ORIGINAL ARTICLE

Expression of Plasma miRNA-221 in Colorectal Carcinoma Patients and its Diagnostic Significance in Comparison with p53 Expression

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SUMMARY

Background: **Colorectal carcinoma development progresses through a sequence of normal mucosa-polyp-carcinoma. Early detection of premalignancy is crucial for improved outcomes. We evaluated the diagnostic performance of plasma miRNA-221 and its feasibility in discriminating premalignant from malignant neoplasms and correlating it with immunohistochemical p53 expression.**
Methods: **A total of 109 plasma samples were collected (76 carcinoma, 14 premalignant, and 19 controls). MiRNA-221 was quantified by qPCR for calculation of ΔCt using RNU6B as endogenous control. p53 immunohistochemical staining was performed on corresponding tissue.**
Results: **Plasma miRNA-221 and p53 in tissues were significantly overexpressed in the malignant group when compared with the premalignant and control groups. Plasma miRNA-221 was increased in late-stage tumors with nodal or distant metastasis. ROC curve construction for distinguishing between malignant and premalignant tumors revealed a cutoff value of 2.97 with 74% sensitivity, 79% specificity, 73.7% positive predictive value and 78.6% negative predictive value (AUC = 0.824; p = 0.001). Plasma miRNA-221 significantly correlated with p53 in cancer samples (r = 0.507).**
Conclusions: **MiRNA-221 could have a diagnostic role in differentiating malignant from premalignant neoplasms and could also serve as a predictive marker indicating tumor progression.**
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INTRODUCTION

Colorectal cancer (CRC) is the third and second most commonly diagnosed cancer in men and women, respectively [1]. CRC is associated with a high-rate of mortality. There were almost 1.4 million cases of CRC worldwide in 2012, leading to approximately 693,900 deaths [1,2]. Early diagnosis of CRC is a crucial step in management of the disease, as the survival rate in ad-

vanced stages is poor. The current diagnostic tools for CRC still depend primarily on conventional methods such as colonoscopy. This is not always an available option in early tumor stages, as this approach is difficult to apply for screening at risk population. To improve the diagnostics of early CRC, recent research has focused on identification of different molecular biomarkers from various types of non-invasive samples, such as plasma and stool [3-5].

MicroRNAs (miRNAs) are small non-coding RNAs that have important roles in gene expression at the post-transcriptional level [6]. Numerous miRNAs located within cancer cells have been recognized as potential biomarkers for different cancers [7]. Of note, many miRNAs extracted from blood samples revealed tumor-specific changes [8]. As a promising novel tool in cancer diagnostics, the relatively high stability of miRNAs in serum or plasma is an important advantage [9]. Plasma miRNA-221 has been identified in some cancers and has been reported to play a role in the epithelial-to-mesenchymal transition which causes tumor invasion [10]. Few studies have examined plasma miRNA-221 in cancer [11,12], and more research is necessary to determine the clinical utility of miRNA-221 in early diagnosis of CRC.

Assessment of p53 in colorectal tissues revealed an increasing pattern of expression from premalignant adenoma to malignant neoplasms [13]. Mutant p53 is associated with poor prognosis in CRC [14]. Colorectal cancer is a disease that passes through the adenoma-carcinoma sequence [15]. It is thus crucial to detect the tumor at the adenoma stage before malignant transformation. The aim of our study was to evaluate the diagnostic performance and feasibility of plasma miRNA-221 in differentiation between malignant tumors and premalignant or non-malignant (or both) tumors in colorectal neoplasms. We also tested the correlation between miRNA-221 and P53 expression in tissues.

MATERIALS AND METHODS

Patients and sample collection

Blood samples were collected in EDTA tubes from 76 CRC patients (61 adenocarcinoma, 14 mucinous adenocarcinoma, and 1 signet ring cell adenocarcinoma), and 14 patients with premalignant colorectal low-grade adenoma. Samples were collected before any surgical or medical treatments. We excluded patients with previous surgical history or patients who had any form of treatment (chemotherapy or radiotherapy). We also excluded patients with high-grade adenoma or carcinoma in situ (for the premalignant samples group). As controls, plasma samples were taken from 19 healthy individuals (Table 1). Adjacent normal colonic mucosa from 19 biopsies were used as controls for p53 expression. Plasma samples were stored at -20°C until miRNA isolation. Patients were diagnosed and treated in the outpatient clinic of The Egyptian National Cancer Institute (Cairo,

Egypt). The study was approved by the Institutional Review Board (IRB) of the National Cancer Institute of Cairo, Egypt (ethical permission number: MD2010014 022.3). Written informed consent was obtained from all subjects.

MiRNA isolation

MiRNA isolation was performed by using the miRNeasy mini kit (Qiagen, Frederick, MD, USA) according to the manufacturer's instructions. The kit includes QIAzol lysis reagent that was added to 200 µL of plasma. MiRNA was eluted in 14 µL of RNase-free water.

Reverse transcriptase reaction

Reverse transcription was performed by using the TaqMan microRNA reverse transcription kit on both miRNA-221 and RNU6B (endogenous control) in a total reaction volume of 15 µL. The reaction was performed according to the following conditions: 16°C for 30 minutes, and 42°C for 30 minutes, 85°C for 5 minutes, and 4°C until sample retrieval.

Real time quantitative PCR

Amplification of both miRNA-221 and RNU6B was performed by using the TaqMan microRNA assay (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. The total reaction volume per single reaction was 20 µl. The reaction mixture was transferred to a 48-well reaction plate and the plate was loaded into a StepOne Real-Time PCR system (Applied Biosystems Foster City, CA, USA). Amplification was performed according to the following conditions: 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds. ΔCt was calculated by subtracting the Ct values of miRNA-221 from the Ct values of the internal control (RNU6B). Mean ΔCt values were compared between cases and controls.

Immunohistochemistry

Immunostaining was performed using an autostainer BenchMark IHC/ISH staining module Ventana (Tuscon, AZ, USA). The anti-p53 mouse monoclonal antibody clone DO-7 (Ventana Medical Systems, Tuscon, AZ, USA) was used. Preparation of one positively charged slide from a representative paraffin block of 4 µm thickness was performed and slides were then arranged in a Ventana autostainer after label printing. Following the end of the run, the slides were washed in tap water for 5 minutes and then dehydrated in ascending grades of alcohol, 5 minutes in each container. Finally, slides were cleared in xylene, and cover slips were applied. Colonic carcinoma positive for p53 was used as a positive-staining control. PBS instead of the primary antibody served as a negative control. The number of positively stained nuclei were recorded in consecutive fields at 400x magnification. The percentage of tumor nuclei expressing p53 was determined by counting 1,000 cells per slide.

Table 1. Patients' characteristics in the malignant, premalignant, and control groups.

Characteristic	Malignant neoplasms	Premalignant neoplasms	Healthy controls
Age (years)			
Average	61	60	59
Gender			
Male	46	7	10
Female	30	7	9
AJCC stage			
I	13	-	-
II	19	-	-
III	34	-	-
IV	10	-	-
Nodal involvement			
Yes	33	-	-
No	43	-	-
Distant metastasis			
Yes	13	-	-
No	63	-	-
Histopathological types			
AC	61	-	-
Mucinous AC	14	-	-
Signet ring cell AC	1	-	-
Adenoma	-	13	-
Hyperplasia	-	1	-

AC - Adenocarcinoma.

Table 2. Median of plasma miRNA-221 in the different groups.

Category	Median	(25th - 75th percentile)
Malignant group	5.8	(2.7 - 9.5)
Premalignant group	2.2	(1.1 - 3.1)
Controls	1.5	(1.2 - 2)

Table 3. Median level of p53 expression in the paired tumor tissue samples.

Category	Median	(25th - 75th percentile)
Malignant group	85%	(32 - 96%)
Premalignant group	20%	(0 - 47%)
Controls	0%	(0 - 0%)

Statistical Analysis

Data was analyzed using IBM SPSS advanced statistics, Version 24 (IBM Corp., Armonk, NY, USA). Comparison between two groups was performed using the Mann-Whitney test (non-parametric *t*-test). Comparison between the three groups was performed using the Kruskal-Wallis test (non-parametric ANOVA) followed by post-hoc tests on a rank of variables used for pairwise comparison. A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic power of plasma miRNA-221 and p53 expression in tissues and to predict cutoff values for these two markers. Pearson's correlation coefficient r value was calculated between plasma miRNA-221 and p53. All tests were two-sided and p-values ≤ 0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 109 plasma samples were collected from colorectal neoplasia patients and healthy controls. They were categorized into the following three groups: malignant, premalignant, and controls. Cancer was staged according to the American Joint Committee on Cancer staging system (AJCC) [16] (Table 1).

Plasma miRNA-221

Median levels of plasma miRNA-221 between malignant, premalignant, and control groups

The median levels of miRNA-221 in plasma were significantly different between malignant, premalignant, and control groups. Plasma miRNA-221 in the malignant group was significantly higher in the malignant group than those of the premalignant and healthy groups (p = 0.001) (Table 2).

Plasma miRNA-221 in relation to tumor stage, nodal, and distant metastasis

Plasma miRNA-221 was significantly higher in the advanced tumor stages (III and IV) than in the early stages (I and II) (p = 0.005) (Figure 1). Similarly, there was significant overexpression of plasma miRNA-221 in neoplasms with lymph-node metastasis when compared with neoplasms without nodal metastasis. Finally, while plasma miRNA-221 was overexpressed (p = 0.038) in metastatic CRC tumors when compared with non-metastatic tumors, the difference was statistically insignificant.

Expression of p53 in corresponding tumor tissues

In the paired tumor tissue samples, there were statistically significant differences in median p53 expression levels between the malignant, premalignant, and control groups (p = 0.001) (Table 3). Interpretation of p53 immunohistochemical results was performed by cell counting, choosing the most cellular area of the tumor, with minimum necrosis or inflammatory cell infiltration
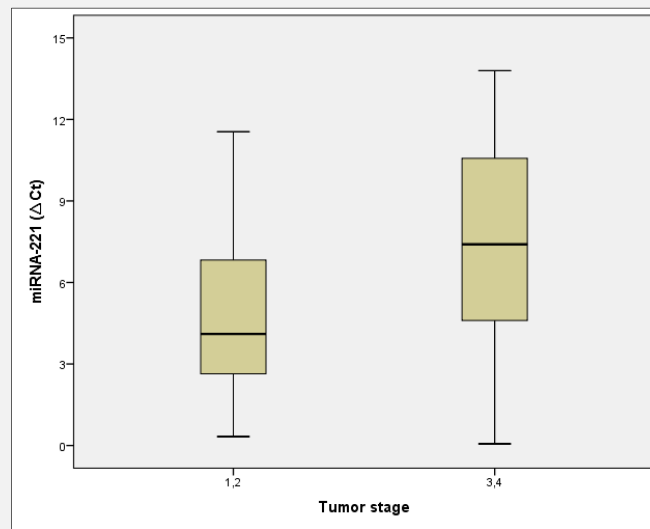
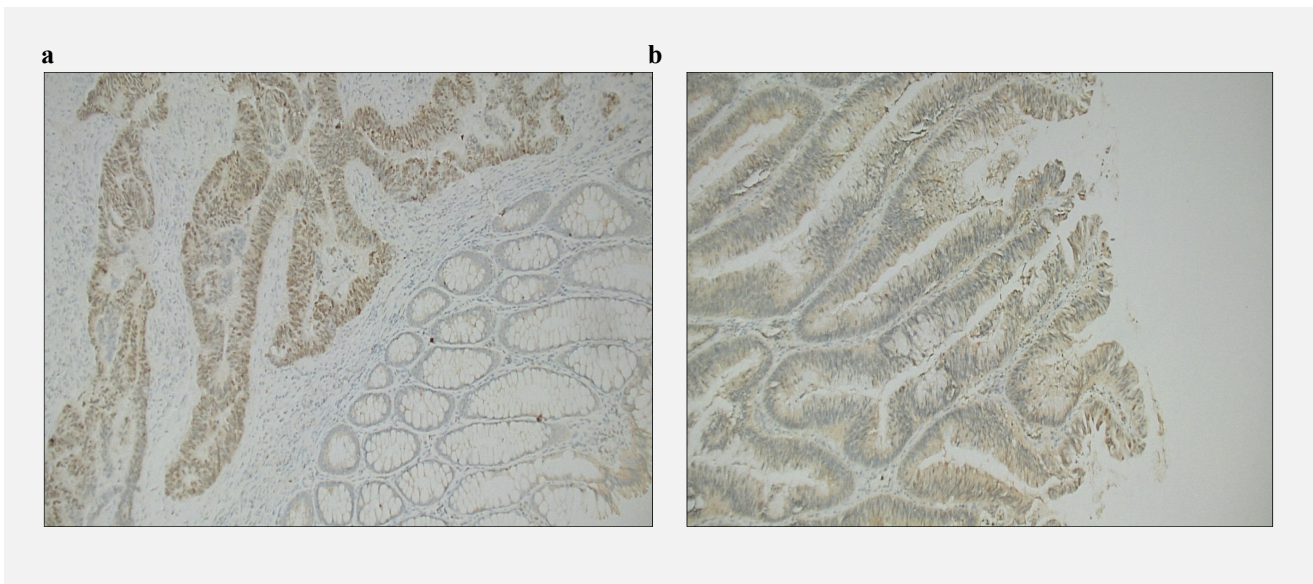**Figure 1. Significant higher plasma miRNA-221 in the advanced cancer stages.**

Figure 2a: A case of adenocarcinoma with strong nuclear positivity for p53. Note the completely negative normal adjacent colonic mucosa on the right [IHCx 100], 2b: A case of tubulovillous adenoma with scarce [5%], weak nuclear positivity for p53 [IHCx 100].

and with the highest nuclear-labeling density (Figures 2a and 2b).

Diagnostic value of plasma miRNA-221 and tissue p53 in differentiation between malignant and pre-malignant tumors

Receiver operating characteristic (ROC) curve analyses were used to assess the diagnostic utility of plasma mi-RNA-221 for discrimination between malignant and premalignant status compared to p53. For miRNA-221 in plasma, at a cutoff value of 2.97, the sensitivity was 74% and the specificity was 79% (AUC = 0.824, 95% confidence interval (CI) 0.737 - 0.912; $p = 0.001$) with a positive predictive value (PPV) of 73.7% and a negative predictive value (NPV) of 78.6%. For p53 expression in tissues, at a cutoff value of 35%, the sensitivity was
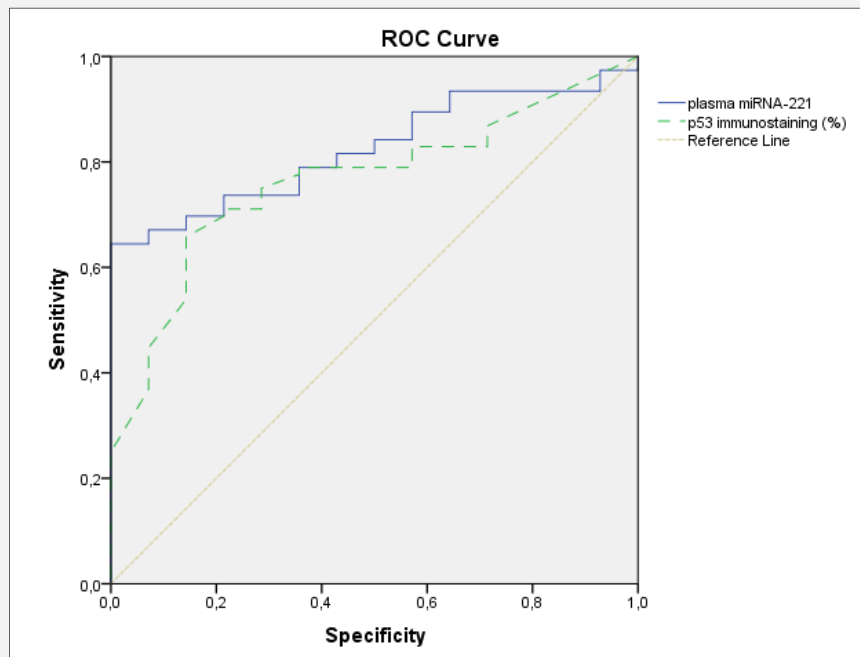
Figure 3. ROC curve comparing the diagnostic utility of plasma miRNA-221 and p53 in differentiation between malignant CRC and premalignant cases.
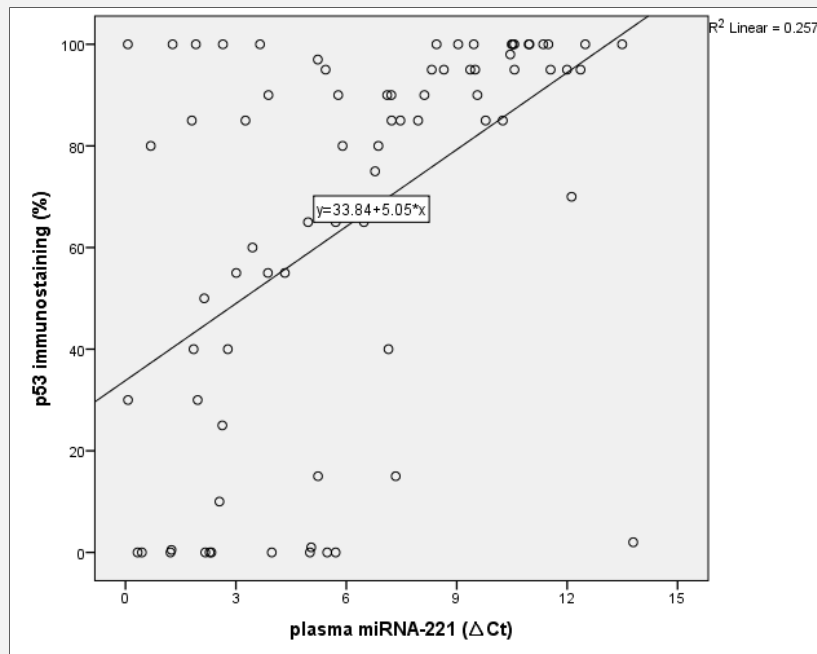

Figure 4. Significant correlation between plasma miRNA-221 and p53 expression in malignancy.

75% and the specificity was 71% (AUC = 0.766, 95% CI 0.652 - 0.880; p = 0.002) with a PPV of 75% and a NPP of 71% (Figure 3).

Correlation between plasma miRNA-221 level and tissue p53 expression
Samples from malignant CRC cases revealed significant correlation between plasma miRNA-221 and p53 expression in tissues (p = 0.001), with a Pearson's correlation value of 0.507 (Figure 4).

DISCUSSION

Plasma miRNAs appear to be useful biomarkers for early detection and diagnosis of CRC. Although research on the diagnostic applicability of plasma miRNAs is still at an early stage when compared with tissue miRNA, plasma miRNA has the advantage of being a non-invasive approach for cancer diagnosis. In this study, we assessed the ability of plasma miRNA-221 to differentiate malignant from premalignant colorectal tumors. Regarding its diagnostic role in early CRC detection, plasma miRNA-221 in the malignant group was significantly upregulated when compared with the premalignant adenoma and control groups (p = 0.001). The same findings were reported by Pu et al., who demonstrated that plasma miRNA-221 can be used as a molecular diagnostic marker in CRC and also as a prognostic marker for overall survival [12]. Similarly, miRNA-221 is upregulated in the plasma of pancreatic cancer patients when compared with premalignant and control groups and may be of possible use in cancer diagnostics and monitoring of tumor dynamics [17]. It is known that miRNA-221 can promote tumor cell proliferation *in vitro* through inhibition of the cell cycle inhibitor CDKN1C/ p57 in CRC cells [18]. This has several consequences for the tumor progression rate in different tumors, such as glioblastomas [19], breast cancer [20], and lung cancer [21].
We tested the diagnostic value of plasma miRNA-221 for discrimination between malignant and premalignant CRC tumors. Using a ROC curve, 2.97 was chosen as the best cutoff with 74% sensitivity and 79% specificity and a PPV of 73.7% and a NPV of 78.6%. In plasma samples from premalignant tumors, there was no significant upregulation of miRNA-221 when compared with controls. The level of stool miRNA-221 was also not significantly upregulated in CRC adenoma or advanced adenoma but significantly upregulated in CRC malignant tumors. This indicates that miRNA-221 is a key regulator of signaling pathways in CRC pathogenesis [22]. Additionally, the diagnostic utility of miRNA-221 in stool from CRC patients was compared with controls and reported a sensitivity of 62% and a specificity of 74% (AUC = 0.73, 95% CI 0.68 - 0.78) [22]. This could be a simple non-invasive method for early detection of adenoma transformation into cancer and could be used to monitor treatment and detect recurrence of malig-

nancy. MiRNA-221 is associated with malignancy recurrence in papillary thyroid carcinoma and prostatic carcinoma [23,24]. We tested plasma miRNA-221 in four patients suspected of clinical recurrence of malignancy (later shown to be negative). Three cases had plasma miRNA-221 levels below the cutoff level (2.1, 1.94, and 2.3), while the last case had a plasma miRNA-221 level above cutoff level (5.3). There was no apparent reason for this upregulation in the latter case.
Additionally, we showed a potential predictive role for plasma miRNA-221. In patients with advanced tumor stages (III and IV), plasma miRNA-221 levels were significantly higher than those in patients at early tumor stages (I and II) (p = 0.005). Similarly, Yau et al. used qPCR to demonstrate a significant trend of increased stool miRNA-221 and miRNA-18a levels from early CRC stages (I and II) to advanced stages (III and IV) [22]. In our study, plasma miRNA-221 was also upregulated in CRC patients with nodal (p = 0.038) and distant metastases (p = 0.177). This could be a reliable predictive marker in understanding tumor behavior, predicting tumor progression, and follow up of treatment protocols.
MiRNA-221 promotes tumor cell migration in CRC cell lines and enhances cell invasion and metastasis *in vitro* by targeting reversion-inducing cysteine-rich protein with Kazal motifs (RECK). RECK is a tumor suppressor that inhibits matrix metalloproteinases and is downregulated in several cancers. It was found that miRNA-221 overexpression caused direct reduction in RECK in CRC cell lines [18]. Furthermore, activation of the signal transducer and activator of transcription (STAT3) signaling pathway can induce CRC tumor initiation and proliferation and simultaneously cause overexpression of miRNA-221 in CRC cells. Interestingly, upregulation of miRNA-221 in CRC causes consecutive activation of STAT3 signaling pathways, resulting in a positive-feedback loop that leads to tumor progression. Disrupting this loop with miRNA-221 inhibitors can promote tumor regression, thus indicating a potential therapeutic role of miRNA-221 in CRC and in guiding follow up of treatment [25].
Plasma miRNA-221 from malignant samples was correlated with p53 expression in the corresponding tissue specimens (r = 0.507). Both miRNA-221 and p53 have the same molecular targets such as MDM2, MDM4, BBC3, and TP53BP2 [12]. As overexpression of p53 is associated with poor survival, correlation of p53 with plasma miRNA-221 could illustrate a prognostic role of miRNA-221. Pu et al. demonstrated an association between elevated plasma miRNA-221 levels and poorer overall survival. We did not perform survival analysis in this study.

CONCLUSION

In summary, we demonstrated a potential diagnostic role of plasma miRNA-221 in the differentiation between malignant and premalignant CRC neoplasms through a non-invasive method. Our results also highlight the predictive role of plasma miRNA-221 in assessment of tumor load and progression, as miRNA-221 was overexpressed in late CRC stages. The diagnostic applicability of plasma miRNA-221 requires further validation.

Declaration of Interest:
No conflict of interest to declare.

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