Original Article
Middle East Journal of Cancer; October 2020; 11(4): 483-492

Hsa-miR-21-mediated Cell Death and Tumor Metastases: A Potential Dual Response During Colorectal Cancer Development

Ehab Maher*, Gamalat Gedawy, Walid Fathy***, Sabah Farouk*, Ahmed Abd El Maksoud****, Adel A. Guirgis*, Hany Khalil*♦**

**Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt*
***Department of Clinical Biochemistry and Molecular Diagnostic, National Liver Institute, Menofyia University, Sebin El-Kom, Egypt*
****Department of Clinical Pathology, Faculty of Medicine, Menofyia University, Sebin El-Kom, Egypt*
*****Industrial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt*

Please cite this article as: Maher E, Gedawy G, Fathy W, Farouk S, Maksoud AA, Guirgis AA, et al. Hsa-miR-21-mediated cell death and tumor metastases: A potential dual response during colorectal cancer development. Middle East J Cancer. 2020;11(4): 483-92. doi: 10.30476/mejc.2020.83146.1139.

Abstract

Background: Colorectal cancer (CRC), caused by abnormal cells growing in the colon or rectum, has a high mortality rate worldwide. On the other hand, microRNAs are small non-coding RNAs that contain approximately 22 nucleotides in length. They are upregulated in a wide range of human cancers such as CRC. MiRNA-21 post-transcriptionally regulates the expression of many tumor suppressor genes such as P53 gene. This indicates that miRNA-21 interacts like oncogenes and is required for CRC development.

Method: The current original study was conducted in the National Liver Institute, Menofyia University, Egypt. We collected a total of 40 blood samples from CRC patients 40 samples from healthy individuals who served as controls. Quantitative real-time PCR detected the levels of miRNA-21 and the fold changes of phosphates-tensin homology (PTEN) gene expression, as a tumor suppressor gene, in blood samples.

Results: The expression levels of miR-21 were upregulated in all obtained samples from patients with CRC in association with aging, gender, and tumor-node-metastasis staging. Furthermore, patients with poor and well-differentiated CRC revealed reduced levels of PTEN gene expression. We observed a putative binding site of miR-21 in PTEN gene sequences. This indicates the direct cleavage between miR-21 and PTEN coding sequence. Prediction analysis for other potential targets identified several malignancy factors and tumor suppressor genes with putative seeding regions for miR-21 such as STAT3, transforming growth factor-beta, tumor necrosis factor-α (TNF-α), and programmed cell death CD4.

Conclusion: The current data exhibited the potential dual role of hsa-miR-21 in regulating cancer progression and showed that hsa-miR-21 is an efficacious biomarker for CRC development and an attractive candidate for CRC treatment during early transformation.

Keywords: Colorectal cancer, Hsa-miR-21, Dual response

♦Corresponding Author:
Hany Khalil, PhD
Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt
P.O. Box: 32897
Tel: + 2048- 2601265
Fax: 2048 – 2601266/2601268
Email: hkhalil74@gmail.com

Received: September 02, 2019; Accepted: February 17, 2020

Introduction

Colorectal cancer (CRC) is a type of cancer commencing at colon or rectum, known as bowel cancer or colon cancer. There are many causes associated with CRC, including aging, obesity, smoking, inflammatory bowel disease, and genetic disorders.[1,2] CRC is the third leading cause of cancer-related morbidity and mortality with an approximate 700.000 annual deaths.[3,4] In Egypt, CRC is the 7th common malignancy occurring in 3% of female cancers and 3.4 % of male cancers. In 2015, The number of colon cancer patients in Egypt, excluding rectal cancer, was estimated at more than 3000.[5] Usually, CRC starts as a benign tumor which is then converted into a malignant tumor invading normal tissues, and ultimately, spreading all over the body.[6] A wide range of diagnostic tests for CRC is currently being employed, including visual exams, stool-based tests, fecal occult blood test, and tumor markers assays such as assessing of carcinoembryonic antigen.[7,8] Basically, a variety of intracellular signaling proteins are activated in CRC evolution such as protein kinase C, the mutant proteins K-Ras/B-Raf in the mitogen activated protein kinases signal, human epidermal growth factor receptor-2, kelch-like ECH-associated protein 1, hippo signaling, nuclear factor erythroid 2-related factor 2, and pro-inflammatory cytokines pathways.[9-12] Other inactivated pathways have been reported through CRC progress, including P53, deleted CRC, and transforming growth factor-beta (TGF-β) signaling cascades.[13,14]

Noteworthy, microRNAs (miRNAs) are small non-coding RNAs: approximately 18-23 nucleotides that can post-transcriptionally regulate the expression of their cleaved messenger RNAs (mRNAs). Hundreds of miRNA genes have been identified in various animals and many of phylogenetically conserved species.[15] In mammalian cells, miRNAs are upregulated upon several microbial infections to modulate a variety of intracellular signaling. Other cumulative evidence indicates that some endogenous miRNAs are able to interact as a tumor suppressor or oncogenes during cancer development. For instance, miR-21 was constantly upregulated in gastric cells caused by Helicobacter pylori infection, leading to CRC disorder.[16] The direct targets of miR-21 include tumor suppressor protein tropomyosin 1 and metastasis suppressor reversion-inducing-cysteine-rich protein.[17] Therefore, in this study, we sought to investigate the expression levels of hsa-miR-21 in Egyptian CRC patients in parallel with the expression profile of tumor suppressor PTEN gene. We also exploited prediction tools to further confirm the direct cleavage between hsa-miR-21 and cellular tumor suppressor genes and anti-inflammatory cytokines. This was done to claim the essential role of miR-21 in the regulation of programmed cell death and inflammatory events.

Materials and Methods

Samples' conditions

We collected a total of 40 blood samples from CRC patients (25 males and 15 females) and 40 blood samples from healthy individuals who served as controls (25 males and 15 females). Blood sample collection from CRC patients was done at the outpatient's clinic of the general surgery department and oncology department, Menofyia University, Egypt. We obtained the approval of the ethics committee and patients consent from December 2016 to February 2018. CRC was diagnosed according to clinical examination, laboratory investigation, ultrasound assay, computed tomography that associated with age,gender, smoking condition, and tumor, node, and metastasis (TNM) staging.[18]

Total RNA extraction and cDNA synthesis

Total RNA was extracted from a fresh blood sample using PureLink® RNA Mini Kit (Ambion, Life Technology, USA) and QIAzol (Ambion, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a high capacity cDNA reverse transcriptase kit according to the manufacturer's instructions (Applied Biosystems, USA). We synthesized cDNA using 1 µg of total RNA that was genteelly mixed with 2 µl reaction buffer, 1 µl free

Table 1. Oligonucleotides sequences used for detecting steady state mRNA for the indicated genes

Description	Primer sequences 5'-3'
PTEN-sense	ACCAGGACCAGAGGAAACCT
PTEN-antisense	GCTAGCCTCTGGATTTGACG
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-antisense	TGGATGCAGGGATGATGTTCT
MiR-21-sense	AGAAATGCCTGGGTTTTTTTGGTT
MiR-21-antisense	TTGGGAATGCTTTTCAAAGAAGGT
MiR-16-sense	GCGGCGGTAGCAGCACGTAAAT
MiR-16-antisense	ATCCAGTGCAGGGTCCGAGG

nucleotides (dNTPs), 1 µl oligo (dT) primer, 1 µl RNase inhibitor, and one unit of reverse transcriptase in a total volume of 25 µl. The programming of the thermal cycler condition was heating at 55 °C for 10 min, 37 °C for 2 hours, and 85 °C for 5 min to stop the reaction.[19]

Quantitative real-time PCR (qRT-PCR) assay

We detected PTEN relative gene expression by use of the Universal TaqMan master mix (Applied Biosystems, Thermo Fisher scientific, USA) according to the manufacture's protocol.

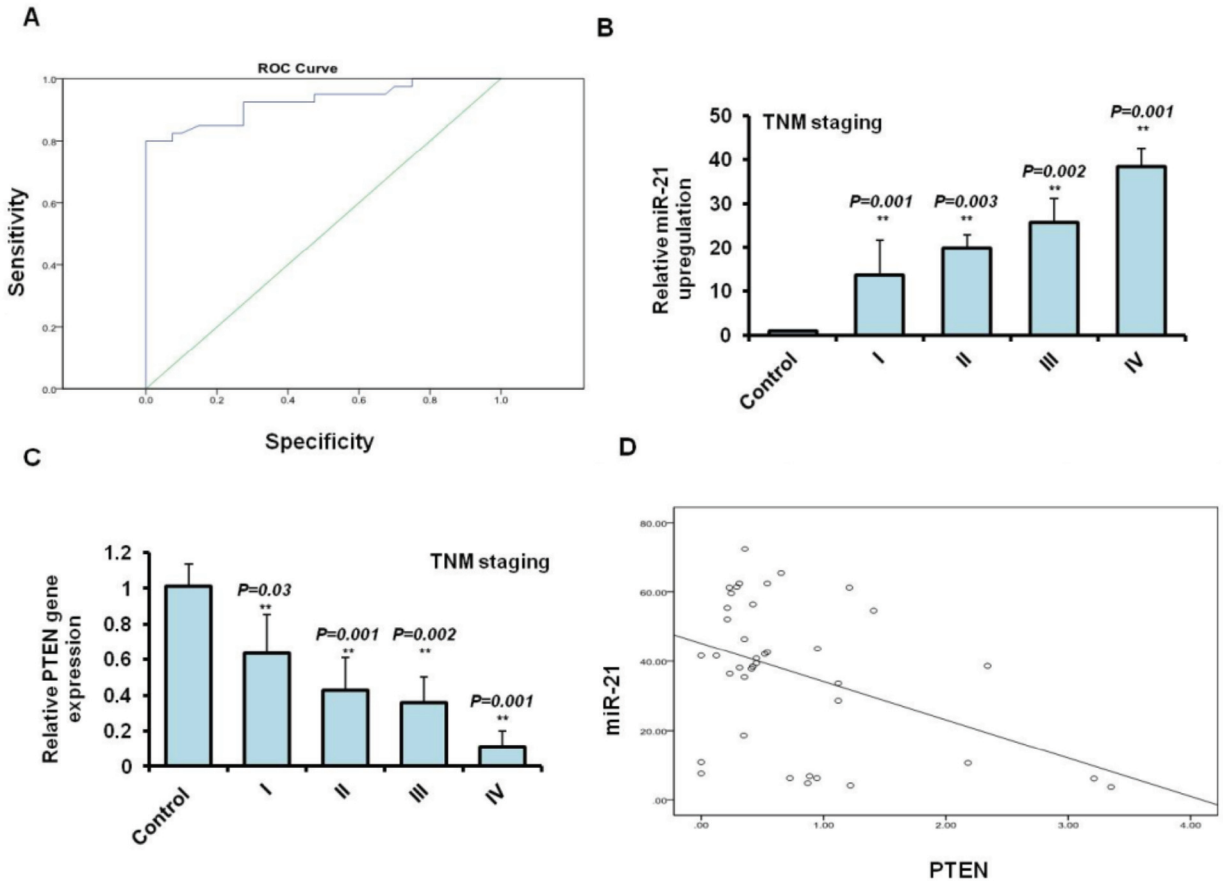


Figure 1. Expression levels of hsa-miR-21 and PTEN gene in obtained blood samples.; (A) ROC curve analysis of hsa-miR-21 indicated by qRT-PCR. (B) The mean expression levels of hsa-miR-21in all CRC obtained blood-samples indicated by qRT-PCR in comparison with control samples. (C) The relative expression levels of PTEN gene in patients with CRC compared with healthy individuals. (D) The represented relationship between hsa-miR-21 expression and PTEN gene expression in each obtained sample. Error bars in qRT-PCR analysis indicate the standard deviation (SD) between collected samples. Student two tailed t-test was performed to obtain significance presented by (*). $P \leq 0.01$ is **, $P \leq 0.05$ is *; CRC: Colorectal cancer.

Table 2. The mean fold changes in miR-21 expression on the samples obtained from CRC patients in comparison with healthy individuals

Studying Conditions	No.	Mi-21 expression Mean fold changes	Standard deviation	Student two tails t-test	*P*-values
Age					
Healthy individuals					
(20-45)	20	1.03	0.17		
(46-80)	20	1.13	0.21		
Patients					
(20-45)	23	19.87	9.5	0.002	≤ 0.01**
(46-80)	17	24.22	9.5	0.001	≤ 0.01**
Gender					
Healthy individuals					
Male	25	1.03	0.21		
Female	15	1.14	0.18		
Patients					
Male	25	20.9	9.2	0.001	≤ 0.01**
Female	15	24.08	10.5	0.002	≤ 0.01**
Smoking					
Healthy individuals					
Nonsmokers	25	1.05	0.21		
Smokers	15	1.11	0.21		
Patients					
Nonsmokers	25	21.76	9.90	0.003	≤ 0.01**
Smokers	15	21.58	9.91	0.003	≤ 0.01**
TNM					
Healthy individuals	40	0.96	0.10		
Patients					
I	12	13.6	8.01	0.002	≤ 0.01**
II	12	19.8	3.10	0.003	≤ 0.01**
III	11	25.76	5.38	0.001	≤ 0.01**
IV	5	38.35	4.20	0.001	≤ 0.01**

**$P \leq 0.01$; TNM: Tumor, node, and metastasis; CRC: Colorectal cancer.

The cycling conditions used for the amplification of the PTEN gene were initial denaturation step at 95º C for 10 min, followed by 40 cycles of 95º C for 15s and 60 º C for 60s. TaqMan Germany provided the primers specific for PTEN and housekeeping GAPDH genes (Table 1).[20]

MiRNA extraction and cDNA synthesis

We extracted MiRNAs from a fresh blood samples using a miRNA extraction kit and QIAzol (Qiagen, USA) according to the manufacturer's instructions. The cDNAs were generated via TaqMan® MicroRNA reverse transcription Kit (Applied Biosystems, Foster City, CA) through mixing 5 µl total miRNAs, 3 µl RT Primer, 1.5 µl 10X RT Buffer, 0.5 µl RNase inhibitor, 0.5 µl dNTP mix, 1 µl MultiScribe™ reverse transcriptase, and 8.5 µl nuclease-free water. The programming of the thermal cycler condition was: holding for 30 min at 16°C and 30 min at 42°C, then heating for 5 min at 85°C to stop the reaction.

qRT-PCR assay for miRNA-21

We showed the levels of miR-21 in the derived samples by TaqMan miRNAs assay (Applied Biosystems, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The parameters of qRT-PCR were performed as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 60s. Table 1 describes the oligoneulcotids

sequences specific for miRNA-21 and miRNA-16 (internal miRNA control).

Prediction tools

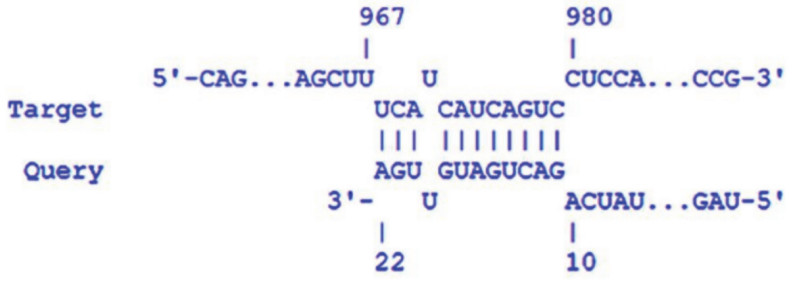
We employed IntaRNA software to test the direct interaction and potential binding site of miR-21 and PTEN genes sequences.[21] PicTare website was used for monitoring and screening the potential targeted genes by miR-21 indicated by the affinity of the binding site and PicTar score at the lowest free energy.[22]

Statistical analysis

Student two-tail t-test determined the significance of miR-21 levels and PTEN relative gene expression were indicated by qRT-PCR. SDS 2-2.2 software was used to analyze the Ct values of the qRT-PCR and to derive the relative miR-21 up-regulation and relative PTEN gene expression using ΔΔCt equations.[23,24,25]

Results

Upregulation of miR-21 in CRC patients is in line with the depletion of PTEN gene expression. Blood samples were collected from CRC patients (n=40) and healthy individuals (n=40) who served as controls. We divided the samples obtained from CRC patients into four groups according to aging condition, gender, smoking case, and TNM staging. The relative expression of both miR-21 and PTEN gene in the derived blood samples was assessed using qRT-PCR with a satisfactory, sensitive, and specific assay as indicated by the ROC curve (Figure 1A). Based on the aging condition, the mean levels of miR-21 significantly increased in all patients' samples (Approximately 20 fold changes) in comparison with healthy individuals; however, aged patients showed the highest levels of miR-21 compared with middle-aged patients (24 and 19 fold changes, respectively). Similarly, females with CRC showed higher miR-21 expressions than male patients (24- and 20-fold changes, respectively). Smoker and non-smoker patients showed negligible differences in terms of miR-21 expression, which increased in both samples by more than 20 folds compared with healthy controls (Table 2). Based on TNM staging, miR-21 gradually increased at the start of CRC development with 13-fold changes during the initial stage of TNM; in the advanced stages of TNM, an almost 40-fold increase was seen (Figure 1B and Table 2). In contrast, the relative gene expression of PTEN was significantly depleted in all patient-derived samples compared with healthy individual samples (Table 3). The lowest expression of PTEN gene belonged to females and aged CRC patients as compared with males and younger patients. Smoking and non-smoking patients showed the same PTEN gene expression level, which was significantly depleted in comparison with healthy controls (Table 3).



Target	Position	Query	Position	Energy
PTEN gene	968 -- 979	hsa-mir-21	11 -- 22	-12.10110

Figure 2. The direct binding site between hsa-miR-21 and PTEN gene sequences. The seeding region of miR-21 presented on PTEN coding sequences is indicated by IntaRNA software which reveals the location of the binding site on both query (miR-21) and target (PTEN) and the lost free energy.

Table 3. The mean fold changes in PTEN gene expression on the samples of CRC patients in comparison with healthy individuals' samples

Studying Conditions	No.	PTEN expression mean fold changes	Standard deviation	Student two tails t-test	*P*-values
Age					
Healthy individuals					
(20-45)	20	1.16	0.22		
(46-80)	20	1.10	0.22		
Patients					
(20-45)	23	0.66	0.65	0.02	≤ 0.05*
(46-80)	17	0.58	0.52	0.001	≤ 0.01**
Gender					
Healthy individuals					
Male	25	1.06	0.27		
Female	15	1.20	0.26		
Patients					
Male	25	0.69	0.6	0.01	≤ 0.01**
Female	15	0.54	0.3	0.002	≤ 0.05*
Smoking					
Healthy individuals					
Nonsmokers	25	1.21	0.21		
Smokers	15	1.15	0.23	0.6	≤ 0.05
Patients					
Nonsmokers	25	0.62	0.44	0.003	≤ 0.01**
Smokers	15	0.61	0.84	0.14	≤ 0.05*
TNM					
Healthy individuals	40	0.01	0.12		
Patients					
I	12	0.68	0.89	0.05	≤ 0.05*
II	12	0.57	0.33	0.01	≤ 0.01**
III	11	0.43	0.16	0.003	≤ 0.01**
IV	5	0.37	0.31	0.005	≤ 0.01**

* $P \leq 0.05$; ** $P \leq 0.01$; TNM: Tumor, node, and metastasis; CRC: Colorectal cancer

During TNM staging, the relative expression of the PTEN gradually decreased in CRC patients compared with healthy individuals (Figure 1C and Table 3). Collectively, as indicated in figure 1D, the qRT-PCR analyses revealed a contradictory relationship between miR-21 and PTEN gene expression profile in CRC patients. Together, these data showed that miR-21 was markedly upregulated in CRC patients in association with the low expression profile of PTEN gene. This reveals the possible regulation of PTEN gene expression, as a tumor suppressor gene, via miR-21 upregulation.

MiR-21 potentially regulates PTEN gene expression. To determine whether miR-21 could interfere with PTEN messenger RNA (mRNA), we utilized IntaRNA software to detect the possible binding site and the direct interaction between miR-21 and PTEN gene. Docking interaction in figure 2 indicates the binding site of miR-21(11-22nt) on PTEN-mRNA at 967-980nt with a low energy of -12.1 kcal/mol. This predicted binding site between miR-21 and PTEN-mRNA indicates the possible interaction and regulation of PTEN gene expression as a result of miR-21 upregulation.

MiR-21 targets tumor suppressor factors to prevent programmed cell death in cancer

To further investigate the other potential targets

Table 4. Potential targeted genes and seeding regions for hsa-miR-21 indicated by PicTar software

Gene category	PicTare Score	Probability	Seeding region	Free energies kcal/mol
STAT3	4.05	0.91-0.91	2056, 2386	-21.8, -17.6
PPARA	3.5	0.73-0.73	995, 1181	-20.9 , -18.01
TGF-β	2.9	0.94	461	-20
TNF	2.5	0.9	475	-16.2
CDC25A	2.1	0.86	868	-19.16
PDCD4	1.55	0.73	399	-17.8

STAT3: Signal transducer and activator of transcription 3; PPARA: Peroxisome proliferator-activated receptor alpha; TGF-β: Transforming growth factor beta; TNF: Tumor necrosis factor; CDC25A: Cell division cycle 25 A; PDCD4: Programmed cell death protein.

of miR-21, we employed PicTar software based on the sequences of homosapiens-miR-21(hsa-miR-21). Our investigation showed a variety of targeted genes at low energy and high PicTar score, including tumor suppressor genes and programmed cell death factors. We detected two seeding regions in the signal transducer and activator of transcription 3 (STAT3) at 2056 and 2386 with the highest PicTar score (4.04) and the lowest free energy observed in both locations (-21.8 and 17.6 kcal/mol, respectively) (Figure 3A and Table 4). The other two binding sites were recognized on the proliferator-activated receptor alpha (PPARA) at 995 and 1181 with free energies of -20.9 and -18.01 kcal/mol, respectively, and a PicTar score of 3.5 (Figure 3B and Table 4). We detected a binding site at the location 461 of TGF-β with a PicTar score of 2.9 and a free energy of -20 kcal/mol (Figure 3C and Table 4). TNF-α showed a binding site with miR-21 at

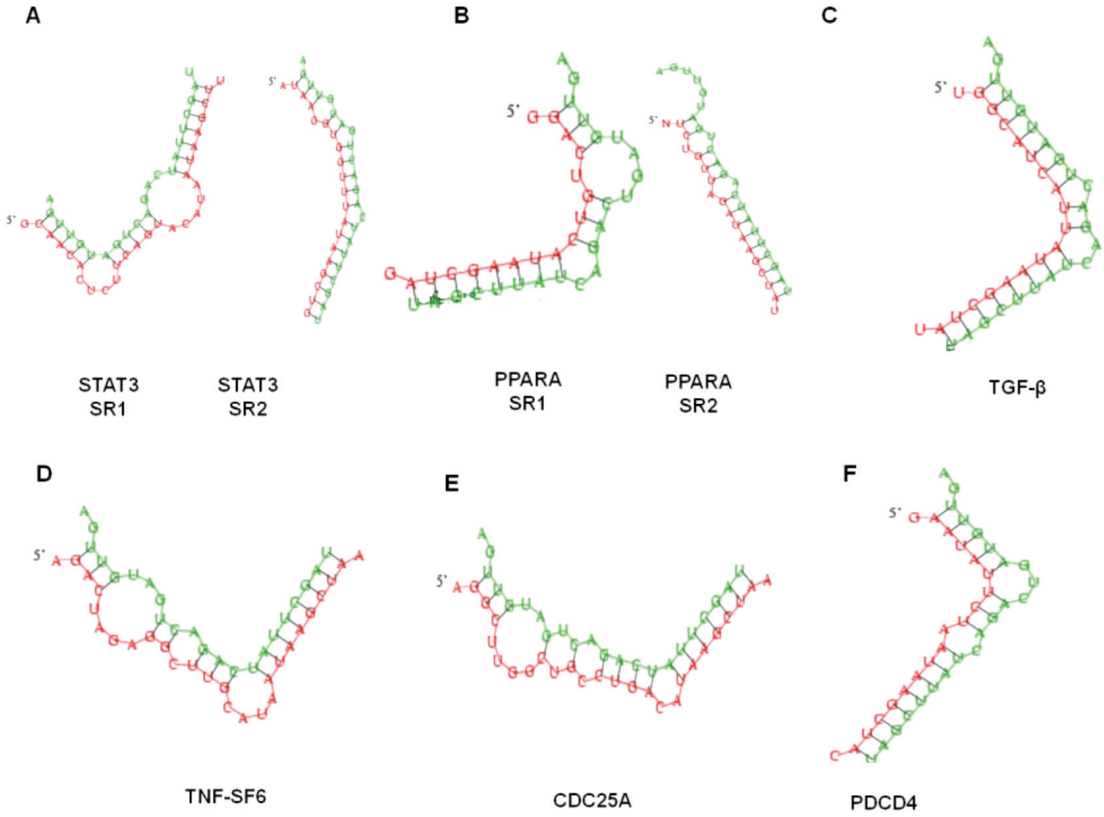


Figure 3. Predicted genes potentially targeted by hsa-mi-R21. The potential seeding regions and binding affinity of hsa-miR-21 on targeted genes carried out by PicTar software; (A) Two seeding regions detected on STAT3 sequences (SR1 and SR2). (B) Two detected SR on PPARA gene sequences. (C) The indicated binding site of miR-21 on TGF-β. (D) TNF-SF6 binding affinity with miR-21. (E) CDC25A-miR-21 potential binding site. (F) PDCD4-miR-21 indicated seeding regions.

STAT3: Signal transducer and activator of transcription 3; PPARA: Peroxisome proliferator-activated receptor alpha ; TGF-β: Transforming growth factor beta; TNF: Tumor necrosis factor; CDC25A: Cell division cycle 25 A; PDCD4: Programmed cell death protein 4

475 with a PicTar score of 2.5 and a free energy of -16.2 kcal/mol (Figure 3D and Table 4). Furthermore, cell division cycle 25 A (CDC25A) revealed one binding site at 868 with a PicTar score of 2.1 and a free energy of -19.6 kcal/mol (Figure 3E and Table 4). Finally, programmed cell death 4 (PDCD4) showed a binding site at 399 with a PicTar score of 1.5 and a free energy of -17.8 kcal/mol (Figure 3F and Table 4). Collectively, these data revealed the crucial impact of hsa-miR-21 on the regulation of programmed cell death and identified hsa-miR-21 as an attractive candidate for cancer treatment, particularly CRC.

Discussion

In the current work, we investigated the expression levels of hsa-miR-21 and PTEN gene expression in patients with CRC and compared them with healthy individuals using qRT-PCR. The expression levels of both miR-21 and PTEN gene correlated with smoking, aging, gender, and TNM staging. IntaRNA-RNA interaction software indicated the direct interference between miR-21 and PTEN gene, revealing the seeding region and binding affinity of both interacted RNAs. Additionally, using online prediction tools, we identified the putative binding sites of miR-21in a variety of tumor metastasis factors and tumor suppression genes. Interestingly, our findings showed that miR-21 was significantly upregulated in all CRC-obtained samples; meanwhile, the highest levels of miR-21 belonged to females and older patients at the late stage of CRC. On the contrary, the relative expression of the PTEN gene significantly decreased in the samples obtained from patients compared with healthy individual samples. Similar to miR-21, the lowest expression of PTEN gene was related to aged and female patients at late TNM staging. IntaRNA software identified a putative binding site in the 3- untranslated region of the PTEN gene,

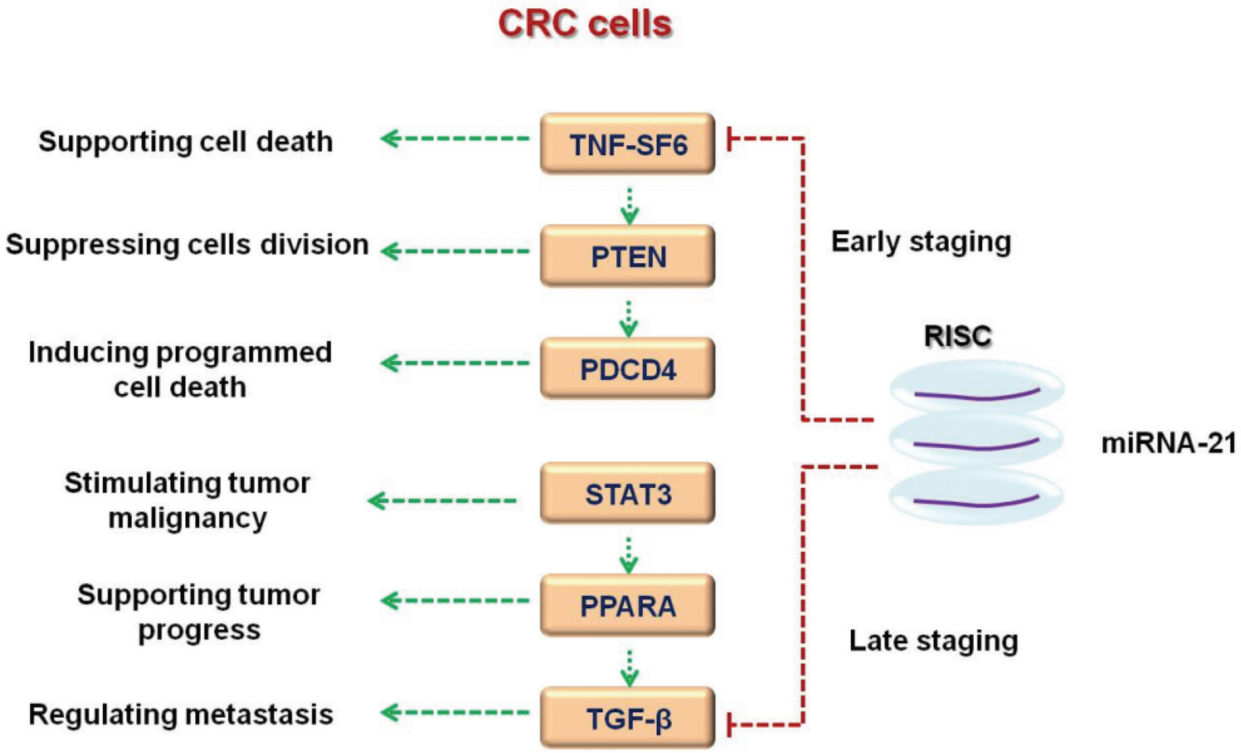


Figure 4. Graphic representation of hsa-miR-21 and its potential targeted genes.The cooperation between miR-21 and RISC in CRC cells through which, the up-regulation of miR-21 can regulate the expression of STAT3 and its associated factors PPARA and TGF-β. To prevent cell death, miR-21 potentially targets TNF-SF6, PTEN, and PDCD4 as predicted by PicTar software.

STAT3: Signal transducer and activator of transcription 3; PPARA: Peroxisome proliferator-activated receptor alpha; TGF-β: Transforming growth factor beta; TNF: Tumor necrosis factor; CDC25A: Cell division cycle 25 A; PDCD4: Programmed cell death protein 4

indicating the direct cleavage of the PTEN gene by miR-21. The PicTar prediction analysis showed multiple genes with binding sites of miR-21, including tumor metastasis factors and tumor suppressor genes. Our data suggested that hsa-miR-21 expression was upregulated in CRC patients according to patients' age, gender, and TNM staging. Furthermore, our data indicated the dual function of miR-21 during CRC evolution through targeting tumor suppressor genes at early initiation and regulating metastasis factors at late TNM staging. The biogenesis pathway of miRNAs includes the incorporation of mature miRNA with RNA induced silencing complex (RISC), where miRNA can post-transcriptionally regulate targeted gene expression. Upregulation of miR-21 in CRC-obtained samples may result in a direct interaction with so many genes, including STAT3, PPARA, TGF-α, and PDCD4 as predicted in our study (Figure 4). Importantly, STAT3 belongs to STAT protein family and is implicated in cancer development through its regulatory role in cell proliferation, malignancy transformation, tumor angiogenesis, and metastasis.[26] Activation of STAT3 signaling was reported to stimulate cellular miR-21during oocyte maturation and human cancer, indicating the potential connection between STAT3 pathway and upregulation of mature miR-21 in CRC.[27,28] The possible dual function of hsa-miR-21 was primarily observed in breast cancer evolution through targeting and regulating STAT3 protein expression.[29] Likewise, the presented prediction data confirmed that a STAT3 gene sequence has two putative binding sites with miR-21. Furthermore, our prediction analysis revealed that tumor metastasis factors PPARA and TGF-β have unique seeding regions for miR-21. In contrast, our findings showed that the programmed cell death factors TFN-SF6, PTEN, and PDCD4 have potential binding sites with miR-21. This corroborates the well-known function of miR-21 in the regulation of cell death during cancer evolution. Taken together, our data showed the over-expressed levels of hsa-miR-21 in Egyptian CRC patients based on their age, TNM stage, and gender. Moreover, miR-21 had a dual function during cancer development and could be considered as an efficacious candidate in cancer diagnostic and therapy during initial stages.

Conflict of Interest

None declared.

References

1. Durko L, Malecka-Panas E. Lifestyle modifications and colorectal cancer. *Curr Colorectal Cancer Rep*. 2014;10:45-54.
2. Simonian M, Khosravi S, Mortazavi D, Bagheri H, Salehi R, Hassanzadeh A, et al. Environmental risk factors associated with sporadic colorectal cancer in Isfahan, Iran. *Middle East J Cancer*. 2018:9(4):318-22.
3. Gandomani H, Yousefi SM, Aghajani M, Mohammadian-Hafshejani A, Tarazoj A, Pouyesh V, et al. Colorectal cancer in the world: incidence, mortality and risk factors. *Biomed Res Ther.* 2017,4(10):1656-75.
4. Lorestani S, Hashemy SI, Mojarad M, Keyvanloo Shahrestanaki M, Bahari A, Asadi M, et al. Increased glutathione reductase expression and activity in colorectal cancer tissue samples: An investigational study in Mashhad, Iran. *Middle East J Cancer.* 2018;9(2):99-104.
5. Ibrahim AS, Khaled HM, Mikhail NN, Baraka H, Kamel H. Cancer incidence in Egypt: results of the national population-based cancer registry program. *J Cancer Epidemiol.* 2014;2014:437971.
6. Kim ER, Chang DK. Colorectal cancer in inflammatory bowel disease: the risk, pathogenesis, prevention and diagnosis. *World J Gastroenterol.* 2014;20(29):9872-81. doi: 10.3748/wjg.v20.i29.9872.
7. Stracci F, Zorzi M, Grazzini G. Colorectal cancer screening: Tests, strategies, and perspectives. *Front Public Heal*. 2014;2: 210.
8. Zygulska AL, Furgala A, Krzemieniecki K, Wlodarczyk B,Thor P. Autonomic dysregulation in colon cancer patients. *Cancer Invest.* 2018;36:255–63.
9. Islam SMA, Patel R, Acevedo-Duncan M. Protein Kinase C-ζ stimulates colorectal cancer cell carcinogenesis via PKC-ζ/Rac1/Pak1/β-Catenin signaling cascade. *Biochim Biophys Acta Mol Cell Res.* 2018;1865(4):650-64. doi:10.1016/j.bbamcr.2018.02.002.
10. Asho KP, Thomas D, Kalaviani K, Vadivel D, Bakthavatchalam V, Ganapasam S. Colorectal carcinogenesis: Insights into the cell death and signal transduction pathways: A review. *World J Gastrointest Oncol.* 2018;10(9):244-59.
11. Buhmeida A, Assidi M, Al-Maghrabi J, Dallol A,

Sibiany A, Al-Ahwal M, et al. Membranous or cytoplasmic HER2 expression in colorectal carcinoma: evaluation of prognostic value using both IHC & BDISH. *Cancer Invest*. 2018;36(2):129-40. doi:10.1080/07357907.2018.1439054.
12. Maksoud AI, Taher RF, Gaara AH, Abdelrazik E, Gaara AH, Abdelrazi KE, et al. Selective regulation of B- Raf dependent K-Ras/mitogen-activated protein by natural occurring multi-kinase inhibitors in cancer cells. *Front Oncol.* 2019; 01220. doi: 10.3389/fonc.2019.01220.
13. Mehlen P, Fearon ER. Role of the dependence receptor DCC in colorectal cancer pathogenesis. *J Clin Oncol.* 2004;22(16):3420-8.
14. Grady WM. Transforming growth factor -β signaling in colorectal cancer. *Curr Colorectal Cancer Rep.* 2007;3:65-70.
15. Kaufman EJ, Miska EA. The microRNAs of *Caenorhabditis elegans*. *Semin Cell Dev Biol.* 2010;21(7):728-37. doi: 10.1016/j.semcdb.2010.07.001.
16. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006;103(7):2257-61.
17. Zhu S, Wu H, Wu F, Nile D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res*. 2008;18(3),350-9.
18. Khalil H, Abd El Maksoud AI, Alian A, El-Hamady WA, Daif AA, Awad AM, et al. Interruption of autophagosome formation in cardiovascular disease, an evidence for protective response of autophagy. *Immunol Invest.* 2019:1-15.doi: 10.1080/08820139.2019.1635619.
19. Khalil H, Arfa M, El-Masrey S, El-Sherbini SM, Abd-Elaziz AA. Single nucleotide polymorphisms of interleukins associated with hepatitis C virus infection in Egypt. *J Infect Dev Ctries.* 2017;11(3):261-8. doi:10.3855/jidc.8127.
20. Khalil H, Abd El Maksoud AI, Roshdey T, El-Masry S. Guava flavonoid glycosides prevent influenza A virus infection via rescue of P53 activity. *J Med Virol.* 2019;91(1):45-55. doi: 10.1002/jmv.25295.
21. Farghaly H, Guirgis A, Khalil H. Heat shock reduces HCV replication via regulation of ribosomal L22 in Alu-RNA molecule dependent manner. *Hepatoma Res.* 2018;4:41.
22. Salehi Z, Akrami H, Sisakhtnezhad S. The effect of placenta growth factor knockdown on hsa-miR-22-3p, hsa-let-7b-3p, hsa-miR-451b, and hsa-mir-4290 expressions in MKN-45- derived gastric cancer stem-like cells. *Middle East J Cancer.* 2018; 9:113-22.
23. Khalil H. Influenza A virus stimulates autophagy to undermine host cell IFN-β production. *Egypt J Biochem Mol Biol*. 2012;30: 283-99.
24. Khalil H, El Malah T, El Maksoud AIA, El Halfawy I, El Rashedy AA, El Hefnawy M. Identification of novel and efficacious chemical compounds that disturb influenza a virus entry in vitro. *Front Cell Infect Microbiol*. 2017;7:304.doi: 10.3389/fcimb.2017.00304.
25. Abd El Maksoud AI, Elebedeey D, Abass NH, Awad AM, Naser GM, Roshdy T, et al. Methylomic changes of autophagy-related genes by Legionella effector Lpg2936 in infected macrophages. *Front Cell Dev Biol*. 2019; 7(390):1-16. doi: 10.3389/fcell.2019.00390.
26. Levy DE, Lee CK. What does Stat3 do? *J Clin Invest*. 2002;109(9):1143-8.
27. Tscherner A, Brown AC, Stalker L, Kao J, Dufort I, Sirard MA, et al. STAT3 signaling stimulates miR-21 expression in bovine cumulus cells during in vitro oocyte maturation. *Sci Rep*. 2018;8(1):11527. doi:10.1038/s41598-018-29874-w.
28. Cao Q, Li YY, He WF, Zhang ZZ, Zhou Q, Liu X, et al. Interplay between microRNAs and the STAT3 signaling pathway in human cancers. *Physiol Genomics*. 2013;45(24):1206-14. doi: 10.1152/physiolgenomics.00122.2013.
29. Zhang C, Liu K, Li T, Fang J, Ding Y, Sun L, et al. miR-21: A gene of dual regulation in breast cancer. *Int J Oncol*. 2016;48(1):161-72.