Molecular Therapy
Nucleic Acids
Review

miR-221/222 as biomarkers and targets for therapeutic intervention on cancer and other diseases: A systematic review

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Among deregulated microRNAs (miRs) in human malignancies, miR-221 has been widely investigated for its oncogenic role and as a promising biomarker. Moreover, recent evidence suggests miR-221 as a fine-tuner of chronic liver injury and inflammation-related events. Available information also supports the potential of miR-221 silencing as promising therapeutic intervention. In this systematic review, we selected papers from the principal databases (PubMed, MedLine, Medscape, ASCO, ESMO) between January 2012 and December 2020, using the keywords "miR-221" and the specific keywords related to the most important hematologic and solid malignancies, and some non-malignant diseases, to define and characterize deregulated miR-221 as a valuable therapeutic target in the modern vision of molecular medicine. We found a major role of miR-221 in this view.

INTRODUCTION

In the last two decades since the discovery of microRNA (miRNA, miR), the knowledge of their biology has considerably increased. The understanding of the role of miRs in different diseases, mostly in cancer, has made miRs attractive tools and targets for novel therapeutic approaches.[1] Functional studies have confirmed that miR deregulation is involved in the onset of many cancers, with a variety of miRs acting as tumor suppressors or oncogenes. miRs have been explored as potential therapeutics, by either miR replacement therapy using miR mimics or inhibition of miR function by antimiRs, and have shown promising behavior in the preclinical settings.[2] The progress of miR therapeutics has been partially due to the increased stability of novel RNA molecules that have improved the development delivery systems/strategies for *in vivo* therapeutic use.[3] So far, even if a variety of preclinical studies with different models of human disease have tested the use of these new-generation therapeutics, few miR-based therapeutics have advanced into clinical testing. These include a mimic of the tumor-suppressor miR-34 for cancer treatment,[4] a locked nucleic acid (LNA) miR-155 antagomiR to treat cutaneous T cell lymphoma, adult T cell leukemia/lymphoma (ATLL), and chronic lymphocytic leukemia (CLL),[5] and an anti-miR miR-122, for treating hepatitis C virus (HCV)-positive hepatitis.[6] More recently, an antisense oligonucleotide targeting miR-221 (LNA-i-miR-221) was approved for first-in-human (FIH) evaluation (NCT04811898), specifically designed to study the safety and tolerability in advanced solid tumor patients. In this systematic review, we discuss the results published from January 2012 to December 2020 in the principal databases (PubMed, MedLine, Medscape, ASCO, ESMO) using the keywords "miR-221" and the name of the specific solid tumor. We specifically describe recent advances in the understanding of the biological role of miR-221 or miR-221/22 cluster, including the evaluation as a potential biomarker, and provide an overview of the current opportunity for miR-221 therapeutics in the treatment of solid tumors and hematological malignancies, some premalignant conditions, and non-cancer-related diseases.

BIOGENESIS OF miR-221 AND ITS DRIVER ROLE IN HUMAN DISEASES IN THE EPIGENETIC VISION OF DISEASE PATHOGENESIS

miRs are small non-coding single-stranded RNAs that regulate protein expression and are involved in many biological events. The biogenesis of miRs is subjected to tight control and their deregulation has been associated with several diseases.[7] Starting from the DNA sequence, miRs are transcribed into primary miRs (pri-miRs) and then processed in precursor-miRs (pre-miRs) before reaching the

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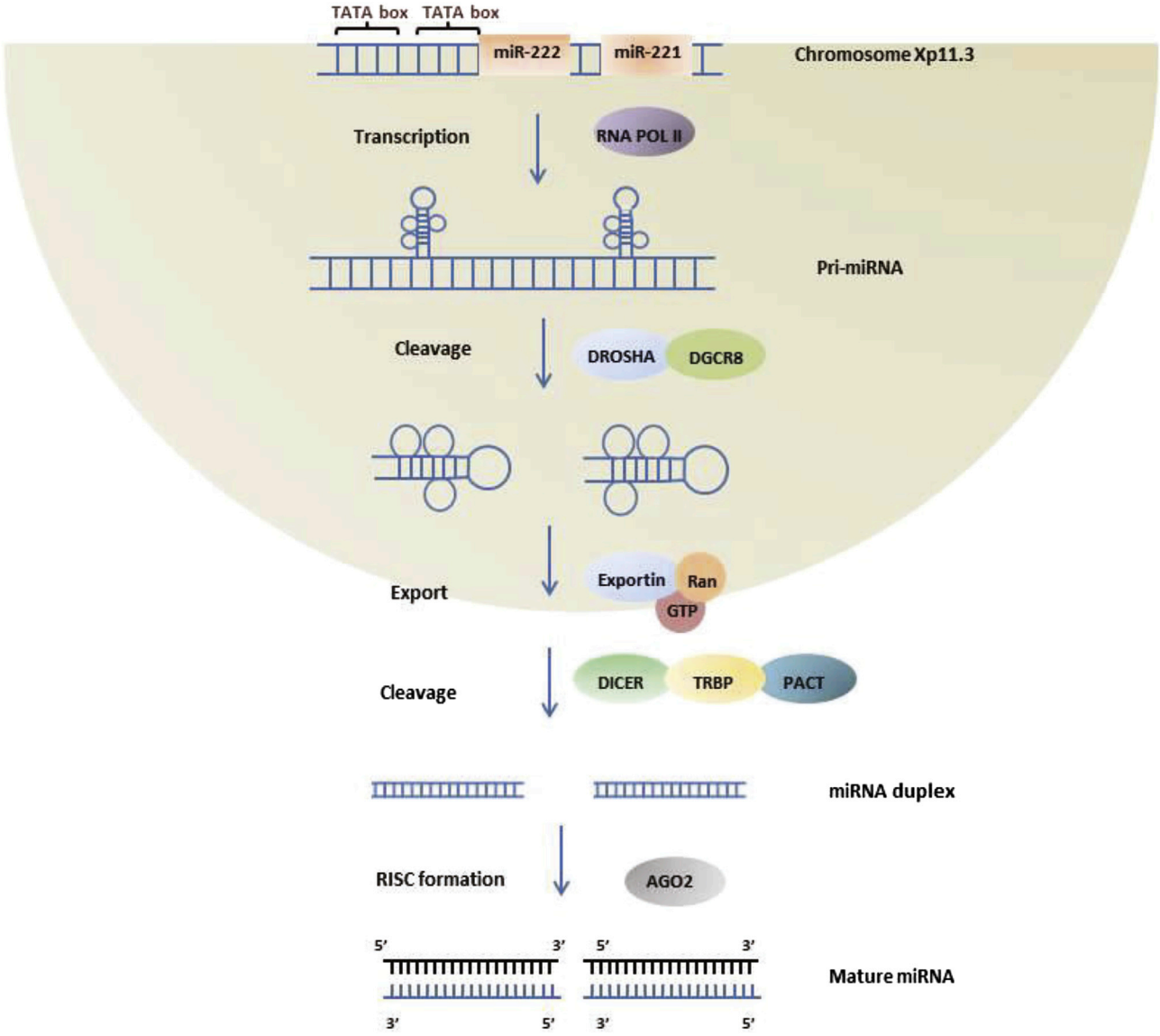

Figure 1. Biogenesis of miR-221/222: miR-221/222 is a gene cluster located on chromosome Xp11.3

The gene of miR-221/222 is transcribed into primary pri-miR-221/222 by RNA polymerase II and then processed to a stem-loop precursor pre-miR-221/222 by the nuclear RNase Drosha in the nucleus. Pre-miR-221/222 is transported to the cytoplasm by the exportin-5 transporter and the endoribonuclease Dicer cleaves it into a double-stranded miR (miR duplex) and the member of the Argonaute (AGO) protein family binds mature miRs to create the RNA-induced silencing complex (RISC).

mature form (Figure 1). This complex process is regulated by canonical and non-canonical pathways involving many interactors.[8] Among the major players are the RNase III family members Drosha and Dicer, which work on the miR processing at the nuclear and cytoplasmic level, respectively, to produce mature miRs, and the member of the Argonaute (AGO) protein family, which binds mature miRs to create the RNA-induced silencing complex (RISC). The regulatory mechanisms underlying the creation of this complex as well as the crosstalk between miRs biogenesis and other cellular pathways have been increasingly investigated.[9,10] In their mature form, miRs can induce the degradation of a target messenger RNA (mRNA) mostly via Watson-Crick base pairing to complementary sequences within the 3′ UTRs. However, the regulation of the gene expression via the interaction with 5′ UTR as well as coding sequence and gene promoters have also been reported in the literature.[11] Intragenic miRs are predominantly processed from intronic regions, while intergenic miRs are transcribed in an independent manner of a host gene and are regulated through their promoters. In the case of different miRs being transcribed as a unique long transcript, they are called "clusters" belonging to the same family.[12] miR-221/222 is an example of a

gene cluster located on chromosome Xp11.3. The promoter region of miR-221/222 includes two canonical TATA boxes on 550 and 190 base pairs upstream from pre-miR-222 and 3 poly-A sequences downstream from pre-miR-221.[13] It has been reported that the expression of this gene cluster is regulated by angiotensin II as well as by a repressive complex including estrogen receptor α and the nuclear receptors NCOR1 and NCOR2.[14,15] miR-221 is encoded and transcribed together with miR-222 as pri-miR, in which the two paralogous miRs are separated by 726 bp and share the same seed nucleotide sequence. The pri-miR transcription is followed by the nuclear processing mediated by Drosha and RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8), leading to the generation of 110-nucleotide pre-miR-221 and pre-miR-222. The pre-miR-221 is then subjected to nuclear-cytoplasm translocation by exportin 5-RanGTP-shuttle system and is cleaved by Dicer into the miR-221 mature form. The 5′ strand of pre-miR-221 produces miR-221-5p (also indicated as miR-221*), while the 3′ strand gives rise to miR-221-3p (usually indicated as miR-221).[16]

The multifaceted implication of miRs in key biological processes like cell proliferation, differentiation, and cell death prompted the attention of research on the development of strategies and experimental approaches enabling the deeper characterization of mechanisms underlying their molecular function, also in an integrative view.[17,18] These studies allowed us to elucidate the role of miRs as biomarkers or therapeutic targets, due to their differential expression in pathological versus physiological conditions, which we discuss more in detail below, and their involvement in the epigenetic regulation behind human diseases.[19] Regarding miR-221, the relationship between this miR and epigenetic changes has been reported in cancer[20] as well as in other conditions, such as cardiovascular[21] and cerebrovascular diseases.[22] A PubMed search for "((miR-221) AND (epigenetic regulation)) AND (human diseases)" resulted in 15 publications, of which two are review articles.[20,23] We report the seminal findings that emerged from these studies concerning the role of miR-221 in the epigenetic vision of human disorders. One study was filtered out because it was not focused on the topic.

Seeley et al. identified miR-221 and miR-222 as epigenetic regulators of macrophage reprogramming induced by stimulation with lipopolysaccharide in mice. They reported that an upregulated expression of both miRs promotes the silencing of inflammatory genes via chromatin remodeling by inducing macrophage tolerization. This event causes immune paralysis and organ damage in patients with sepsis.[24]

The investigation of epigenetic influences by selected miRs in Parkinson's disease was instead investigated by Tatura et al., who focused their work on miR expression profiling in gyri cinguli. miR-221 was one of the five miRs whose upregulation was correlated to the decrease of the transcript level of *SNCA*, *PARK2*, and *LRRK2* genes, which are normally involved in the physiological cellular function.[25]

Wahlang et al. detected an increased expression of miR-221 by analyzing the environmentally driven epigenetic modifications induced by the exposure to polychlorinated biphenyls (PCBs) in primary human endothelial cells (ECs) and by providing novel findings to explain the mechanisms behind PCB toxicity associated with vascular diseases.[26]

A series of five studies conducted by Hromadnikova et al. provided post-partum epigenetic profiling of miRs previously associated with cardio- or cerebrovascular pathologies, including miR-221. This analysis, performed in women with pregnancy-related complications, highlighted the association between miR-221 upregulation and gestational hypertension (GH). The aberrant expression profiles of seven miRs, including miR-221, in patients with GH only, provided a list of biomarkers for the identification of patients with a higher risk of later development of cardio-cerebrovascular diseases.[27] On the other hand, the studies conducted on umbilical cord blood in GH, preeclampsia (PE), and fetal growth restriction (FGR) highlighted the downregulation of miR-221-3p in severe PE and FGR by demonstrating the importance of evaluating the epigenetic changes that characterize each pregnancy-related complication and that may be indicative of subsequent diseases.[22,28–30]

El-Daly et al. investigated the involvement of miRs in the epigenetic switch causing colorectal cancer (CRC) cells transformation via nuclear factor κB (NF-κB) and STAT3 signaling pathways. Among the miRs included in the study, they found that miR-221 upregulation induces NF-κB/STAT3 constitutive activation through the inhibition of PDLIM2, which is normally implicated in intra-nuclear degradation of a subunit of NF-κB and proteasome degradation of STAT3.[31]

In their vision of miR-mediated epigenetic regulation, Ravegnini et al. investigated the role of miR-221/222 in gastrointestinal stromal tumor (GIST) susceptibility. They identified the variant rs17084733, located in the 3′ UTR of the *KIT* oncogene, as a potential biomarker of KIT-WT GIST and clarified that this variant acts as a sponge that competes with miR-221/222 binding. This polymorphism, together with the rs75246947 (T>A) variant on pri-miR-222, was associated with GIST susceptibility.[32]

To investigate the epigenetic mechanisms underlying the lineage homeostasis in breast epithelium, Ke et al. reported the ability of miR-221 to sustain breast cancer (BC) hierarchy in normal and malignant cells. They found that overexpression of miR-221 in normal BC cells induces stem-like cells to differentiate in luminal cancer cells and epithelial-mesenchymal transition via ATX1 targeting.[33]

Coarfa et al. pointed out the epigenetic regulation of miR-221/222 in prostate cancer (PCa) cells. They reported that miR-221 is involved in the control of the androgen receptor axis and it is suppressed in metastatic PCa. The evidence that miR-221 expression level could be restored by treatment with the pan-histone deacetylase (HDAC) inhibitor vorinostat and the DNA methyltransferase inhibitor 5-azacytidine highlighted the importance of the combination between conventional therapies and epigenetic therapies for PCa treatment.[34]

LONG NON-CODING RNA AND miR-221

The most explored long non-coding RNA (lncRNA) in correlation with miR-221 was *GAS5*. In CRC, a predictive value of miR-221 and *GAS5* has been reported in the prognosis of CRC. Specifically, in CRC tissues, plasma, and exosomes, *GAS5* was upregulated while miR-221 was downregulated and the receiver operating characteristic (ROC) curves showed their diagnostic value. Overexpression of *GAS5* in CRC cells induces miR-221 downregulation and decreased proliferation, migration, and invasion of cells.[35] Similarly, *GAS5* suppresses cell growth and epithelial-mesenchymal transition (EMT) in osteosarcoma by regulating the miR-221/aplasia Ras homologue member I (ARHI) pathway. In this disease, levels of *GAS5* and ARHI were significantly lower, while miR-221 was higher. As above, the reported overexpression of *GAS5* inhibited proliferation, migration, and EMT in osteosarcoma cells by direct binding to miR-221 and subsequent decrease that in turn enhances ARHI expression. miR-221 mimics or ARHI siRNA reverted these biological effects *in vivo*, suggesting that *GAS5* functions as a competing endogenous RNA (ceRNA) for miR-221 to suppress cell growth and EMT in osteosarcoma.[9] In pancreatic cancer (PC), it has been reported that *GAS5* reverses EMT tumor stem cell-mediated gemcitabine resistance and metastasis by directly targeting and suppressing miR-221 and *SOCS3*. The overexpression of *SOCS3* reverses these biological effects in PC cells and xenografts, confirming that *GAS5* acts again as a ceRNA for miR-221, mediating EMT and tumor stem cell self-renewal by miR221/SOCS3 pathway regulation.[36] In BC, the *GAS5*/miR-221-3p/DKK2 axis has been related to the modulation of ABCB1-mediated adriamycin resistance. Again, *GAS5* acts as an endogenous sponge by competing for miR-221-3p binding to regulate its target DKK2, which consequently inhibited Wnt/β-catenin pathway activation. *In vivo*, *GAS5* overexpression enhances the antitumor effect induced by adriamycin.[37] Similarly, activity exerts *GAS5* in follicular thyroid carcinoma (FTC). In FTC tissues and cells, *GAS5* was downregulated while miR-221 was upregulated. *GAS5* acts as a sponge of miR-221-3p that, through its direct target CDKN2B, induces G0/G1 arrest and inhibition of cell proliferation.[38] In non-small cell lung cancer (NSCLC), the binding sequence of *GAS5* for miR-221-3p was confirmed. *GAS5* expression was lower in NSCLC tissues and cell lines in which *GAS5* overexpression increased proliferation, migration, and invasion while the restoration of miR-221-3p reversed these effects. The biological activity of *GAS5* in NSCLC is partially mediated via *IRF2* expression and miR-221-3p repression.[39]

Zinc finger protein (ZNF)281 has a tumor-suppressive role in NSCLC. It has been reported that the expression level of *ZNF281* is lower in cancer tissue compared with non-cancerous tissues, which predicts poor survival. Moreover, *ZNF281* levels are negatively associated with miR-221 expression but positively associated with phosphatase and tensin homolog (*PTEN*) mRNA expression levels. In addition, miR-221 and PTEN are negatively associated. In NSCLC cells, *ZNF281* overexpression induces cell apoptosis and inhibition of cell proliferation, which, in this cell context, could account for miR-221 downregulation and consequent PTEN upregulation.[40] Another lncRNA investigated in NSCLC linked to miR-221 is HOX transcript antisense RNA (*HOTAIR*), the expression of which has been correlated to clinical stage of cancer. Specifically, the expression of *HOTAIR* was lower in stage I and II patients than in those with stage II and IV, which negatively correlated with miR-221 levels. Alternately, *HOTAIR* and miR-221 silencing or overexpression confirmed a negative regulation of miR-221 on *HOTAIR*.[41]

Similarly, the lncRNA *TUG1* was found to be downregulated in NSCLC non-responders to platinum-based treatment and reflected poor survival. Enhanced cisplatin chemosensitivity has been described in xenografted NSCLC resistant to cisplatin stably transfected with *TUG1*. The authors hypothesize that *TUG1* could work as a ceRNA, increasing PTEN expression, which in this study was confirmed as a direct target of miR-221.[42]

Moreover, *TUG1* silencing inhibits cell viability and promotes apoptosis in acute myeloid leukemia (AML) by targeting the miR-221/KIT axis. In these cells, *TUG1* and *KIT* were upregulated while miR-221 was downregulated. In this experimental setting, authors demonstrated that both *TUG1* and *KIT* were targeted by miR-221 with 3′ UTR-binding sites.[43]

In renal cell carcinoma (RCC), the lncRNA T cell leukemia lymphoma 6 (*TCL6*), expression of which has been evaluated to be deficient in RCC tissues and associated with worse overall survival (OS) and progression-free survival (PFS), sensitizes RCC cells to paclitaxel-induced apoptosis through the direct targeting to miR-221.[44] In addition, the lncRNA *LINC00671* expression was reduced in RCC tissues and correlated to the poor OS and disease-free survival (DFS). In RCC, cells have been reported in which *LINC00671* expression suppressed progression via regulating the miR-221-5p/SOCS1 axis.[45]

The lncRNA *LOC642852* (also known as *LINC00205*) is overexpressed in ovarian cancer (OC) and omental/peritoneal metastases compared with effusion specimens. The opposite has been reported for miR-221 and miR-22 expression. OC cells following *LOC642852* knockout were significantly less invasive compared with controls. By the use of bioinformatics tools, the authors identified that miR-221-3p has binding sites to both the transcription corepressor (TLE) family member 3 and to *LOC642852*, leading them to hypothesize that ceRNA is related to TLE3 and *LOC642852*. This hypothesis was reinforced through the identification of lower TLE3 expression in *LOC642852* KO cells.[46]

Moreover, the lncRNA *CASC15* has been described as a predictor of unfavorable OC prognosis. It has been detected with lower expression in tissues and OC cells, and is closely correlated with advanced tumor-node-metastasis (TNM) stage, moderate/poor differentiation, larger tumor size, and poorer OS and PFS compared with high *CASC15* expression. In addition, *CASC15* had diagnostic value to distinguish tumors tissues from non-tumors. *CASC15* overexpression induced inhibition of OC cell proliferation, colony formation, migration, invasion, cell-cycle progression, and promoted apoptosis. Luciferase reporter and biotin pull-down assays confirmed that *CASC15* strictly

Table 1. Chemical modification and proprieties of ASOs developed for mRNA targeting

ASO chemistry	Modification	Properties	Characterization of the molecules (reference)	Application for miR-221 inhibition (reference)
PS linkage	replaces the non-bridging oxygen group with a sulfur atom	increased nuclease stability and improved PK	Eckstein[51]	Callegari et al.[52]
(1) 2′-O-methyl	modifications at 2′-ribose	improved RNA affinity and nuclease resistance	Cummins et al.[53]	Park et al.[54]
(2) 2′-O-methoxyethyl				
(3) 2′-fluoro				
PNA	the pseudo-peptide backbone is composed of unnatural N-(2-aminoethyl)glycine units	resistance to both nucleases and proteases; hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices	Gambari[55]	Finotti et al.[56]
			Nielsen et al.[57]	
			Demidov et al.[58]	
			Patel[59]	
LNA	links the 2′-oxygen and 4′-carbon of ribose	reducing the conformational flexibility of nucleotides increases their binding affinity	Obika et al.[60]	Oieni et al.[61]
				Di Martino et al.[62]
				Gallo Cantafio et al.[63]
constrained ethyl	LNA methylated analog	reducing the conformational flexibility of nucleotides increases their binding affinity	Watts[64]	
Gapmer	central region of deoxyribonucleotides (gap) flanked by 2′-modified sugar ring bases (wings)	RNase H-mediated target degradation	Jaschinski et al.[65]	

interacts with miR-221, which, by its direct downstream target ARID1A, exerted its tumor-suppressive effect in OC.[47]

In hepatocellular carcinoma (HCC), the *C1QTNF1-AS1*/miR-221/SOC3 axis has been identified. Again miR-221 acts as ceRNA for *C1QTNF1-AS1* and targets SOC3 with direct binding. In HCC tissues and cells, *C1QTNF1-AS1* and SOC3 were upregulated while miR-221 was downregulated. *In vitro*, miR-221 silencing inhibited cell proliferation, migration, and invasion and the JAK/STAT signaling pathway, while promoting cell apoptosis. *In vivo*, upregulation of *C1QTNF1-AS1* induces tumor growth inhibition through miR-221 downregulation.[48,49] Similarly, *RNF185-AS1* has been identified as a novel lncRNA, highly expressed in HCC tissues and correlated with TNM stage, distant metastasis, and poorer OS. In HCC cells, *RNF185-AS1* promotes proliferation, metastasis, and invasion, acting as a sponge for miR-221-5p and concomitant integrin β5 inhibition, while miR-221 inhibitors or integrin β5 overexpression rescued these functions.[50]

EVOLVING APPROACHES FOR miR-221 TARGETING IN THE NOVEL SCENARIO OF RNA THERAPEUTICS

The targeting of miRs has been investigated in this last decade by the use of different approaches mainly consisting of the use of antisense oligonucleotides (ASOs). They are short, synthetic, single-stranded oligonucleotides that can bind to mature miR targets or pre-miR via Watson-Crick base pairing, affecting miR expression. These ASOs act as inhibitors forming a duplex with the miR guide strand and preventing the miR from binding to its target. Consequently, downstream protein expression will be restored or modified through several distinct mechanisms. Improvements in the clinic translation of these agents have been significantly constrained by inadequate target engagement, insufficient biological activity, and off-target toxic effects. Over the years, modifications of ASOs chemistry have been introduced to address most of these issues, including modified backbones and 2′-ribose that conferred enhanced pharmacological and therapeutic properties (Table 1). These chemical modifications, together with clarification of the mechanism of action of ASOs, have given a boost to the translation of ASO-based strategies into therapy.

The first oligonucleotide approved by the US Food and Drug Administration (FDA) as an agent to treat cytomegalovirus (CMV)-induced chorioretinitis was fomivirsen, a 21-mer phosphorothioate (PS) oligo targeting the mRNA that encoded the CMV immediate-early (IE)-2 protein, which is required for viral replication. Then, an antisense 20-mer PS 2′-methoxyethoxy (MOE) gapmer targeting the coding region of the *APOB* mRNA and reducing LDL-C levels was approved by the FDA, but, due to the high risk of liver toxicity, to date it is only available for patients under the restricted Kynamro Risk Evaluation and Mitigation Strategy program. An exemplary case study is the ASO nusinersen, a 18-mer PS 2′-O-MOE antisense oligonucleotide with methyl-modification at the carbon-5 of all cytidines. The oligo induces the inclusion of exon 7 in the Survival Motor Neuron (*SMN*) *1* and *SMN2* mRNA by targeting and blocking an intron 7 internal splice site, making it a candidate as a promising drug in infants with types 1, 2, and 3 spinal muscular atrophy (SMA).[66] The successful preclinical studies followed by well-designed clinical studies led to its approval, sparking great hopes for the potential use of this new class of drugs for several human conditions. The first-ever miR therapeutic,

rapidly moving from bench to clinic, is to date in phase II clinical trials. It is a short LNA drug called miravirsen, able to inhibit miR-122 for the treatment of HCV infection.

Regarding different strategies applied to specifically inhibit miR-221, preclinical investigations of a variety of oligonucleotides specifically directed against the oncogenic miR-221 have been reported. Park et al. explored nine chemistries for miR-221 silencing in HCC and concluded that a cholesterol-modified isoform of anti-miR-221 (chol-anti-miR-221) exhibited improved pharmacokinetics (PK) properties and liver tissue distribution compared with the unmodified oligonucleotide. Moreover, there were significantly reduced miR-221 levels in the liver within a week of intravenous administration together with chol-anti-miR-221 accumulation in HCC tumor cells, enhancing the survival in orthotopic mice.[54]

An anti-miR oligonucleotide against miR-221 was also investigated by Callegari et al. in a mouse transgenic model of liver tumorigenicity that overexpresses miR-221. It was a 23 mer 2′-O-methyl RNA oligonucleotide (AMO) with PS bond, specifically designed to target miR-221 *in vivo*, delivered nude in saline solution by intravenous injection. By the use of their animal model, the authors demonstrated an antitumor activity induced by miR-221 targeting with AMO.[52]

An advanced further method has been used to deliver peptide nucleic acids (PNA) targeting miR-221-3p based on a macrocyclic multivalent tetra argininocalix[4]arene used as a non-covalent vector for anti-miR-221-3p PNAs. The high delivery efficiency, low cytotoxicity, maintenance of the PNA biological activity, and easy preparation make this vector a candidate for a universal delivery system for this class of nucleic acid analogs, as has been recently reviewed by Finotti et al.[56]

Even though with innovative chemical modification, the progression into the clinic of ASOs has been questioned by different authors for their susceptibility to nucleases and fast clearance, the experts in the field have been induced to search for delivery systems to specifically target tissues. These attempts included nano-carriers with different chemistry (i.e., neutral lipids, PEGylation) or conjugation to different moieties, non-viral nano-carriers, and, to overcome immunogenicity, biomimetic nanovesicles (NVs). For example, an ASO anti-miR-221 encapsulated in biomimetic NVs has been developed by Oieni et al.[61] for the delivery of a miR-221 inhibitor to human mesenchymal stem cells, avoiding the degradation by lysosomes that cause most of the failures to deliver NV cargoes in the cytoplasm.

Although major optimizations of nano-carriers and ASO encapsulation have been attempted, the strategies based on the use of delivery systems lack tissue-specific targeting and do not allow a scalable production process.

In contrast, in the last years, an original LNA-miR-221 inhibitor has been deeply investigated, and it is currently evaluated in an ongoing FIH study (NCT0481189). This is a novel 13-mer LNA oligonucleotide with a fully PS-modified backbone that showed significant antitumor activity, a strong and efficient miR-221 downregulation, and, consequently, its canonical target modulation, in different preclinical tumor models.[62] Moreover, this oligonucleotide, named LNA-i-miR-221, revealed very favorable PK proprieties in all animal species tested, including mouse, rat, and monkey.[63] The detection of the LNA-i-miR-221 in tumor tissues as well as in body fluids showed a rapid and wide tissue distribution, which, together with the biological activity, makes it an optimal agent for miR-221 targeting.[63] Furthermore, this ASO did not show any behavioral changes or organ-related toxicity in mice, in monkeys,[62,63,67,68] and in rats.[69–71] To date, it is the most advanced investigational drug, targeting miR-221, in early clinical development in cancer patients.

Recently, it has been reported that anticancer drugs can change the miRNA expression profiles and may exert their effect through upregulation of tumor-suppressing miRNAs and downregulation of onco-miRs. Table 2 lists agents, including natural compounds and chemotherapeutic drugs, that promote cell apoptosis and/or cell-cycle arrest in preclinical cancer models via miR-221 downregulation[72–84]

DEREGULATED EXPRESSION OF miR-221 IN HEMATOPOIETIC MALIGNANCIES

Myeloma

miRNA therapeutics, including miRNA mimics[87–89] or non-coding RNA (ncRNA) targeting,[90–93] have been extensively explored in multiple myeloma (MM) disease, offering a novel opportunity for personalized treatment. Among miRs involved in MM pathogenesis, the cluster miR-221/222 is of high potential interest as a therapeutic target. Indeed, by analyzing miR expression on a panel of MM and plasma cell leukemia (PCL), Di Martino et al. showed that miR-221/222 was strongly upregulated in MM patients compared with healthy donors.[94] In particular, the authors showed that the highest levels of miR-221/222 were observed in MM patients carrying t(4; 14) and belonging to translocation and cyclin (TC) groups 2, 4, and part of TC3, overall accounting for >50% of all MM. These findings were of translational relevance given the high-risk features of patients carrying t(4; 14), which therefore represent an ideal target population to investigate biological effects induced by miR-221/222 inhibition. These data were also validated by analyzing miR-221/222 on a panel of MM cell lines. Indeed, the highest levels of miR-221/222 were found in OPM2 and NCI-H929 cells, both t(4; 14), while the lowest levels were found in t(4; 14)-negative U266 and RPMI-8226 cells. Furthermore, miR-221/222 expression is inversely correlated with sensitivity to melphalan in MM cells. Indeed, melphalan-resistant U266/LR7 cells showed the highest miR-221/222 expression, while melphalan-sensitive cell lines, such as NCI-H929, U266/S, and AMO1 cells, did not display any miR-221/222 expression change after drug exposure.[68] Moreover, higher miR-221/222 expression was found in the dexamethasone-resistant cell line MM1R compared with its parental sensitive cell line MM1S.[95]

Importantly, inhibition of miR-221/222 by specific inhibitors in t(4; 14)-positive MM cells induced antitumor activity *in vitro* and

different murine models of human MM. Mechanistically, these effects were accompanied by upregulation of the relevant targets of miR-221/222, such as p27, p57, and p53 upregulated modulator of apoptosis (PUMA), which all together exert a critical role as tumor suppressors in cancer cells. Moreover, miR-221/222 inhibition was able to overcome melphalan resistance in a synergistic manner, triggering MM cell apoptosis.[68] In particular, anti-MM activity induced by inhibition of miR-221/222 *plus* melphalan induced a significant upregulation of pro-apoptotic BCL2 binding component 3 (BBC3)/PUMA protein as well as modulation of SLC7A5/LAT1 and ABCC1/MRP1, two drug influx-efflux transporters. In addition, anti-miR-221/222 was able to restore dexamethasone sensitivity in MM1R cells *in vitro* and *in vivo*. Indeed, mice transplanted with MM1R stably expressing anti-miR-221/222 and treated with dexamethasone showed lower tumor burden and increased survival compared with control mice.[95]

Other hematological malignancies

In the last decades, a growing body of evidence suggests the involvement of several miRs in normal and pathological hematopoiesis, diagnosis, and prognostic evolution. In this context, miR-221 has been frequently identified as deregulated across both acute and chronic malignancies. For instance, essential thrombocythemia (ET) is a myeloproliferative neoplasm characterized by thrombocytosis, megakaryocytic proliferation and increased tendency to bleeding and thrombosis. The hyperactivation of the JAK/STAT pathway due to JAK2V617F, MPL (MPL proto-oncogene, thrombopoietin receptor) or calreticulin (CALR) mutations has a central role in ET pathogenesis; nevertheless, a minority of ET patients still lack a molecular marker.

Navarro et al. performed miR profiling in platelets from 19 ET patients with and without JAK2V617F mutations in order to identify a specific miR signature in an ET JAK2V617F-negative subset. They demonstrated that, among 28 miRs differentially expressed and involved in the JAK/STAT pathway, miR-221 was strongly upregulated and specifically targeted two negative regulators of JAK/STAT pathway, SOCS1 and SOCS3, proposing miR-221 as a new potential target in JAK2V617F-negative ET.[96]

The diagnostic and pathogenetic role of miR-221 was also explored in several hematological malignancies. miR-221 was found to be upregulated in nodal marginal zone lymphoma (NMZL) versus follicular lymphoma (FL) and could be proposed as a diagnostic marker in NMZL, whose differential diagnosis from other small B-cell neoplasms is hampered by the lack of specific criteria.[97]

Szymczyk et al. identified miR-221 as a predictor of OS in CLL patients. They demonstrated a negative correlation between miR-221 levels and cytogenetic risk and β-2-microglobulin levels, concluding that a low expression of this miR results in more CLL.[98]

Chronic myeloid leukemia (CML) is a clonal hemopoietic disorder characterized by a reciprocal translocation between the long arms of chromosomes 9 (chr9) and 22 (chr22) that accounts for 15% of all leukemia cases in the Western world.[99] The regulatory role of miR-221 in CML progression is controversial and depends on miR-221 targets, cell type, and cell differentiation stage. A miR profiling study performed in different stages of CML showed overexpression of miR-221 in more immature blasts. Using *in silico* target prediction software, miR-221 target genes, involved in cell-cycle, growth-inhibition, and p53 signaling pathways, were identified, suggesting that miR-221 may be involved in CML pathogenesis.[100]

In another work, it has been demonstrated that miR-221 might act as a chemosensitivity regulator in CML. Specifically, miR-221 through STAT5 inhibition would reduce reactive oxygen species (ROS) production and DNA damage, maintaining genome stability and preventing the generation of mutations involved in drug resistance.[101]

T acute lymphoblastic leukemia (T-ALL) is a rare, aggressive, and orphan malignancy. Despite intensive treatment regimens, refractory/relapsed patients have a poor prognosis. So far, no immunotherapeutic strategies are available, even if new promising therapeutics are in an advanced phase of preclinical development.[102] More efforts in the field are therefore needed. Gimenes-Teixeira et al. evaluated miR expression profiles in T-ALL samples with and without CD56 expression and correlated miR expression with patient outcomes. Interestingly, among 56 miRs differentially expressed, miR-221 was found to be upregulated in T-ALL/CD56+ and in leukemic blasts compared with normal T cells, and its expression level was found to be predictive for shorter OS.[103]

Early T cell precursor (ETP) acute lymphoblastic leukemia (ALL) is a relatively new subgroup of T-ALL, characterized by an immature and myeloid immunophenotype and is associated with a poor prognosis. Through expression profiling, differentially expressed miRs in ETP-ALL compared with non-ETP T-ALL and mature CD3+ cells from healthy donors were identified and miR-221 was found to be one of the most highly expressed in ETP-ALL. Furthermore, the expression of miR-221 was associated with CD34 expression and a more immature immunophenotype, indirectly supporting the hypothesis that ETP is an early immature T-ALL subtype.[104]

Moses et al. demonstrated that overexpression of miR-221 resulted in sustained blast proliferation and exposure of leukemic cells to stromal components of the microenvironment, in particular, to bone marrow-derived stromal cells (BMSC) or osteoblasts, which would reduce mir-221 level in ALL cells, suggesting a potential bone marrow niche regulation of miR-221.[105] Furthermore, miR-221 aberrant expression plays a key role in early myeloid hematopoiesis through KIT downregulation and may modulate the growth of KIT+ leukemic cells. Taking advantage of the miR-ON reporter system, which allows the quantification of miR at single-cell level based on fluorescent protein expression, Rossetti et al. evaluated temporal changes in the level of miR-221. They demonstrated that, depending on cell-specific context, miR-221 is able to affect

Table 2. List of known antitumor agents inhibiting miR-221 and/or miR-221/222 cluster

Name	Class	Mechanism of action	Type of tumor	Reference
Resveratrol	natural compound	inhibition of miR-221 by NF-κB (RELA Proto-Oncogene, NF-KB Subunit) activity regulation	melanoma	Wu et al.[72]
Metformin	oral hypoglycemic drug	downregulation of miR-221 caused G1-phase arrest via p27 upregulation	pancreatic cancer	Tanaka et al.[73]
Myrothecine A	mycotoxin	inhibits cell proliferation by miR-221 downregulation and influences p27 protein level	HCC	Fu et al.[74]
Ginsenoside Rg3	natural compound	inhibits cell viability and proliferation via miR-221 downregulation, upregulation of TIMP3 and PI3K/AKT/MAPK/ERK inactivation	oral squamous cell carcinoma	Cheng and Xing [85]
13-*cis*-RA and 1,25-VD3	vitamin A analogs/vitamin D3	cotreatment of 13-*cis*-RA and 1,25-VD3 significantly downregulate miR-221 expression and induce an increase of TIMP3 protein suppressing TNF-α-mediated cell invasion in PDAC	pancreatic cancer	Cheng et al.[86]
Oroxin B	natural compound	induces antitumor effect by PTEN upregulation and PI3K/Akt signaling pathway inactivation through downregulating the expression of miR-221	HCC	Melendez-Villanueva et al.[76]
Vinblastine/vinorelbine/ vincristine	vinka alkaloids	treatment with vincristine, vinblastine, and/or vinorelbine induces TP53 upregulation and miR-221 downregulation	BC	Mavrogiannis et al.[77]
Indole-3-carbinol	natural compound	induces miR-21and miR-221 and -222 leading to PTEN/AKT pathway repression	HCC	Wang et al.[78]
Oleuropein	natural compound	reduces cell viability and improves apoptosis through increase of miR-125b, miR-16, miR-34a, p53, p21, and TNFRS10B expression level and downregulation of bcl-2, mcl1, miR-221, miR-29a, and miR-21	BC	Asgharzade et al.[79]
Curcumin	natural compound	promotes cell apoptosis and inhibits cell proliferation via miRs modulation. Downregulation of oncogenic miR-21, miR-221, and miR-222, and upregulation of tumor-suppressive miR-192-5p and miR-215	lung cancer	Lelli et al.[80]
Capecitabine and ixabepilone	chemotherapeutic drugs	induce upregulation of tumor-suppressor miRs (miR-122a, miR-145, and miR-205) and downregulation of onco-miRs (miR-296 miR-221, miR-210, miR-21, and miR-10b)	BC	Yao et al.[81]
α-pinene	natural compound	induces cell-cycle arrest via downregulation of cyclin-dependent kinase 1 and miR-221 levels and upregulation of CDKN1B/p27, γ-H2AX, phosphorylated ATM, phosphorylated Chk2, and phosphorylated p53	HCC	Xu et al.[82]
Chrysin	natural compound	induce cell apoptosis via miR-221 downregulation	gastric carcinoma	Mohammadian et al.[83]
Mibolerone and dihydrotestosterone	androgen receptor agonist	repression of miR-221, which is involved in PC development	PC	Segal et al.[84]

13-*cis*-RA, 13-*cis*-retinoic acid; 1,25-VD3, 1,25-dihydroxyvitamin D3.

cell fate through c-KIT regulation in both normal hematopoiesis and undifferentiated myeloblasts.[106]

Acute leukemia with ambiguous lineage accounts for 4% of total ALL cases and is associated with a poor prognosis. With the purpose of classifying acute leukemia with ambiguous lineage and distinguishing between a predominant myeloid or lymphoid origin, miR profiling was performed on 16 patients with leukemia of ambiguous lineage and 12 patients with AML, B acute lymphoblastic leukemia (B-ALL), or T-ALL. Five miRs previously reported to be associated with AML or ALL have been identified, including miR-221, indicating that leukemia of ambiguous lineage is not a unique entity but can be assigned to ALL or AML subtypes using miR expression profiling.[107]

The mutation of FMS-like receptor tyrosine kinase 3 (*FLT3*) occurs in about one-third of patients with AML and its internal tandem mutation (ITD) correlates to poor prognosis.

The lncRNA *SOCS2-AS* was found overexpressed in FLT3-ITD+ AML patients compared with FLT3-ITD-AML patients and negatively correlated with miR-221 expression in FLT3-ITD+ AML patients. Zhang et al. mechanically demonstrated that *SOCS2-AS* acts as a proto-oncogene and promotes FLT3-ITD+ AML progression by regulation of STAT5 pathway through miR-221 sponging.[108]

Deng et al. analyzed two datasets of miR profiling in AML patients, discovering that miR-221 was upregulated in both models. To further understand the biological function of miR-221 in AML leukemogenesis, they evaluated, both in silico and *in vitro*, the potential role of miR-221 in epigenetic pathway regulation, and demonstrated the existence of NCL/miR-221/NF-κB/DNMT1 epigenetic regulatory network that modulates p27kip1 and p15INK4B. Finally, to evaluate the antileukemic activity, they designed peptide gold nanoparticles carrying the aptamer AS1411 and anti-miR-221, which synergistically suppress AML cell growth *in vitro* and *in vivo* without any evidence of toxicity.[109]

In summary, taking into account the deregulated expression and key biological role across hematological malignancies, miR-221 represents a potential therapeutic target for nucleic acid-based therapeutics in several onco-hematological conditions.

DEREGULATED miR-221 EXPRESSION IN SOLID TUMORS

Lung cancer

Early detection of lung cancer using non-invasive and reliable biomarkers is still an unmet medical need. Indeed, for several specific circulating miRs and mainly miR signatures, diagnostic and prognostic significance have been found in early-stage lung cancer, before clinical manifestation of the disease.[110] Zhou et al. reported a six-plasma miR panel, including miR-221-3p, that could discriminate lung cancer patients. Specifically, miR-19-3p, miR-21-5p, and miR-221-3p were significantly upregulated in exosomes extracted from lung cancer plasma in an Asian population.[111] Zhu et al. performed a biomarker screening followed by a validation phase to identify specific serum miRs as potential diagnostic markers for detection of lung cancer in Asian patients. By polymerase chain reaction (PCR) array, the expression of serum miRs was examined in naive lung cancer patients, benign pulmonary disease patients, and healthy controls, identifying a signature, a combination of four miRs (miR-23b, miR-221, miR-148b, and miR-423-3p) that may be considered as a novel biomarker in this patient setting. Nevertheless, limitations of this study are the small sample size, disregard of smoking status, as well as stage of the disease. The percentage of the early stages was limited, so the results should be considered carefully.[112]

In another study, six selected miRs, including miR-221, although not among the most deregulated, were confirmed to be significantly expressed in stage I/II adenocarcinoma patients compared with healthy controls.[113]

Regarding different NSCLC histology, Zhang et al. investigated different miR expression profiles and their significance in lung cancer patients with adenocarcinoma or squamocellular carcinoma. Comparing miR expression profiles with paired adjacent non-neoplastic lung tissue, increased expression of miR-221 was demonstrated in squamous cell histology. Moreover, squamous carcinoma patients with lymph node metastasis and advanced stage were associated with higher expression of miR-221.[114]

Immunotherapy and molecularly targeted therapies have become a focal point in NSCLC, revolutionizing the survival history of lung cancer. However, acquired resistance against these treatments to date represents a difficult issue. Overcoming this resistance is a challenge that requires novel genomic approaches to understand the drug-resistance repertoire and improve the treatment of lung cancer patients.

miR-221 has a key role in tumor inhibition because the downregulation of PTEN as a consequence of miR-221 overexpression results in strong activation of the PI3K/AKT pathway, which promotes cell transformation. In this context, miR-221 could be a promising therapeutic target for tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) resistance in NSCLC because it induced the downregulation of p27kip1, PTEN, and tissue inhibitor of metallopeptidases (TIMP) 3.[115] Acunzo et al. showed that miR-130a, whose expression is reduced in lung cancer cell lines, reduces TRAIL resistance in NSCLC through the downregulation of miR-221 and miR-222 by targeting mesenchymal epithelial transition (MET), offering another possible strategy in NSCLC therapy.[116] In addition, miR-221 is upregulated in cisplatin-resistant lung cancer cells, where it negatively regulates the oncosuppressor *PTEN* and activates the PI3K/AKT pathway. Therefore, in this context, miR-221 might play a key role in the modulation of cisplatin chemosensitivity, leading to the development of targeted treatments against platinum resistance in NSCLC.[117]

Upregulation of miR-221/222 has been associated with multidrug resistance or altered response to chemotherapy. Antoniali et al. demonstrated that the inhibition of apurinic/apyrimidinic endonuclease 1 (APE1), a DNA repair enzyme implicated in genome stability, tumor progression, and chemoresistance, correlates with the decrease of miR-221/222 expression, opening up a new potential therapeutic strategy.[118]

Li et al. demonstrated that miR-221-3p is upregulated in extracellular vesicles (EVs) secreted by multi-drug-resistant lung cancer cells, by targeting the HMBOX1, activating the AKT/mTOR pathway, and promoting tumor progression.[119]

A recent study reported that ZNF281 overexpression, a tumor-suppressive lncRNA, causes the upregulation of PTEN and the downregulation of miR-221, leading to the promotion of cell apoptosis and

the inhibition of cell proliferation in NSCLC cells.[40] Instead, another study reported that LEF1-AS1, a recently characterized oncogenic lncRNA, is overexpressed in NSCLC patients. It correlates negatively with the expression of PTEN but positively with the expression of miR-221, promoting cancer cell proliferation and suppressing apoptosis.[120]

Yin et al. performed gain and loss of function experiments, demonstrating a significant overexpression of miR-221 in NSCLC tissues compared with normal tissues. Moreover, authors transfected SPCA1 and H1299 cell lines with miR-221 mimics or inhibitors, highlighting that the upregulation of miR-221 promotes cell proliferation, migration, invasion, and downregulation of TIMP2, concluding that miR-221 acts with an oncogenic role in NSCLC by targeting *TIMP2*.[121]

Another study confirmed that miR-221-3p is overexpressed in NSCLC and in cell lines inducing proliferation and invasion through the negative regulation of p27, and promoting G1/S-phase progression.[122]

Sun et al. investigated the effects of miR-221 on the proliferation of NSCLC cells through the study of lncRNA *HOTAIR*. miR-221 negatively regulated the expression of *HOTAIR*, inducing apoptosis of NSCLC cells.[41]

Targeted therapies using epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have prolonged the survival of patients with EGFR mutations, but the development of acquired resistance is very frequent and it may be caused by different mechanisms. Jang et al. demonstrated that Adenine Nucleotide Translocase-2 (ANT2) contributes to EGFR-TKI resistance in NSCLC. ANT2 is overexpressed in patients exhibiting poor responses compared with those showing excellent responses. Therefore, ANT2 inhibition sensitizes NSCLC cells to EGFR-TKI inhibitors by downregulating the levels of miR-221/222, consequently restoring PTEN expression and inactivating the PI3K/AKT pathway. This mechanism provides a rationale to restore the responsiveness of EGFR-TKI-resistant cells in NSCLC.[123]

Xu et al. described that high expression of miR-221 is also predictive of severe esophageal toxicity induced by radiation in patients with stage IIIB–IV NSCLC disease treated with chemo-radiation therapy (CRT). Specifically, high serum levels of miR-221 are involved in inflammation and tumor growth and are linked to a poor prognosis. Then miR-221 serum dosage during the first 2 weeks of CRT therapy could anticipate and avoid the occurrence of any serious adverse events.[124]

BreC

Highly invasive and metastatic BC is nowadays the leading cause of death among women with malignant diseases. The identification of appropriate therapies to reduce the risk of tumor recurrence and progression is a mainstay in patients with localized BC. In this scenario, miRs recently emerged as a key regulator of the complex mechanisms that underlie BC therapeutic resistance and susceptibility. BC is a heterogeneous disease, including several molecular subtypes underlying different prognoses and responses to treatments. Among them, estrogen-receptor (ER)-negative BCs (HER2+ and triple negatives) have a higher mortality rate and strongly need the discovery of new specific tumor biomarkers as both predictive and potential therapeutic targets. The majority of BCs (about 70%) are ER positive, also known as luminal subtypes, with a better prognosis that nevertheless suffers from resistance to hormonal therapy. The cluster miR-221/222 is frequently overexpressed in BC, resulting in tumor aggressiveness. High levels of miR-221 have been related to the invasiveness of BC cells and advanced clinical stages.[90] In particular, the miR-222 expression has been related to BC progression and drug resistance, whereas miR-221 downregulates certain oncosuppressor genes.[91] Moreover, high expression of miR-221 has been related to lymph node metastasis, distant metastasis, and poor prognosis.[92]

Triple-negative BC

Triple-negative BC (TNBC) represents 15% of all BCs. An interesting theory described by Kaushik et al. suggests the involvement of miR-221 as a mediator of local dissemination in TNBC, arguing that a therapeutic intervention might mitigate the degree of tumorigenesis and metastatic spread of human TNBC.[125] In addition, TNBC is closely related to cancer neoangiogenesis in which the main actor is represented by the NF-κB/c-Rel family of transcription factors. NF-κB also plays a primary role in regulating immune response, inflammation, cell proliferation, apoptosis, and cancer. According to recent evidence, miR-221 downregulates A20 (NF-κB inhibitor) expression and increases both c-Rel and connective tissue growth factor (CTGF; a central mediator of tissue remodeling), leading to cell growth and migration. These effects can be abrogated using the LNA-i-miR-221,[126] suggesting therefore a potential path to overcome cancer progression, antagonizing in this disease setting the main mechanism of tumor growth and invasiveness.

The urokinase plasminogen activator surface receptor (UPAR) isoform 2 has been associated with malignancy in TNBC and positively related to the expression of miR-221, targeting of which could significantly improve TNBC therapy.[127] Ye et al. reported that the transfection of BC cells with an anti-miR-221 ASO promotes the cytotoxicity of cisplatin, inducing apoptosis by targeting the Bim-Bax/Bak axis.[128] This feedback could particularly benefit BRCA-mutated patients, in whom cancers are known to be more susceptible to platinoids.

miR-221/222 expression is also elevated in basal-like BCs (BLBCs), which represent an even more aggressive phenotype characterized by the absence of expression of hormonal and HER2 receptors and by increased expression of basal cytokeratins CK5/6 and CK14. A correlation between TNBC and BLBC has been found in about 80% of cases, to further demonstrate the extreme heterogeneity within these subgroups. Proteomic analysis indirectly demonstrated downregulation of two tumor-suppressor genes, the suppressor of cytokine signaling 1 (SOCS1) and cyclin-dependent kinase inhibit 1B

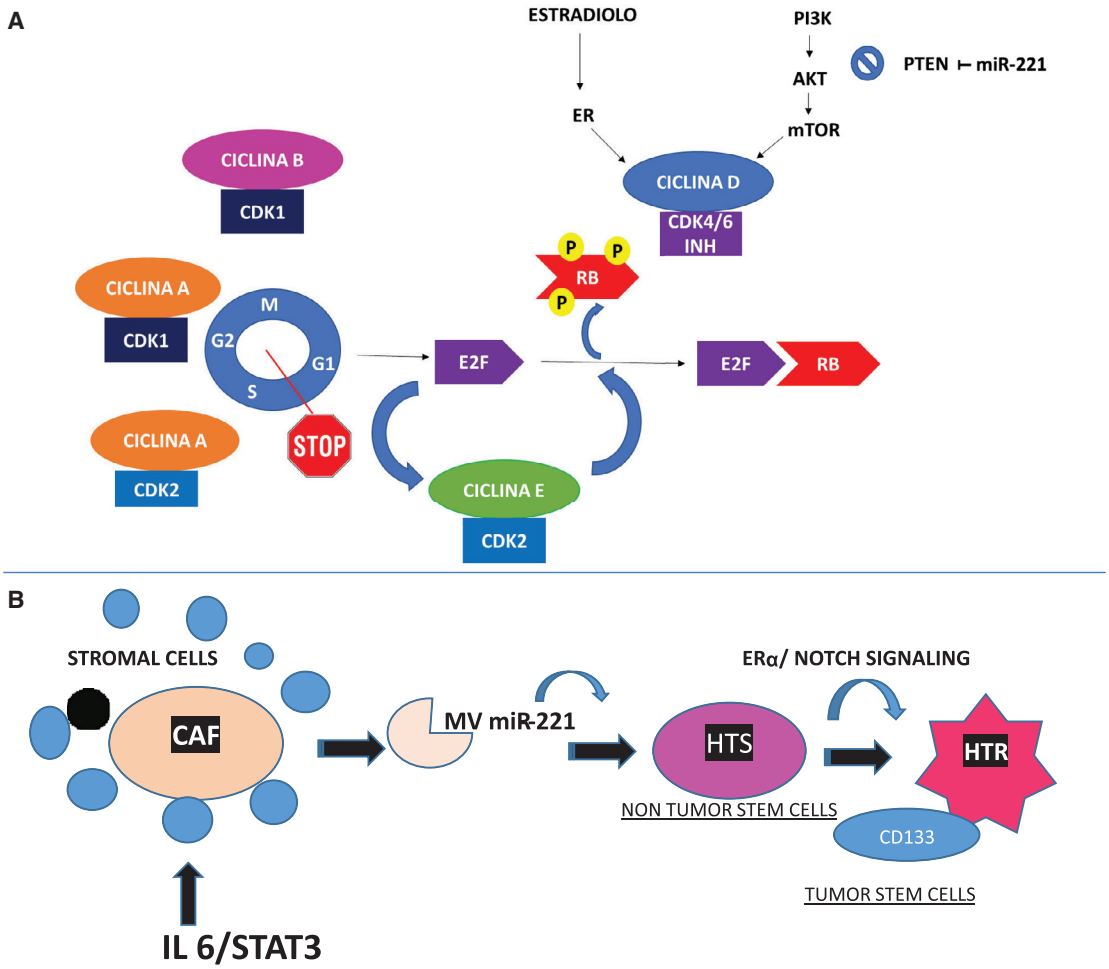

Figure 2. MiRNA-221 activity in HER2+ and Luminal BC

(A) miR-221 targets PI3K-AKT-mTOR pathway in HER2+ BC. When PTEN works correctly, it helps to control the cell cycle, but when it is not functioning at full speed, cells multiply in an uncontrolled way, turning into tumor formations. PTEN inhibits the PI3K-AKT-mTOR pathway downstream of HER2, leading to resistance to various therapies like CDK 4/6 inhibitors. miR-221 facilitates tumor proliferation downregulating PTEN thus inducing EMT. miR-221 downregulates PTEN, thus inducing EMT. (B) miR-221 promotes hormonal resistance in luminal BC. IL6/Stat3 pathway drives the proliferation of CAFs and production of oncomiR-221 MVs. MVs horizontally transfer miR-221. miR-221 promotes conversion of non-CSC (ER high) into therapy-resistant CSC (ER low) by Notch3 upregulation and ER signaling suppression. This leads to CD133hi/ERlo/Notch hi CSCs.

(CDKN1B) by miR-221/222 direct targeting, contributing to the aggressiveness in control of BLBC. In MDA-MB-231 cells, miR-221/222 inhibitors significantly suppressed cellular migration, invasion, and G1/S cell-cycle progression.[129] Moreover, miR-221/222 plays an important role in BC progression and metastasis through the negative regulation of the direct target adiponectin receptor 1 (ADIPOR1), a gene involved in EMT and cell invasion. This was particularly evident in BLBC patients, where ADIPOR1 was observed with a lower expression than in luminal subtypes. Overexpression of ADIPOR1 in the basal-like cell line, MDA-MB-231, attenuated cell invasion and negatively regulates EMT, providing an additional node by which miR-221/222 induces EMT. Consequently, miR-221/222 inhibition offers an alternative therapeutic option to reduce invasiveness and EMT in basal-like subtypes.[130]

HER2 positive BC

About 13%–15% of BCs show HER2 overexpression, which is a resistance factor to endocrine therapy but is also predictive of treatment response by anti-HER2 monoclonal antibodies (mAbs). To date, the primary and acquired resistance to the anti-HER2 trastuzumab is the major obstacle for clinical application. It has been reported that miR-221 inhibits apoptosis, induces trastuzumab resistance, and promotes metastasis of HER2-positive ($HER2^+$) BCs. This mechanism of resistance was mediated by the downregulation of the oncosuppressor PTEN, which involves the hyperactivation of the PI3K pathway. Although further investigations are needed, combined HER2 and miR-221 targeting could overcome drug resistance and improve the treatment of $HER2^+$ BC.[131]

According to Wang et al., PTEN is downregulated in TRAIL-resistant cells and thus induces EMT and enhances invasiveness. Silencing miR-221 in MDA-MB-231 TRAIL-resistant cells reversed resistance to TRAIL partially by targeting PTEN[132] (Figure 2A).

The knockdown of *PTEN* transcription by miR-221 has also a significant effect on BC resistance to Adriamycin. miR-221 was elevated in tumor tissue compared with nearby non-tumor samples, as well as in a BC cell line with Adriamycin resistance (MCF-7/ADR) compared with its parental line (MCF-7) and the normal breast epithelial cell line (MCF-10A). Enforced expression of miR-221 promoted Adriamycin resistance and sustained cell survival. miR-221 silencing improved the sensitivity to chemotherapy, resulting in enhanced apoptosis in resistant BC cells.[133]

It has been hypothesized that carcinoma cells that have undergone EMT acquire cancer stem cell properties, including self-renewal, chemoresistance, and radioresistance. Zinc finger E-box-binding homeobox 1 (ZEB1) is a transcription factor that promotes tumor invasion and metastasis by inducing EMT.[134] Anastasov et al. observed a significant decrease in migration capacity, including reduction of miR-221 and ZEB1 levels, and EMT marker expression, by using MEK1 inhibitor (TAK-733) in combination with radiation therapy in BC cells. Novel therapy design including miR-221 inhibitors can be seriously considered for the heavily pre-treated advanced BCs (e.g., TNBC and HER2$^+$), which are recognized as highly migrative and resistant to radiation treatment alone.[135]

Notably, radiotherapy also induces an upregulation of miR-221/222 in the blood of BC patients and their levels are affected by cardiovascular disease. miR-221/222 cluster is highly expressed in vascular smooth muscle cells (VSMCs) and ECs, inducing migration, proliferative, and anti-apoptotic effects in VSMCs, whereas it has antiproliferative, anti-migration, and pro-apoptotic effects in ECs. Moreover, miR-221 overexpression has been shown to induce cardiac hypertrophy *in vitro* and to promote heart failure. Indeed, miR-221/222 cluster is significantly upregulated in patients with hypertrophic cardiomyopathy. Therefore, miR-221/222 modulation by anticancer treatments seems to be deeply involved in cardiotoxicity and their inhibition could provide an interesting perspective on cardioprotection in these patients.[136]

Luminal BC

miR-221/222 are known to be involved in acquired endocrine resistance and related to poor clinical outcomes in ER-positive (ER$^+$, also known as luminal) BC. Knockdown of miR-221 and/or miR-222 sensitizes BC cells to tamoxifen, inducing cell growth arrest and apoptosis. This effect is mediated by upregulation of metalloproteinase-3 (TIMP3), via inhibition of miR-221/222, which represents a promising therapeutic approach for this specific BC subtype.[137]

Sansone et al. demonstrated that microvesicles (MVs) derived from cancer-associated fibroblasts (CAFs) transfer miR-221 to promote hormonal therapy resistance (HTR) in models of luminal BC. CAF-derived MVs horizontally transferred miR-221 to tumor cells and, in combination with hormone therapy, activated an ER^{lo}/Notch^{hi} feedforward loop responsible for the generation of CD133^{hi} cancer stem cells (CSCs). Importantly, MVs from patients with HTR metastatic disease expressed high levels of miR-221 promoted by the IL6-pStat3 pathway. CAFs can promote HTR, leading to post-transcriptional downregulation of ER expression by MV-mediated transfer of miR-221, suggesting that CAF-derived MVs could generate *de novo* HTR disease via an miR-221-mediated conversion of non-CSCs into therapy-resistant CSCs[138] (Figure 2B).

Rehman et al. reported that 14-3-3ζ overexpression enhanced MAPK/c-Jun signaling, increasing miR-221 transcription, which in turn inhibited p27-CDKI translation with consequent promotion of cell proliferation in transgenic mouse models of breast tumors and in patients is associated with high-grade cancers.[139] In addition, combining the expression information of 14-3-3ζ, miR-221, p27, and Ki-67 allowed better prediction of the tumor grade and prognosis compared with Ki-67 alone evaluation.[139]

Finally, most high-grade luminal invasive carcinomas (Lum-ICs) express β4 integrin, overexpression of which in cancer cells supports tumor progression. In human primary tumors with different β4 integrin expression and grade, low miR-221/222 levels have been inversely correlated to β4 integrin expression and tumor proliferating index. miR-221/222 with a post-transcriptional mechanism modulated β4 integrin, ADAM17, and STAT5, affecting a more aggressive behavior of Lum-IC cancer cells.[140]

In patients in early-stage ER$^+$ BC and on endocrine therapy, high value of tricho-rhino-phalangeal syndrome-1 (TRPS-1), a GATA transcription factor in the regulation of EMT, has been correlated with longer OS and PFS.[141] According to Stinson et al., the TRPS-1 expression can be repressed by miR-221/222, leading to reduction of E-cadherin and gain of vimentin.[142] Promoting EMT through miR-221/222 may contribute to the more aggressive clinical behavior of basal-like BCs, which can be counteracted by inhibiting miR-221/222 cluster.

Gastrointestinal cancers

Esophagus and gastric

Esophageal carcinoma (EC) is one of the most aggressive tumors of the gastrointestinal district and urgently needs new therapeutic targets and also biomarkers for its early diagnosis.[143–145] Staniz et al. evaluated the correlation between impaired miR expression and common risk factors for EC. In EC tissues of 23 patients were observed overexpression, among other miRs, of miR-221 ($p = 0.001$) compared with healthy tissue.[143] Duodeno-gastro-esophageal bile reflux is a risk factor for EC because induces oxidative stress and the production of ROS in esophageal squamous cells, which, in turn, promote oncogenesis.[146,147] Matsuzaki et al. reported high levels of miR-221/222 and activity of nuclear bile acid receptor/farnesoid X receptor (FXR) in esophageal cells exposed to bile acids. At the same time, they detected low levels of p27Kip1 and CDX2. p27Kip1 is a known miR-221/222 target that inhibits the proteasome-mediated degradation of the

transcription factor CDX2. By incubating esophageal cancer cells with miR-221 and miR-222 inhibitors, they observed an increase in p27Kip1 and CDX2 levels, resulting in reduced tumor cell growth. This work opens up the prospect of targeting miR-221 and miR-222 as a therapeutic target in the treatment of EC.[146]

Chemoresistance is a major obstacle in the effective treatment of EC. Wang et al. studied the role of miRs in the context of chemotherapy resistance and reported overexpression of miR-221 in EC tissue samples. Consequently, they questioned whether there was an association between miR-221 and resistance to chemotherapy in these patients. They found that miR-221 activates the WNT/β-catenin pathway by regulating the expression of DKK2, a WNT/β-catenin antagonist, thereby inducing cell proliferation, confirming an oncogenic role of miR-221 in EC. Moreover, it was observed that EC tissue samples exposed to 5-fluorouracil (5-FU) treatment showed a decrease in DKK2 and an accumulation of β-catenin, while inactivated β-catenin was mainly localized in EC tissue without prior chemotherapy treatment.[148] In conclusion, the authors suggested that an increased expression of miR-221 influences chemoresistance, therefore potentially acting as a biomarker to predict chemoresistance in EC.

Gastric cancer (GC) is the fifth cause of cancer in the world. Surgery and systemic chemotherapy presently represent the standard approach of this aggressive disease. Several studies have shown the role of miRs in all stages of carcinogenesis and GC progression. These small ncRNAs are involved in different biological processes of GC, from cell proliferation and differentiation to apoptosis.[149–151]

miR-221 has been proved to have a key role in the development of GC as reported by Zhang et al.[152] In particular, they demonstrated that miR-221 and miR-222, through the modulation of tumor suppressor PTEN, regulate growth, invasiveness, and radioresistance of GC cells.

Ning et al. identified one of the molecular mechanisms by which miR-221/222 promotes the growth of GC.[153] Specifically, they reported that GC cells have an increased expression of miR-221 and miR-222, but also reduced expression of its direct target hepatocyte growth factor activator inhibitor 1 (HAI-1), which inhibits the growth factors involved in carcinogenesis. Overexpression of miR-221 and miR-222 has been shown to inhibit the expression of HAI-1, and thus promote cell proliferation and invasive capacity.

Liu et al. explored the interaction between miR-221, miR-222 and the tumor-suppressor gene RECK, and specifically the relationship between *Helicobacter pylori* infection and the expression of miR-221/222 cluster.[154] The authors showed that *H. pylori* increased the expression of miR-221 and miR-222 by targeting RECK and then regulating its expression. The authors concluded that miR-221/222-RECK axis is an important path modulating *H. pylori* infection-related GC development.

Recent attention has been paid to the study of bone marrow mesenchymal stem cells (BM-MSCs) as promoters of an immunosuppressive microenvironment that favors the growth and proliferation of various tumor stem cells.[155–158] In this context, Ma et al. described the overexpression of miR-221 in GC-MSC exosomes that correlated with the TNM stage of GC.[159]

miR-221 and miR-222 overexpression has been also detected in GC tissue-derived mesenchymal stem cells (GC-MSCs) compared with GC-MSCs from their adjacent non-cancerous tissues.[160] GC-MSCs, by transferring exosomal miR-221 to gastric cancer cells, favor tumor progression and development of metastases. Cai et al. identified the plasma expression of three miRs, including miR-221, significantly increased in GC patients compared with in healthy controls ($p < 0.05$), suggesting their use as potential biomarkers.[161]

The role of miR-221 as a potential biomarker for GC diagnosis has also been proposed by Liu et al.[150] The authors identified overexpression of miR-221 in 88% of gastric tumors compared with adjacent non-tumor tissues. Furthermore, they observed a close association between the expression of miR-221 and greater aggressiveness of the tumor, resulting in a worse prognosis. Patients with higher miR-221 expression levels had a 5-year survival rate of 34 months, compared with 43 months for patients with low miR-221 levels ($p = 0.018$). A statistically significant correlation was also identified between the expression of miR-221 and the advanced clinical stage ($p = 0.021$), local invasion ($p = 0.008$), and lymph node metastases ($p = 0.039$), without any correlation with age, sex, or cell differentiation.

Effatpanah et al. showed an increased expression of miR-221 and miR-21 ($p = 0.012$) in early GC tumor cells (patient TNM stage I–II) compared with paired adjacent non-tumor tissue samples. The increased expression of miR-21 and miR-221 were not associated with gender, age, tumor size, lymph node, or metastatic involvement. So, both miRs had the potential role as novel biomarkers for early GC detection.[162]

Further evidence about miR-221/222 cluster overexpression in GC, and their role in tumor development and growth, came from the study of Hong et al.[163] The authors evaluated advanced GC patients treated with radical gastric resection and sequentially adjuvant systemic chemotherapy. During long-term follow-up, no statistically significant correlation between the expression of miR-221/222 and the tumor invasion or lymph node metastases was identified. However, overexpression of miR-221/222 correlated with a worse prognosis and a reduced 5-year survival rate.

Pancreatic cancer

PC is a devastating malignancy, whose prognosis remains impressively poor. It is the deadliest cancer worldwide. The expression of miR-221 is significantly upregulated in PC cell lines, higher in invasive PC cell lines (SW-1990, Panc-1, and Miapaca-2) than in a non-aggressive PC cell line (Bxpc-3). Panc-1 and SW-1990 cells transfected with miR-221/222 mimics showed higher growth and invasion capacity, decrease in the number of G1 phase cells, and lower apoptotic rate compared with the miR-221/222 inhibitor-transfected

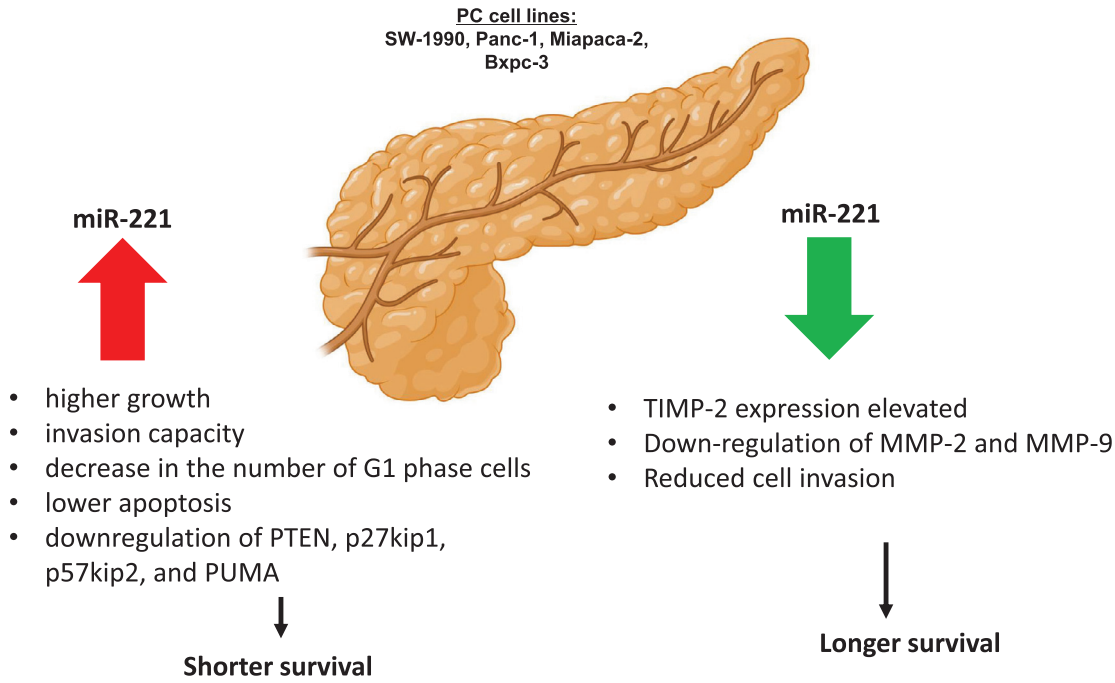

Figure 3. miRNA-221 regulation in pancreatic ductal adenocarcinoma

miRNA-221 is upregulated in pancreatic adenocarcinoma cell lines such as SW-1990, Panc-1, Miapaca-2, and Bxpc-3. When they are transfected with miR-221, mimics show an increased capacity of growth and invasion, a reduced number of G1-phase cells, and a lower apoptotic rate, which also leads to a downregulation of tumor-suppressor genes, such as pTEN, p27kip1, p57kip2, and PUMA. On the other hand, when miR-221 levels are reduced, TIMP2 expression is increased. TIMP2 inhibits MMP2 and MMP9, which are involved in cell proliferation.

cells.[164] TIMP2 was identified as a direct target of miR-221/222, whereas, in cells with reduced levels of miR-221/222, TIMP2 expression was elevated and its silencing induced significant upregulation of MMP-2 and MMP-9, important factors that influence cell invasion, confirming that PC cell invasion was dependent on miR-221/222 regulation. In 24 pancreatic ductal adenocarcinoma (PDAC) tumor tissues higher expression of miR-221 has been detected compared with pancreatic ductal epithelial cells and adjacent normal pancreatic tissue, together with downregulation of PTEN, p27kip1, p57kip2, and PUMA, the main miR-221 targets[165] (Figure 3). miR-221 inhibition in MiaPaCa-2 cells reversed expression levels of these mRNAs and proteins. Moreover, in this study, it has been found that patients with low miR-221 expression had a longer survival compared with the patients with high expression of miR-221, although the difference was not statistically significant due to a low number of patients evaluated in the study.[165] In addition, two patients were still alive at the publication time, surviving for more than 5 years after diagnosis, who had low expression of miR-221. Based on this evidence, high expression of miR-221 could be a prognostic factor for the aggressiveness and poor survival of PC patients, and the downregulation of miR-221 can inhibit the PC progression.

More evidence related miR-221 overexpression to the decrease of the sensitivity to 5-FU in both PANC-1 and PATU8988 cells by directly binding to retinoblastoma gene (RB1) 3′-UTR, suggesting that higher expression of miR-221 increases 5-FU resistance and then reduces the therapeutic effect.[166]

Hepatocellular cancer

HCC is a widespread disease characterized by a poor prognosis.[167] Nowadays, in the first-line treatment for advanced HCC, TKIs like sorafenib represent the main treatment strategy, but other therapies like bevacizumab plus atezolizumab are new promising approaches.[168] Despite that, the prognosis of HCC remains poor.

In HCC, miR-221 is of particular interest since it has been reported to be involved in tumorigenesis, growth, and apoptosis inhibition by constitutive activation of NF-κB,[169] inhibition of SOCS3,[170] and DKN1C/p57 and CDKN1B/p27 expression.[171] In the Yoon et al. study, formalin-fixed paraffin-embedded (FFPE) HCC tissue specimens were collected and compared with non-neoplastic liver cells of 115 patients.[172] miR-221 was significantly overexpressed in HCC, and higher expression was associated with a shorter time to disease progression. Furthermore, the multivariate analysis demonstrated that miR-221 was an independent predictor of tumor recurrence after resection, leading authors to conclude the potential prognostic value, specifically as a predictor of recurrence after resection. Similar results were reported by Sharker et al., confirming the upregulation of miR-221 in serum of HCC patients and its potential use as a

non-invasive diagnostic biomarker for early prediction of HCC among HCV^+ patients.[173]

In the Chen et al. study, 135 specimens of HCC tissues were collected, and the expression levels of miR-221 were compared with adjacent tissues by quantitative PCR.[174] miR-221 was found to be overexpressed in 62 (of 135) HCC cases, and levels of miR-221 were associated with tumor size, numbers of nodes, and microvascular invasion. The overexpression of miR-221 was also linked with smaller OS, and the Cox regression suggested miR-221 as an independent predictor of poor prognosis.

In the Karakatsanis et al. study, the authors compared tissues from 60 patients affected by HCC, 21 with intrahepatic cholangiocarcinoma (ICC) and 98 healthy controls.[175] Once again, miR-221 expression was significantly higher in HCC tissues and correlated with cirrhosis, suggesting an early miR-221 upregulation in the tumorigenesis process. Additionally, the expression of miR-221 was associated with tumor stage and poor prognosis. Multivariate Cox regression analysis has revealed miR-221 as an independent predictor of OS in HCC patients, supporting the further investigation of miR-221 upregulation in HCC as a serum biomarker with potential prognostic value. Accordingly, in Li et al.'s study, significant upregulation of serum miR-221 was observed in 46 HCC patients compared with healthy controls.[50,176] Moreover, the authors highlighted a correlation between higher serum levels of miR-221 and advanced tumor stage, indicating a possible link between serum miR-221 levels and prognosis. Similar results were reported in the study of Zekri et al. performed in HCC patients on top of HCV infection.[177]

In Ghosh et al.'s study, four circulating miRs, including miR-221, analyzed in 38 patients with HCC showed superior sensitivity with respect to alpha-fetoprotein (AFP) on clustering HCC from non-HCC patients, by ROC analysis.[178] Specifically, miR-221 was found to be significantly upregulated in the exosome of HCC patients compared with chronic hepatitis/non-HCC.

Of particular interest was Feng Li et al.'s study.[179] The authors investigated miR-221 expression of HCC and adjacent non-cancerous tissues, showing a significant correlation between miR-221 levels and poor prognosis. By the use of poly-lactic-co-glycolic (PLGA) acid-based nanoparticle, miR-221 or inhibitors were delivered into HCC cells. HCC cells transfected with nanoparticle/miR mimic complexes showed an increase in tumor growth and invasion capacity. Subsequently, HCC cells with higher miR-221 expression were transfected with nanoparticle/miR-221 inhibitor, and, in these cells, a reduction of miR-221 accounted for a significant reduction of growth and invasion. Finally, an *in vivo* model with luminescent HCC cells subcutaneously injected into naked mice was used to evaluate both nanoparticle/miR-221 mimic and inhibitor activity. HCC cells transfected with nanoparticle/miR-221 mimic complex showed increased tumor growth compared with controls, whereas xenograft tumors transfected with the nanoparticle/miR-221 inhibitor were significantly smaller, indicating a reduction in cell proliferation (Figure 4A).

Finally, miR-221 could have a role in mediating sorafenib resistance. Fornari et al. evaluated the resistance to sorafenib induced by miR-221 in mice models, supporting a possible mechanism for this effect through the inhibition of apoptosis mediated by Caspase-3 (Figure 4B).[180]

Thus, the treatment of HCC with miR-221 inhibitors could have a clinical and crucial role in the future, but large trials are needed to verify this hypothesis. Of course, miR-221 is a valuable marker for predicting HCC prognosis and progression, as well as a potentially relevant target for miR-based interventions in this still incurable disease.

Biliary tract cancer

Biliary tract carcinoma (BTC) is a heterogeneous group of malignant cancers, including carcinomas of the gallbladder, ampulla of Vater, and intrahepatic, hilar, and distal cholangiocarcinoma.[181–183] BTCs are infrequent tumors (<1% of all tumors) with highly aggressive features in most cases. The preferred treatment is surgery when possible, with radiotherapy only in selected cases and generally systemic chemotherapy.[182]

Among BTCs, miR-221 is one of the most deregulated miRs. Correa-Gallego et al. observed in patients with ICC an increased expression of miR-221 compared with healthy controls. Specifically, high concentrations of miR-221 were observed, both in the neoplastic tissue and in the plasma of patients, supporting their use as valid biomarkers for early diagnosis. Furthermore, the expression of miR-221 was significantly higher in poorly differentiated tumors than in moderately differentiated tumors ($p = 0.016$), while no statistically significant difference was found between miR-221 expression and tumor characteristics such as size, lymph node involvement, metastatic spread, and resection status, either in patients undergoing hepatic resection or in inoperable patients.[184]

A close correlation was also identified between miR-221 and extrahepatic cholangiocarcinoma (EHCC). Li et al. reported an overexpression of miR-221 in EHCC tumor cells that correlated with lymphatic metastasis ($p = 0.042$), advanced clinical stages ($p = 0.033$), and decreased DFS rate ($p = 0.032$). No correlation was observed between miR-221 level and sex, age, tumor size, histopathological types, cell differentiation, and Bismuth classification ($p > 0.05$).[185]

Finally, by assessing the expression of miRs in two cholangiocarcinoma cell lines, Okamoto et al. identified four miRs that influenced sensitivity to gemcitabine. The authors observed that miR-221 is decreased in gemcitabine-resistant cells. The molecular mechanisms of chemoresistance to gemcitabine have been explored and, in particular, miR-221 has been reported to induce caspase-3/7 and inhibit PIK3R1 in HuH28 cells.[186]

Urogenital cancers

Bladder cancer

Bladder cancer (BlC) is the 10^{th} most common cancer worldwide and, unfortunately, one of the leading causes of mortality from

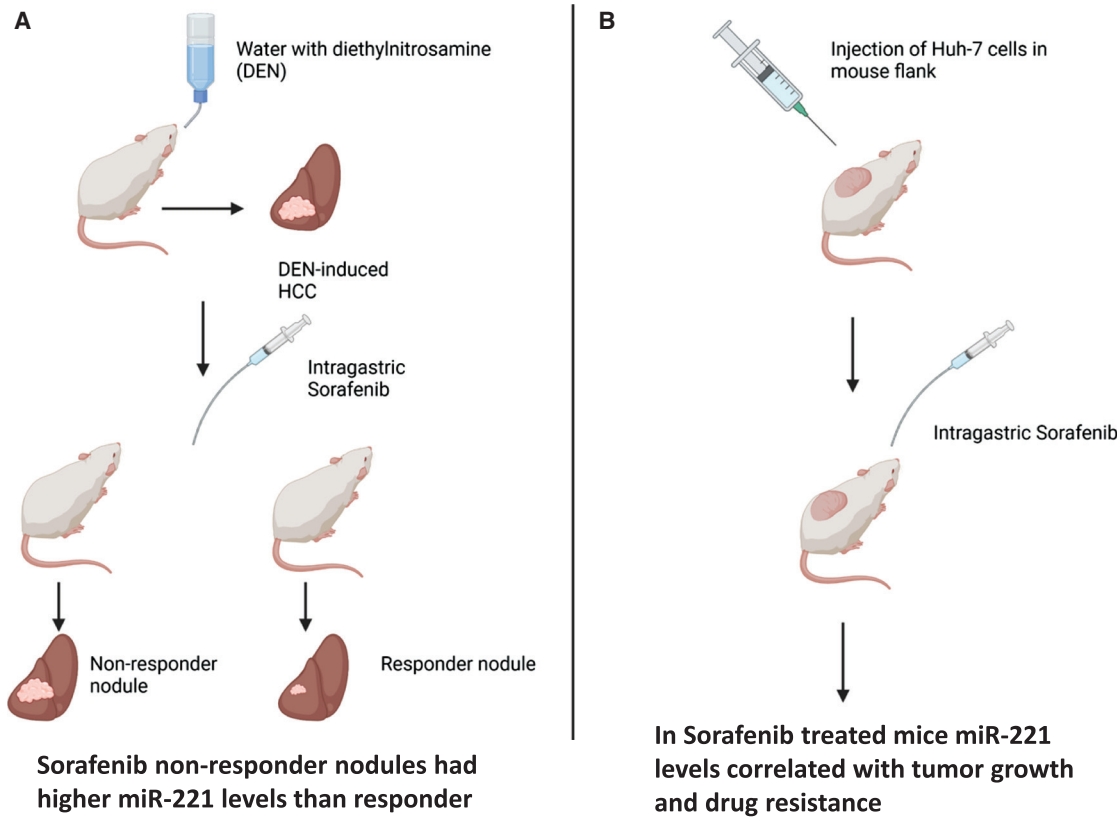

Figure 4. Models of sorafenib reistance in HCC

(A) miR-221 role in HCC proliferation and sorafenib resistance. Sorafenib non-responder nodules show higher levels of miR-221. (B) miR-221 inhibition in HCC overcomes sorafenib resistance. In mice treated with sorafenib, miR-221 levels correlated with tumor growth and drug resistance. Created with BioRender.com.

neoplasms.[187] As in other human cancers, deregulated expression of miRs has been demonstrated in BlC. miR-221 expression was confirmed to be higher in bladder tumors compared with normal bladder tissues, and, in particular, in invasive muscle disease, in high-grade tumors, and short-term relapse disease. Tsikrika et al. suggested that miR-221/222 overexpression may help to discriminate BlC from normal bladder tissues. It has been demonstrated that the miR-221/222 cluster promotes both cell proliferation and survival through PTEN and PUMA suppression and, also, through the activation of the PI3K/AKT/mTOR pathway in the bladder carcinogenesis.[188] Among urothelial tumors, one of the most common post-transcriptional changes is reported to be the methylation of N6-methyladenosine (m6A). According to Han et al. methyltransferase-like 3 (METTL3) was found to accelerate the maturation of pri-miR-221/222 by a pathway involving m6A, resulting in the reduction of PTEN. The suppression of PTEN was the crucial step leading to cancer cell proliferation.[189] Moreover, the expression of miR-221 is regulated by the high-mobility group AT-hook 1 (HMGA1), which is overexpressed in BlC. Liu et al. reported that the expression of miR-221 forecasts a poor prognosis and the downregulation of HMGA1 prevents invasion and migration of tumor cells by inducing autophagy through the modulation of the miR-221/TP53INP1/p-ERK axis. These data support the role of miR-221 as a potential therapeutic target in patients with BlC.[190] The expression of miR-221 has been shown to contribute significantly to the development of the EMT phenotype in human BlC cells. These findings support the role of miR-221 in the transforming growth factor (TGF) β1-induced EMT process in BlC cells by suppressing STMN1.[191,192] Finally, miR-221 was found to promote immune escape of BlC cells by silencing the pro-apoptotic factor Bcl-2 and PUMA. Indeed, PUMA expression is regulated by transcription factors such as p53, c-MYC, and FoxO3a but also by post-transcriptional factors, including the miR-221/222 cluster. Fu et al. reported that the inhibition of miR-221 in BlC cells reduced the expression of VEGF-C, MMP-2, and MMP-9, resulting in inhibition of tumor cell proliferation.[193]

Prostate Cancer

miR evaluation was of interest also in the Prostate cancer PCa setting, to support an unmet need, relative to prognostic biomarkers, predictive value, diagnostic factors, and therapeutics implementation strategies. Among promising miRs explored in PCa, miR-221 was revealed as one of the most strongly deregulated.[194] Specifically, the expression of circulating miR-221 has been correlated with PCa progression,

suggesting its use as an innovative non-invasive biomarker.[195] Based on this evidence, a novel diagnostic and prognostic tool has been proposed, named miQ, comprising evaluation of circulating miR-221-5p and other miRs. miQ demonstrated a high accuracy to predict diagnosis (area under the curve [AUC] = 0.931, $p < 0.0001$), tumor invasion (AUC = 0.895), metastasis (AUC = 0.827), and OS ($p = 0.0013$, Wilcoxon test HR = 6.5) in PCa.[196]

The role of miR-221 in tumorigenesis and progression in PCa is still unclear, and it appears variably under- or overexpressed, performing as oncogene or tumor suppressor, depending on context and cell-type specificity. Zedan et al. described the overexpression of miR-221-3p plasma levels, among other miRs, in patients affected by PCa versus healthy donors, while miR-221 plasma levels decreased after first-cycle treatment start with docetaxel or abiraterone.[197] Low levels of miR-221 have been linked to a protective factor for relapse-free survival (RFS) (HR = 0.68; 0.51–0.89, $p = 0.005$) in young PCa, related to low Gleason score, and the absence of extraprostatic extension and lymphatic invasion.[198] miR-221/222 directly targets caspase-10 mRNA, exerting an anti-apoptotic function[199] in LNCaP and PC3 cells. In addition, miR-221 promotes aggressiveness of PC3 cells by downregulating DIRAS3, a predicted miR-221 target, supporting EMT[200] and regulating cell-cycle distribution via SIRT1 exerting a tumor-promoting action.[201] Kneitz et al. correlated lower expression of miR-221-3p with higher rate of PCa-related death. miR-221 induced tumor suppression by direct targeting SOCS3 and IRF2 oncogenes that negatively regulate JAK/STAT signaling through STAT1/STAT3-mediated activation, a pathway active only in androgen-independent, SOCS3-positive cell line, as evaluated on PC-3 and DU-145 PCa cells.[202] Moreover, miR-221 has been demonstrated to reduce angiogenesis via downregulation of oncogenic c-kit[203]; cell proliferation targeting Bim1, SOCS3, and IRF2[204]; migration; and invasion targeting ecm29.[205] Krebs et al. reported miR-221-sensitized cells toward TRAIL-induced inhibition of proliferation and induction of apoptosis through the downregulation of the well-known miR-221 target gene SOCS3 and the novel target PIK3R1 identified in PCa cells.[206] Moreover, miR-221-3p acted as an escape mechanism from TKIs in PC3 cells, in which sunitinib exposure induced an interferon-related gene signature together with a significant upregulation of miR-221-3p/miR-222-3p.[207]

Kiener et al. explored the functional role of miR-221-5p, an miR generated from the same stem-loop precursor as miR-221-3p and able to have the same target or not, in androgen-dependent and androgen-independent PCa cell line models.[208] Highly aggressive PC-3M-Pro4 cells overexpressing miR-221-5p had reduced cell extravasation and metastatic potential in the zebrafish model and decreased tumor burden in an orthotopic mouse model. Opposite results have been reported by Shao et al., in which, specifically, miR-221-5p promoted proliferation by targeting the tumor suppressor SOCS1 and MAP/ERK survival pathway in PC3 cell lines and nude mice PCa xenografts.[209]

Pudova et al.[210] hypothesize a role for lymphatic dissemination, supported by low expression of miR-221-5p and miR-221-3p in N1 PCa, compared with N0. According to Kurul et al., downregulation of miR-221 was significant in patients with biochemical recurrence compared with non-recurrent patients, with 70% sensitivity and 71% specificity, associated with TMPRSS2-ERG gene fusion expression, a poor prognosis marker indicating the role of miR-221 as prognostic factor and therapeutic indicator.[211] Furthermore, miR-221 was also involved in castration-resistant PCa development and in androgen receptor regulation.[212] This interaction is mediated by epigenetic modulation targeting HECT-domain-containing E3 ubiquitin-protein ligase 2 (HECTD2) and Ras-related protein Rab-1A (RAB1A).[213] Zheng et al. also observed a role in neuroendocrine differentiation through interaction with DVL2.[214] Finally, Krebs et al. suggest an miR-221-3p value in escape mechanism from VEGFR2 inhibitors and hypothesize a theragnostic role in predicting TKI inhibitor response.[207]

In conclusion, the number of relevant studies presented demonstrate the great interest in miR-221 and its application in diagnostic, prognostic, and therapeutics research areas. Among these studies, there is a substantial variability, maybe related to different methodology, molecular heterogeneity, and sample collection. Further research and insights will be essential to enable the clinical application of this promising miR in PCa.

Renal cell carcinoma Renal cell carcinoma (RCC)

Renal cell carcinoma 979 Renal cell carcinoma (RCC) is responsible for about 2%–3% of all cancers in adults. While the prognosis of early-diagnosed disease is up to 90% at 5 years, metastatic disease is associated with a poor prognosis. Therefore, new biomarkers that would allow improvement of risk stratification and prognosis, and prediction of the response to treatments are urgently needed.[215,216] As for most cancers, abnormal expression of miR-221 is also involved in the process of carcinogenesis and disease progression via the regulation of cell proliferation, migration, and invasion in RCC.[217] Szabò et al. investigated the miR-21 and miR-221 expression in renal tumor tissues. They reported that that both miRs were overexpressed (and co-expressed in 79.2%) in tissue samples of RCC compared with normal renal tissue ($p < 0.001$ and $p < 0.05$ for miR-21 and miR-221, respectively).[218] Notably, levels of these miRs correlated with the severity of the pathological stage ($p < 0.001$). Furthermore, elevated circulating expression levels of miR-221 have been associated with poor prognosis, especially a reduced OS (48 versus 116 months; $p = 0.024$).[219] miR-221 has been involved in the EGFR-RAS-RAF-MEK pathway and its inhibition was associated with reduced cell invasion capacity and secretion of MMP 2 and 9.[218,219] miR-221 and miR-222 overexpression has also been correlated with the activation of the EGFR/MAPK pathway, especially in patients with RCC at intermediate or high risk of relapse.[220] Lu et al. showed that miR-221 directly inhibits the molecular tumor suppressor TIMP2. The authors demonstrated that the blockade in kidney cancer of miR-221 by miR-221 inhibitor was able to increase TIMP2 levels, thus contributing to the integrity of the cell membrane. These evidences prompted miR-221 to be retained as a therapeutic target in kidney cancer treatment.[221]

Khella et al. assessed the expression profile of miRs in patients with RCC under sunitinib as first-line therapy. They reported upregulation of miR-221 and miR-222 in patients with short-term responses to sunitinib (PFS $\leq$ 12 months). Although TKI resistance is a multifactorial process, these data suggest that miR-221 may be considered a biomarker that is useful to predict response to sunitinib in metastatic RCC.[222] García-Donas et al. also reported an interesting association between abnormal expression of miRs, including miR-221, and progression during therapy with TKIs.[223] In contrast, Kovacova et al., in an attempt to validate some miRs as predictive biomarkers of therapeutic responses, showed a positive association between response to treatment and miR-221 expression.[224] Liu et al. confirmed that miR-221 is significantly upregulated in RCC tissues and that patients with high levels of miR-221 had a poor prognosis.[225] According to Dias et al. miR-221 plasma levels are increased in patients with RCC. Furthermore, the data suggest that patients with higher levels of miR-210, miR-221, and miR-1233 presented the highest risks of renal-cancer-specific death. These results support the applicability of miR-221 combined with miR-210 and miR-1233 as prognostic biomarkers for RCC.[226]

Gynecological cancers

Ovarian Cancer

Ovarian Cancer (OC) is a gynecological malignancy characterized by a very low survival rate.[227] Worldwide, this type of cancer represents approximately 3% of all cancers in women and is the fifth most common cause of cancer-associated death in the female population.[228] Most miRs are down- or upregulated in OC tissues, and their expression levels can distinguish the malignant from the non-malignant ovarian epithelium.[229]

Hong et al. showed that miR-221 decreases after cytoreductive intervention and increases when the disease relapses.[230] Recent findings suggested that the miR-221 was upregulated in OC and in particular in larger tumor size, deeper tumor invasion, and higher International Federation of Gynaecology and Obstetrics (FIGO) stage ($p = 0.0076$).[231] Increased expression levels of miR-221 were also associated with poor patient prognosis and reduced DFS ($p = 0.0014$) and OS ($p = 0.0058$).[228] The apoptotic protease activating factor 1 (APAF-1) gene, an important component of the apoptosome, playing a role in response to cellular stress such as DNA damage, hypoxia, and oncogene activation, is a direct target of miR-221. In the study of Li et al., APAF-1 protein expression was negatively associated with miR-221 expression and was found to be downregulated in human OC tissues and cell lines SKOV3, OVACR-3, A2780, and 3AO. Patients with high expression of miR-221 had a lower PFS and OS rate than patients with low expression of miR-221, confirming the oncogenic role of miR-221 in this malignancy. In OC cell lines, transfection with the miR-221 inhibitor induced APAF1 protein overexpression, inhibition of cell proliferation, migration and invasion, and finally apoptosis induction. The miR-221-APAF1 axis represents a new potential diagnosis and therapeutic biomarker in OC.[228]

Bcl-2-modifying factor (BMF) is an interesting miR-221 target explored in OC where it plays a role in promoting apoptosis by binding anti-apoptotic proteins. Xie et al. reported that the high expression of miR-221 in OC tissues reduced the apoptosis of tumor cells by decreasing the expression of the pro-apoptotic factor BMF and promoted proliferation, occurrence, and development of OC, confirming that miR-221 acts as a cancer promoter.[227]

Amini-Farsani et al. showed that miR-221/222 upregulation in OC led to altered response to chemotherapy, particularly by inhibiting cisplatin-induced cytotoxicity, a mechanism of chemoresistance. The authors demonstrated that knockdown or inhibition of miR-221/222 enhances chemosensitivity of cancer cells to cisplatin by upregulating phosphatase and tensin homolog (PTEN) through PI3K/AKT pathway repression. These findings suggest that miR-221/222/PTEN/PI3K/AKT is a promising prognostic and therapeutic target in OC.[232]

Other studies have also found that the upregulation of miR-221 may have an anticancer effect, correlated with higher OS in epithelial OC patients.[231] Wu et al. showed that high expression levels of miR-221 were associated with significantly longer survival and a better prognosis in patients affected by OC. Therefore, overexpression of miR-221 suppressed cell proliferation and cancer progression by targeting and downregulating ADP-ribosylation factor (ARF4), a member of the Ras superfamily of small G proteins. This study demonstrated that miR-221 had a tumor-suppressive role, it was significantly downregulated, and inversely correlated with ARF4 in epithelial ovarian cancer (EOC) tissues, suggesting that restoration of miR-221 could be a new potential therapeutic strategy in OC.[233]

In conclusion, the oncogenic role of miR-221 in OC is not clear and more investigation is needed to elucidate whether its inhibition has therapeutic potential.

Cervical cancer

Cervical cancer (CC) is one of the most common cancer in women in developed countries. The major risk factor associated with CC is persistent infection with high-risk human papillomavirus (HPV). Significant decrease in the incidence of CC has been enriched by widespread vaccination against HPV, periodic cancer screening, and early surgical treatment lead, but CC remains one of the most common diseases causing mortality in women.[234,235]

Recently, many studies have been focused on the role of miRs in HPV infections, which are involved in the immune response regulating type I interferon (IFN). Lu et al. described that miR-221 is overexpressed in serum samples of patients with HPV 16 infection as well as in human cervical squamous cell carcinoma (CSCC), suggesting that miR-221 may be involved in the host-virus response of HPV16 infection. The authors demonstrated that miR-221 inhibited HPV 16 E1-E2-mediated DNA replication *in vitro* through positively regulating IFN-I production and interferon-stimulated gene (ISG) expression mainly through targeting SOCS1.[236]

Recent evidence shows that miR-221 is involved in metastasis development in CC. Wei et al. showed that miR-221-3p is overexpressed in

lymphatic metastasis of patients with CC compared with non-metastatic CC. Enforced miR-221-3p expression induced EMT and SiHa and HeLa cell migration and invasion. In contrast, in these cells, inhibitors of miR-221-3p drastically reduced EMT and decreased cell migration and invasion mediated by Twist homolog 2 (TWIST2), a key transcription factor binding to the promoter of miR-221-3p. miR-221-3p directly targets the 3′ UTR of *THBS2*, downregulation of which contributes to CC progression. In CC tissues, particularly with lymphatic metastasis, miR-221-3p and TWIST2 were increased while THBS2 was decreased, so, mechanistically, TWIST2 induces miR-221-3p expression, which in turn suppresses THBS2.[237] Moreover, it has been demonstrated by Zhou et al. that miR-221-3p is transferred by CSCC-secreted exosomes into human lymphatic ECs (HLECs) and mouse lymphatic ECs (MLECs), promoting HLEC and MLEC migration and tube formation and facilitating lymphangiogenesis. The authors also demonstrated that miR-221-3p promotes lymphangiogenesis and lymph node metastasis *in vivo*, downregulating vasohibin-1 (VASH1) expression in HLEC CSCC-secreted exosomes.[238]

Recently, Pan et al. demonstrated that the upregulation of miR-221/222 promotes CC by repressing MBD2 and MeCP2. In this study, it the expression of MeCP2 and MBD2 in CC tissues samples was analyzed by qRT-PCR, proving a significant reduction of the expression levels of both mRNAs that, together, account for the upregulation of miR-221/222. To further elucidate the relation between the increased levels of miR-221/222 and the promotion of CC by repressing MBD2 and MeCP2, the authors used different CC cell lines, including C33A, HeLa, and CaSki, confirming these pieces of evidence. However, the underlying mechanism by which miR-221/222 regulates MBD2 and MeCP2 is not elucidated.[239] Moreover, Yang et al. also correlate the miR-221/222 upregulation and AT-rich interactive domain-containing protein 1A (ARID1A) downregulation in CC tissues compared with normal cervical tissues. *In vitro* in both HeLa and SiHa cells, miR-221 and miR-222 overexpression significantly increased cell viability, increased the proportion of cells in S phase, and enhanced invasion. In contrast, ARID1A overexpression abrogated these effects induced by miR-221 and miR-222 overexpression, suggesting the miR-221/222-ARID1A axis as a potential therapeutic target.[240]

Endometrial cancer

Endometrial cancer is the most common gynecological cancer. Estrogen expression is a classic etiological factor for endometrial tumorigenesis.[241] In this tumor, the role of miR-221 has been explored by Penolazzi et al. The authors demonstrated that miR-221 is correlated with obesity and ERα/PR expression through the HIF1-α and SLUG pattern. Tissue samples from post-menopausal women affected by type 1 EC or endometrioid endometrial adenocarcinoma were collected and classified into two subcategories: ERα+/PR+ with more benign prognosis and ERα−/PR− with poorer prognosis. While the percentages of HIF1-α and SLUG-positive samples in the ERα+/PR+ and ERα−/PR− groups were comparable, the obesity factor, evaluated by the body mass index (BMI) measurement, affected the ERα+/PR + group more. In addition, miR-221 levels were significantly higher in the obese (OB) than non-OB patients and, also, in this case, obesity affected the ERα+/PR+ group more. In EC, miR-221 seems to be a nodal point of a molecular system with mutual regulation between ERα, PR, HIF1-α, SLUG, and miR-221, particularly enhanced by metabolic events that occur in obesity.[242]

Melanoma

Melanoma is the most aggressive cutaneous malignancy, but is mainly curable in the early stage. The diagnosis of metastatic disease leads to a very poor prognosis. Due to aggressive evolution and resistance to chemo- or radiotherapy, melanoma disease is well known for high mortality. The study of the molecular mechanism to identify novel therapeutic opportunities and prognostic markers is of major interest in melanoma. Among the most dysregulated oncogenic miRs in melanoma, the miR-221/222 cluster has been explored both *in vitro* and *in vivo* studies and has been also reported an important role as a biomarker. Ping Li et al. evaluated the link between serum levels of miR-221 and the prognosis of melanoma patients. The authors observed higher serum levels in patients with melanoma compared with healthy controls, which correlated with a lower 5-year OS and RFS rates, suggesting that miR-221 is a potential molecular biomarker for predicting the prognosis of cutaneous malignant melanoma patients.[243]

Wu and Cui investigated the effect induced by resveratrol on melanoma cells, a natural compound found in grapes, berries, chocolate, red wines, and so forth, with anticancer activity. Its role as an anti-oxidative, anti-infective, and anti-inflammatory molecule has already been studied. In two melanoma cell lines (A375 and MV3), the antitumor effects through the NF-κB pathway's inhibition mediated by miR-221 expression decrease have been reported.[72] The binding motif of NF-κB in the promoter region of miR-221 has been specifically identified using a JASPAR database and luciferase experiments. Another regulatory link involved in melanomas cell death is HOXB7/PBX2-miR-221/222-c-FOS. In particular, the HOXB7/PBX2 dimer acts as an miR-221/222 positive transcriptional regulator. The homeobox (HOX) genes are a family of transcription factors that clarify cells identified during early development. Through the highly conserved sequence, the homeodomain, HOX proteins bind to DNA and cofactors such as PBX and MEIS stabilize this interaction. Previous studies showed that c-FOS, an miR-221/222 target, together with c-JUN, a member of the complex activator protein-1 (AP-1), acts as either a pro- or anti-apoptotic factor in different cell types, including tumor cells. Errico et al. demonstrated that the HOXB7/PBX2 dimer acts as a positive transcriptional regulator of the oncogenic miR-221 and -222, which directly target c-FOS, a key apoptosis regulator. Comparison of endogenous levels in normal melanocytes and melanoma cell lines has shown an inverse correlation of miR-221/222 and c-FOS expressions. Treatment with HXR9, a peptide designed as a competitive inhibitor of the HOX/PBX dimerization, resulted in a marked activation of caspase-3 *in vitro* and reduced melanoma growth in a xenograft mouse model.[244]

Sarcomas

Sarcomas are tumors with poor prognosis and few therapeutic options. Yang et al., analyzing levels of miR-221 in tumor tissues of 108 patients with osteosarcoma, detected statistically significant higher miR-221 levels in tumor tissues compared with adjacent normal tissues ($p = 0.001$). Specifically, higher miR-221 levels were detected in patients with distance metastasis versus the absence of metastasis ($p = 0.01$) and in patients with advanced clinical stage versus early stage ($p = 0.006$). Higher levels of miR-221 were also related to shorter RFS and OS ($p = 0.001$).[245]

Zhao et al. reported miR-221 overexpression in 79 osteosarcoma patients with respect to the 94 enrolled (84.04%), showing significantly higher levels in recurrent tumors versus primary tumors ($p = 0.023$) and in recurrent and primary tumors compared with normal tissues ($p = 0.031$ and $p = 0.012$, respectively). miR-221 overexpression was also related to Enneking clinical stage and lung metastasis ($p = 0.016$ and $p = 0$ .004, respectively).[246]

Gong et al. reported high expression of miR-221 in osteosarcoma tissues related to clinical stage (IIB/IIIA versus IIA, $p = 0.017$), metastasis ($p = 0.038$), and pre-operative chemotherapy responses ($p < 0.001$), thus suggesting miR-221 as a prognostic factor.[247]

Andersen et al. analyzed miRs in 101 samples of osteosarcoma, from 23 patients included in a discovery cohort and 78 in a validation cohort. miR-221-3p overexpression was almost significantly associated with time to metastasis ($p = 0.0863$) and with poor prognosis ($p = 0.0074$).[248]

Different studies explored the upregulation of miR-221 to influence cell proliferation, migration, and invasion, by different mediators such as suppressing PTEN and so activating PI3K/Akt pathway,[249] or suppressing FBXW11 through Wnt pathway activation,[250] or inducing cisplatin resistance through downregulation of the tumor-suppressor gene *PPP2R2A*. This last induced activation of the AKT pathway, whose inhibition has been reported to restore cisplatin sensitivity.[251,252] In addition, the downregulation of miR-221 increased activity of CDKN1B/p27, a canonical miR-221 target, along with decreased caspase-3 and Bax, while levels of Bcl-2, cyclin D1, cyclin E, Snail, and Twist1 are increased in osteosarcoma cell lines.[253] Moreover, it has been reported in osteosarcoma tumors that reduced levels of GAS5, a tumor-suppressor gene with a specific activity of miR-221 inhibition and Aplasia Ras Homology member I (ARHI) upregulation that negatively affect proliferation, migration, and EMT. Conversely, overexpression of miR-221 reduces ARHI but not *GAS5* in osteosarcoma cells *in vivo*.[254]

In models of alveolar rhabdomyosarcoma human xenografts, miR-221 suppression by PAX3-FOXO1 fusion gene and inhibition of proliferation and invasion by downregulating CCND2, CDK6, and ERBB3 have been observed.[255]

Ihle et al. analyzed 18 samples of gastrointestinal stromal tumors, reporting miR-221 downregulation compared with six samples of normal smooth muscle tissue. In three *KIT*-mutated GIST cell lines, apoptosis increase after miR-221-3p cell transfection along with reduced levels of p-AKT, AKT and BCL2 were observed.[256]

Finally, in Kaposi' sarcoma, it has been reported that Kaposin B and c-Myc inhibit miR-221 promoter, reducing its levels and, in turn, angiogenesis.[257] Figure 5 summarizes different pathways involved in carcinogenesis in sarcomas.

In summary, the role of miR-221 in sarcomas needs more investigation considering that is positively regulated in osteosarcoma but negatively in rhabdomyosarcoma, GIST, and Kaposi' sarcoma. The use of miR-221 as a therapeutic target needs to be better elucidated in these human diseases.

Glioblastoma

Glioblastoma multiforme (GBM) is a rare neoplasm of the central nervous system associated with a poor prognosis. The front-line therapy for GBM is radiotherapy followed by adjuvant treatment with temozolomide.[258]

To date, many authors have investigated the role of miRs in the genesis of GBM, and in particular the involvement of miR-221 and its potential role as a biomarker and therapeutic target.

Some authors have investigated the expression of miR-221 in GBM tissues compared with non-tumor tissues to find a correlation between miR-221 and GBM growth and prognosis.

In the studies by Visani et al.[259] and Toraih et al.,[260] the authors compared miR-221 expression in GBM and adjacent non-tumor tissues in FFPE glioblastoma samples. Both studies confirmed the overexpression of miR-221 in GBM tissues, while Slaby et al.[261] and Lakomy et al.[262] have previously reported the downregulation of miR-221 levels in FFPE-dissected GBMs compared with normal tissues (probably non-optimal tissue because of non-matched controls). Moreover, Ilhan-Mutlu et al. reported no significant difference in miR-221 levels between initial and recurrent glioblastoma.[263]

Chen et al. studied the expression of miR-221 in FFPE glioblastoma tissues without O6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation. The authors found overexpression of miR-221 in MGMT-unmethylated GBM tissues, and this was significantly associated with poor prognosis in Cox multivariate analysis.[264]

Higher serum miR-221 levels of patients affected by GBM compared with healthy controls have been found, with a significant correlation between serum levels and the presence of GBM suggesting that high serum miR-221 levels could be a predictor of the worst prognosis.[265–267]

The role of miR-221 as a GBM tumor promoter has been deeply investigated, confirming that, in this tumor, miR-221 also induced

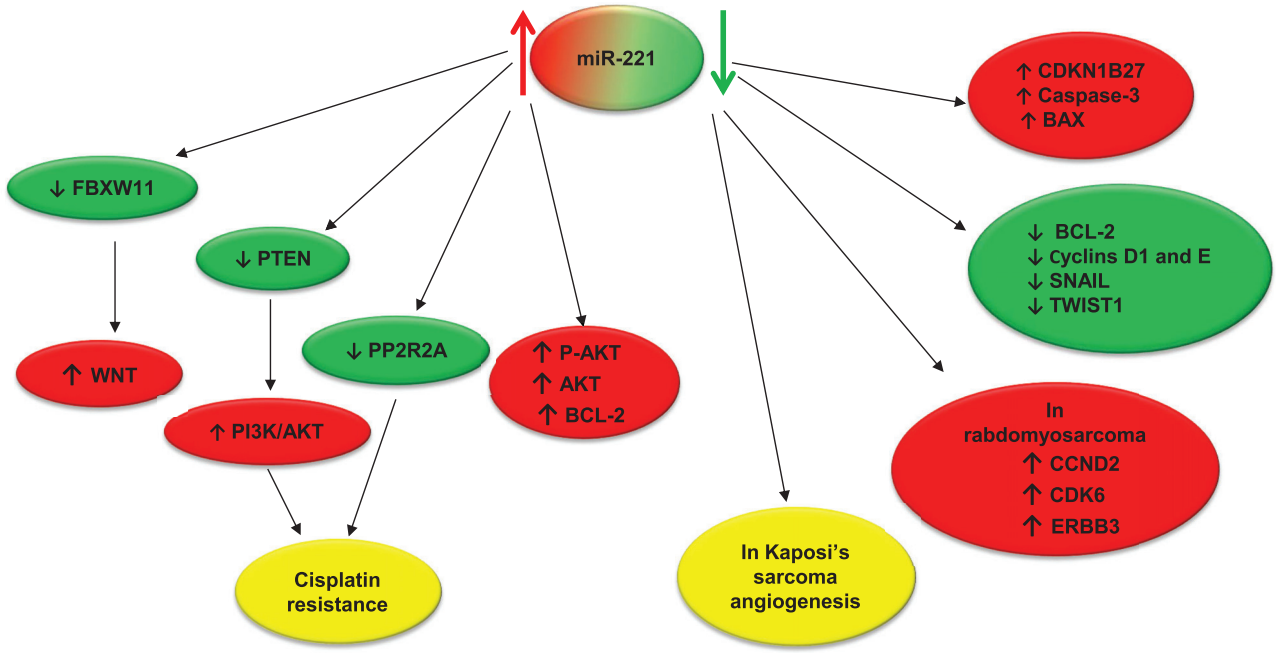

Figure 5. Different pathways involved in sarcomas carcinogenesis

Up- or downregulation of miR-221 can cause different effects in terms of oncogenesis or oncosuppression. In osteosarcomas, upregulation of miR-221 activates P-AKT, AKT, and BCL-2 as well as inhibiting FBXW11, which in turn upregulates Wnt. PTEN and PP2R2A upregulation is also induced by miR-221 together with cisplatin resistance; downregulation of miR-221 upregulates CDKN1B27, Caspase-3, and BAX, and downregulates BCL-2, Cyclins D1 and E, SNAIL, and TWIST1, determining oncosuppression. Downregulation of miR-221 also causes angiogenesis in Kaposi sarcoma and activates CCND2, CDK6, and ERBB3 in alveolar rhabdomyosarcoma. ↑, upregulation; ↓, downregulation.

angiogenesis, tumor growth, and invasiveness *in vitro*, obtained by transfection of GBM cells and non-tumor cells with miR-221 or anti-miR-221, respectively. Hao et al. demonstrated a potential role of miR-221 in the genesis of glioblastoma inhibiting connexin 43 (Cx43).[268] Antisense-miR-221 oligonucleotide (AS-miR-221) transfection into human glioblastoma cells induced miR-221 downregulation and Cx43 upregulation, which suggests a feasible role of miR-221 in the growth of glioblastoma by Cx43 inhibition. miR-221 was upregulated, inducing the ability to form colonies, while anti-miR-221 transfection reduced it.[269] The delivery of miR-221 mimics significantly enhanced cell growth, whereas miR-221 inhibitor transfection reduced it through PTEN modulation.[270] Likewise, Tokudome et al. demonstrated the overexpression of miR-221 in GBM cells treated with radiotherapy and subsequent PTEN downregulation, suggesting PTEN as an important tumor suppressor in these cells that could be inhibited by miR-221 after radiation therapy.[271]

Xu et al., by the use of nude mice inoculated with overexpressing miR-221 glioblastoma, demonstrated a superior growth capacity to the glioblastoma cells with miR-221 silenced, supported by inhibition of cytokine signaling-3 (SOCS3) and promotion of p-JAK2/JAK2 and p-STAT3/STAT3 activity.[272] Figure 6 recapitulates the main pathways involving miR-221 in GBM.

Finally, the role of miR-221 in mediating resistance to temozolomide or radiation therapy has been also investigated. Li et al. demonstrated for the first time a connection between miR-221 expression and radioresistance, indicating that knockdown of miR-221 significantly increased radiosensitivity of glioblastoma cells.[273] Moreover, temozolomide resistance in glioblastoma cell cultures has been related to DNM3 gene inhibition, and anti-miR-221 inhibits cell growth and restores temozolomide sensitivity.[274] Quintavalle et al. reported that miR-221 reduces MGMT expression in GBM and the transfection of GBM cells with anti-miR-221 modulates the sensitivity to temozolomide.[275] Areeb et al. reported that miR-221 increased expression in GBM cells treated with temozolomide and radiotherapy correlated to downregulation of EGFR.[276]

In conclusion, the oncogenic role of miR-221 and its use as a therapeutic target in GBM is clear and it could be a feasible biomarker, probably linked to poor prognosis; however, its involvement in the development of radio- and chemoresistance is still unclear.

DEREGULATED miR-221 EXPRESSION IN NON-ALCOHOLIC STEATOHEPATITIS

In this section, we provide a brief review of the role of miR-221 in chronic metabolic diseases, specifically non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Although

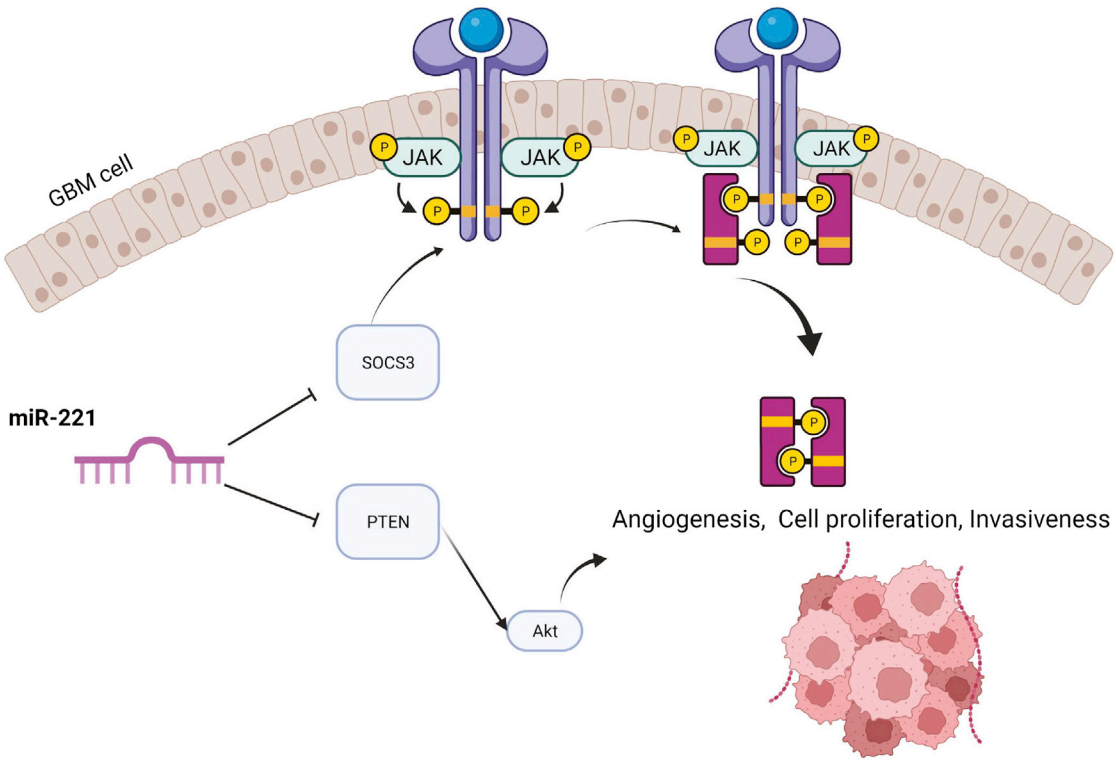

Figure 6. Main pathways involving miR-221 in GBM
miR-221 inhibits SOCS3 and PTEN and promotes STAT and AKT activity, inducing cell proliferation.

around 2,000 miRs have been identified, only a few of them play a key role in NAFLD.[277] Epigenetic alterations depend on events that occur at the molecular level, which include DNA methylation, histone acetylation, and regulation of ncRNA dependents. Among these, miR-221 plays a fundamental role in the evolution of NAFLD toward inflammation (NASH), fibrosis, and finally cirrhosis and HCC.[278,279]

miR-221 exerts tight control on several aspects of the hepatic lipid metabolism,[280] and miR-221 alterations can lead to NAFLD and its subsequent progression to more advanced stages.[281] Recent evidence has shown that miR-221 is overexpressed in hepatic tissue and in the bloodstream. In several *in vivo* studies using NASH-related animal models, miR-221 was overexpressed.[282] In the Tryndyak et al. study, it has been observed that upregulation of miR-221 in the liver is strongly associated with severity of NAFLD-related patho-morphological changes of the liver. Interestingly, plasma levels of miR-221 were also found to be significantly correlated with the severity of NASH-induced liver injury[283] (Figure 7A).

miR-221 is involved in cell-to-cell interactions, specifically between hepatocytes and hepatic stellate cells (HSCs), whose cross-talk resulted in fibrogenesis and liver damage progression. Chronic liver damage and inflammatory signals are the main cause of the HSC activated state that induces hepatic fibrogenesis. This induces the formation of fibrous tissue (fibrogenesis), which progressively leads to liver carcinogenesis. miR-221 is expressed in hepatocytes, in HSC, and is secreted into the bloodstream with a potential role as a biomarker in this disease stage[284] (Figure 7B). miR-221 performs several biological functions in the pathogenesis of NAFLD and NASH, making it an attractive therapeutic target. In an experimental model, Ogawa et al. reported that miR-221 was upregulated in 26 patients with NASH in a fibrosis-dependent manner. They observed it was correlated with type I collagen A1 (Col1A1) and alpha-smooth muscle actin (α-SMA) expression in activated primary mouse HSCs. Moreover, mice affected by hepatic fibrosis induced by thioacetamide administration or fed a methionine choline-deficient diet had increased levels of miR-221 compared with controls.[285] Currently, miR-221 is still under investigation for its potential in the prediction and induction of NAFLD progression. These conditions in which there is a dysregulation of lipid metabolism lead to oxidative stress causing tissue damage. miR-221 is involved in the control of oxidative stress and in maintaining the balance of the oxidative system. In particular, miR-221 plays a negative role in endothelial nitric oxide synthase (eNOS) resulting in downregulation of SIRT1 (Sirtuina1), an enzyme with deacetylase activity that exerts an anti-inflammatory action.[286]

High miR-221 expression in the subcutaneous abdominal adipose tissue biopsies was correlated to high BMI and fasting insulin

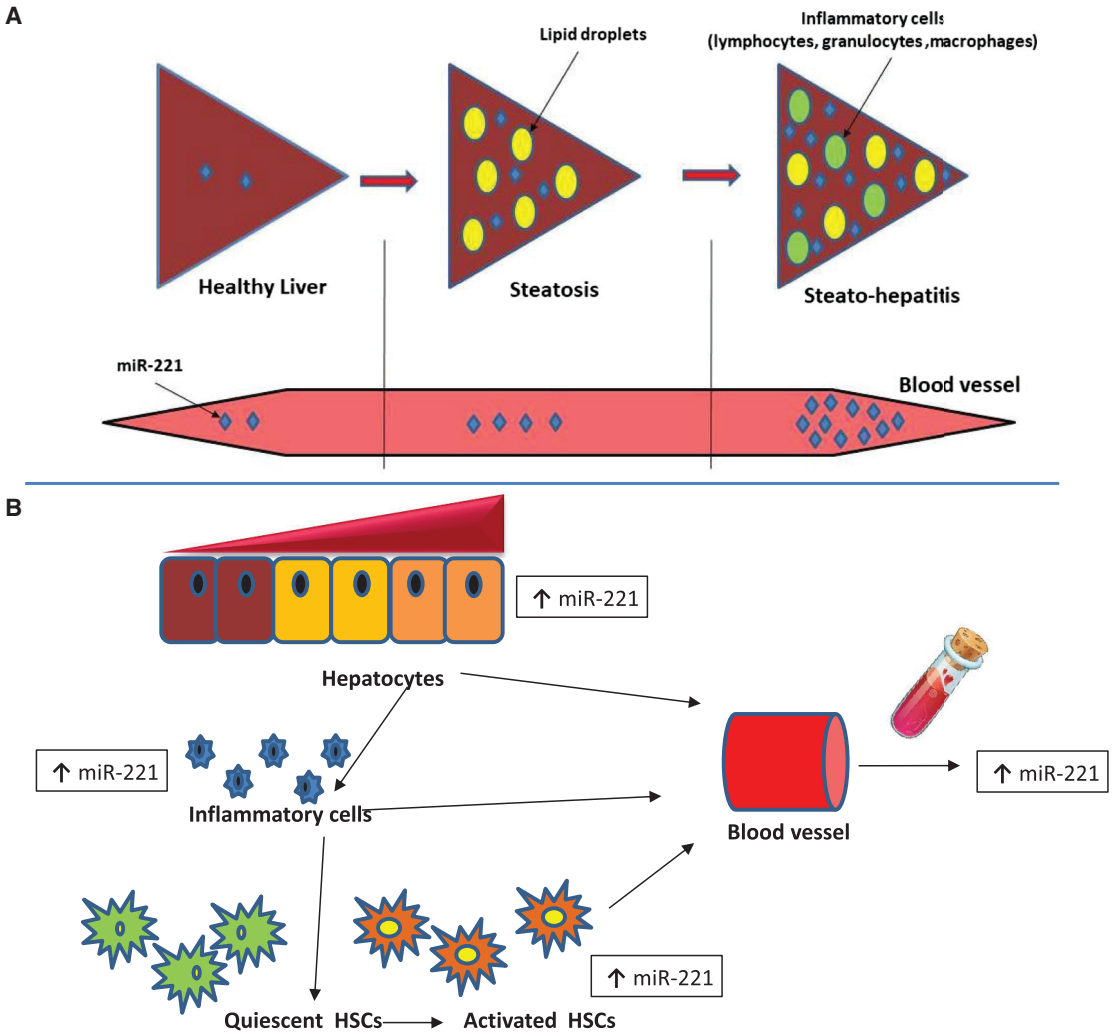

Figure 7. miR-221 deregulation in non-alcoholic steatohepatitis

(A) Implication of miR-221 in the pathogenesis of NAFLD. miR-221 is upregulated in NASH. Plasma levels of miR-221 have been found to be significantly correlated with the severity of NASH-induced liver injury. (B) miR-221 involved in the interaction between hepatocytes and hepatic stellate cells (HSCs). Chronic liver damage and inflammatory signals are the main cause of the HSC activated state that induces hepatic fibrogenesis. miR-221 is expressed in hepatocytes in HSC, and is secreted into the bloodstream with a potential role of a biomarker in this disease stage.

concentrations in non-diabetic patients.[287] Enforced overexpression of miR-221 in cultured human pre-adipocytes upregulated several proteins involved in fat metabolism and directly downregulated the adiponectin receptor 1 (ADIPOR1) and the transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1), known to promote insulin sensitivity and angiogenesis, respectively. Moreover, miR-221 was associated with accelerated hepatic proliferation and hepatic regeneration in mouse models where liver regeneration was surgically induced by hepatectomy.[288] Adeno-associated virus-mediated overexpression of miR-221 in the mouse liver led to rapid S-phase entry of hepatocytes during liver regeneration mediated by the downregulation of known targets p27 and p57 and the novel target Aryl hydrocarbon nuclear translocator (Arnt) mRNAs, which contributed to the pro-proliferative activity of miR-221.

Finally, Jiang et al. showed hepatic deletion of miR-221/222 resulted in a significant reduction of liver fibrosis, lipid deposition, and inflammatory infiltration in the methionine and choline-deficient diet (MCDD) or chronic carbon tetrachloride (CCl4)-treated mouse models.[285] On the other hand, the hepatic steatosis and fibrosis were dramatically worsened when miR-221/222 were re-expressed in knockout animals by controlling the expression of target gene Timp3, suggesting that anti-miR-221/222 could reduce steatohepatitis with prominent antifibrotic effect in NASH.[289]

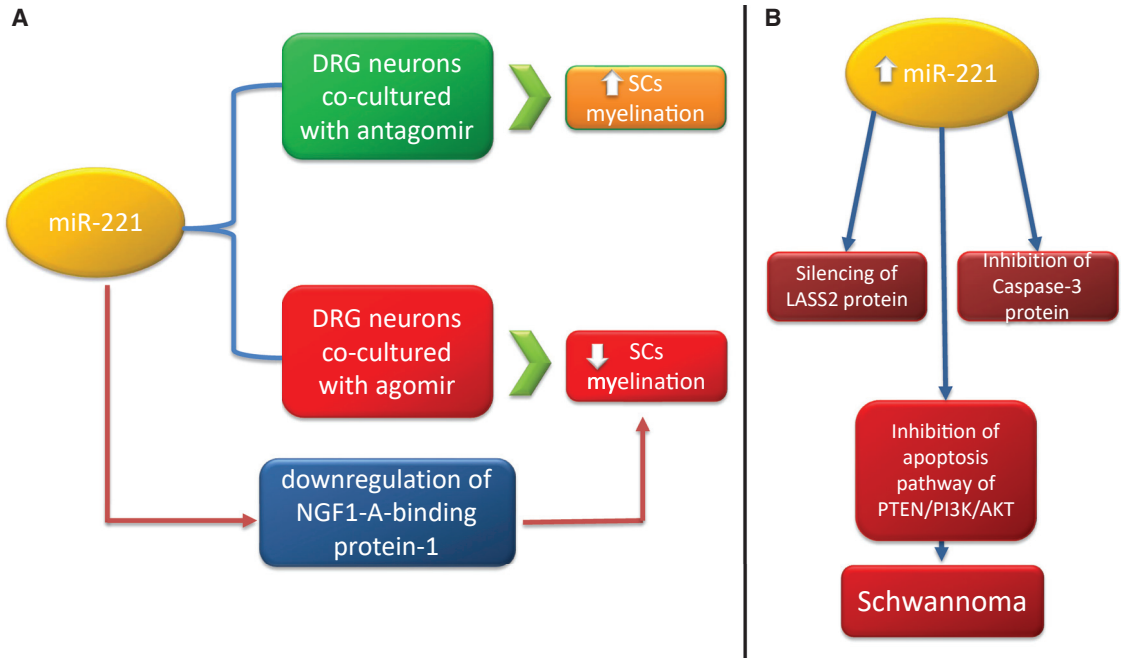

Figure 8. Deregulated expression of miR-221 in demyelinating diseases

(A) The role of miR-221 in SC myelination induced through NGF1-A-binding protein-1 downregulation. (B) The role of miR-221 in schwannoma pathogenesis acting in both silencing LASS2 protein and inhibiting apoptosis. DRG, dorsal root ganglion; SCs, Schwann cells; LASS2, longevity assurance homolog 2 protein.

DEREGULATED EXPRESSION IN NEUROPATHIES WITH SPECIAL EMPHASIS ON DEMYELINATING DISEASES

Both in central nervous system (CNS) and peripheral nervous system (PNS), miR profiles are crucial during the process of neurogenesis since they influence neuronal plasticity.[290] It has been demonstrated that demyelinating diseases affecting both the CNS and PNS are caused by a deregulation of miRs. To this aim, animal models have clarified that miRs intervene in cell proliferation. Nakata et al. performed quantitative expression profile of 277 miRs in the spinal cord of a model of canine degenerative myelopathy (DM). Among them, the overexpression of three miRs *in vitro* significantly increased the proportion of cells with mutant superoxide-dismutase1 (SOD1) aggregation, suggesting a role of these miRs in the inhibition of ubiquitination of misfolded SOD1, leading to further progression of degenerative processes in the DM pathology. The pivotal role of miR-221 in the demyelination of the CNS and PNS was investigated by Zhao et al., identifying its role in promoting Schwann cell (SC) proliferation and migration. miR-221-3p represses SC myelination when co-cultured with dorsal root ganglion cells *in vitro*. Its concentration decreases after 7 to 14 days from the nerve damage, thus testifying to the important contribution to myelination and protection against the onset of gliomas. Agomir and antagomir of miR-221-3p were transfected in SCs that were suddenly co-cultured with dorsal root ganglion (DRG) neurons, obtained from rats. Immunochemistry staining (IHC), assessed 2 weeks after co-culture to detect the expression of the myelinic basic protein, highlighted diminished myelination in cultures subjected to miR-221 agomir treatment. Interestingly, SCs co-cultured with antagomir of miR-221 had increased myelination. In addition, miR-221 downregulated nerve growth factor 1-A (NGF1-A)-binding protein-1 (Nab1), which is a crucial factor in SC myelination[291] (Figure 8A). Moreover, the role of miR-221 in the pathogenesis of schwannoma has been investigated. Schwannoma is a benign malignancy of the PNS, which often has vestibular localization, and determines subacute symptoms, such as hearing loss, dizziness, facial hypoesthesia, and tinnitus. It explains about 8% of intracranial malignancies and about 80% of cerebellopontine angle neoplasms.[292] Although its origin is benign, vestibular schwannoma could also determine brain compression and, finally, an evolutive fatal intracranial hypertension. Different studies corroborate the hypothesis of the central role of miR-221 upregulation in determining the proliferation of SCs, which could act by targeting the longevity assurance homolog 2 (LASS2) protein,[293] which is the principal miR-221 target in SCs.[294] Moreover, another mechanism of SC proliferation has been reported, due to the inhibition of Caspase-3 protein and of the apoptosis pathway PTEN/PI3K/AKT, which is also miR-221 mediated[292] (Figure 8B). Future studies are needed to evaluate whether miR-221 may work as a predictive biomarker in these neuropathies. It should be tested in intervention studies (i.e., clinical trials), which should have the power to assess if the miR-221 expression is modified by a specific treatment and if the interaction miR-221 treatment/expression is associated with a better prognosis in these patients.

TRANSLATIONAL DATA AND PK

As reported above, the deep dysregulation of miR-221 plays an important role in several tumors and inflammatory diseases, and its expression level is correlated to neoplastic stage and prognosis. Therefore, miR-221 could be considered as a diagnostic and prognostic biomarker for these diseases and a valuable target for miR-based therapeutic strategies. An important point to take into account is the tumor heterogeneity concerning miR-221 expression within the same tumor sample. This is essential for comparing the miR expression of different tumor subtypes or when miR expression is used to stratify treatment or infer any prognostic information. Veryaskina et al. analyzed the expression levels of 16 miRs, including miR-221, in four different areas of 33 BC tumors (tumor center, opposite tumor peripheral sites, and tumor border) and normal tissue. Comparative analysis of miR-221 expression levels revealed that miR-221 is significantly higher in samples from the four intra-tumoral areas than in normal tissue; however, there are statistically significant differences in its expression levels between tumor samples.[295] Translational data of other miR-targeted drugs, including phase I and phase II trials, have been reported in this decade, including the first cancer-targeted miR drug, MRX34 (MiR Therapeutics), a liposome-based miR-34a mimic, the LNA miR-122 inhibitor (miravirsen, Santharis Pharma), or RG-101 (Regulus Therapeutics). In particular, MRX34 completed phase I clinical trials in a subset of patients with refractory advanced solid tumors (NCT01829971), providing proof of concept for miR-based cancer therapy.[4,296] MRX34, with dexamethasone premedication, showed a manageable toxicity profile in most patients but low clinical activity. Because of serious immune-mediated adverse events (AEs), with four patient deaths, the study was suspended early. Instead, miravirsen demonstrated in a phase-2a trial[297] the potential drug-like property of LNA-class oligonucleotides, together with low systemic toxicity in HCV-infected subjects (NCT01200420), including headache, fatigue, and nausea. This trial concluded that the use of miravirsen in patients chronically infected with HCV genotype 1 had prolonged dose-dependent reductions in HCV RNA levels without viral resistance. Regulus Therapeutics developed an N-acetylgalactosamine conjugated oligonucleotide anti-miR-122 compound, named RG-101, for the treatment of chronic HCV infection that has been tested in phase Ib clinical trial[6] with encouraging results characterized by viral load reduction in all treated patients within 4 weeks, and prolonged virological response in three patients for 76 weeks of observation. Similarly, the LNA miR-155 antagomiR (MRG-106 or Cobomarsen) developed by MiRagen, is involved in phase I (NCT02580552) and phase II (NCT03713320) clinical trials to treat cutaneous T cell lymphoma, ATLL, and CLL.[5] Regarding the oncogenic miR-221, despite several investigations having been performed by the use of different miR-221 inhibitors, only one molecule is going to clinical translation and is now under investigation in the FIH trial registered on ClinicalTrials.gov (NCT04811898), specifically designed to study the safety and tolerability of the LNA-i-miR-221 in advanced malignancies. LNA-i-miR-221 showed a powerful antitumor activity and upregulation of canonical targets *in vitro* and *in vivo* in clinically relevant murine models of human MM, identifying a molecular scenario that might be predictive of efficacy.[62,68,94] The antitumoral activity was evaluated in MM xenografts in severe combined immunodeficiency (SCID)/non-obese diabetic (NOD) mice after 2 weeks of intravenous (IV) treatment, establishing 25 mg/kg as the therapeutic dose. *In situ* hybridization (ISH) assay demonstrated LNA-i-miR-221 detectability in animal and tumor tissues where no acute toxicity or inflammation was observed.[63,71] To evaluate the safety, bioavailability, and human dose calculation, LNA-i-miR-221 has been evaluated in three animal species (mouse, rat, and monkey) in non-Good Laboratory Practice (GLP) and GLP conditions, as required by regulatory authorities for clinical translation of this class of compound.[298] In addition, for the quantification of LNA-i-miR-221 in biological fluids new methods based on mass spectrometry technologies have been developed and validated. PK studies in SCID mice, cynomolgus monkeys, and Sprague-Dawley rats showed rapid absorption, with maximum distribution and extensive tissues uptake, short half-life, optimal tissue bioavailability and systemic clearance, with minimum urinary excretion.[298] This performance has been ascribed to a high fraction of LNA-i-miR-221 bound to plasma proteins in all animal species evaluated by authors and ranging from 98.2% to 99.05%.[70] These safety kinetic profiles are in agreement with the PK proprieties of other third-generation ASOs carrying a PS backbone, whose chemistry drives plasmatic stability, protein binding, tissue bioavailability, and CNS distribution.[299,300] It is well known that, for all oligonucleotides, as well as for LNA-i-miR-221, tissue distribution occurs via receptor-mediated endocytosis, and liver and kidney are the organs in which were found the highest concentrations compared with lymph nodes, bone marrow, and adipocytes.[301] All findings from LNA-i-miR-221 preclinical studies showed safety concerns in vital organ functions, biochemistry, clinical signs, and parameters, demonstrating strong evidence for safe novel therapeutic opportunities of LNA-i-miR-221 and providing the chance for immediate clinical translation in different human cancers.

CONCLUSIONS AND PERSPECTIVES

The role of miR-221 as oncomiR, targeting important mRNA involved in main survival pathways (Table 3), has been extensively explored and a large amount of data are presently available. All the available evidence supports the potential role of miR-221 as a novel diagnostic, prognostic biomarker, and therapeutic target in many malignancies. The detection of miRs in body fluids can be considered a novel device for liquid biopsy that requires the availability of accurate quantification methods, so becoming a new biomarker to improve the evaluation of the individual response to drugs in the personalized treatment process. However, the development and commercialization of new diagnostic and therapeutic devices based on circulating miRs requires a long process still in its infancy.[302] The advantages of liquid biopsy for real-time evaluation of the disease outcome or drug response are already known, related to minimal invasiveness and accessibility to the sample, but large replication studies are needed

Table 3. miR-221 targets in cancers and other human diseases

Cancer	Target
Glioblastoma	Cx43, SOCS3
Hepatocarcinoma	p27, p57, HDAC6, BMF, NF-κB, SOCS3,
Pancreatic cancer	PTEN, p27, p57, PUMA, TIMP2, SOCS3
Esophago-gastric cancer	PTEN, p27, CDX2, DKK2, HAI-1
Prostate Cancer	p27, DIRAS3, SOCS3, IRF2
Bladder Cancer	PTEN, PUMA, STMN1
Breast Cancer	p27, TRPS1, A20, Bim-Bax/Bak, SOCS1, ADIPOR1, PTEN, ZEB1, TIMP3, β4 integrin, ADAM17, STAT5
Chronic lymphocytic leukemia	p27
Chronic myeloid leukemia	p27, STAT5
Multiple myeloma	p27, p57, PTEN, PUMA, ABCC1/MRP1, SLC7A5/LAT1
Essential thrombocythemia	SOCS1, SOCS3
Lung cancer	p27, PTEN, TIMP2, TIMP3
Renal cell carcinoma	TIMP2
Ovarian cancer	APAF-1, BMF, PTEN, ARF4
Cervical cancer	SOCS1, THBS2, MBD2, ARID1A
Melanoma	c-FOS
Sarcomas	p27, PTEN, CCND2, CDK6, ERBB3, ARHI
Neuropathies	Nab1, LASS2
NASH	ADIPOR1, ETS1, p27, p57, Arnt, TIMP3

for validation of miRs as a biomarker platform. Moreover, as for other miRs, miR-221 could have a role in several physiological conditions, including modulation in interindividual variability in drug response correlated to differences in the levels of circulating miRs or in addition to the role of genetic variants in the miR genes.[303] In fact, miR pharmacogenomics studies highlighted the role of post-transcriptional factors and expression levels in influencing the regulation of genes involved in the PK and pharmacodynamics (PD) of many drugs.[304] In addition, miR-221 has been explored as a fine-tuner of chronic liver injury being upregulated during the fibrosis and cirrhosis process caused by multiple etiologies, advising its use as a circulating biomarker. In this context, the inhibition of miR-221 has shown promising results in terms of the suppression of fibrogenic gene signatures *in vitro* and *in vivo*, suggesting its targeting for HCC prevention. In addition, a pivotal role of miR-221 has been described in the demyelination of CNS and PNS, suggesting a new role in biological processes involving inflammation that deserves to be investigated. Efforts are presently being made for the clinical development of the novel miR-221 inhibitor, LNA-i-miR-221, providing a new investigational clinical option. The preliminary phase I study results appear to be of great interest to move toward further phases of clinical development to better define and characterize the effectiveness and safety profile in specific neoplasms. The major novelty will be to consider LNA-i-miR-221 in chemoprevention or anti-inflammatory strategies, opening a novel research avenue on the translational relevance as therapeutic targets against early premalignant processes and non-malignant diseases.

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AUTHOR CONTRIBUTIONS

M.T.D.M. wrote the manuscript, and coordinated and revised all sections. P.F.T. and P.S.T. conceptualize and revised the manuscript. Each of the other authors contributed by writing a single section.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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