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Current Status and Perspectives Regarding LNA-Anti-miR Oligonucleotides and microRNA miR-21 Inhibitors as a Potential Therapeutic Option in Treatment of Colorectal Cancer

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ABSTRACT

Colorectal cancer (CRC) is among the leading causes of cancer-related death, principally due to its metastatic spread and multifactorial chemoresistance. The therapeutic failure can also be explained by inter- or intra-tumor genetic heterogeneity and tumor stromal content. Thus, the identification of novel prognostic biomarkers and therapeutic options are warranted in the management of CRC patients. There are data showing that microRNA-21 is elevated in different types of cancer, particularly colon adenocarcinoma and that this is association with a poor prognosis. This suggests that microRNA-21 may be of value as a potential therapeutic target. Furthermore, locked nucleic acid (LNA)-modified oligonucleotides have recently emerged as a therapeutic option for targeting dysregulated miRNAs in cancer therapy, through antisense-based gene silencing. Further work is required to identify innovative anticancer drugs that improve the current therapy either through novel combinatorial approaches or with better efficacy than conventional drugs. We aimed to provide an overview of the preclinical and clinical studies targeting key dysregulated signaling pathways in CRC as well as the therapeutic application of LNA-modified oligonucleotides, and miR inhibitors in the treatment of CRC patients. J. Cell. Biochem. 9999: 1–12, 2017.

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MiRNAs (miRNA) are a class of small non-coding 19–25 nucleotide-long RNAs that play an important role in biological processes. The first identified miRNA, namely Lin-4, was indentified in 1993 in *Caenorhabditis elegans*; in which it was proposed to be involved in its growth and development. In 2000, the second miRNA, let-7, was identified in the same organism. The human homologue, let-7 was characterised in 2000 [Barh et al., 2010]. Colorectal cancer (CRC) is the third most common (malignant) cancer worldwide and one of the leading causes of cancer-related deaths in women, and men in many countries [Edwards et al., 2010; Society, 2016]. Surgical resection is one option in the treatment for CRC, although most patients are diagnosed at a late stage. Colon cancer may metastasize, via the veins or lymph vessels; thus, and hence may be found in the blood at an early stage [Minsky et al., 1988]. Apart from surgical resection, adjuvant chemotherapy, and radiation are often used in patients with CRC. The chemotherapy for CRC include: fluoropyrimidines, *irinotecan*, and *oxaliplatin*, that are cytotoxic drugs. Standard treatments of malignant CRC, that are often used in combination include: *FOLFOX* (*5-fluorouracil*, *leucovorin*, and *oxaliplatin*) or *FOLFIRI* (*5-fluorouracil*, *leucovorin*, and *irinotecan*). The MoAb *bevacizumab (avastin), cetuximab (erbitux), and panitumumab (vectibix)* have been approved by the FDA, and are also used for the treatment of metastatic CRC [Aka et al., 2017]. Most recently, aflibercept (ziv-aflibercept) as a decoy receptor (or VEGF Trap) [Jitawatanarat and Ma, 2013; Ducreux et al., 2017] plus FOLFIRI was shown significant improvements in patients with metastatic CRC [Gaya and Tse, 2012]. In addition, treatment with nab-paclitaxel may also be a potential strategy for overcoming drug resistance in CRC [Ducreux et al., 2017]. Recently the antiangiogenic agents Brivanib alaninate (BMS-582664), Ramucirumab (IMC-1121B), Vatalinib (PTK787/ZK222584), and Cediranib (AZD2171) have been approved or in phase III trials for the treatment of Metastatic CRC [Gaya and Tse, 2012]. Preclinical and clinical studies have provided evidence that aflibercept showed significant improvements in overall survival. Trials with this new agent are continuing, and may lead to an expansion of the therapeutic options available to patients with cancer [Gaya and Tse, 2012]. However, drug resistance, and tumor recurrence are still common [Gong et al., 2015]. We have explored the potential importance of miR-21 as a miRNA biomarker for CRC and evaluated the potential benefits associated with inhibiting this miRNA in patients with CRC.

CURRENT AND NOVEL TK INHIBITORS IN CRC

Cetuximab (an IgG1 Monoclonal antibody) and *panitumumab* (a fully human IgG2 monoclonal antibody) are monoclonal antibodies (MoAb) that target the epidermal growth factor receptor (EGFR), and have now been widely used for the treatment of malignant CRC. However, only 10–20% of patients show clinical improvement with these agents, and patients with *KRAS* mutations are particularly resistant [Di et al., 2008]. The significance of other RAS mutations, as they are very well established predictive factors (i.e., KRAS exon 3,4 and NRAS exon 2,3 mutations, the so-called RAS panel) [Cicenas et al., 2017]. Patients treated with *cetuximab* and *panitumumab* have an improved response when miR-21 is inhibited [Lankat-Buttgereit et al., 2004; Lankat-Buttgereit and Goke, 2005, 2009; Frankel et al., 2008a; Allen and Weiss, 2010; Allgayer, 2010; Fassan et al., 2011; Fischer et al., 2011; Kanaan et al., 2012; Kim and Cha, 2012; Mlcochova et al., 2013]. *PTEN* normally inhibits the *PI3/AKT* pathway, which is one of the key dysregulated pathways in CRC, and is often mutated in CRC [Sood et al., 2012]. *EGF* activates *S6kinase1* via the *PI3K-Akt-mTOR* signaling pathway and stimulates the degradation of *PDCD4* [Matsuhashi et al., 2014].

Afatinib (BIBW 2992), is a highly selective inhibitor for *EGFR* and human EGFR-2 (HER2), is currently being used in Phase I trials for a variety of advanced solid tumors. Patients with metastatic CRC still have disease progression, in spite of receiving oxaliplatin or irinotecan [Guan et al., 2014].

Necitumumab (LY3012211; IMC-11F8) is a second-generation recombinant fully human IgG1 monoclonal antibody against EGFR. Elez and colleagues used this in combination with irinotecan in a Phase 2 of CRC patients with a *KRAS* wild-type. Patients were given *mFOLFOX* or *necitumumab* and *mFOLFOX-6* [Elez et al., 2016]. It was shown that the combination of drugs is better tolerated in patients with previously untreated metastatic CRC.

Erlotinib (Tarceva, OSI-774) is an orally active, reversible inhibitor of HER1/EGFR tyrosine kinase. It has been tested in a number of Phase 2 and 3 trials in advanced CRC [Kaori et al., 2006]. Recent data suggest that erlotinib ruins patients with *KRAS*-mutated advanced CRC while it may provide profit to those with *KRAS*-WT CRC [Vincent et al., 2017].

Gefitinib (ZD1839, Iressa®) is a reversible tyrosine kinase EGFR inhibitor. It has currently been assessed in Phase 2 trials. The combination of *irinotecan or capecitabine* and *gefitinib* in the second phase of advanced CRC treatment showed no added benefits, and furthermore showed toxic effects [Yang et al., 2011].

Zalutumumab (HuMax-EGFR) is the fully human IgG1 monoclonal antibody against EGFR. Phase 1 study of *zalutumumab* and *irinotecan* in CRC was conducted after the failure of *irinotecan,* and *cetuximab* use.

Lapatinib (GW 572016) is an orally active dual inhibitor of *EGFR-TKI* and *HER-2* [Montemurro et al., 2007]. A recent Phase 2 trial in advanced solid tumors, including CRC, has been conducted. This represents the efficacy of this compound in solid tumors. Its toxicity is still being examined in a series of Phase 2 studies for CRC.

INHIBITORS IN RAS-RAF-MEK-ERK PATHWAY

XL281 (BMS-908662) is a potent oral RAF serine/threonine kinases inhibitor currently being assessed in Phase 2 trials. It is only used in combination with *cetuximab* in patients with a KRAS mutation or *BRAF* [Dickson et al., 2015].

Pimasertib (MSC1936369B) is an *MEK* inhibitor in Phase 2 trials. It is used in combination with *FOLFORI* in patients with tumors and harmful mutations of *KRAS* [De et al., 2012].

Selumetinib(AZD6244) is an oral selective inhibitor of *MEK1/2*. It has recently entered Phase 2 trials for CRC studies. A comparison has been made between oral *capecitabine* and *selumetinib* in advanced CRC in second and third stages of chemotherapy, which showed a good tolerance and efficacy. The most common adverse effects of

selumetinib were reported to be skin inflammation, diarrhea, and weakness [Tentler et al., 2010; Morelli et al., 2012].

Vemurafenib (PLX 4032) is an oral selective inhibitor of the mutant BRAF Kinase. This is found only in a small proportion of patients with CRC [Patrawala and Puzanov, 2012]. In the Phase 1 trial, this drug was used against CRC. Relatively weak results were obtained in patients with advanced CRC with mutations in *BRAF*.

Sorafenib (Nexavar) is also an oral *RAF* serine/threonine kinases (Raf-1, wild-type B-Raf) used as multi-target kinase inhibitors [Woo et al., 2017]. At first, it was found to be an inhibitor for *RAF*, but is also an inhibitor of *VEGFR* and *PDGFR*

MEK 62/ARRY-438162 is the best potential inhibitor for *MEK1/MEK2*. It is already in phases 1 and 2 trials for many advanced solid tumors with mutations in *KRAS, NRAS,* and *BRAF* [Merla and Goel, 2012].

INHIBITORS OF PI3K-Akt-mTOR PATHWAY

This is considered to be an important drug target being involved in the growth and survival of cells. Abnormalities of this pathway are involved in the causation of many cancers, and genetic mutations in this pathway are observed in many malignancies. Mutation of *PI3 K* can be seen in 25% of CRCs, and mutations in *PTEN*, *AKT2*, and *PDK1* are important causes of CRC [Leavy, 2017]. MiR-21 also plays a role in many tumor suppressors inhibitions involved in CRC.

(1) *MK-2206* is an inhibitor for *AKT*. This with *selumetinib* is in Phase 2 trials for patients with advanced CRC. This study aims to inhibit pathways involving *PI3K-AKT* and *RAS-MAPK* simultaneously. *MK-2206* is generally well tolerated, although acne, nausea, hyperglycemia, and diarrhea have been reported [Do et al., 2015].

(2) *Everolimus (RAD001)* is a derivative of *rapamycin*. *Rapamycin* is an oral inhibitor of *mTOR* path that is used in many countries for the prevention of invasive solid tumors. A non-randomized Phase 2 trial test was conducted for 50 malignant-CRC patients for whom the first chemotherapy was unsuccessful [Merla and Goel, 2012; Do et al., 2015].

(3) *Temsirolimus* (TEM) is a novel, water-soluble inhibitor of the mammalian target of rapamycin (mTOR) pathway. The drug is currently in Phases 1 and 2 of trials for advanced CRC [Heudel et al., 2017]. The combinations of traditional chemotherapy drugs with targeted therapies have shown a greater treatment efficiency in various studies. Nevertheless, no observed activities in preclinical studies were reported in the clinical results. In trials, *CAIRO2* (*capecitabine*, *irinotecan*, and *oxaliplatin* in advanced CRC) and *PACCE* (*panitumumab* in the study related to the emergence of advanced CRC), by adding EGFR antibody to the combination of chemotherapy and *bevacizumab*, significantly reduced progression-free survival (PFS) [Merla and Goel, 2012]. Targeted therapy is rapidly improving, but the lack of specific biomarkers to determine the course of treatment in patients who took advantage of this approach is still an unresolved problem.

Resistance to *cetuximab* and *panitumumab* in patients with *KRAS* mutations is well recognized. However it appears that the treatment of patients with KRAS mutations by using *EGFR* antibody has not been very successful. It indicates that there are other potential factors for resistance. A number of studies have shown that *BRAF* mutations (about 10% in CRC) in the *KRAS* gene downstream are markers of resistance to *cetuximab* or *panitumumab*. *BRAF* in patients with this mutation has a weaker response than the antibodies against EGFR. Routine testing of *BRAF* has not found its status for clinical applications. In a number of studies, the lack of *PTEN* in malignant CRC and the lack of strong response is diagnosed when treated with *EGFR* drugs. Mutations in *PIK3CA* gene and the lack of *PTEN* expression for resistance to *cetuximab* at some levels of CRC cells are also anticipated [Merla and Goel, 2012].

MiR-21 IN CARCINOGENESIS

MiR-21, which is described as an oncomir with clinical significance. MiR-21 is encoded by MIR21 gene (located on human chromosome 17q23.2). The mature sequence of miR-21 has been preserved during evolution [Lagos-Quintana et al., 2001; Kanellopoulou and Monticelli, 2008; Dong et al., 2009; Roy et al., 2009]. MiR-21 is associated with different types of cancer, including breast, ovarian, cervical, colon, lung, liver, brain, esophageal, prostate, pancreas, and thyroid cancers [Flatmark et al., 2004; Ghadjar et al., 2006; Asangani et al., 2008; Ghadjar et al., 2009; Spizzo et al., 2009; Hrasovec and Glavac, 2012; Vicinus et al., 2012; Li et al., 2013; Toiyama et al., 2013; Huang et al., 2013b]. The first target genes of miR-21 involved in cancer development include *TPM* and *PTEN* [Meng et al., 2006; Zhu et al., 2007]. Other miR-21 targets are tumor suppressors, including *PTEN* [Meng et al., 2007], *PDCD4* [Asangani et al., 2008], *Tropomyosin (TPM1)* [Papagiannakopoulos et al., 2008], *Sprouty 1* [Thum et al., 2008], *Sprouty 2* [Sayed et al., 2008], *Bcl2* [Wickramasinghe et al., 2009], *RECK* [Gabriely et al., 2008], *IL-12p35* [Lu et al., 2009], *JAG1* [Hashimi et al., 2009], *HNRPK* [Papagiannakopoulos et al., 2008], *BTG2* [Liu et al., 2009], *TGFBRII* [Kim et al., 2009], *TAp63* [Papagiannakopoulos et al., 2008], *P12/CDK2AP1* [Zheng et al., 2011], *MEF2C* [Yelamanchili et al., 2010], *ANP32A, SMARCA4* [Schramedei et al., 2011], *RhoB* [Sabatel et al., 2011], and *hMSH2* [Valeri et al., 2010].

MiRNAs have important regulatory functions. Increased expression of certain atypical miRNAs have been observed specifically as miR-21, −17-92, −15, −16, −141, let-7, miR-103, and miR-107 in tumor growth, cancer formation, or response to chemotherapeutic agents in a variety of malignancies [Calin et al., 2002; Chan et al., 2005; Hammond, 2006; Meng et al., 2006; Roldo et al., 2006]. In the last five years, the effect of miR-21 in CRC has been to shown to affect the growth of this type of cancer. Many studies were conducted with information about tumors that were surgically removed from patients with CRC, while clinical studies of miR-21 and *PDCD4* in the past indicated that inhibitory strategies against miR-21, and intervention strategies with *PDCD4*/ miR-21 reaction or maintaining *PDCD4* expression can be a very strong approach to cancer treatment in the future. MiR-21 is upregulated in most cancer cell lines and tumors that include: breast, colon, lung, pancreas, stomach, and prostate tumors. It is predicted that it functions as a miRNA oncogene [Nedaeinia et al., 2017b]. The expression of miR-21 increases the resistance to chemotherapy in a large number of colon cancer cells that are enriched with undifferentiated cancer stem/stem-like cells (*CSCs/CSLCs*). Several studies have been carried

out to determine the relationship between mesenchymal transition (EMT) and cancer; their results have shown that the EMT plays a pivotal role in the metastasis, invasion, and recurrence of cancer, besides drug resistance. This trend shows that miR-21 plays an important role in growth regulation and CSC/CSLC development [Meyerhardt and Mayer, 2005]. Some reports have shown that mesenchymal cells share the same gene expression and phenotype profile with *CSCs*. This probability has enhanced the correlation between cancer and EMT. MiR-21 represents itself more in epithelial tissues such as CRCs. Knockdown of miR-21 in cancer cells damages the growth process, induces apoptosis, and also reduces migration and invasion of cancer cells. Overexpression of miR-21 increases activity of T-cell factor/lymphoid enhancer factor (TCF/LEF). Amplification of *c-Myc* and *cyclin D* expression happens by increasing the ability to form spheres in vitro and tumor in mice with severe combined immunodeficiency (SCID) [Yu et al., 2013].

MiR-21 plays an important role in the development of malignancies. Thus, it is not surprising that miR-21 is involved in various biological processes. These biological processes include cell proliferation and apoptosis. These processes may be done by targeting regulatory proteins [Nedaeinia et al., 2016]. The main objective of the current study of downregulation miR-21 in chemotherapy-resistant colon cancer cells is to induce differentiation of *CSC/CSLC* and apoptosis, and increase susceptibility to conventional and non-conventional treatment regimens.

ROLE OF *PDCD4* CORRELATED TO miR-21

Recently, it has been found that miR-21 can target *PDCD4* tumor suppressor gene expression during the post-transcriptional stage [Nedaeinia et al., 2016]. MiR-21 or targeting *PDCD*4 (tumor-suppressor), which is localizes to chromosome 10q24, has a negative regulatory effect on the cell cycle and increases proliferation of epithelial cells in the post-transcriptional stage [Nedaeinia et al., 2016, 2017b]. Analysis of the protein sequence data reveals that *PDCD4* comprises 469 amino acids with two main domains in C-terminal and N-terminal, including two conserved α-helical MA-3 domains [Lankat-Buttgereit and Göke, 2003] (Fig 1). *PDCD4*, with a molecular weight of 54 kDa, is a tumor suppressor which inhibits tetradecanoyl phorbol acetate (*TPA*). Malignant transformers that stimulate and increase tumor progression [Cmarik et al., 1999] induce *TPA*. *PDCD4* are involved in protein–protein interactions of *eIF4G* and *eIF4A*, and the repression of translation [Yang et al., 2004; Jansen et al., 2005; Zakowicz et al., 2005]. The regulatory role of *PDCD4* is on the following factors: *P21* [Zakowicz et al., 2005], *Cdk4*, *ornithine decarboxylase* [Cmarik et al., 1999], *carbonic anhydrase II* [Lankat-Buttgereit et al., 2004], and *JNK/c-Jun/AP-1*[Goke et al., 2004]. Recently, it has been shown that *PDCD4* inhibits the invasion of receptor-dependent gene expression Urokinase-receptor–(*u-PAR*)–gene, which plays a role in invasion and intravasation through the stimulating *Sp1/Sp3* motif [Allgayer, 2010]. In earlier studies, it has been shown that *PDCD4* reduction particularly occurs in lung cancer and CRC, with poor prognosis for the patient [Bitomsky et al., 2004; Chen et al., 2003]. There is relatively little information about the mechanisms that regulate the expression of *PDCD4* in cancer. Preliminary studies suggest that the factors involved in the regulation of *PDCD4* expression include Topoisomerase inhibitors [Mudduluru et al., 2007], *COX-2* inhibitors [Onishi et al., 1998], *Myb*, and *Akt* [Schlichter et al., 2001]. Some studies have shown that *PDCD4* is regulated by proteasome degradation in response to mitogen [Palamarchuk et al., 2005]. Too many mechanisms lead to the reduction of this important tumor suppressor in the deadly cancer that needs to be investigated. Taking advantage of *PDCD4* as a therapeutic target is not only of interest to many researches but should also be considered as a selection [Soejima et al., 1999]. Yang et al., showed that *PDCD4* can reduce the regulatory activation mechanisms of c-Jun through the inhibition of mitogen-activated protein kinase kinase1 expression (*MAP4K1*) in the *JNK* (Jun N- terminal kinase) gene upstream gene. Overexpression of dominant negative mutated *MAP4K1* gene inhibits the activation of c-Jun and invasion of matrigel in colon cancer cells. In a similar way, invasion of metastatic colon cancer cells of *RKO* to matrigel is inhibited via induction of the *PDCD4*. In addition, this group showed that *PDCD4* is capable of inhibiting matrix metalloprotease 2 (*MMp2*) and other additional *MMp* activations [Dorrello et al., 2006].

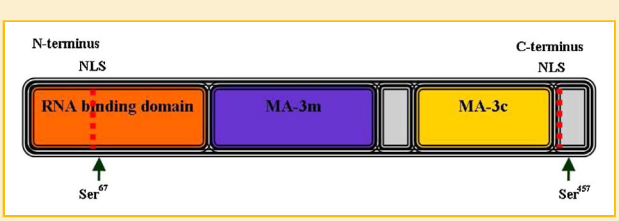

Fig 1. Structure of *PDCD4*.

INTERACTIVE ROLE OF miR-21 IN *PDCD4* GENE INHIBITION

Studies on the target sequence of miR-21 in the region of 3′-UTR *PDCD4* show that it has a negative regulatory effect on *PDCD4* by specific sequence of 249–228 nucleotides of the target 3′-UTR *PDCD4*, and it induces the processes of proliferation and invasion in CRC. In vivo studies show the relevance of these findings with studies on patients with CRC tissue removed by surgery, implying that there is a significant correlation between high levels of miR-21 and low protein content of *PDCD4*.

Furthermore, *PDCD4* tumor suppressor leads to increased suppression, malignant transformation [Yang et al., 2003], tumorigenesis, tumor progression, apoptosis [Cmarik et al., 1999], and increased cell aging [Yang et al., 2006]. It is interesting that miR-21 act as an anti-apoptotic factor in glioblastoma cells [Chan et al., 2005]. Previous studies show that miR-21 can inhibit apoptosis using *Bcl-2* regulation in mice cancer models [Si et al., 2007], and prevent apoptosis induced by Gemcitabine through inhibitory effect on *PTEN* and *PI-3* Kinas pathway [Meng et al., 2006]. Based on recent studies, it seems that miR-21 has a negative regulatory effect on *PDCD4*. Moreover, miR-21 may have anti-apoptotic effects, at least in part, which acts through a negative regulation on *PDCD4*. It has been shown that *PDCD4* inhibits induction of invasion and

intravasation. This can be obtained by inhibition of matrix-metalloproteinase effect and *u-PAR* gene. Of course, there are multiple mechanisms and targets for miR-21 in addition to *PDCD4*, which are probably stimulated with tumor cell invasion. However, these mechanisms also need to be investigated further.

Generally, it seems that in addition to miR-21, multiple regulatory mechanisms occur on *PDCD4* at different levels. Like the negative regulatory effect, it is due to *PDCD4* phosphorylation, and degradation by *B-TRCP* that ubiquitin ligase and proteasome are created, followed by mitosis stimulation [Palamarchuk et al., 2005]. MiR-21 targets, including anti-apoptotic proteins, *Bcl2*, *PDCD4*, tropomyosin (*TPM1*), and tumor suppressor (*SERPINB5*) of Maspin and those which suppress cell growth, invasion, and metastasis are: metalloproteinase inhibitor (*RECK*), reversion inducing cysteine-rich protein with kazal metalloproteinases in addition to tissue inhibitors (*TIMP3*) of metalloproteinas (*SPRY2*), which regulate cell growth, branching, and migration. Frankel et al. [2008a] used anti-miR-LNA to antagonize miR-21 in MCF-7 cells which significant increase, including tumor suppressor protein or *PDCD4*. This is indicated by later tests with affymetrix chips and 737ntranscripts. Mudduluru et al. [2007] investigated 71 colorectal tumors and normal tissue around them, together with 42 adenomas by *PDCD4* using immunohistochemistry. *PDCD4* protein was not found in the nucleus, and was weakly expressed in the cytoplasm of tumor tissues. Whilst in normal tissue of *PDCD4* protein was found at high levels in the nucleus. They observed in the case of adenoma an intermediate status with both nucleus and cytoplasm. Several recent studies indicate some of the cellular functions of these proteins [Nedaeinia et al., 2016].

MiR-21 is considered to be prognostic marker for the return of diseases. Results of studies on clinical data show that there is a correlation between the increased tendency of miR21 and disease recurrence in patients who have had surgical resection. When patients in Stage II were analyzed as subgroups, the presence level of miR21 in the samples was higher than that of patients whose disease had relapsed. The analysis of more samples will finalize the conclusions of the clinical data. However, it is interesting that the upregulation of miR-21 as a sign of poor prognosis for CRC has remained as a finding [Kang et al., 2002]. In recent data overexpression of miR-21 in conjunction with the return of the disease in its early stages were not only consistent the detection but also provided a better role of miR-21 as a sign of recurrence in early stages.

miR-21 may be a useful prognostic marker for a wide range of cancers. Up-regulation of miR-21 in the most aggressive and malignant tumors such as gliomas, breast tumors, colon adenocarcinomas, pancreatic cancer, and gastric cancer are often linked with a poor prognosis for the patients [Yan et al., 2008]. Transfer of miR-21 by tumor-derived exosomes can also increase oncogenic transformation into target cells away from the rudimentary tumors without direct secretion by cancer cells [Hosseini et al., 2016; Nedaeinia et al., 2017a]. This can be used as a diagnostic tool. Exosomes seem to have important roles in cell-to-cell relevance [Nedaeinia et al., 2017a].

ROLE OF miR-21 AND *RASA1* IN *RAS* SIGNALING PATHWAYS

RAS activity is regulated by two types of molecules—*RAS*-GEFs and *RAS*-GAPs. *RAS*-GAPs can hydrolyze GTP and belay it as *RAS*-GDP to deactivate *RAS* protein, and also turn off *RAS* signaling pathways. *RASA1* belongs to the *RAS*-GAP family, which has 16 members. MiR-21 controls *RASA1* haste. *RAS*-GTP levels, which show the activity of *RAS* protein, were investigated in high/low miR-21 and *RASA1* cells. Overexpressed miR-21 increases *RAS*-GTP activity so that downregulation of miR-21 or upregulation of *RASA1* can reduce *RAS*-GTP activity. High levels of *RAS*-GTP suggest RAS protein activity, which in turn can strengthen signaling pathways in cells, including *RAS-Raf-MAPK* and *RAS- PI3K-AKT* pathways, while promoting tumor cell proliferation and anti-apoptosis [Gong et al., 2015]. Change in proteins *Raf-1, KRAS, AKT, ERK1/2, P-ERK1/2*, and *P-AKT* in cells with decreased expression of miR-21 or overexpressed *RASA1* less than cells with overexpressed miR-21 and decreased *RASA1*. These findings suggest that miR-21 can activate *RAS* signaling pathways by help of reduced *RASA1* expression, therefore playing an important role in the accession of cell proliferation, anti-apoptosis, and formation of cancer cells [Gong et al., 2015].

MiR-21 could also have important functions in tumor development and the progression of colon cancer by targeting *RASA1*. These data provide a newly developed targeted therapy for colon cancer. This is especially important in view of the drug resistance observed with colon cancer-targeted drug *cetuximab* or *panitumumab*, thought to be the leading cause of cancer recurrence Therefore, MiR-21 and *RASA1* may prove to be valuable candidates for new therapeutic agents to conquer *cetuximab* or *panitumumab* resistance. A study by Wang et al. [2008] shows that downregulating tumor suppressor gene *PDCD4* leads to E-cadherin inhibition and activation of transcription dependent on *b-catenin/Tcf*, which simulates cell proliferation, invasion, and inhibition of apoptosis in the colon carcinoma. Further studies explain most mechanisms by which it turns up. Increased presence of zinc-finger proteins Snail, arising from knockdown *PDCD4* which activates B-catenin, in turn stimulates *c-Myc* and *u-PAR* expression, which are responsible for tumor invasion. Reduced miRNA expression, along with the expression of *RAS* oncogenes, can develop carcinoma in vivo conditions. It seems that the overall decline in miRNA increases tumorigenesis [Palamarchuk et al., 2005]. In addition, more than 50% of miRNA genes in areas related to cancer or vulnerable areas are in fragile sites, which can avert the miRNA performance in cancer [Wang et al., 2008]. Talotta et al. [2009] discovered a new regulatory pathway which contained *RAS*, *AP1* transcription factor, miR21, and *PDCD4*. They have shown that *RAS* activates *AP1*, which in turn stimulates upregulation in miR21 and leads to downregulation in *PDCD4*. Consequently, *PDCD4* acts as an *AP1* negative regulator, which completes a positive auto-tuning loop. The *PDCD4*/miR21 regulation mechanism in *RAS* pathway in the process of tumor formation is very important. It was recently shown that *PDCD4* plays a role in another pathway of colon tumor formation, for example, *APC*, *b-catenin*, and T-cell factor (Tcf).

THERAPEUTIC APPLICATION OF miRNAs

The treatment of CRC currently comprises, cytotoxic chemotherapies and biological agents. Based on their role in oncogenic and tumor suppressor, miRNAs are used in the early diagnosis, cancer prognosis, or determining response to treatment [Talotta et al., 2009].

To investigate the potential use of miRNAs in the treatment of CRC, recently some studies have been conducted on the role of *CCL20* cytokines reduction in CRC cell lines with upregulation of miR-21 [Vicinus et al., 2012]. *CCR6* and *CCL20* interactions that might promote the formation of colorectal liver metastasis lead to proliferation and migration. This provides a potential basis for novel remedy strategies [Ghadjar et al., 2009] (Table I).

Permanent use of conventional chemotherapy is associated with toxic substances that cause death. *CSC/CSLC* is considered as an undifferentiated population, which can perform self-renewal within a tumor that is mainly responsible for the bulk of the tumor mass. They are resistant to radiation and chemotherapy, and can also stimulate the tumor and cause it to grow and progress, leading to its invasion in the body and finally its relapse [Barh et al., 2010]. *CSCs/CSLCs* that represent *CD44, CD166, CD133,* and epithelial-specific antigen surface markers separate from solid tumors including colon tumors, which usually have specific surface epitopes. CSCs/CSLCs are present not only in adenoma before malignancy but also in normally appearing colonic mucosa, while *CSC/CSLC* population increases with the advancing. Moreover, CRC may increase with rising growth [Barh et al., 2010]. As natural stem cells, *CSCs/CSLCs* grow slowly; cells are likely to remain viable with the chemotherapy process. Therefore, *CSC/CSLC* growth rate in tumor increases after chemotherapy. It has been demonstrated that this pathway has led to metastasis through *CSC* induction. There are several methods that have been tested for miRNA inhibition, which include: (1) Anti-miRNA oligonucleotide (AMO) methods in which RNA sequences are target RNA complement that bind to it for inhibition. (2) Small interfering RNA (siRNA) methods that lead to breakdown in target miRNA based on sense and antisense property in the RISC complex [Sharifi et al., 2014]. Ribozyme methods (RNA, which has catalytic properties) used to break in target RNA. Locked nucleic acid (LNA) method to inhibit target RNA. Based on these methods, some efforts have been made to treat some malignancies [Trang et al., 2008; Yamamichi et al., 2009b]. Because of the extended topic of antisense, here the best and latest methods of LNA application are examined. LNA is a new class of modified oligonucleotides [Krutzfeldt et al., 2005]. LNAs include a group of RNA analogues in which the Furanose rings are locked in LNA monomers in a spatial structure of endo/N-type C3 similar to that of RNA. Its ribose ring is attached with a methylene bridge between O-2′ and C-4′, while it locks the ribose ring in the structure ideal for Watson–Crick base pairing. So LNA is defined as 2′-O, 4′-C-Methylene-ß-d-ribofuranosyl [Yelamanchili et al., 2010; Sabatel et al., 2011; Schramedei et al., 2011] (Fig. 2).

Several studies have revealed that LNA modifies oligonucleotides to make a hybrid with their target RNA molecules, showing high thermal stability (LNA fine-tunes the recognition of DNA and RNA) [Orom et al., 2006]. This is a direct result of the ability of LNA monomers to twist the conformation of adjacent DNA monomers toward the RNA-like N-type conformation in DNA/LNA mixmers. LNA anti-miR are a modified RNA, 19–25 nt that binds complementary RNA with unprecedented affinity and specificity to silence endogenous miRNA by Watson–Crick base pairing.

LNA oligonucleotides can enter the cell using the appropriate gene delivery method. They are stable and non-toxic with good aqueous solubility and effective antisense performance within the cell and they do not cause an immune response. These properties have led LNA oligonucleotides to be used as a strong and useful silencing tool based on special antisense in gene therapy [Cowland et al., 2007; Fabbri et al., 2008]. Recent developments also provide a new attitude for the regulation of gene expression by non-coding RNAs. For treating malignancies, miRNAs can lead to a major breakthrough in cancer treatment. Therefore, they are aimed at providing and expanding new therapeutic approaches for the treatment of cancer, and creating a new way to inhibit cancer by

TABLE I. Clinical Trials Base on miR Inhibitors

Study	Intervention	References	Status
Study of EZN-2968 weekly in adult patients with advanced solid tumors or lymphoma	Drug: Intravenous EZN-2968 (anti-HIF-1α LNA AS-ODN)	NCT00466583	Completed
EZN-3042 administered with re-induction chemotherapy in children with relapsed acute lymphoblastic leukemia (ALL)	Drug: EZN-3042|drug: cytarabine|drug: doxorubicin|drug: prednisone|drug: vincristine|drug: pegaspargase|drug: methotrexate	NCT01186328	Terminated
Multiple ascending dose study of SPC4955 in healthy subjects	Drug: SPC4955|drug: SPC4955|drug: SPC4955|drug: SPC4955|Drug: SPC4955|drug: Saline 0.9%	NCT01365663	Completed
SPC3649 multiple dose study in healthy volunteers	Drug: SPC3649|drug: saline	NCT00979927	Completed
Safety study of SPC3649 in healthy men	Drug: SPC3649	NCT00688012	Completed
Multiple ascending dose study of miravirsen in treatment-naïve chronic Hepatitis C subjects	Drug: miravirsen|drug: saline	NCT01200420	Completed
Miravirsen in combination with telaprevir and ribavirin in null responder to pegylated-interferon alpha plus ribavirin subjects with chronic Hepatitis C virus infection	Drug: miravirsen|drug: telaprevir|drug: ribavirin	NCT01872936	Active, not recruiting
Miravirsen study in null responder to pegylated interferon alpha plus ribavirin subjects with chronic Hepatitis C	Drug: miravirsen sodium	NCT01727934	Active, not recruiting
Drug interaction study to assess the effect of co-administered miravirsen and telaprevir in healthy subjects	Drug: miravirsen sodium|drug: telaprevir	NCT01646489	Completed
Long-term extension to miravirsen study in null responder to pegylated interferon alpha plus ribavirin subjects with chronic Hepatitis C	Drug: miravirsen	NCT02031133	Active, not recruiting
Long-term extension study of miravirsen among participants with genotype 1 chronic Hepatitis C (CHC) who have not responded to pegylated-interferon alpha plus ribavirin	–	NCT02508090	Active, not recruiting
SPC2996 in chronic lymphocytic leukaemia	Drug: SPC2996	NCT00285103	Completed

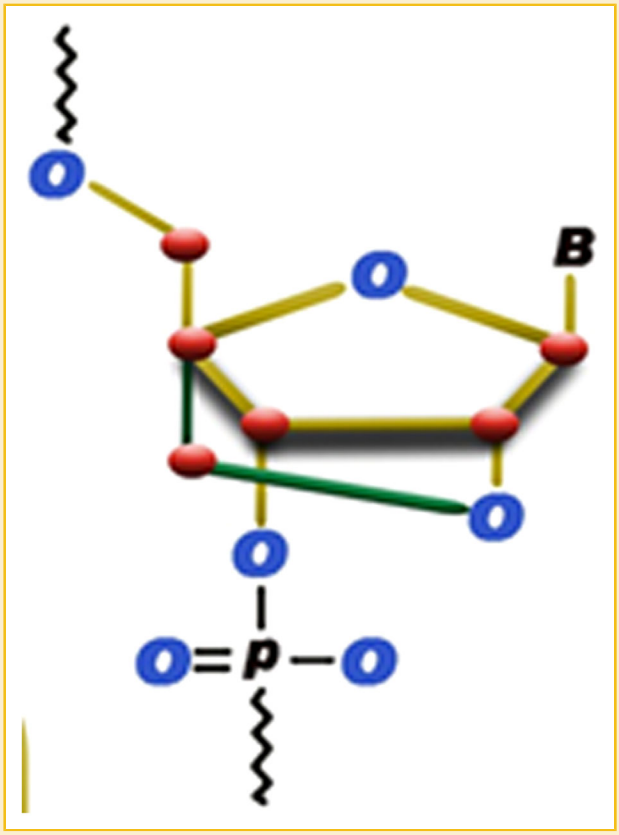

Fig 2. Chemical structure of LNA (locked nucleic acid).

microRNA oncomirs using LNA technology, which has been developed recently (Table II). In addition, LNA has important physicochemical properties that are important for the development of safe and effective drugs for therapeutic purposes. It has a great tendency for combination with RNA target molecules, cellular absorption, and good biological release. Since the human desire has long been the treatment of incurable diseases and increased life expectancy, miRNAs could be used to treat cancer. This will lead to a great development in the treatment of cancer. miRNA is obviously important in cancer treatment for regulating key cancer genes like *RAS* (let-7), *PTEN* (miR-21), and *Bcl-2* (miR-15/16). Using different oligonucleotides anti-miRs with different chemical changes, several oncogenic miRNAs have been evaluated as potential targets for therapy, especially miR-17-92 cluster, let-7, and miR-21 [Orom et al., 2006]. The high thermal stability of LNA oligonucleotide probes and their ability to detect mismatch imply that oligonucleotides with LNA are valuable tools for strong miRNA diagnosis and the prediction of cancer. In addition, the high metabolic stability of LNA with an accurate diagnosis of miRNA features shows that LNA-anti-miR can be good and useful for the development of new therapeutic approaches based on miRNA related to cancer. LNA antagonists have been applied in numerous in vitro and in vivo studies. For example, miRNA expression alterations are linked in both the initiation and progression of human tumorigenesis, while miRNAs that behave as oncogenes could become molecular targets for cancer therapy through antagonism by anti-miR oligonucleotides. Two novel drug compound—*EZN-2968* and *EZN-3042*—that antagonize the *HIF-1*α and *Survivin*, respectively, are currently being evaluated in clinical trials for cancer therapy. SPC-4955 is an inhibitor of the protein necessary for the formation of plasma LDL cholesterol particles. This has the potential to be used in the treatment of patients with hyperlipidemia. *SPC5001* is currently being evaluated in Phase 1 clinical trials in the treatment of healthy subjects and subjects with familial hypercholesterolemia (FH). *SPC3649*, also known as miravirsen, shows that with pharmacological inhibition of miR-122, Hepatitis C replication can be repressed [Lindow and Kauppinen, 2012]. *SPC2996* is an LNA antisense molecule against *Bcl-2*, in patients with relapsed or refractory chronic lymphocytic leukemia.

TABLE II. Preclinical Studies About LNA-anti-miR Oligonucleotides

miRNA	Disease	Antisense chemistry	Model	Reference
miR-21	Breast cancer, glioblastoma, colorectal cancer, thyroid cancers, and non-small-cell lung cancers	LNA	Cell lines, surgically excised tumors, in vivo(mice)	Chan et al. [2005]; Papagiannakopoulos et al. [2008]; Frankel et al. [2008b]; Yamamichi et al. [2009a]; Cottonham et al. [2010]; Zhou et al. [2010]; Frezzetti et al. [2011]; Nedaeinia et al. [2016]
miR-31	Colorectal cancer, oesophageal adenocarcinoma, gastric cancer, colon carcinoma	LNA	Cell lines, surgically excised tumors,	Valastyan et al. [2009]; Cottonham et al. [2010]; Panarelli and Yantiss [2011]; Lynam-Lennon et al. [2012]; Xu et al. [2013]
miR-125b	Prostate cancer, stomach cancer, colon cancer, pancreases cancer, bladder cancer, and ovary cancer, breast cancer	LNA	Cell lines, surgically excised tumors	Bloomston et al. [2007]; Iorio et al. [2008]; Sorrentino et al. [2008]; Baffa et al. [2009]; Veerla et al. [2009]; Ueda et al. [2010]; Frampton et al. [2011]; Nana-Sinkam and Croce [2011]; Shi et al. [2011]; Giangreco et al. [2013]
miR −155	B-cell lymphomas, breast cancer, pancreatic neoplasia	LNA	Cell lines, surgically excised tumors, in vivo(mice)	Iorio et al. [2008]; Habbe et al. [2009]; Fabani et al. [2010]; Zhang et al. [2012c]
miR-221/222	Liver tumorigenesis, prostate carcinoma, glioblastoma, breast cancer, hepatocellular carcinoma,	LNA, 2′-O-methyl–	Cell lines, surgically excised tumors, in vivo(mice)	Galardi et al. [2007]; Mercatelli et al. [2008]; Zhao et al. [2008]; Zhang et al. [2009]; Pineau et al. [2010]; Zhang et al. [2010]; Obad et al. [2011]; Park et al. [2011]; Zhang et al. [2012a]
miR-19a	Breast cancer, colon carcinoma, gliomas	LNA	Cell lines, surgically excised tumors, in vivo(mice)	Liang et al. [2011]; Ouchida et al. [2012]; Zhang et al. [2012b]; Jia et al. [2013]
miR-92	Colon cancer, acute leukemia	LNA	Cell lines, surgically excised tumors	Tanaka et al. [2009]; Tsuchida et al. [2011]
miR-10b	Breast oncogenesis, glioblastoma stem cells, pancreatic ductal adenocarcinoma	LNA	Cell lines, surgically excised tumors	Frampton et al. [2011]; Guessous et al. [2013]; Yigit et al. [2013]

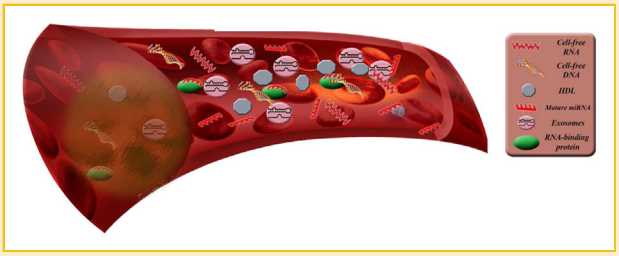

Fig 3. Extracellular circulation systems of miRNAs.

APPLICATION OF miRNAs IN CANCER DIAGNOSIS

Tumor cells use different approaches, including exosomes, for the transfer of genetic information, including miRNA transfer to surrounding cells to inhibit the growth of tumor cells [Nedaeinia et al., 2017a]. In addition, circulating miRNAs may modulate the immune response [Skog et al., 2008]: for example, microvesicles derived from human colon cancer and melanoma tumor can support the growth, and evasion to the immune system by differentiation of monocytes toward myeloid suppressive cells secreting *TGFb* [Skog et al., 2008]. Circulating miRNAs were found to detect cancer in early stages of development. Huang et al. [2013a] studied patterns of miRNAs in the blood stream in the early stages of CRC. The usefulness of circulating miRNAs as biomarkers for a wide range of cancer such as CRC (Fig. 3). It is remarkable that circulating miRNA profiles distinguish adenoma from healthy controls with 73% sensitivity and 79% specificity. These data show that cell-free miRNAs are confirmed to act as a biomarker for the early diagnosis of tumors [Valenti et al., 2007], while miRNA-17-3p is considered as a diagnostic marker for CRC. Stool is another biological material in which miRNA is preserved and studied. The most important known miRNA has been observed to have an increased expression, including miR-21, miR-203, miR-126, and miR16, while miRNAs with decreased expression are miR-320 and miR-192. Low expression of hsa-miR-16 and hsa-miR-126b in stool with sensitivity of 91% and specificity of 72% can be used to detect the presence of CRC [Brase et al., 2010].

CONCLUSION

CRC is the third most common cause of death worldwide, which highlights the need for identification of novel biomarkers to identify patients at earlier stages or detect novel anticancer agents for the treatment of CRC patients. Increasing evidence shows that miRNA can be used as a promising class of potential therapeutic target. On the basis of this information, LNA-modified oligonucleotides are suggested as a therapeutic option for targeting dysregulated miRNAs in cancer, although further studies are needed to explore the value of this approach in the treatment of CRC.

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