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MicroRNAs Used in Combination with Anti-Cancer Treatments Can Enhance Therapy Efficacy

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Abstract: MicroRNAs (miRNAs), a recently discovered class of small non-coding RNAs, constitute a promising approach to anti-cancer treatments when they are used in combination with other agents. MiRNAs are evolutionarily conserved non-coding RNAs that negatively regulate gene expression by binding to the complementary sequence in the 3'-untranslated region (UTR) of target genes. MiRNAs typically suppress gene expression by direct association with target transcripts, thus decreasing the expression levels of target proteins. The delivery to cells of synthetic miRNAs that mimic endogenous miRNA targeting genes involved in the DNA-Damage Response (DDR) can perturb the process, making cells more sensitive to chemotherapy or radiotherapy. This review examines how cells respond to combined therapy and it provides insights into the role of miRNAs in targeting the DDR repair pathway when they are used in combination with chemical compounds or ionizing radiation to enhance cellular sensitivity to treatments.

Keywords: Apoptosis, Cell cycle checkpoints, DNA-Damage Response, DNA Repair, microRNAs, Tumor resistance.

1. INTRODUCTION

Mammalian cells rely on a highly regulated and complex pathway to respond to DNA damage caused by genotoxic stress, which is commonly referred to as DNA-Damage Response (DDR), and includes damage sensors, mediators, signal transducers and effectors involved in cell cycle checkpoint activation, DNA repair, and apoptosis. Following genotoxic stress, the DDR pathway is post-transcriptionally regulated through selective mRNA stabilization or decay or translational control [1]; in this context, microRNAs (miRNAs) have emerged as important regulators of gene expression of key components of the DDR pathway. DNA damage can regulate miRNA expression at both the transcriptional and post-transcriptional levels as well as at miRNA biogenesis in an ataxia telangiectasia mutated (ATM)-dependent manner [2].

Alterations in the expression levels of DDR genes are frequently associated with resistance to conventional cancer therapies that rely on efficient DNA repair and/or alterations in cell cycle checkpoints and apoptosis regulation. The possibility of restoring the correct expression levels through miRNA targeting has recently been investigated as a potential therapeutic approach. The overexpression of tumor-suppressive miRNAs or inhibition of oncogenic miRNAs has been shown to have therapeutic potential in model systems [3]. Here we summarize recent findings linked to the emerging role of miRNAs in targeting the DDR pathway when they are used in combination with chemotherapic agents or ionizing radiation (IR) as a new strategy to enhance the cytotoxic effects of cancer treatments in tumor resistant cells.

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1.1. DNA-Damage Response

To ensure genomic stability, the DDR must be able to recognize all types of DNA structural alterations, such as double-strand breaks (DSBs), stalled replication forks, nicks, and gaps. When cells are unable to properly repair DNA, apoptosis or senescence pathways may be triggered to eliminate the possibility of passing on damaged or unrepaired genetic material to its progeny.

DSBs represent the most deleterious DNA lesions to cells since they can lead to cell death if they are left unrepaired and can cause chromosomal translocations or genomic instability if they are repaired incorrectly. Upon DSBs are recognized, members of the phosphatidylinositol-3-OH kinase (PI(3)K)-like family, such as ATM (DNA-PKcs (DNA-dependent protein kinase catalytic subunit) and ATR (ataxia telangiectasia and Rad3 related) [4] relay and amplify the damage signal to effector proteins that in turn activate cell cycle checkpoints, regulate transcription, translation, and metabolism, and activate the appropriate DNA repair process, or seal the fate of the cells, such as apoptosis or senescence [5, 6]. Following DNA DSB induction, ATM undergoes spatial relocalization and catalytic activation. It is subsequently rapidly recruited to DNA damage sites; here ATM phosphorylates specific serines or threonines on many downstream protein substrates, including Ser-139 of the histone variant H2AX present in nucleosomes surrounding DSB sites, thereby regulating the DDR mechanism [7]. p53, which maintains genomic integrity by transactivating target genes involved in cell cycle arrest, DNA repair, apoptosis

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and senescence, is one of the proteins phosphorylated by ATM [8, 9]. In mammalian cells, the MRN complex (Mre11, Rad50, Nbs1) is one of the first complexes to be recruited to DSB sites where it acts as a damage sensor that can also form a physical bridge spanning the DSB ends [10]. The MRN complex recognizes DSBs and binds to the DNA surrounding the lesion, resecting the DNA around the break in a 5'–3' dependent direction, and producing long 3′ ends that are recognized by the Replication Protein A (RPA) [10]. RPA is displaced by BRCA2, which binds to RAD51 and initiates repair by homologous recombination (HR) [11]. This pathway promotes accurate DNA repair by using an undamaged homologous sequence as its template. DSBs recognized and tethered by the MRN complex can also be repaired by the non-homologous end-joining pathway (NHEJ), which employs the products of the Ku70/80 heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, XRCC4, DNA ligase IV and XLF (XRCC4-like factor, also called Cernunnos) [12]. NHEJ directly joins the two ends of a DSB regardless of the sequence template at the break's exposed ends, making it error-prone but available at all times during the cell cycle. In contrast to NHEJ, HR is active only in the S and G2 phases of the cell cycle, when an intact sister chromatid sequence is available as a template for error-free DSB repair.

DNA structural alterations consisting in base and nucleotide damage are repaired by base excision repair (BER) and nucleotide excision repair (NER) pathways. The former repairs damaged bases, abasic sites, and single strand breaks (SSBs); the latter repairs DNA lesions that distort the DNA helical structure. The proteins of the NER/BER pathways seem to be connected to the DSB repair pathway; indeed ATM and ATR recruitment and their phosphorylation are negatively affected in cells defective in DDB2 or XPC functions, and DDB2 and XPC promote DNA repair through the BRCA1- and RAD51-dependent pathway [13]. While the mismatch repair (MMR) pathway is involved in the repair of small insertions, deletions and base-base mismatches caused by base deamination, oxidation, methylation, and replication errors [14, 15], the Fanconi Anaemia (FA) repair pathway is involved in repairing interstrand cross-links at replication forks, possibly by facilitating ATR- and ATM-dependent checkpoint signaling and activating HR repair [16].

2. MICRORNAs

MiRNAs are endogenous single stranded non-coding RNAs (18-24 nt), which act as post-transcriptional modulators of gene expression by interacting with 3'-untranslated regions (UTR) of target genes. Mature miRNAs recognize their target mRNAs by base-pairing interactions between nucleotides 2-8 of the miRNA (the seed region) and complementary nucleotides in the 3'UTR of mRNAs (referred to as miRNA response elements, MREs). MiRNAs typically repress translation and/or induce destabilization and decay of mRNAs [17]. Recruitment of the deadenylase and decapping complexes is required both for translational repression and mRNA degradation; the subsequent exonucleolytic digestion is required for mRNA decay [18].

MiRNAs play a physiological role in a variety of important biological and pathological processes, as well as in cellular stress. It is estimated that more than half of DNA damage checkpoint genes and DNA repair genes contain conserved miRNA target sites [19]. Genes that are key regulators or essential for cell function may have high rates of transcription and low rates of translation [20]. In this context, the negative translational modulation by miRNAs allows the cell to make many mRNA copies but to have a low and carefully controlled amount of protein [21].

MiRNA expression profiling has revealed drastic changes in the expression of multiple miRNAs in human cancers; indeed a variety of miRNAs are specifically overexpressed or lost in tumors compared to that in normal tissues. Oncogenic miRNAs, called oncomiRs, promote tumour development through the negative regulation of tumour suppressor genes, and tumour suppressor miRNAs, called anti-oncomiRs, are able to inhibit tumour growth by targeting oncogenes [22].

MiRNAs involved in the DDR pathway play a fundamental role in tumor formation and progression. Three members of the miR-34 family - miR-34a, miR-34b, and miR-34c - have been shown to inhibit genes involved in controlling cell cycle progression and apoptosis [23]. It has been demonstrated that miR-17/20 and miR-221/222 clusters target cell cycle regulators thus controlling cell cycle checkpoints and progression [24, 25]. To date, increasing numbers of miRNAs target genes involved in DDR pathways have been functionally validated (Table **1**).

3. THERAPEUTIC RESISTANCE OF CANCER CELLS

Therapeutic resistance is the primary factor that limits the effectiveness of some cancer therapies such as chemotherapy and radiotherapy. Anticancer treatments target proliferating cancer cells by interfering with DNA replication, thereby generating lethal DNA damage. Some cancer treatments such as chemotherapeutic drugs, ionizing radiation (IR) and topoisomerase II poisons can induce DSBs both directly and indirectly via single-stranded DNA (ssDNA) lesions, which induce replication fork collapse leading to DSB formation. DNA damage caused by drugs or IR and the inherent DNA repair capacity of tumour cells are important factors that determine therapeutic outcome.

3.1. Tumour Resistance Linked to DDR Gene Alterations

Proficient DSB repair is required for cellular resistance to agents that induce DSBs in the S-phase, such as IR, topoisomerase poisons (e.g. camptothecin and etoposide), and poly ADP ribose (PARP) polymerase inhibitors. Several tumors have, however, a strong DSB repair capacity meaning that therapy has low efficacy against cancer cells. Cells that overexpress RAD51 exhibit resistance to radiotherapy and chemotherapy [73-75] and are correlated with poor clinical outcome in lung, prostate, and breast cancers [76-78]. RAD51 expression is increased during breast cancer progression and metastasis, and the subsequent knockdown of RAD51 has been found to repress cancer cell migration *in vitro* and to reduce primary tumor growth in a

Table 1. MiRNA-mRNA interactions in the DDR pathway functionally validated by luciferase assay.

miRNA	Gene targeted	DDR pathway	Type of cells	Ref.
miR-100	*ATM*	DNA damage signalling, DNA repair, cell cycle, apoptosis	Malignant glioma cells	[26]
miR-101			Lung cancer cells	[27]
miR-27a			Gastric cancer cells	[28]
miR-181a			Glioblastoma multiforme cells	[29]
miR-26a			Breast cancer cells	[30]
miR-18a			Colorectal cancer cells	[31]
miR-203			Neuroblastoma cells	[32]
miR-421			Neuroblastoma cells	[33]
miR-182	*BRCA1*	DNA repair	Breast cancer cells	[34]
miR-1255b			Breast/ovarian cancer cells	[35]
miR-9			Ovarian cancer cells	[36]
miR-638			Breast cancer cells	[37]
miR-146a/b-5p			Breast cancer cells	[38]
miR-1245	*BRCA2*	DNA repair	Breast cancer cells	[39]
miR-148b*			Breast/ovarian cancer cells	[35]
miR-101	*DNA-PKcs*	DNA repair	Malignant glioma cells	[27]
miR-424*	*FANCF*	DNA repair	Lung cancer cells	[40]
miR-383	*GADD45G*	DNA repair, cell cycle, apoptosis	Breast cancer cells	[41]
miR-15b/16-2	*PPM1D*	DNA Damage sensing	Bronchial epithelial cells	[42]
miR-16			Breast and osteosarcoma cancer cells	[43]
miR-155	*RAD51*	DNA repair	Breast cancer cells	[44]
miR-96-5p			Not available	[45]
miR-96			Breast cancer cells	[46]
miR-193b*			Breast/ovarian cancer cells	[35]
miR-103/107			Osteosarcoma cells	[47]
miR-7	*XRCC2*	DNA repair	Colorectal cancer cells	[48]
miR-125b	*P53*	Cell cycle checkpoint, apoptosis	Neuroblastoma cells	[49]
miR-504			Colorectal carcinoma cells	[50]
miR-25/miR-30d			Lung adenocarcinoma cells	[51]
miR-19b			Breast and cervical cancer cells	[52]
miR-150			Lung cancer cells	[53]
miR-509-5p	*MDM2*	p53 stabilization	Cervical/hepatoma cancer cells	[54]
miR-660			Lung cancer cells	[55]
miR-490-3p	*CCND1*	Cell cycle	Lung cancer cells	[56]
miR-545			Lung cancer cells	[57]
miR-138	*CCND3*	Cell cycle	Hepatocellular carcinoma	[58]
miR-630	*CDC7*	Cell cycle	Lung cancer cells	[59]
miR-509-3p	*CDK2*	Cell cycle	Lung cancer cells	[60]
miR-545	*CDK4*	Cell cycle	Lung cancer cells	[57]
miR-224	*CDKN1A (p21)*	Cell cycle	Lung cancer cells	[61]

(Table 1) Contd….

miRNA	Gene targeted	DDR pathway	Type of cells	Ref.
miR-451	*c-MYC*	Cell cycle	Lung adenocarcinoma cells	[62]
miR-144	*BAX*	Apoptosis	Lung cancer cells	[40]
miR-200bc/429	*BCL2*	Apoptosis	Gastric cancer cells	[63]
miR-181			Chronic lymphocytic leukemia	[64]
miR-204			Neuroblastoma cells	[65]
miR-24-2			MCF-7 cells	[66]
miR-181a			Leukemia cells	[67]
miR-363	*MCL-1*	Apoptosis	Breast cancer cells	[68]
miR-181a	*PRKCD*	Apoptosis	Cervical cancer cells	[69]
miR-221	*PTEN*	Apoptosis	Breast cancer cells	[70]
miR-222	*PUMA*	Apoptosis	Oral squamous cell carcinoma	[71]
miR-424*	*STAT5A*	Apoptosis	Lung cancer cells	[40]
miR-155	*TP53INP1*	Apoptosis	Lung cancer cells	[53]
miR-200bc/429	*XIAP*	Apoptosis	Gastric cancer cells	[63]
miR-24			Lung cancer cells	[72]

syngeneic mouse model *in vivo* [79]. BRCA1 and BRCA2 are key components of the HR repair pathway and integral parts of the cellular DNA damage response. Mutations restoring BRCA1/BRCA2 function lead to a loss of sensitivity to cisplatin in human ovarian tumors [80]. Overexpression of DNA-PKcs, which is closely associated with tumor cell growth, poor prognosis, and the clinical therapeutic outcome, is frequently found in various cancers [81-83]. Increased expression of XRCC2 and XRCC4, involved respectively in HR and NHEJ repair, has been detected in lung cancer cells [84]. The down-regulation of the core MMR recognition protein complex, human mutS homolog 2 (hMSH2) and 6 (hMSH6) causes resistance to 5-FU [85]. Defects in MMR proteins have been associated with reduced or absent benefits from 5-FU adjuvant chemotherapy [86], topoisomerase inhibitors chemotherapy [87, 88], and alkylating agents [87; 89]. Examples exist of increased expression of genes associated with nucleotide excision repair (NER) in resistant cells and consequent increases in repair activity. The over-expression of excision repair cross-complementation group 1 ERCC1 protein has been linked to poor responses to chemotherapy in numerous cancer types, including non-small cell lung cancer, squamous cell carcinoma, and ovarian cancer [90-92]. Barckhausen *et al.* [93] demonstrated that drug-induced resistance in melanoma cells is a result of p53-dependent up-regulation of the NER genes XPC and DDB2, which stimulate the repair of DNA interstrand cross-links arising from O(6)-chloroethylguanine.

Many tumors display alterations in cell cycle progression that cause aberrant cell growth. Oncogenic alterations of cyclin-dependent serine/threonine kinases (CDKs), cyclins (CCNs) and inhibitors of cyclin-dependent kinases (CDKIs) have been reported in more than 90% of human cancers [94]. Tumor cell lines expressing higher levels of cyclin CCND1 have demonstrated greater resistance to cytotoxic drugs with respect to cells expressing lower levels [95, 96]. Overexpression of CCND1 in a human fibrosarcoma cell line has been shown to confer resistance to methotrexate [97]; conversely, suppression of CCND1 has been shown to potentiate the response of human pancreatic cancer cells to cisplatinum [98]. *CDKN2A/2B* deletion confers a poor prognosis in diffuse large B cell lymphoma under rituximab-CHOP (R-CHOP) chemotherapy [99]. *MYC* gene encodes for c-Myc, an oncogenic transcription factor involved in cell cycle progression, which is found to be deregulated in many human tumors, and frequently associated with tumor progression [100]. C-Myc is significantly upregulated in docetaxel-non-responding lung adenocarcinoma tissues in comparison with docetaxel-responding tissues [62].

Up-regulation of anti-apoptotic genes or down-regulation of pro-apoptotic genes is frequently observed in human tumors. Aberrant expression of members of the Bcl-2 (B-cell leukemia/lymphoma-2) family is, for example, strongly associated with resistance to chemotherapy and radiation [101-103]. Myeloid cell leukemia-1 (MCL-1) is an anti-apoptotic Bcl-2 family member that is often overexpressed in breast tumors and has been reported to play an important role in regulating drug resistance in various types of cancer [68]. X-linked inhibitors of apoptosis proteins (XIAP), which belong to the family of inhibitors of apoptosis proteins (IAPs), have been described as a chemoresistance factor in mammalian cancer [104].

4. MiRNAs TARGETING DDR GENES TO INCREASE RADIO/CHEMO-SENSITIVITY OF TUMOR RESISTANT CELLS

The disruption of DNA-damage response pathways via chemotherapeutic compounds used as monotherapy or in conjunction with radiotherapy has led to promising results in the clinical setting with regard to the treatment of various cancers. Inhibitors of the DDR pathway have been shown to have a great potential for chemo- and radio-sensitization of numerous cancers. Resistant cancers do not, however, respond to therapy as a consequence of the altered expression of genes that confer resistance to drugs or IR. Resistant cancer cells rely on efficient DNA repair, alterations in cell cycle checkpoints and apoptosis regulation, and no inhibitors have been successfully used until now.

The approach that several current research studies are attempting is that of combining normal chemotherapy and radiotherapy using the RNA interference (RNAi) technique to specifically knock-down the expression of the drug- or IR-resistance genes. During gene silencing the resistant cells transiently become sensitized to the anti-cancer treatment. The therapeutic delivery to cells of synthetic miRNAs that mimic endogenous miRNAs that modulate genes involved in the DDR pathway has, therefore, been considered an appropriate approach to treating resistant cancer cells. MiRNA mimics, called miRNA precursor molecules (pre-miR), are, thus small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNA molecules, and they enable miRNA functional analysis by up-regulation of miRNA activity. Pre-miR molecules are carefully designed and modified to ensure that the correct strand, representing the desired mature miRNA, is taken up into the RNA-induced silencing-like complex (RISC) responsible for miRNA activity (Fig. **1**). The delivery into cells of synthetic miRNAs that mimic endogenous miRNAs targeting genes involved in DDR can impair the process, making cells more sensitive to IR. An interval of 24-48h represents the time that is necessary for miRNA-transfected cells to show alterations in the expression level of target genes as a consequence of miRNA action. This time interval after transfection has been calculated on the basis of experiments described in the literature and of our own previous data [28, 40]. Pre-miRNAs rather than mature miRNAs are usually employed for therapeutical purposes. MiRNA precursors are double-stranded RNA molecules composed of a guide strand that is identical to the mature miRNA and a passenger strand that is partially or fully complementary to the guide strand [105]. Chorn *et al.* [106] recently demonstrated that modified single-stranded miRNA mimics also exhibit significant miRNA seed-based activity in cultured cells. The efficacy of single-stranded miRNA mimics was, however, reduced with respect to that of double-stranded ones, suggesting that further chemical modifications could be developed to enhance the targeting activity of single-stranded miRNA mimics. The experimental methods to examine the interaction between the microRNA and its targeting site(s) in the mRNA are important for understanding microRNA functions. Their interaction should be validated by luciferase assay to ensure that miRNA targets the exact mRNA. Luciferase reporter vectors, including recently introduced ones, are designed to test miRNA-mediated gene silencing. Cells are co-transfected with luciferase constructs containing the 3'UTRs of target genes (wild type/mutant) and with pre-miR™ miRNA precursor molecules. Cell lysates are collected 24h after transfection and the effect of miRNA on the luciferase gene expression can be seen as changes in the relative luciferase activity. Importantly, this assay does not reveal whether an interaction occurs endogenously, and unless care is taken with the miRNA concentration and promoter selection for the reporter, it does not reveal whether it could happen under physiological conditions. The functional interaction between miRNA and target transcript should, therefore, be validated under physiological conditions in miRNA-transfected cells by measuring the expression level of miRNA target genes using quantitative real-time PCR (qRT-PCR) and western blotting.

Transfecting mammalian cells with exogenous small interfering RNAs (siRNAs) has been adopted as a technology for targeted gene silencing. SiRNAs are double-strand RNAs of 21 to 23 nucleotides that can function in translational repression by binding to mRNAs with perfect sequence complementarity and causing cleavage of the mRNAs. One siRNA cleaving its mRNA target may provide robust gene silencing of target mRNA expression while a single miRNA leading to translational repression generally does not lead to robust gene silencing of its target. Indeed, the power of miRNA-based gene silencing may be due to the fact that one miRNA targets multiple genes, many of which participate in common signalling pathways. Furthermore, several different miRNAs may target a single mRNA [107]. One major concern linked to the therapeutical use of siRNAs is the potential for off-target effects, depending on the translational repression by siRNAs of unintended mRNA targets. MiRNA mimics carry the same sequence as naturally occurring endogenous miRNAs and are therefore expected to target the same set of genes, while siRNAs are exogenously introduced and no endogenous siRNAs have been identified in mammals. As cells already express the miRNA in question, the administration of a miRNA to cells can unlikely induce adverse events. Another concern linked to the therapeutic use of siRNAs is their potential to be immunogenic; they can indeed induce high levels of inflammatory cytokines and type I interferons, in particular interferon-alpha, after systemic administration in mammals and in primary human blood cell cultures [108].

The combination of miRNA delivery with chemotherapy or IR has recently been proposed as a strategy to improve therapy efficacy in patients with cancers refractory to standard therapies. To date, several studies have shown that miRNAs may be a useful modality to restore DDR gene networks in different cancer cell lines and *in vivo* models, rendering cells more sensitive to anti-cancer treatments.

4.1. Sensitizing Tumor Resistant Cells Through miRNAs Targeting DNA Repair Genes

The inability of tumor cells to properly repair some types of DNA damage render them more sensitive than normal cells to DNA-damaging agents. Some cancer cells that rely

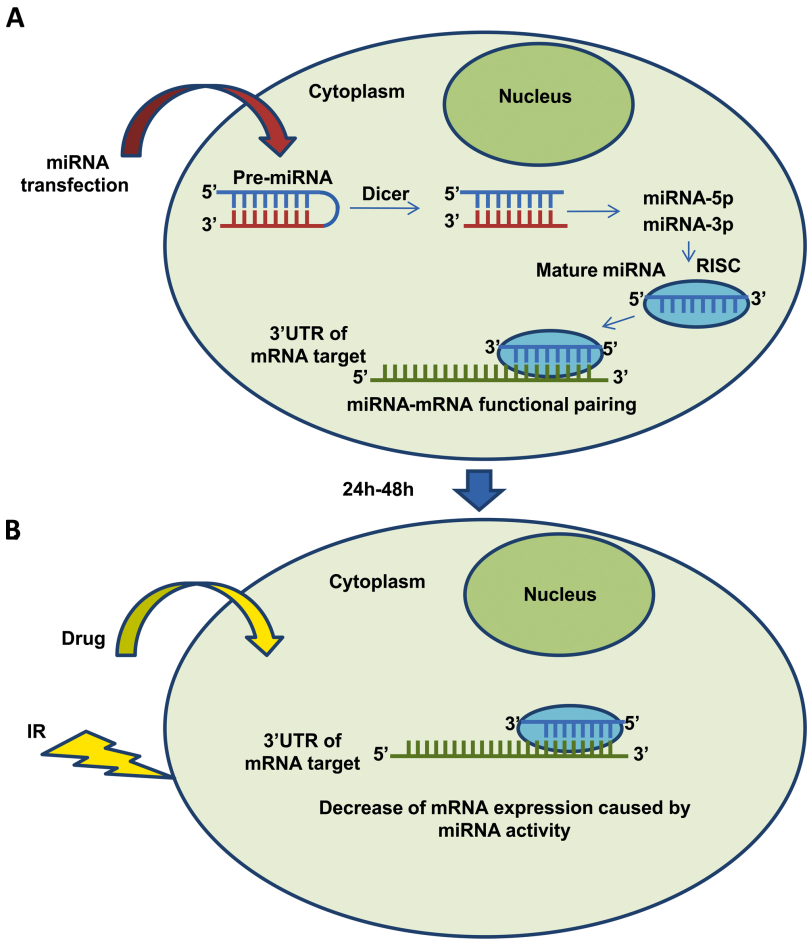

Fig. (1). Therapeutical approach for resistant cancer cells. **A**) Cells are transfected with miRNA mimics targeting DDR genes. **B**) After transfection (24-48h) cells are treated with chemotherapic drugs or ionizing radiation (IR).

on high repair efficiency due to the over-expression of DNA repair genes are nevertheless resistant to conventional therapy. There are several examples of miRNAs targeting DNA repair genes combined with cancer therapy described in the literature. MiR-26a enhanced the *in vivo* radiosensitivity of glioblastoma multiforme cells by targeting ATM and reducing their DNA repair ability [30]. The microRNA-mediated down-regulation of ATM by miR-421 leads to clinically manifest tumor radiosensitivity of oral carcinoma cells [109]. The down-regulation of ATM and DNA-PKcs by direct targeting of miR-101 sensitizes glioma cell lines to ionizing radiation [27]. Inhibition of H2AX expression by either miR-24 or miR-138 promotes cellular sensitivity to IR and/ or genotoxic agents [110, 111]. MiR-155 has been demonstrated to be a direct regulator of RAD51 and to enhance the radiosensitivity of breast cancer cells [44]. Chemosensitization to DNA damaging agents has been observed when miR-103/107 were overexpressed in several cell lines [47]. These miRNAs directly target and regulate RAD51 and RAD51D, which are essential proteins for the HR repair system. Overexpression of BRCA1 in breast cancer cells has been reported to lead to an increased resistance to cisplatin [112]. MiR-638 down-regulates BRCA1 expression sensitizing breast cancer cells to DNA-damaging agents [37]. MiR-9 mediates the down-regulation of BRCA1 and impedes DNA damage repair, increasing the sensitivity of ovarian cancer cells to cisplatin [36]. MiR-218 regulates cisplatin chemosensitivity in breast cancer by targeting BRCA1 [113]. MiR-16 inhibits the DNA damage-mediated induction of Wip1 in human breast cancer cells (MCF-7), sensitizing them to doxorubicin [43]. MiR-21 has been demonstrated to negatively regulate mismatch repair genes MLH1 and MSH2, increasing the therapeutic efficacy of 5-FU in colorectal tumors [114].

4.2. Sensitizing Tumor Resistant Cells Through miRNAs Targeting Cell Cycle Genes

Tumor resistant cells commonly have cell cycle alterations as in the case of cisplatin-resistant cells in which cell-cycle kinases WEE1 and CHK1 are frequently overexpressed. It has been shown that miR-155 and the miR-15 family sensitized cisplatin-resistant cells to apoptosis by targeting WEE1 and CHK1 that regulate the G_2/M checkpoint in the cell cycle [115]. MiR-122 sensitizes hepatocellular carcinoma cells to chemotherapeutic drugs by modulating the expression of the anti-apoptotic gene Bcl-w and the cell cycle related gene cyclin B1 [116]. MiR-133a

sensitized colorectal cancer cells to chemotherapeutic drug treatment with doxorubicin or oxaliplatin by targeting the RFFL gene which regulates the p53/p21 signaling pathway [117]. MiR-224 targets p21Waf1/CIP1 [61], which is responsible for cell cycle control, blocking the transition from phase G1 to phase S. The down-regulation of miR-27a could sensitize gastric cancer cells to drugs by decreasing cyclin D1 expression and up-regulating p21expression [118]. Cells overexpressing miR-383, which targets the DDR gene GADD45G, exhibited a more severe cell death than control cells exposed to cisplatin treatment [41].

4.3. Sensitizing Tumor Resistant Cells Through miRNAs Targeting Apoptotic Genes

Tumor cells resistant to conventional therapy frequently show a decreased susceptibility to drug-induced apoptosis as a consequence of an up-regulation of anti-apoptotic proteins and a down-regulation of pro-apoptotic proteins. Several reports have shown that the miR-181b, miR-451, miR-497 and miR-200bc/429 cluster sensitize the cells to platinum induced cell death by down-regulating the expression of anti-apoptotic BCL-2 gene whose overexpression causes drug resistance [63, 64, 119]. MiR-129 triggers apoptosis by suppressing BCL2 and enhancing the cytotoxic effect of 5-fluorouracil in colorectal cancer cells [120]. MiR-204 significantly increased sensitivity to cisplatin and etoposide in neuroblastoma cells by targeting BCL-2 [65]. Xie *et al.* [72] demonstrated that miR-24 overexpression can overcome apoptosis resistance in cancer cells via down-regulation of XIAP expression in TRAIL-resistant lung cancer cells. In gastric and lung cell lines, the miRNA cluster miR-200bc/429 was shown to promote apoptosis by targeting BCL2 and XIAP, which sensitized resistant lines to vincristine as well as to cisplatin [63]. MiR-221 conferred resistance to trastuzumab in breast cancer cells by targeting PTEN, and suppression of miR-221 or restoration of PTEN expression reversed the malignant phenotypes of breast cancer cells [70]. MiR-181a negatively regulated the expression of PRKCD, a pro-apoptotic protein kinase, thereby inhibiting irradiation-induced apoptosis and decreasing G2/M block [69]. MiR-181a sensitizes, moreover, a multidrug-resistant leukemia cell line K562/A02 to daunorubicin by targeting BCL-2 [67]. MiR-363 reversed the resistance of the MDA-MB-231 breast cancer cell line to cisplatin by targeting anti-apoptotic gene Mcl-1 [68]. MiR-222 targeted PUMA to sensitize human oral squamous cell carcinoma cells to cisplatin [71].

5. THERAPEUTIC DELIVERY OF miRNAs TARGETING DDR GENES

The efficacy of the combined use of miRNAs targeting DDR genes together with chemo or radiotherapy has been demonstrated in mice. PEG-coated iron nanoparticles have been successfully used as delivery systems for miR-16 to reduce adriamycin resistance in a mouse gastric cancer model [121]. A xenograft of miR-101-overexpressing cells in mice yields tumors that shrink to a greater extent with respect to those in controls after IR [27]. Cortez *et al.* [122] recently demonstrated that the therapeutic delivery of a liposomal loaded with miR-200c mimics in combination with fractioned irradiation enhanced radiosensitivity in the lung cancer cells of xenograft mice by directly regulating the oxidative stress response genes PRDX2, GAPB/Nrf2, and SESN1 in ways that inhibited DNA double-strand break repair, increased the levels of reactive oxygen species, and up-regulated p21. Kasinski *et al.* [123] demonstrated that the systemic nanodelivery of miR-34 suppressed tumor growth leading to survival advantage. The therapeutic delivery of miR-205 mimics via nanoliposomes can sensitize the tumour to radiation by inhibiting DNA damage repair in a xenograft model [124]. Mir-205 combined with gemcitabine conjugated micelles in a pancreatic tumor model of gemcitabine resistant cells was found to produce significant inhibition of tumor growth [125]. MiR-34a, which inhibits tumor growth by regulating multiple tumor-related genes including p53 and cyclin D1, has been successfully incorporated in nanoparticle formulations to reduce cell proliferation and delay lung tumor growth in p53 deficient tumors [126]. MiR-34a incorporated together with paclitaxel into cationic solid lipid nanoparticles enhanced anti-cancer therapy in mice [127]. MiR-34a and doxorubicin can be efficiently encapsulated into hyaluronic acid-chitosan nanoparticles and delivered to breast tumor cells or tumor tissues and enhance the anti-tumor effects of the drug by suppressing the expression of non-pump resistance and anti-apoptosis proto-oncogene Bcl-2 [128]. MiR-129 enhances 5-FU cytotoxicity by suppressing BCL2 in a mouse colorectal tumor xenograft model [120].

Scarce data concerning the therapeutic delivery of miRNAs targeting genes involved in DDR are available with regard to humans. To date, MRX34 is the only miR-34a replacement therapy being assessed by a phase 1 clinical trial in patients with primary liver cancer or liver metastasis from other cancers using miR-34 mimic and the liposomal delivery technology [129]. The MRX34 phase 1 clinical study on solid tumors and hematological malignancies is expected to be completed shortly. The primary objectives of the clinical trial were to establish the maximum tolerated dose and the recommended phase 2 dose for future clinical trials. The secondary objectives were to assess the safety, tolerability and pharmacokinetic profile of MRX34 as well as to evaluate its biological activity and clinical outcomes. Further studies are critical to discover better delivery methods and improved oligonucleotide modification to enhance the performance of miRNAs as therapeutic agents in human patients.

CONCLUSION

MiRNAs which affect chemo- and radio-resistance could improve targeted miRNA-based therapeutic strategies and enhance the clinical outcome of conventional tumor therapy. MiRNAs as therapeutical agents have several advantages: they are, in fact, small, stable, can be delivered systemically and must enter into the cytoplasm of target cells to be active. As a result, the delivery of miRNA mimics seems to face less of a hurdle compared with protein-encoding DNA [130]. MiRNA mimics have the same sequence as naturally occurring miRNAs and are, therefore, expected to target the same set of mRNAs that are also regulated by natural miRNAs with little off-target effects. Moreover, since the

genes targeted by a single miRNA are commonly related and function in a comparable cellular process, the targeting of a single miRNA can result in a combinatorial effect of gene expression changes in related target genes. Potential side effects of miRNA therapy must, nevertheless, be considered. Since miRNAs typically target hundreds of transcripts, there is a limit on the effect of a miRNA on an individual protein and miRNA modulation might have beneficial effects in one cell type but harmful ones in another. Moreover, in view of the fact that the majority of preclinical models have used mice with different degrees of immunosuppression, the interaction between the host immune system and miRNAs should be considered since it may improve or weaken the therapeutic effects of a particular miRNA [131]. Other limits associated to the use of miRNAs as therapeutic agents are their systemic delivery linked to degradation by nucleases, renal clearance, failure to cross the capillary endothelium, and cellular uptake of sufficient amounts of synthetic oligonucleotides to achieve sustained target inhibition [132-134]. In addition, the molecule's negative charge of miRNAs prevents their penetration through the cell membrane, although conjugation of miRNAs to cationic carriers should improve cell penetration and also prevent enzymatic cleavage of the miRNA, as has been reported for siRNAs [135]. Finally, the tumor microenvironment heterogeneity significantly enhances the efficacy of drug therapy, probably contributing to the development of drug resistance in some contexts [136].

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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