mixtures list in Table1. Livak module (2– Delta Delta CT) or the comparative CT method used to generate folding result based on the result of calibrator-RNU-43 and resulted controls samples that subjected to the same assay.

Table1. miR20a-q-PCR Reaction mixture adopted by the current study.

Reaction component	Volume
Master Mix	5µl
Forward primer	0.5µM
Reverse Primer	0.5 µM
Nuclease Free Water	3µl
cDNA	1 ng/µl
Total volume	10
Aliquot per single reaction	9µl of master mix per tube + 1µl of cDNA template

RESULTS AND DESCUSSION

All processed samples from 60 patients and 40 controls subjected to qPCR assay and dynamically normalized, the threshold established at 0.026 at cycle 1, with fluorescence cutoff of 5% as an exclusion, the r^2 correlation coefficient for miR20a for all samples which exceed the 0.99 which represent good quality indicator (2), the Cp values for the patients samples were at range of 19.19 – 38.24 and at range of 20.576 to 31.842 for controls. The amplification assay of miR20a handled at 95°C for 15s followed by 55°C for acquiring green dye, and at 72°C for 15s, these steps repeated for 50 cycles, the melting temperature were at range of 75.82 – 76.31°C for all poles of current study which generated by dissociation curve, No reported results gained from the negative controls which added as a quality indicator, the dissociation curve and amplification are listed in Figure 1 and Figure2 respectively.

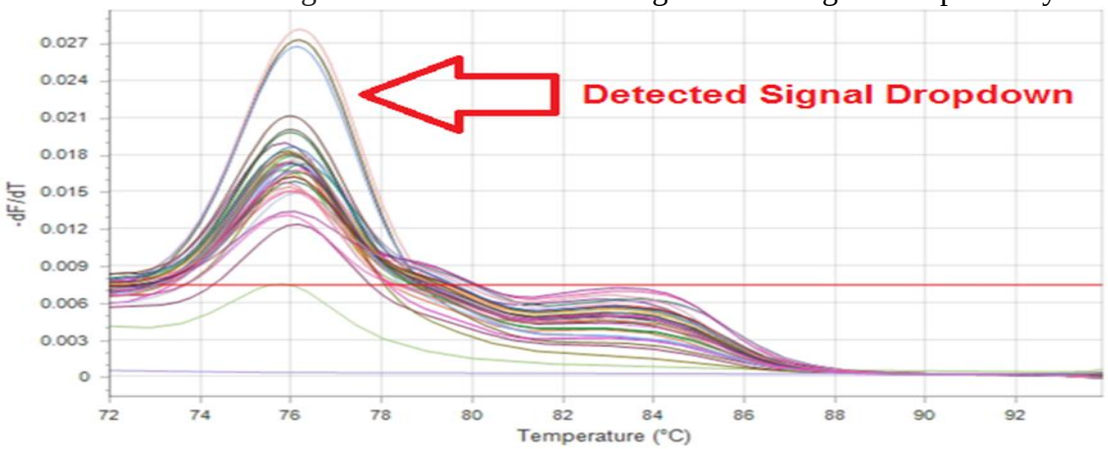

Figure 1. miR-20a Dissociation curve which established at threshold of 0.007 that represented as horizontal red line (melting curve).

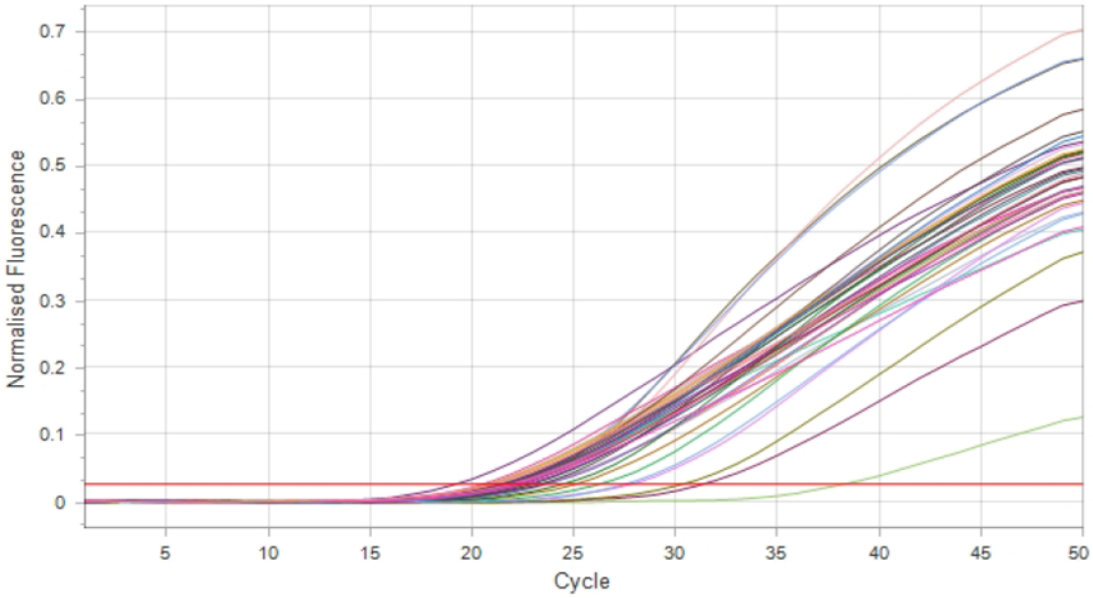

Figure2. Normalized fluorescence of miR20a assay (Y axis) in corresponding to the Cycles (X axis), the first amplification exceeds the threshold of 0.026 (represented as horizontal red line) at cycle 19.9.

Livak method selected to report the data resulted from the qPCR assay for the miR20a in comparison to calibrator (RNU43), the resulted folding for 22 (36.66%) patient samples ranged from 0.007 to 0.7 which is below 1 folding level, the resulted folding for 26 patients' samples (43.33%) ranged from 1.06 to 7.83 which consider below 10 folding levels, 8 samples (13.33) were at range of 11.39- 35.21 below 50 fold level, one sample (1.66%) reported at folding 67.34 below 100 folding level, and 3 samples (5%) were above 100 folding level. The reported data of current study for 60% of non-cancerous controls (24 samples) below one folding level at range of 0.011645 to 0.47, 25% of controls (10 samples) were at range of 1.12 to 1.95 below 3.2 folding level, and 15 % of controls samples ranged from 3.44 to 15.6067 which higher than 3.2 folding level. The overall range of folding for patients' samples was at range 0.007 to 170.4 and at range of 0.011645 to 15.6067, all folding results for all samples of the two current poles compared using SPSS illustrated in Figure3.

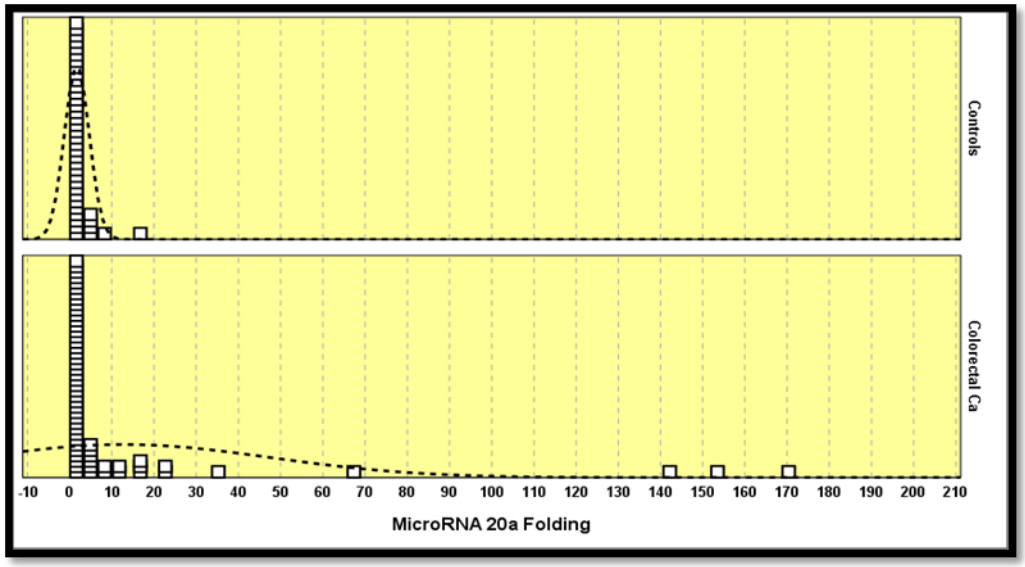

Figure3. folding level comparison between non-cancerous controls and CRC patients

All resulted folding, percentages and numbers of controls and CRC samples clarified in Table 1, P values revealed using Chi-square test and students-t-test at 0.05 level, the P value of Chi-square test was 0.102 higher than 0.05 and 0.047 (smaller than 0.05) for student-t-test, the

mean of CRC samples was higher than the controls when compared together (Patients: 12.7801< controls: 2.9489), the same findings concluded when the standard deviation (SD) of CRC samples compared to control samples (34.7397 > 2.9489). The summary of statistical analysis for miR20a samples poles listed in Table2.

Table2. folding level abundance among CRC patients' samples and non-cancerous controls samples.

miR ID	Folding level	CRC		Controls		P value
		No	%	No	%	
MicroRNA 20a Folding	<1.0F	22	36.7	24	60.0	0.102
	1.0---	11	18.3	7	17.5	
	2.0---	6	10.0	1	2.5	
	3.0---	5	8.3	4	10.0	
	4.0---	1	1.7	1	2.5	
	5.0---9.9	3	5.0	2	5.0	
	=>10.0F	12	20.0	1	2.5	
	Mean± SD (Range)	12.7801±34.7397 (0.00684-170.3951)		1.6910±2.9489 (0.011645-15.6067)		0.047#

*Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.
#Significant difference between two independent means using Students-t-test at 0.05 level.

The folding level of all CRC samples compared to the mean controls, the comparison revealed that 46.66% of CRC patients (28 samples of 60) had higher fold of miR20a genetic expression than 1.6909 controls mean which indicate that these samples are in up-regulated expression of miR20a, however 6 control samples (15%) showed higher folding level ranged from 3.449- 15.60569 which when excluded from the mean calculation, the results greatly differ when the folding level compared individually for CRC samples, the mean of the control samples that used for comparison reduced from 1.6910 to 0.74441, on that basis, 22 CRC samples were lower than control mean and 38 CRC samples were higher than the control mean. Mean based comparison illustrated in Table3.

Table3. Mean Based comparison for miR20a folding level in CRC patients

miR20a Status	Comparison with 1.6910	MiR20a status in corresponding to the new mean	Percentage % (UP, Down)
Up-regulation	28	38	(48.66, 63.33)
Down-regulation	32	22	(53.33, 36.66)

Statistical analysis was performed to establish a link between the folding level among ages and gender, however the result were not significant and above level of 0.05 of Students-t-test and ANNOVA test as showed in Table4.

Table4. Statistical analysis of miR20a folding level among age and gender of the patients and control groups.

		MicroRNA 20a Folding				P value
		Colorectal Cancer		Controls		
		No	Mean ± SD	No	Mean ± SD	
Age (years)	23---29	2	2.68069±1.69777	5	1.36742±2.66870	0.558
	30---39	12	4.57771±6.56690	11	1.09777±0.93527	0.097
	40---49	14	14.69067±37.29230	11	2.13977±4.60753	0.281
	50---59	20	3.19156±7.83272	11	2.27170±2.72474	0.711
	60---66	12	36.41760±61.69977	2	0.09970±0.01016	0.437
	P value		0.088		0.796	
Gender	Male	34	10.89678±26.81461	20	2.17455±3.81997	0.156
	Female	26	15.24289±43.45005	20	1.20735±1.66303	0.157
	P value		0.635		0.306	

#Significant difference between two independent means using Students-t-test at 0.05 level.
^Significant difference among more than two independent means using ANOVA-test at 0.05 level.

The prognostic role of miR20a investigated widely, miR20a is one of oncomiR that involved in cancer progression and cell proliferation along with other microRNAs molecules that belong to the same or different clusters, miR20a showed to be up-regulated in solid and hematopoietic cancers, miR20a mainly involved in CRC by up-regulation the TGF-signaling cascade, however, several studies showed that the level of miR20a reduced when the tumor removed surgically (5, 25, 27), however, the up-regulation/down-regulation of miR-20 genetic expression in detected tumors is not consistent as result of different number cell lines used to detected these molecules along with limited samples population (21**,** 36). The current study with both comparison listed in table 2 showed that not all samples were in up-regulation status, due to limited samples size, the sequence profile for these samples not reveled which may contain a mutation in the template sequence or effected by different environmental parameters ranged from bacterial and viral infection, any not reported medical treatment, food habits and involvement of immunological pathways, this statement applicable to the non-cancerous control 6 controls samples that detected with high folding level taking in consideration that the samples collected during the SARS-COV-2 pandemic and many individuals had the ability to be a symptomatic carriers, or had not reported metabolic disorders (10**,** 13**,** 19**,** 25**,** 27). Furthermore, the level of miRNAs varied in response to multiple factors, such as type of diets, diets can had both promoting and protective effects, including dietary diet and other vegetable based diet, each type of food had specific active compound that may had effect, furthermore, it has been mentioned that miRNAs can pass through species via milks or plants parts which it had some level of miRNAs which is mostly conserved among species; miRNAs by that can increase the already existed level of host miRNAs or could effect on the increasing or decreasing the level of genetic expression, such interference is described as horizontal transfer of small RNAs molecules (6, 9, 21, 22, 36).

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