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Clinical Significance of Mir-221 in Colorectal Cancer

Amal F. Gharib[1,2], Abdel Rhman Nasser Alghamdi[3], Mosfer Nasser Alghamdi[4], Ahmed El Askary[2,5] and Wael H. Elsawy[6*]

[1]Department of Medical Biochemistry, Faculty of Medicine, Zagazig University, Egypt.
[2]Department of Clinical Laboratory, College of Applied Medical Science, Taif University, KSA.
[3]Department of Family Medicine, College of Medicine, Taif University, KSA.
[4]Department of Gastro and Hepatology, Alhada Armed Forces Hospital, Taif, KSA.
[5]Department of Medical Biochemistry, Faculty of Medicine (New Damietta), Al Azhar University, Egypt.
[6]Department of Clinical Oncology and Nuclear Medicine, Faculty of Medicine, Zagazig University Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. The Initial manuscript was prepared by author WHE, the study was conceptualized and designed by authors AFG, ARNA and AEA. The final data preparation was done by authors AFG, WHE and MNA and the statistical analysis completed by author WHE and final data analysis and interpretation finally assembled by authors WHE, AFG, AEA. All the contributing authors have taken part in manuscript editing and review.

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ABSTRACT

Aim: MicroRNA-221 plays an important role in a number of human malignancies. The present study was conducted to shed light on the role of mir-221in colorectal cancer as regards the initiation of malignant process and progression.
Methods: Real-time reverse transcription–polymerase chain reaction was used to determine the levels of mir-221 in 92 patients with colorectal cancer and in their adjacent non-cancerous tissues and to explore the relation between mir-221 level and clinical and pathological features of the disease.

**Corresponding author: E-mail: whelsawy@gmail.com;*

Results: Mir-221 was up-regulated in 90.2% (83/92) of colorectal cancer tissue samples compared with their adjacent non-cancerous tissue samples. The high expression of mir-221 was significantly correlated to tumor size and infiltration, clinical stage and lymph node metastasis. Univariate and multivariate analyses showed that over expression of mir-221 was an unfavorable prognostic indicator for overall survival in colorectal cancer patients.
Conclusion: Our findings suggest mir-221 may be used as a prognostic marker in CRC and it needs more studies to be used as a potential diagnostic marker and as a molecular target for targeted cancer therapy.

Keywords: Colorectal cancer; mir-221; RT-PCR.

1. INTRODUCTION

Colorectal cancer (CRC) is the third prevalent cancer type worldwide and the main cause of mortality from malignant tumors in both sexes [1,2]. In spite of the fact that CRC incidence has been decreasing due to decreased exposure to social risk factors, the five years survival rate is unsatisfactory 64.9% necessitating the need for early detection [3,4].

MiRNA is a kind of endogenous small RNA with a length of around 25 nucleotides, which plays various important roles in early development, fat metabolism, cell differentiation and other biological activities [5]. The expression of miRNAs is closely related to the occurrence and development of human malignancies. miRNAs participate in the proliferation, apoptosis, and invasion of tumor cells either as anti-oncogenes or oncogenes [6].

MicroRNA-221 (miR-221), encoded on human chromosome X, is overexpressed in many aggressive carcinomas [7-9]. Abnormal overexpression of miR-221 strongly facilitates tumor cell growth by inducing cell lines in vitro to progress into the S phase of the cell cycle [10].

Several studies have discovered that miR-221 is significantly up-regulated in cell lines [11], plasma or serum [12-15], and tissues of many human malignancies [16-20].

Elevated expression of miR-221 in many types of carcinomas is obviously related to an easier tumor invasion [10,17,21-23], a larger size of tumor [13,17,23] earlier distant metastasis [17,22,24], and shorter time to recurrence and shorter survival [24-26].

Studies have revealed a close association between the level of miR-221 expression and clinic pathological tumor features including tumor–node–metastasis (TNM) stage, local invasion, metastasis, prognosis, radiosensitivity and anticancer drug resistance [22,27] but the role of miR-221 in colorectal progression and metastasis is still unclear.

The current study was designed to compare miR-221 expression in colorectal cancer tissues and adjacent normal tissues, to study the prognostic significance of miR-221expression and its relation to the clinic pathological characteristics of the tumors.

2. PATIENTS AND METHODS

Tissue samples from primary colorectal cancer and adjacent non-cancerous tissue at least 3 cm from tumorous tissue were obtained surgically from 92 patients with histo-pathologically proven colorectal cancer, who underwent curative surgical resections at colorectal cancer unit, Faculty of Medicine, Zagazig University between June 2012 and September 2017. All patients with previous malignancy or with previous chemotherapy, immunotherapy or radiation therapy were excluded from the study.

After surgery tissues were flash-frozen and stored at -80°C in liquid nitrogen. Patients were staged according to the American Joint Committee on Cancer (8^{th} edition) [28]. The study was approved with the medical ethics committee of our institution. Informed consent was obtained from all individual participants included in the study.

2.1 RNA Extraction and Quantitative Real-Time RT–PCR

TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions, to isolate RNA from 20-30 mg of colorectal samples and their adjacent non-cancerous normal tissues. The RNA quantity and quality was assessed by Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

TaqMan® miRNA (Applied Biosystems, Foster City, CA, USA) and specific gene primers were used to synthesize the complementary DNA of MiR-221 and RNA U6 (internal control) from total RNA.

Reverse transcription (RT) primer sequences (Invitrogen) were 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACGAAACCCA-3′ (for miR-221) and 5′-CGTTCACGAATTTGCGTGTCAT-3′ (for RNA U6). The reaction mixture of RT consisted of 50 nmol / l RT primer, 1 × RT buffer, 3.33 U/µl MultiScribe ® reverse transcriptase, 10 ng total RNA, and 0.25 mmol / l of deoxynucleotide triphosphate and 0.25 U/µl RNase inhibitor in a total volume of 7.5 µl (Purchased from Applied Biosystems). Reactions were incubated in a 96-well plate for 30 min at 16°C, followed by 30 min at 42°C and 5 min at 85°C, followed by 4°C.

Reactions were carried out in 96-well plates in a total volume of 10 µl (0.67 µl RT products, 1 × TaqMan® Universal PCR master mix, and 1 µl TaqMan® miRNA assay primer and probe mix). Real-time polymerase chain reaction (PCR) was performed using the 7500 real-time PCR system (Applied Biosystems).

The program of cycling consists of preliminary denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and elongation at 70°C for 30 s, followed by a final elongation step at 60°C for 10 min.

All samples were processed in triplicate. When the fluorescence passed a fixed threshold, this cycle number is defined as the threshold cycle (CT). The relative amount of miR-221 to U6 was calculated using equation $2^{-\Delta CT}$,

where, $\Delta CT = (C_{T\ miR\text{-}221} - C_{T\ U6})$. [29]

2.2 Statistical Analysis

Statistical analysis was performed with SPSS® version 23.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Data were expressed as mean ± SD. Differences in expression levels of miR-221 between groups were compared using Student's *t*-test. Kaplan–Meier curves were used to calculate the survival of the patients and the curves were compared by the log-rank test. The Cox proportional hazards regression model was used to examine the joint effect of co-variables.

3. RESULTS

Tissues samples obtained by surgery from 92 patients with colorectal cancers and another sample obtained from normal tissues adjacent to the tumor and located at least 3-5 cm from the tumor were obtained for each patient. Our study consisted of 49 Males and 43 females with a mean age ± SD (52.5 ± 6.4). MiR-221 was up-regulated in 90.2% (83/92) of colorectal cancer tissue samples compared with their adjacent non- cancerous tissue samples. The mean Δ*C*T value of mir-221 ± SD in colorectal cancer samples was 2.46± 0.32, compared with 0.86 ± 0.14 in their adjacent non-cancerous tissue samples ($P < .001$). The correlations between the clinical and pathological data of patients and miR-221 expression levels (Table 1).

Advanced Clinical stage III ($P = .018$), invasion of the tumor (T) ($P = .006$) and lymph nodes metastasis ($P = .032$), all showed significant correlation with high mir-221 expression. Meanwhile, no significant correlation was noticed between age, gender, and degree of tumor differentiation and level of mir-221 expression.

At the time of survival analysis (March 2019), fifteen patients out of ninety-two (16.3%) are still alive. The range of follow-up period was (11- 65 months) and the median duration of follow-up was 42 months. During follow-up distant metastasis was observed 52 patients as the first recurrence, 16 patients had a local recurrence, while both distant metastasis and local recurrence was noted in 4 patients as the first relapse.

According to the mean level of mir-221 (2.46 Δ*C*T), patients were divided into patients with low mir-221 expression (n=44) and those with high expression (n = 48). The survival between low and high expression groups is shown in Fig. 1. The 4-year survival rates were 44% in patients with low expression and 35% in those with high expression with corresponding median survival times of 41 and 35 months respectively ($P = 0.016$).

The univariate analysis revealed that patients the female gender having worse prognosis; (hazard ratio [HR] 0.435; P = 0.23), high-level miR-221 expression (HR 2.422; $P = 0.016$), tumor size and infiltration (T) (HR 5.652; $P < 0.001$), tumor stage (TNM) (HR = 3.462; $P = 0.008$), lymph node metastasis (HR 2.956; $P = 0.006$) and

degree of tumor differentiation (HR 5.165; *P* = 0.011) all were predictive factors for prognosis in patients with colorectal cancer (Table 2).

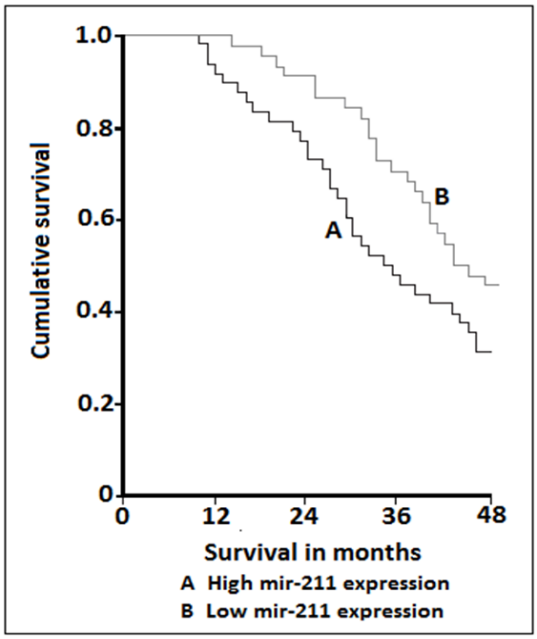

Fig. 1. Kaplan–Meier survival curve for patients with colorectal cancer; the 4-year survival rate and the median survival time were significantly lower in patients with high miR-221 expression compared with patients with low miR-221 expression (*P* = 0.016, both comparisons; log-rank test)

Multivariate Cox regression analyses revealed that the high level of mir-221 expression was the only unfavorable factor, independent of other factors as tumor size and infiltration (T) (HR 4.268; *P* < 0.001), tumor stage (TNM) (HR 3.225; *P* = 0.016), lymph node metastasis (HR 2.685; *P* = 0.026) and degree of tumor differentiation (HR 3.678; *P* = 0.032) (Table 3).

4. DISCUSSION

Colorectal cancer (CRC) is one of the most common cancers worldwide, accounting for 10% of all new cancer cases and over 1.23 million deaths per year [1].

Early-stage CRC usually has a favorable prognosis but unfortunately about two-thirds of patients present with locally advanced or metastatic disease [30].

Significant progress in molecular pathogenesis and treatment of CRC has been made in the last decade, but advanced stages CRC is still difficult to treat because CRC is a group of diverse heterogeneous diseases arising through various molecular pathways. The prognosis and response to treatment are determined by this heterogeneity that made a big challenge to study the molecular basis of the disease. To date, there are a few molecular markers as KRAS, microsatellite instability, the mutation status of BRAF and PICK3CA that are used in treatment decisions and in clinical patient management. To better define CRC patients with aggressive and lethal disease, we need to identify new molecular biomarkers.

The understanding of gene regulation has been dramatically changed since the discovery of miRNAs; research findings have emphasized the important role of miRNAs in the molecular biology of cancer.

miRNAs are a group of small non-coding RNA molecules that regulate gene expression at the post-transcriptional level. There are more than 2500 miRNAs have been identified in humans (miRBase database 20.0) [31].

In particular, miR-221 has been demonstrated to be an important oncogenic miR in many cancers [12–21], but the associations between miR-221 expression levels, clinicopathological characteristics, and survival in patients with gastric cancer remain mir-221, particularly has been shown to play an important role in many cancers [7-11], but its levels of expression, clinical, pathological characteristics and survival in patients with CRC still understudied.

Although gene expression profiling has revealed several molecular changes associated with CRC [32,33]. Biological markers that precisely predict progression and prognosis are not yet available.

In the current study, mir-221 was over expressed significantly in CRC samples compared with adjacent non-cancerous tissue samples; this increase was accompanied by advanced clinical stage, large tumor size with local infiltration, lymph node metastasis and poor survival.

Our results are in agreement with other studies. Liu et al. demonstrated that human CRC tissues had higher levels of mir-221 than non-tumor colon tissues [34]. Yau et al. also reported that miR-221 levels were significantly higher in 40 CRC tumors compared with their respective adjacent normal tissues and miR-221 showed a significant rise in the trend from normal control to

Table 1. Clinical and pathological features in colorectal cancer patients and mir-221 expression levels

Clinical and pathological parameters	No.	miR-221 (ΔC_T) mean ± SD	*P*
Age			
< 60 years	48	2.38 ± 0.16	NS*
≥ 60 years	44	2.70 ± 0.25	
Gender			
Male	49	2.54 ± 0.33	NS*
Female	43	2.42 ± 0.16	
Differentiation grade			
G_1-G_2	40	2.28 ± 0.24	NS*
G_3-G_4	52	2.89 ± 0.23	
Stage			
I-II	56	2.18 ± 0.14	*P* = 0.018
III	36	3.09 ± 0.32	
T			
T_1-T_2	62	2.09 ± 0.12	*P* = 0.006
T_3	30	3.17 ± 0.46	
LNs			
Negative	30	2.14 ± 0.16	*P* = 0.032
Positive	62	2.92 ± 0.34	

**NS, not statistically significant (P ≥ 0.05, Student's t-test)*

Table 2. Univariate Cox proportional hazard regression analyses of the relationship between clinical-pathological characteristics and levels of mir-221

Variable		Hazard ratio (HR)	*P*
Age	≥ 60 vs < 60 years	0.678	NS
Gender	Male vs female	0.435	0.23
Level of mir-221 expression	High vs low	2.422	0.016
Tumor size and infiltration (T)	T1+T2 vs T3	5.652	*< 0.001*
Tumor stage (TNM)	I +II vs III	3.462	*0.008*
Lymph node metastasis	+ve vs -ve	2.956	*0.006*
Degree of tumor differentiation	GI + GII vs GIII + GIV	5.165	*0.011*

Table 3. Multivariate Cox proportional hazard regression analyses of the relationship between clinical-pathological characteristics and levels of mir-221

Variable		Hazard ratio (HR)	*P*
Age	≥ 60 vs < 60 years	0.725	NS
Gender	Male vs female	0.571	NS
Level of mir-221 expression	High vs low	2.321	0.024
Tumor size and infiltration (T)	T1+T2 vs T3	4.268	*0.001*
Tumor stage (TNM)	I +II vs III	3.225	*0.016*
Lymph node metastasis	+ve vs -ve		*0.006*
Degree of tumor differentiation	GI + GII vs GIII + GIV		*0.011*

late CRC (P<0.0001). The levels of stool mir-221 are significantly higher in subjects with stage I+II and stage III+IV CRC compared to normal controls [35]. miR-221 down regulated in CRC and its level was higher in patients with liver metastases in comparison with nonmetastatic patients [36]. In addition Liu et al. show the predictive value of mir-221 in the prognosis of colorectal cancer (CRC) and its effects on CRC cell proliferation, migration, and invasion. Mir-221 expression was associated with tumor size, TNM stage, Duke stage, LNM,

local recurrence rate, and distant metastases [37]. Liao et al. studied survival using Cancer Genome Atlas data indicated that increased expression of mir-221 was associated with lower survival in CRC patients [38]. Tao et al. [39] found that high levels of mir-221is associated with a more tumor phenotype and decreased time to recur after surgery in CRC patients. Gramantieri *et al*. also found the same previous results but in patients with hepatocellular carcinoma [22]. Sun et al. noticed that up-regulated mir-221 expression significantly with deep local invasion and advanced TNM stage in CRC [32].

Molecular mechanisms that link mir-221 over expression to cancer and their functional targets have been identified and revealed by several studies. It is known that the progression of cancer cells from G0/G1 to S phase in the cell cycle is promoted by mir-221, it also facilitates cell proliferation by down-regulates p27Kip1 and p57Kip2. Both are tumor suppressors and cell-cycle inhibitors related to the protein family kinase inhibitors [13,40,41].

It is also has been proofed the common oncogenic protein, c-Kit, is down streaming mir-221 in gastrointestinal stromal tumors (GIST) [42], papillary thyroid carcinoma [43], prostate cancer [44] and leukemic cells [45].

One miR may have more than 100 targets [46] and a single mRNA may be regulated by a number of miRs, this means that mir-221induced mechanisms of tumor genesis and development are not understood [47].

5. CONCLUSION

In conclusion, the present study confirmed the over expression of mir-221 in CRC tissue samples in comparison with normal adjacent non- cancerous tissue samples. It also showed a relation between the up-regulation of mir-221 and tumor infiltration, progression and poor prognosis in CRC. Our findings suggest mir-221 may be used as a prognostic marker in CRC and it needs more studies to be used as a potential diagnostic marker and as a molecular target for targeted cancer therapy.

CONSENT

Informed consent was obtained from all individual participants included in the study.

ETHICAL APPROVAL

This study was carried out in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethical committee of Zagazig University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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