

# The Dysregulation of mir-21 and mir-143 as a clinical Marker for Cancer Stem Cells in Tissue Samples of Iraqi Male Patients with Gastrointestinal Sarcoma

Zahraa K. Zedan<sup>1</sup> and Salah H.A.AL-Amer<sup>2</sup>

<sup>1</sup>Department of molecular and medical Biotechnology, College of Biotechnology, Al-Nahrain University, Iraq

<sup>2</sup>Department of Surgery and Obstetrics, College of Veterinary Medicine, Al-Qasim Green University, Iraq

## ABSTRACT

A few examinations have perceived that disease undifferentiated cells (CSCs) are a biomarker for Colon malignant growth treatment; in any case, its meaning of guess is flighty, and there is a need expected for new biomarkers to delineate patients and administer those ideal treatments by considering planned of hereditary and epigenetic contrasts including MicroRNAs. This study distinguished Colon-CSCs explicit markers utilizing an immunohistochemistry articulation marker (CD44). A qRT-PCR is utilized for estimating the declaration of mir-21 and mir-143 in colon malignant growth tissue taken from Iraqi guys with colon disease. The Colon CSCs were described by utilizing Immunohistochemical (IHC) staining of formalin-fixed, paraffin-implanted tissue segments consuming explicit markers in forty tissue tests of colonic disease patients gathered from the Oncology Department, Baghdad Teaching Hospital, Baghdad, Iraq. A coordinated neoplastic colonic tissue was with a non-neoplastic colonic epithelial cell line was utilized as control. The complete RNA was removed from the example cells and changed over completely to cDNA utilizing the Stem-Loop method, and the articulation was estimated by ongoing PCR (RT-PCR) utilizing the Stem-circle strategy, SYBR Green, and explicit groundworks, then collapsing investigation. CD44 was altogether profoundly communicated in all cases. The connection was genuinely non-critical between the two gatherings. The statement of miR-21 was overexpressed in CC patients at both early (I-II) and high level stages (III-I). Results additionally showed that miR-143 was altogether down-controlled in the two phases. Mir-21 and mir-143 assume a basic part in managing many objective qualities and pathways that are ensnared in cancer expansion, intrusion, apoptosis, and metastasis, as well as medication obstruction. MiR-21 and miR-143 could be significant biomarkers utilized in CC determination, visualization and to be a likely restorative objective for CC.

**Key words:** Colon cancer; CSCs; CD44; miR-21; miR-143; RT-PCR.

## INTRODUCTION

The colorectal disease is one of the generally recorded malignancies. There has been gathering proof on the side of the disease immature microorganisms (CSC) considered malignant growth which suggests that CSCs are focal in the commencement of disease.<sup>1</sup> In many instances of CC-related passing brought about by metastases to the liver, primarily certify to the growth entering the digestive wall and conveyance through the lymphatics to lymph hubs and fundamentally to far off organs by means of the circulation system, This has expected to the ID and comprehension of hereditary and epi-hereditary engaged with the acceptance and support of pluripotency of

immature microorganisms, and markers from pluripotent to separated cells, the distinguishing proof and focusing of these

**Address for correspondence:** Salah H.A.AL-Amer  
Department of Surgery and Obstetrics, College of  
Veterinary Medicine, Al- Qasim Green University, Iraq

**E-mail address:** salah\_hassan43@yahoo.com

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markers might prompt superior techniques for early discovery of colon disease (CC) as well as to achieve novel treatment and novel therapy for CC and other disease types. Regardless of the possibly high clinical importance of Cancer Stem Cells, little is had some significant awareness of the prognostic meaning of the declaration of putative CSC markers in colorectal diseases.<sup>4</sup> Many examinations have been affirmed and recognized that numerous hereditary markers including OCT4, SOX2, NANOG, KLF4, SALL4, C-Myc, STAT3, ALDH, and CD133 have indisputable relationship with malignant growth, pluripotency, ordinary foundational microorganisms, CSCs, and chemo-obstruction. A class of little non-coding RNAs, called microRNAs, has come into accentuation. MicroRNAs are little with 18 to 24 nucleotides long that control the interpretation at the post-transcriptional level by restricting to explicit mRNA.<sup>7</sup> Studies announced that numerous microRNAs are unpredictable controlled in Colon malignant growth this study zeroed in on specific microRNAs that have significant oncogenic capabilities in CC as many examinations detailed including miR-21,<sup>8</sup> which is over-communicated in numerous other strong cancer types and this microRNA plays significant parts in disease commencement, movement, and metastasis,<sup>9</sup> while other MicroRNAs have growth silencer capability in CC including miR-143 that have been displayed to work in growth concealment, in light of the fact that their decreased articulation has been seen in the vast majority of the human disease cell lines tried and especially so in colon tumors, by focusing on ERK5, which is connected with cell development. This study aimed to isolate and characterized CCSCs to identify the location and distribution of target cells by staining with a specific antibody combined with the investigation of the expression of miR-143 and miR-21 in the newly diagnosed and chemo-resistance Iraqi patients with colon cancer.

## METHODS

### Subjects

Forty patients with colonic malignant growth who were analyzed by histopathological assessment at various stages from September 2019 to February 2020 were signed up for Oncology Department, Baghdad Teaching Hospital; Baghdad, Iraq. Counting 24 guys and 16 females, their mean age was gone from 47.8 - to 67.7 years. The incorporation standards were as the followings: Eighteen patients with CC were recently analyzed and affirmed by the pathology, without medical procedure, chemotherapy, radiotherapy, or different therapies, and 21 of CC patients was chemo-obstruction with cutting edge stage. The rejection models were as per the following: different illnesses, for example, irresistible infections, diabetes type 1 and 2, threatening cancers, extreme liver and kidney sickness, pneumonic fibrosis, bone metabolic sicknesses, auxiliary renal hypertension, fundamental resistant sicknesses, and dangerous growth confusions. Fifty solid workers were laid out as controls, their age was matched to patients.

Tissues were fixed in 10% formalin at room temperature for 14 hours and then, at that point, flush with running faucet

water for 60 minutes. Tissues implanted in the paraffin cycle are called parchedness and are achieved by going the tissue through a progression of expanding liquor focuses. The miRNA level in patients was examined in new tissue utilizing Trizol (TRI Reagent®; ZYMO RESEARCH, USA). A coordinated neoplastic colonic tissue with a non-neoplastic colonic epithelial cell line was utilized as control.

### Immunohistochemical Staining of Formalin-fixed, Paraffin-embedded Tissue Sections

Deparaffinize slides were acted in 2 changes of xylene, then, at that point, slides were moved to 100 percent liquor, for 2 changes, and afterward move once through 95%, 70%, and half alcohols separately. The endogenous peroxidase action was impeded by hatching segments in a 3% H<sub>2</sub>O<sub>2</sub> arrangement in methanol at room temperature, the slides were flushed in 300 ml of PBS for 2 changes, 5 min each. A citrate support strategy was accustomed to performing antigen recovery to expose the antigenic epitope. 100 µl of impeding support (10% fetal cow-like serum in PBS) was added onto the areas than in counter acting agent weakening cushion of ox-like serum egg whites in PBS was applied to segments followed by washing step by PBS. Arranged Sav-HRP forms then applied to the segments, brooded, and furthermore followed by washing PBS step. Then pre-arranged DAB substrate arrangement was applied to the segments on the slides to uncover the shade of immunizer staining and washing with PBS. At last, the counterstain of the slides was finished by submerging sides in Hematoxylin after that the slides were flushed in running regular water the technique was finished by All Hycult Biotech. For immunohistochemical staining the essential antibodies were utilized: Invitrogen against CD44/H-CAM, Clone: Hermes-1, Thermo Scientific™, Thermo Fisher Scientific Austria.

### Primers that used in this Study

The primers were designed according to the manufacturer, the stem loop for miR-21 and miR-143 was designed according to the data base of <http://www.mirbase.org>. The forward and reverse primer was used according to the manufacturer Takara Bio Inc., Shiga, Japan.

### Preforming the Expression of miR-21 and miR-143

The RNA was detached from tissue tests utilizing extraction Direct-zol™ RNA MiniPrep, Zymo-Research/USA. As per the producer's guidelines, the RNA was eluted in 30µl of sans nuclease water. The Prime Script™ RT reagent Kit cDNA combination response was utilized. Momentarily, switch record response comprised of 2 µl 20X prime content response cradle, 0.5 µl engineered stem-circle preliminary, 4.5 µl without nuclease water, and the last grouping of 100 ng/µl of complete RNA, individually. RT response was performed utilizing a SaCycler-48 warm cycler, Sacace, Italy, at 42°C for 15 minutes, trailed by heat-inactivation at 85°C for 1 moment, and put away at 4°C. For quantitative PCR (qPCR), a 20 µl PCR response blend was arranged utilizing

KAPA-SYBR® FAST-PCR Master Mix, KAPA, USA. For serious 10 µl of 2 X SYBR Green expert blends, 4 µl of cDNA, 0.5 µl of each forward and turn around preliminary blend were added then finished the volume to 20 µl by adding sans nuclease water. The U6 housekeeping quality was utilized as the endogenous control. A qPCR was performed at 95°C for 7 minutes for compound initiation shadowed by 45 patterns of 95°C for 10 seconds and 60°C for 1 moment. Finally, the liquefying bend investigation was accomplished the partition elements of dsDNA during cycles with expanding denaturing temperature.

### Statistical analysis

The fold change was calculated by the equations,  $\Delta CT = CT$  of target gene – CT of U gene,  $\Delta\Delta CT = \Delta CT$  of each sample - average control  $\Delta C$  and the Fold change =  $2^{-\Delta\Delta Ct}$ , respectively.

### RESULTS

This study was included 40 cases of CC classified in to 18 (45 %) had early stage (I–II) and 22 cases (55%) are chemo resist with advanced stage (III- IV) stage for the disease.

### Immunohistochemical Staining of Formalin-fixed, Paraffin-embedded Tissue Sections

The expression of CD44s in tumor cells was demonstrated in figure 1. CD44 showed a higher rate of expression in all cases. The correlation was statistically non-significant between the two groups.\

### Gene Expression of miR-21 and miR-143

The gene expression level of miR-21 and miR-143 were tested using RT-PCR after converting the RNA into cDNA using specific stem loop primer that lengthen the targeted miRNA and the resulted curves were shown in (figure-1) then the folding were calculated using the Livak method.<sup>13</sup>

The outcomes showed that miR-21 was overexpressed in CC patients at both early (I-II) and high-level stages (III-IV) as well as in chemo oppose patients than in controls p=0.001. The relationship between's quality articulations of a miR-21 level was upregulated in patients with the chemo-oppose progressed stage (stage III and IV) by giving fold change 26.9 than in patients with beginning phase (stage I and II), the overlay change was 13.72 as contrasted and (Table 1).

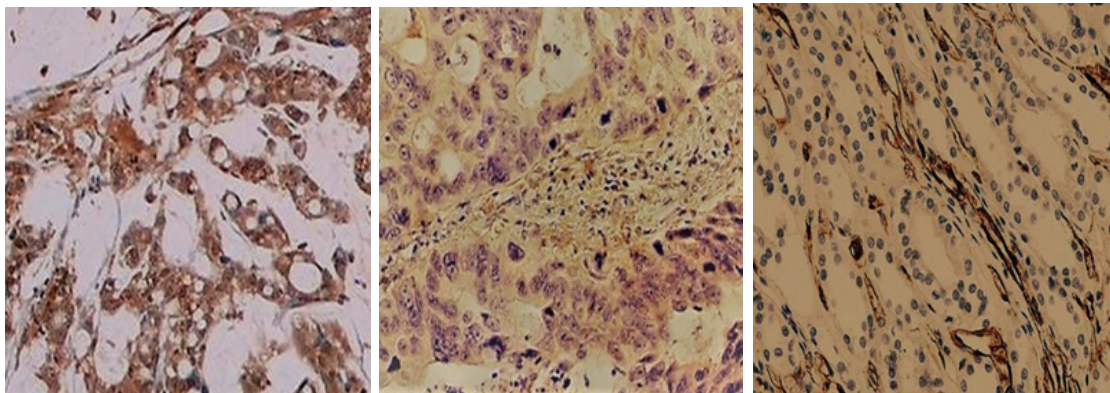


Fig. 1: Immunohistochemical section of the Colon cancer showing strong CD44 expression in; membranous and focal cytoplasmic staining (400×)

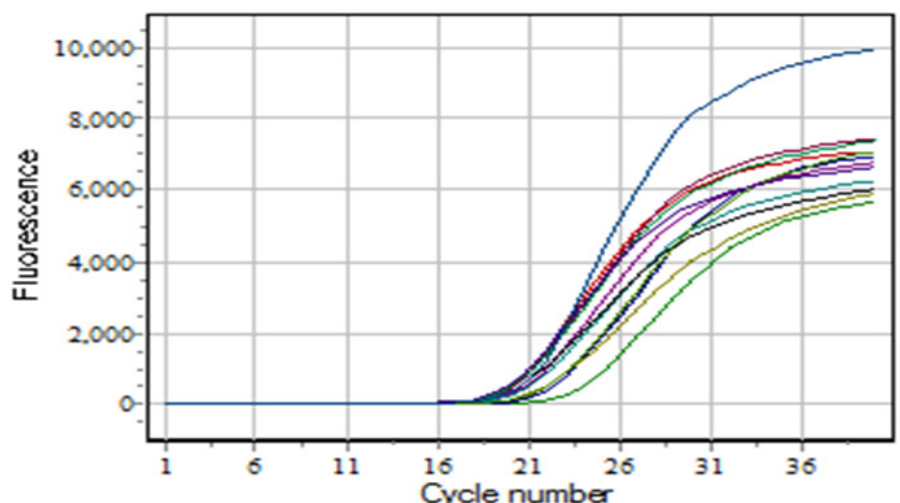


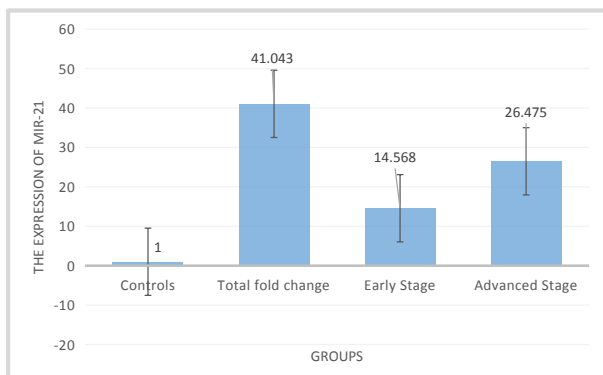
Fig. 2: The results of RT-PCR curves

**Table 1:** Gene expression of miR-21 comparison between patients and control

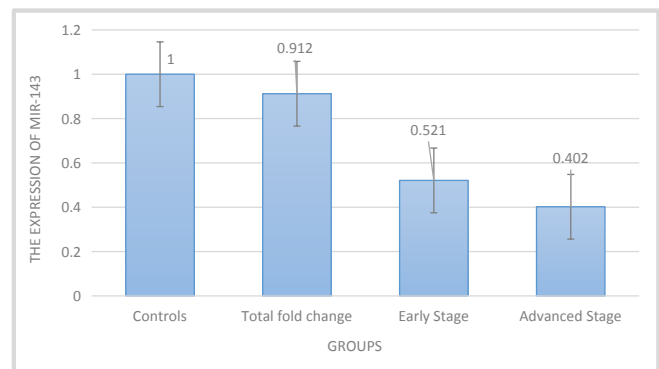
miR-21	mean of $\Delta Ct \pm SE$ for controls	mean of $\Delta Ct \pm SE$ for patients	$\Delta \Delta Ct$	P value	Folding
Early stages	3.5 ± 0.09	-1.91 ± 0.08	-2.26	0.001	13.72
Advanced stages	3.5 ± 0.09	-2.95 ± 0.15	-3.32	0.001	26.9

Table 2: Fold change of miR-143 comparison between early and advance stages of CC patients.

miR-143	mean of $\Delta Ct \pm SE$ for controls	mean of $\Delta Ct \pm SE$ for patients	$\Delta \Delta Ct$	P value	Folding
Early stages	-0.25 ± 0.02	2.03 ± 0.06	2.33	0.001	0.691
Advanced stages	-0.25 ± 0.02	2.81 ± 0.03	1.114	0.001	0.603



**Fig. 3:** The expression of miR-21 in early and advance stages



**Fig. 4:** The expression of miR-143 in early and advance stages

The relationship between's quality articulations of the miR-143 level was down-directed in patients with chemo as opposed to the progressed stage (stage III and IV) by giving fold change 0.603 than in patients with beginning phase (stage I and II), the overlay change was 0.691 as looked at (Table 2). There was a huge expansion in miR-21 in the aggregate, early and high-level phases of CC patients with overlap changes of 41.043, 14.568, and 26745 separately. There was a significant diminishing in the statement of miR-145 in selected bunches with overlap changes of 0.912, 0.521, and 0.402 Vs 1.00, separately.

## DISCUSSION

Disease-related passing from colorectal malignant growth is common because of the improvement of cutting-edge metastasis. Almost 70% of all patients determined to have CC contribution conceivably helpful medical procedure, a big part of those patients present with cutting edge nearby illness or metastases a few prescient variables exist, including clinical organizing game plan, a more unambiguous marker for CC with high metastatic potential would give valuable data to surveying adjuvant treatments (15). The commitment of colon malignant growth immature microorganisms to cancers advancement is broadly acknowledged, yet the connection of individual CSC markers articulation to infection anticipation is as yet not totally clear (16). CD44 articulation genuinely

critical, while many examinations detailed, rectal cancers showed a lower pace of CD44 articulation (33.3%) than colonic growths (66.7%) which is steady with the consequences of [15] (17) who found a lower pace of CD44 articulation in the rectum (44.4%) than colon (64.4%). Nonetheless, different examinations showed higher pace of CD44 articulation in rectum (48%) than colon (21.2%) (16), rectum (53.8%) and colon (30.8%) (18), and in rectum and left colon (53.8%) every then right colon (45.2%) (19). Many examinations found a higher pace of CD44 which likewise affirms our discoveries (16, 4). This study expressed that CD44 is of practical significance for malignant growth inception, and movement; support of the properties of CSC joined with CD133 could be valuable to distinguish putative colorectal CSCs. The articulation levels of all examined miRNAs fundamentally varied between cancer and non-growth adjoining mucosa. Up-guideline of miR-21 and downregulation of miR-143 were more huge markers in malignant growths than in adenomas. The outflow of miR-21 was significantly overexpressed in CRC patients when contrasted and ordinary examples and noticed a significant distinction in miR-21 articulation between stages I and II and stages III and IV with higher articulation levels. As many examinations affirm that miR-21 has been reliably demonstrated to be dysregulated in CRC (20-23). Large numbers of the early examinations relating miR-21 to CRC have been acted in vitro on laid out cell lines (24,25). A review revealed that the Infection of pre-miR-21

lentiviral vector in CRC cell lines (HT-29, Colo206f, LIM 1863, SW480, and DLD1 cells) can prompt significant cell expansion, improve transitory and obtrusive properties, and lessening apoptosis. On the other hand, the wreck of miR-21 could significantly reduce cell expansion, restrain attack, movement, and increment apoptosis subsequently, viewed as a significant cancer oncogene in CRC (26). This concentration likewise reposted the down-guideline of miR-143 in the two new and high-level phases of CC patients. Check-point kinase CHEK2 has likewise been recommended to be applicant target qualities of miR-143 with potential oncogenic capabilities, record factors like MYCN, FOS, YES, and FLI, cell-cycle advertisers, for example, cyclin D2 and CDK3, and mitogen-enacted protein kinase pathway (MAPK) transduction proteins (27). The overexpression of miR-21 and down-guideline of mir-143 assume a basic part in the improvement of adenoma might be fundamental. In this way, miR-21 and miR-143 could be biomarkers valuable for a quest for chemo-preventive specialists for the improvement of colon malignant growth.

## CONCLUSIONS

The roles of miR-21 and miR-143 in CC, evidence from the current studies suggest that both of the MicroRNAs may perform significantly in CC, and plays a critical role in regulating many target genes and pathways that are implicated in tumor proliferation, invasion, apoptosis, and metastasis, as well as drug resistance. MiR-21 and miR-143 could be important biomarker used in CC diagnosis, prognosis and to be a potential therapeutic target for CC. Further studies will be needed to reveal the mechanisms involving miR-21 and miR-145 in the early phase of colon cancer development.

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