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ORIGINAL RESEARCH

# MicroRNA Expression Levels and Histopathological Features of Colorectal Cancer

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## Abstract

**Introduction** Non-coding RNAs have opened a new window in cancer biology. MicroRNAs (miRNAs), as a family of non-coding RNAs, play an important role in the gene regulation. The aberrant expression of these small molecules has been documented to involve in colorectal cancer (CRC) pathogenesis. This study aimed to examine the expression of miRNAs in CRC and to correlate their expression levels with histological markers (Ki-67 and CD34).

**Materials and Methods** Tumor tissues and matched normal adjacent tissues were collected from 36 patients with newly diagnosed CRC. Immunohistochemical (IHC) staining of tumor tissues was performed for Ki-67 (proliferation) and CD34 (angiogenesis) markers, and the immunoexpression staining scores were obtained. A polyadenylation SYBER Green quantitative real-time PCR technique was used to quantify the expression of a panel of five CRC-related miRNAs (hsa-miR-21, 31, 20a, 133b, and 145). Histopathological (H) scores and miRNA expression levels were correlated with clinicopathological features including the degree of differentiation, staging, and lymphovascular invasion.

**Results** Our results showed the significant difference between the two groups for the expression level of hsa-miR-21, hsa-miR-31, hsa-miR-145, and miR-20a ($P < 0.001$), but not for hsa-miR-133b ($P = 0.57$). Further analysis revealed an inverse significant correlation between hsa-miR-145 and Ki-67 ($r = -0.942$, $P < 0.001$). While a positive correlation was observed between hsa-miR-21 and Ki-67 ($r = 0.920$, $P < 0.001$), and hsa-miR-21 and CD34 ($r = 0.981$, $P < 0.001$). Also, a positive correlation between hsa-miR-31 and Ki-67 ($r = 0.913$, $P < 0.001$), hsa-miR-31 and CD34 ($r = 0.798$, $P < 0.05$), hsa-miR-20a and Ki-67 ($r = 0.871$, $P < 0.001$), and hsa-miR-20a and CD34 ($r = 0.890$, $P < 0.001$) was found.

**Conclusion** Dysregulation of miRNAs and correlation with molecular histopathology indicate a biological role for miRNAs in various cellular processes including cell proliferation and angiogenesis in CRC development. On the other hand, the pattern of miRNA expression and its correlation with histological markers are potentially valuable to apply as diagnostic biomarkers for CRC.

**Keywords** CD34 · Colorectal cancer · Ki-67 · MicroRNA

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## Introduction

Colorectal cancer (CRC) is the most common malignancy with a high mortality worldwide. Even though new advances in prognostic and therapeutic approaches decrease CRC mortality rates, the patients are frequently diagnosed with poor clinical outcome [1–3]. Tumor tissue markers currently have valuable applications in the detection and treatment of CRC in the early stage. A number of tumor markers have even the potential to modify diagnostic and treatment settings by selecting the suitable chemotherapeutic strategies in CRC patients [4–6].

Generally, various clinical, histological, and molecular features have been reported as valuable markers for CRC prognosis and diagnosis. The clinical value of these factors potentially might be independent or mutually dependent [7]. For instance, the histopathological markers have increasingly been applied to classify the malignancy into distinct subtypes. On the other hand, the biological characteristics of tumors could be served to predict the disease status and select a right therapeutic approach. Moreover, the molecular technology has incorporated new biomarkers along with histological and molecular biomarkers [8]. It has been recognized that the key molecular mechanisms involving carcinogenesis such as aberrant cell proliferation and angiogenesis can be affected by various signaling pathways [9].

Recently, a group of non-coding RNAs named microRNAs (miRNAs) have been suggested as post-transcriptional regulators in a number of malignancies. These endogenous small biomolecules involve in fine-tune regulation of gene expression in various cellular processes including cell proliferation, differentiation, and apoptosis [10, 11]. Notably, ever more evidences indicate that the dysregulation of miRNAs plays a biological role in tumor developmental events of CRC. Additionally, these miRNAs have received increasingly attention as potential biomarkers for diagnosis and prognosis of the malignancy [4, 12–14].

This study aimed to quantitatively examine the expression of miRNAs in CRC and to correlate their expression levels with histological markers (Ki-67 and CD34).

## Materials and Methods

### Sample Collection

In this study, we used 36 colorectal cancer patients. All tumors and their adjacent normal tissues were collected from colorectal cancer patients. Some clinicopathological features of CRC patients were illustrated in Table 1. The tumor-node-metastasis (TNM) stage of tumors was determined according to the standard TNM classification system of the International Union Against Cancer (7th edition) (http://www.uicc.org/). This project was approved by the ethical and research committee (IR.IUMS.REC 1394.26649). All of tissue biopsies were divided into two different tubes containing RNAlater and formalin. Tissues containing RNAlater were subjected to molecular examinations while formalin-fixed, paraffin-embedded (FFPE) tissue blocks were used for histopathological investigations.

### Histological Analysis

Tumor tissue sections from the FFPE blocks were studied for colon cancer characterization by hematoxylin and eosin (H&E) staining. Also, immunohistochemistry (IHC) staining for proliferation (Ki-67) and angiogenesis (CD34) markers was conducted as described previously [15]. Briefly, the proliferation index was calculated as the percentage of cells stained with Ki-67 or CD34. For CD34, in cases with interstitial or scattered cells, the cells were cross-checked to classify the target cells on a separate slide. The histopathological score (H score) was described as a score from 1 to 100 based on the quantity of stained cells and severity of staining.

### RNA Extraction

The samples were processed and total RNA was isolated from the frozen tissue samples by TRIzol Reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA, USA) according to the

**Table 1** Clinicopathological characteristics of CRC patients

| Variable | Clinicopathologic parameter | Number of samples ($n = 36$) |
|---|---|---|
| Age | $\geq 55$ | 15 |
| | $< 55$ | 21 |
| Gender | Male | 29 |
| | Female | 17 |
| TNM stage | II | 18 |
| | III | 15 |
| | IV | 3 |
| Tumor size | Size $< 2$ | 5 |
| | $2 \leq$ size $< 3.5$ | 15 |
| | $3.5 \leq$ size $\geq 5$ | 12 |
| | Size $> 5$ | 4 |
| Localization | Colon | 15 |
| | Rectum | 21 |
| LVI | Positive | 22 |
| | Negative | 14 |
| Differentiation | Well | 6 |
| | Moderate | 27 |
| | Poor | 3 |

*CRC* colorectal cancer, *TNM* tumor-node-metastasis, *LVI* lymphovascular invasion manufacturer's protocol. Then, extracted RNA was treated using RNase-free DNase I and stored at − 80 °C, as described previously [16].

### Quantitative Real-Time PCR for Detecting Mature miRNAs

Five microRNAs were selected by virtue of being published as CRC-related miRNAs and established as potential tissue biomarkers for a number of cancers. The expression of the aforementioned miRNAs was measured by a quantitative poly A reverse transcription real-time polymerase chain reaction (q-PCR) using specific primers (Table 2) [17, 18]. All PCR reactions were performed in triplicate and the mean $C_t$ data were concluded using cycle threshold settings. The relative expression levels of miRNAs were normalized to that of RNU6B, as internal control using Rotor-gene Q software. The normalization was completed by the equation: $\log_{10}(2^{-\Delta\Delta C_t})$, in which $\Delta C_t = C_{\mathrm{tmiRNA}} - C_{\mathrm{tcontrol}}$.

### Statistical Analysis

The SPSS software version 22.0 was used for statistical analysis. Data were analyzed using Student's *t* test in independent experiments. At first, the normality was checked using Kolmogorov-Smirnov test. Furthermore, Pearson correlation coefficient was used to examine the relationship between Ki-67 and CD34 expression in samples based on miRNA expression level. The level of significance for statistical test was 0.05.

## Results

### Immunohistochemical Markers

The tissue expression of Ki-67 and CD34 markers was analyzed in all of the 36 CRC patients by immunohistochemistry (IHC) staining. Immunoexpression of marker Ki67 with high histopathological (H) score was noticeable in late-stage tumors (96.28 ± 0.79) (Fig. 3).

Moreover, CD34 marker was positive in the late stages with different scores (38.82 ± 0.611). Comparison of Ki-67 and CD-34 immunohistochemical expression between tumors with low and high degrees of the cell differentiation (well vs. poor) presented $P = 0.07$ and $P = 0.12$, respectively. Further statistical analysis showed that there is no significant correlation between stages II, III, and IV and the tumor markers ($P > 0.05$).

**Table 2** Primer sequences used for qRT-PCR in this study

| Gene name | 5′ → 3′ nucleotide sequence |
|---|---|
| miR-20a | CTGCTGGCGAATTAGTAGACCA |
| miR-21 | *CGGCGTAGCTTATCAGACTGATG* |
| miR-31 | *AGGCAAGATGCTGGCATAGCT* |
| miR-145 | *GTCCAGTTTTCCCAGGAAUCCCT* |
| miR-133b | CATCCATCCCGATCTTCTAGCA |

### Expression of miRNAs in Tissue Samples

Our data showed that four studied miRNAs (miR-145, miR-31, miR-21, miR-20a) were differentially expressed in CRC patients compared to controls (Table 3 and Figs. 1 and 2). Independent Student's *t* test showed the significant difference between the two groups in miR-21, miR-20a, miR-31, and miR-145 expression levels ($P < 0.001$). Among the abovementioned miRNAs, the expression level of miR-21, miR-20a, and miR-31miR-145 showed a significant up-regulation, while that of miR-145 showed a significant down-regulation in CRC compared to the normal group ($P < 0.05$). However, the expression level of miR-133b was not significant between the CRC and control groups ($P = 0.57$).

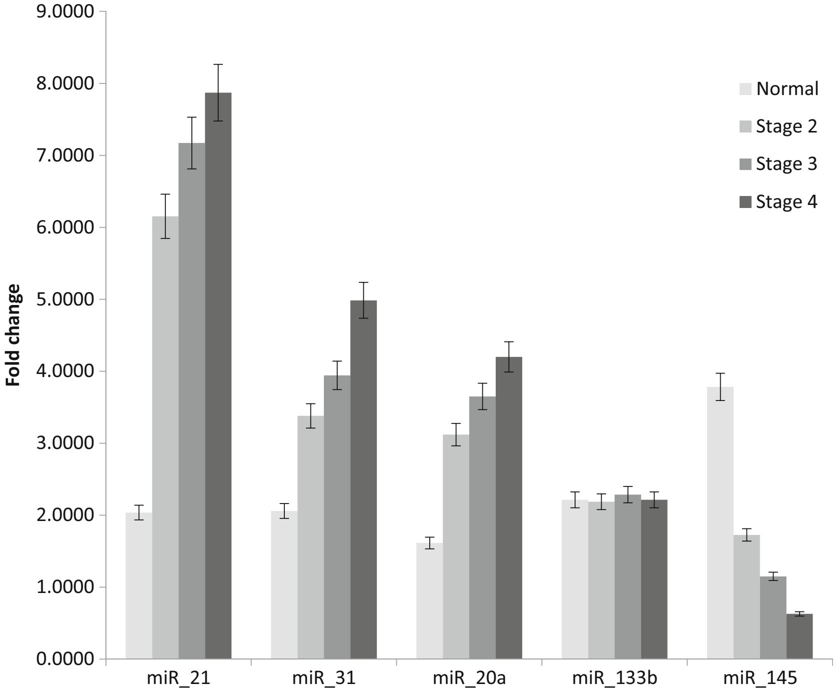
### Correlation of Histopathological Markers with miRNA Expression Level

The correlation between the expression levels of Ki-67 and CD34 and miRNAs was evaluated by the Pearson test in CRC tissues. According to our analysis, the expression level of miR-145 demonstrated a significant reverse correlation with the immunohistochemical markers Ki-67 ($r = -0.942$, $P < 0.001$) and CD34 ($r = -0.836$, $P < 0.001$) (Fig. 3a, b). While a positive correlation was observed between miR-21 and Ki-67 ($r = 0.920$, $P < 0.001$), and miR-21 and CD34 ($r = 0.981$, $P < 0.001$) (Fig. 4a, b). Also, a positive correlation between miR-31 and Ki-67 ($r = 0.913$, $P < 0.001$), miR-31 and CD34 ($r = 0.798$, $P < 0.05$), miR-20a and Ki-67 ($r = 0.871$, $P < 0.001$), and miR-

**Table 3** Descriptive statistics of miRNAs and Ki-67 and CD34 markers

| Variables | Category | Minimum | Maximum | Mean ± SD |
|---|---|---|---|---|
| miR-21 | Normal | 0.9 | 1.5 | 1.26 ± 0.16 |
| | Tumor | 2.8 | 3.86 | 3.4 ± 0.29 |
| miR-31 | Normal | 1.5 | 2.34 | 2.05 ± 0.15 |
| | Tumor | 1.1 | 2.9 | 2.1 ± 0.42 |
| miR-20a | Normal | 0.9 | 1.3 | 1.06 ± 0.103 |
| | Tumor | 2.8 | 3.06 | 2.95 ± 0.082 |
| miR-133b | Normal | 0.11 | 0.34 | 0.26 ± 0.074 |
| | Tumor | 0.14 | 0.4 | 0.32 ± 0.085 |
| miR-145 | Normal | 0.13 | 0.42 | 0.26 ± 0.075 |
| | Tumor | 0.12 | 0.34 | 0.27 ± 0.064 |
| Ki-67 | Tumor | 94 | 97.5 | 96.28 ± 0.79 |
| CD34 | Tumor | 37.5 | 40 | 38.82 ± 0.611 |

**Fig. 1** *Box plot of miRNA expression level in different stages of colorectal tumor and normal groups.* The plot shows minimum and maximum and the first, second, and third quartiles of hsa-miR-21, hsa-miR-20a, hsa-miR-31, hsa-miR-145, and hsa-miR-133b in tumor and normal groups



20a and CD34 ($r = 0.890$, $P < 0.001$) was found (Figs. 5a, b and 6a, b). Whereas there was no significant correlation between miR-133b with Ki-67 ($r = 0.09$, $P = 0.57$) and CD34 ($r = 0.122$, $P = 0.47$) (Fig. 7a, b).

## Discussion

Commonly, a better understanding of the tumor behavior and its aggressiveness can be achieved by examining the molecular and histological expression of multiple markers [16–18]. The investigation and identification of tumor markers are imperative to evaluate the cancer prognosis, as well as deciding on appropriate treatment regimen. A number of molecular and histological features have frequently been reported as clinical markers for CRC prognosis and diagnosis [19–21].

Histopathological markers have been applied to predict the disease status and select a right therapeutic approach. These markers may also be served as valuable tools for the prognosis

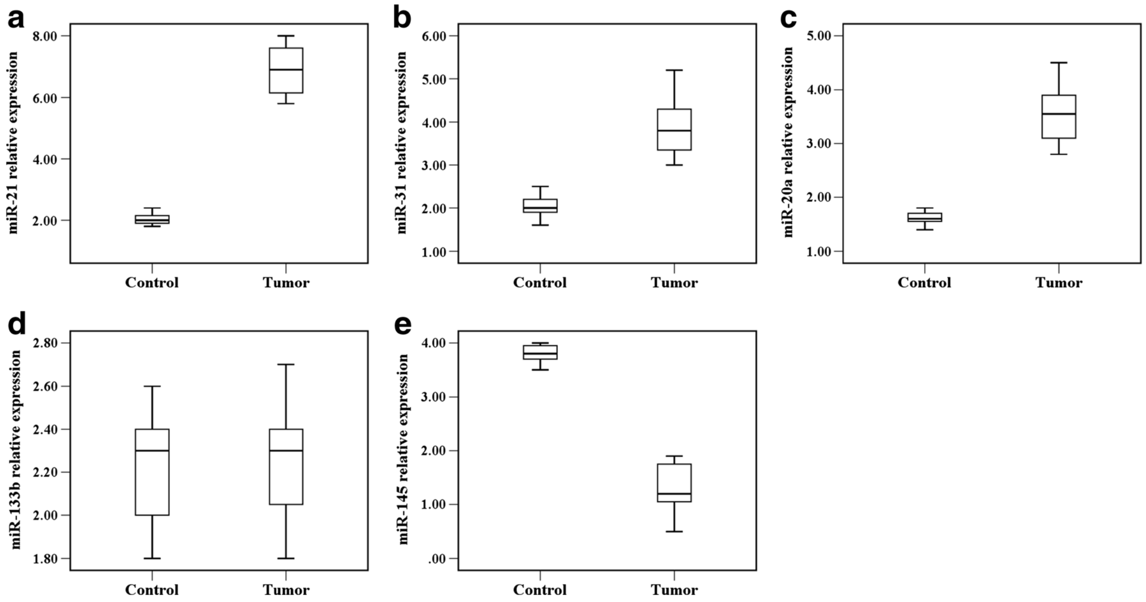


**Fig. 2** *The differential expression level of hsa-miR-21 (a), hsa-miR-31 (b), hsa-miR-20a (c), hsa-miR-133b (d), and hsa-miR-145 (e) in tumor tissues in comparison with normal tissues*

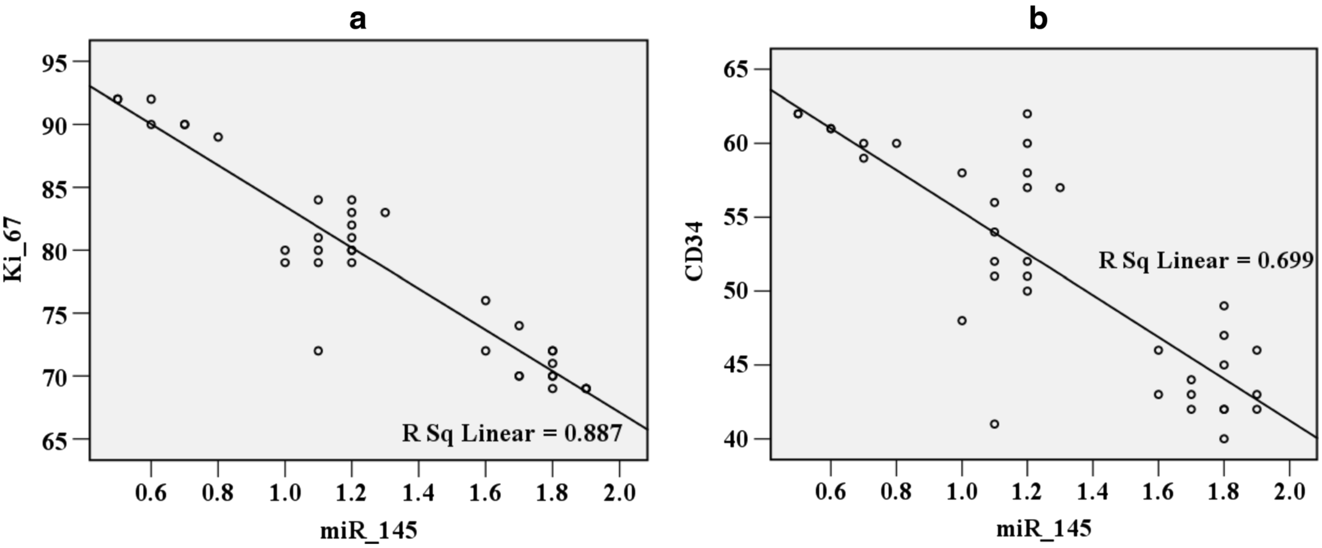


**Fig. 3** *Scatter plot of the hsa-miR-145 with tissue Ki-67 and CD34.* A significant inverse correlation between hsa-miR-145 and Ki-67 ($r = -0.942$, $P < 0.05$) was found (**a**). A significant inverse correlation between hsa-miR-145 and CD34 ($r = -0.836$, $P < 0.05$) was also found (**b**)

and decision-making of adjuvant therapies [22]. For instance, evaluating the cell proliferation and angiogenesis by immunohistochemistry (IHC) has been completed as the objective of many cancer investigations. IHC has frequently been used as a useful technique for histologic antigen recognition, classification of tumor types, clinical diagnosis, and evaluation of cancer invasion [23], though the expression of histologic markers and correlation with clinicopathological parameters have been reported to be contradictory in some malignancies [24, 25]. Alternatively, the detection of carcinogenesis-related gene dysregulations could create a new molecular-based staging system, improving the accuracy of cancer detection and prognosis. On the other hand, the molecular technology has incorporated new biomarkers along with histological and molecular biomarkers [8]. MicroRNAs (miRNAs) have been demonstrated to play a key role in colorectal cancer signaling pathways. Thus, the dysregulation of these molecules has repeatedly been shown in the cancer development and progression [26, 27]. In recent decades, several studies investigated the cancer-related miRNAs for their clinical application as potential diagnostic and prognostic biomarkers [28].

The oncogenic or suppressive roles of miRNAs in cancer initiation and progression have been accepted in several studies [29, 30]. On the other hand, the function of miRNAs in cellular processes such as cell proliferation and angiogenesis has been revealed to be varied depending on cancer type. The biological significance of miRNAs in CRC was revealed by many studies supporting their regulatory roles for a range of target genes (TPM1, PTEN, and bcl-2) [31–33].

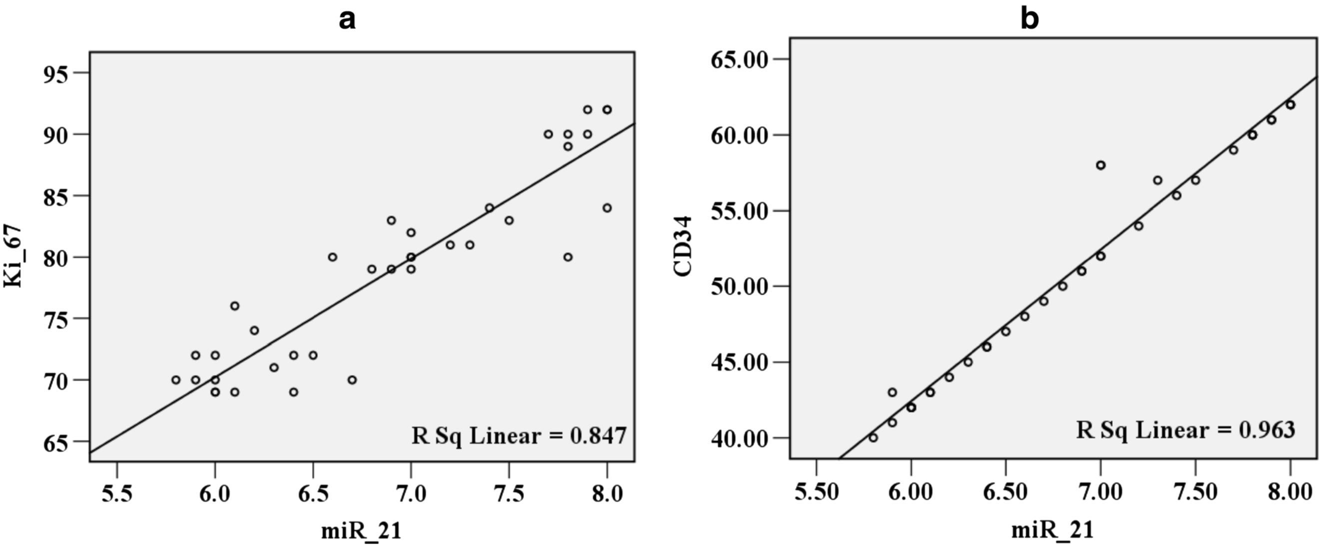


**Fig. 4** Scatter plot of the hsa-miR-21 with tissue Ki-67 and CD34. A significant positive correlation between hsa-miR-21 and Ki-67 ($r = 0.920$, $P < 0.05$) was found (a). A significant positive correlation between hsa-miR-21 and CD34 ($r = 0.981$, $P < 0.05$) was also found (**b**)

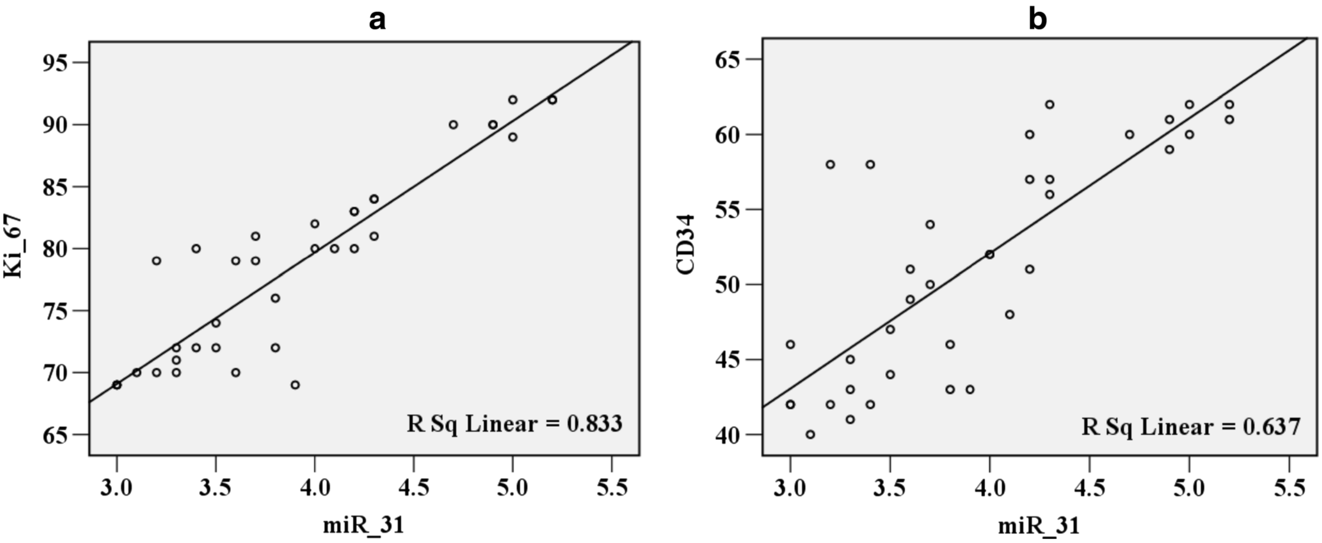


**Fig. 5** *Scatter plot of the hsa-miR-31 with tissue Ki-67 and CD34.* A significant positive correlation between hsa-miR-31 and Ki-67 ($r = 0.913$, $P < 0.05$) was found (**a**). A significant positive correlation between hsa-miR-31 and CD34 ($r = 0.798$, $P < 0.05$) was also found (**b**)

To the best of our knowledge, this is the first study that examines the relationship between miRNA expression levels and immunoexpression of cell proliferation and angiogenesis markers in colorectal carcinoma. In this study, we analyzed the expression levels of five CRC-related miRNAs and CD34 and Ki-67 markers in colorectal tumor tissues. Our results showed that the expression levels of hsa-miR-21 ($P < 0.001$), hsa-miR-20a ($P < 0.001$), and hsa-miR-31($P < 0.05$) were significantly higher in colorectal tumors than those in normal adjacent tissues. Other analysis showed a high powerful correlation between the expression level of hsa-miR-21 and Ki-67 ($r = 0.920$, $P < 0.001$) and CD34 ($r = 0.981$, $P < 0.001$).

Hsa-miR-21, as a well-known oncogenic miRNA (oncomiR), has been demonstrated to involve in the cell proliferation and angiogenesis [34]. Several studies revealed the miR-21 up-regulation and its correlation with carcinogenesis and poor prognosis in colorectal cancer [35, 36]. Hsa-miR-21 participates in tumor angiogenesis by targeting several genes, such as tumor suppressor phosphatase and tensin homolog (PTEN), hypoxia-inducible factor-1 (HIF-1), and tumor suppressor tropomyosin I (TPM1) [37, 38]. In addition, HIF-1 is a potent inducer to regulate vascular endothelial growth factor (VEGF) expression through transcriptional activation [39]. VEGF has been confirmed as a major mediator in the tumor vascularization and angiogenesis [40, 41]. Some researchers showed that elevated expression of hsa-miR-21 in tumor cells can lead to the up-regulation of HIF-1a and VEGF, facilitating angiogenesis [38].

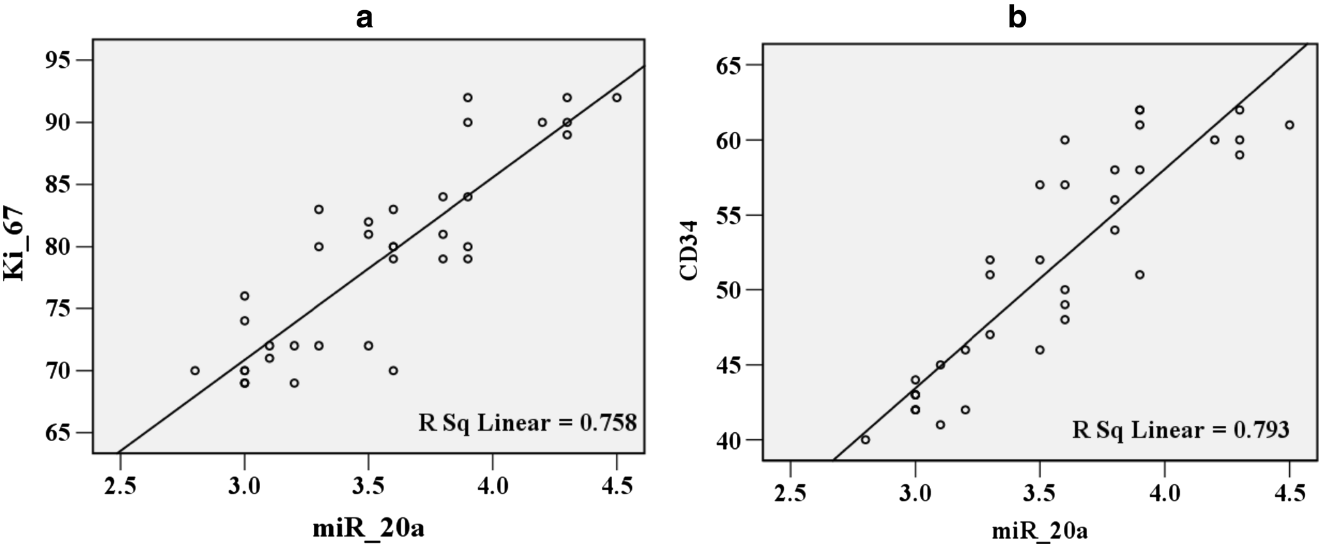


**Fig. 6** *Scatter plot of the hsa-miR-20a with tissue Ki-67 and CD34.* A significant positive correlation between hsa-miR-20a and Ki-67 ($r = 0.871$, $P < 0.05$) was found (**a**). A significant positive correlation between hsa-miR-20a and CD34 ($r = 0.890$, $P < 0.05$) was also found (**b**)

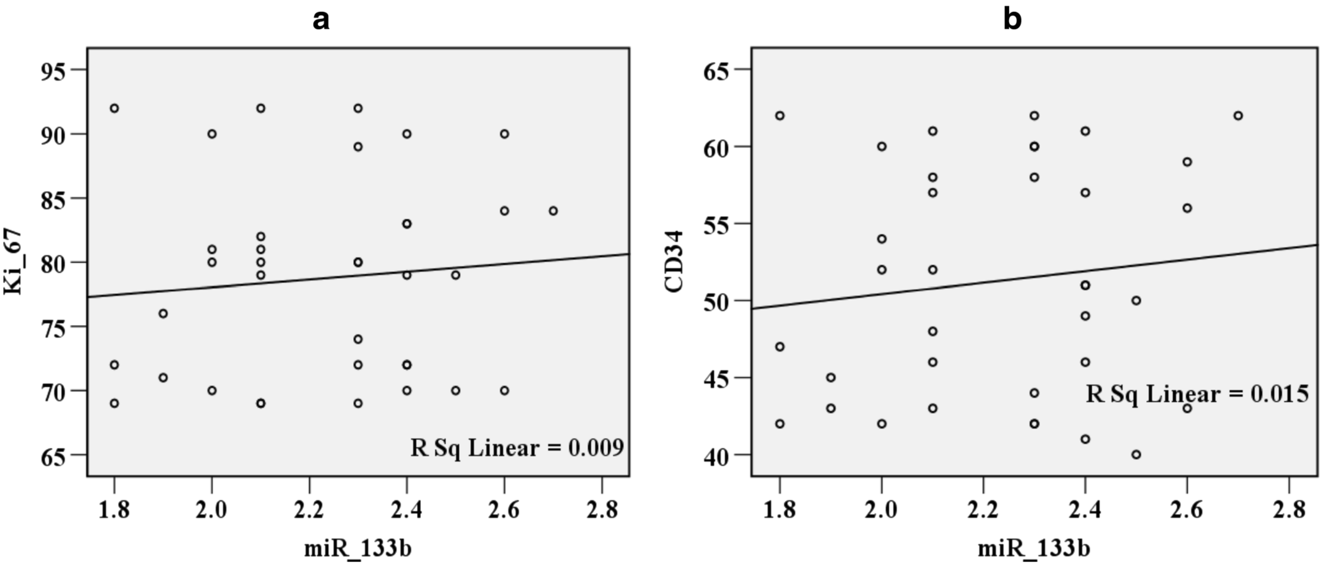


**Fig. 7** *Scatter plot of the hsa-miR-133b with tissue Ki-67 and CD34.* No significant correlation between hsa-miR-133b and Ki-67 ($r = 0.096$, $P = 0.57$) (**a**) or between hsa-miR-133b and CD34 ($r = 0.122$, $P = 0.47$) (**b**) was found

PTEN, as a potential target of hsa-miR-21, is also involved in multiple downstream cellular processes, including cell proliferation and apoptosis. This suppressor gene is the upstream regulator of both PI3K/AKT and MEK/ERK signaling pathways [42]. The PI3K/AKT and ERK signaling pathways have been revealed to play crucial roles in various intracellular cascade events including tumor growth and angiogenesis. Based on this background, hsa-miR-21 potentially induces the cell proliferation and inhibits apoptosis by down-regulation of the target genes [43, 44].

Also, there was a significant difference in the expression level of hsa-miR-20a in tumor patients than healthy subjects. A positive correlation between hsa-miR-20a and Ki-67 ($r = 0.871$, $P < 0.001$) and CD34 ($r = 0.890$, $P < 0.001$) in CRC tissues was also found. The overexpression of this angiogenic miRNA (angiomiR) has been reported in various kinds of human cancer such as CRC [45]. Hsa-miR-20a, as well, could act as an oncomiR in the epithelial-mesenchymal transition (EMT) process and cell invasion. Nonetheless, the molecular mechanisms underlying the function of hsa-miR-20a in CRC development remain to be completely clarified. Hsa-miR-20a, as a post-transcriptional regulator of gene expression, has been demonstrated to play a role in cancer angiogenesis and metastasis through down-regulating of some suppressive genes such as Smad4 [46]. Therefore, hsa-miR-20a-dependent Smad4 suppression functionally could lead to VEGF expression and cancer progression [47]. Hsa-miR-20a also functions in a non-cell-autonomous manner in which it could promote tumor angiogenesis by targeting anti-angiogenic proteins thrombospondin-1 (Tsp1) and connective tissue growth factor (CTGF) [48, 49]. This miRNA has been shown to regulate the multiple steps of vascularization, including the expression of angiogenic factors, matrix breakdown, endothelial cell proliferation, cell migration, and vessel formation [50].

Moreover, our data revealed a positive correlation between hsa-miR-31 and Ki-67 ($r = 0.913$, $P < 0.001$), and CD34 ($r = 0.798$, $P < 0.05$). It has been reported that hsa-miR-31 plays an imperative role in the carcinogenesis and its overexpression promotes colorectal cancer.

Hsa-miR-31could intervene tumor angiogenesis through targeting FIH1 (factor inhibiting hypoxia-inducible factor 1). FIH1 is a multi-functional hydroxylase with downstream targets including HIF1A (hypoxia-inducible factor 1 alpha) and NOTCH. Suppression of FIH1 by hsa-miR-31 potentially could up-regulate HIF1A, resulting in up-regulation of VEGF and promoting angiogenesis [51].

Our findings suggested the prospective molecular pathology based on miRNA dysregulation underlying the cell proliferation and angiogenesis in CRC development.

According to our analysis, hsa-miR-145 was significantly down-regulated in CRC tissues compared to adjacent normal tissues. As well, the expression level of hsa-miR-145 showed a significant reverse correlation with the both immunohistochemical markers Ki-67 ($r = -0.942$, $P < 0.001$) and CD34 ($r = -0.836$, $P < 0.001$). A number of studies showed that hsa-miR-145 was down-regulated in several types of cancers including colon cancer [52–54].

HIF-1a, VEGF, and ribosomal protein S6 kinase beta-1 (p70S6K1), as three genes with essential roles in angiogenesis and tumor development and progression [39, 41], have previously been established to be targeted by hsa-miR-145 [54, 55]. Moreover, p70S6K1 overexpression in colon cells expressing hsa-miR-145 restores hsa-miR-145-inhibiting HIF-1a and VEGF levels, suggesting this miRNA could control HIF-1a and VEGF expression by targeting p70S6K1.

Taken together, our results supported this hypothesis that hsa-miR-145 could inhibit tumor growth and angiogenesis by targeting p70S6K1 followed by regulating HIF-1a and VEGF expression [56–58]. However, additional investigations are needed to validate our findings and elucidate the possible mechanisms of miRNA regulation during colorectal tumorigenesis.

## Conclusion

In conclusion, the miRNA expression levels correlate with various biological processes including cell proliferation and angiogenesis. MiRNAs potentially contribute to CRC development through regulating various key angiogenic or oncogenic signaling pathways. On the other hand, the pattern of miRNA expression and its correlation with histological markers are valuable for cancer detection and predict a prognosis in patients with CRC.

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**Compliance with Ethical Standards** This project was approved by the ethical and research committee (IR.IUMS.REC 1394.26649).

**Conflict of Interest** The authors declare that they have no conflict of interest.

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