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Evaluation of Plasma-microRNA320 level among Colorectal Cancer Iraqi Patients

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Abstract:

Colorectal cancer (CRC) Patients showed different expression patterns of miRNAs which are involved in carcinogenesis in comparison to healthy controls individuals, miRNAs are involved in tumor progression and development of metastases. We investigate the expression profile of microRNA 320 and ~~to~~ quantify the expression level abundance among colorectal cancer patients in comparison to the healthy control group. A total number of 60 plasma samples was collected from CRC patients along with 40 plasma samples from healthy controls and subjected to relative quantification using qPCR assay with a specified set of primers designed using stem-loop strategy. The resulting folding level revealed 2 distinguished patterns of expression; 31 samples (51.66%) were higher than the folding mean level of control (Up-regulation) and 29 samples (48.33%) were below the folding mean level (Down-regulation). The relation of folding level among healthy controls and patient groups showed a P value of 0.0001 using the Pearson Chi-square test. The Area under curve (AUC) was determined as 0.730 with a P-value of 0.0001 with a 95% confidence interval, the lower bound was 0.632 and the upper bound was 0.827.

Keywords: Colorectal Cancer, microRNA 320, Down-regulation, Up-regulation.

تقييم مستوى الحامض النووي الرايبوزي المايكروي-320 في بلازما مرضى سرطان القولون والمستقيم العراقيين

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الخلاصة:

أظهر مرضى سرطان القولون والمستقيم نمطًا مختلفًا في مستويات التعبير الجيني عن الحمض النووي الرايبوزي المايكروي التي تشارك في التسرطن مقارنة بأفراد السيطرة الأصحاء ، وتشارك جزيئات الحمض النووي الرايبوزي المايكروي في تحول الخلايا السرطانية وتطور الورم ، هدفت هذه الدراسة الى التحري عن طبيعة تعبير الحمض النووي الرايبوزي المايكروي320 وقياس مستوى تعبيره كميا في المصابين بسرطان القولون والمستقيم ومقارنتها مع مستويات التعبير في مجموعة الأشخاص الأصحاء. أستخلصت 60 عينة بلازما من مصابي سرطان القولون والمستقيم بالإضافة الى 40 عينة بلازما من أشخاص سليمين وتم مقارنة كمية التعبير في كلا المجموعتين عن طريق تقنية تفاعل البلمرة المتسلسل الكمي بأستخدام مجموعة من

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البوادئ المتخصصة والتي صممت خصيصا بإستخدام تقنية بنية الحلقة الجذعية. أظهرت نتائج مستويات التعبير نمطين مميزين للتعبير حيث كانت نسبة التعبير في 31 عينة والتي تمثل نسبة (51.66%) أعلى من معدل التعبير في عينات الأصحاء مما يشير الى إرتفاع تعبير الحمض النووي الرايبوزي المايكروي 320 في عينات هؤلاء المرضى بينما كانت نتائج 29 عينة والتي تمثل نسبة (48.33%) أقل من معدل التعبير في الأشخاص الأصحاء مما يؤشر إنخفاض تعبير الحمض النووي الرايبوزي المايكروي 320 في عينات هؤلاء المرضى. أظهرت علاقة مستوى التعبير الجيني بين الاشخاص الأصحاء ومجموعات المرضى قيمة P قدرها 0.0001 باستخدام اختبار مربع كاي–بيرسون وتم تحديد AUC على أنها 0.730 بقيمة P تبلغ 0.0001 مع فاصل ثقة 95٪ ، وكان الحد الأدنى 0.632 والحد الأعلى 0.827.

Introduction:

Worldwide, colorectal cancer is the third most diagnosed type of cancer based on the estimation of the international agency for research on cancer and the second leading main cause of cancer deaths based on the published data in 2020 for 36 types of reported cancers in 185 countries [1, 2, 3]. In 2021, WHO reported 1.93 million cases of CRC with 935.000 confirmed death, such statistics make the CRC as the third most diagnostic type of cancer [1, 4]. CRC is a complicated disease and is affected by several factors, microRNAs (miRs) appeared as an important factor involved in the regulation process of many genes since 2002 when miR15 and miR16 were involved in chronic lymphocytic leukemia [5, 6]. Such molecules exert their effects by increasing or buffering the genetic expression of specified genes via targeting mRNA transcripts in a complementary manner; acting as onco-miRNAs or tumor-suppressor miRNAs [7]. miR320 family is considered as one of the important miRs that affect CRC in a way or another, miR320 molecules encoded via the upstream region of cell cycle RNA polymerase III subunit D that resemble a remarkable conserved area within RNA polymerase III enzyme, miR 320 family showed to have significant roles in human health and multiple diseases [8, 9,10]. The miR320 family includes miR-320a, miR320b, miR-320d, miR320C, miR320e, among those, miR320a and miR320b are closely related in their sequence which is different by only one nucleotide [8, 9, 11]. There are many reported genes that are considered to be a favorite target for miR320a/320b molecules and mainly include Neuropilin-1, β-catenin, and Rac-1; all of these genes are known to promote invasion, proliferation, and metastasis [12]. The miR320 is considered a potential biomarker and its relation with various diseases has been investigated and it found that miR320 could be detected in the extracted plasma samples of CRC patients in down-regulation status [8,13]. miR320a is considered as representative of the miR320 family and exerts a suppressive effect through targeting the neuropilin 1 in CRC, with a vast range of targeted genes in other diseases and other types of cancers which clarify the complex network of genetic regulation, the specific fingerprint that distinguishes such molecules simplified by its expression pattern in relation to a specific tissue site and type of collected fluid sample and in relation to a different type of disease, however, The great majority of reported findings are consistent on down-regulation status of miR320a in collected plasma samples from CRC patients [14, 15, 16, 13, 17, 18, 19]. This study aimed to investigate the expression pattern of miR320 (up-regulation or down-regulation) in CRC Iraqi patients in comparison to a control group of healthy individuals and ~~to~~ expression patterns of miR320 in CRC plasma samples since there is not much-reported data about the specific expression fingerprint of miR320 in the plasma samples of Iraqi CRC patients.

Materials and Methods

Samples collection:

Total number of 60 CRC patients were included in the study along with 40 healthy individuals as control group after acquiring their approval to be enrolled in the study, these samples were collected from Gastroenterology and Hepatology Hospital in Baghdad and from private labs at Al-Karadha region and Al-Kindi Street which referred by ~~a~~ private physicians.

RNA Extraction

Workflow procedure for miR320 quantification was established by collecting plasma samples from 60 CRC patients and 40 healthy individuals, 0.4ml of plasma was collected from 5ml of venous blood samples after subjecting all these samples to centrifugation followed by the addition to 0.6ml of Triazol reagent, spin-column method used as an additional step for miRs purification [20, 21]. All extracted samples were checked with Qunatus fluorometer (Promega, USA) using Qunatifluor RNA system to evaluate the resulting purity and concentration.

Complementary DNA Synthesis

M-MLV reverse transcriptase (M1701, Promega, USA) was used in cDNA synthesis, the concentration of the resulted cDNA was at range12-15 ng/µl for patients control samples, the cDNA synthesizing reaction mixture consisted of 2µl of M-MLV Reaction Buffer, 2µl of dNTPs, 0.25µl RNAsin, 0.5µl of M-MLV reverse transcriptase enzyme, and 5.25µl of nuclease-free water to achieve 10µl working solution.

Primers designing and quantitative PCR assay

Primers were designed using the stem-loop structure method [22, 23], the primer was synthesized by Macrogen (South Korea), GoTaq qPCR master mix kit (A6010, Promega, USA) used for quantitative detection using Bryt™ Green dye. Table 1 shows the primers set used in the miR320 quantification procedure amplification procedure which was designed by primer designing algorithm primer premier 3 software, miR320 retrieved from miRBASE [24] using stem-loop designing module, these primers have already been used in several published articles for quantification procedure via qPCR, RNU-43 used as reference gene for normalization purposes [25,26]. Livak method (2– Δ Δ CT) was used to analyze the MIC RT-PCR (Australia) Cqs for the controls and patients [27].

Table 1: Nucleotide sequence of the primers that included in the quantification procedure.

Primer ID	Nucleotide sequence
miR-320-RT	5`GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCA CCAGAGCCAAC TTGCCC-3`
miR-320-F	5`-GGGAAAAGCTGGGTTGAGA-3`
RNU43-RT	5`GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAA TCAG-3`
RNU43-F	5`-GTGAACTTATTGACGGGCG-3`
Universal Primer	5`-GTGCAGGGTCCGAGGT-3`

Statistical Analysis

The statistical analysis was performed using the statistical package of SPSS-27 (Statistical Packages for Social Sciences- version 27), the significance of differences for different means of quantitative data was tested using the Students-t-test to calculate the differences between

two independent means, ANOVA test method was used to analyze the difference among independent means. The significance of the difference among qualitative data was tested using the Pearson Chi-square test. The significance of statistical analysis was considered whenever the P-value was equal to or less than 0.05. The Receiver Operating Characteristic (ROC) curve method is considered to determine the possibility to exploit any parameter as a screening or diagnostic tool specific for the CRC and to calculate the cut-off value, the explanation of the area under the curve (AUC) values is considered fair if the result is < 0.9 (good equal to 0.8 or higher) and > 0.7 (Poor equal to 0.6 or higher).

Results and Discussion

All samples were collected from patients who did not receive any kind of surgical, radiological, or chemical therapies to avoid any possible effect on the expression profile of miRNAs since the level of miRNAs re-established again to the normal level when the affecting cause was eliminated [28]. The extracted 60 CRC plasma patients samples and 40 control samples were subjected to qPCR-miR320 assay after optimization procedures, the threshold of 0.153 was established automatically at cycle 1 with a dynamic normalization and extensive fluorescence cutoff of 5% as exclusion, the correlation coefficient (r2) was higher than 0.99 for all analyzed samples which represent a good quality indicator [29], the threshold of the melting curve was 0.007 which started at 73.95°C, reaction parameters considered withhold for 5 min at 95°C, followed by 60 cycles at 95°C for 15s, 55°C for 15s for acquiring on green and 72°C for 15s, the resulted in Cq ranged from 29.33 to 51.41 for the tested CRC plasma samples and from 32.48 to 49.49 for the tested controls plasma samples, no reported results gained from the negative controls which added as a quality indicator.

Among 60 tested CRC plasma samples, the revealed folding data which analyzed via the Livak method were at the range of 0.000579 to 652494.005, among them, 15 CRC samples (25%) were at folding range of 0.000579 - 0.62885 below 1 folding level, 7 samples (11.66%) were at folding range of 1.6799 - 3.26938 below 4 folding levels, 7 samples (11.66%) were at the range of 8.5294 - 24.7780 below 25 folding levels, 3 samples (5%) were at the range of 8.5294 - 24.7780 below 25 folding levels, additionally, the folding level exceeds the 50 folding levels for the rest of CRC samples; 10 samples (16.66%) were at the range of 52.2903 - 921.776 below 1000 folding level, 18 samples (30%) were at folding range of 1040.250 - 652494.0052 which is higher than 1000 folding level and exceed 100000 folding level for 4 samples, mean based analysis showed that CRC samples folding level was 39877.3 which is much higher than the mean of 27.3074 for the tested controls plasma samples, folding level compression and distribution listed in Figures 1 and 2 represent a mean-based comparison between the folding mean of patients and controls. The statistical analysis of folding level among study poles revealed a significant P-value using Pearson Chi-square test of 0.0001 < 0.05 level, number of tested samples for each fold, and their percentage shown in Table 2.

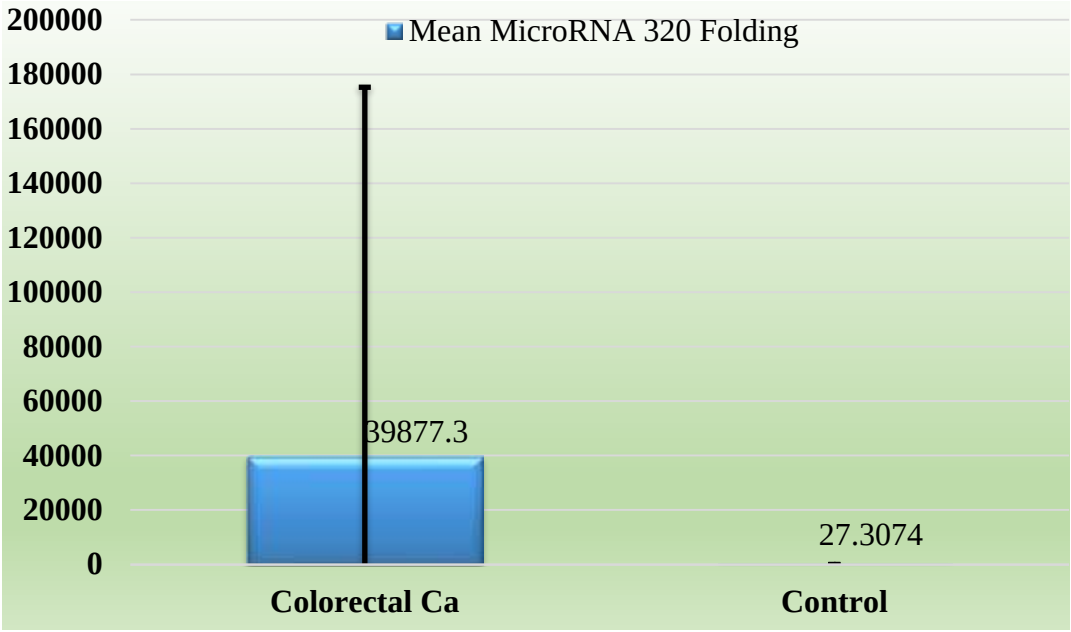

Figure 1: mean based comparison between CRC patients and healthy controls

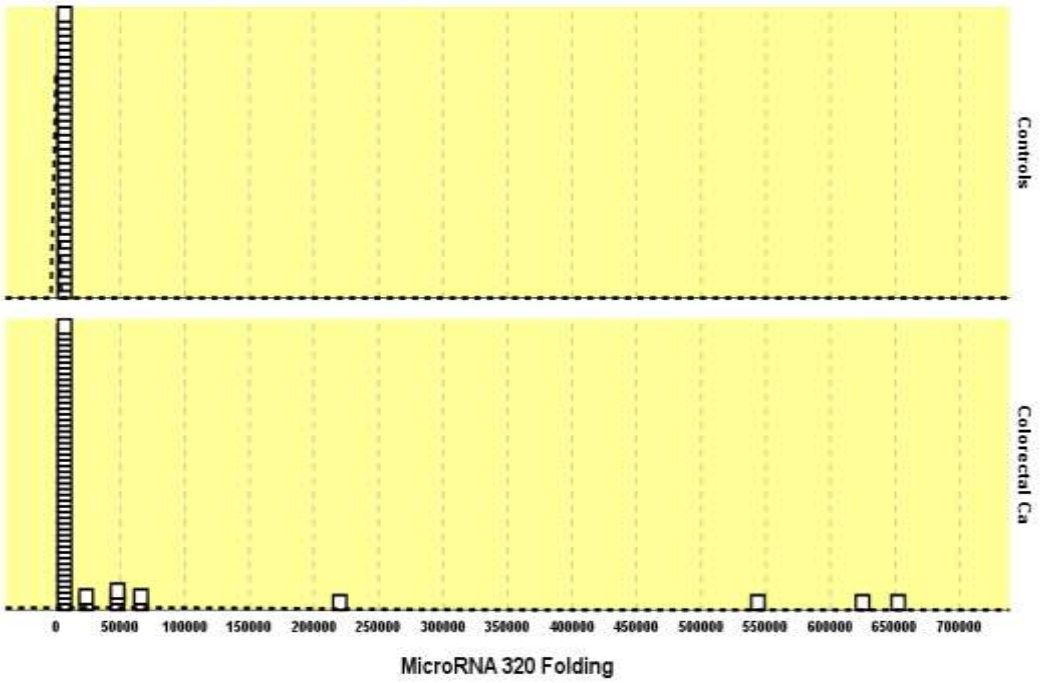

Figure 2: Folding level representation of Controls processed samples and Patients processed samples.

Table 2: Statistical analysis summary of miR320 folding level among CRC and Controls samples.

mIR ID	Folding Level	Colorectal Ca		Controls		P value
		No	%	No	%	
MicroRNA 320 Folding	<1.0F	15	25.0	23	57.5	0.0001*
	1.0---	3	5.0	-	-	
	2.0---	3	5.0	-	-	
	3.0---	1	1.7	1	2.5	
	4.0---	-	-	2	5.0	
	5.0---9.9	1	1.7	6	15.0	
	=>10.0F	37	61.7	8	20.0	
	Mean±SD (Range)	39877.3±135414.15 (0.00058-9223.372)		27.3074±84.2470 (0.001107-452.6038)		0.066

*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.

Among 60 CRC samples, 29 samples (48.33%) were below the folding mean of controls when compared individually, such samples considered to be in down-regulation status at range of 0.000579 - 24.778 < 27.30744, however, 31 samples (51.66%) were above the folding mean of controls at range of 52.29031 - 652494.0052 > 27.30744 and considered to be in up-regulation status, the miR320 is type of miR320 family and used as representative in of miR320 family and it reported to be in down-regulation status [13], however, there are another version miR320a referred as miR320b; another member of miR320 family who share 99% homology with miR320a with one different nucleotide, although miR320b pose a different effects, miR320b had high expression level among CRC patients rather than low expression level as indicated with miR320a, both miR320a/320b work in homologous competition on the same genes that affected in CRC cases which include Neuropilin-1, β-catenin and Rac-1; all of these genes are known to promote invasion, proliferation and metastasis [12], although of complexity of explanation, miR320a and miR320b could not be excluded because of major role in CRC through controlling the genes that related to CRC uncontrolled cellular growth [30, 31]. Statistical analysis showed no significant P values related to gender, however, significant P values of 0.020 (using student t-test) were gained in relation to 30968.0765 folding means of CRC patients at age 23-29 years old as illustrated in Table 3.

Table 3: Statistical Analysis of miR320 folding level in relation to ages and gender groups of CRC and Controls analyzed samples.

Parameters		MicroRNA 320 Folding				P-value
		Colorectal Cancer		Controls		
		No	Mean±SD	No	Mean±SD	
Age (years)	23---29	2	30968.0765±24439.44	5	13.37690±29.83929	0.020#
	30---39	12	327.41461±531.45449	11	8.35065±10.32188	0.060
	40---49	14	586.69192±1539.53588	11	65.97688±147.52977	0.277
	50---59	20	57359.814±149130.024	11	18.83332±55.59562	0.216
	60---66	12	97613.637±228042.97	2	0.32186±0.44252	0.569
	P value		0.322		0.524	
Gender	Male	34	32931.089±116869.452	20	13.86925±41.26198	0.215
	Female	26	48960.835±158413.034	20	40.74563±111.74043	0.175
	P value		0.653		0.319	

#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 revealed Area under the curve (AUC) for miR320 was 0.730 at a P-value of 0.000.1, the standard error was 0.050, the lower and upper bound revealed at a 95% confidence interval of 0.632 and 0.827 respectively, the result considered as Fair since the AUC is greater than 0.6 and less than 0.8, the current result indicates that miR320 could be either upregulated which give an indicator about the dominant effect of miRNA320b, or down-regulation level that provides an indicator about the dominant effect of miR320a.

Conclusions

MicroRNAs emerge as one of the tools that can be implemented to enhance the cancer-based diagnostic efforts along with other commonly used markers, here, we showed that miR320a and b have a distinctive expression pattern that differs from the expression pattern among healthy non-cancerous individuals and ~~on~~ that basis, it can be implemented to be used as a biomarker.

Ethical Clearance

This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific Research, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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