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REVIEW ARTICLE

The role of miRNA -31-3p and miR-31-5p in the anti-EGFR treatment efficacy of wild-type K-RAS metastatic colorectal cancer. Is it really the next best thing in miRNAs?

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Summary

Colorectal cancer (CRC) is one of the most common cancers worldwide with a high incidence and mortality. Although many treatment options are available in stage IV disease, the clinical outcome is still minimal. The primary treatment problem in metastatic colorectal cancer (mCRC) is early liver metastases that occur in more than 50% of patients. First-line treatment in mCRC is a combination of chemotherapy plus targeted therapies like Cetuximab or Bevacizumab, depending on K-RAS status. The decision of which regimen to choose is difficult because almost half of the patients do not receive second-line treatment due to complications or death. To avoid exposing non-responding patients to inefficient and harmful therapies new robust biomarkers are needed. Ongoing studies have demonstrated constantly that microRNAs (miRNAs) could become suitable biomarkers for screening and treatment response. In CRC, miR-31-3p and miR-31-5p dysregulation seems to have a particular role in evaluating treatment response from anti-EGFR therapy.
In this review, we will present up to date information on the role of miRNA-31-3p and miR-31-5p in CRC with a particular focus in treatment response of metastatic K-RAS wild-type CRC treated with anti-EGFR molecules.

Key words: *anti-EGFR, biomarker, miRNA-31, metastatic colorectal cancer, treatment response, wild-type K-RAS*

Introduction

CRC still represents a major problem of heath worldwide. The latest data of GLOBOCAN for 2018 show more than 1.8 million new cases of CRC and about 881.000 deaths [1]. CRC incidence ranks third in men and second in women, and fourth in men and third in women in mortality. One in four patients at diagnosis will present liver or lung metastases, and half of the patients who get operated will develop metastases some time during their follow up. With a survival rate of about 30 months in metastatic disease, CRC represents a significant health problem, even though survival rates continue to increase [2,3].

Metastases are the central motif for mortality in stage IV CRC patients. In the early stages, surgery alone or surgery associated with chemo-

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therapy can be performed with curative intent [4]. There are a handful of target therapies in stage IV CRC. Bevacizumab, a vascular endothelial growth factor (VEGF)-targeted therapy, and also Cetuximab or Panitumumab which are anti-epidermal growth factor receptor-targeted therapies, while when associated with chemotherapy are very active. Guidelines recommend association of 5-Fluorouracil, Oxaliplatin, Irinotecan and Capecitabine in different sequences for mCRC [5-7].

In first-line mCRC treatment, the use of anti-EGFR antibodies Cetuximab and Panitumumab is dependent on K-RAS wild-type status. Patients who are K-RAS mutated do not have any clinical benefit from anti-EGFR therapy [2]. The objective response of patients with wild-type K-RAS tumors who receive anti EGFR treatment is about 70%, while the remaining 30% do not benefit from this therapy [8]. Moreover, clinical oncologists have to consider the common side effects of Cetuximab and Panitumumab. Skin toxicity [9] represents the most significant side effect of these therapies. Because toxicity decreases the quality of life, exposing patients to inefficient treatment it should be avoided with the usage of personalized approaches.

Despite modern management, monitoring and surveillance performed by using imaging techniques and cancer-specific markers, metastases of CRC are hardly identified [10]. Classical screening methods and prognostic markers are in continuous development, but more drastic measures are in need for early detection and stratification of patients.

Ongoing research data proposed a series of biomarkers and molecular targets that could be useful in clinical practice for monitoring and treatment stratification. Part of useful biomarkers include: carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA 19-9), microsatellite instability (MSI), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS), tumor protein p53 to circulating DNA, BRAF, PIK3CA, PTEN, EGFR, HER-2 amplification, epiregulin and amphiregulin overexpression, circulating tumor cells (CTCs) and miRNAs. However, many of the markers presented above are not currently in use in the day-to-day patients care because of their cost and lack of sensitivity and specificity. Moreover, their detection and assessment often represent a big issue [4,11].

Considering their high stability in tissue, blood, and urine, miRNAs have attracted a great deal of attention as a new class of biomarkers. They are small noncoding RNA molecules that regulate protein-coding gene expression in physiological status, but by their alteration of expression, they can contribute to developing of many pathologies, including cancer [12]. Previous data have demonstrated that miRNAs could be used as a unique class of accurate biomarkers to characterize tumor phenotype and its evolution [13]. On this line, alteration of expression of both forms of mature miR-31 including miR-31-3p and miR-31-5p [14] are associated with advanced CRC and inadequate response to anti-EGFR therapy [15,16]. Moreover, recent discoveries have shown that these biomarkers could be considered as stable indicators of effective treatment of stage IV CRC [17].

In this review, we focused on evidence about the predictive role of miR-31-3p and miR-31-5p in wild type K-RAS mCRC anti-EGFR therapy. We critically assessed their role for evaluating the opportunity of using Cetuximab or Panitumumab in first-line chemotherapy schedules, taking into consideration both the lack of objective response and the significant side effects.

Short overview about miRNAs

MiRNAs (miRNA, miR) are short (20-25 nucleotides in length) non-coding RNAs [13]. They could be called as master modulators of the human genome because they are responsible for negative regulation of the expression of about 50-60% of protein-coding genes (PCG) [18]. Important features of miRNAs consist of their multi-target capacity to modulate up to 200 mRNAs, but also a single mRNA target can be modulated by different miRNAs [19,20]. MiRNAs are key players in maintaining the physiological status of normal cells, but by altering their expression by gain or loss of function, miRNAs are responsible for developing pathological status, including cancer. In cancer, two classes of miRNAs, defined as tumor suppressor miRNAs (TS-miR) and oncomiRs are responsible for alteration of gene expression for the two most important classes of PCG in cancer, known as oncogenes and tumor suppressor genes, leading to proliferation, metastasis and drug resistance [21].

Considering the crucial role of miRNAs in cell cycle regulation, a worldwide research effort has been undertaken to identify and characterize as many miRNAs as possible. Currently, there are about 2815 mature human miRNAs included in the latest data of miRNA database, miRBase Release 22 (http://www.mirbase.org/). The biogenesis of miRNAs is quite complex, involves many cellular pathways [22], and it is conducted in multiple phases. First, miRNA biogenesis starts in the nucleus, by RNA polymerase II, where is synthesized a primary miRNA precursor (pri-miRNA) of hun-

dreds or thousands of nucleotides. Pri-miR is then processed by the ribonuclease RNase III enzyme (Drosha) and DGCR8 (DiGeorge syndrome critical region 8) protein in a smaller transcript (~70 nucleotides), called pre-miRNA. After that is transferred into the cytoplasm by nuclear receptor exportin-5, pre-miRNA is processed by Dicer complex enzymes to a mature miRNA duplex of about 20-25 nucleotides and then to a single-stranded mature miRNA. To become active, mature miRNA is then loaded in Argonaut protein (AGO2) and RNA-induced silencing complex (RISC). After that is loading in RISC, miRNA will function as a guide to identify a specific area in the 3' untranslated region (UTR) of mRNA transcripts by sequence complementarity. This miRNA-mRNA binding leads to translational repression or mRNA degradation, and this process is also known as RNA interference (siRNA) (Figure 1) [23].

The role of miRNA-31-3p and miRNA-31-5p in human cancers

MiRNA-31, including its mature forms miRNA-31-3p and miRNA-31-5p, has a dual role, both oncogenic and tumor-suppressing, being disrupted in many human cancers [24]. MiR-31 is involved in the migration and invasion in breast and colorectal cancers [25,26]. In CRC, miR-31 activates the RAS signaling pathway by inhibiting the RAS p21 GTPase activating protein 1. This property confers cancer cell growth and stimulates tumorigenesis. High expression of miR-31 is correlated with advanced disease and worst clinical outcome in metastatic CRC [14,27].

The association of miR-31 with K-RAS or BRAF pathway in CRC was previously mentioned by Nosho et al [28] and recently by Lundberg et al [29]. Kent and Joshua also stated that high expression of miR-31 is associated with K-RAS and BRAF mutation in pancreatic cells [30]. In cervical cancer, miR-31-3p overexpression was associated with clinical response [31] while high levels of miR-31-3p were found in breast cancer-associated fibroblasts (CAF) compared with normal fibroblasts [32].

Both mature forms of miR-31, miR-31-3p and miR-31-5p, are up-regulated in oral cancers. A meta-analysis included seven independent studies of miRNAs in cancer tissues and matched non-cancer tissue, pointed out the role of both miR-31-3p and miR-31-5p as biomarkers for a specific signature of head and neck cancers [33]. MiR-31 plays an intricate role in human cancer function both as oncomiR and tumor suppressor miR (TS-miR) (Figure 2).

The molecular mechanisms involving miR-31 are not completely elucidated. However, as it has been extensively presented in recent studies [34,35], miR-31 acting as a TS-miR, targets genes involved in specific pathways like AR (androgen re-

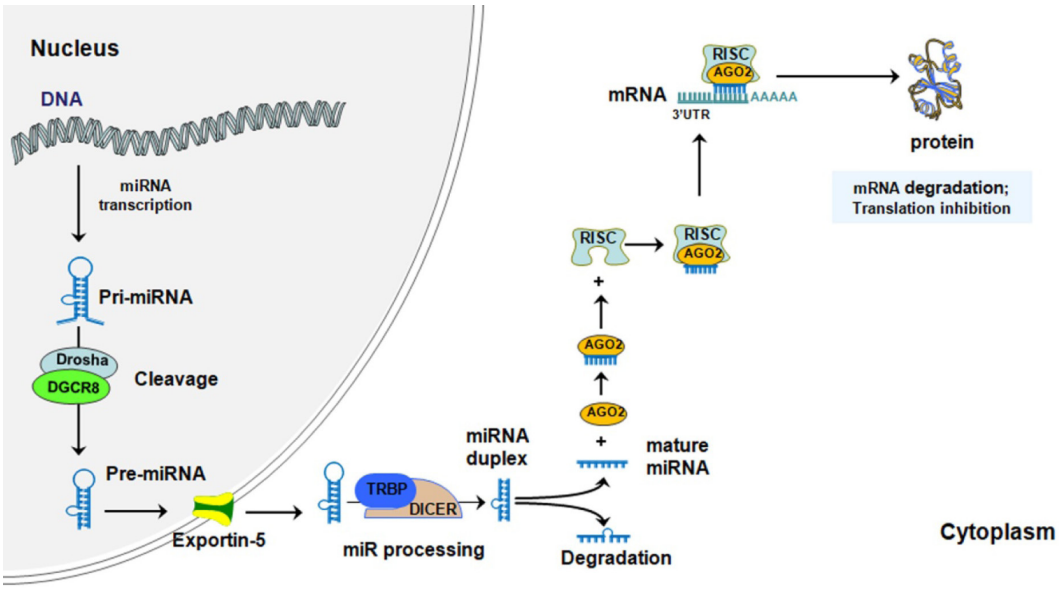

Figure 1. MiRNA biogenesis. The biogenesis of miRNA starts in the nucleus, where is synthesized a long primary miRNA (pri-miRNA) precursor. Further, pri-miRNA is processed to a shorter pre-miRNA of about 70 nucleotides followed by its export in the cytoplasm by Exportin-5 protein. In the cytoplasm, pre-miRNA is processed to a short miRNA duplex with a long hairpin transcript called pri-miRNA that is further processed to a smaller transcript of 70 nucleotides. After it is exported in the cytoplasm, pre-miRNA is processed to a mature miRNA and loaded in an enzymatic complex including AGO2 and RISC enzymatic complex. By targeting the 3' UTR of mRNA target, miRNA-RISC will coordinate translational repression or mRNA degradation.

ceptor), cell cycle, DNA repairing, PI3K/AKT, Rho/Rock and NF-κB. On the opposite side, miR-31 has an oncogenic role, whose gain of function leads to activating of several pathways, including WNT, HIF, MEK5/ERK5, TGF-β/BMP, Hippo, Rac1, NF-κB and RAS/MARK/ERK1/2.

Although the role of miR-31 was associated with colorectal tissues, no study about its role as possible blood biomarker in CRC has presented yet.

Clinical implications of miRNA-31-3p in CRC

Metastatic CRC is a very heterogeneous disease. Recent data suggest that the side of the tumor is a predictive factor for clinical outcome [36]. Although this data needs to be interpreted with caution because of the lack of randomized clinical data, guidelines recommend taking into account the primary tumor localization for treatment. Patients with left-sided CRC seem to have a better response to anti-EGFR therapy. On the other hand, patients with right-sided CRC benefit more from anti-VEGF [37,38].

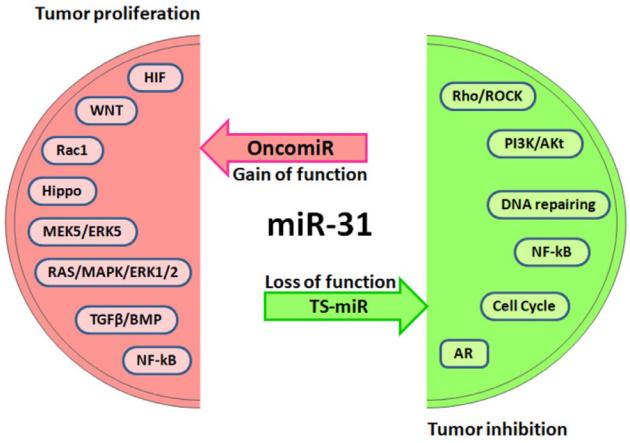

Figure 2. The intricate role of mir-31 in CRC, as oncomiR, whose gain of function lead to tumor proliferation, and as Ts-miR, whose loss of function results in lack of tumor inhibition.

Data correlated from the FIRE-3 trial [39] proves that low miR-31-3p expression could differentiate patients who benefit more from Cetuximab than Bevacizumab in wild-type K-RAS mCRC. The study analyzed the expression of miR-31-3p (low/high) from 164 patients receiving FOLFIRI plus Cetuximab and 176 patients receiving FOLFIRI plus Bevacizumab and linked the results with overall survival (OS) and progression-free survival (PFS). Considering tumor side, patients with left-sided tumors had a more significant benefit from FOLFIRI associated with Cetuximab vs. FOLFIRI +Bevacizumab regardless of their level of miR-31-3p expression. The response rate was higher in patients with miR-31-3p low expression. Association of Cetuximab with low miR-31-3p in wild-type RAS mCRC showed no harmful effect in patients with operable liver metastases [40]. However, the data of the study stated contradictory information with that of the NEW EPOC trial although it failed to observe a clear association between miR-31-3p expression and response to Cetuximab.

Another study aimed at evaluating miR-31-3p/5p expression regarding time to progression in wild type K-RAS mCRC treated with Cetuximab [16]. It has been demonstrated that both miR-31-3p and miR-31-5p are strongly associated with time to progression in patients treated with Cetuximab, but not Panitumumab. Preliminary work on finding miRNAs that could predict anti-EGFR efficacy revealed that miR-31-3p could become a biomarker in wild type K-RAS/BRAF patients. The endpoint of the study was evaluating survival. These analyses need to be conducted on large series of patients [41]. The study of Manceau et al [15] suggests the possible implication of miR-31-3p in deciding the treatment of wild type K-RAS mCRC that is refractory to chemotherapy. This study was the first to correlate miR-31-3p expression with the prediction of response to anti-EGFR therapy and its as-

Table 1. Studies evaluating the role of mir-31 in CRC

First author	*Year*	*Sample size*	*Tissue sample*	*K-RAS*	*BRAF*	*Anti -EGFR therapy*	*PFS*	*OS*	*Ref*
Laurent-Puig P et al	2018	340	FFPE	Yes	Yes	Cetuximab	Yes	Yes	[39]
Pugh S et al	2017	149	FFPE	Yes	Yes	Cetuximab	Yes	Yes	[40]
Manceau G et al	2014	132	FFPE/FF	Yes	Yes	Cetuximab & Panitumumab	Yes	No	[15]
Mlcochova J et al	2015	93	FFPE	Yes	No	Cetuximab & Panitumumab	No/ TTP	No	[16]
Mosakhani N et al	2012	99	FFPE	Yes	Yes	Cetuximab & Panitumumab	No	Yes	[41]
Ramon L et al	2018	189	FFPE	No	No	No	No	No	[17]

PFS: progression-free survival, OS: overall survival, FFPE: formalin-fixed, paraffin-embedded

sociation with PFS. However, the limitations of the study were that no control arm existed for patients without anti-EGFR therapy. Table 1 summarizes the studies evaluating the role of miR-31-3p in CRC.

Considering the high stability of miRNAs, Ramon et al [17] demonstrated that using standardized RT-qPCR assay was possible to quantify the expression of miR-31-3p from FFPE tumor tissue. The method was able to differentiate low versus high miR-31-3p expression in a robustly and accurately manner making it more accessible for clinical use.

Recently a company has developed a miRNA kit for detecting miR-31-3p from FFPE samples using RT-qPCR [42]. As far as we know this is the first commercial kit with direct use in clinics for patients with wild type K-RAS mCRC. This kit can identify the patients who will most benefit more from Cetuximab or Panitumumab in first-line treatment. It can also be used, if suitable, in second- and third-line therapy (Integragen).

Clinical implications of miRNA-31-5p in CRC

The assessment of miR-31-5p levels and the clinical efficacy of anti-EGFR antibodies therapy in patients with mCRC showed that high expression is connected with shorter PFS. Besides, patients with no mutations in KRAS, NRAS, or BRAF present shorter PFS in the high versus low expression group. There was no significant difference in OS between the two groups. In contrast, there was no significant difference in PFS or OS in the high/low miRNA-31-3p expression groups [43].

MiRNA 31- 5p was proven to have oncogenic properties in both CRC cell lines and primary colorectal tumors [34]. Nosho et al [28] demonstrated an association between high expression of miR-31-5p, BRAF, RAS mutation, and proximal location, after multivariate logistic regression analysis of a database of 721 patients with CRC. Furthermore, the authors showed that inhibition of miR-31-5p led to a decrease in BRAF target protein by suppression of RAS p21 GTPase-activating protein 1(RASA1). The up-regulation of the signaling pathway may confer resistance to anti-EGFR antibodies therapy. On this line, Slattery et al have shown, in a large population-based data, that high miR-31-5p expression is associated with more advanced tumor stage [44].

Tumors that express miR-31 present elevated epithelial-mesenchymal transition, TNFα/NFκB, TGF β and IFN -α/γ gene expression and downregulation of MYC target. These features provide immune evasive and tissue invasive capabilities that may become the biological basis for aggressive disease. A high miR-31-5p expression was associated to important clinicopathological features including advanced stage, right-sided tumor site, sessile serrated adenoma, low differentiation grade, microsatellite instability, and mutated BRAF and K-RAS. A total of 1993 samples were analyzed to investigate the value of miRNA-31-5p as well as its precursor miR-31HG as a prognostic factor. MiR- 31 tumors were more probable to be at an advanced cancer stage, right-sided, have a low differentiation level and BRAF/V600 mutations. Patients with stage II tumors that expressed high miR-31 had a 5-year relapse-free survival (RFS) 49% compared to 77% for those with normal miR-31. The status of miR-31 was linked with inferior outcome when stratifying for adjuvant chemotherapy. DFS in stage II and III patients with high expression of miR-31 was inferior (5-year DFS 0% and 42%). Kaplan- Meier analysis revealed that miRNA-31 expression conferred a worse outcome in all consensus molecular subtypes (CMS) groups [45].

A study by Sang Bum Kim addressed the issue of the cellular response to radiation, depending on miR-31-5p expression [46]. They transfected a miR-31-5p mimics or inhibitor into immortalized human colonic epithelial cells, and then subjected the cells to gamma-irradiation. The results were surprising because miR-31-5p mimics sensitized the colonic cells while the miR-31-5p inhibitor induced a protective role from irradiation. In this context, miR-31-5p mimic inhibits mismatch repair gene hMLH1 expression after irradiation. On the contrary, miR-31-5p inhibitor leads to an increase in the level of hMLH1 gene expression.

Concerning the role of miR-31-5p in immunotherapy, this mature form of miR-31 did not associate with Panitumumab treatment response after the failure of Cetuximab in patients with wild type K-RAS mCRC [47]. High expression of miR-31-5p was only associated with low PFS in Cetuximab-treated patients like previous data from the literature.

Previous data indicated that miR-31-5p/miR-31-3p are involved in CRC development, by targeting EpHA2 and EpHB2 ephrin receptors of the protein-tyrosine kinase family, modulating stem-like properties, and progression of CRC cells [48]. The specific molecular mechanism of miR-31-5p was analyzed in both colorectal tissue and cell lines. Recent data have shown that miR-31-5p promotes cell proliferation, migration and metastasis as well as apoptosis and cell cycle arrest in CRC by targeting NUMB protein [49]. Moreover,

Table 2. Studies evaluating the role of mir-31 in CRC

First author	*Year*	*Sample size*	*Tissue sample*	*K-RAS*	*BRAF*	*Anti-EGFR therapy*	*PFS*	*OS*	*Ref*
Igarashi H et al	2015	102	FFPE	Yes	Yes	Cetuximab & Panitumumab	Yes	Yes	[43]
Nosho K et al	2014	721	FFPE	Yes	Yes	No	No	Yes	[28]
Eide PW et al	2018	1993	FFPE /Cell lines/TCGA	Yes	Yes	No	No (RFS)	Yes	[45]
Kiss I et al	2016	26	FFPE	Yes	No	Cetuximab & Panitumumab	Yes	No	[47]
De Robertis et al	2018	1663	cell lines, TCGA	No	No	No	No	Yes	[48]
Peng H et al	2019	30	FFPE/cell lines	No	No	No	No	No	[49]
Slattery ML et al	2016	1893	FFPE	Yes	Yes	No	No	Yes	[50]
Slattery ML et al	2015	1141	FFPE	Yes	Yes	No	No	Yes	[44]
Choi YW et al	2016	535	FFPE	Yes	Yes	No	No	No	[51]
Mlcochova J et al	2015	93	FFPE	Yes	No	Cetuximab & Panitumumab	No /TTP	No	[16]

FFPE: formalin-fixed, paraffin-embedded, FF: frozen tissue, TCGA: The Cancer Genome Atlas, RFS: relapse-free survival, TTP: time to progression, PFS: progression free survival, OS: overall survival, K-RAS: Kirsten RAT Sarcoma virus, BRAF: v-Raf murine sarcoma viral oncogene homolog B

the role of miR-31-5p in CRC survival has been proved on a large microarray data, including 1893 samples [50]. Interestingly, overexpression of miR-31-5p was correlated to the risk of dying for colon cancer between microsatellite unstable (MSI) and microsatellite stable (MSS) tumors, but with increased survival for MSI compared with MSS rectal tumors.

From another point of view, Choi et al [51] were investigating the miRNA expression signature between BRAF mutated CRC samples compared with those with K-RAS mutations. Data from this study have highlighted that miR-31-5p presented the highest expression level among ten miRNAs of interest. This miRNA signature is associated with BRAF mutation but not with K-RAS mutation in CRCs, being involved in the modulation of both WNT and MAPK signaling pathways. Table 2 summarizes the studies evaluating the role of miR-31-3p in CRC.

Conclusions and perspectives

As summarized in this review, mir-31-3p and mir-31-5p are involved in various biological processes of CRC, such as proliferation, migration, and invasion. As promising biomarkers in mCRC for prognosis and efficacy evaluation, these miRNAs are of great use to find the best-personalized treatment schedule. As a result, miR-31-3p and miR-31-5p provide a new perspective for the evaluation of first-line treatment of mCRC with wild type K-RAS, despite the lack of inconclusive results from the studies presented above. However, new more extensive studies focused on validation and standardization of miR-31-3p and miR-31-5p are needed before their consideration as biomarkers for clinical use. Further, the integration of these miRNAs information with clinical data in multicentric trials could sustain better tailoring of the personalized medicine for CRC patients. Not eventually validated guidelines and clear protocols are the key to branching this knowledge into clinically compatible applications, standardization programs, and clinical trials for rapid insertion in clinical practice.

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Authors' contribution

All authors contributed equally to this work.

Conflict of interests

The authors declare no conflict of interests.

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