HHS Public Access

Author manuscript

Future Oncol. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

Future Oncol. 2015 June ; 11(12): 1791–1808. doi:10.2217/fon.15.83.

An update on miRNAs as biological and clinical determinants in colorectal cancer: a bench-to-bedside approach

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Abstract

Colorectal carcinogenesis represents a sequential progression of normal colonic mucosa from adenoma to carcinoma. It has become apparent that miRNA deregulation contributes to the initiation and progression of colorectal cancer (CRC). These oncogenic or tumor-suppressive miRNAs interact with intracellular signaling networks and lead to alteration of cell proliferation, apoptosis, metastasis and even response to chemotherapeutic treatments. This article aims to review the cutting edge progress in the discovery of the role of novel mechanisms for miRNAs in the development of CRC. We will also discuss the potential use of miRNAs as biomarkers for early diagnosis and prognosis of CRC. Furthermore, with advancements in RNA delivery technology, it is anticipated that manipulation of miRNAs may offer an alternative therapy for CRC treatment.

Keywords

carcinogenesis; clinical application; colorectal cancer; diagnosis; miRNA; predictive biomarkers; prognosis

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Financial & competing interests disclosure

This work was supported by grants R01 CA72851, CA181572, CA184792 and U01 CA187956 from the National Cancer Institute, NIH, a pilot grant from Charles A Sammons Cancer Center, and funds from the Baylor Research Institute to A Goel. This work was also supported by grants from the National Natural Science Foundation of China (no. 81372615 and no. 81201884), Shanghai Science and Technology Development Fund (no. 12140902300 and no. 12410707400), Shanghai Health System Outstanding Young Talent Training Plan (no. XYQ2013118) and Shanghai Tenth people's Hospital CLIMB project for outstanding Young Clinical Investigators (W Weng). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

Colorectal cancer (CRC) is one of the leading malignancies in the western countries, and is the second leading cause of cancer-related deaths in the USA. The pathogenesis of CRC usually follows a protracted stepwise sequence from benign polyps to malignant adenocarcinoma and distant metastasis [1]. Considering that it may take up to a decade or longer for this disease to fully develop and it is a truly preventable disease if diagnosed early, there is an urgent need for developing potential biomarkers for early detection and disease prevention, as well as therapeutic targets for treatment, leading to an overall improvement in reducing the mortality burden a ssociated with this disease.

Although the etiology of CRC is multifactorial, genetic and epigenetic alterations remain the central theme and fundamental mechanisms underlying its pathogenesis. The aberrant expression or deregulation of oncogenes and tumor suppressor genes leads to the malignant transformation of normal intestinal epithelial cells by several molecular processes including cell proliferation, a poptosis, invasion, angiogenesis, multidrug resistance and maintenance of genetic and genomic stability [2].

Discovery of miRNAs has opened a new frontier of cancer research and provides an unprecedented potential for the development of diagnostic biomarkers, as well as identification of novel miRNA-based treatment targets that can influence downstream genes and signaling pathways. A growing body of published studies has recently shown that miRNAs are involved in regulation of cancer-related genes through post-transcriptional processing, indicating that miRNAs play an important role during colorectal carcinogenesis. In this review, we will discuss global differences in miRNA expression in colorectal tumors, followed by discussion for the function of specific miRNAs in carcinogenesis and their potential diagnostic, prognostic and therapeutic applications for the management of CRC in the clinic.

miRNA expression is frequently & consistently altered in CRC

Regulation of normal function of intestinal epithelial cells is controlled by miRNAs. Therefore, it is not surprising that dysregulation of miRNA expression has been linked to various human cancers. Several groups have reported that global miRNA expression patterns are frequently altered in CRC [3,4]. Based upon hundreds and thousands of scientific studies, several miRNA-related databases have been curated, such as miRNA body map analysis [5] and Human MicroRNA Disease Database or HMDD [6], which hold expression profiles of miRNAs from several hundred patients with CRC and normal healthy subjects. The differential expression of selected miRNAs in tumor versus healthy, control tissues is clearly highlighted in these databases. In this review, we searched NIH’s PubMed database for literature searches from 2012 to 2015, and have summarized all the key miRNAs that have been reported to be differentially expressed in CRC (Table 1). Based on these expression studies, the aberrant expression of miRNAs and their downstream intracellular gene targets, provide us with the global landscape of molecular events that contribute to colorectal carcinogenesis, as well as highlight the potential of specific miRNAs that can be used as candidates for development of disease biomarkers.

The functional role of miRNAs in the initiation of CRC development

Just in a short time span during the last decade, plethora of scientific studies have been gathered that described a mechanistic role for altered miRNAs expression patterns in the initiation of CRC. Notwithstanding the challenge to adequately review each of these studies within the scope of this review article, we will summarize some of recent important findings on specific miRNAs and their critical role in CRC development (Table 1 & Figure 1).

One of the first miRNAs to be implicated in colon cancer was miR-143. miR-143 is particularly interesting because it possesses tumor suppressive activity and its expression is substantially reduced in several human cancers, especially in CRC [45,63–64]. Since downregulated miR-143 expression can frequently precede *APC* gene mutations, it is possible that miR-143 downregulation is essential for the initiation of CRC development [65]. Furthermore, reduced expression of miR-143 was also observed in dextran sulfate sodium (DSS)-induced chronic inflammation mouse model [66]. As inflammation is known to provide a procarcinogenic milieu for tumorigenesis, decreased levels of miR-143 indicate an early step in malignant transformation of normal colonic epithelium. It is also noted that constitutively active KRAS mutations, which occurs in half of all CRCs, repress miR-143 expression via its downstream effector RREB1 [67], suggesting that downregulation of miR-143 is indispensable for the subsequent CRC development. In addition, other studies have implicated tumor suppressive role of miR-143 by uncovering its target genes such as IGF-IR [43], TLR2 [45], D44, KLF5, KRAS and BRAF [68], and DNMT3A [69].

Recently, miR-31 has been shown to contribute to the initiation of CRC. Necela *et al.* [70] used quantitative PCR arrays to compare miRNA expression in tumors and normal colonic epithelial cells isolated from distal colons of chronically-inflamed mice and APC(Min/+) mice. miR-31 expression was upregulated in all these tumor types. Furthermore, miR-31 levels were found to be increased during inflammatory bowel disease-associated neoplastic transformation, suggesting that the elevated levels miR-31 is indicative of probably an early event in CRC development.

Colorectal carcinogenesis follows a stepwise sequence where normal colonic epithelium transforms into adenomatous polyps followed by progression to malignant carcinoma. Revealing temporal pattern of miRNAs expression alterations during progression in the well-established normal-adenoma-adenocarcinoma sequence will help us to understand the functional role of miRNAs in the initiation of CRC development. Bartley *et al.* [71] analyzed 69 matched specimens of adenomas with low and high grade dysplasia, adenocarcinomas and normal mucosa by using miRNA microarrays. These investigators clustered differentially expressed miRNAs into two patterns of early (type 1) and late (type 2) expression pattern. Type 1 pattern showed miRNAs that were differentially expressed between normal mucosa and low-grade dysplasia, while type 2 pattern revealed miRNAs alterations between late stage versus early stage CRCs. Since Wnt signaling is considered one of the driver pathways in colorectal carcinogens, these authors also analyzed the correlation between expression of type 1 miRNAs and β-catenin localization in the cancer cells. Not surprisingly, they discovered that expression alterations in type 1 miRNAs preceded nuclear β-catenin accumulation, and some of these miRNAs targeted various

component of Wnt signaling pathway. APC mutations are known to trigger Wnt signaling activation. However, since APC mutations are uncommon in colorectal adenomas, these type 1 pattern miRNAs targeting Wnt signaling probably contributed to the initiation of CRC. Another group used similar strategy to compare miRNA expression in cancer tissues and colon tissues from chronically inflamed mice and APC (Min/+) mice. Eight miRNAs were differentially expressed in tumors due to presence of germline APC mutations, while expression of other seven miRNAs was altered due to chronic inflammation [70]. Among these miRNAs, miR-215 and miR-133a were found to belong to type 1 pattern miRNAs, suggesting these two miRNAs have important role in the initiation of CRC development. In addition, the effect of DNA mismatch repair (MMR) status on miRNA expression pattern has also been of interest considering MMR-deficient cancers constitute approximately 15% of all neoplasms, and these patients undergo different clinical management [72]. One of the studies showed that several miRNAs in early stage of disease are important in both MMR-deficient and MMR-proficient CRC. However, four miRNAs (miR-31, miR-552, miR-592 and miR-224) were differentially expressed in MMR-deficient and proficient CRC [73]. Our group also found that Lynch syndrome CRCs displayed a unique miRNA profile compared with sporadic microsatellite instability tumors, suggesting MMR status affects miRNA expression pattern in the transition from normal epithelia to colonic neoplasia [74].

miRNAs affect the invasion & metastasis in CRC

• Angiogenesis

Angiogenesis, which represents the growth of new blood vessels, is an essential component for CRC growth and distant metastasis [75]. Furthermore, tumor [76] angiogenesis is a multi-step process precisely regulated by a number of pro- and anti-angiogenic molecules. In the past decades, several studies have identified a number of growth factor receptor pathways that promote tumor angiogenesis. One of the major pathways involved in this process is the VEGF family of proteins and receptors. Previous studies demonstrated a role for miRNAs in the regulation of angiogenesis and the VEGF pathway [77]. miR-126 was reported to regulate angiogenesis by interfering with VEGF signaling pathway in different cell types [78–81]. In CRC, lower miR-126 expression was associated with poor survival [38,82]. Reduced miR-126 expression was in part caused by promoter methylation of its host gene, EGFL7. Treatment with 5-aza-CdR restored miR-126 expression in cancer cell lines and thereby led to decreased VEGF expression, suggesting miR-126 may be a potential therapeutic target for CRC [38]. Other studies have reported several important miRNAs such as miR-27b [29], miR-143 [44] and miR-145 [51], all of which interact with components of VEGF signaling pathway in CRC angiogenesis. Collectively, discovery of pro- and antiangiogenic miRNAs may possibly be used as diagnostic markers and potential therapeutic targets in the near future.

• Cell invasion

During CRC development, neoplastic cells may acquire the ability to invade or spread to distant organs through complex processes including directional activation of proteolytic enzymes, epithelial-to-mesenchymal transition (EMT) and translocation of cancer cells. Matrix metalloproteases (MMPs) belong to zinc-dependent protease family, and are

responsible for pericellular proteolysis for the regulation of neoplastic invasion. One such miRNA, let-7c was found to directly destabilize the MMP11 mRNA, leading to the suppression of cellular migration and invasion in CRC cells [24]. With forced expression of miR-146a in CaCo-2 and HT-29 colon cancer cells, a decrease in mRNA and protein expression levels of MMP-16, and a lower gelatinase activity in a gelatin zymography was also observed [52]. EMT is a highly conserved cellular process which allows polarized, immotile epithelial cells to transform into motile mesenchymal cells. CRC cells undergoing EMT may represent higher metastatic potential and stem cell characteristics. A reversible biological process called mesenchymal-to-epithelial transition (MET), is also an important step for tumor metastasis since MET allows colon cancers to regain epithelial properties and facilitates spread of metastatic cells to distant organs. miR-200 cluster was reported to be involved in the regulation of EMT-MET plasticity in colon cancer. Our group found liver metastasis tissues showed higher expression of miR-200c compared with primary CRCs. The invasive front in primary CRC tissues revealed low expression of miR-200c, and induced overexpression of miR-200c in colon cancer cells contributed to MET through increased E-cadherin and reduced vimentin expression [83]. Another group showed the expression pattern of miR-200 family in EMT and MET in the metastasis cascade. These investigators found decreased expression of miR-200 family members at the invasive front of adenocarcinomas but increased miR-200 level at regional lymph node metastases and vascular carcinoma. Furthermore, the expression of miR-200 was uniform from the tumor core to the tumor-normal interface. These results revealed miR-200 was downregulated when cancer cell underwent invasion, but its expression was restored at metastatic sites [84]. Ascl2, a downstream target of Wnt signaling, is known to regulate EMT and MET programs in colon cancer cells. However, the mechanisms underlying this process still remain unknown. Recently, Tian *et al.* found Ascl2 to regulate EMT-MET plasticity through modulation of various miR-200 family members including, miR-200a/b/c, miR-429 and miR-141 [85]. Another key miRNA involved in EMT is miR-34a. It was reported that miR-34a and its target gene snail1, which is an EMT driver gene, forms a forward-feedback loop to promote epithelial–mesenchymal transition in CRC [86]. Likewise, another group revealed an active IL-6R/STAT3/miR-34a loop for EMT in CRC cell lines, and also found upregulation of p-STAT3, IL-6R and Snail in colitis-associated intestinal tumors in miR-34 deficient mice [87].

- **Intravasation, circulation & extravasation**

As a part of metastatic process, CRC cells must enter the bloodstream, defined as intravasation, followed by their dissemination to other parts of the body. The precise mechanisms underlying this process require collagenases, MMPs, urokinase plasminogen activator (uPA), uPA receptor (uPAR) and EMT [88]. Therefore, miRNAs that can modulate cellular invasion are also involved in regulating CRC cell intravasation. Oncogenic miR-21, for example, plays a role in intra vasation through downregulation of the tumor suppressor Pdcd4 [89]. In cancer cells, CD151 was reported to regulate adhesion-dependent signaling and postadhesion events, including intravasation and extravasation [90]. Several miRNAs including miR-22 [91], miR-124 [92] and miR-506 [93] were reported to target CD151 and subsequently cause inhibition of CD151 expression. Furthermore, other essential enzymes

required for more efficient intravasation including collagenases, and extracellular matrix proteins such as collagen and laminin gamma-1 were found to be targeted by miR-29c [94].

Once tumor cells enter the bloodstream, they utilize specific mechanisms to avoid shear stress and immune system attack [95]. Cancer cells can be eliminated by innate immune cells like natural killer cells. Currently, tumor-derived exosomes are shown to be of functional relevance for tumorigenesis, as these evade immune attack. Since these vesicles derived tumor cells contain biological active miRNAs which target components critical for the function or maturation of immune cells. In breast cancer, for example, tumor-derived exosomes containing miR-146a, miR-29a and miR-21 affect lymphocyte development and function [96]. In CRC, exosome-associated miRNAs including let-7a, miR-1229, miR-150, miR-21, miR-23a and miR-1246 were expressed much higher than healthy subjects [97]. Among these miRNAs, miR-23a was found to be a strong repressor of transcription factor BLIMP-1 which promotes cytotoxic T lymphocyte activity [98]. In addition, miR-21 was shown to be a negative mediator of signal transduction downstream of TCR in T-lymphocytes. miR-21 inhibition revealed enhanced IFN-Γ production and strong activation in response to TCR engagement [99]. HLA-G is a ligand for both natural killer cells and cytotoxic T lymphocyte, which promotes the activity of both types of immune cells. Recently, miRNAs including miR-148 family and miR-133a have been identified to target HLA-G, which prevents malignant cells from elimination by immune effector cells [100]. Likewise, miR-155 is required for adaptive and innate immunity and miR-17 cluster for adaptive differentiation of B cells and conventional T cells [101]. Furthermore, loss of miR-155 accelerated tumor growth, as well as simultaneously impaired activation of tumor-associated macrophages [102]. Taken together, miRNA-based immune therapy may provide a promising treatment for CRC patients in the near future.

So far, a complete and comprehensive knowledge with regards to the mechanistic role of miRNAs in extravasation is poorly understood. Recently, miR-214 was found to modulate extravasation in melanoma. In an *in vitro* assay, miR-214 overexpression in melanoma cells resulted in a two- to three-fold more efficient transendothelial migration. *In vivo*, CMRA-labeled miR-214 overexpressing or silenced cells were injected via tail vein in nude mice, causing a two- to three-fold increased extravasation for miR-214 overexpressing cells and approximately 50% reduction in extravasation ability in miR-214-silenced cells, compared with controls [103]. Similar results and mechanistic data have been observed for miR-31 in breast cancer [104].

- **Metastatic colonization**

Metastatic colonization is the final step during cancer progression. In CRC, circulating cancer cells predominantly affect and lodge in the liver, but rarely in the bone or brain. This is essentially due to direct portal seeding from the colon and rectum to the liver, as well as the unique features of hepatic blood flow that pivotally contribute to this metastatic pattern [105,106]. Additionally, several other studies have demonstrated a role for the chemokine receptor CXCR4, CCR6 in the dissemination of CRC to the liver [107–109]. miR-126 was found to be downregulated in metastatic colon cancer cells, and overexpression of miR-126 inhibited colon cancer cell viability and reduced tumor cell migration and invasion capacity

through negative regulation of CXCR4 expression [35]. Based on the 'seed and soil' theory [110], cancer stem cells may provide the 'seeds' for CRC in the liver. The stem cell properties ensure proliferation of cancer cells or dormancy in a new environment. Ma *et al.* compared miRNA profiles of human embryonic stem cells and CRC cells and found that both types of cells shared the common endogenous miR-26b. miR-26b was downregulated in both ESC and Lovo cells. Over-expression of miR-26b significantly suppressed CRC cell growth and induced apoptosis *in vitro* and *in vivo*. Since miR-26b may regulate stem-cell properties of cancer cells, it has been hypothesized to play a central role in CRC metastasis [111]. Another group isolated populations of cancer stem cells that possessed $CD133^{+}$/$CD44^{+}$ and $CD133^{-}$/$CD44^{-}$ surface phenotypes from SW1116 colon cancer cells. It was interesting to observe that compared with parental cells, cancer stem cells SW1116 showed differential expression of various miRNAs, including miR29a and miR29b. Recently, miR-146a was found to regulate asymmetrical cell division of cancer stem cells via a Snail-miR-146a-β-catenin feedback loop, causing disruption of stem cell pool balance and promoting tumor growth at the metastatic sites [112].

In general, colorectal carcinogenesis results from the cumulative effect of multiple genetic and epigenetic alterations. In past decades, accumulating evidence has shown that miRNAs are involved in carcinogenesis by targeting various onco- or tumor-suppressive genes including CRC. Figure 1 illustrates an overview of CRC pathogenesis, as it relates to the involvement of miRNAs. A better understanding of the biological impact of these key miRNAs on colorectal tumorigenesis, as well as their clinical impact will be crucial for our understanding and management of this malignancy in the future.

Clinical application of miRNAs

• Serum & fecal miRNAs serve as promising biomarkers for CRC diagnosis

As an ideal approach for CRC screening, the method must possess a very high degree of sensitivity and specificity for the early detection of cancer. Since fecal occult blood test (FOBT) is safe and acceptable to patients, it has been widely used as noninvasive screening tool for CRC. However, FOBT has relatively poor sensitivity and specificity for CRC diagnosis and FOBT screen only reduces 16–25% of relative risk associated with CRC-related mortality [113]. In an effort to identify better approaches for CRC screening, miRNAs have emerged as solid contenders for development as noninvasive biomarker substrates. Measurement of expression of miRNAs in circulating blood provides a novel and promising early diagnostic option for CRC screening. Several studies till date have now demonstrated that various human tumors possess unique miRNAs expression profiles in serum or plasma. Furthermore, some circulating miRNAs have been found to be packaged and protected in extracellular vesicles, called 'exosomes' or 'microvesicles'. Therefore, high concentration of miRNAs can be discovered in highly stable, cell-free form in the peripheral blood [114,115].

Recently, several circulating miRNAs have been shown to be potential biomarkers for CRC diagnosis (Table 2). Adenoma is the precancerous lesion in CRC patients, and the early and accurate diagnosis for adenoma may reduce risk of CRC mortality. A panel of multiple plasma miRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b,

miR-532 and miR-652) was found to distinguish polyps from controls with an AUC value of 0.87 [116]. A meta-analysis study compared diagnostic accuracy of currently published circulating miRNAs in discriminating CRC from controls. This study found miR-21 had a higher diagnostic accuracy for CRC diagnosis (AUC = 0.803) [117]. Our group also revealed serum miR-21 expression robustly distinguished adenoma (AUC = 0.81) and CRC patients (AUC = 0.92) from healthy control individuals [118]. Giraldez *et al.* used a genome-wide miRNA expression profiling assay to display 13 differential plasma miRNAs in 123 patients (63 with CRC and 60 with advanced adenomas) and 73 healthy individuals. These miRNAs were then used for validation in an independent cohort of 135 CRC patients. Six miRNAs, including miR-18a, miR-19a, miR-19b, miR-15b, miR-29a and miR335, were confirmed to be significantly upregulated in CRC patients compared with normal controls. The AUC value of each miRNAs' ROC ranged from 0.8 to 0.7 [119]. Although circulating miRNAs are emerging as promising biomarkers for CRC, several technical factors may probably have impact on the accurate measurement of miRNAs including hemolysis, storage temperature and time [120]. Zanutto *et al.* found miR-378 level was not influenced by hemolysis levels of plasma samples [121]. However, Zanutto and our group found miR-21 level correlated with the degree of hemolysis in serum samples. Another factor that influences the results of circulating miRNAs is endogenous control. So far, there is no consensus control that can normalize the value of miRNA, making the results incomparable. Hu *et al.* found miR-1228 was the most stable endogenous control across eight cancer types and three independent control cohorts [122].

Exosomal miRNAs have recently been attracting a lot of interest as potential biomarkers for CRC diagnosis. Exosomes derived from CRC may carry tumor-specific genetic materials that transmit signaling between tumor and stromal cells in the tumor microenvironment. Therefore, serum exosomal miRNAs levels are probably more robust for CRC diagnosis, compared with free circulating miRNAs. A study of 88 primary CRC patients and 11 healthy controls was performed to identify candidate exosomal miRNAs. Seven miRNAs were found upregulated in exosome-enriched fractions of serum samples; however, the level of these miRNA was not dependent on tumor stage. Two of these miRNAs, miR-23 and miR-1246, showed higher sensitivity for detection of stage I CRCs (95 and 90%), compared with CEA and CA-199 (15,10%), suggesting their promising potential as substrates for the development of noninvasive CRC diagnostics [97].

To date, much attention has been focused on miRNAs in tissue and circulation, and to a lesser extent in stool (Table 2). We recently developed a new method called direct miRNA analysis to analyze the fecal miRNA levels. We also reported that fecal miRNAs can be effectively extracted and analyzed from clinical specimens collected in FOBT kits. Furthermore, we noted that miR-21 and miR-106a had a higher expression in patient with adenomas and CRC than in healthy controls [154]. Several other studies have validated these findings and have also reported that miRNAs can be efficiently extracted from stool samples and can be possibly exploited for distinguishing healthy individuals from patients with colorectal adenomas and cancers [123,155–156]. Wu *et al.* reported that stool miR-92a level, but not miR-21, was increased in patients with adenomatous polyps compared with healthy subjects. Moreover, fecal miR-92a expression showed higher sensitivity for distal

compared with proximal CRC, and possessed higher sensitivity for advanced adenomas than minor polyps. Furthermore, decreased levels of fecal miR-92a expression levels were noted after removal of the tumor or advanced adenomas, supporting the specificity of these miRNAs for the presence of a colorectal polyp or cancer [123]. Recently, this research group analyzed 424 stool samples from patients with colorectal adenomas and cancers, inflammatory bowel disease and healthy controls. These assays were performed in both pre- and postsurgery fecal specimens, following up detection after removal of lesions. These results demonstrate the feasibility of fecal-based miR-135b as a potential biomarker for both advanced adenoma and CRC [157].

• miRNA biomarkers for determining prognosis & predicting treatment response in CRC patients

To date, management of CRC is dependent on clinico-pathologic features including TNM stage, histology and tumor margin involvement [158]. Although pathological tumor staging is an important consideration for treatment selection, treatment response among patients with same stage has varied significantly. One of the tragic realities is that the chemotherapeutic drugs that are currently used to treat cancer patients are highly toxic, and their effectiveness varies unpredictably from patient to patient – mainly due to subtle differences in genomic makeup of each patient and the molecular characteristics of their tumor. In particular, development of resistance to chemotherapeutic drugs presents a major challenge in terms of poor response and survival – and remains a critical hurdle while treating patients with advanced CRC. Many of these patients eventually develop resistance to such treatments, leading to tumor relapse or recurrence. In view of this, there is an urgent clinical need for development of tools or (bio)markers that can help stratify which patients will respond to specific drugs or not, so that others can be spared from being needlessly exposed to such toxic and expensive therapeutic drugs, and triaged for alternative treatment options. Accumulation of studies supports the use of molecular biomarkers to refine estimates of prognosis and tailoring of adjuvant and neo-adjuvant treatment to individual patients [159]. The unique miRNA expression patterns in CRC have the potential to be used as prognostic and predictive factors in determining and predicting their clinical outcomes.

Although this field is relatively young, there are several studies that suggest the key role of miRNAs in determining prognosis and predicting responses to chemotherapeutic treatments. EMT-related miRNAs play an important role in CRC progression and metastasis. As mentioned previously, we found that miR-200c modulated EMT in human CRC metastasis [83]. We have performed another three-phase study in which we analyzed 446 colorectal specimens and found serum miR-200c serves as a good prognostic biomarker for CRC. High serum miR-200c was found in stage IV patients compared with its levels in stage I–III CRC patients. Furthermore, serum miR-200c emerged as an independent predictor for lymph node metastasis and tumor recurrence [145]. Diaz *et al.* determined the expression of miR-200c in 127 surgically-resected patients with stage I–III CRC. The higher expression of miR-200c correlated with longer overall survival. Furthermore, in the subgroup of patients treated with fluoropyrimidines, the higher levels of miR-200c showed longer overall survival, as well as improved disease free survival, suggesting miR-200c as a potential predictive biomarker for chemotherapeutic response [152].

Likewise, miR-21 has been shown to be a robust prognostic biomarker for CRC. A meta-analysis revealed that higher expression of miR-21 predicts poor overall survival [160]. The pooled hazard ratios were 1.76 (95% CI: 1.34– 2.32) in this report. Another clinically significant biomarker is miR-625, which was found to be decreased, indicating unfavorable prognosis for CRC patients [161]. Table 2 summarizes some of the recently published miRNA biomarkers that are associated with clinical outcomes in CRC patients. As of now, several miRNA biomarkers have been developed for the predicting therapeutic response in CRC patients. For instance, miRNAs including miR-222 [162], miR-297 [163], miR-19b [164] and miR-224 [165] have been demonstrated to confer CRC cells with multidrug resistant ability in cell culture studies. miR-126 has been known to be an angiogenesis-specific miRNA, and the expression levels of miR-126 were noted to be higher in metastatic CRC patients that responded to capecitabine and oxaliplatin (XELOX) treatment, compared with those that failed to show any response. Furthermore, progression-free survival was 11.5 months in patients with higher expression of miR-126, compared with 6 months in those with lower expression of this noncoding RNA [151]. Other studies have showed that increased levels of miR-10b and miR-192/215 predict response to 5-FU-based chemotherapy while low levels of miR-19a indicate resistance to FOLOFOX treatment. Finally, there are data suggesting that miR-200a, miR-200c, miR-141 and miR-429 expression levels may identify CRC patients who stand to benefit from fluoropyrimidine-based treatments and suffering from CRC [166].

- **miRNAs as potential therapeutic targets in CRC**

A wealth of published data has shown that blocking the expression of specific onco-miRNAs or restoring the levels of suppressive-miRNAs in CRC cells can result in inhibiting tumor growth, angiogenesis and metastasis, as well as enhancement of response to various chemo and radiation therapies – providing a rational for the development of miRNA-based targeted CRC therapeutic strategies. However, the major hurdle in this arena is safe delivery of these therapies to the target tissues with less/ minimal side effects to the surrounding normal tissues. Several viral and nonviral strategies have been reported in previous studies. A novel RNA delivery system, immune-liposomes, is currently being developed for cancer treatment. Immunoliposomes are coupled with antibodies which allow for more efficient and focused targeting of tumor tissues by binding to tumor-specific receptors. The distribution of immune-liposomes in tumor-bearing animals has shown their higher concentration in tumor tissues compared with significantly lower concentrations in normal tissues [167]. Sonoporation is another delivery technology used for gene therapy. During this process, the delivery of the intended therapy is facilitated via creation of temporary pores in the cell membranes induced by ultrasonic waves [168]. The first clinical trial utilizing such a sonoporation and microbubbles approach for local therapeutic delivery was recently published by Kotopoulis and colleagues [169]. Another challenge with miRNA-based therapy is ‘off target’ effects. Since miRNAs target plethora of mRNAs in context of signaling networks, it is possible that undesired toxicity and even immune reactions may manifest, making it more difficult to achieve desired results. Furthermore, rapid degradation and blocked uptake of miRNAs by nucleases or other unknown cellular mechanisms can also potentially affect the clinical impact of such an approach. To be on the safe side, lowest optimal concentrations of miRNAs along with effective target delivery systems need to be

developed and validated prior to considering such tools for safe and efficacious treatment offering for patients with colorectal and other cancers.

Thus far, no clinical trials for miRNA replacement therapy in patients with CRC have been reported. However, *in vivo* studies have shown that miRNAs are promising therapeutic targets for CRC treatment. Liu *et al.* delivered lentiviral-based vectors expressing miR-221 and miR-222 sponges in an animal model of DSS-induced colitis. Treatment with miR-221 and miR-222 significantly decreased tumor number and size. Furthermore, markedly reduced advanced adenomas were found in DSS-mice treated with miR-221/222 [17]. In another study, investigators developed a robust nonviral delivery method for *in vivo* miRNA administration. After systemic or local application of polyethylenimine (PEI)-miR-145/-33a complex, the mouse xenograft tumors were significantly inhibited [170]. Another promising therapy is antiangiogenesis-based miRNA replacement. A special modified, dicetyl phosphate-tetraethylenepentamine-based polycation liposomes (TEPA-PCL), were used as delivery system of miR-92a. After transfection of human umbilical vein endothelial cells (HUVECs) with miR-92a-TEPA-PCL complex, the cytoplasm of HUVECs was filled with miR-29a. Furthermore, HUVECs showed impaired capability of forming capillary tubes, suggesting miR-29a-TEPA-PCL transfection system is a candidate antiangiogenesis treatment for CRC [171].

Conclusion & future perspective

Dysregulation of miRNAs is now being regarded as a critical step for the pathogenesis of CRC. There are convincing data that altered expression of miRNAs facilitates tumor initiation, proliferation and angiogenesis, confers resistance to chemotherapy and enhances metastasis in colon cancer cells through interaction with intracellular signaling networks. From a clinical perspective, there is no doubt that miRNAs will be important diagnostic and prognostic biomarkers for CRC (Table 2 & Figure 2). Given the ever-expanding number of miRNAs, it is a challenge to accurately determine which of these miRNAs can be used for the earlier diagnosis, risk assessment, prognosis prediction and determining response to chemotherapeutic treatments in patients suffering with CRC. In addition, there is a growing level of interest for miRNAs as potential therapeutic targets. miR-21, for example, not only serves as a promising biomarker for CRC but also as potential therapeutic target since miR-21 is involved in both cancer development and immune regulation. Although the safety and efficacy of delivering miRNA therapeutics effectively to the target tumor tissues in miRNA-based therapeutic approaches, there is undeniable body of data already gathered indicating that miRNAs do offer a promising substrate for the development of biomarkers aimed for earlier diagnosis, determining prognosis and predicting response to chemotherapeutic treatments in patients suffering from CRC.

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EXECUTIVE SUMMARY

miRNA expression is frequently & consistently altered in colorectal cancer

- Global miRNA expression patterns are altered in colorectal cancer (CRC), and existing data suggest that these alterations can occur in a cancer-specific manner.
- The functional role of miRNAs in the initiation of CRC development has been studied, and there is a growing interest in associating miRNA-mediated transcriptional repression of downstream target genes.
- Several miRNAs such as miR-143 and miR-31 contribute to the initiation of CRC.
- miRNA expression profiles are altered during progression in the normal adenoma carcinoma sequence.
- MMR status affects miRNA expression pattern in the transition from normal epithelia to colonic neoplasia.

miRNAs affect the invasion & metastasis in CRC

- Specific miRNAs and families promote tumor angiogenesis by targeting VEGF signaling pathways.
- miR-200 clusters mediate epithelial–mesenchymal transition in CRC.
- Several miRNAs contribute to the development of CRC during intravasation, circulation extravasation and metastatic colonization.

Clinical application of miRNAs

- Serum and fecal miRNAs serve as promising biomarkers for CRC diagnosis.
- miRNA biomarkers are being developed for determining prognosis and predicting treatment response in CRC patients.
- Several miRNAs have been identified as potential therapeutic targets for CRC.

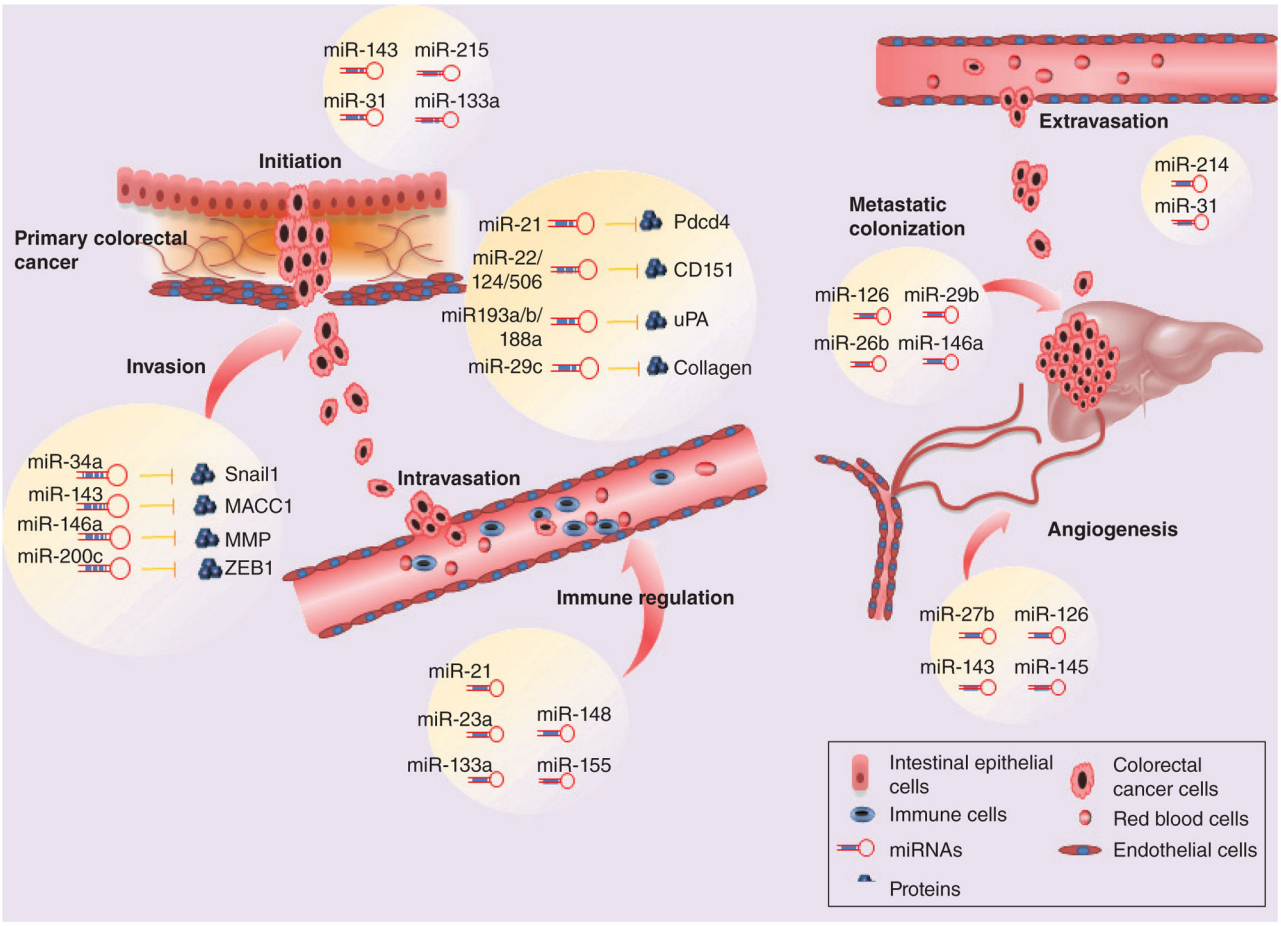

Figure 1. A network of miRNAs involved in the multiple steps of the development of colorectal cancer

Colorectal carcinogenesis follows a stepwise sequence where normal colonic epithelium transforms into adenomatous polyps followed by progression to malignant carcinoma. Specific miRNAs such as miR-143 involve in the initiation of colorectal cancer. Neoplastic cells may acquire the ability to invade or spread to distant through complex processes including directional activation of proteolytic enzymes, epithelial-to-mesenchymal transition and translocation of cancer cells. miR-146 promote invasion via targeting MMP proteins and miR-200 family contributed to epithelial-to-mesenchymal transition. As a part of metastatic process, colorectal cancer cells must enter the bloodstream. miR-21, miR-29c and miR-22, among others, modulate colorectal cancer cell intravasation. Once tumor cells enter the bloodstream, they utilize specific mechanisms to avoid immune system attack. Tumor-derived exosomes containing miR-146a, miR-29a and miR-21 affect lymphocyte development and function. Metastatic colonization is the final step during cancer progression. It was demonstrated miR-126, miR-26b and miR-146 promote tumor growth at the metastatic sites. Furthermore, angiogenesis, which represents the growth of new blood vessels, is also an essential component for colorectal cancer growth and distant metastasis. miR-27b, miR-143, miR-126 and miR-145 were shown as a critical role in the regulation of angiogenesis and the VEGF pathway.

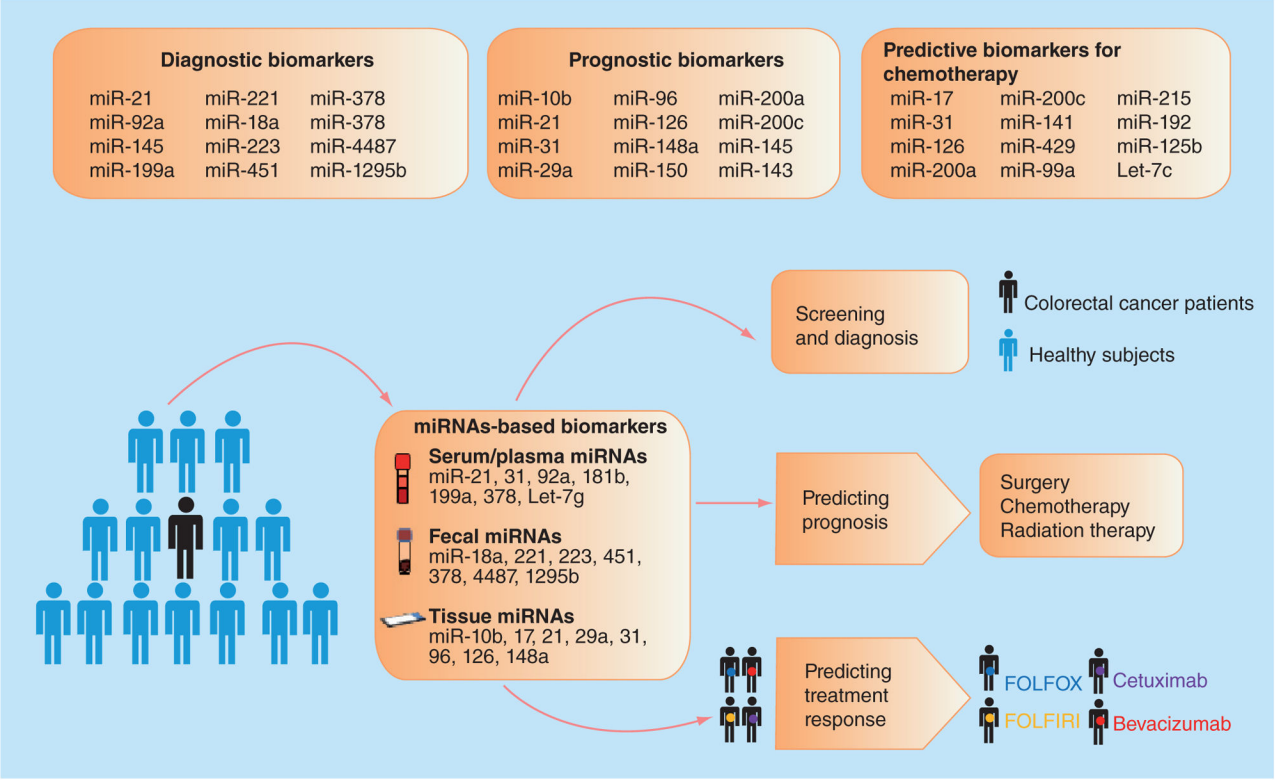

Figure 2. The clinical application of miRNAs in colorectal cancer

Colorectal cancer-related miRNAs can be extracted from tissue, blood or stools samples and analyzed by real-time PCR, microarray or next-generation sequence. These circulating miRNAs, tissue or fecal-based miRNAs provide useful tools of diagnostic and prognostic information.

Table 1

The novel functional role of miRNAs in the colorectal cancer progression.

miRNA	Target genes	Phenotype(s) affected	Ref.
Upregulated miRNAs			
miR-17	*PTEN*	Cell invasiveness	[7]
miR-31	*SATB2*	Proliferation, invasion and migration	[8]
	RASA1	Tumor growth	[9]
	RhoBTB1	Proliferation	[10]
miR-92a	*PTEN*	Migration	[11]
miR-96	*TP53INP1/FOXO1/ FOXO3a*	Proliferation	[12]
miR-103/107	*DAPK/ KLF4*	Cell motility, cell–matrix adhesion and cell–cell adhesion	[13]
miR-130b	*Integrin β1*	Migration and invasion	[14]
miR-153	*FOXO3a*	Cell invasiveness and response to chemotherapy	[15]
miR-181a	*WIF-1*	Migration and invasion	[16]
miR-221/222	*RelA*	Proliferation and colony formation	[17]
miR-224	*PHLPP1/2*	Proliferation, tumor growth	[18]
miR-301a	*SOCS6*	Proliferation, migration and invasion, tumor growth	[19]
miR-362	*E2F1/USF2/PTPN1*	Proliferation, cell cycle	[20]
miR-429	*SOX2*	Apoptosis	[21]
miR-574	*Qki6/7*	Proliferation, migration and invasion, differentiation and cell cycle	[22]
miR-708	*CDKN2B*	Proliferation and invasion	[23]
Downregulated miRNAs			
let-7c	*MMP11*	Migration and invasion	[24]
miR-7	*EGFR*	Response to cetuximab	[25]
	YY1	Proliferation, apoptosis, cell cycle	[26]
miR-18a	*CDC42*	Migration, cell cycle, apoptosis	[27]
miR-27a	*SGPP1*	Proliferation, apoptosis and cell migration	[28]
miR-27b	*VEGFC*	Proliferation, colony formation	[29]
miR-29c	*GNA13*	Migration and invasion	[30]
miR-30a	*IRS2*	Proliferation, migration and invasion	[31]

miRNA	Target genes	Phenotype(s) affected	Ref.
miR-30b	*KRAS*/*PIK3CD*/*BCL2*	Proliferation and tumor growth	[32]
miR-100	*RAP1B*	Cell growth and invasion and induce apoptosis	[33]
miR-124	*PRRX1*	Response to radiosensitivity	[34]
miR-126	*CXCR4*	Proliferation, migration and invasion and cell cycle	[35,36]
	IRS-1	Proliferation, migration, invasion and cell cycle	[37]
	VEGF	Migration and invasion, tumor growth	[38]
	RhoA/*ROCK*	Cell cycle, invasion, tumor growth	[39]
miR-133b	*CXCR4*	Invasion and apoptosis	[40]
miR-139	*AMFR*/*NOTCH1*	Migration and invasion	[41]
	NOTCH1	Tumor growth	[42]
miR-143	*IGF1R*	Proliferation	[43]
	IGF1R	Proliferation, migration, angiogenesis and chemosensitivity	[44]
	TLR2	Invasion and migration	[45]
	HK2	Metabolism	[46]
	MACC1	Invasion and migration	[47]
miR-144	*mTOR*	Proliferation, response to rapamycin	[48]
miR-145	*fascin-1*	Proliferation, migration and invasion	[49]
	catenin δ-1	Tumor growth	[50]
	N-RAS, *IRS1*	Proliferation, migration and invasion	[51]
miR-146a	*MMP16*	Migration and invasion	[52]
miR-185	*STIM1*	Migration and invasion	[53]
miR-199a	*DDR1*	Proliferation, colony formation, cell cycle, invasion and migration	[54]
miR-202	*ARL5A*	Tumor growth	[55]
miR-203	*TYMS*	Response to 5-fluorouracil	[56]
miR-212	*MnSOD*	Migration and invasion	[57]
miR-375	*PIK3CA*	Tumor growth	[58]
miR-378	*VIMENTIN*	Tumor growth and invasion	[59]
miR-399	*MDM2*	Apoptosis, senescence, migration and invasion	[60]
miR-455	*RAF1*	Proliferation and invasion	[61]
miR-497	*IGF1-R*	Proliferation, invasion and apoptosis	[62]

Upregulation and downregulation of miRNAs is defined by differential expression between cancer vs matched normal tissues.

Table 2

The clinical implication of miRNAs in colorectal cancer.

miRNA	Specimen	Dysregulation	n	Remark	Ref.
Diagnostic biomarkers					
miR-21	Serum	↑	49	CRC vs healthy control	[117]
miR-92a	Feces	↑	246	Higher sensitivity for advanced adenoma than minor polyps	[123]
miR-92a	Serum	↑	697	Early stage of colorectal cancer	[124]
miR-145	Tissue	↓	202	Lymph node metastasis	[125]
miR-199a	Serum	↑	116	Associated with deep wall invasion	[126]
miR-221/18a	Feces	↑	595	CRC vs healthy control	[127]
miR-223/451	Feces	↑	45	CRC vs healthy control	[128]
miR-378	Serum	↓	181	CRC vs healthy control	[121]
miR-4487/1295b	Feces	↓	56	Decreased in early stage (I/II)	[129]
miR-21/let-7g	Serum	↑	202	Diagnosis of CRC, with relatively high sensitivity and specificity compared with CEA and CA-199	[130]
miR-31/92a/181b/203		↓			
miR-126/141/21	Serum	↑	224	Early stage liver-metastatic CRC	[131]
miR-409-3p	Plasma	↑	241	Early stage of CRC	[132]
miR-7/93		↓			
miR-18a/19a/19b/15b/29a/335	Plasma	↑	392	Early diagnosis of CRC	[119]
Prognostic biomarkers					
miR-10b	Tissue	↑	88	Associated with poor prognosis	[133]
miR-10b	Serum	↑	169	Associated with distant metastasis	[134]
miR-17	Tissue	↑	96	Associated with poor prognosis	[135]
miR-21	Tissue	↑	301,764	Associated with poor prognosis	[136,137]
miR-21	Serum	↑	342	Associated with poor prognosis	[118]
miR-31	Tissue	↑	1353	Associated with BRAF mutation and poor prognosis	[138]
miR-29a	Tissue	↓	169	High risk of recurrence in stage II colon cancer	[139]
miR-96	Tissue	↓	80	Associated with poor prognosis	[140]
miR-126	Tissue	↓	92	Associated with poor OS	[82]

miRNA	Specimen	Dysregulation	n	Remark	Ref.
miR-148a	Tissue	↓	273	Prognostic/predictive biomarker for stage III and IV CRC	[141]
miR-148a	serum	↓	30	Predictive for the risk of recurrence of early stage colon cancer	[142]
miR-150	Tissue	↓	239	Associated with poor prognosis	[143]
miR-200a	Tissue	↓	328	Associated with poor prognosis	[144]
miR-200c	Serum	↓	446	Prediction for metastasis	[145]
miR-145/31	Tissue	↑	1141	Associated with advanced CRC	[146]
miR-200b/215/451a		↓			
miR-532-3p/331/195/17/142-3p/15b/532/652	Serum	↑	128	Distinguished polyps from controls	[116]
miR-431/15b/139-3p		↑		Distinguished stage IV CRC from controls	
miR-21/20a/miR-103a-/106b miR-143/215	Tissue	↑ ↓	638	Associated with poor prognosis	[147]
Predictive biomarkers for chemotherapy					
miR-17	Tissue	↑	425	Poor response to chemotherapy	[148]
miR-31	Tissue	↑	102,87	Anti-EGFR therapy, associated with shorter PFS	[149,150]
miR-126	Tissue	↓	89	Associated with responding to XELOX	[151]
miR-200a/200c/141/429	Tissue	↓	127	Higher level associated with longer OS and DFS in stage II patients treated with fluoropyrimidines	[152]
miR-99a/Let-7c/miR-125b	Tissue	↓	183	Selection of patients with *KRAS* wild-type mCRC	[153]

CRC: Colorectal cancer; DFS: Disease-free survival; mCRC: Metastatic colorectal cancer; OS: Overall survival; PFS: Progression-free survival.