FROM "JUNK DNA" TO CLINICALLY RELEVANT TOOLS FOR CANCER DIAGNOSIS, STAGING, AND TAILORED THERAPIES: THE INCREDIBLE CASE OF NON-CODING RNAS

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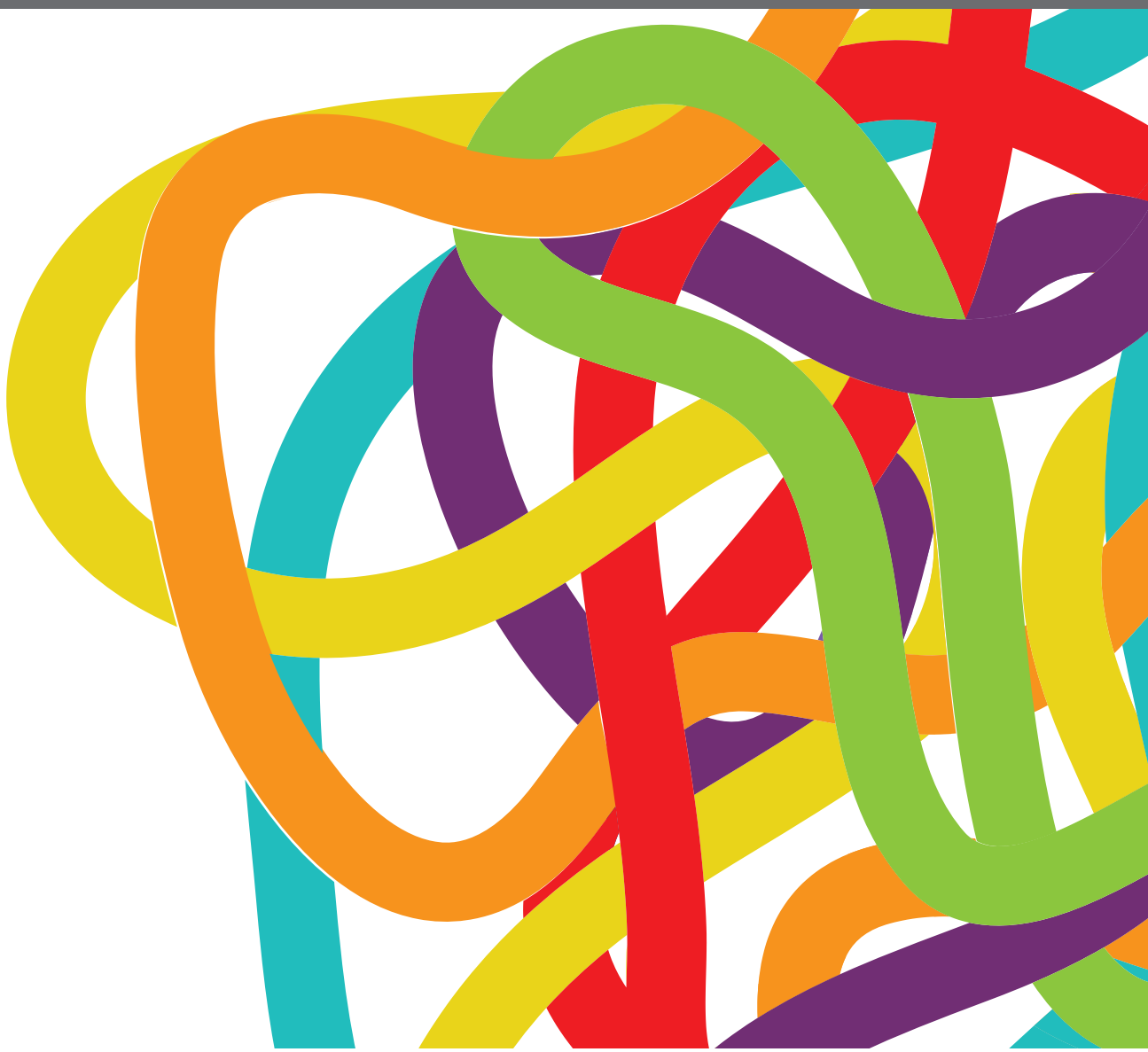

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FROM "JUNK DNA" TO CLINICALLY RELEVANT TOOLS FOR CANCER DIAGNOSIS, STAGING, AND TAILORED THERAPIES: THE INCREDIBLE CASE OF NON-CODING RNAS

Topic Editors:
Marilena V. Iorio, Fondazione IRCCS Istituto Nazionale dei Tumori and IFOM (Fondazione FIRC Oncologia Molecolare), Italy
Dario Palmieri, The Ohio State University, United States

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EDITORIAL
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Editorial: From "Junk DNA" to Clinically Relevant Tools for Cancer Diagnosis, Staging, and Tailored Therapies: The Incredible Case of Non-Coding RNAs

Marilena V. Iorio[1,2*†] *and Dario Palmieri*[3*†]

[1] Molecular Targeting Unit, Experimental Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, [2] IFOM (Fondazione FIRC Oncologia Molecolare), Milan, Italy, [3] Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH, United States

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Edited and reviewed by:
Giuseppe Giaccone,
Georgetown University, United States

***Correspondence:**
Marilena V. Iorio
marilena.iorio@istitutotumori.mi.it;
marilena.iorio@ifom.eu
Dario Palmieri
Dario.palmieri@osumc.edu

†These authors have contributed equally to this work

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Editorial on the Research Topic

From "Junk DNA" to Clinically Relevant Tools for Cancer Diagnosis, Staging, and Tailored Therapies: The Incredible Case of Non-Coding RNAs

In recent years, a growing body of evidence indicates that cell genetic behavior and fate is not merely driven by the limited number (∼21,000) of protein-coding genes and their regulatory regions. Conversely, non-coding genomic regions, originally labeled as "junk DNA," have been demonstrated to be transcriptionally active (although not translated into proteins) and to play causal roles in cell physiology and pathology.

It is now well-known that these non-coding RNAs (ncRNAs) are not transcriptional noise, but they are critically involved in a number of processes such as differentiation, development, inflammation, immune response, and cancer. Their biological relevance has been confirmed by comparative genome studies demonstrating that species degree of complexity correlates with the number of non-coding genes more than protein-coding genes.

However, the biological mechanisms through which ncRNAs exert their functions have only been partially elucidated. This is especially the case of the about 9,000 small (under 200 bp) ncRNAs, and particularly microRNAs, which inhibit gene expression at post-transcriptional level by preventing translation of complementary mRNA by binding to their 3'UnTranslated Regions (UTRs). On the other hand, the long (200 bp−100 kb) ncRNAs family, encompassing more than 40,000 members, still requires extensive effort to obtain a comprehensive understanding of their molecular details and mechanisms of action.

As far as microRNAs are concerned, several groups have demonstrated their causal role in cancer pathogenesis, and extensive studies have attempted to modulate the small non-coding transcriptome (by abrogating or recovering the expression of oncogenic and tumor-suppressive miRNAs, respectively) as a therapeutic approach for cancer. Unfortunately, the promise of microRNA-based anti-cancer drugs is still far from the clinical use, especially due to the lack of appropriate cancer-specific delivery systems. Conversely, the accurate quantification of microRNAs from cancer patients, both in neoplastic lesions and in liquid biopsies, might represent an invaluable tool for tumor classification, staging, and to provide patients with the most appropriate clinical care.

In this Research Topic, Laprovitera et al. provide a method for the accurate and absolute assessment of multiple microRNA levels in paraffin-embedded tissues using EvaGreen-based droplet digital PCR technology, potentially applicable also to miRNAs circulating in biological fluids and in multiple subcellular compartments such as exosomes and microvesicles. This approach would allow the discovery and the validation of miRNAs as biomarkers in a number of different tumor types (Laprovitera et al.). In fact, to date, one of the drawbacks of the comparison of multiple studies for the validation of microRNAs as biomarkers is represented by the absence of a standardized and absolute quantification method. This concern is specifically raised by Lo Russo et al. while analyzing the potential clinical impact of microRNA quantification in tumors, blood samples and pleural effusions of Malignant Pleural Mesothelioma (MPM) patients. In an extensive literature analysis, the authors describe the great potential of miRNAs from both a diagnostic and therapeutic point of view. However, because of the heterogeneity of the analyzed studies, the authors urge a coordinated collaboration among research and clinical groups to implement miRNA-based diagnostic/prognostic systems in the clinical settings (Lo Russo et al.).

Despite the development of several new therapeutic protocols and targeted drugs, including an ever-growing number of small molecules with enhanced efficacy in the treatment of cancer patients of different type, resistance still represents an extremely frequent phenomenon. When resistance to treatments arises, cancer patients switch to different (and potentially less effective or more toxic) approaches until they run out of options. For these reasons, it is crucial that clinicians can identify those patients who could benefit of specific drugs, sparing them unnecessary toxicities and providing them with the best possible clinical treatment. Non-coding RNAs represent valid biomarkers predictive of response to several different conventional and new-generation anti-neoplastic treatments. Plantamura et al. specifically reviewed the relevance of microRNAs as modulators of the cellular DNA damage response (DDR) by targeting DNA-repair genes such as ATM, BRCA1/2, and DNA-PK. Since many chemo- and radio-therapeutic agents act by inducing damages to the genomic DNA, a dysfunctional DDR (dependent on microRNA-dysregulation) could lead to potential sensitivity/resistance to these treatments (Plantamura et al.).

In the review from Hahne and Valeri an extensive analysis of non-coding RNAs involved in the resistance to anti-cancer drugs in gastrointestinal tumors was performed. The review reports evidence of the role of both microRNAs and long non-coding RNAs as central hubs for the development of drug resistance mechanisms, including those related to DDR. However, as indicated by the authors, the current potential clinical relevance for microRNAs and other non-coding RNAs is represented by their role as tissues or biofluids biomarkers that could potentially show, in a cost-effective way, their utility to monitor patient response or forecast treatment resistance (Hahne and Valeri).

The role of non-coding RNAs in cancer drug resistance and the extensive network of interactions between ncRNAs and anti-neoplastic therapies are also the subject of the review from Corrá et al. This study contains an extremely interesting network analysis, focused on miRNAs and long non-coding RNAs playing the most central role in chemoresistance. Notably, the authors also focused on ncRNAs associated to a limited number of drugs, and generated a clustering analysis that could potentially help in identifying the cross-talk between non-coding RNAs and multiple treatment options (Corrá et al.).

Dragomir and Calin provided an extensive review of a novel and extremely interesting class of non-coding RNAs, called circular RNAs (circRNAs). A limited number of biological functions for this class of RNAs have been clearly described so far, including their ability to "sponge" microRNAs and preventing their ability to modulate gene expression. Noteworthy, the authors describe how this ncRNA family could be either common driving mechanism of oncogenesis, or common byproduct/end-products. For these reasons, they suggest to treat circRNAs, especially those found in serum/plasma, with caution, especially based on the experience obtained in the last years with microRNAs, until a better understanding of their biogenesis, secretion, and molecular roles is gained. This approach will prevent supplementary errors and data misinterpretation to be considered before their use as cancer biomarkers (Dragomir and Calin). In line with the growing interest toward circRNAs, an original research article from Shao et al. showed that specific circular RNAs where differentially expressed in pancreatic ductal adenocarcinoma (PDAC) cell lines and in the plasma of PDAC patients, suggesting a potential role as predictive biomarkers with a causal role in the sensitivity to gemcitabine.

In summary, this Research Topic covered multiple basic, technical, and clinical issues regarding the multiple classes of ncRNAs and highlights their impact both in our understanding of cancer biology and in their relevance as biomarkers predictive of clinical outcome or response to therapies.

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REVIEW
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MicroRNAs for the Diagnosis and Management of Malignant Pleural Mesothelioma: A Literature Review

Giuseppe Lo Russo[1†], Anna Tessari[2†], Marina Capece[2], Giulia Galli[1], Filippo de Braud[1,3], Marina Chiara Garassino[1] and Dario Palmieri[2*]*

[1] Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, [2] Department of Cancer Biology and Genetics, the Ohio State University, Columbus, OH, United States, [3] Department of Oncology and Hemato-Oncology, Università degli Studi di Milano, Milan, Italy

Malignant pleural mesothelioma (MPM) is a rare and aggressive tumor with a variable incidence among different countries. Occupational asbestos exposure is the most important etiological factor and a very long latency period is widely reported. In the early phase of the disease, clinical signs are absent or not specific. For this reason, the diagnosis is frequently achieved only in the advanced stages. The histopathological diagnosis *per se* is also very complex, and no known factor can predict the prognosis with certainty. Nonetheless, current survival rates remain very low, despite the use of standard treatments, which include surgery, chemotherapy and radiotherapy. The identification of new prognostic and/or diagnostic biomarkers, and the discovery of therapeutic targets is a priority and could lead to a real significant impact on the management of malignant pleural mesothelioma. In this scenario, the role of microRNAs is becoming increasingly relevant, with the promise of a quick translation in the current clinical practice. Despite the relative novelty of this field, the number of works and candidate microRNAs that are present in literature is striking. Unfortunately, to date the microRNAs with the most clinical relevance for MPM are still matter of debate, probably due to the variety of approaches, techniques, and collected samples. Although specific microRNAs (e.g., let-7, miR-15 and miR-16, miR-21, miR-34a, and the miR-200 family) have been reported several times from different groups, the heterogeneity of published data reinforces the need of more comprehensive and unified studies on this topic. In this review we collect and discuss the studies focused on the involvement of microRNAs in different aspects of MPM, from their biological role in tumorigenesis and progression, to their possible application as diagnostic, prognostic and predictive biomarkers. Lastly, we examine their potential value as for the design of therapeutic approaches that could benefit MPM patients.

Keywords: miRNAs, malignant pleural mesothelioma biomarkers, diagnosis, prognosis, therapy

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Yisong Wang,
National Institutes of Health (NIH), United States

Reviewed by:
Massimiliano Agostini,
Università degli Studi di Roma Tor Vergata, Italy
Victor C. Kok,
Asia University, Taiwan

***Correspondence:**
Giuseppe Lo Russo
giuseppe.lorusso@istitutotumori.mi.it
Dario Palmieri
dario.palmieri@osumc.edu

[†]These authors have contributed equally to this work

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare form of cancer originating from mesothelial cells of the pleura and generally characterized by a poor prognosis. The highest incidence is reported in the sixth and seventh decade of life. This tumor is more common in males than in females. The overall survival (OS) is about 10 months for advanced disease, with a 5% 5-years survival rate. Globally, MPM is responsible for 4% of cancer deaths in both men and women (1, 2).

A cause-effect relationship to asbestos exposure is widely reported, with symptoms that become often evident after a long latency period. Because of this, a peak in the incidence of MPM is awaited around 2030, due to the high exposure to asbestos in past years in several countries (3). Other recognized risk factors are radiation exposure, genetic mutations and the exposition to Simian Virus 40 (4).

The most common subtype of MPM is the epithelioid subtype (55–65%), followed by biphasic (15–20%), and sarcomatoid (10–15%) forms (5). The median OS is strongly influenced by histology, with lower survival rates for sarcomatoid patients in comparison with epithelioid ones (6).

The diagnosis of MPM displays several layers of complexity. Firstly, symptoms and imaging analyses are not disease-specific. Moreover, the cytological examination of pleural fluid is frequently possible only in advanced stages, and leads to specific diagnosis in a minority of cases (7–9). Pleural biopsy is the gold-standard diagnostic tool, but it can be affected by adverse events like pleural bleeding, infections, empyema, and pneumothorax (10). The histopathological diagnosis *per se* is also very difficult because of the lack of immunohistochemical markers with high specificity, and it requires the presence of a particular combination of positive/negative markers evaluated by an expert pathologist, especially when the goal is the differential diagnosis of MPM subtypes (11, 12).

In recent decades, the identification of specific molecular targets and genetic alterations has radically changed the therapeutic paradigms for different types of cancer, but has not significantly affected the natural history of MPM. In MPM both the role of surgery and radiotherapy is controversial. Since 2003, the only treatment that has clearly shown an improvement of patients survival is the standard chemotherapy with platinum and pemetrexed (13, 14). Both the use of different targeted biological agents and immunotherapy with anti-CTLA4 did not show a relevant efficacy even if many other checkpoint inhibitors (anti-PD1/PD-L1) and new generation compounds are still being investigated (13, 14).

In this difficult context, new prognostic or predictive biomarkers, new diagnostic approaches, and therapeutic targets are needed and could have a significant impact in the clinical management of MPM. Among these, the role of microRNAs (miRNAs) is becoming increasingly relevant. MiRNAs are small non-coding RNAs of about 22 nucleotides, playing an important role in post-transcriptional regulation of the expression of all human genes. For this reason, miRNAs affect any cellular process, including cell proliferation, apoptosis, and migration (15, 16). Altered expression of specific miRNAs has been associated with multiple human diseases, including cancer. Notably, differential expression of miRNAs in healthy vs. cancer tissues of different origins has been described, confirming the causal role of miRNAs in multiple aspects of cancer pathogenesis, ranging from tumor establishment to progression, metastasis and resistance to therapies (15, 16). Therefore, specific miRNA expression signatures may correlate with different patient prognosis or response to therapeutic approaches. Nonetheless, miRNAs can be quantified in multiple biological fluids, such as blood, cerebrospinal fluid, urine, and saliva. Also in these cases, specific signatures for cancer vs. normal patients have been identified. Altogether, these features make miRNAs ideal candidates as prognostic, predictive and diagnostic biomarkers (16–18).

Finally, the modulation of miRNA expression, by inhibiting those with oncogenic properties or rescuing the tumor-suppressive ones, represents a new exciting topic in the development of novel anti-cancer therapies (15–18).

The aim of this review is to describe the role of miRNAs in MPM with a specific focus on the research state of the art and on the potential translation in the current clinical practice.

BIOLOGICAL ROLES OF MIRNAS IN MPM

MiRNA Signatures

The first observations regarding the biological role of miRNAs in MPM date back to 2009. Using miRNA microarray technique (Agilent human miRNAs V2) and normal human pericardium tissues as controls, Guled et al. demonstrated a different miRNA expression profile between MPM and non-cancer tissues. By analyzing 17 MPM samples and testing 723 miRNAs, they showed a lower expression of let-7e, miR-7-1, miR-9, miR-34a, miR-144, miR-203, miR-340, miR-423, miR-582, and a higher expression of let-7b, miR-30b, miR-32, miR-195, miR-345, miR-483-3p, miR-584, miR-595, miR-615-3p, miR-1228 in neoplastic tissues compared with normal ones. The majority of these miRNAs were either located in chromosomal areas generally known as aberrant in MPM, or were targeting well-described genes involved in MPM tumorigenesis. Over-expressed miR-30b, miR-32, miR-483-3p, miR-584, and miR-885-3p target tumor-suppressor genes such as *CDKN2A*, *RB1,* and *NF2*. Conversely, down-regulated miR-9, miR-7-1, and miR-203 target *EGF*, *HGF*, *JUN,* and *PDGFA* oncogenes (19).

Balatti et al. evaluated miRNA expression profile in 5 human normal pleural mesothelial short-term cell cultures (HMCs) and 5 MPM tissue samples, with microarray approach. The comparative analysis of miRNA expression showed that miR-17-92 cluster and its paralogs, called miR-17-5p, 18a, 19b, 20a, 20b, 25, 92, 106a, 106b, were strongly up-regulated. Furthermore miR-7, miR-182, miR-214, and miR-497 were showed to be dysregulated in MPM. Intriguingly, these miRNAs were predicted (and later partially confirmed) to target genes involved in the regulation of cell cycle progression (20).

Ramirez-Salazar et al analyzed, using PCR Array (384 miRNAs), specimens obtained from 4 patients with pleural chronic inflammation, 5 patients with mesothelial hyperplasia, 5 patients with MPM and 4 normal controls with the aim to identify tumorigenesis-related miRNAs and their biological networks. MiR-101-3p and miR-494 were down-regulated in pleural chronic inflammation and mesothelial hyperplasia, respectively. In MPM tissues a reduction of miR-181a-5p, miR-101-3p, miR-145-5p, and miR-212-3p expression was observed. The down-regulation of these miRNAs resulted in increased levels of the mesenchymal transition-associated molecule FZDA, the transcription factor ETS1 and the signaling-activation molecule MAPK1, which have strong oncogenic functions. This suggested a possible association between pleural inflammation, hyperplasia and tumorigenesis (21).

Ak et al. also used PCR Array to compare miRNA signatures in 18 MPM and 6 non-cancer pleural tissue samples obtained from patients with benign asbestos-related pleural effusion. The study found 11 over-expressed miRNAs in MPM (let-7a, let-7d, miR-20a, miR-92a, miR-125a-5p, miR-152, miR-155, miR-193b, miR-320, miR-484, and miR-744). The authors further evaluated miRNA-mRNA interactions and found eight significant pathways targeted by miRNAs, including two related to NOTCH signaling. Compared to benign asbestos-related pleural effusion, *MET* was the most overexpressed gene in MPM (22).

In the study by Walter et al., NanoString technique was used to evaluate expression of 800 miRNAs from 24 formalin-fixed paraffin-embedded MPM samples. The principal aim was to define the impact of miRNA expression on the MDM2-P14/ARF (CDKN2A)-TP53 pathway, taking into account the differential immunohistochemical MDM2 expression (score 0 vs. score $\geq$1) in MPM tissues. Eleven miRNAs suppressing *CDKN2A* (miR-29a, miR-29b, miR-29c, miR-125a, miR-125b, let-7a, let-7c, let-7d, let-7e, let-7g, miR-340), 17 miRNAs inhibiting *TP53* (miR-29a, miR-29b, miR-29c, miR-125a, miR-125b let-7a, let-7c, let-7d, let-7e, let-7g miR-34a, miR-145, miR-185, miR-19b, miR-218, miR-22, miR-27b) and 18 miRNAs targeting *MDM2* (miR-29a, miR-29b, miR-29c, miR-125a, miR-125b miR-34a, miR-145, miR-185, miR-140, miR-223, miR-23b, miR-142, miR-191, miR-331, miR-605, miR-548d, miR-374b, miR-383) were down-regulated in MDM2-expressing MPM. Since MDM2 and CDKN2A expression regulates *TP53* and may contribute to its inactivation, the authors concluded that TP53 may be suppressed by miRNAs depending on expression pattern, whereas the impact of miRNAs on CDKN2A and on MDM2 itself is mild (23).

Very recently, the same group published another paper based on the same case series. In this work the authors focused their attention on a small subset of miRNAs regulating key enzymes involved in DNA damage repair. Specifically, the pathways reported as mostly de-regulated were TP53 (let-7b-5p and miR-143-3p), PARP1 (miR-21-5p, miR-223-3p, miR-302d-3p), and RAD52 (miR-106a-5p, miR-106b-5p, miR-20a-5p) (24).

Lastly, Kim et al. investigated the global expression profile of miRNAs in distinct subpopulations of a MPM cell line (MS1). Their results showed that a subset of miRNAs is able to define the most aggressive cell subpopulations. ErbB-2 receptor tyrosine kinase signaling was the most involved pathway and *DDIT4* and *ROCK2* the most involved target genes. The specific miRNA signature defining aggressive subpopulations included over-expression of miR-3198-1, miR-3198-2, miR-4497, miR-138-1, miR-4304, miR-1281, miR-489, miR-4745, miR-301a, miR-3935, and down-regulation of miR-148b, miR-484, miR-584, miR-425, miR-197, miR-629, miR-183, miR-4485, miR-4443, and miR-1246 (25).

Single miRNAs or miRNA Families

In regards to the role of single miRNAs or miRNA families in the pathogenesis of MPM, a wide literature has been published to date (**Table 1**) (26–42, 44–54).

Pass et al. analyzed 12 MPM cell lines (9 neoplastic and 3 normal) and 142 MPM tumor samples. In MPM cell lines, the authors demonstrated that the over-expression of miR-29c decrease invasion, migration, proliferation, and colony formation. Furthermore, miR-29c over-expression mediated epigenetic mechanisms through the down-regulation of *DNMT1* and *DNMT3A* and the up-regulation of demethylating genes. The increased level of miR-29c in tumor samples was related with a better outcome after surgery. The authors hypothesized that miR-29c could play a tumor suppressive role in MPM and thus it may be a potential new therapeutic target (36).

Ivanov et al. reported that miR-31 plays a similar role in MPM. The loss of expression of miR-31 due to the deletion of miR-31 gene in chromosome 9p21.3 is a common aberration in the aggressive forms of MPM. The investigators demonstrated that miR-31 as well as miR-29c is able to block migration, proliferation, and invasion in MPM cell lines. Moreover, low miR-31 levels were associated with high levels of protein phosphatase 6 (PPP6C) which were related with radio and chemo-resistance. According to this study, the re-introduction of miR-31 in MPM patients could be another potential therapeutic approach (37).

One of the most studied miRNA family in MPM is represented by miR-34 (38–42).

Ghawanmeh et al. examined the effects of docetaxel and radiotherapy on MPM cell lines. In the M28K cells, radiotherapy induced miR-34a expression, cell cycle arrest and cell death (38). Kubo et al demonstrated that the epigenetic silencing of miR34b/c due to methylation, is crucial in the pathogenesis of MPM. In MPM cell lines the authors showed that physiologic miR-34b/c levels had anti-proliferative effects and that the forced over-expression of miR-34b/c had a pro-apoptotic effect (39).

The studies by Tanaka et al and by Maki et al confirmed these observations (40, 41). The first article reported that the down-regulation of miR-34a induced cell proliferation and invasion in MPM cells because of the consequent up-regulation of *c-MET* and *BCL-2* (40). The second paper demonstrated that high levels of miR-34b/c increased radiation-induced apoptosis and suggested that miR-34b/c could be used as radiosensitizing agent in MPM (41). Finally, Menges et al., proved *in vivo* that the inactivation of *CDKN2a* and *NF2* causes the development of MPM in mouse models. These tumors were characterized by TP53/miR-34a-dependent activation of c-MET, which correlated with high aggressiveness and presence of cancer stem cells (42). Some years after these studies, Yamamoto et al reported that miR-379/411 cluster directly target *IL-18* gene, whose over-expression was associated with drug resistance in MPM cell lines. *In vitro*, the introduction of miR-379 and miR-411 (with the consequent *IL-18* silencing) reduced invasiveness and increased chemosesnsitivity of MPM cells (53).

A large body of evidence has demonstrated the role of let-7 family in MPM pathogenesis. MiRNAs of this group (including more than 10 different members) have all a similar structure and have a huge number of functions and targets. Firstly, Khodayari et al. showed that EphrinA1 signaling inhibits MPM tumor growth by repressing RAS proto-oncogene family through let-7a. EphrinA1 is a specific ligand of the EphrinA2 receptor, which is over-expressed in most cancer cells, including MPM. In this work, the authors demonstrated that EphrinA1

TABLE 1 | Biological roles of miRNAs in MPM.

miRNA	Target genes/pathways	Cell function	References
miR-1	*PIM1, TP53, BAX, P16/21, BCL2*	Apoptosis, proliferation, migration, invasion	(26, 27)
Let-7a/b	EphA1 signaling, *RAS*, *PARP*, Procaspases 3, Twist, b-catenin, *AKT*, *TP53*	EMT, apoptosis, proliferation, migration, invasion	(23, 24, 28–30)
miR-15a/16	*BCL-2, CCDN1, PD-L1, FGF* axis	Radio/chemo-sensitivity, apoptosis, proliferation, colony formation	(31–33)
miR-17-5p	*KCNMA1*	Migration	(34)
miR-21-5p	*PARP1, MSLN*	Proliferation	(24, 35)
miR-29c-5p	*TP53, DNMT1, DNMT3A*	Methylation, proliferation, colony formation, migration, invasion	(23, 24, 36)
miR-31	*PPP6C*	Radio/chemo-sensitivity, proliferation, colony formation, migration, invasion	(37)
miR-34a/b/c	*BCL-2, c-MET, CDKN2A, NF2, TP53*	Radio/chemo-sensitivity, apoptosis, methylation, proliferation, colony formation, migration, invasion	(38–43)
miR-126	*ACL, PDK, IRS1, HIF1α, EGFL7*	Autophagic flux, mitochondrial function, methylation, proliferation, migration, invasion	(44–46)
miR-137	*YBX1*	Proliferation, migration, invasion,	(47)
miR-145	*OCT4, ZEB1*	Proliferation, migration, invasion, colony formation,	(48)
miR-193a-3p	*MCL1, PD-L1*	Apoptosis, cell death, proliferation	(49)
miR-205	*ZEB1, ZEB2*	EMT, migration, invasion	(50)
miR-223	*PARP1, MDM2, TP53, JNK* signaling, *STMN1*	Tubulin acethylation, proliferation, migration	(23, 24, 51)
miR-302b	*MCL1*	Apoptosis, proliferation	(52)
miR-379, miR-411	*IL-18*	Chemo-sensitivity, proliferation, invasion	(53)

binding to its receptor EphrinA2 suppresses MPM tumor growth through up-regulation of miR let-7a and the subsequent down-regulation of RAS proto-oncogenes family (28). Two years later the same group reported that the targeted delivery of miR let-7a, encapsulated in liposomal nanoparticles conjugated with EphrinA1, inhibits migration, proliferation, and tumor growth in MPM and NSCLC cell lines. This observation suggests a new possible therapeutic approach potentially useful especially in neoplasms overexpressing EphrinA2 receptor (29).

Sohn et al. focused their attention on another member of let-7 family. By transfecting let-7b synthetic mimic in H28, H2452, and MSTO-211H MPM cell lines treated with ursolic acid, they found that the up-regulation of let-7b was critically involved in ursolic acid induced apoptosis. The over-expression of let-7b increased the activity of ursolic acid leading to PARP and caspase 3 cleavage, and down-regulation of Twist, β-catenin and pAKT with a consequential sub-G1 cell accumulation and block of the epithelial to mesenchymal transition (EMT) (30).

In regards to the role of EMT in MPM pathogenesis, Fassina et al. have given an important contribution. They collected 109 MPM tissue samples (58 epithelioid, 25 biphasic, and 26 sarcomatoid) and showed that there is a switch in the expression from epithelial to mesenchymal markers going through the less aggressive epithelioid forms to the more aggressive biphasic and sarcomatoid histotypes. Moreover, overexpression of miR-205 in mesothelial (MeT-5A) and MPM cell lines (H2452 and MSTO-211H) caused a reduction in the expression of mesenchymal (*ZEB1* and *ZEB2*) and an increment of epithelial (*E-cadherin*) markers, which ultimately led to the inhibition of migration and invasion processes (50).

Using microarray transcriptional profiling and having normal pleural tissue as control, Xu et al. analyzed 25 MPM tissue samples and found lower miR-1 levels in neoplastic tissues. Accordingly, reduced proliferation and increased apoptosis of MPM cell lines (H513 and H2052) upon overexpression of miR-1 was observed. This suggests that miR-1 may act as tumor suppressor in MPM (26). These data were confirmed in the subsequent study by Amatya et al, where transfection of miR-1 and miR-214 mimic led to the down-regulation of the proto-oncogene PIM1 and to the inhibition of cell growth, invasion and migration (27).

Reid et al. firstly showed that miR-15/16 family is down-regulated in MPM tumor tissues and cell lines, and has a tumor suppressive role in MPM. In their experience, restoring miR-15/16 expression in MPM cell lines caused a reduction of cell proliferation and increased chemosensitivity. These phenomena were correlated with the down-regulation of specific genes such as *CCND1* and *BCL-2*. Using xenografts models, the authors described a relevant antitumoral activity for miR-16 mimic packaged in intravenously-administered "minicells" (31). Very recently, the same group demonstrated that miR-15a, miR-16, and also miR-193a-3p contribute to the regulation of programmed death ligand 1 (PD-L1) expression in MPM causing its down-regulation (32). Moreover, Schelch et al. observed a down-regulation of the fibroblast growth factor (FGF) axis after transfection with miR-15/16 mimics. The restoration of

miR-15/16 caused growth reduction in MPM cell lines and the combined inhibition of BCL-2 (another miR-15/16 target) resulted in a synergistic activity (33).

Cioce et al. focused their attention on miR-145, showing that treatment of MPM cell lines with miR-145 agonists reduced the protumorigenic power of MPM cells and increased the sensibility to pemetrexed. These data were confirmed in animal models, in which restoration of miR-145 expression inhibited tumor growth. The authors found that miR-145 targeted *OCT4* reducing its level and the level of its transcriptional target *ZEB1*. Higher OCT4 levels were associated with resistance to chemotherapy and with tumor growth (48).

As described by Tomasetti et al., miR-126 displays an oncosuppressive role in MPM cells by targeting *IRS1*, leading to impaired mitochondrial function and cell growth. Moreover, they demonstrated that miR-126 initiates a metabolic program, which implies high autophagic flux and HIF1α stabilization, playing a protective role in MPM (44, 45). Andersen et al., analyzing MPM tumor tissues and non-neoplastic controls, showed that DNA-hypermethylation down-regulates miR-126 and its host gene *EGFL7* leading to a reduction in patients survival in MPM (46).

Birnie et al. identified reduced levels of miR-223 in MPM patient specimens. The authors demonstrated that miR-223 targets *STMN1*, a microtubule regulator that has been associated with MPM. Moreover, they displayed that STMN1 is also regulated by the JNK signaling. The overexpression of miR-223 in MPM cell lines reduced STMN1 levels with a consequential induction of tubulin acetylation and reduction of cell motility. Furthermore, miR-223 levels grew and STMN1 levels decreased after the re-expression of the JNK isoforms in JNK-null murine embryonic fibroblasts. Finally, STMN1 levels decreased after JNK signaling activation (51). As reported by Walter et al., miR-223 is also down-regulated in MPM expressing MDM2, a negative regulator of TP53 (23).

Williams et al. found a significant decrease of the levels of miR-192 and miR-193a-3p in MPM tumor samples compared with non-cancer tissues. In MPM cell lines, transfection of miR-193a-3p mimic induced apoptosis and reduced cell proliferation causing reduction of the expression of the anti-apoptotic protein MCL1, frequently over-expressed in MPM. These data were confirmed in xenograft models in which the use of minicells containing miR-193a-3p mimics reduced tumor growth and increased apoptosis (49). In MPM, MCL1 is also downregulated by miR-302b. Khodayari et al. demonstrated that the treatment with ephrinA1 leads to the over-expression of miR-302b, which inhibits MCL-1 expression with a consequential induction of apoptosis and reduction of cells proliferation (52).

Cheng et al demonstrated that KCa1.1, a calcium-activated potassium channel subunit alpha 1 encoded by the KCNMA1 gene, is a target of miR-17-5p. KCa1.1 was overexpressed in MPM cells lines and MPM tissues compared with non-cancer samples. Moreover, the transfection of MPM cells with miR-17-5p mimic reduced the expression of KCa1.1 and blocked MPM cells migration (34).

De Santi et al., using Next Generation Sequencing (NGS) and the "miR-CATCH" method (based on biotinylated DNA antisense oligonucleotides that capture mRNA), identified miR-21-5p as a functional regulator of mesothelin (MSLN) gene expression. Moreover, they demonstrated that treatment with miR-21-5p mimic may decrease the proliferation in MPM cell lines (35). In a different study, the same group suggested that miR-126, miR-15b, miR-145, miR-185, miR-197, and miR-299 play a role in the regulation of cell metabolism in MPM. Comparing miRNA expression profile of 96 MPM patients with 10 non-cancer controls they found a significant down-regulation of these miRNAs in MPM. The top five pathways significantly affected by the deregulated miRNAs were: fatty acid biosynthesis, focal adhesion, MAPK, P53, and WNT signaling pathway (54).

Finally, few months before the submission of our review, Johnson et al., using MPM cell lines,127 MPM tissue samples (3 different cohorts), and 23 pleural or pericardium tissue controls, showed that miR-137 can exhibit a tumor-suppressive role in MPM by targeting Y-box binding protein 1 (YBX1). YBX1 knockdown significantly reduces tumor growth, migration, and invasion of MPM cells (47).

TISSUE EXPRESSION OF MIRNAS AS PROGNOSTIC AND DIAGNOSTIC BIOMARKERS IN MPM

The previously cited paper by Pass et al is the first work proposing microRNAs as potential prognostic biomarkers in MPM. Using a custom miRNA expression analysis platform, a training set of 44 and a test set of 98 MPM tumor samples were analyzed. In both training and test sets, higher levels of tissue miR-29c was shown to be an independent prognostic factor for higher OS and time to progression (TTP) after surgery (36). Using qRT-PCR, Matsumoto et al measured miR-31 expression in 25 tissue samples obtained from MPM patients and in 20 tissues of patients with reactive mesothelial proliferations (RMPs). They displayed that the expression of miR-31 was reduced in MPM compared with RMPs. However, the up-regulation of miR-31 was associated with the presence of sarcomatoid component and with worse prognosis in patients affected by this histological tumor subtype (55). Likewise, in the study by Busacca et al., low cancer tissue levels of miR-17-5p and miR-30c were associated with better OS in sarcomatoid MPM patients. Moreover, miR-30c was described as differentially expressed in the three MPM histotypes (56). Lastly, in the study by Fassina et al., the tissue levels of miR-205 were reported as lower in the more aggressive biphasic and sarcomatoid MPM histotypes and higher in the epithelioid forms characterized by better prognosis (50). Obviously, all these studies also suggest a role of miR-31, miR-17-5p, miR-30c, and miR-205 in the differential histopathological diagnosis of MPM.

The prognostic role of miRNAs in MPM has been demonstrated by different other studies (**Tables 2–4**) (24, 47, 54, 73–75, 78, 79).

In particular: high miR-137 (47) and miR-1, miR-335-5p, miR-566 (24) tissue levels have been correlated to poor prognosis, while the high tissue expression of miR-146a-5p, miR-378a-3p, miR-451a, miR-1246 (24), and of miR-16, miR-486 (75) was positively related with a better outcome.

TABLE 2 | miRNAs associated with oncogenic mechanisms in MPM.

References	Samples								Assay				miRNA
	MPM tissue	Cytological component of pleural effusion	Serum	Plasma	Cellular fraction of peripheral blood	Other tumor	Non-cancer related thoracic disease	Normal control	Microarray	PCR array	qRT-PCR	ISH	
Guled et al. (19)	17							1	X				Low: let-7e, miR-7-1,-9,-34a,-144,-203,-340, -423,-582. High: let-7b, miR-30b,-32,-195,-345,-483-3p,-584,-595,-615-3p,-1228
Ramirez Salazar et al. (21)	5						9	4		X			Low: miR-18a-3p,-101-3p,-145-5p,-181a-5p,-212-3p,-501-3p,-517b-3p,-596,-627,-671-3p,-766-3p. High: let-7-g-5p, miR-18a-5p,-34a-3p,-135b-5p,-196b-5p,-302b-3p,-622
Xu et al. (26)	25							6	X		X		Low: miR-1,-206,-483-5p. High expression: miR-155
Reid et al. (31)	60							23			X		Low: miR-16,-15a,-15b,-195
Cioce et al. (48)	29 + 6 + 36						12	14 + 36	X		X		Low: miR-145
Birnie et al. (51)	17	26					10	6			X		Low: miR-223

TABLE 3 | miRNAs associated with MPM diagnosis.

References	Samples								Assay				miRNA	Specificity	Sensitivity
	MPM tissue	Cytological component of pleural effusion	Serum	Plasma	Cellular fraction of peripheral blood	Other tumor	Non-cancer related thoracic disease	Normal control	Microarray	PCR array	qRT-PCR	ISH			
Ak et al. (22)	18						6			X			let-7a, let-7d, miR-20a,-92a,-125a-5p,-152,-155,-193b,-320, -484,-744	≥80%	≥80%
Benjamin et al. (57)	7 + 32 + 16 + 14					97 + 113 + 23 + 49			X		X		Low: miR-200c,-192. High: mi-193a-3p	94%	100%
Gee et al. (58)	15 + 100					10 + 32			X		X		Low: miR-141,-200b,-200c,-203, -205,-429	>80%	>80%
Andersen et al. (59)	10 + 52							5 + 14		X	X		Low: mir-126,-143,-145,-652	>80%	>80%
Santarelli et al. (60)	10 + 27							5 + 27		X	X		Low: mir-126		
Cappellesso et al. (61)	51	29					40 + 24				X		Low: miR-126. High expression: miR-19a,-19b,-21,-25	87%	86%
Cappellesso et al. (62)	41	26				40 + 26					X		High: miR130a	67%	77%
Santarelli et al. (60)			44				196	50			X		Low: mir-126		
Santarelli et al. (63)			45 + 18			42	99 +50	44 +20			X		Low: mir-126		
Gayosso-Gómez et al. (64)			11			36		45	High throughput sequencing				High: miR-92b-5p,-96-5p,-185-5p,-409-5p,-1271-5p,-1292-5p,-4791		
Kirschner et al. (65)	18		30	5 + 15			10	3 + 14 +7	X		X		High: miR-625-3p	≥80%	≥80%
Bononi et al. (66)			10 + 20				10 + 15	10 + 14	X		X		High: miR-1281,-32-3p,-197-3p		
Weber et al. (67)				21 + 22			21 + 44			X	X		Low: miR-132-3p	61%	86%
Matboli et al. (68)			60				20	20			X		High: miR-20a,-548a-3p	87.5%	100%
Cavalleri et al. (69)				23 (exosomes)			19		Open Array		X		Low: miR-30e-3p,-103a-3p	80%	95.5%
Weber et al. (70)					23 + 23		17 + 17	25	X		X		Low: miR-103a-3p	76%	78%
Weber et al. (71)					43		52				X		Low: miR-103a-3p	81%	95%
Muraoka et al. (72)			48				21	41			Digital methylation PCR		High methylation: miR-34b/c		

TABLE 4 | miRNAs associated with MPM prognosis.

References	Samples								Assay				miRNA
	MPM tissue	Cytological component of pleural effusion	Serum	Plasma	Cellular fraction of peripheral blood	Other tumor	Non-cancer related thoracic disease	Normal control	Microarray	PCR array	qRT-PCR	ISH	
Mairinger et al. (24)	24								X				miR-1, 146a-5p,-335-5p,-378a-3p,-451a,-566,-1246
Pass et al. (36)	44+98								X		X		miR-29c-5p
Fassina et al. (50)	74										X	X	miR-205
De Santi et al. (54)	52 + 16								X		X		let-7c-5p, miR-151a-5p
Johnson et al. (47)	115 + 12							23			X		miR-137
Matsumoto et al. (55)	25										X		miR-31
Busacca et al. (56)	24								X		X		miR-17-5p,-30c
Kirschner et al. (73)	64 + 43								X		X		miR-21-5p,-23a-3p,-30e-5p,-31-5p,-221-3p,-222-3p
Truini et al. (74)	27 + 30							4	X		X		let-7c, miR-99a,-125b
Mozzoni et al. (75)	32			32			15 + 14		X		X		miR-16, miR-17, miR-126, miR-486
Tomasetti et al. (76)			45			20		56			X		mir-126
Lamberti et al. (77)			14				10			X	X		miR-191, miR-223. High: miR-25,-26b,-29a,-101,-335, -433,-516

Kirschner et al. identified a 6-miRNA signature (miR-Score) predictive of higher OS in patients with MPM who underwent extrapleural pneumonectomy with or without induction chemotherapy, including miR-21-5p, miR-23a-3p, miR-30e-5p, miR-221-3p, miR-222-3p, and miR-31-5p.14 (73). The 6-miR-Score has been subsequently modified first into a 2-miR-Score for use in diagnostic chemo-naïve specimens (78), than in a combined 2-miRNA-clinical score prognostic in both chemo-naïve and treated patients (79). The 2-miR-Score includes miR-221-3p.

De Santi et al. also identified a 2-miRNA prognostic signature. In the 52 MPM tissue samples analyzed, higher levels of let-7c-5p and miR-151a-5p were associated with poorer OS. These data were confirmed in a second cohort of 16 fresh/ frozen MPM (54).

Finally, using a microarray platform and 27 tissue samples obtained from un-resected MPM patients, Truini et al. performed a whole miRNA profiling and selected mir-99a, let-7c, and miR-125b as potential prognostic miRNAs. The signature was tested on public miRNA sequencing data from 72 MPM patients with available OS and validated by RT-qPCR in an independent set of 30 MPM patients. The authors found that the down-regulation of the miR-99a/let-7/miR-125b miRNA cluster was able to predict poor outcome in unresected MPM (74).

In regards to the potential role of miRNA tissue expression as diagnostic biomarkers, a large number of studies have been published (**Tables 2–4**) (19–22, 26, 27, 48, 51, 57–60).

Guled et al. identified specific miRNA profiles in tumor and non-cancer tissues, associated with specific histopathological subtypes (19).

Using microarrays, Benjamin et al. identified a different miRNA tissue expression profile between different types of cancer and MPM. MiR-193-3p levels were higher in MPM, while miR-192 and miR-200c levels were higher in lung primary adenocarcinomas and pleural metastases. In a blinded validation set of 68 samples the assay had a specificity of 94% and a sensitivity of 100% (57).

With the aim to identify a miRNA signature able to distinguish between MPM and lung adenocarcinoma, Gee et al., using microarrays, analyzed 15 MPM and 10 lung adenocarcinoma tissue samples. The results were validated by RT-qPCR in 32 lung adenocarcinoma and 100 MPM samples, respectively. MiR-141, miR-200b, miR-200c, miR-203, miR-205, and miR-429 were down-expressed in MPM and resulted able to discriminate MPM from lung adenocarcinoma (58).

Santarelli et al. tested fresh or frozen biopsies of MPM for the expression of 88 miRNAs and compared the results with non-cancer tissue controls. They found that miR-126 was significantly down-regulated in neoplastic tissues (60).

Ak et al. showed a significant up-regulation of multiple miRNAs in MPM tissue samples, and demonstrated that let-7a, miR-125a-5p, miR-320, and miR-484, were able to discriminate MPM from benign asbestos related diseases (22).

Andersen et al. demonstrated a potential diagnostic value of miR-126, miR-143, miR-145, and miR-652 in MPM. They screened with a (RT-qPCR)-based platform the expression of 742 miRNAs in 5 MPM tissue samples of patients previously treated with chemotherapy, 5 preoperative diagnostic biopsies of patients with MPM and 5 non-neoplastic pleura samples obtained from patients with MPM diagnosis after chemotherapy treatment. The author showed that miR-126, miR-143, miR-145, and miR-652 levels were significantly reduced in MPM samples compared with non-cancer pleural tissues. The results were validated by RT-qPCR in a cohort of 40 independent MPMs. However, we have to take into account that chemotherapy may induce changes in the miRNA expression both in neoplastic and non-cancer tissues and this is the biggest limitation of this study (59).

EXPRESSION OF MIRNAS IN PLEURAL EFFUSION

The detection and the quantification of miRNAs in pleural effusion cells have a great potential for the identification of new minimally-invasive diagnostic biomarker (**Tables 2–4**).

Firstly Birnie et al., using RT-qPCR, showed that in comparison with non-cancer specimens, miR-223 levels were significantly reduced both in the cellular component of the pleural effusion of MPM patients and in MPM tissue samples. They compared 6 non-neoplastic with 17 neoplastic tissue samples and 10 pleural effusion specimens obtained from patients with benign pleural diseases with 26 coming from MPM patients (51).

In a first work from Cappellesso et al., analyzed the expression of 15 selected miRNAs in one normal mesothelial (MeT-5A) and two neoplastic (H2052 and H28) MPM cell lines using RT-qPCR. MiRNAs were also tested in 51 MPM and 40 non-neoplastic pleural tissue samples, and validated in 29 neoplastic and 24 non-neoplastic pleural effusion cytologic specimens. Compared with non-neoplastic controls, miR-19a, miR-19b, and miR-21 were over-expressed, and miR-126 was under-expressed in tumor samples. The authors concluded that miRNAs were detectable in the cytologic component of MPM pleural effusion, and especially the combination of miR-21 and miR-126 could be useful in the MPM diagnosis, reporting 86% sensitivity and 87% specificity (61).

In a second study, the authors investigated the significance of miRNAs in the differential diagnosis between lung adenocarcinoma and MPM pleural effusion. A pool of selected miRNAs was analyzed by RT-qPCR in 41 vs. 40 tissue samples and in 26 vs. 27 cytological pleural effusion specimens obtained from MPM and lung adenocarcinoma patients, respectively. The authors showed that miR-130a, miR-141, miR-193a, miR-205, miR-375, and miR-675 were differentially expressed in the two tumors, but only miR-130a was significantly overexpressed in MPM compared with lung adenocarcinoma. The sensitivity and specificity of miR-130a quantification in the differential diagnosis were 77 and 67%, respectively (62).

MIRNA EXPRESSION IN SERUM, PLASMA AND CELLULAR FRACTION OF BLOOD

It has been demonstrated that miRNAs are secreted in blood and serum, where they can be found both as soluble/protein associated, or included in lipid vesicles such as exosomes.

For mechanisms that are still not completely known, cancer cells release a higher amount of circulating miRNAs, whose composition reflects the one present in the secreting cells. For this reason, the detection and quantification of circulating cancer-derived miRNAs might represent an extremely valuable tool for the management of different tumor types, including MPM (**Tables 2–4**) (15–18).

Using RT-qPCR Santarelli et al. compared the serum levels of miR-126 obtained from 50 healthy controls, 196 asbestos-exposed, and 44 MPM patients. The authors reported that cut-off values of miR-126 could significantly differentiate asbestos-exposed subjects from healthy controls and from MPM group. Moreover, the association between low levels of miR-126 and high levels of the specific MPM markers such as soluble mesothelin-related peptides (SMRPs) was able to identify subjects with high risk to develop MPM (60).

One year later the same group published another paper with the aim to investigate the accuracy and precision of circulating miR-126 quantification as clinical biomarker. The authors evaluated miR-126 serum levels in 56 healthy subjects, 20 non-small-cell lung cancer and 45 MPM patients, using both absolute and relative qRT-PCR methods. MiR-126 serum levels were reduced in both tumor types and associated with worse prognosis in MPM. Moreover, the quantification of miR-126 differentiated MPM from both normal controls and non-small-cell lung cancer, but it was not able to discriminate healthy controls from non-small-cell lung cancer (76).

In a third paper, Santarelli et al. combined the quantification of circulating miR-126 with SMRPs and methylated thrombomodulin promoter (Met-TM) serum determination. A total of 44 healthy controls, 99 asbestos-exposed, and 44 MPM patients were evaluated. The combination of high SMRP and Met-TM levels with low levels of miR-16 was evaluated as the best method to distinguish MPM from the other two groups. Moreover, in non-neoplastic subjects, the association between high SMRP levels and high Met-TM or low miR-16 levels, increased significantly the MPM risk. These data were confirmed in a validation cohort of 20 healthy controls, 50 asbestos-exposed subjects, 18 MPM, and 42 lung cancer patients (63).

With the aim to discriminate between lung adenocarcinoma and MPM diagnosis, Gayosso-Gómez et al. studied miRNA profile of serum samples obtained from healthy subjects ($N = 45$), lung adenocarcinoma ($N = 36$), and MPM patients ($N = 11$). Among known miRNAs, in comparison with normal controls, 12 miRNAs were overexpressed in lung adenocarcinoma, and 7 in MPM. Three of these were up-regulated only in MPM (miR-92b-5p, miR-409-5, and miR-1292). These differences could be very useful in the differential diagnosis process (64).

The potential role of increased circulating levels of miR-625-3p as biomarker for MPM has been showed by Kirschner et al. Firstly, using microarray analyses, the authors tested 90 miRNAs previously reported as associated with MPM in plasma samples of a cohort of 5 MPM patients and 3 healthy subjects, and found 15 miRNAs with higher levels in MPM patients compared with controls. Using qRT-PCR, the results were validated in a second cohort of plasma samples obtained from 14 non-neoplastic subjects and 15 MPM patients, and in a third cohort of serum samples obtained from 10 patients with asbestosis and 30 with MPM. In the three cohorts, only the high concentration of miR-625-3p was always able to discriminate between MPM and non-MPM patients. The up-regulation of miR-625-3p in MPM was also confirmed in a forth cohort of tissue samples (6 normal pericardial and 18 MPM tissues) (65).

Lamberti et al. collected serum samples from 14 patients with MPM and 10 patients affected by non-cancer-related pleural effusions, and performed a miRNA profiling using low-density microarray Real Time PCR system. They found two miRNAs exclusively expressed (miR-516 and miR-29a), two miRNAs down-regulated (miR-223 and miR-191), and five miRNAs up-regulated (miR-335, miR-25, miR-26b, miR-101, and miR-433) in MPM samples compared with non-cancer controls. Patients with MPM were divided into two miRNA serum signature groups: signature A (patients with more than 3/9 up-regulated miRNAs or 3/9 up-regulated miRNAs and miR-516 unchanged or not recordable) and signature B (patients with at least 3/9 down-regulated or unchanged miRNAs and/or miR-29a down-regulated). MPM patients with signature B had longer OS in comparison with patients with signature A (17 vs. 7 months). The authors also reported that signature A was associated with sarcomatoid or biphasic histology (5/5 patients), nevertheless they did not report the statistical value. However, this study displayed the limitations of a low number of patients enrolled and the use of patients with pleural effusions as controls instead of healthy subjects (77).

In the study by Bononi et al., serum circulating miRNAs from 10 healthy subjects, 10 asbestos-exposed and 10 MPM patients were analyzed with microarray and validated by qRT-PCR in a second cohort of 14 healthy controls, 15 asbestos-exposed, and 20 MPM patients (30 serum samples were previously used for microarray analysis). In MPM patients they found up-regulation of miR-1281 in comparison to both healthy subjects and asbestos-exposed patients, up-regulation of miR-32-3p and miR-197-3p in comparison only to asbestos-exposed patients and up-regulation of miR-32-3p, miR-197-3p, and miR-1281 in comparison only to healthy subjects. The authors concluded that these three miRNAs could be proposed as new MPM diagnostic biomarker (66).

Weber et al used TaqMan Low Density Array Human MicroRNA Cards to analyze 377 miRNAs in plasma samples obtained from 21 asbestos-exposed and 21 MPM patients. The results were validated in a second cohort of 44 asbestos-exposed and 22 MPM patients using RT-qPCR. The authors showed that miR-132-3p was significantly down-regulated in MPM and only this miRNA resulted able to well discriminate between MPM and asbestos-exposed patients with a reported specificity of 61% and sensitivity of 86%. MiR-126 was also reported as down-regulated in MPM but only in the validation cohort. The authors calculated a specificity of 86% and a sensitivity of 77% for the combined down-regulation of the two miRNAs used as diagnostic biomarker. Nevertheless, it is not clear why miR-126 was not reported as down-regulated also in the discovery cohort of this study (67).

Mozzoni et al. aimed to identify a miRNA signature helpful as diagnostic biomarker for asbestos-exposed and MPM patients. The authors collected tissue and plasma samples from patients

affected by MPM (32 cases), asbestosis (14 cases), and other non-cancer pulmonary diseases (15 cases, used as controls). MiR-16, miR-17, miR-126, and miR-486 levels were quantified in plasma and tissues using qRT-PCR and all resulted decreased both in patients with asbestosis and in MPM, compared to controls. The levels of miR-486, miR-17, and miR-16 were significantly correlated in MPM tissue and plasma samples. Moreover, the tissue expression of miR-16 and miR-486 and the plasma levels of miRNA-16 were positively related with OS (75).

Lastly, Matboli et al., in a very recent paper, showed that the quantification of serum miR-548a-3p and miR-20a levels is a promising new diagnostic tool in MPM management. MiR-20a and miR-548a-3p were assessed in sera of healthy controls, asbestos-exposed and MPM patients using qRT-PCR. Their expression was positive in 91.6 and 96.7% MPM cases respectively, with a 100% of sensitivity as diagnostic MPM biomarker when used in combination (68).

Diagnostic approaches very different from those described above in this section have been used in some other works (69–72).

The epigenetic silencing of miR-34b/c plays a crucial role in the pathogenesis of MPM and in about 90% of MPM cases miR-34b/c is downregulated by DNA methylation (39). Using a digital methylation specific PCR assay, Muraoka et al. analyzed serum samples of 41 healthy controls, 21 asbestos-exposed and 48 MPM patients and demonstrated that a high degree of methylation of miR-34b/c in serum-circulating DNA is associated with MPM diagnosis and with higher MPM stage in patients with previous MPM diagnosis (72).

Cavalleri et al., using an OpenArray method, investigated the expression of 754 miRNAs in the plasmatic extracellular vesicles of 19 asbestos-exposed and 23 MPM patients, and found 55 differentially expressed miRNAs. Among these, 16 were confirmed by RT-qPCR in the validation phase. MiR-30e-3p, miR-98, miR-103a-3p, miR-148b, and miR-744 were the best discriminating miRNAs, and the combination of miR-30e-3p and miR-103a-3p was reported as the most discriminating one with a specificity of 80.0% and a sensitivity of 95.5%. This study is the only one using miRNA quantification in plasma exosomes. This new diagnostic approach is very interesting because it may provide a huge number of information about miRNA release mechanisms but it has the disadvantage of being very expensive (69).

The role of miR-103 family as diagnostic biomarker has been previously shown also by Weber et al. In two consecutive works, the authors used a totally different diagnostic technique based on the identification and quantification of miRNAs in the cellular fraction of human peripheral blood (70, 71). In the first pilot study, published on 2012, the investigators enrolled 17 asbestos-exposed and 23 MPM patients. Analyzing a panel of 328 miRNAs with microarrays, they found the low expression of miR-20a and miR-103. Quantitative-RT-PCR was used for validation phase in 25 healthy subjects, 17 asbestos-exposed and 23 MPM patients and confirmed only miR-103 as significantly down-expressed in the cellular fraction of MPM patients' blood. The authors calculated a specificity of 76% with a sensitivity of 78%, and a specificity of 71% with a sensitivity of 83% for the discrimination of MPM from healthy and asbestos-exposed subjects, respectively (70).

In the second study, the authors evaluated the performance of the combination of miR-103a-3p and mesothelin quantification as diagnostic biomarker in MPM. The analysis was performed on 52 asbestos-exposed and 43 MPM male patients. Mesothelin concentration was determined in plasma samples using ELISA test whilst the levels of miR-103a-3p was assessed in the blood cellular fraction using RT-qPCR. For the discrimination between asbestos-exposed and MPM patients miR-103a-3p, mesothelin and the combination of both showed 89, 74, 95% and 63, 85, 81% of sensitivity and specificity, respectively (71).

POTENTIAL THERAPEUTIC ROLE

Since their discovery, miRNAs have always been considered as one of the most interesting therapeutic prospects for cancer treatment. Their ability to target multiple cell pathways and the important regulatory role they play in almost all the mechanisms underlying cell replication and tumor progression, have made scientists to believe that they could be widely exploited to increase anti-tumor response and to reduce drug resistance. Considering that a huge number of miRNAs are down-regulated in many cancer types, the great part of the tested therapeutic strategies are based on the possibility to restoring the miRNAs function, often through the delivery of the down-expressed miRNAs inside the tumor cell. In MPM this new therapeutic approach has been experimented in cell lines and mouse models. Various miRNAs (let-7a, miR-16, miR-34b-c, miR-126, mir-145, miR-193a-3p) and various delivery systems have been tested obtaining interesting results in terms of tumor growth inhibition (29, 31, 33, 42–44, 48, 49).

However, despite these interesting premises, to date the results of only one clinical trial (NCT02369198) are available in human subjects. In this phase I, open-label, dose-escalation study, the authors aimed to investigate the safety, the optimal dose and the activity of TargomiRs in MPM patients. TargomiRs are minicells (EnGeneIC Dream Vectors) loaded with miR-16 mimic and targeted against EGFR. The drug was designed with the aim to restore the frequent down-expression of miR-16 in MPM. Twenty-seven patients (with diagnosis of EGFR positive MPM progressed after chemotherapy) were enrolled between September 2014 and November 2016 (26 patients were treated). The investigators found that 5×10^9 intravenous TargomiRs once weekly was the maximum tolerated dose. The most common adverse events were transient lymphopenia (96%), hypophosphataemia (65%), and increased transaminase levels (23%). Cardiac events (18%) occurred in five cases including one case of ischaemia and one case of Takotsubo cardiomyopathy. The drug showed early signs of activity. The median OS was 200 days (95% CI 94-358) with 5% of partial response, 68% of stable disease, and 27% of progressive disease registered as best response (80). The toxicity profile and the initial activity signs, make the development of this drug interesting, especially in association with chemotherapy and or immunotherapy.

Sayeed et al. investigated the potential role of dietary phytochemicals as possible preventive and/or therapeutic tool in MPM in a very interesting review (81). Nevertheless, at the present, only one dietary phytochemical (ursolic acid) (30) has been shown to have miRNA regulatory activity in MPM. The research in this field is at a very preliminary level and a completed opinion cannot be expressed yet.

CONCLUSIONS

Our review strongly supports the idea that the detection, quantization and analysis of miRNAs in MPM tissues and biological fluid samples have a great potential both from diagnostic and therapeutic point of view. Nevertheless, the literature analysis showed multiple limitations in the discussed studies.

One potential explanation for the strong diversity of data obtained from different studies is the heterogeneity in the quantification methods and in the type of samples and controls used. In particular, the adoption of various technical approaches among the analyzed studies, based on extremely different chemistries, represent a limitation for the identification of miRNA candidates with a consistent differential expression in diverse analyzed populations. It is desirable that the use of RNA high throughput sequencing systems will provide more reliable and reproducible data in future investigations, with a more clear clinical application.

Furthermore, in some studies data are poorly defined and some important information are not provided and/or the statistical analysis is not adequate. In order to improve these weaknesses, it is critical that future studies will use more uniform controls for their quantitative evaluations. In particular, while some studies involved normal healthy patients, others included in the same control population patients affected from non-cancer pulmonary disease or asbestos-exposed non-cancer patients. This last approach might lead to misidentification (or lack of identification) of microRNAs whose expression is also altered in these pathological statuses. For these reasons, there is limited reproducibility in the available results, which strongly affects the possibility of meta-analysis of published studies.

Finally, future studies would strongly benefit from the inclusion of additional clinic/pathological parameters of the included patients, such as histotype, disease status, and treatments. Stratification of patients based on these additional parameters might allow a better characterization and classification of MPMs.

However, despite these critical points, several miRNAs and/or miRNA families able to modulate crucial cell functions such as methylation, autophagy, apoptosis, proliferation, invasion, migration, and chemo/radio-resistance have been recently discovered and their knowledge has been deepened also in MPM. The role of various miRNAs as diagnostic or prognostic biomarkers in MPM has been confirmed in more than one study and in some cases it is becoming more and more solid (miR-16, miR-103, miR-126, miR-145, and miR-200c), and could pave the way to their clinical testing, both as diagnostic/prognostic markers, and as therapeutic agents.

Initial data on the use of miRNA replacement therapy in humans are starting to be published and the preliminary results in MPM patients are encouraging (82, 83). However, these therapeutic approaches are still lacking appropriate clinical validation, and several issues will have to be solved before they could be considered for the clinical practice. In particular, it is still unclear how miRNA-based therapies, using mimics or inhibitors, could affect the physiology of normal cells, and there is a still-unmet need of cancer-specific delivery systems that could limit undesired effects of these treatments.

In conclusion, this literature review, by highlighting the extreme heterogeneity of studies analyzing the role of microRNAs in MPM, wants to urge a coordinated collaboration between the main international groups working in this research field. In fact, the future scenario of MPM patients will see relevant clinical improvements only through coordinated efforts of multiple basic and translational research studies.

AUTHOR CONTRIBUTIONS

GL, AT, and DP participated in the designing of the manuscript and draft it. MC, GG, FdB, and MG participated in drafting the manuscript and critically revised it. GL, AT, and DP designed and coordinated the manuscript. All the authors read and approved the final manuscript.

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METHODS
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Cancer Site-Specific Multiple microRNA Quantification by Droplet Digital PCR

Noemi Laprovitera[1], Maria Grzes[1,2], Elisa Porcellini[1] and Manuela Ferracin[1]**

[1] Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy, [2] Department of Molecular Biology, Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, Lesznowola, Poland

Archival formalin-fixed paraffin-embedded (FFPE) tissues represent an extraordinary source of smallRNAs, including microRNAs (miRNAs). Contrary to other RNA molecules, miRNAs are stable, nuclease-resistant and quantifiable even in low quality samples. The accurate assessment of miRNA levels in archival samples is of great interest for many pathological conditions, including cancer. In human tumors, microRNA expression is type-specific and can be used as diagnostic, prognostic or response-to-treatment biomarker. In this study, we provide a method for multiple miRNA quantification in 96-well plates, using EvaGreen-based droplet digital PCR technology and miRCURY LNA miRNA assays. This approach allows the absolute quantification of a customizable panel of miRNAs at the same time and under identical experimental conditions, to be used for diagnostic or prognostic applications.

Keywords: microRNA, FFPE (formalin-fixed paraffin-embedded), droplet digital PCR (ddPCR), cancer, EvaGreen chemistry

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Marilena Valeria Iorio, Istituto Nazionale dei Tumori (IRCCS), Italy

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Gabriele Multhoff, Technische Universität München, Germany
Kenneth K. W. To, The Chinese University of Hong Kong, Hong Kong

****Correspondence:***
Elisa Porcellini
elisa.porcellini3@unibo.it
Manuela Ferracin
manuela.ferracin@unibo.it

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INTRODUCTION

MicroRNAs (miRNAs) are important molecules involved in post-transcriptional regulation of gene expression. Their crucial role in human cancer is well documented (1). MicroRNA expression profiles can distinguish different cancer types and contribute to cancer sub-classification (2). The pattern of miRNA expression can be used to infer the origin site of metastatic tumors (3–5). In addition, the value of microRNAs as molecular biomarkers in formalin-fixed paraffin-embedded (FFPE) specimens and body fluids, and the many applications of miRNA quantification for diagnosis and prognosis in several human cancers were also described (6). MicroRNAs are highly stable molecules that resist to RNAase activity in FFPE specimens, which are the most widely available clinical archival samples, biological fluids, and also microvescicles and can be easily isolated from all these tissues (7–9).

Droplet Digital PCR (ddPCR) is a relatively newborn technology that has been used for several applications, such as rare DNA mutation detection, copy number variation analysis and absolute nucleic acid quantification (10–12). This assessment can be performed using both dsDNA-binding dye EvaGreen-based chemistry or TaqMan probe-based assays.

Droplet digital PCR technology (Bio-Rad patent) consists in sample partitioning into nanoliter-sized water-in-oil droplets, which generates thousands of multiple individual reactions measured as endpoint PCRs. Droplets are then individually analyzed by a fluorescence detector and classified as positive if fluorescence is detected (i.e., the target sequence is included) or negative if not. Finally, the number of positive droplets is used to estimate the target concentration with the

application of Poisson correction. Specifically, the real concentration could be underestimated because this technology cannot distinguish droplets with multiple copies of the target molecule. Poisson correction solves this problem, by estimating the number of multiple-target droplets on droplet total number (10, 13).

This powerful technology has many applications in research and diagnostics (12, 14). Droplet digital PCR overcomes the limits of quantitative PCR (qPCR) in performance and accuracy, eluding several problems connected to qPCR methodology, such as the need of reference genes for normalization or replicate samples (15). In a previous study, we assessed the overall precision and accuracy, as well as intra- and inter-assay reproducibility, of EvaGreen-based ddPCR with above standard results (16). Using ddPCR it is also possible to evaluate the expression of specific miRNAs that circulate in biological fluids, including blood (17).

METHODS

Objectives and Validation of the Method

In this article we describe a tool for miRNA multi-assay quantification using RNA obtained from FFPE tissues and EvaGreen-based droplet digital PCR technology. Multiple miRNA quantification with ddPCR technology in the same plate has never been performed and could be quite challenging because the optimal annealing temperature and primer amount could change between different miRNA primer sets (16, 18). For multiple miRNA quantification, we had to use the same annealing temperature and primer amount. To achieve this goal, we selected the experimental conditions that were most efficient for the majority of the single assays we tested in the past (18, 19), which were 58°C annealing temperature and 1 μL primer amount. Then, we designed pre-spotted custom plates (96-well format) with 92 different miRCURY LNA miRNA primers (Qiagen, former Exiqon). Using this approach, we were able to assess the expression of 92 different miRNAs at the same time, using the same amount of primer and the same PCR conditions (16, 18).

Detailed Protocol

Here we present a detailed protocol of our approach for multiple miRNA quantification with ddPCR, with the sequential steps necessary to use this method.

RNA Extraction From FFPE Samples

We collected 4–5 tissue slices 10–20 μm thick from 14 diagnostic archival formalin-fixed paraffin embedded (FFPE) blocks from 14 different tumor types. The study was approved by the local ethical committee (Comitato Etico Indipendente dell'Azienda Ospedaliero-Universitaria di Bologna, Policlinico S.Orsola Malpighi). All subjects gave written informed consent in accordance with the Declaration of Helsinki. Pathological characteristics of cancer patients are detailed in **Supplementary Table 1**.

With the assistance of a Haematoxylin and Eosin (H&E) stained section, we dissected the best tumor area and placed in 2 mL Eppendorf tubes (cat. no. H0030120094).

Total RNA can be isolated using any standard method and commercial kit currently available. We describe herein a protocol for the isolation of RNA from FFPE samples using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion/ThermoFisher, cat. no. AM1975). In detail, FFPE tumor sections underwent paraffin removal, by adding 1 mL of 100% xylene and incubating at 50°C for 3 min. After centrifugation, xylene was removed without disturbing the pellet; this was then washed twice with 1 mL of 100% ethanol and left to air dry 45 min at room temperature, to remove any residual ethanol. Protease digestion was performed adding 200 μL of Digestion Buffer and 4 μL of Protease enzyme. Samples were left to incubate for 15 min at 50°C and 15 min at 80°C in heat blocks. The Isolation mix was then prepared, mixing 240 μL of Isolation Additive with 550 μL of 100% ethanol, and added to the sample. All this mixture was then passed through a filter cartridge and collected in a collection tube through centrifugation at 10000 g for 30 s. The filter was then washed firstly with 700 μL of Wash 1 solution and then with Wash 2/3 solution and centrifuge to remove any residual fluid. A mixture containing 6 μL of 10X DNase buffer, 4 μL of DNase and 50 μL of Nuclease free water was added to the center of the filter and left to incubate 30 min at room temperature. After repeating the washes with Wash 1 and Wash 2/3 solutions, filter underwent to the elution of RNA with 60 μL of nuclease-free water.

RNA quality and quantity were assessed by Nanodrop spectrophotometer (ThermoFisher) and frozen at −80°C.

cDNA Synthesis

The conversion of RNA to cDNA was performed using the Universal cDNA synthesis kit II (Exiqon, cat. no. EX203301PR), following the manufacturer's protocol.

RNA was diluted to a concentration of 5 ng/μL using nuclease-free water. The mixture for reverse transcription was prepared for each sample mixing: 2 μL of 5x Reaction Buffer, 4.5 μL of nuclease free water, 1 μL of enzyme mix, 0.5 μL of synthetic RNA (Sp6) spike-in and 2 μL of diluted RNA (10 ng of total RNA). The reaction was performed in a conventional thermal cycler and comprised an incubation step (60 min at 42°C), enzyme heat inactivation (5 min at 95°C) and a holding step (4°C forever). Resulting cDNA was then conserved at −20°C in 1.5 mL LoBind DNA Eppendorf tubes (cat. no. H 0030 108 051).

cDNA Dilution

Just before the next steps, cDNA was diluted 1:50. A further dilution of 1:10 (final 1:500) was prepared to assess one very abundant microRNA (miR-21-5p) and the internal control assay UniSP6, in order to avoid the saturation of the positive droplets.

miRNA Plate Setup

Pre-spotted custom plates including 89 different cancer-specific miRNA primer sets were designed using miRCURY LNA Custom PCR Panel (Qiagen, former Exiqon) in the 96-well format (cat. no. 339330, cat. no. 339332). Three additional assays for small non-coding RNAs (SNORD44, SNORD48 e snRNAU6) were

included as reference. The remaining wells contained two inter-plate calibrator assays (UniSp3), a control plate assay (UniSP6) and a no template control (NTC) well.

Pre-spotted custom plates have both forward and reverse PCR amplification primers in an amount that would be sufficient for one qPCR reaction. We tested these primers as individual assays (16), and verified that this amount is excessive for EvaGreen-based ddPCR, because of the broad fluorescence amplitude of the negative signal. Half the amount was the best solution for ddPCR testing. Thus, we split the plate primers in two plates, as described in the next step.

Droplet Generation and PCR

Droplet digital PCR workflow was performed using a miRNA quantification protocol that was recently developed (6, 18) using a 20 μL volume of PCR mix containing 10 μL of 2X QX200 ddPCR EvaGreen Supermix (Bio-Rad, cat. no. 1864034), 6 μL of nuclease-free water and 4 μL of diluted cDNA.

To split into two plates the pre-spotted primers, we re-suspended each well in a double amount of EvaGreen and water and transferred half amount in a second plate (Eppendorf, cat. no. H 0030 128 605). At the end of this step, we obtained two 96-well plates with half amount of primers in a volume of 16 μL, both ready for the addition of the diluted cDNA, which was performed with a multichannel pipet.

Each ddPCR assay mixture was loaded into a disposable DG8 Cartridge (Bio-Rad, cat. no. 1864008) located into a cartridge holder (Bio-Rad, cat. no. 1863051). Then, 70 μL of droplet generation oil for EvaGreen (Bio-Rad, cat. no. 1864005) was loaded into each of the eight oil wells. The cartridge was then covered with a DG8 Gasket (Bio-Rad, cat. no. 1863009) and placed inside the QX200 Droplet Generator (Bio-Rad, cat. no. 1864002). Upon completion of droplet generation, the droplets were carefully transferred to a new Eppendorf blue twin.tec 96-well PCR plate (cat. no. H 0030 128 605). The plate was heat-sealed with a pierceable aluminum foil (Bio-Rad, cat. no. 1814040) into the PX1 PCR Plate Sealer (Bio-Rad, cat. no. 1814000) and placed in a thermal cycler. Thermal cycling conditions were: 95°C for 5 min, then 40 cycles of 95°C for 30 s and 58°C for 1 min and three final steps at 4°C for 5 min, 90°C for 5 min and a 4°C infinite hold. It is mandatory to use a ramping rate of 2°C/second in every step.

Droplet Reading and Data Analysis

After PCR is completed, the sealed plate was transferred into the plate holder of the QX200 Droplet Reader (Bio-Rad, cat. no. 1864003). Using QuantaSoft software (Bio-Rad), the analysis was set up and started in order to analyze the droplets with an optical detector. At the end of the plate reading, the resulting data were analyzed with QuantaSoft software v1.7. Specifically, from the 2D amplitude plot, the positive droplets in each well were selected using the lasso tool. This function allows to manually select the positive cloud by drawing a circle around it, and finally obtain the miRNA amount (copies/μL). Using the events tab, the number of positive and total generated droplets were evaluated. The general performance of EvaGreen ddPCR consented to obtain a total number of 18,000–21,000 droplets per well.

RESULTS

Reproducibility

To demonstrate the reproducibility of this multiple quantification approach, the same sample was assessed twice using the procedure above described. Pearson correlation analysis was performed to assess the correlation between replicates. Comparing the miRNA expression in the two samples the *p*-value resulted highly significant ($P < 0.0001$). Pearson r was found to be 0.9537 (0.92–0.97 95% confidence interval), indicating a strong reproducibility of the methodology (**Figure 1**).

Application and Effectiveness

Using this multiple miRNA quantification tool, we evaluated the miRNAs expression of 14 FFPE samples from different tumor types (liver, skin, breast, gastric, endometrium, testis, GI-neuroendocrine, prostate, urothelial, kidney, colon, pancreas, lung, ovary).

Our custom plate was designed to contain the most cancer-specific miRNAs: specifically, 89 cancer-specific miRNAs and 3 reference genes.

As shown in **Figure 2**, we were able to obtain a good separation between positive and negative droplets for all the miRNA assays in our panel. The above detailed experimental conditions allowed a reliable and efficient quantification of all the targets in the same experiment. In **Figure 3** we represented some 2D plots of different miRNA assays: the shape of the positive clouds could vary between targets, but it is still easily selectable with the above mentioned QuantaSoft lasso tool.

We obtained an average total number of droplets of 20,000 (**Figure 4**), thus confirming the high sensitivity of the method and accurate detection even of rare targets.

One of the many applications of this tool is represented by the analysis of miRNA expression profile in cancer. By

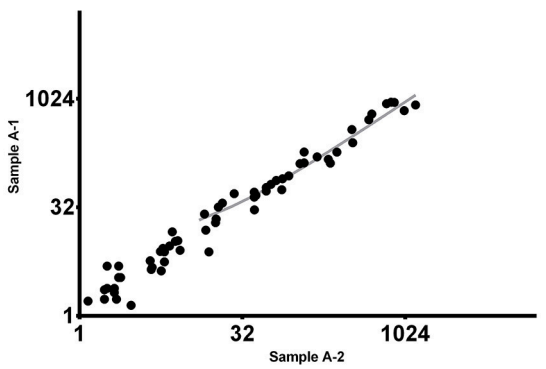

FIGURE 1 | Correlation between replicates. The same 92-miRNA panel was assessed twice in the same sample. Results were highly concordant (Pearson $r = 0.9537$). Each dot represents a microRNA. The miRNA absolute expression is presented as copies per microliter of the amplification reaction mixture. Axis scale is log2 transformed.

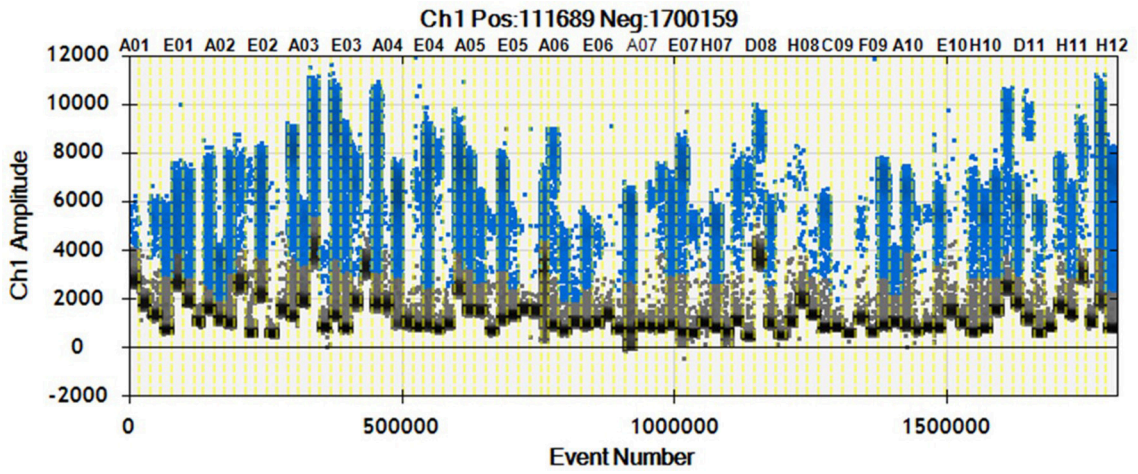

FIGURE 2 | 1D Plot representation. Droplet distribution for different miRNA assays evaluated in the same sample. 1D Plot representation shows positive (blue) and negative (black) droplet amplitudes. The fluorescence amplitude can change according to the assay.

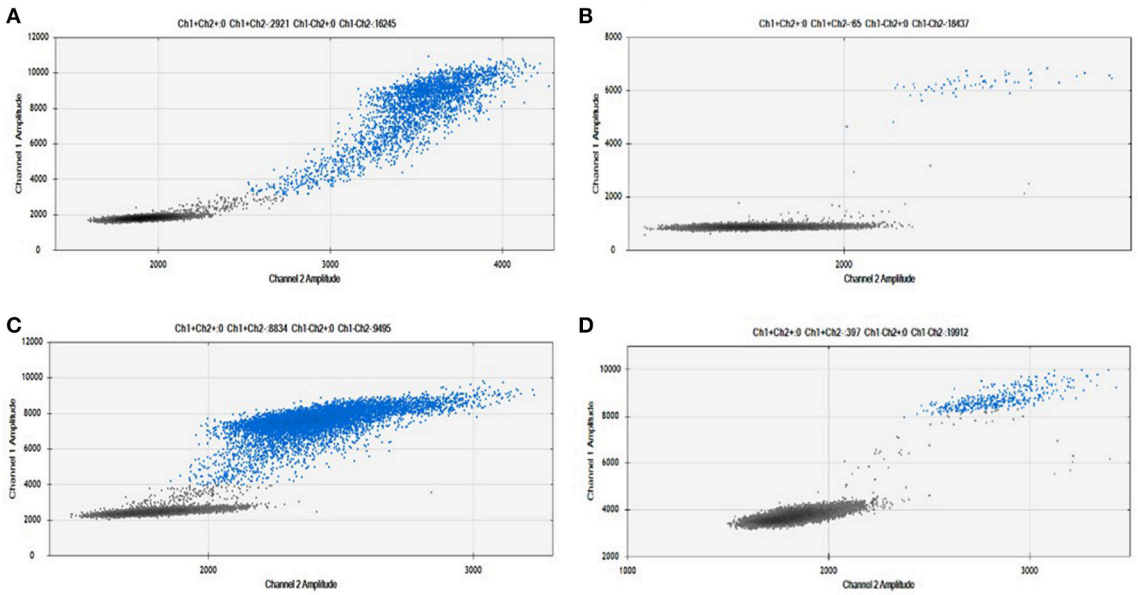

FIGURE 3 | Positive droplet selection in 2D Plot. Bi-dimensional droplet plots of four illustrative miRNA assays: **(A)** RNU6, **(B)** miR-149-5p, **(C)** miR-24-3p, and **(D)** miR-210-3p. Droplet clouds could have different appearance and shapes, but droplet positive selection is always possible.

analyzing different cancer types, we were able to validate the expected miRNA signatures, in agreement with our previous data obtained with microarray technology (4). The miRNA profile of 14 different tumor samples is shown in **Figure 5**. Although we could not directly compare the miRNA quantification provided by a probe hybridization-based technology (Agilent microRNA microarray) and a digital PCR technology, we analyzed the correlation in microRNA quantification between these two approaches, after data normalization, and observed a highly significant correlation of the data ($p < 0.0001$, Spearman $r > 0.7$) for all the tested cancer types.

DISCUSSION

Advantages and Limitations

Our miRNA multi-assay tool suits the need for a simultaneous absolute quantification of different miRNA targets. The detection of up to 92 different targets all at once and in the same ddPCR experiment, consents to adjust the use of this method for any user need.

We demonstrated that using EvaGreen based-ddPCR and our PCR conditions the quantification of all targets was efficient and reproducible, despite starting from low quality material as FFPE tissue. This novel approach can be particularly

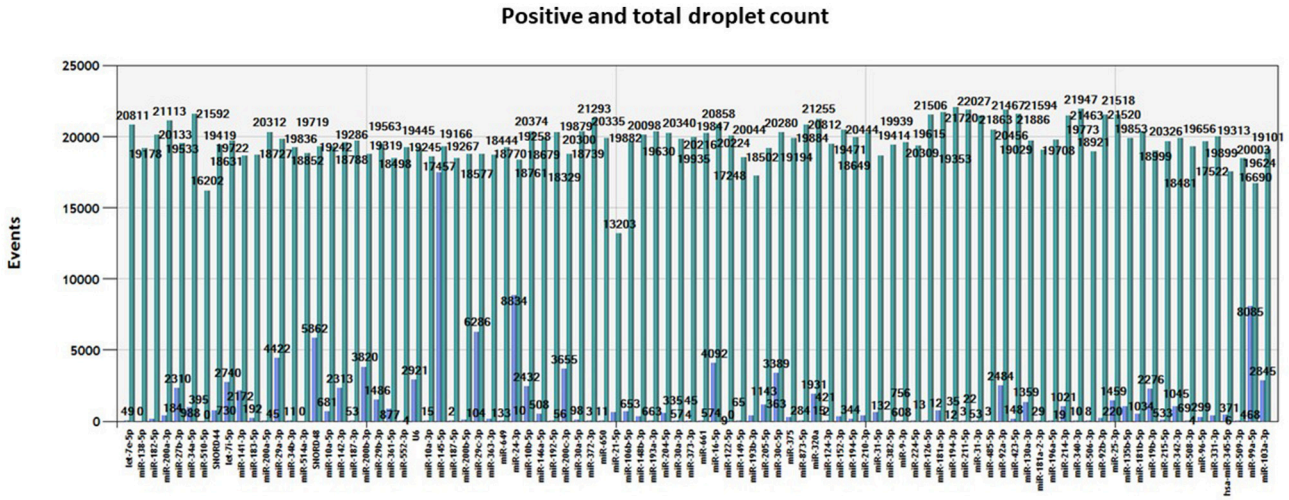

FIGURE 4 | Number of events. Total number of positive (blue) and negative (green) droplets visualized in QuantaSoft software (Bio-Rad) for each miRNA assay assessed in the same tumor sample.

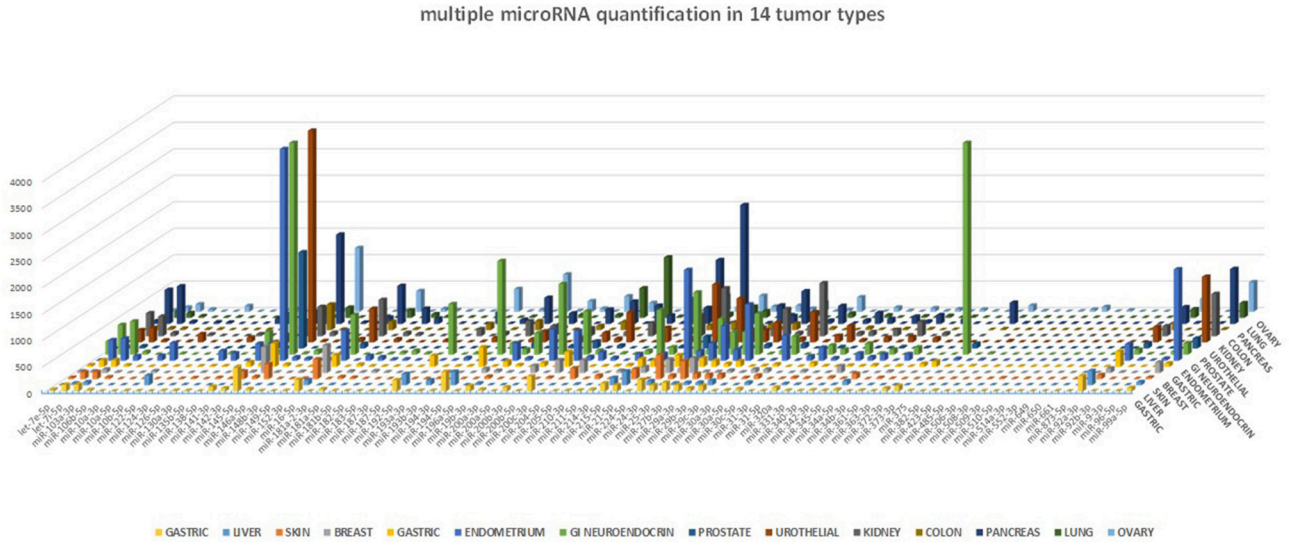

FIGURE 5 | miRNA profile in human cancers. The 89-miRNA expression profile (normalized on reference genes) across 14 different cancer types, as detected by our ddPCR multi-assay method. Each tumor displays a specific miRNA expression profile.

appropriate when the miRNAs are expressed at low levels and wouldn't be detected by standard quantitative PCR. Other studies have already proved ddPCR superior accuracy, which overcomes the need of replicates and reduce the experimental costs (15, 16).

Since this tool provides the quantification of up to 92 miRNAs per experiment, it is necessary to identify a focused custom panel of interest. It is important to pay attention to the step of droplet selection during the analysis, and carefully distinguish the positive from the negative droplets. This is a critical step and must be done analyzing each well one-by-one in the 2D amplitude plot and selecting the positive cloud manually with the available software tools.

Even if in some cases the separation could be further optimized (e.g., changing the annealing temperature or the amount of primer), for all the assays that were tested in our experiment a good separation between positive and negative droplets was obtained.

The method described in this paper represents a remarkable innovation. Indeed, the multiple miRNA quantification with ddPCR in the same experiment has never been described. In addition, the possibility to extend this approach to archival samples could support and improve disease biomarker discovery and validation.

Given the sensibility and flexibility of this method, possible applications include: discovery or validation of

miRNA biomarkers; miRNA quantification in every type of human tissue, including FFPE, biological fluids and fresh tissue; absolute quantification of multiple miRNAs at the same time in subcellular compartments (e.g., exosomes and microvescicles).

AUTHOR CONTRIBUTIONS

NL, MG, and EP carried out the experiments and drafted the initial manuscript. MF reviewed the results and wrote the paper. All authors reviewed and approved the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00447/full#supplementary-material

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REVIEW
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MicroRNAs and DNA-Damaging Drugs in Breast Cancer: Strength in Numbers

*Ilaria Plantamura, Giulia Cosentino and Alessandra Cataldo**

Molecular Targeting Unit, Research Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

MicroRNAs are a class of small non-coding regulatory RNAs playing key roles in cancer. Breast cancer is the most common female malignancy worldwide and is categorized into four molecular subtypes: luminal A and B, HER2+ and triple-negative breast cancer (TNBC). Despite the development of multiple targeted therapies for luminal and HER2+ breast tumors, TNBC lacks specific therapeutic approaches, thus they are treated mainly with radio- and chemotherapy. The effectiveness of these therapeutic regimens is based on their ability to induce DNA damage, which is differentially resolved and repaired by normal vs. cancer cells. Recently, drugs directly targeting DNA repair mechanisms, such as PARP inhibitors, have emerged as attractive candidates for the future molecular targeted-therapy in breast cancer. These compounds prevent cancer cells to appropriate repair DNA double strand breaks and induce a phenomenon called *synthetic lethality*, that results from the concurrent inhibition of PARP and the absence of functional BRCA genes which prompt cell death. MicroRNAs are relevant players in most of the biological processes including DNA damage repair mechanisms. Consistently, the downregulation of DNA repair genes by miRNAs have been probe to improve the therapeutic effect of genotoxic drugs. In this review, we discuss how microRNAs can sensitize cancer cells to DNA-damaging drugs, through the regulation of DNA repair genes, and examine the most recent findings on their possible use as a therapeutic tools of treatment response in breast cancer.

Keywords: breast cancer, DNA repair, DNA damage response, DNA-damaging drugs, microRNAs

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Edited by:
Anna Rita Migliaccio, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Shiv K. Gupta, Mayo Clinic, United States
Cristiana Tanase, Victor Babes National Institute of Pathology, Romania

****Correspondence:***
Alessandra Cataldo
alessandra.cataldo@istitutotumori.mi.it

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GENERAL OVERVIEW

MicroRNAs

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs that regulate gene expression at post-transcriptional level. Mature miRNAs are single strand molecules of ∼18–25 nucleotides (nt), transcribed by RNA polymerase II/III as long primary transcripts with a hairpin structure, called pri-miRNAs. Pri-miRNAs are then cleaved in the nucleus into ∼60 nt long molecules (pre-miRNAs) by the Microprocessor, a multi-protein complex comprising the RNase III enzyme DROSHA and its cofactor DGCR8 (1, 2). As pre-miRNAs, these molecules are specifically recognized by the nuclear export machinery, mainly composed of Exportin-5 and Ran-GTPase, and exported to the cytoplasm where their processing is completed. The dsRNA stem of pre-miRNAs is asymmetrically cleaved by the second multi-domain RNAase III enzyme DICER into a short nucleotide duplex (3). During this step, the transactivation-responsive RNA-binding protein

(TRBP) mediates the assembling of the miRNA-induced silencing complex (miRISC), favoring DICER and Argonaute protein (AGO1, AGO2, AGO3 or AGO4) interaction (4). The miRISC complex selects the mature miRNA (guide strand), which then guides the machinery to the target mRNA (5). MiRNA/mRNA interaction occurs by the recognition between the "seed" region at the miRNA 5′UTR and its complementary sequence on the 3′UTR of the designated mRNA. The result of the pairing is, either the translational repression or transcript degradation, in dependence of the complementary degree between the two sequences (6). After the discovery of the small RNA lin-4 function in the larval development of *Caenorhabditis elegans* in 1993, many researchers had started to investigate the regulatory potential of these small molecules (7). To date, it is well-known that miRNAs participate in almost every biological process in mammals, including cancer (8). Several mechanisms alter miRNA expression in cancer such as genomic aberrations, epigenetic changes, dysfunction of the processing machinery, alteration of transcription factor expression, among others (9).

In cancer, miRNAs can act as tumor suppressors or oncogenes (oncomiR). Functionally, miRNAs with a tumor suppressor role target oncogenes and are generally downregulated in cancer cells (e.g., miR-205 and miR-34 in breast cancer). While, oncogenic miRNAs target tumor suppressor genes and are usually upregulated in tumor cells (e.g., miR-21, miR-155, and miR-221/222 in breast cancer) (10).

MiRNA capability to regulate several target genes involved in oncogenic mechanism such as proliferation, progression, metastasis, and therapy response, makes these small molecules fascinating candidates as therapeutic tools. In fact, recent studies have been focused on develop new strategies to make possible the miRNA-based therapy approach. Generally, miRNAs can be reintroduced in cancer cells using miRNA *mimics* or be inhibited by *anti-miRs*. During the last years, new methods to deliver and to stabilize miRNA *mimics* and *anti-miRs* have been developed, some of which are currently in clinical trials.

MiRNA *mimics* and *anti-miRs* can be delivered with lipid carriers, for instance the miR-34-based therapy MRX34 (Mirna Therapeutics) deliver miR-34 *mimic* sequence through the lipid carrier NOV40. MRX34 is the first miRNA-based therapy undergoing in a clinical trial for cancer treatment. During 2013, patients with lymphoma, melanoma, multiple myeloma and liver, small cell lung and renal carcinoma were enrolled in a phase I clinical trial. Unfortunately, despite the promising results obtained with the partial response of 3 patients and stable disease in other 14 patients, the trial was terminated in September 2016 due to severe and lethal immune-related adverse reactions occurred in some patients (clinicaltrials.gov:NCT01829971) (11, 12). Additionally, EnGeneIC Delivery Vehicle (EDV) nanocells (also called TargomiRs) coated with epidermal growth factor receptor (EGFR)-specific antibodies are currently in a phase I trial to deliver miR-16 mimics in patients with malignant pleural mesothelioma and NSCLC (clinicaltrials.gov: NCT02369198), current preliminary results show that the treatment is well-tolerated. MiRNA *mimics* can be also conjugated to *N*-acetyl-D-galactosamine (GalNAc) particles, improving their uptake into cells through clathrin-mediated endocytosis. Moreover, RG-125 (Regulus Therapeutics), a GalNAc-conjugated containing an *anti-miR*-103/107 sequence, recently entered in clinical investigations to treat non-alcoholic steatohepatitis (NASH). In addition, a phase I trial in HCV-infected patients was initiated to evaluate the response of RG-101 (Regulus Therapeutics), a *N*-acetyl-D-galactosamine (GalNAc)-conjugated *anti-miR* targeting miR-122. Finally, in November 2015, a LNA-modified anti-miR-155 (MRG-106) has begun to test in a phase I clinical trial to treat patients with cutaneous T-cell lymphoma (clinicaltrials.gov: NCT02580552).

Breast Cancer

Breast cancer represents one of the most common malignancies worldwide and a leading cause of cancer-related death in women (13). Biological and genomic characterizations have described breast cancer as a highly heterogeneous disease, according to histological and molecular features, and responsiveness to therapy (14).

Clinically, breast cancers are firstly categorized, according to the expression of three receptors routinely assessed by immunohistochemistry assay in the following subtypes: estrogen and progesterone receptor positive (ER+, PR+), human epidermal growth factor receptor positive (HER2+) and triple-negative (ER–, PR–, HER2–) malignancies (15, 16). This classification provide valuable clinical information, mainly to choice the first line treatment, in addition to the histological grade, clinical stage, patient's age and menopausal status. The advent of high throughput technologies, such as microarray-based transcriptomic analysis, has provided new sources of information regarding breast cancer biology. Gene expression profiling of breast cancers identified five intrinsic molecular subtypes: hormone receptors positive luminal A and luminal B, HER2-enriched, basal-like and normal-like. These subtypes differ in incidence, prognosis and responsiveness to therapy (17–19). Luminal B (ER+, PR+, HER2–, Ki67+) usually present higher clinical grade than luminal A (ER+, PR+, HER2–, Ki67–) tumors, and some of them also express HER2 receptor. The HER2-enriched (HER2+, ER–, PR–) most frequently present high grade and node positive, whereas the basal-like (HER2–, ER–, PR–) subgroup mainly comprises triple-negative breast cancers (TNBCs) and frequently shows BRCA1 mutations, both germinal and sporadic (20).

An additional intrinsic subtype described more recently is the claudin-low subtype, sorted mainly from the TNBC subgroup and characterized by stem cell-like features (21, 22). A more detail classification based on molecular portrait of TNBCs have been provided in 2011 by Lehmann and colleagues through the identification of six subtypes with distinct gene expression patterns and response to treatment: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M),

Abbreviations: DDR, DNA damage response; NER, nucleotide excision repair; BER, base excision repair; MMR, mismatch repair machinery; DSBs, Double Strand Breaks; SSBs, Single-Strand Breaks; NHEJ, non-homologous end joining; HR, homologous recombination; ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth factor receptor 2; TNBC, triple negative breast cancer; miRNAs, microRNAs; IR, ionizing radiation.

mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) subtype (23). High level of cell cycle and DNA damage response genes are expressed by BL1 and BL2 subtypes, which are preferentially sensitive to cisplatin. Epithelial-to-mesenchymal transition and growth factor pathways are enriched in the M and MSL subtypes, which respond to PI3K/mTOR and abl/src inhibitors. The LAR subtype, characterized by androgen receptor (AR) signaling and shorter relapse-free survival, is sensitive to bicalutamide (an AR antagonist). During the last years, next generation sequencing (NGS) has significantly improved the molecular characterization of breast carcinomas, providing data on gene mutations, DNA copy number variations, DNA methylation and miRNA expression patterns. Important examples of such studies are The Cancer Genome Atlas (TCGA) project, the International Cancer Genome Consortium (ICGC) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (24, 25). Information gathered from these studies are particularly useful to the clinical practice, because they provide a list of new molecules, which can be potentially targeted or exploited for therapy interventions to improve drug efficacy (26).

The role of miRNAs in breast cancer was deeply investigated in the last decades. The first miRNA signature in breast cancer was described by Iorio et al. (27), followed by several studies that have demonstrated a functional role of miRNAs in the disease. One of the most studied miRNA in breast cancer is miR-21, which acts as an oncomiR mediating cell survival, proliferation, invasion, and metastasis (28). MiR-9, targeting E-cadherin and regulating the EMT process, is recognized as a metastamiR in breast cancer (29); as well as miR-10b, one of the first metastamiRs described in a breast cancer model (30). Conversely, many miRNAs have been identified as tumor suppressors in breast cancer. MiR-205 and miR-125a, for example, modulate the expression of HER3 and HER2 oncogenes, respectively (31, 32). Moreover, it has been reported that miR-205 and miR-200 family have an important anti-tumorigenic role by targeting ZEB1 and ZEB2, suppressing EMT process (33).

DNA Repair Mechanisms

Genomic instability is well-recognized as one of the hallmarks of cancer (34). Many studies have demonstrated that breast cancer cells have defective DNA damage response (DDR) mechanisms. In general, when DNA damage occurs, cells repair the errors and continue to proliferate; otherwise, the damage can cause mutations or chromosomal rearrangements, which induce tumorigenesis. The DDR is a complex system activated upon DNA damage, shaped by the activity of DNA damage signal transduction, DNA repair mechanisms, cell cycle checkpoints and apoptosis signaling pathways (35). DDR regulates DNA repair by inducing the following molecular processes: detection of damage sites, recruitment of repair factors and repair of DNA lesions. DDR machinery is divided into DNA damage sensors, signal transducers and effectors. DDR can use different mechanisms to repair DNA damage. In particular, two mechanisms are designated to remove damaged and modified nucleotides: (1) Nucleotide Excision Repair (NER) which works on helix-distorting and transcription-blocking lesions (i.e., UV-induced pyrimidine dimers) and (2) Base Excision Repair (BER) which removes single nucleotides by methylation, alkylation, deamination, or oxidation (36, 37). Other DDR mechanism participates in the recognition of incorrect insertions or deletions of nucleotides during DNA synthesis, which can lead to microsatellite instability and cancer. These errors affect the canonic DNA sequence and induce base mismatches that cause the distortion of DNA helix. The Mismatch Repair Machinery (MMR) operates through MSH2 and MLH1, which form heterodimers with MSH3 or MSH6 and MLH3, PMS2, or PMS1, respectively (38, 39). Many environmental agents can cause other types of DNA damage such as: Double-Strand Breaks (DSBs) or Single-Strand Breaks (SSBs) (40). SSBs and modified bases are the most common DNA damage, approximately in 1 day occurs 20,000 events per cell, but are usually repaired via BER mechanism (41). Instead, DSBs induce the recruitment of MRE11–RAD50–NBS1 (MRN) complex, which activates the serine/threonine-specific kinases ATM which allows its auto-phosphorylation (pATM) and the phosphorylation of the Ser139 of histone H2AX (γH2AX) in response to DNA damage signals. γH2AX recruits additional pATM molecules and DDR proteins, such as p53-binding protein 1 (53BP1), at DSB site, to generate a nuclear foci (42, 43). In response to DSBs, ATM kinase promotes the phosphorylation of many proteins, in particular Chk2 kinase, one of the most important effectors of ATM (44, 45). On the other hand, ATR recruit Chk1 kinase after stalled replication-forks and SSBs induced by UV (46, 47). ATM and ATR induce the phosphorylation of multiple proteins to activate downstream DNA repair pathways and induce cell cycle arrest, apoptosis or senescence when damage repair was not efficient (48). DSBs are repaired by two mechanisms: (1) Non-Homologous End Joining (NHEJ), active during all phases of the cell cycle but its activation occur mainly in G0/G1 phases, or (2) Homologous Recombination (HR), which acts in the S/G2 phases of the cell cycle (49, 50). In particular, NHEJ resolve double-stranded DNA breaks by enhancing Ku protein binding, as well as the recruitment and activation of DNA-PK. NHEJ complex includes DNA ligase IV, XRCC4, and XLF/Cernunnos protein which promotes a direct ligation of the ends of DSBs. However, this mechanism can induce some alterations, such as deletions or mutations of DNA sequences at the DSB site or around it (40). HR comprehends a set of proteins, including BRCA1, BRCA2, RAD51, and PALB2 that allow the restoration of the original DNA sequence at the damage site. Briefly, the DNA sequence near to DSB is deleted and the sequence on the homologous sister chromatid is used as a template to synthesize new DNA at the DSB site (37, 49). Many studies have demonstrated that miRNAs regulate, at transcriptional and post-transcriptional levels, the DNA damage sensor, signal transducer and effector genes in cancer cells. For example, miR-182, miR-181a/b, miR-28, and miR-146 have been demonstrated to target BRCA1 in breast cancer cells. The DDR gene RAD51 is modulated by miR-155, miR-107, miR-221/222; whereas ATM is targeted by miR-181a/b and miR-18a. Moreover, in breast cancer cells, miR-125b and miR-34a are able to control the expression of TP53, the main cell cycle regulator

(51). **Figure 1** shows a schematic representation of DNA damage repair mechanisms and some relevant miRNAs involved in the modulation of DNA repair genes. Therefore, miRNAs represent an important regulatory mechanism of DNA repair pathways and a novel source to exploit DDR gene/miRNA interactions for clinical purposes as potential biomarkers and therapeutic tools.

In the next sections, we will review how miRNAs could influence the response to DNA-damaging drugs in breast cancer therapy. Indeed, several studies have reported a key role of miRNAs in the responsiveness to DNA-damage based therapies, by modulating the expression of genes involved in the initiation, activation and maintenance of DDR mechanisms.

DNA DAMAGING DRUGS IN BREAST CANCER THERAPY

The strategies of breast cancer treatment include surgery, radiation, chemotherapy, hormone therapy, and biological targeted therapy. Patients with hormone receptor positive (ER+, PR+) tumors receive hormone therapy (i.e., tamoxifen and aromatase inhibitor), whereas patients affected by HER2+ tumors receive anti-HER2 targeted therapy (i.e., trastuzumab and pertuzumab) (52). Thus, the biggest challenge is represented by the clinical management of TNBCs, mainly due to the lack of targeted drugs. Indeed, the standard therapy for these tumors still remains cytotoxic chemotherapy (53). In term of response to chemotherapy, luminal A tumors show lower responsiveness, luminal B are more responsive than luminal A but less than HER2-enriched and basal-like, which are the subgroups with the higher response rate.

In breast cancer management, radio- and chemotherapy exert their effects by causing DNA damage, and are usually used as first-line drugs in combination with hormone and target therapies. Ionizing radiation (IR), anthracyclines, platinum compounds, and taxanes usually induce DSBs and SSBs, the efficacy of current DNA-damaging drugs is correlated with the capabilities of cancer cells to resolve and repair DNA lesions. Cancer cells are highly proliferative in comparison with normal cells, this feature increases their susceptibility to DNA damage exposure in the S phase of the cell cycle (20). However, the main problem of radio- and chemotherapy is the development of acquired resistance along the drug administration.

Radiotherapy treatment, based on the administration of a specific amount of energy, induces the activation of multi-staged processes which enhance tumor cell death. In particular, DSBs promote chromosomal alterations and affect cell division, contributing to cell death or mutation (54). Ionizing radiation (IR), such as X-rays, can extend cell damage by direct DNA breaks or indirectly through the creation of free radicals (55, 56). Tumors receiving a large total dose of radiations sometimes develop radioresistance, which eventually leads to treatment failure. The acquired radioresistance can be associated with altered expression of cell cycle molecules and DDR effectors, such as the overexpression of cyclin D1 and the constitutive activation of DNA-PK and AKT, respectively (57).

Chemotherapy is the most common therapy for cancer. Chemotherapeutic agents promote tumor cell death by direct cytotoxicity, activation of host immune response, inhibition of cell proliferation and induction of apoptosis (58). After cytotoxic agents administration, DNA damage is the first event sensed by the cellular stress response machinery, triggering the activation of effector systems, such as apoptosis (59). Unfortunately, resistance to chemotherapy can occur at many levels including DNA repair, cell cycle regulation and evasion of apoptosis (60).

Recently, the identification of BRCA-associated DNA repair mechanisms, frequently impaired in TNBCs, led to the development of specific DNA damage target therapies.

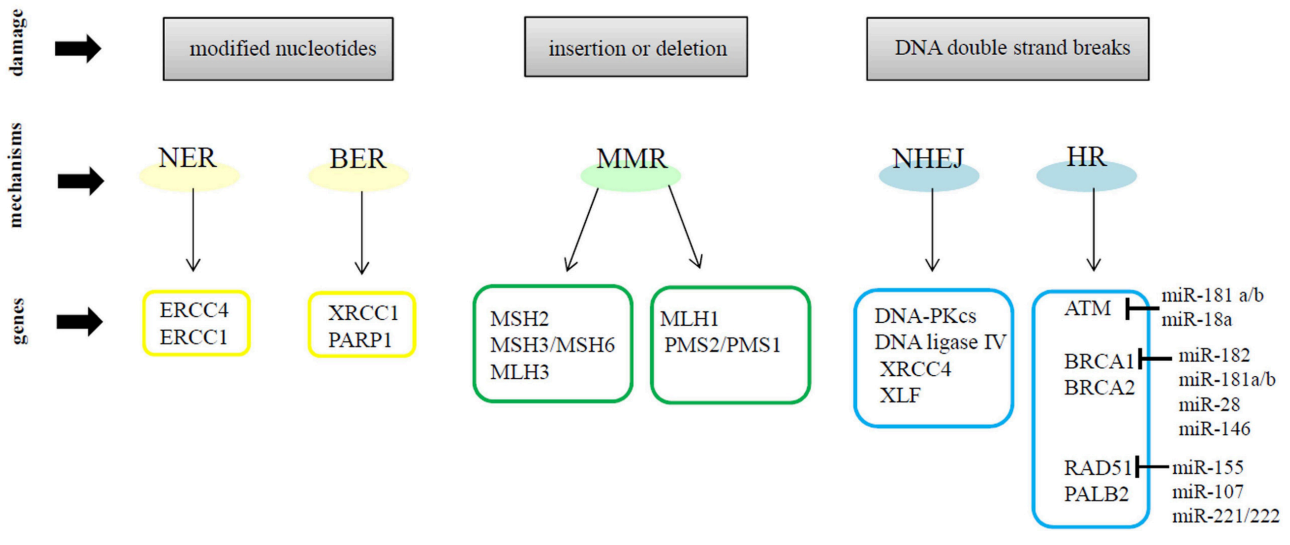

FIGURE 1 | A schematic representation of DNA repair mechanisms and some relevant miRNAs involved in the modulation of DNA repair genes.

BRCA genes, involved in DNA repair through HR after DSBs, are altered in sporadic and hereditary breast cancer; notably, in sporadic tumors BRCA1 mutations are rare (<5%) while ∼10% of TNBC patients present germline mutations in BRCA1/2 which increase breast cancer risk about 60–70% (61, 62). In the last years, many groups have deep investigated the role of defective HR mechanisms in cancer: the so-called "BRCAness" status is defined as the presence in tumor cells of alternative mechanisms impairing BRCA1/2 functions, or the alteration in HR genes. BRCAness is a phenocopy of BRCA1/2 mutations, in fact, HR mechanisms result defective although tumor cells do not carry mutations in BRCA1/2 genes (63).

As above clarified, for patients affected by TNBCs, targeted therapies are not currently available and chemotherapy may lead the acquisition of resistance in the later stages of the disease (64). In the last years PARP inhibitors have emerged as a possible therapeutic approach, especially when cancer cells lack functional alleles of the genes BRCA1 or BRCA2 (65). Poly-[ADP-Ribose]-Polymerase-1 (PARP-1) is a crucial molecule involved in the activation of the DNA-damage response. PARP-1 is a nuclear protein implicated in various processes involving DNA-related transactions. PARP-1 recognizes DNA-damage sites and creates long chains of poly-ADP-ribose, required for the appropriate recruitment of DNA repair enzymes (66). When DNA damage occurs, PARP-1 is rapidly recruited to the altered DNA and converts nicotinamide adenine dinucleotide (NAD) into ADP-ribose polymers (PAR) by attracting XRCC1, a scaffold protein which stabilizes or stimulates compounds involved in single-stranded breakage (67). PARP-1 is composed of three functional domains: the amino-terminal DNA-binding domain composed of two zinc fingers, which is necessary to bind the DNA breaks; the automodification domain, which allows the enzyme to PARate itself, and the c-terminal catalytic domain where ADP-ribose subunits are transferred from NAD+ to proteins acceptors (67). PARP inhibitors are able to block the catalytic PARP domain competing with NAD, and impairing the single-stranded DNA breakage repair activity. This mechanism induces apoptosis through the accumulation of damaged DNA in the cells. Functionally, PARP inhibitors (e.g., olaparib, talazoparib, rucaparib, and veliparib) prevent cancer cells from appropriately repair DNA damages, consequently genotoxic stress results in cancer cell death (65). Cells with BRCA1/2 wild-type can still repair the damage through HR, whereas mutated BRCA1/2 cells strictly depend on PARP activity for DNA repair. The inhibition of PARP in absence of functional BRCA genes results in *synthetic lethality*. As well-known, chemotherapy alters pathways involved in DNA damage repair; for this reason, PARP inhibitors can sensitize tumor cells to chemotherapy and radiotherapy and can induce *synthetic lethality* in tumors from patients with hereditary or sporadic mutations in BRCA1/2 genes. Different PARP inhibitors have been evaluated in preclinical studies and in clinical trials as mono or combination therapies for breast cancer patients, particularly for TNBC. In a BRCA1-deficient breast cancer mouse model, the combination of a PARP inhibitor with cisplatin or carboplatin increases the recurrence-free and overall survival, indicating that PARP inhibitors can improve the efficacy of DNA-damaging compounds (68). Moreover, Hay T. and colleagues have shown that daily treatment with olaparib for 28 days in mice with $BRCA2^{-/-}$ mammary epithelium caused a significant regression of 46/52 tumors (69).

In clinics, different trials on breast cancer patients with BRCA1/2-defective tumors demonstrated that PARP inhibitors, such as olaparib, enhance the therapeutic response when administrated as single agents or in combination with platinum compounds.

In a first study of phase I, 60 patients, affected by different tumor types including breast cancer, were enrolled and treated with olaparib. Among these, 22 patients had BRCA1 or BRCA2 mutations. Results showed that olaparib has the capability to inhibit PARP with limited adverse effects in comparison with conventional chemotherapy, however an antitumor activity was observed only in patients carrying BRCA mutations (70). In a phase II study, two cohorts of 27 patients with confirmed BRCA1 or BRCA2 mutation-advanced breast cancer were enrolled and treated with two different doses of olaparib. The first cohort had an overall response rate (ORR) of 41% and in the second cohort an ORR of 22%; moreover no particular toxicity has been reported (71).

Furthermore, when olaparib had been combined with paclitaxel in a cohort of 19 patients with metastatic TNBC in phase I study, an ORR of about 30–40% has been observed. Particularly seven patients had a confirmed partial response and one patient remained stable with olaparib monotherapy without progression (72). To date, phase III trials are ongoing to investigate the use of olaparib in the metastatic and neoadjuvant setting, for patients with mutations in BRCA1/2 (62). Finally, a randomized phase II trial that recruited patients with TNBC and/or BRCA mutations, treated with cisplatin alone or in combination with rucaparib, showed that both treatment groups present a similar disease-free survival at 1 year follow-up (∼76%), and rucaparib did not add significant toxicity to the cisplatin regimen (62, 73).

Results of clinical studies with PARP inhibitors have shown promising results in advanced breast cancer, but there is still an urgent need to identify suitable patients who may actually benefit from this treatment. Further investigations to find new strategies to efficiently impair DNA repair mechanisms in breast cancer patients could enhance the response to radio-, chemo-, and PARP inhibition therapies.

MICRORNAS REGULATE DNA REPAIR GENES AND RADIO-CHEMOTHERAPY RESPONSIVENESS

Alterations in DNA repair mechanisms and in miRNA expression are both features of cancer development and progression (28, 74). As reported in this review, genotoxic agents, causing DNA damage, are commonly used for radio- and chemo-therapeutic treatments in breast cancer. MiRNA up- or down-modulation is often involved in the regulation of DNA repair mechanisms (75) and it is currently known that miRNAs can regulate responsiveness to drugs (76). Thus, the alterations of miRNA expression involved in DDR mechanisms play an important role

in responsiveness to radio- and chemotherapy. Recently, our group has shown that miR-302b expression in breast cancer cell lines induces cisplatin sensitivity, reducing cell viability and proliferation in response to the treatment (77). E2F1, a master regulator of the G1/S transition, is directly targeted by miR-302b. Moreover, this miRNA, through the negative regulation of E2F1, indirectly downregulates ATM, the main cellular sensor of DNA damage, affecting cell-cycle progression following cisplatin treatment. As a result, miR-302b impairs the capability to repair damaged DNA upon cisplatin treatment, enhancing apoptosis in breast cancer cells (77).

Accordingly, another group has demonstrated that miR-302 family is able to sensitize breast cancer cells to radiotherapy; in particular Liang et al. showed that the decreased expression of miR-302a induces radiotherapy resistance and the reintroduction of miR-302a expression enhances radiotherapy sensitivity in *in vitro* and *in vivo* breast cancer models, abrogating the expression of AKT1 and RAD52 (78).

Gasparini et al. revealed that miR-155 overexpression reduced RAD51 levels in human breast cancer cells, which affects the response to IR and impairs the efficiency of HR repair enhancing IR sensitivity both in *in vitro* and *in vivo* models. Moreover, a series of TNBC patients with high levels of miR-155 and low expression of RAD51 revealed a significant association with a better overall survival. Thus, miR-155 expression can be considered as a prognostic biomarker which allows to identify TNBC patients who will likely be responsive to IR-based therapeutic approach (79). It was broadly demonstrated the over-expression of the oncomiR miR-21 in tumors with relevant consequences in cell cycle, DNA damage repair, apoptosis, autophagy, and hypoxia of cancer cells during irradiation. Indeed, cell cycle progression is influenced by miR-21 through the induction of DNA damage in G2 checkpoint and the miRNA inhibition (by anti-miR-21 administration) reduced the G2/M block and induced apoptosis following radiation treatment in breast cancer (80).

Of note, the overexpression of the miR-205, an oncosuppressive miRNA, increases the response to tyrosine kinase inhibitors, lapatinib and gefitinib in preclinical breast cancer models (81). Recently, it has been described that enhanced expression of miR-205 sensitizes breast cancer cells to radiation by regulating ZEB1 and affecting DNA repair. Indeed, miR-205 directly targets Ubc13, a protein involved in the homologous recombination. Moreover, the authors demonstrated that the delivery of miR-205 mimics, by nanoliposomes in a xenograft model, has a therapeutic effect sensitizing tumor to radiation (82).

MiR-18a is upregulated in breast cancer cell lines and patient tissues, interestingly its ectopic expression downregulates ATM. This phenomenon in breast cancer cells reduced the DNA damage repair ability, the efficiency of HR and sensitized cells to radiation treatment (83). Wip1 is a regulator of DNA damage signaling pathways, in particular it inhibits the phosphorylation of some DNA repair factors, including ATM, Chk1, Chk2, p53, and others. Zhang et al have demonstrated that miR-16 targets Wip1, affecting DNA repair and sensitizing breast cancer cells to doxorubicin treatment (84). Furthermore, using a TNBC *in vivo* model, Wang et al. demonstrated that miR-96 reduces the expression of REV1 and RAD51 and consequently inhibits tumor growth after cisplatin treatment. Thus, miR-96 is a potent cisplatin sensitizer *in vivo* (85).

Finally, diverse studies have focused their attention on miRNAs directly targeting BRCA genes in breast cancer. BRCA1 and BRCA2 are tumor suppressor genes important for the HR mechanism, which is the process involved in the repair of DNA DSBs. For instance, miR-218 directly targets BRCA1 and that its restored expression in cisplatin resistant breast cancer cell lines sensitizes cells against the drug, affecting DNA damage (86). Moreover, miR-638 overexpression increases sensitivity to DNA-damaging agents, ultraviolet (UV) and cisplatin, and reduces proliferation, invasive ability, and DNA

TABLE 1 | miRNAs involved in the chemo- and radio-responsiveness, through the regulation of DNA repair genes.

microRNA expression	Gene target	Drug response
miR-302b overexpression	E2F1 and ATM	Cisplatin sensitivity (77)
miR-302a overexpression	AKT1 and RAD52	IR sensitivity (78)
miR-155 overexpression	RAD51	IR sensitivity (79)
miR-21 downregulation	G2/M block	IR sensitivity (80)
miR-205 overexpression	Ubc13	IR sensitivity (82)
miR-18a overexpression	ATM	IR sensitivity (83)
miR-16 overexpression	Wip1	Doxorubicin sensitivity (84)
miR-96 overexpression	REV1 and RAD51	Cisplatin sensitivity (85)
miR-218 overexpression	BRCA1	Cisplatin sensitivity (86)
miR-638 overexpression	BRCA1	UV and Cisplatin sensitivity (87)

TABLE 2 | miRNAs involved in the PARP inhibitors response, through the regulation of DNA repair genes.

microRNA expression	Gene target	Drug response
miR-182 overexpression	BRCA1	IR and PARP inhibitors sensitivity (88)
miR-182 overexpression	CHEK2	PARP inhibitors sensitivity (89)
miR-107 and miR-122 overexpression	RAD51	PARP inhibitors sensitivity (90)
miR-103 and miR-107 overexpression	RAD51 and RAD51D	PARP inhibitors sensitivity (91)
miR-96 overexpression	RAD51 and REV1	PARP inhibitors sensitivity (85)
miR-181 overexpression	ATM and BRCA1	PARP inhibitors sensitivity (92)
miR-21 overexpression	MSH2	PARP inhibitors sensitivity (92)
miR-664b-5p overexpression	CCNE2	PARP inhibitors and chemo-sensitivity (93)
miR-151-5p overexpression	SMARCA5	PARP inhibitors sensitivity (94)

repair capabilities, by down-regulation of BRCA1 in TNBC cells (87). **Table 1** summarizes miRNAs involved in the chemo- and radio-responsiveness, through the regulation of DNA repair genes, in breast cancer.

MICRORNAS REGULATE DNA REPAIR GENES AND PARP INHIBITOR RESPONSE

As reported above, PARP inhibitors represent one of the most innovative approaches in the development of anti-breast cancer therapies. However, whether miRNAs could influence sensitivity to PARP inhibitors has not been deeply investigated yet. Indeed, few reports have described the role of miRNAs in the modulation of PARP inhibitor response. Here, we report the main results about this regulation in breast cancer. In 2011, Moskwa et al. have demonstrated that breast cancer cells overexpressing miR-182 are more sensitive to IR and PARP inhibitors via BRCA1 targeting and impairment of DNA repair. These results were also confirmed in *in vivo* models; mice injected with breast cancer cells overexpressing miR-182 showed a reduced tumor growth when treated with PARP inhibitor olaparib (88). Moreover, it has been demonstrated that CHEK2, another gene involved in the HR, is a direct target of miR-182-5p. This regulation enhances the sensitivity to a PARP inhibitor in breast cancer cells (89). As reported by Neijenhuis et al. and Huang et al. miR-107, miR-222 and miR-103 regulate the DDR and sensitize tumor cells to PARP inhibitors in breast cancer cell lines, targeting RAD51 and impairing HR (90, 91). It is also known that miR-96 targets RAD51 and REV1, and that the overexpression of this miRNA in breast cancer in *in vitro* models results in improved sensitivity to PARP inhibitors (85). In this context, it has also been demonstrated that TGFβ regulates DNA repair genes and responsiveness to PARP inhibitors. Liu et al. have shown that two TGFβ-targeted DNA-repair genes, ATM and BRCA1, both regulated by miR-181, and MSH2 targeted by miR-21, contribute to TGFβ-induced sensitivity to PARP inhibition (92). More recently, the role of miR-664b-5p has been investigated. This miRNA is a tumor suppressor and results upregulated upon PARP inhibitor plus chemotherapy treatments. Thus miR-664b-5p has an important role in the regulation of PARP inhibitors to increase chemosensitivity by targeting CCNE2 in BRCA1 not-mutated TNBC (93). Furthermore, an interesting mechanism involving miR-151-5p and its target SMARCA5, an ISWI family member with an important role in DSB repair (94), has been proposed. Indeed, Tommasi et al. reported the possibility of

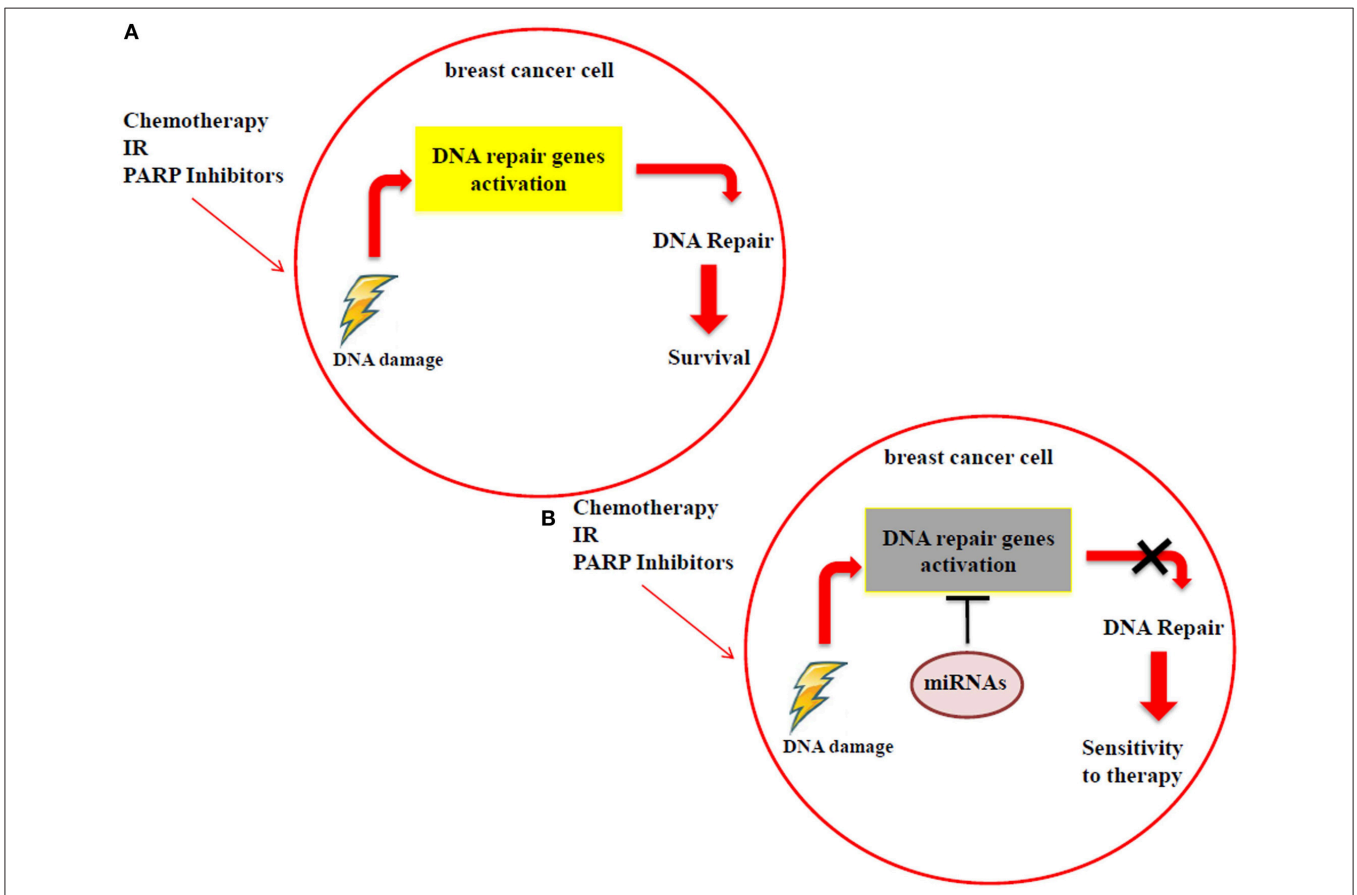

FIGURE 2 | (A) Breast cancer cells can still repair the DNA damage, caused by radiotherapy, chemotherapy and PARP inhibitor treatments, using DDR mechanisms. **(B)** MiRNAs impair the activation of DDR mechanisms by targeting DNA repair genes, improving the sensitivity to radiotherapy, chemotherapy and PARP inhibitors.

considering the overexpression of PARP1 and miR-151-5p as predictive biomarkers, useful to correctly select sporadic breast cancers for treatment with PARP inhibitors.

Table 2 summarizes the miRNAs involved in the PARP inhibitor response, through the regulation of DNA repair genes, in breast cancer.

CONCLUSION

Many studies have reported that miRNA modulation in breast cancer, by using *in vitro* and *in vivo* models, can be exploited to achieve a higher response to the DNA-damaging drugs, as radiotherapy, chemotherapy and PARP inhibitors. These small RNAs have the ability to directly target DNA repair genes, thus resulting in the impairment of DNA repair mechanisms. When breast cancer cells are treated with radiotherapy, chemotherapy or PARP inhibitors, the resulting DNA damage could be still repaired through the activation of specific DNA repair genes, such as ATM, BRCA1/2, RAD51, DNA-PK etc., thus cells survive and continue to proliferate (**Figure 2A**). Specific miRNAs, targeting DNA repair genes, are able to impair the mechanisms involved in repairing the DNA damage and to promote a higher sensitivity to the treatments in breast cancer cells (**Figure 2B**). For this reason, miRNAs could be exploited as predictive biomarkers and therapeutic tools to increase the response to DNA-damaging drugs. Considering PARP inhibitors, they are currently not in clinical practice for breast cancer and the inclusion criteria to treat patients using these drugs in clinical trials are that tumor cells are BRCA 1/2 mutated. We can speculate that the miRNAs involved in the regulation of DNA repair genes could represent a novel strategy to mimic the BRCAness phenotype, making tumor cells BRCA1/2 wild type more responsive to PARP inhibitors. However, in this context it is relevant to underline that currently the feasibility of a miRNA-based therapy in clinics has not been demonstrated yet, either alone or in combination with standard therapies. Technical and practical issues still need to be solved, such as the toxicity, off-target effects and systemic delivery. Indeed, for the clinical practice it will be necessary to identify the best strategy to deliver miRNAs directly to the tumor, i.e., by conjugation with antibodies or specific nanoparticles, thus avoiding unwanted off-target effects in healthy cells.

AUTHOR CONTRIBUTIONS

IP, GC, and AC wrote and edited the manuscript. IP revised the new strategies of breast cancer treatment. GC revised the breast cancer and microRNAs sections. AC revised DNA damage section and the recent findings about microRNAs and DNA-damaging drug responsiveness.

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REVIEW
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The Network of Non-coding RNAs in Cancer Drug Resistance

*Fabio Corrà, Chiara Agnoletto, Linda Minotti, Federica Baldassari and Stefano Volinia**

Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

Non-coding RNAs (ncRNAs) have been implicated in most cellular functions. The disruption of their function through somatic mutations, genomic imprinting, transcriptional and post-transcriptional regulation, plays an ever-increasing role in cancer development. ncRNAs, including notorious microRNAs, have been thus proposed to function as tumor suppressors or oncogenes, often in a context-dependent fashion. In parallel, ncRNAs with altered expression in cancer have been reported to exert a key role in determining drug sensitivity or restoring drug responsiveness in resistant cells. Acquisition of resistance to anti-cancer drugs is a major hindrance to effective chemotherapy and is one of the most important causes of relapse and mortality in cancer patients. For these reasons, non-coding RNAs have become recent focuses as prognostic agents and modifiers of chemo-sensitivity. This review starts with a brief outline of the role of most studied non-coding RNAs in cancer and then highlights the modulation of cancer drug resistance via known ncRNAs based mechanisms. We identified from literature 388 ncRNA-drugs interactions and analyzed them using an unsupervised approach. Essentially, we performed a network analysis of the non-coding RNAs with direct relations with cancer drugs. Within such a machine-learning framework we detected the most representative ncRNAs-drug associations and groups. We finally discussed the higher integration of the drug-ncRNA clusters with the goal of disentangling effectors from downstream effects and further clarify the involvement of ncRNAs in the cellular mechanisms underlying resistance to cancer treatments.

Keywords: non-coding RNAs, chemoresistance, drug sensitivity, miRNA, lncRNA, cancer, gene networks

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Marilena Valeria Iorio, Istituto Nazionale dei Tumori (IRCCS), Italy

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Francesco Russo, Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark
Ajaikumar B. Kunnumakkara, Indian Institute of Technology Guwahati, India

****Correspondence:***
Stefano Volinia
s.volinia@unife.it

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miRNAs AND DRUG RESISTANCE IN CANCER

Chemotherapy represents the primary treatment for both early and advanced tumors. However, drug resistance seriously limits the potency of conventional chemotherapeutics and novel biological agents, this constitutes a major obstacle in the treatment of cancer (1). Then, a lot of effort is aimed to identify new biomarkers, and to assess and predict the response of patients to drugs (2). Cancer drug resistance is referred as intrinsic, if tumors demonstrate to be insensitive to therapeutic agents before treatment, otherwise it is defined acquired if tumor becomes resistant during the treatment. The acquisition of resistance to several types of anticancer drugs can be due to the expression of transporters that eject drugs from cells, resulting in multidrug resistance (3). Nevertheless, several other mechanisms are involved in resistance, including insensitivity to apoptosis induced by drugs, increased repair of damaged DNA, decreased intracellular accumulation of therapeutics, and induction of mechanisms capable of drug detoxification (1). Recent data showed that other than by genetic and epigenetic changes, such as base mutations, amplifications, methylation and

other post-translational modifications, drug resistance might also be due to non-coding RNA (ncRNAs) (4). The bulk of the human transcriptome, excluding the ribosomal and mitochondrial RNA, is represented by non-coding transcripts, including the most studied miRNAs and the newly discovered long non-coding RNAs (lncRNA) (5). MicroRNAs (miRNA) are small non-coding RNA molecules (18–22 nt in length) that act as negative regulators of gene expression through modulation of multiple target mRNAs, by inhibition of translation (6–9). A number of miRNA genes are located within intronic regions of genes, both coding or non-coding for proteins and can be transcriptionally regulated through their promoters (10). Other miRNAs are found either within exons, including 3′ UTRs of mRNAs, or clustered with other miRNA genes (11). Since their discovery (12, 13), the number of annotated miRNAs in the human genome has grown rapidly and they regulate a variety of cellular processes, including apoptosis (14), differentiation (15) and cell proliferation. miRNA deregulation has been demonstrated in cancer (16–19). The role of miRNAs in controlling cellular proliferation, differentiation and apoptosis, and their location at sites of translocation breakpoints or deletions (20), suggests that they might function as tumor suppressors or oncogenes (21–23). Profiles of miRNA expression differ between normal and tumor tissues, and among tumor types (18, 24–27). The association of miRNAs with cancer was first revealed in chronic lymphocytic leukemia (CLL), upon the discovery that miR-15a and miR-16-1 were frequently deleted or down-regulated (16, 28), and that their expression was inversely correlated to that of BCL2 (29). Since then, numerous studies have provided evidence for changes in microRNA expression in oncogenesis: different cancer pathways can converge to affect the same miRNAs and conversely a single miRNA can control an entire transcriptional program, affecting a lot of target genes. The deregulation of miRNAs is linked to cancer progression and clinical outcome (30), and miRNAs have been proposed as potential diagnostic markers, prognostics factors, and therapeutic targets (27, 31–33). When aberrant microRNA expression is directly involved in carcinogenesis (21), the inhibition of selectd microRNAs may have therapeutic implications. Modified antisense oligonucleotides have been designed *ad-hoc* and have proven effective at inhibiting microRNA function *in vivo* in mice (34, 35). The association of microRNA expression with cancer prognosis, therapeutic outcome and response to therapy, independently of other clinical covariates has been documented (25, 26, 36, 37), and selected miRNAs may influence cancer response to chemotherapy (38). The prognostic potential of microRNAs has been demonstrated for CLL (37), lung cancer (39), pancreatic cancer (25), and neuroblastoma (40) among others. One of the firsts observation on a possible link between miRNAs and drug resistance was reported in breast cancer (BC) suggesting that increased sensitivity of patients to anthracycline-based chemotherapy was related to deletion of chromosome 11q, a region containing MIR125B1 (41). The effect of miRNAs on chemotherapy was systematically studied by Blower et al. (42) on NCI-60, a panel of 60 cancer cell lines, used by the National Cancer Institute to screen >100,000 chemical compounds for anticancer drug sensitivity (20, 38, 42). Overall, miRNAs can mediate drug resistance through multiple pathways, including: (i) cell cycle and proliferation control, (ii) survival and/or apoptosis signaling pathways, (iii) DNA repair systems, (iv) specific drug targets, (v) adenosine triphosphate–binding cassette (ABC) transporter proteins, and/or drug metabolism, (vi) the epithelial–mesenchymal transition (EMT) process (4, 6, 43, 44). For example, miR-15b, miR-16 and miR-22 have been documented as mechanisms in chemotherapy resistance (45, 46). Cell cycle deregulation by miRNAs can induce resistance in cancer cells, as confirmed for miR-224 (47). Also, miR-24 and miR-508-5p can directly target enzymes involved in drug metabolism (48, 49). In addition to the mechanisms described above, modulation of epithelial-mesenchymal transition (EMT) can exert an effect on cancer cell resistance. Importantly, once cancer cells undergo EMT, chemo-resistance is increased and metastasis can occur (50, 51). Normal stem cells are already more resistant to drug treatment due to over-expression of drug efflux pumps and anti-apoptotic proteins (52). In this context, miR-34, miR-125b, miR-140, and miR-215 have an important role in conveying drug resistance to cancer stem cells (2). Chemotherapy can induce EMT and modulate the expression of miR-448 to promote cancer cell progression (53); conversely miR-29c or miR-224 have recently been shown to regulate the EMT process (54). miRNome dysregulation in relation to chemotherapy has been described for the most common tumor types: breast, ovarian, lung, prostate, gastric and colon cancer, squamous and hepatocellular carcinoma (HCC), cholangiocarcinoma, neuroblastoma and various types of leukemia (55). Overall, these studies highlight the complexity of adaptive/selective mechanisms in the establishment of resistance to cancer therapies.

lncRNAs AND DRUG RESISTANCE IN CANCER

lncRNAs have been linked to cancer progression and metastasis (56), and recently intensive research has been devoted to the molecular dissection of their roles, as well as to their diagnostic and prognostic significance (57). lncRNAs are mRNA-like transcripts 200 nt to ∼100 kb in length lacking significant open reading frames. lncRNAs can be transcribed by RNA polymerase II (RNA pol II), poly-adenylated and located within nuclear or cytosolic fractions (58). lncRNAs can be divided into different categories: if overlapping with any transcript on sense or anti-sense strand lncRNAs will be classified as (i) sense or (ii) antisense respectively. When its expression is initiated along with a neighboring transcript, sense or antisense, that is proximal, (iii) bidirectional. When deriving from an intronic region, (iv) intronic or (v) intergenic if placed between two genes (53). Generally, lncRNA expression levels appear to be lower than those of protein-coding genes (54), and lncRNAs might be preferentially expressed in specific tissues (59). As to their functions, lncRNAs can regulate the expression of genes in close proximity (cis-acting regulation) or can target distant transcriptional activators or repressors (trans-acting) (53, 60). Their mechanisms of action are still diverse, and have been associated with a spectrum

of biological processes, for example, epigenetics, alternative splicing, nuclear import, structural components, precursors to small RNAs and regulators of mRNA decay (60–63). Thus lncRNAs can regulate cellular functions such as chromosome dosage compensation (64), imprinting (65), cell cycle progression (66) and differentiation (67). Aberrant regulation of lncRNAs is reported in a variety of diseases, including cancer (68–71). Accumulating reports of misregulated lncRNA expression across numerous cancer types suggest that also this class of ncRNA can act in oncogenesis and tumor-suppression (72). A number of useful databases providing molecular information on lncRNAs are available (73). Loss of imprinting and redirecting chromatin remodeling complexes (74), induction of metastasis (75), depletion of miRNAs as "molecular decoy" or "miRNA sponge" (76) and direct inactivation of tumor suppressor genes (77) have been referred to specific lncRNAs. Preliminary studies commenced to report the value of ncRNAs as potential biomarkers in clinical settings (78, 79) and their roles in drug resistance (80).

A NETWORK ANALYSIS: THE MOST CENTRAL ncRNAs IN CHEMORESISTANCE

In recents years, an increasing number of studies have been reported on ncRNAs, target gene modulation, and affected drug functions, pharmacogenomics or chemoresistance. With the aim to facilitate the classification of ncRNAs and drug targets, some databases have been developed, such as NRDT (81) or PharmacomiR (82), collecting all information about ncRNA-target gene-drugs. There are large numbers, and growing, of both ncRNAs and cancer drugs, thus the combinations between members of the two groups are very difficult to manage in a traditional review or interpretate in a database. Therefore we decided to use machine-learning systems and to study the RNA-drug interactions using a network-based approach. Basically, we took from KEGG database all approved drugs used for cancer therapy. Then, we searched in PubMed all recent studies (published from 2011 onwards) investigating ncRNAs in chemoresistance. This selection was performed by batch analysis of PubMed-NCBI (National Center for Biotechnology Information) using as major topics the drugs from KEGG, ncRNA and chemoresistance. The result of this screening was manually curated in order to avoid and remove papers with generic statements and not direct links between ncRNAs and drugs. Only the investigations that proved (by *in vitro*/*in vivo*) experiments the existence of a direct association between ncRNAs and chemoresistance were then analyzed using a machine-learning tool. We thus built a network of non-coding RNAs starting from a human-curated selection of papers and applied an *ad-hoc* data mining approach to dissect the network and identify the most important ncRNA/cancer drugs interactions and cliques. We obtained a fully connected network of 388 drug/ncRNA interactions (edges) and 5 unconnected pairs (**Supplementary Image 1**). We then went on with studying the network, which had 227 nodes: 150 miRNAs, 35 lncRNAs and 42 drugs. Three graph theory measures were considered to define the most relevant non-coding RNAs associated to therapeutics resistance: (i) degree, indicating the number of links that an ncRNA had with different nodes (here drugs) (ii) betweenness centrality, a measure of centrality in the network based on shortest paths (iii) closeness centrality, related to the distance between the ncRNA and all the other nodes in the network. Then, we ranked the nodes (drugs and ncRNAs) and edges (combinations) in the network and collected the combinations from ncRNAs with a degree >3 and a central position (closeness centrality > 0.26 and betweenness centrality >0.003) (**Figure 1** and **Supplementary Table 1**). Finally, we performed a community structure analysis using Glay and Cytoscape (83) to identify different clusters of ncRNAs and drugs. The clusters were converted to subnetworks for convenient visualization. The visual separation of clusters was improved by overlaying the community structure on a graphic layout addressing specific topology (**Figure 2**).

We wish to add a cautionary note to our reviewing effort. Even in the genome-wide studies (a minority among those we included in this review) for a number of conscious or unconscious reasons, scientists often end up chasing the most "popular" ncRNAs among others of "lesser pedigree." Thus there is potentially a positive bias toward well-known ncRNAs in the overall scheme, and therefore in the final network. For this reason, we decided to keep all associations and, although the "degree" (number of associated drugs for an ncRNA) is important, we tried to avoid biased selections and included in our review all ncRNAs/pairs.

Here we commence with describing the ncRNAs that are most prominent in relation to chemoresistance, as detailed in (**Figure 1**).

miR-21 has the highest scores (degree, betweenness centrality and closeness centrality) as it was associated with several drugs. The MIR21 gene is located at17q23.2, a region frequently amplified in several tumors (84, 85). Its overexpression has been observed in most cancer types and modulates the resistance toward apoptosis-inducing drugs (86–91). Down-regulation of miR-21 sensitizes cancer cells *in vitro* to different chemotherapeutics, including cisplatin, etoposide and doxorubicin (92–94). On the other hand, some drugs can induce alterations in miR-21 levels: e.g. soladosine can inhibit lung cancer cell invasion through miR-21 down-regulation, via PI3K/Akt signaling pathway (95). Interestingly, exogenous Epstein Barr virus modulates the PI3K/Akt pathway through LMP1, thus enhancing miR-21 expression and contributing to cisplatin reduced response in nasopharyngeal carcinoma (96). Moreover, miR-21 delivered by exosomes augmented malignancy in recipient cells and conferred paclitaxel resistance to ovarian cancer cells (97). There was also a report for enhancement of anticancer activity when Cao et al. reported that miR-21 induction sensitized gastrointestinal tumor cells to imatinib (98).

miR-34a was reported to be downstream of p53 and to function as a tumor suppressor (99). It is down-modulated in colorectal cancer (CRC) (100). In 5-Fluorouracil (5-FU)-resistant colon cancer cells ectopic expression of miR-34a inhibited cell growth and attenuated the resistance to 5-FU through down-regulation of SIRT1 and E2F3 (101), inhibition of LDHA (102) and of c-Kit, thus reducing stem cell factor (SCF)-induced

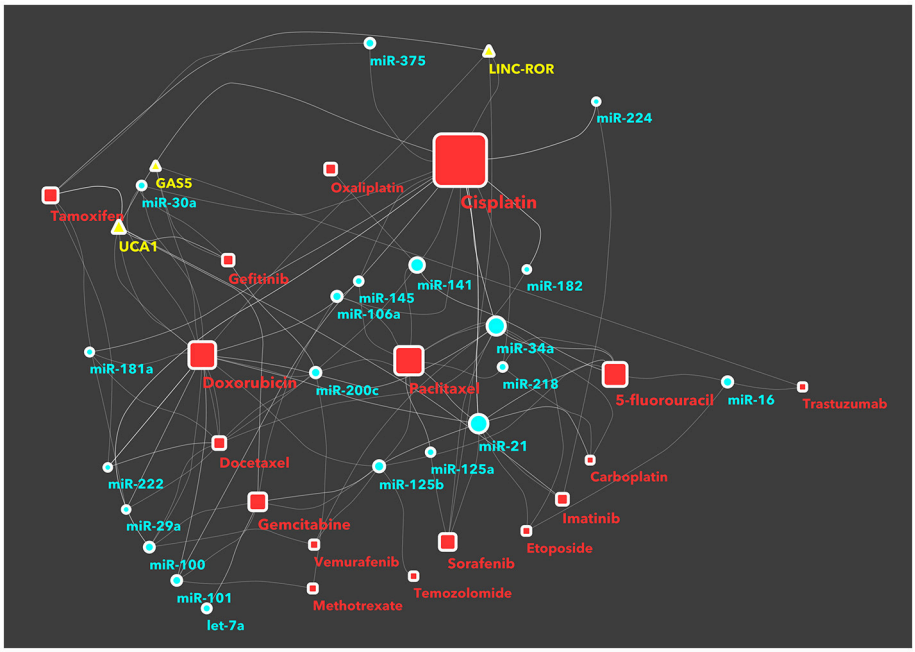

FIGURE 1 | The network of non-coding RNAs and anti-cancer drugs. Each link between a drug and an ncRNA indicates a study in literature, investigating on the specific chemoresistance involvement of that ncRNA in cancer. The nodes (a ncRNA or a drug) shown in this figure have a degree >3, a central position in the network (expressed as betweenness centrality in the network description) >0.003, or a closer position relative to the companion drug (expressed as closeness centrality >0.26). The full network, with all nodes, is reported in supplemental information as **Supplementary Image 1**. Drugs are represented as red squares, miRNAs as light blue circles and lncRNAs as yellow triangles. The size of a node is proportional to its betweeenness centrality, while the size of a node name is proportional to its degree.

migration/invasion (103). Yang et al. demonstrated that miR-34 targets BCL2 and sensitizes HCC cells to sorafenib (104). In osteosarcoma cell lines miR-34a has been tested in combination with celecoxib: the treatment showed decreased cell viability, migration and invasion through regulation of the Notch1/ROCK1-PTEN-Akt-GSK-3β axis (105). Moreover, miR-34 could enhance the therapeutic efficacy of paclitaxel in resistant prostate cancer (106). Its overexpression enhanced cisplatin sensitivity, as confirmed in gastric cancer, by targeting MET (107) and in lung cancer, through the p53/MICN axis (108). Conversely, Pu et al. found that miR-34a overexpression in osteosarcoma promoted resistance to several drugs (doxorubicin, etoposide, carboplatin, cisplatin), via repression of AGTR1 (109).

The **lncRNA Urothelial Cancer-Associated 1 UCA1** gene is located at 19p13.12 (110). Different transcriptional isoforms have been reported, UCA1 (1.4 kb), UCA1a (2.2 kb) and CUDR (2.7 kb), generated by alternative splicing and poly-adenylated. UCA1 is the most abundant isoform in various malignant tumors such as bladder cancer, BC and HCC (110–113). UCA1 could promote drug resistance by directly binding to miR-204, miR-18a and miR-16 (114). UCA1 emerged as a competitive endogenous RNA (ceRNA) of multi-drug resistance associated protein 1 (MDR1), inducing resistance to imatinib in CLL cells by sequestering miR-16 (115). Overexpression of UCA1 up-regulated MDR1, resulting in imatinib resistance, whereas its silencing had the opposite effect (116). In bladder cancer, UCA1 enhanced chemoresistance to cisplatin by regulating Wnt signaling (117) and to cisplatin/gemcitabine through modulation of miR-195a (118). Recent studies reported that UCA1 regulates tamoxifen resistance in BC (119). Liu et al. demonstrated that the knockdown of this lncRNA could revert resistant phenotype and increase tamoxifen sensitivity through inhibition of the Wnt/β-Catenin pathway, thus further confirming the oncogenic role of UCA1 in BC (120). Moreover, UCA1 was shown to be released in exosomes by tamoxifen resistant BC cells and increased tamoxifen resistance in ER-positive recipient cells (121).

The members of **miR-125** family (miR-125a, miR-125b-1 and miR-125b-2) play an important role in tumorigenesis and are potential biomarkers for cancer diagnosis, treatment and prognosis in clinical settings (122). MIR125A gene is on chromosome 19, while two separate loci on chromosomes 11 and 21 harbor MIR125B1 and MIR125B2, respectively (123). miR-125b expression has been found negatively correlated with 5-Fluorouracil resistance in HCC (124), while resistance to pharmacological treatments with gentamicin, cetuximab, doxorubicin and temozolomide by miR-125b still remains controversial (88, 125–127). miR-125b regulates the resistance to paclitaxel in colon cancer cells, in association with miR-125a (128). Recent data strongly supports a relevant role for miR-125b in conferring taxol resistance in BC, via suppression of pro-apoptotic BCL2 antagonist killer 1 (Bak1) (129). In contrast,

in chondrosarcoma, overexpression of miR-125 enhanced the sensitivity to doxorubicin by directly targeting ERBB2-mediated glucose metabolism (130). miR-125a overexpression increased the response to paclitaxel in cervical cancer, through STAT3 down-modulation (131). Sorafenib treatment in HCC showed restoration of mir125 levels by sirtuin-7 and p21/p27 signaling blockage inhibiting cell cycle progression (132). In AML cells, via mubritinib, miR125a inhibited the ERBB pathway and cell cycle proliferation and progression, suggesting that miR-125a increased the sensitivity to the drug (133).

The MIR100 gene is at 11q24. Deregulation of **miR-100** has been reported in drug resistance; however, miR-100 expression can be either over-expressed or under-expressed in diverse cancers (134). In ovarian cancer, miR-100 targets mTOR therefore reverting the cell's chemoresistance toward cisplatin (135) and chondrosarcoma (136). In pancreatic cancer, miR-100 mimics inhibit proliferation and increase sensitivity to cisplatin by targeting FGFR3 (137). Recently, it has been shown that down-modulation of miR-100 could increase β-tubulin class V expression, promoting tumor cells proliferation, with implications for paclitaxel resistance (138). Also, miR-100 reduced ATM levels in a human glioma cell line (M059J) and could sensitize tumor cells to ionizing radiation (139). *In vitro*, miR-100 also induced the differentiation of BC stem cells expressing a functional ER (140). Furthermore, in CRC cells miR-100, together with miR-125b, negatively regulated Wnt/β-catenin signaling, and restored responsiveness to cetuximab (125). On the other hand, in mutant p53 pancreatic carcinoma, miR-100 up-regulation was related to gemcitabine resistance (88). In accordance, the exosomes-mediated intercellular transfer of miR-100, from drug resistant BC cells, could lead to resistance in sensitive cells (141).

miR-200c acts as a tumor suppressor, and could inhibit the initiating steps of metastasis; a negative correlation with *ZEB* factors has been reported, suggesting that this miRNA-mediated regulatory pathway influences EMT (142–147), potentially modulating drug resistance in advanced tumors. miR-200c reverses resistance of lung cancer cells, both to chemotherapeutics, like methotrexate (148), and to targeted drugs, like crizotinib (149) and gefitinib (146, 150). In breast and renal cancers, miR-200c could be involved in resistance or re-sensitization to microtubule-targeting drug (151–153).

miR-141 is another member of the miR-200 family, also involved in EMT, invasion, migration and drug resistance (154). miR-141 overexpression contributes to acquired chemoresistance, for both *in vitro* and *in vivo* models. The initiation factor 4E (EIF4E) mRNA is a target of miR-141, that is involved in drug-induced apoptosis, conferring resistance to docetaxel-sensitive BC cells (155). miR-141 regulates cisplatin sensitivity in non-small lung cancer cells via PDCP4 inhibition and its inhibition increases cisplatin-induced apoptosis (156). In oesophageal squamous cell carcinoma, miR-141 was highly overexpressed in 5-Fluorouracil and oxaliplatin resistant cells and contributed to acquired chemo-resistance via PTEN (157). Moreover, in HCC cells, miR-141 was shown to confer resistance to 5-Fluorouracil through the inhibition of KEAP1, thereby reactivating the NRF2-dependent antioxidant pathway (158). Li et al. discovered that miR-141 together with other miRNAs like miR-16 contribute to prostate cancer chemoresistance via an exosome network (159).

Two homologous microRNAs, **miR-221** and **miR-222,** are generally considered having an oncogenic activity (160). The expression of miR-221 and miR-222 is highly up-regulated in HER2/neu-positive human BCs resistant to endocrine therapy, compared with HER2/neu-negative tissue samples (161); also, in BC patients miR-222 is elevated in chemoresistant tissues after surgery, compared with the pre-neoadjuvant samples (162). miR-221/222 reduce the protein level of the cell cycle inhibitor p27Kip1, conferring tamoxifen (161) and doxorubicin resistance (162). Also, secreted miR-221/222 could serve as signaling molecules and mediate communication of tamoxifen resistance (163). Aberrant expression of miR-222 is tightly related to poor overall survival (164) and affect oncogenic signaling pathways associated with resistance to different drugs (165). miR-222 also mediated BC cells resistance to adriamycin via PTEN/Akt/FOXO1 (164). Furthermore, the exosome mediated release of miR-222, miR-100 and miR-30a contributes to the same effect on docetaxel and doxorubicin: loss of responsiveness in BC cells (141). In oesophageal and prostate cancers, miR-221 could modulate 5-Fluoruracil resistance via the Wnt/β-catenin-EMT pathway (166) or RB1 (167), respectively.

miR-101 (168, 169) has a relevant role in autophagy. Targeting the autophagy process is a promising therapeutic strategy to improve chemotherapy efficiency. In BC cells miR-101 inhibits basal autophagy, as well as etoposide- and rapamycin-induced autophagy, thus sensitizing cancer cells to 4-hydroxytamoxifen (4-OHT)-mediated cell death (170). In HCC, miR-101 sensitizes cell lines to cisplatin-induced apoptosis by targeting Mcl-1 (171). Likewise, miR-101 inhibits autophagy and enhances chemo-sensitivity to doxorubicin of osteosarcoma cells *in vitro* (172). In pancreatic cancer, miR-101 up-regulation reverts gemcitabine resistance by inhibiting the expression of ribonucleotide reductase M1 (RRM1) (173). Moreover, recent studies demonstrate that miR-101 interacts with lncRNA MALAT1 in regulatory networks that modulate cisplatin and temozolomide resistance, in lung cancer (174) and glioblastoma (80), respectively.

The **miR-15/16** gene cluster in chromosome 13 (13q14) is deleted or down-regulated in some cancer types (21). This somatic alteration was reported to occur early in cancer development and could represent a target for intervention (21). miR-16 expression is affected by several drugs: in gastric cancer cell lines etoposide and 5-Fluorouracil could increase the levels of miR-16, both *in vitro* and *in vivo*, and the up-regulation of miR-16 is modulated by p38 MAPK signaling pathway (175). In BC, lapatinib and trastuzumab are reported to regulate miR-16 via PI3K/Akt (176). Noteworthy, the altered expression of both miR-15a/16-1, due to the CXCR4 inhibitor BL-8040 induced the apoptosis of AML blasts by down-regulating ERK, BCL2, MCL1 and cyclin-D1 (177).

The lncRNA **GAS5**, originating from the Growth Arrest-Specific 5 gene, is down-regulated in multiple cancers. GAS5 inhibits proliferation and promotes apoptosis, thus playing a tumor suppressor role (178). Several studies confirmed GAS5

as an mTOR effector, and its expression was directly correlated with chemoresistance. Thus, enhancing GAS5 expression may improve the effectiveness of rapalogues, as confirmed both in prostate tumor cells and in mantle cell lymphoma cells (179, 180); also, the down-modulation of GAS5 caused resistance to trastuzumab in BC (181). In lung adenocarcinoma cells resistant to EGFR inhibitors, GAS5 enhance gefitinib-induced cell death, via down-regulation of IGF1R (182). Lastly, in bladder transitional cell carcinoma GAS5 inhibited malignant proliferation and chemotherapy resistance to doxorubicin, partly acting via BCL2 (183).

miR-106a, a member of the miR-17 family, is associated with poor prognosis, invasion and metastasis (184). In ovarian cancer (OV), miR-106a inhibited cell survival and cisplatin resistance, through downregulation of MCL1 (185); conversely expression of miR-106a was higher in cisplatin-resistant OV. miR-106a may be involved in the modulation of cisplatin-induced apoptosis by regulating PDCD4 (186). In non-small cell lung cancer, miR-106a also confers cisplatin resistance, by targeting adenosine triphosphatase-binding cassette A1, an ABC transporter (187). Otherwise, by targeting autophagy, miR-106a enhances sensitivity of lung cancer cells to SRC inhibitors, including saracatinib and dasatinib, expliciting once more the context-dependent function of miRNAs (188). Further, dysregulation of miR-106a conferred resistance to paclitaxel in OV; its modulation resensitized resistant cells by targeting BCL10, caspase-7, and ZEB1 (189). Down-modulation of miR-106a was reported in gentamicin resistant hepatoma, participating to EMT via the PDGF-D/miR-106a/Twist1 pathway; notably, in HCC patients, miR-106a and Twist1 were associated with PDGF-D expression (190).

miR-375 is involved in a positive feedback loop with ER in BC (191) and its re-expression is sufficient to sensitize tamoxifen-resistant cells. Furthermore, miR-375 partly reversed the EMT process: metadherin (MTDH) was identified as a direct target of miR-375 and tamoxifen-treated patients with higher MTDH had a higher risk of relapse (192). Another miR-375 target is HOXB3; miR-375 inhibited cancer stem cells (CSCs) phenotype and tamoxifen resistance by regulating CSCs, through degradation of HOXB3 (193). Epigenetically down-regulated miR-375 in HER2-positive BC could induce trastuzumab resistance by targeting IGF1R (194). 9-cis retinoic acid (Alitretinoin) modulated the expression of miR-375 in BC depending on ER status: thus, miR-375 was inhibited in ERα-positive cells while highly induced in ERα-negative cells (195). The deregulation of miR-375 was also observed in other malignancies: in medullary thyroid carcinomas (MTC) miR-375 was the strongest up-regulated miRNA (196). Vandetanib is a tyrosine kinase inhibitor for the treatment of patients with recurrent or metastatic MTC that are unresectable, and/or symptomatic (197). Interestingly, miR-375 over-expression associated with SEC23A down-regulation could improve the efficacy of vandetanib (196). Thus, the expression levels of miR-375 and SEC23A pointed to vandetanib sensitivity and could be evaluated as predictive indicators for efficacy of vandetanib in MTC. Analogously, up-regulation of miR-375 increased the cisplatin-sensitivity of gastric cancer cells by regulating ERBB2 and phospho-Akt (198).

A role in chemoresistance modulation has emerged for putative tumor-suppressor **miR-145** (199). miR-145 targeting of MDR1 helps to restore drug efficacy in resistant cells and *in vivo* models of bladder cancer and BC (200, 201). Moreover miR-145 confirmed its role in reducing chemoresistance also with paclitaxel (202) and doxorubicin (203), possibly via regulation of EMT.

miR-218 has a physiological role in neuron development and its loss of expression is involved in neurodegeneration (204). In BC, it acts as a risk factor in ductal carcinoma *in situ* (DCIS) (205). In association with platinum compounds, miR-218 and miR-205 inhibit tumorigenesis and overcome chemoresistance in lung cancer (206). In prostate cancer, miR-218 up-regulation inhibited tumor growth and increased chemo-sensitivity to cisplatin, by negatively regulating BCAT1 (207). Furthermore miR-218 mediated autophagy and was associated with positive response to paclitaxel in resistant endometrial carcinoma (208). It also promoted apoptosis and caused cell cycle arrest in CRC by targeting BIRC5, thus possibly enhancing first-line 5-FU treatment. Also, miR-218 through targeting the enzyme thymidylate synthase (TS), enhanced 5-FU cytotoxicity in CRC cells (209).

The **let-7** family members are down-regulated in lung (210), gastric (211), colon cancer (212) and in Burkitt's lymphoma (213). Loss of let-7 was associated with the shortened post-operative survival of patients with lung cancer (210). The altered expression of let-7a could increase chemoresistance to epirubicin (214) and cytarabine (215). Furthermore, let-7a expression has demonstrated to influence chemoresistance, due to maintained treatment with gemcitabine, in pancreatic cancer patients (216, 217). Several studies have reported that let-7a acts as a tumor suppressor in renal cell carcinoma (RCC), by targeting c-Myc (218). let-7b and let-7e are down-regulated in glioblastoma and ovarian cancer, respectively and promote resistance to cisplatin by acting on the same target Cyclin D1 (219, 220). Reduced levels of both let-7b and let-7c could determine the intrinsic chemoresistance to 5-FU in RCC, possibly via AKT2 (221). Clinically, 5-FU-based chemotherapy is considered moderately effective in RCC due to rare response and severe toxicity (222); transfection of let-7b or let-7c potentiated the efficacy of 5-FU *in vitro* at tolerable concentrations. Moreover, let-7c up-regulation contributed to sensitize lung cancer cells with acquired cisplatin resistance, by involving ABCC2 and Bcl-XL (223). Interestingly, a combination of **miR-224 and let-7i**, reduced imatinib resistance in CML, probably through targeting the ST3GAL IV sialyltransferase (224).

miR-30a was found to act as an oncosuppressor, but could also promote tumor progression in several types of cancer (225). The same dual activity was described for drug resistance. In ovarian and lung carcinoma miR-30a interacted with cellular receptors (EDNRA and EGFR) and played an important role in overcoming the acquired resistance (226, 227), also via exosomes (141). **miR-181a** is down-regulated in glioma and lung cancer, while its up-regulation is involved in metastasis and invasion in breast and oral squamous carcinomas (228). Prostate cancer patients undergoing maintained treatment with taxane develop resistance to the therapy. Recently, Armstrong

et al. discovered that miR-181a overexpression contributes to docetaxel and cabazitaxel resistance in prostate cancer cells (229). The role of miR-181a in cisplatin resistance is apparently dual: in cervical squamous cancer, it could induce chemoresistance, partly by down-regulating PRKCD (230), while it could reverse cisplatin resistance in tongue squamous cell carcinoma, acting through Twist1 (231).

miR-182 is overexpressed in a broad range of tumor types. Clinical studies associated miR-182 with increased aggressiveness and poor survival (232). miR-182 was also found to have a role in chemoresistance. Acting as a negative regulator of PDCD4, it determined a reduction of sensitivity to cisplatin and paclitaxel in OV (233) and to cisplatin in lung cancer (234). Further, in HCC miR-182 was directly correlated *in vitro* and *in vivo* with cisplatin resistance, possibly by regulating TP53 (235).

In inflammatory bowel disease (IBD) and in cancer, **miR-224** has an important function. By targeting p21, it participated in cell cycle regulation at the G1/S checkpoint (236). miR-224 could induce resistance to cisplatin in lung and ovarian cancer cell lines (47, 237). In contrast, miR-224 promoted cisplatin sensitivity in

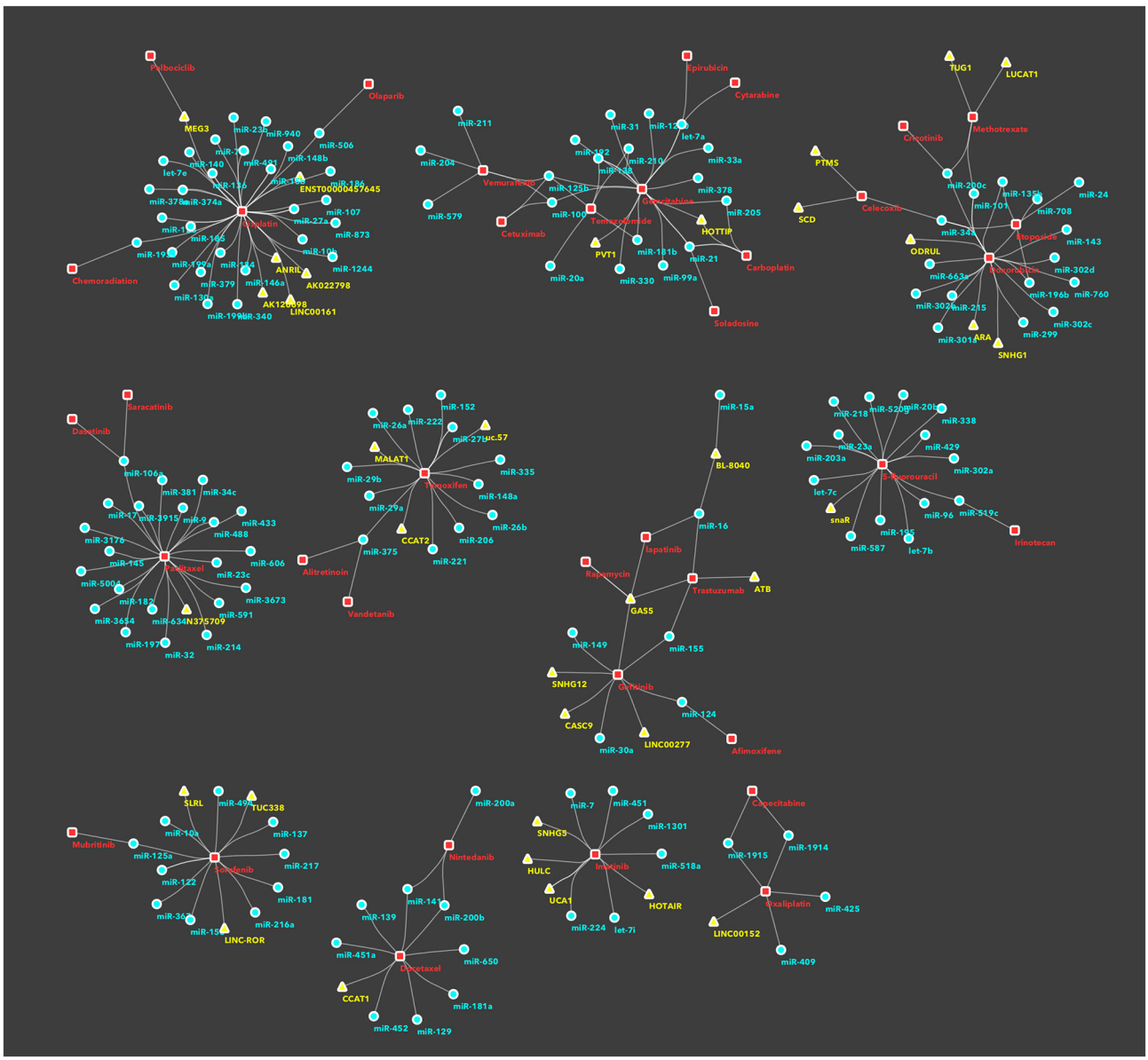

FIGURE 2 | Subnetworks/clusters of non-coding RNAs/drugs associations, according to community analysis. This figure depicts disjoint subnetworks corresponding to the different clusters in the whole network (**Supplementary Image 1**) and identified using the community analysis tool in Cytoscape. This is a simplification of the involvement of ncRNAs in drug resistance, as an ncRNA, or a drug, is represented only in a single cluster/subnetwork. For sake of completeness, all interactions are described in the main text and presented in the **Supplementary Table 1**.

osteosarcoma resistant cells by targeting Rac1 (238). miR-224 was related with CRC progression and the response to 5-fluorouracil through KRAS-dependent and -independent mechanisms (239).

Finally, **miR-29** family members are miRNAs that can play different roles in cancer (240). For example, they can contribute in BC to the acquisition of doxorubicin resistance by inhibition of PTEN/AKT/GSK3β (241). Conversely, miR-29b exerts a tumor suppressor activity in tamoxifen-resistant BC cells (242).

The lncRNA Regulator Of Reprogramming **LINC-ROR** is involved in the regulation of the pluripotent stem cells reprogramming. Its expression suppresses the induction of p53 after DNA damage and is associated with tumor progression, EMT and metastasis (243). LINC-ROR is significantly up-regulated in BC, resulting in chemotherapy tolerance and enhanced invasiveness (244). In tamoxifen-resistant BC cell lines, down-regulated LINC-ROR could inhibit EMT and enhance the sensitivity to tamoxifen by increasing miR-205 (245). A relevant study on cancer tissues from BC patients demonstrated that inhibition of LINC-ROR reversed resistance to tamoxifen by inducing autophagy (246). Moreover, LINC-ROR could mediate for sorafenib chemosensitivity in HCC, through the realease of extracellular vesicles (247).

DRUGS/NON-CODING RNAs SUBNETWORKS

Non-coding RNAs can regulate several protein targets or molecular pathways that lead or inhibit drug resistance according to tumor type, stage and class of drug (248). Above we discussed the ncRNAs with the most prominent roles in the literature as measured using network statistics. There are though many ncRNAs which have been described only in association to one or few more drugs: for these rare ncRNA/drug combinations we performed a clustering analysis of the whole network and identified less than a dozen of groups. The ncRNA/drug combinations are described below as subnetworks and are visualized in (**Figure 2**). The **Supplementary Table 2** details the effects of ncRNAs on chemoresistance.

Subnetwork 1: Gefitinib, Afimoxifene, Rapamycin, Trastuzumab, Lapatinib, BL-8040

Gefitinib is a selective inhibitor of the Epidermal Growth Factor (EGFR) protein. It is used to treat solid tumors, as non small cell lung cancer (NSCLC). It acts by inhibiting the anti-apoptotic Ras signaling cascade (249). Recent studies confirmed also that the loss of regulation of ncRNAs is involved in chemoresistant acquisition (250, 251). The GAS5 lncRNA is implicated in chemoresistance modulation of several different drugs included into this subnetwork (179–182). Another interesting lncRNA present in this group is the Small Nucleolar RNA Host Gene 12 (SNHG12), that plays an oncogenic role in various cancers (252). Moreover, SNHG12 overexpression is implicated in multidrug resistance (included gefitinib resistance),by sponging miR-181a and thus activating the MAPK/Slug pathway (253). This confirms also the involvement of miR-181a in the regulation of chemoresistance. miR-16 has been previously described, and in cancers it may regulate the response to trastuzumab and lapatinib. This miRNA plays an important role in inhibiting cell proliferation and potentiting drug effects (176). Furthermore, in leukemia miR-16 in combination with miR-15 interacts with new phase II drug (177). miR-124 has a role in neuronal differentiation (254) and may modulate resistance to gefitinib and afimoxifene: miR-124 down-regulation could reverse afimoxifene induced autophagy in BC through regulation of Beclin-1 protein (255), while in lung cancer miR-124 depletion plays a role in gefitinib resistance by regulating SNAI2 and STAT3 expressions (256). A prolonged treatment with Gefitinib dramatically reduced the expression of miR-155 and miR-200c. The depletion of these miRNAs may contribute to the decrease in the sensitivity to gefitinib (150). Intriguingly, trastuzumab positively regulates miR-155 and as a consequence, this micro RNA negatively regulates ErbB2 and the malignant cell transformation of breast epithelial cells (257).

Subnetwork 2: Cisplatin, Olaparib, Palbociclib, Chemoradiation

Cisplatin is a platinum compound classified as alkylating like agent that interferes with DNA replication and is used to treat several solid malignancies (258). The efficacy of cisplatin in cancer therapies is limited by the acquired resistance, that can lead to therapeutic failure and tumor recurrence (259). It was demonstrated that cisplatin-resistant cancer cells present an altered expression pattern of ncRNAs (260–281). Among them, miR-451 is known to exert a critical role in the pathogenesis and the development of several types of cancers, including CRC, glioblastoma and NSCLC. miR-451 is located on chromosome 17q11.2, in close proximity of ERBB2 (17q12) (282). miR-451 enhances cisplatin sensitivity in lung cancer cells through regulation of Mcl-1 (283); furthermore, it is involved in the resistance to imatinib in CML patients (284). Another ncRNA present in this network is miR-20a, a member of miR-17 family, which has an oncogenic role and is involved in leukemia and CRC (285). Moreover, Zhou et al. established that miR-20a expression in glioma cells was negatively correlated to Temozolomide sensitivity by targeting DNA methyltransferase (DNMT1) (286). In gastric cancer, miR-20a negatively regulates cylindromatosis (CYLD) expression, thus inducing cisplatin resistance (287). miR-15b had a dual role in oral tongue squamous cell carcinoma (TSCC) and lung adenocarcinoma; through the regulation of TRIM14 it was implicated in the reversion of cisplatin resistance in TSCC (288), while it decreased sensitivity to cisplatin by targeting PEBP4 in lung adenocarcinoma (289). Similarly, Chen et al. discovered the involvement of miR-136 as a tumor suppressor, which targeted E2F1 gene and reversed cisplatin resistance in glioma cells (290). On the contrary, in ovarian cancer miR-136 might induce chemoresistance through the inhibition of apoptosis, while promoting the repair of cisplatin-induced DNA damage (291). miR-27 has a well-defined role: in lung adenocarcinoma cells it contributed to cisplatin resistance by suppressing Raf Kinase Inhibitory Protein RKIP (292). Strikingly, in esophageal

cancer miR-27 was associated with the transformation of normal fibroblasts to cancer-associated fibroblasts (293). The same ncRNA could have a role in the sensibilization to different drugs: e.g., miR-506-3p, which is up-regulated in ovarian cancer, has an important function in sensitizing cancer cells to both olaparib and cisplatin (294). Another interesting example is miR-193a-3p that can contribute to the inhibition of chemoradiation and of cisplatin resistance through PSEN1 and p73, respectively in esophageal tumor (295) and osteosarcoma (296). These findings confirmed an oncosuppressor activity for miR-193a-3p (297). miR-199 also may act as either a potential tumor suppressor or oncogene depending on cellular context (298). Consequently, epigenetic silencing of miR-199b-5p may contribute to raise cisplatin resistance via loss of control in cell cycle regulation (299) or miR-199a-3p may enhance cisplatin sensitization by downregulating TFAM (300). Interesting situations emerged when comparing miRNAs from the same family: i.e. down regulation of both let-7 members (let-7b and let-7e) controlled cisplatin resistance through down-modulation of cyclin D1 (219, 220). lncRNAs are an eterogeneous class of non coding RNAs and several studies demonstrated that their dysregulation could affect chemoresistance modulation as much as miRNAs (301–303). Maternally expressed 3 (MEG3) lncRNA that acts as a growth suppressor in tumor cells and selectively regulates p53 target (304), does not have a defined role in chemotherapy. Nevertheless, its up regulation seems to enhance cisplatin resistance in lung cancer (305). Meanwhile, palbociclib can determine the increment of MEG3 expression in a dose dependent manner, yielding to an increase anticancer outcome (306). Controversely, lncRNAs might also modify drug responsiveness exerting a miRNAs sponge activity acting as ceRNAs. Wang et al. demonstrated that downregulation of ANRIL lncRNA enhanced cisplatin citotoxicity via let-7a in nasopharyngeal carcinoma (307). These findings further confirm the role of let-7 family as inhibitors of chemoresistance.

Subnetwork 3: Paclitaxel, Saracatinib, Dasatinib

Subnetwork 3 incorporates several non-coding RNAs related with paclitaxel. This antineoplastic drug is a taxol derivative that blocks cell cycle progression by targeting beta-tubulin. Paclitaxel causes inhibition of mitosis and triggers the apoptotic process or the reversion of cell cycle. Paclitaxel is used to treat a number of solid cancers that include lung, ovarian, breast and pancreatic tumors (308). A number of studies produced evidence that loss of non-coding RNAs regulation can modify chemoresistance to taxol (202, 309–313). miR-182 is often up-regulated in cancers; it can enhance cell proliferation, invasion and it plays an important role in drug resistance. Two different studies found that miR-182 overexpression, by negatively regulating programmed cell death 4 (PDCD4), was involved in chemoresistance exacerbation of lung and ovarian cancers to cisplatin and paclitaxel, respectively (233, 234). Qin et al. demonstrated also that miR-182 expression increases cisplatin resistance of HCC cell by targeting TP53INP1 (tumor protein 53-induced nuclear protein 1) (235). miR-214, through targeting activating protein 2 (AP-2), contributes to regulate molecular processes in melanoma (314). Despite its role, miR-214 function in chemoresistance is still not clear: it could enhance sensitivity to cisplatin in esophageal cancer (315), or promote paclitaxel and carboplatin resistance in ovarian cancer (89). miR-9 may influence cell growth, cell cycle and it is often downregulated in cancer (316). miR-9 down-regulation is one of the key mechanisms accounting for paclitaxel resistance in ovarian carcinoma (317); while high expression of miR-9 in CD133+ glioblastoma cells activates MDR1 gene and imparts Temozolomide (TMZ) resistance (318). miR-17-5p isan oncogenic miRNA, member of the miR-17∼92 cluster, which plays an important role in the control of cell cycle progression (319). Despite its oncogenic role, miR-17-5p can promote paclitaxel-induced apoptosis by increasing p53 expression in BC cells (320). The same ncRNA may also influence resistance to different drugs. It is the case of miR-106a that can enhance paclitaxel resistance through apoptosis inhibition (189) or promote sensitivity of lung cancer cells to Saracatinib and Dasatinib (188). In addition, the secretion of miRNA in exosomes is involved in paclitaxel resistance of prostate cancer (159).

Subnetwork 4: Sorafenib, Mubritinib

To treat HCC in advanced status the multikinase inhibitor Sorafenib is the only validated therapy, but tumor response rates to this drug are quite low (321). Several miRNAs, including miR-137 (322), miR-367-3p (323), and miR-125a (131, 133) or lncRNA such as LINC-ROR (247) are involved in the regulation of HCC-Sorafenib treatment efficacy. Tang et al. demonstrated that the simultaneous silencing of miR-21, miR-153, miR-216a, miR-217, miR-494, and miR-10a-5p overcome sorafenib resistance *in vitro* and *in vivo* models of HCC (324). Azumi et al. found also that up-regulation of miR-181a increased sorafenib resistance, by blocking a MAPK signaling factor (RASSF1) in HCC cells (325). miR-122 is highly expressed in the liver, where it has been implicated as a regulator of fatty-acid metabolism. This ncRNA was significantly reduced in sorafenib-resistant HCC cells. Xu et al. demonstrated that miR-122 restoration increases sensitivity to sorafenib and induces apoptosis by repressing IGF1R (326). miR-122 is also involved in the control of arginine transport by targeting the solute carrier family 7 (SLC7). Arginine is the substrate for nitric oxide (NO) synthetase and as a result, loss of miR-122 in HCC cells causes an increment of intracellular NO and resistance to sorafenib (327). Moreover, knock-down of TUC338 lncRNA increased expression of RASAL1 protein in HCC, inhibited tumor growth and sensitized cells to sorafenib (328). Sorafenib is also used in the treatment of renal carcinoma (RCC), where SRLR (sorafenib resistance-associated lncRNA in RCC) was found up-regulated in sorafenib-resistant RCCs and contributed to sorafenib tolerance (329).

Subnetwork 5: Docetaxel, Nintedanib

Docetaxel is a drug that promotes cell apoptosis after its interaction with beta-Tubulin metabolism and Bcl-2 phosphorylation. It is used to treat late-stage and metastatic BC, head and neck cancer, stomach cancer, prostate cancer

and NSCLC (330). This subnetwork underlines the role of miR-129, a miRNA with tumor suppressor activity in several cancers (331). Lu et al. confirmed the role of this miRNA also in reducing drug resistance: miR-129 in gastric cancer cells reverses cisplatin-resistance through inhibition of P-gp expression (332). Nevertheless, another study demonstrated that miR-129 overexpression may be implicated in BC and docetaxel resistance, mainly through CP110 inhibition (333). Up-regulation of miR-141 and miR-181a (155, 229) also could contribute to docetaxel resistance, while down-regulation of miR-29a and miR-451 inhibited this process (334). Similarly to miR-200c, miR-200b has also a role in drug response: loss of miR-200b regulated autophagy in lung adenocarcinoma and was associated with resistance to docetaxel (335). Nintedanib inhibited VEGFR and consequently angiogenesis (336, 337). Nintedanib is also capable of reverting the resistance to gefitinib promoted by miR-200b and miR-141 (338). Dongqin et al. found that miR-451 down-regulation induced c-Myc expression, an event related to docetaxel-resistance (339). The role of miR-139 in cancer is still not clear (340), but by targeting NOTCH1, it could mediate cell sensitivity to docetaxel and 5-FU, respectively in breast (341) and CRC (342). Chen et. al. reported that miR-30a was related with docetaxel resistance in BC by horizontal exosomes transfer (141). Aberrant expression of CCAT1 lncRNA had a sponging effect on miR let-7c and, as a consequence, promoted chemoresistance to docetaxel in lung adenocarcinoma (343). This last evidence is intriguing, since it is also reported that let-7c up-regulation inhibited chemoresistance to 5-Fluorouracil in renal carcinoma (221) and sensitized resistant lung carcinoma cell (A549) to cisplatin (223).

Subnetwork 6: Gemcitabine, Temozolomide, Cetuximab, Carboplatin, Cytarabine, Epirubicin, Soladosine, Vemurafenib

Gemcitabine is a synthetic nucleoside analog used to treat various carcinomas and several investigations confirm that ncRNAs can modulate gemcitabine action (344, 345). Cao et al. demonstrated that miR-192 regulated gemcitabine and cisplatin resistance in lung adenocarcinoma through modulation of apoptosis (346). miR-192 together with miR-215, was found to be a positive regulator of TP53 (347). In glioblastoma miR-138 is involved in cell death mechanisms that promote chemoresistance to temozolomide (348), an alkylating drug similar to gemcitabine, that enhances cell apoptosis of tumor cells. Moreover miR-138 aberrant expression can provide a basis for novel gemcitabine chemoresistance markers in bladder and pancreatic ductal carcinoma (88, 349). Furthermore, miR-138 was implicated in the pathogenesis of chronic myeloid leukemia and its clinical response to imatinib (350). Cetuximab is a monoclonal antibody with a mechanism of action different from gemcitabine, however, miR-100 over-expression may promote chemoresistance against both treatments (88, 125). Depending on the cellular context both up-regulation and down-regulation of an ncRNA could lead to chemoresistance. In our review, the role of miR-205, which regulates EMT (351), emerged as one of such cases. It is apparent that miR-205 upregulation causes inhibition of chemoresistance to gemcitabine in pancreatic cancer (352), but Zarogoulidis et al. demonstrated that miR-205 and miR-218 were associated with carboplatin resistance in lung cancer (206). miR-181b over-expression increased gemcitabine resistance (353), whereas miR-181b was involved in temozolomide sensitivity in glioma by targeting MEK1 (354). Lee et al. found that hypoxia-induced miR-210 (355) could potentially reverse temozolomide resistance in glioblastoma (356). Another investigation discovered that miR-210, in association with miR-21, miR-99a, miR-100, miR-125b, and miR-138 may serve as biomarkers of gemcitabine resistance in pancreatic cancer (88). In this subnetwork we find also some important miRNAs that were previous described: miR-21-5p (91) miR-125b-5p (89, 125, 127), and let-7a (214–217). The HOTTIP lncRNA (HOXA transcript at the distal tip) can promote cancer progression and gemcitabine resistance in pancreatic cancer (357). Finally the overexpression of BC200 lncRNA has a role in the induction of cell death by carboplatin in ovarian cancer (358). Furthemore, miR-204 is highly induced by vemurafenib in resistant melanoma cells and tissues, as much as miR-211 (359). Although belonging to the same family, the expression of miR-204 is high in amelanotic melanoma cells, and acts as an effector of vemurafenib's anti-motility activity. Conversely, miR-211 which is induced in melanotic melanoma cells, mediates and potentiates the increase in pigmentation due to vemurafenib; this adaptive response *de facto* limits its efficacy (360). miR-204 inhibits the migration/invasion of melanoma cells with a potency similar to that of miR-211 and, more importantly, it acts in the cellular contexts in which miR-211 is absent (359). Fattore et al. demonstrated that miR-579-3p is strongly downregulated in melanoma and loss of BRAF and MDM2 regulation leads to chemoresistance to targeted therapy (361).

Subetwork 7: Oxaliplatin, Capecitabine

Oxaliplatin is used for the treatment of CRC and has been compared with other platinum compounds used for advanced cancers, such as cisplatin and carboplatin. Oxaliplatin in combination with capecitabine (XELOX) is a first-line treatment of CRC, hile for CRC in advanced stages is common to use oxaliplatin in combination with 5-FU (FOLFOX) (362). Several studies demonstrated that miRNAs modulate the chemoresistance to these drugs. In particular, Hu et al. found that circulating miR-1914-3p and miR-1915-3p are down-regulated in patients with chemoresistant CRC. Consequently, up-regulation of these miRNAs *in vivo*, could partially restore CRC cells sensitivity to XELOX treatment (363). Furthermore, miR-425-5p ihnibition reversed oxaliplatin resistance both in HTC116-resistant cells lines and xenograft models by modulating the expression of PDCD10 (364). Tan et al. observed a negative correlation between miR-409-3p and resistance to Oxaliplatin in CRC resistant cells (365). Moreover, as a putative miRNAs modulator, also long intergenic noncoding RNA (LINC00152), can be involved in chemosensitivity of Oxaliplatin in CRC. LINC00152 increases the chemosensitivity becoming an endogenous RNA competitor for miR-193a-3p and ErbB receptor tyrosine kinase 4 (ERBB4) (366).

Subnetwork 8: 5-Fluorouracil, Irinotecan

5-Fluorouracil (5-FU) is a widely used therapeutic agent for treating a range of cancers, including advanced CRC (367), liver and BCs. It interferes with DNA replication by interrupting the synthesis of pyrimidine thymidine and thereby leading to cell cycle arrest or cell death (368, 369). In the 5-FU metabolic pathway, the enzymes dihydropyrimidine dehydrogenase, thymidylate synthase, thymidine phosphorylase and methylenetetrahydrofolate reductase are important to determine resistance (370). miRNAs are altered in CRC (26) and targeting tumor-associated genes (23, 371–373). Moreover, miRNAs are promising tumor biomarkers for CRC screening (27) and are also responsible for 5-fluorouracil drug resistance (374). In particular miR-587 (369), miR-195 (375), miR-149 (376), miR-203 (377), miR-129 (378), and miR-218 (209) are involved in 5-FU response. While miR-20b (379) and miR-519c (380) influence 5-FU and Irinotecan (only miR-519c) resistance in CRC. Another interesting miRNA is miR-302a, belonging to the miR-302-367 cluster, which includes miR-302b, miR-302c, miR-302a, miR-302d, and miR-367. This cluster was first identified in human embryonic stem cells (hESCs) and human embryonic carcinoma cells (hECCs) and it has been reported to help maintaining stemness and reprogramming somatic cells into induced pluripotent stem cells (381). Recently, *in vitro* models have pinpointed its role in chemoresistance: miR-302a exerts its function through inhibition of IGF1R and of downstream Akt signaling: events associated with enhanced 5-FU-induced cell death in colon cancer cells (370). The up-regulation of miR-96 has been reported in several cancers (382, 383) and conversely low expression levels of miR-96 have been associated with poor clinical outcomes in CRC patients (384). miR-96 modulated 5-FU sensitivity in CRC cells by promoting apoptosis through reduction of the anti-apoptotic regulator XIAP and the p53 stability regulator UBE2N (ubiquitin-conjugating enzyme E2N) (385). miR-23a antisense enhanced 5-fluorouracil chemosensitivity in CRC cells, by acting on the APAF1/Caspase-9 apoptotic pathway (386), while miR-23a over-expression provided 5-FU resistance in a subtype of CRC (387). Like let-7c, also present in this subnetwork, let-7b resulted important for development of 5-FU chemoresistance in RCC (221). miR-34a also plays a role in resistance to 5-FU and to vemurafenib (102, 103, 388). The expression profile of lncRNAs was investigated in 5-FU-resistant colon cancer cell lines and snaR was confirmed to be downregulated (389); this loss increases cell viability after 5-FU treatment, suggesting that this lncRNA has a potential role as a negative regulator in drug response (390). miR-204 is significantly attenuated in CRC (391) and has a relevant function in this cancer as tumor-suppressive miRNA, through direct targeting of HMGA2. The miR-204/HMGA2 axis notably modulated cell proliferation and positively influenced CRC sensitivity to 5-FU (392).

Subnetwork 9: Doxorubicin, Methotrexate, Etoposide, Crizotinib, Celecoxib

Most of non-coding RNAs disregulations related to doxorubicin, methotrexate and etoposide play a role in chemoresistance exacerbation or inhibition. They are involved in several pathways that regulate cell growth, autophagy, apoptosis and cell proliferation (393–401) or miR-34a (105), lnc-SCD and lnc-PTMS (402) modulates the effects of celecoxib. Both doxorubicin and etoposide block DNA replication by topoisomerase II inhibition: thus causing errors in DNA synthesis and promoting apoptosis in cancer cells. They are often used to treat cancers including breast, bladder, ovarian, prostate and leukemia (403). The human miR-135a is encoded by two genes localized on chromosomes 3 and 12. It may have contradictory effects promoting or repressing cell migration and invasion in colon, melanoma, breast and prostate cancer cell lines (404). This subnetwork shows a relation between miR-135 and miR-196b; upregulation of these two miRNAs is reflected in ABCB1 increment. This pattern conferred resistance to genotoxic agents like etoposide and doxorubicin in leukemia cancer cells (405), an interesting result that confirms the pro-oncogenic role of miR-196b. Its over-expression has been reported in different types of leukemia (406), in the maintenance of stem cell properties and chemoresistance in CRC (407), and in castrate-resistant prostate cancer (408). Novel insights in improving the effectiveness of chemotherapy emerged with miR-708, miR-101-3p, and miR-29b. Their regulation could enhance chemosensivity of drug targeted genes involved in responses like autophagy or apoptosis (172, 409, 410). miR-29b is generally the most highly expressed ncRNA in the miR-29 family. Up-regulation of miR-29b is common in the majority of human cancers where it affects tumor progression (411). miR-29b increases etoposide and paclitaxel induced toxicity in ovarian cancer, this effect being linked to Mcl-1 (410, 412). Very interesting was the case of the miR-200 family members that include miR-200c. The expression of this miRNA was inversely correlated with the chemoresistance to antioneoplastic drugs like Doxorubicin, Crizotinib and Methotrexate. miR-200c improved drug sensivity targeting TrkB and Bmi1 in BCs (151), ZEB1, and EZH2 in lung cancer cells (148, 149). Furthermore, Ham et al. found that overexpression of LUCAT1 lncRNA promotes methotrexate resistance through miR-200c (413). A very interesting loop, if considered that miR-200c up regulation contributes to restore methotrexate sensivity. The identification of ncRNA effects on cancer drugs could promote the development of novel approaches. For example, Xu et al. found that co-delivery of miR-101 and doxorubicin suppressed malignant properties of HCC (414). The role of miR-215, as well as that of his homologous miR-192 (subnetwork 6), in cancer is ambiguous. These two miRNAs exert cell growth and migration-promoting effects in gastric cancer (415) and are positive regulators of p53, playing an important role in multiple myeloma (347). Furthermore, a recent study has confirmed that miR-215 overexpression leads to the development of doxorubicin resistance in HCC and is also associated with bad prognosis in HCC patients harboring mutated p53 (416). In another case, Doxorubicin was shown to affect the subcellular localization of lncRNAs and to enhance their functional effects. For example, Shen et al. discovered that SNGH1 was retained in the nucleus as a consequence to doxorubicin treatment, in turn leading to accumulation of p53 in the nucleus and to the enhancement of p53-dependent apoptosis (417).

Subnetwork 10: Tamoxifen, Vandetanib, Alitretinoin

Tamoxifen, a selective modulator of estrogen receptor, is an effective first-line endocrine therapy that significantly improved relapse overall and relapse-free survival for many ER+ and endocrine-responsive patients. However, a significant proportion of the advanced ER+ BC patients do not respond (418). Recurrence occurs in approximately 40% of patients (419). As pinpointed in this subnetwork, seven miRNAs could sensitize cells to tamoxifen and might serve as potential therapeutic approaches for overcoming tamoxifen-resistance in BC: miR-27b, miR-375, miR-148a, miR-152, miR-206, miR-26a, miR-26b. Conversely, only three miRNAs confered tamoxifen resistance: miR-221, miR-222, miR-335. Lastly, aberrant expression of lncRNAs has also been linked to cancer progression and metastasis (56, 420). In the complex network of ER signaling, lncRNAs are emerging as critical determinants of hormone action. As opposed to miRNAs, high expression of lncRNAs, namely LINC-ROR (248, 249), MALAT1, CCAT2, was often associated with tamoxifen treatment failure in BC: their knock-down improved tamoxifen responsiveness in BC cells while uc.57 lncRNA promoted drug sensitivity. miR-27b had a different expression pattern between tamoxifen-sensitive vs. -resistant BC cell lines (421). In particular, miR-27b was found to be down-regulated in breast tumor tissues from tamoxifen-resistant patients (422) and high levels of miR-27b correlated with poor prognosis in BC (423, 424). CSC generation and EMT are essential events in tumor cell invasion and metastasis, both present in resistance to tamoxifen (425, 426). Of note, miRNAs have been associated with EMT and resistance to standard therapies. A direct target of miR-27b in modulating drug resistance and EMT is HMGB3 (427), an oncogene that can modulate drug resistance, proliferation and metastasis (428). Notably, while tamoxifen repressed miR-27b expression, estrogen induced miR-27b in BC cells (422). We already illustrated above miR-375, that can modulate the sensitivity/resistance of drug treatments in different cancers, including BC (193) and MTC (196). At the same time an anticancer treatment like alitretinoin may exert a regulatory action on miR-375 expression in BC cells (195). miR-148a and miR-152 reduced tamoxifen resistance in ER+BC via direct down-regulating the activated leukocyte cell adhesion molecule (ALCAM) (429). miR-206 was elevated in ER+BC cell lines (161) and its knock-down induced resistance to tamoxifen, while its overexpression reduced it by regulating G1/S-related proteins (430). miR-26a/b levels were lower in tamoxifen-resistant ER+BC and the inactivation of miR-26a/b decreased tamoxifen responsiveness of cancer cells (431). Additionally, miR-26 was found to be frequently downregulated in HCC and correlated with poor survival. miR-26b significantly suppressed the NF-κB signaling and dramatically enhanced chemo-sensitivity of HCC to doxorubicin by targeting TAK1 and TAB3, two positive regulators of NF-κB pathway (432). Subnetwork 10 also includes miR-221 and miR-222. These two miRNAs have a bivalent role in drug resistance across different cancer types. In this subnetwork miR-221/221 were found to enhance tamoxifen resistance (161, 163, 433). miR-335, promoted estrogen signaling, resulting in increased potency of tamoxifen. Additionally, tumor cells with acquired tamoxifen resistance did not show miR-335 nor ESR1 expression (434). The Metastasis associated in lung adenocarcinoma transcript 1 lncRNA (MALAT1), is over-expressed in several human malignancies, including ER+BC (435). High MALAT1 levels were also associated with tamoxifen treatment failure by regulating the transcription and splicing of ESR1, thus affecting ER signaling (436). Accordingly, MALAT1 may serve as an oncogenic lncRNA in pancreatic cancer, by promoting EMT, decreasing chemosensitivity to anticancer drugs and accelerating tumor angiogenesis (437). The CCAT2 lncRNA is overexpressed in BC, with the highest expression in lymph node negative patients. However, its expression levels are informative solely for a subgroup of patients, namely for lymph node positive patients that received adjuvant 5-fluorouracil, cyclophosphamide and methotrexate chemotherapy: high levels of CCAT2 suggested that patients would not benefit from CMF (438). Tamoxifen-resistant cells present a higher level of CCAT2 compared with sensitive cell, and knockdown of CCAT2 improved their response to tamoxifen (420). The levels of transcribed ultraconserved region uc.57 are lower in BC tissues than in precancerous breast tissues. uc.57 overexpression down-modulated BCL11A and reduced tamoxifen resistance in BC cells MCF7R by inhibiting the PI3K/AKT and MAPK signaling pathways (439).

Subnetwork 11: Imatinib

Imatinib (IM) is a 2-phenyl-amino-pyrimidine, an ATP-competitive tyrosine kinase inhibitor (TKI) and one of the most potent inhibitors of ABL1. Imatinib was approved for clinical treatment of CML but the problem of drug resistance encouraged the development of new TKI generations (440). Various ncRNAs have been associated with imatinib in CML, either as enhancers or inhibitors. HOX Antisense Intergenic RNA (HOTAIR) is located in the antisense strand of the HOXC gene locus, flanked by HOXC11 and HOXC12 (441). HOTAIR expression levels correlated with metastasis in BC and its loss was linked to decrease in invasion potential (442). HOTAIR lncRNA was up-regulated in CML patients with high levels of MDR1. Moreover, the knockdown of HOTAIR led to down-regulation of MDR1 resulting in higher sensitivity to imatinib; an involvement of HOTAIR in the PI3K/Akt pathway was also proposed (443). HULC is located at 6p24.3 and its transcript is a ∼500 nt long, spliced and poly-adenylated lncRNA that localizes to the cytoplasm (444). The impact of HULC in hematologic malignancies is not clear yet, but it could act as a sponge for miRNA-372 in acute lymphoblastic leukemia (445). Moreover, HULC is involved in K562 cells survival and its silencing leads to increased apoptosis in CML cells by up-regulating PI3K/AKT signaling and c-Myc (446). Colorectal cancer, gastric cancer and melanoma show aberrant expression of SNHG5 (447, 448). SNHG5 lncRNA promotes imatinb resistance in CML and, although the mechanism may be complex, it seems to act as a competing endogenous RNA for miR-205-5p (449). UCA1 lncRNA located at 19p13.12, has an important role in drug resistance (114, 116). let-7i cooperates with miR-224 to revert imatinib resistance in CML (224). miR-1301 is involved in human

cancers but shows an ambiguous behavior (450, 451). It can target the Ran GTPase Activating Protein 1 (RanGAP1) mRNA, as demonstrated by inverse correlation in CML patients: the RanGAP1 protein down-regulation or an increased miR-1301 are beneficial for the sensitivity to imatinib (452). miR-7 acts as an inhibitor in hepatocellular and pancreatic carcinomas (453, 454) possibly regulating the PI3K/AKT pathway, which is also downstream of BCR-ABL (455). In fact, over-expression of miR-7 in K562 cells, exhibit a significant inhibition of proliferation and increase of apoptosis via inhibition of BCR-ABL/PI3K/AKT signaling. Another report showed that miR-7 could work in synergy with imatinib to sensitize K562 (456). As the last ncRNA in this subnetwork, miR-518a is down-regulated in imatinib-resistant gastrointestinal stromal tumor (GIST) and PIK3C2A was identified as the relevant specific target (457).

Non-connected RNA and Drug Nodes

Few ncRNA and drug combinations are not connected to the main network (and also obviously not to the subnetworks). One of these drugs is dactilosib, an imidazoquinoline derivative under phase II trial that works as dual inhibitor of PI3K and mTOR. It might improve conventional drug treatments and overcome some intrinsic adverse reactions of rapamycin and its derivates (458). Deng et al. studied dactilosib in AML and discovered that it caused up-regulation of miR-1-3p and consequent down-regulation of its targets involved in apoptosis, migration and multidrugs resistance. Moreover inhibition of miR-1-3p could interfere with dactilosib anti-proliferation effects (459).

In several human cancers miR-144 and miR-451 were identified as tumor suppressor ncRNAs (460). In terms of chemoresistance, miR-144 reversed 5-FU and imatinib resistance respectively in HCC (461) and leukemia (462). In addition, miR-144 might promote cisplatin sensitivity in prostate cancer (463) and in thyroid carcinoma (464). Whereas miR-144-3p contributed to sunitinib resistance in RCC by targeting ARID1A, a cancer gene involved in chromatin remodeling (465). Reduction of ARID1A expression could also serve as a predictive biomarker for trastuzumab resistance in BC (466). Although breast and ovarian cancer have comparable levels of HER2/ErbB2 expression patterns, pertuzumab treatment is more effective in BC. Wuerkenbieke et al. investigated this effect and found miR-150 knockdown in ovarian cancer; this might contribute to enhance pertuzumab resistance (467). ncRNAs can also be related to side-effects occurring upon cancer treatment: vascular events are a serious problem in CML patients treated with tyrosine kinase inhibitors like nilotinib. Recent findings suggest that nilotinib decreases levels of miR-3121-3p resulting in higher levels of IL-1β and adhesion molecules in vascular endothelial cells. (468). miR-132-5p expression, via CYP1A2 modulation, could reduce flutamide-induced hepatic cell toxicity (469). Finally, in a matrix *in vitro* screen of several miRNAs and drugs in BC, miR-126 augmented the potency of CDK4/6 or PIK3CA inhibitors in MCF7 (Luminal) and MDA-MB-453 ($HER2^+$) cell lines (470).

AUTHOR CONTRIBUTIONS

SV contributed to design and critical revision. FC, LM, and FB performed collection and curation of data. FC assembled data and wrote the article. LM and FB contributed to network analysis. CA contributed to network analysis and writing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00327/full#supplementary-material

Supplementary Image 1 | The complete ncRNA/drug network. The figure illustrates the connected network of all drug/ncRNA interactions (edges) and 5 unconnected pairs. The network represents a human curated selection of papers listed in PubMed to identify the most important ncRNAs/cancer drugs interactions and cliques.

Supplementary Table 1 | ncRNA/drug network indices. The Excel file contains the measures for all nodes (drugs/ncRNAs) in the network.

Supplementary Table 2 | ncRNA/drug interaction effect on chemoresistance. This table shows, for each subnetwork, the non-coding RNAs that influenced chemoresistance, or drug sensivity, as reported in our review. Red represents inhibition, and green enhancement, of the relative process. We indicated all drugs in the subnetworks and the related ncRNAs behaviors. Keys: ↑ ncRNA up-regulation, ↓ ncRNA down-regulation.

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REVIEW
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Non-Coding RNAs and Resistance to Anticancer Drugs in Gastrointestinal Tumors

Jens C. Hahne[1] *and Nicola Valeri*[1,2]*

[1] *Division of Molecular Pathology, The Institute of Cancer Research, London, United Kingdom,* [2] *Department of Medicine, The Royal Marsden NHS Trust, London, United Kingdom*

Non-coding RNAs are important regulators of gene expression and transcription. It is well established that impaired non-coding RNA expression especially the one of long non-coding RNAs and microRNAs is involved in a number of pathological conditions including cancer. Non-coding RNAs are responsible for the development of resistance to anticancer treatments as they regulate drug resistance-related genes, affect intracellular drug concentrations, induce alternative signaling pathways, alter drug efficiency *via* blocking cell cycle regulation, and DNA damage response. Furthermore, they can prevent therapeutic-induced cell death and promote epithelial–mesenchymal transition (EMT) and elicit non-cell autonomous mechanisms of resistance. In this review, we summarize the role of non-coding RNAs for different mechanisms resulting in drug resistance (e.g., drug transport, drug metabolism, cell cycle regulation, regulation of apoptotic pathways, cancer stem cells, and EMT) in the context of gastrointestinal cancers.

Keywords: non-coding RNA, lncRNA, microRNA, anticancer drugs, gastrointestinal tumor, cancer therapy, resistance

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Massimiliano Agostini, Università degli Studi di Roma Tor Vergata, Italy

***Correspondence:**
Nicola Valeri
nicola.valeri@icr.ac.uk

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INTRODUCTION

Gastrointestinal (GI) cancer encompasses a heterogeneous group of tumors that affect the digestive tract system (1). These include cancers of the esophagus, stomach, gallbladder, liver and biliary tract, pancreas, small intestine, colon, rectum, and anus. GI cancer is the most common form of cancer responsible for nearly 25% of all new cancer diagnosis and responsible for most of cancer-related death (around 30% of all cancer-related death) worldwide (2, 3).

Chemotherapy is, alongside with surgery and radiation therapy, one of the main treatments for cancer (4–12). Many chemotherapeutic agents have successfully prolonged overall and progression-free survival of GI cancer patients (13–17). In addition, a better understanding of the biology and mechanism underpinning GI cancer initiation and progression is leading to more personalized treatments. Indeed, identification of well-defined molecular subtypes and/or molecular profiling of somatic mutations offer the opportunity to further optimize the efficacy of treatments through tailored approaches (18–21).

Despite major improvements in the management of GI cancer patients, resistance to therapies arises almost inevitably at some point during the treatment and chemoresistance is one of the main challenges in cancer therapy (22). Drug resistance can be caused by gene mutations, abnormal DNA repair, alteration in cell cycle regulation, cell death inhibition (mostly caused by deregulated apoptotic signaling pathways), reduced drug efficacy, and enhanced drug clearance (22, 23). Furthermore, the epithelial–mesenchymal transition (EMT) process and the presence of tumor stem cells have

been identified as causes of drug resistance (24–27). The complex molecular mechanisms of chemoresistance have not been fully elucidated yet and a better understanding of drivers of primary and secondary resistance to chemotherapy will likely result into improved patients' survival. Increasing evidence points toward the role of non-coding RNAs as a central hub for treatment resistance. Therefore, this review outlines the role of non-coding RNAs for the different drug resistance mechanisms involved in GI cancer therapy failure. **Table 1** summarized the non-coding RNAs discussed in this review; and in **Figures 1**–**7**, the role for each of these non-coding RNAs in the context of the different GI tumors is illustrated.

NON-CODING RNAs

In human tissues, the amount of non-coding RNAs is more than three times higher compared to the amount of protein-coding RNAs (189). Non-coding RNAs are a large family that includes more than 16 categories of long and short RNA molecules (**Table 2**); among them transfer RNAs (tRNAs), ribosomal RNAs

TABLE 1 | Overview about non-coding RNAs involved in resistance to anticancer drugs in gastrointestinal tumors.

Non-coding RNA	GI cancer type	Causing drug resistance *via*	Reference
lncRNA AK022798	Gastric cancer	Increasing the expression of *ABCB1* gene	(28)
lncRNA ANRIL	Gastric cancer	Increasing the expression of *MDR1* gene	(29, 30)
lncRNA ARA	Liver cancer	Reduced G2/M cell-cycle arrest; reduced apoptosis rate; deregulation of MAPK-pathway	(31, 32)
lncRNA-ATB	Liver cancer	Increased expression of ZEB1 and ZEB2; induced EMT	(33)
lncRNA CCAL	Colorectal cancer	Increasing the expression of *ABCB1* gene; increased activity of Wnt/β-catenin pathway	(34)
lncRNA H19	Liver cancer esophageal cancer	Upregulation of membrane glycoprotein p95; elevating the expression of *MDR1* gene by increasing promoter methylation; increasing telomere length	(35–37)
lncRNA HOTAIR	Liver cancer Colorectal cancer Pancreatic cancer Gi stromal tumor	Increased expression of PRC2 complex members; genome-wide changes in transcription process due to epigenetic chromatin silencing; downregulation of p21(WAF/CIP1); repression of G1/S cell-cycle arrest; increased proliferation rate; reduced DNA-damage response	(38–41)
lncRNA HOTAIR	Colon cancer Pancreatic cancer Gastric cancer esophageal cancer	Transformation of stem cells into cancer stem cells due to activation of *OCT4*, *RNF51*, *CD44*, and *CD133* gene expression; increased activity of Wnt/β-catenin pathway; modulation of chromatin organization leads to reduced efficiency of the mismatch repair system; increased MSI; reduced apoptosis rate; inhibition of the expression of miR-126 and activating the PI3K-AKT-mTOR pathway (in gastric cancer)	(42–48)
lncRNA HOTTTIP	Pancreatic cancer	Increased expression of transcription factor HOX13; cell cycle deregulation	(49, 50)
lncRNA HULC	Liver cancer	Increased activity of Wnt-β-catenin; increased expression of USP22 and SIRT1; reduced expression of miR-6825-5p, miR-6845-5p, miR-6886-3p; increased autophagy pathway	(51)
lncRNA HULC	Gastric cancer	Induced EMT; suppressed apoptosis	(52, 53)
lncRNA LEIGG	Gastric cancer	Induced EMT	(54, 55)
lncRNA linc-ROR	Pancreatic cancer	Inhibition of p53; inhibition of the expression of miR-200 family; increased expression of the transcription factor ZEB1; induced EMT	(56, 57)
lncRNA linc-ROR	Liver cancer	Preventing the binding of miR-145 to pluripotent factors OKT-4, NANOG, and SOX2 resulting in increased expression of these transcription factors necessary for sustain stem cell character	(58, 59)
lncRNA LOC285194	esophageal cancer	Cell-cycle deregulation; blocking non-apoptotic cell death pathway	(60)
lncRNA MALAT-1	esophageal tumor	Binds miR-107 and miR-217; reduced activity of the ATM-CHK2 signaling pathway; reduced cell-cycle arrest and cell death as response to DNA damage; increased expression of transcription factor B-Myb	(61–63)
lncRNA MALAT-1	Pancreatic cancer	Increased expression of cancer stem cell marker CD133; increased expression of pluripotent factors OCT4, NANOG, and SOX2; induced EMT; repression of G2/M cell-cycle arrest; reduced apoptosis rate	(64–66)
lncRNA MALAT-1	Gastric cancer	Sequestering of miR-23b-3p; increased expression of ATG12; increased autophagy	(67)
lncRNA MIR100HG	Colon cancer	Increased activity of Wnt-β-catenin pathway	(68)
lncRNA MRUL	Gastric cancer	Increasing the expression of *MDR1* gene	(69)
lncRNA PANDAR	Gastric cancer Colorectal cancer Hepatocellular cancer cholangiocarcinoma	Interacts with the transcription factor NF-YA resulting in reduced translation of proapoptotic genes—leading to reduced apoptosis rate and increased proliferation	(70–74)
lncRNA PVT1	Gastric cancer esophageal cancer Pancreatic cancer Colon cancer Liver cancer	Induced EMT	(75–77)

(*Continued*)

TABLE 1 | Continued

Non-coding RNA	GI cancer type	Causing drug resistance *via*	Reference
lncRNA PVT-1	Gastric cancer	Increasing the expression of *MDR1* gene	(29, 30)
lncRNA TUC338	Hepatocellular cancer	Inhibiting the RASAL-1 pathway	(78)
lncRNA TUG1	esophageal cancer Gastric cancer Colorectal cancer Hepatocellular cancer cholangiocarcinoma	Increasing the expression of *Bc-2* gene; reducing the expression of cyclin-dependent protein kinase, caspase-3, caspase-9, and Bax; decreasing G0/G1 arrest during cell cycle; reducing apoptosis rate; inducing EMT	(79–85)
lncRNA UCA1 (identical with lncRNA CDUR)	Liver cancer Colorectal cancer Pancreatic cancer Gastric cancer esophageal cancer	Sequestering microRNAs (miR-216b in liver cancer; miR-204-5p in colorectal and esophageal cancer; miR-27 in gastric cancer); increase expression of lncRNAs (HULC; H19); increased activity of Wnt-β-catenin pathway; increased activity of PI3K-AKT-mTOR pathway; increased phosphorylation of tumor suppressor retinoblastoma; increased expression of c-myc; increased cell-cycle progression; increased expression of antiapoptotic protein Bcl-2; reduced expression of PARP (in gastric cancer); reduced apoptosis rate. In liver cancer, additional effects: transformation of stem cells into cancer stem cells due to increased c-myc expression; increasing telomere length	(35, 86–96)
lncRNA URHC	Liver cancer	Reduced expression of the tumor suppressor ZAK; increased proliferation rate; reduced apoptosis rate	(97)
lncRNA-34a	Colon cancer	Increased activity of Wnt-β-catenin pathway; increased activity of NOTCH pathway; increasing the self-renewal of cancer stem cells	(98, 99)
miR let-7 family	Pancreatic cancer	Induced EMT	(100)
miR let-7a	Pancreatic tumors	Increased expression of RRM2	(101)
miR let-7g	esophageal cancer	Increased expression of ABCC10	(102)
miR let-7i	esophageal cancer	Increased expression of ABCC10	(102)
miR-100	Colon cancer	Increased activity of Wnt-β-catenin pathway	(68)
miR-101	Liver cancer	Increased expression of EZH2; increased activity of Wnt-β-catenin pathway; increased expression of Mcl-1; reduced apoptosis rate	(103–105)
miR-10b	Colorectal cancer	Increased expression of antiapoptotic protein Blm	(106)
miR-103/107	Gastric cancer	Reduced expression of tumor-suppressor caveolin-1; activation of Ras-p42/p44 MAP pathway; reduced apoptosis rate	(107–109)
miR-106a	Gastric cancer	Reduced expression of FAS; reduced apoptosis rate	(110, 111)
miR-1182	Gastric cancer	Increased expression of hTERT	(112)
miR-122	Liver cancer	Increased expression of ABC proteins; increased expression of cyclin G1; reduced G2/M cell-cycle arrest; reduced DNA repair; reduced apoptosis rate	(113, 114)
miR-124	Pancreatic cancer Liver cancer	Reduced expression of SLC16A1	(115)
miR-125b	Colon cancer	Increased activity of Wnt-β-catenin pathway	(68)
miR-1246	Pancreatic cancer	Reduced expression of cyclin-G2; deregulated cell-cycle	(116)
miR-129	Colorectal cancer	Increased expression of antiapoptotic protein Bcl-2	(117)
miR-1291	Pancreatic cancer	Increased expression of ABCC1	(118)
miR-130b	Liver cancer	Reduce expression of tumor protein 53-induced nuclear protein 1	(119)
miR-1307	Pancreatic cancer	Reduced apoptosis rate	(120)
miR-133a	esophageal cancer	Increased expression of GSTP1	(121)
miR-145	Colon carcinoma	Increased expression of ABCB1	(122)
miR-147	Colon cancer	Induced EMT; increased phosphorylation of AKT; increased activity of PI3K-AKT-mtor pathway; increased activity of TGF-β pathway	(123)
miR-155	Colorectal cancer	Inhibition of MSH2, MSH6, and MLH1	(124)
miR-15b	Gastric cancer	Increased expression of antiapoptotic protein Bcl-2	(125)
miR-16	Gastric cancer	Increased expression of antiapoptotic protein Bcl-2	(125)
mir-17-5p	Colorectal cancer	Reduced expression of PTEN expression; activation of AKT-mtor pathways	(126)
miR-17-5p	Pancreatic cancer	Reduced expression of BIM	(127)
miR-1915	Colon cancer	Increased expression of BCL-2	(128)
miR-192	Colon cancer	Reduced expression of thymidylate synthase; altered cell-cycle control at multiple levels; prevent progression into the S-phase	(129)
miR-193b	Hepatocellular cancer	Increased expression of Mcl-1	(130)
miR-195	Colorectal cancer	Increased expression of antiapoptotic protein Bcl-2L2	(131)

(*Continued*)

TABLE 1 | Continued

Non-coding RNA	GI cancer type	Causing drug resistance *via*	Reference
miR-199a-3p	Liver cancer	Reduced G1/S cell-cycle arrest; increased expression of mtor and c-Met; reduced apoptosis rate	(132, 133)
miR-19a	Gastric cancer	Reduced expression of PTEN expression; activation of AKT-mtor pathways	(134)
miR-19b	Gastric cancer	Reduced expression of PTEN expression; activation of AKT-mtor pathways	(134)
miR-200a	Pancreatic cancer	Induced EMT	(100)
miR-200b	Pancreatic cancer	Induced EMT	(100)
miR-200c	Pancreatic cancer	Induced EMT	(100, 135)
miR-203	Colorectal cancer	Reduced expression of ATM; impaired DNA repair; reduced apoptosis rate	(136)
miR-205	Pancreatic cancer	Increased expression of pluripotent factors OKT3, OKT8, and CD44	(137)
miR-21	Colorectal cancer	Inhibition of MSH2 and MSH6; reduced G2/M cell-cycle arrest; reduced apoptosis rate; increasing the number of undifferentiated cancer stem cells	(138, 139)
miR-21	Pancreatic cancer	Reduced cell-cycle arrest; reduced expression of PTEN; activation of AKT-mtor pathway; increased expression of antiapoptotic protein Bcl-2; increased cell proliferation; reduced apoptosis rate	(140, 141)
miR-21	Liver cancer Gastric cancer	Reduced expression of PTEN expression; activation of AKT-mtor pathways	(142–144)
Synergistic action of miR-21 miR-23a miR-27a	Pancreatic cancer	Reduced expression of the tumor suppressors PDCD4, BTG2, and NEDD4L; deregulated cell-cycle; reduced apoptosis rate	(145, 146)
miR-211	Pancreatic tumors	Increased expression of RRM2	(147)
miR-215	Liver cancer	Reduced expression of dihydrofolate reductase; reduced expression of thymidylate synthase	(148)
miR-215	Colon cancer	Reduced expression of thymidylate synthase; altered cell-cycle control at multiple levels; prevent progression into the S-phase	(129)
miR-215	Gastric cancer	Reduced expression of retinoblastoma 1; altered cell-cycle control	(149, 150)
miR-22	P53-mutated colon cancer	Reduced expression of PTEN expression; activation of AKT-mtor pathways	
miR-221	esophageal cancer	Reduced expression of DDK2; activation of Wnt/β-catenin pathway; induced EMT	(151, 152)
miR-223	Liver cancer	Increased expression of ABCB1	
miR-223	Pancreatic cancer	Induced EMT	(153)
miR-223	Gastric cancer	Reduced expression of FBXW7; altered cell-cycle control	(154)
miR-224	Colon cancer	Induced EMT; increased phosphorylation of AKT und ERK; increased activity of PI3K-AKT-mtor pathway; increased activity of ERK pathway; activation of NF-κB; and EGFR dependent pathways	(155)
miR-23a	Microsatellite instable colon cancer	Increased expression of ABCF1	(156)
miR-25	Gastric cancer	Reduced expression of FOXO3a, ERBB2, and FBXW7; cell-cycle deregulation; reduced apoptosis rate	(157–160)
miR-26b	Liver cancer	Increased activation of NF-κB	(161, 162)
miR-27a	Liver cancer	Reduced expression of dihydropyrimidine dehydrogenase	(163)
miR-27b	Liver cancer	Increased expression of CYP1B1; reduced expression of dihydropyrimidine dehydrogenase	(163, 164)
miR-27b	Pancreatic cancer	Reduced expression of CYP3A4—resulting in cyclophosphamide resistance due to missing drug activation	(165)
miR-297	Colorectal cancer	Increased expression of ABCC2	(166)
miR-29a	Pancreatic cancer Liver cancer	Reduced expression of SLC16A1	(115)
miR-29b	Pancreatic cancer Liver cancer	Reduced expression of SLC16A1	(115)
miR-31	Colorectal cancer	Cell-cycle deregulation; reduced apoptosis rate	(167, 168)
miR-320	Colon cancer	Increased expression of SOX4; inhibition of p53 mediated apoptosis; reduced expression of FOXM1 and FOXQ1; cell-cycle deregulation	(169, 170)
miR-338-3p	p53 mutant colorectal cancer	Reduced expression of mtor; increased autophagy; and reduced apoptosis rate	(171)
miR-34a	Colon cancer	Increased expression of antiapoptotic protein Bcl-2	(172)
miR-365	Colon cancer	Increased expression of antiapoptotic protein Bcl-2	(173)
miR-374b	Pancreatic cancer	Increased ATP7A expression	(174)
miR-378	Liver cancer	Increased expression of CYP2E1	(175)

(*Continued*)

TABLE 1 | Continued

Non-coding RNA	GI cancer type	Causing drug resistance *via*	Reference
miR-409-3p	Colon cancer	Increased expression of Beclin-1; increased autophagy pathway	(176)
miR-451	Colon cancer	Increasing the self-renewal of cancer stem cells; increased expression of ABCB1	(177)
miR-494	Colon cancer	Reduced expression of dihydropyrimidine dehydrogenase	(178)
miR-503-5p	Colorectal cancer	Reduced expression of apoptotic protein PUMA	(179)
miR-508-5p	Gastric cancer	Increased expression of ABCB1; increased expression of transcription factor ZNRD1	(180)
miR-519d	Liver cancer	Reduced expression of G1-checkpoint CDK inhibitor p21; reduced apoptosis rate	(181)
miR-522	Colon cancer	Increased expression of ABCB5	(182)
miR-92b	Colon cancer	Reduced expression of SLC15A and SLC15A1	(183)
miR-939	Gastric cancer	Increased expression of SLC34A2; activation of Ras/MEK/ERK pathway	(184)
miR-96	Colorectal cancer	Reduced expression of antiapoptotic proteins XIAP and UBE2N	(185)
svRNAb	All GI tumors	Reduced expression of CYP3A4	(186)
vRNA hvg-1	All GI tumors	Transporting drugs away from the target and drug sequestration	(187, 188)
vRNA hvg-2	All GI tumors	Transporting drugs away from the target and drug sequestration	(187, 188)

GI, gastrointestinal; vRNA, vault RNA; lncRNA, long non-coding RNA; miR, microRNA; EMT, epithelial–mesenchymal transition.

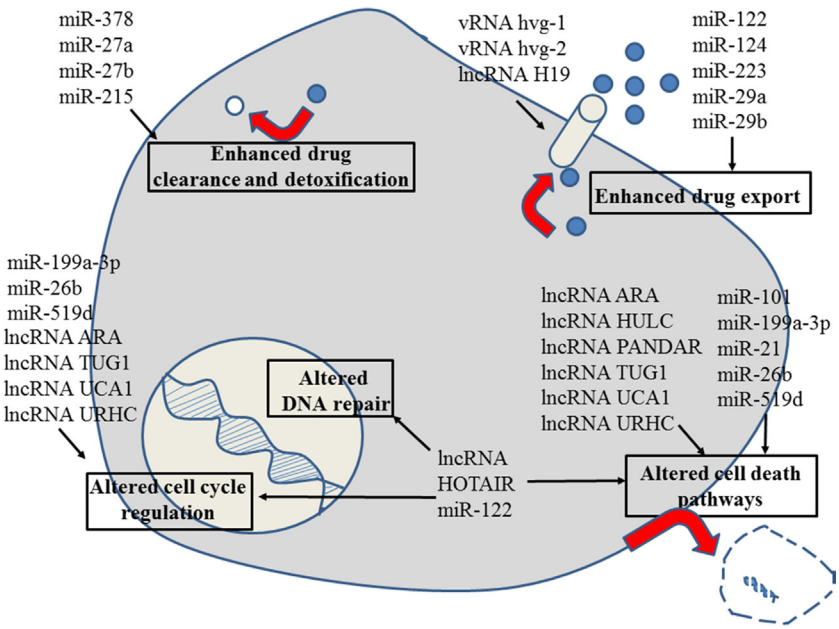

FIGURE 1 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in liver cancer. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

(rRNAs), small nucleolar RNAs (snoRNAs), endogenous small interfering RNAs (endo-siRNAs), sno-derived RNAs (sdRNAs), transcription initiation RNAs (tiRNAs), miRNA-offset-RNAs (moRNAs), circular RNAs (circRNAs), vault RNAs (vRNAs), microRNAs, small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), extracellular RNAs (exRNAs), piwi-interacting RNAs (piRNAs), small Cajal body RNAs (scaRNAs), long intergenic non-coding RNAs (lincRNAs), and long non-coding RNAs (lncRNAs), all of which are not coding for known proteins (190–211).

Long non-coding RNAs (lncRNAs) and microRNAs are the most studied non-coding RNAs playing a role in anticancer drug resistance and will be covered in this review.

LncRNAs are composed of more than 200 nucleotides. They are important regulators during development and pathological processes (212–216). LncRNAs are pivotal in regulating gene expression by binding to chromatin regulatory proteins and they are able to alter chromatin modification as well as transcriptional or posttranscriptional gene regulation by interacting with other RNAs and proteins (217–219). Recently, a crosstalk and strong linkage between lncRNA and microRNAs has been identified (220). It has been shown that lncRNA stability can be reduced by interaction with specific microRNAs and, *vice versa,* lncRNAs act as microRNA decoys sequestering microRNAs from the intracellular cytosol and leading to reexpression of microRNA target genes (220). Furthermore, lncRNAs can promote gene expression

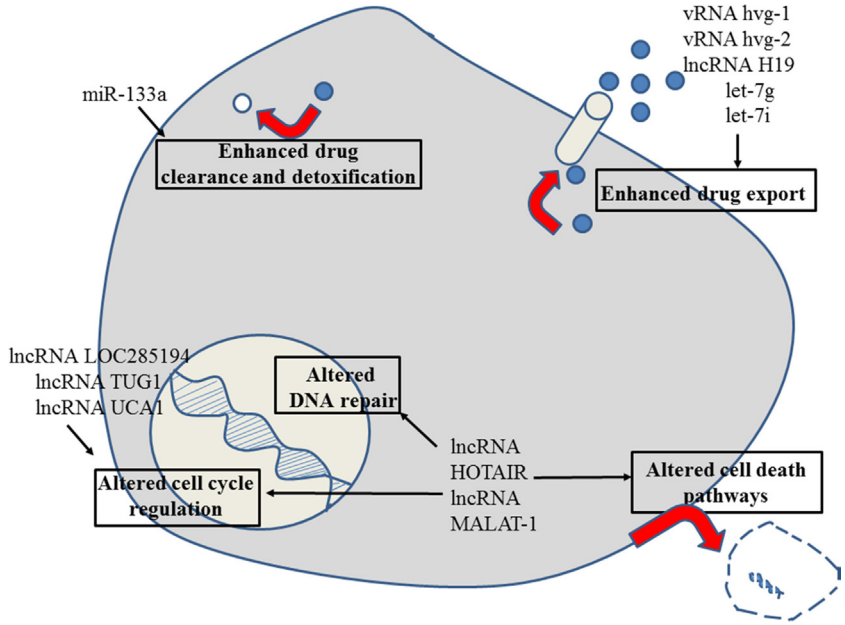

FIGURE 2 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in esophageal cancer. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

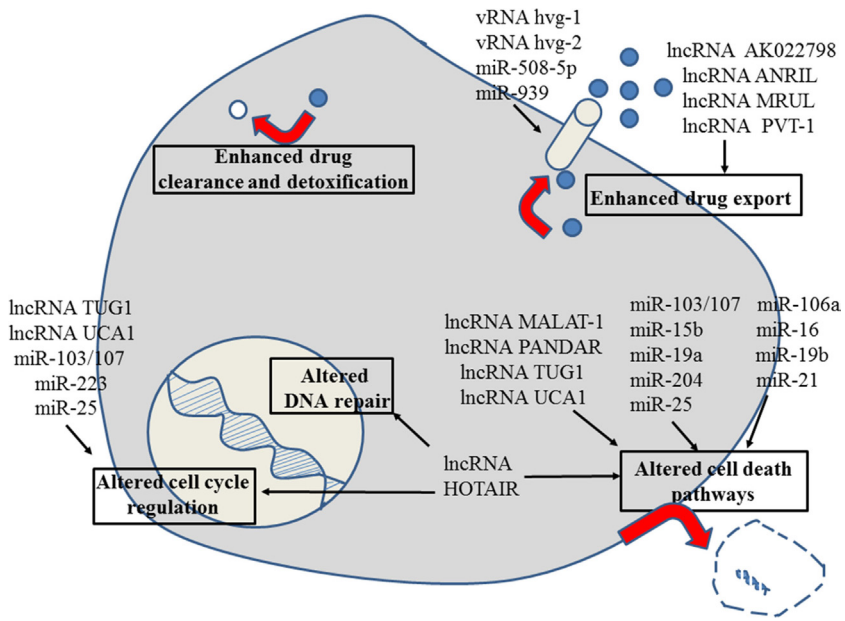

FIGURE 3 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in gastric cancer. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

by competing with microRNAs for specific binding sites in the non-coding regions of mRNAs and prevent the transcriptional repression caused by microRNAs (220). Interestingly some lncRNAs can be processed into microRNAs (220) suggesting a plastic interaction among different classes of non-coding RNAs.

MicroRNAs are short RNA transcripts of 18–24 nucleotides. They are responsible for fine tuning cell homeostasis by controlling gene expression at posttranscriptional level (221–223). Due to the fact that each microRNAs can have several target mRNAs, the interaction of one microRNA with various target mRNAs results in direct deregulation of different target proteins acting simultaneously in regulation of diverse cellular pathways (224, 225). Therefore, variation in microRNA expression can result in reduced mRNA levels ultimately resulting in changes

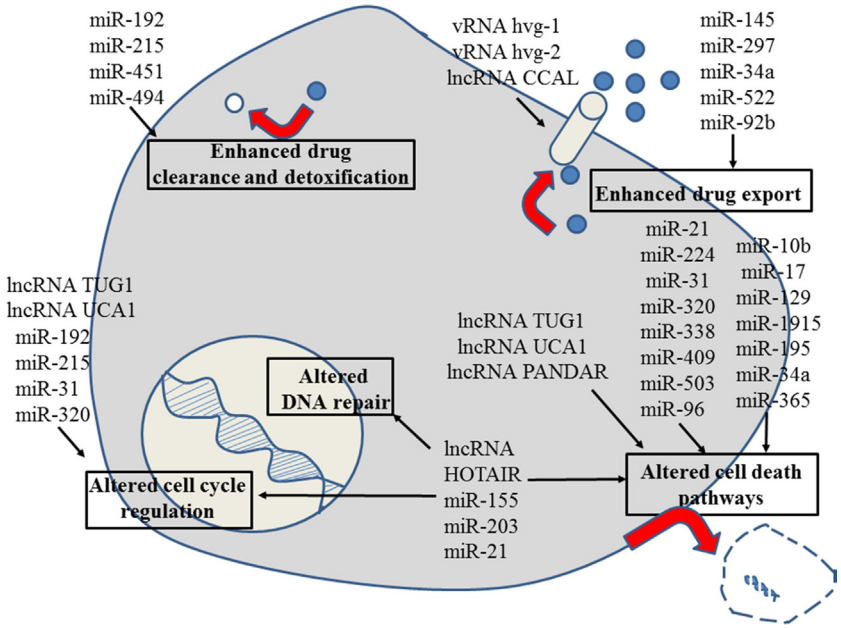

FIGURE 4 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in colon and colorectal cancer. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

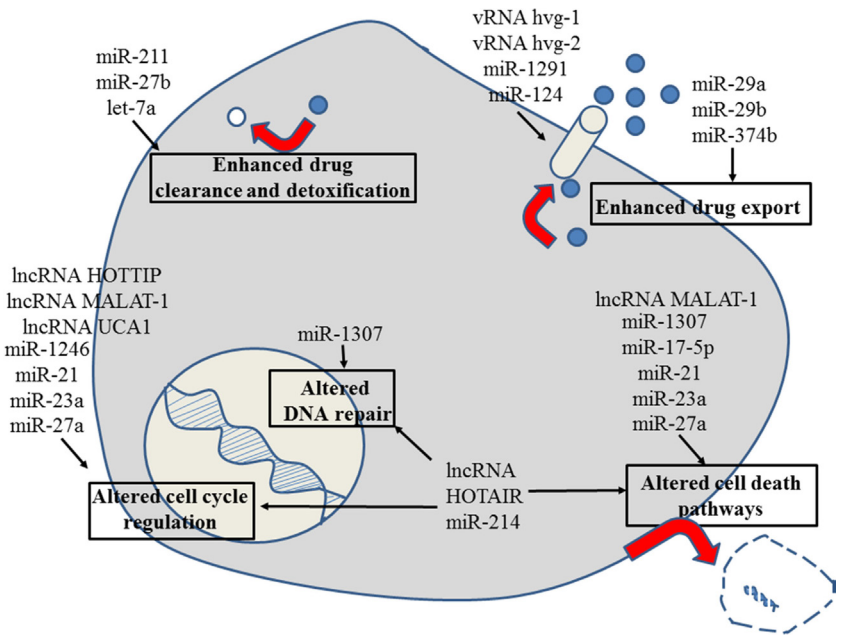

FIGURE 5 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in pancreatic cancer. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

in protein levels within the cell (225, 226). MicroRNAs expression patterns are tissue specific (227) and often define the physiological status of the cell (228). Strong clinical and preclincial evidence suggests that microRNA aberrant expression plays a role in several diseases including cancer, infectious, neurodegenerative, and immune-related diseases (229–240). Analysis of microRNA expression patterns represents a promising tool for cancer diagnosis, prognosis and treatment prediction. MicroRNAs have been extensively studied in monitoring treatment resistance in consideration of their high stability in

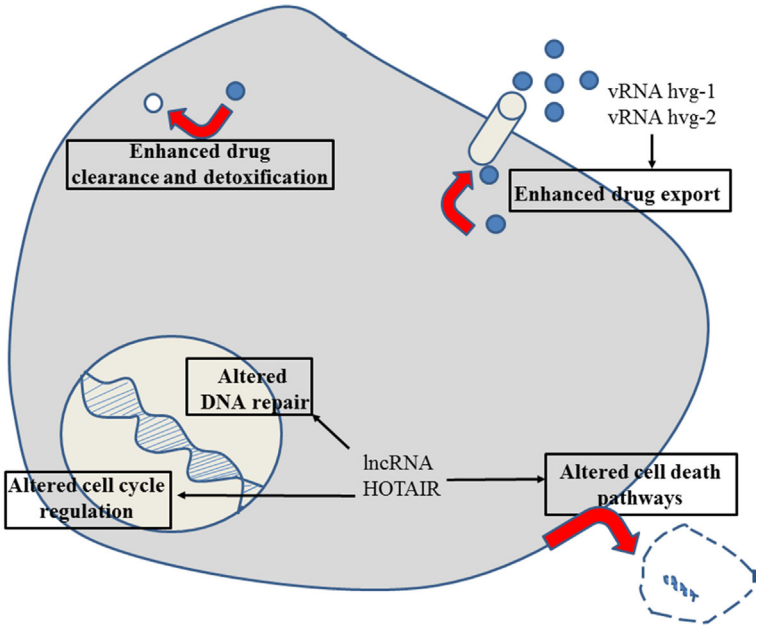

FIGURE 6 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in gastrointestinal stromal cancer. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

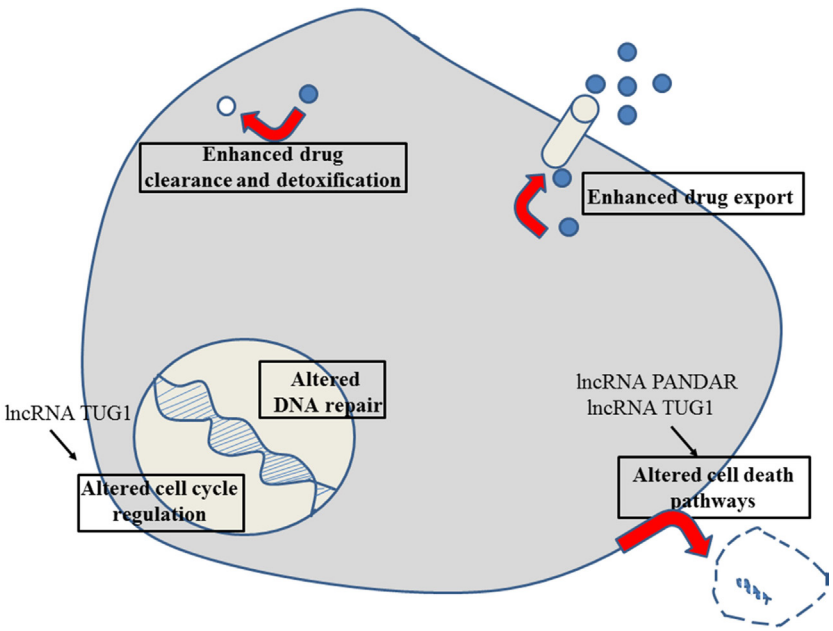

FIGURE 7 | Role of non-coding RNAs for the different reasons that can cause resistance to anticancer drugs in cholangiocarcinoma. For details about target genes and regulated protein expression by the non-coding RNAs, see text.

tissues and body fluids. In blood, microRNAs are included in RNA-binding multiprotein complexes and/or exosomes and their short length makes microRNAs less prone to degradation and improves their stability under different sample storage conditions in blood (224, 230, 236, 240).

GENERAL PRINCIPLES OF DRUG RESISTANCE

Drug resistance is classified into intrinsic and acquired. Primary drug resistance is pre-existing and renders cancer cells immune

TABLE 2 | Overview about the different categories of non-coding RNA molecules.

Name	Biological role
Circular RNA (circRNA)	Involved in forming RNA-protein complex that regulate gene transcription; involved in regulating gene expression at posttranscriptional level by acting as miRNA sponge
Endogenous small interfering RNA (endo-siRNA)	Involved in repression of transposable elements, chromatin organization as well as gene regulation at transcriptional and posttranscriptional level
Extracellular RNA (exRNA)	Involved in intercellular communication and cell regulation
Long intergenic non-coding RNA (lincRNA)	Involved in gene expression *via* directing chromatin-modification complexes to specific target regions; lincRNAs located in the cytoplasm function as scaffold to bring together proteins and other RNA categories (especially mRNAs and miRNAs)
Long non-coding RNA (lncRNA)	Involved in regulation of gene expression *via* binding to chromatin regulatory proteins; involved in regulating gene expression at posttranscriptional level by acting as microRNA decoys; some lncRNAs are processed into microRNAs
MicroRNA	Involved in fine tuning cell homeostasis by controlling gene expression at posttranscriptional level
miRNA-offset-RNA (moRNA)	Unknown
piwi-interacting RNA (piRNA)	Involved in maintain germline integrity by repressing transposable elements; involved in mRNA deadenylation
Ribosomal RNA (rRNA)	Component of the ribosomes; involved in protein synthesis
Small Cajal body RNA (scaRNA)	Component of the Cajal bodies; involved in the biogenesis of small nuclear ribonucleoproteins and by this influence splicing of pre-mRNAs
Small interfering RNA (siRNA)	Involved in RNA interference pathway as part of antiviral defense
Small nuclear RNA (snRNA)	Component of the spliceosome; involved in splicing of pre-mRNAs during posttranscriptional modifications
Small nucleolar RNA (snoRNA)	Component of the Cajal bodies; involved in modification and processing of snRNA, rRNA and tRNA precursors as well as in mRNA editing
sno-derived RNA (sdRNA)	Component of the Cajal bodies; involved in alternative splicing of mRNAs; some sdRNAs control gene expression at posttranscriptional level
Transcription initiation RNA (tiRNA)	Involved in regulation of RNA polymerase II dependent transcription
Transfer RNA (tRNA)	Involved in transporting amino acids to the ribosomes during translation
Vault RNA (vRNA)	Component of the vaults (large ribonucleoprotein complexes in cytoplasm); unknown function

against the therapy from the very beginning. In contrast, acquired (secondary) drug resistance develops during therapy due to adaptive processes of the tumor (22, 241–244). Different mechanisms are involved in primary and acquired drug resistance and relate to non-coding RNAs dysregulation.

Deregulation of Proteins Involved in Drug Metabolism

One reason for drug resistance can be found on the level of drug transport. Reduced influx or increased efflux of chemotherapeutics result in lower intracellular drug concentrations and promotes therapy failure (241). Altered drug metabolism is another possible cause for drug resistance. Drug metabolism is a complex pathway composed of multiple proteins for detoxification of foreign compounds (e.g., chemotherapeutics) normally neither produced nor present in a cell (245). This pathway can be subdivided into modification (phase I reaction), conjugation (phase II reaction), and excretion (phase III reaction) (246). Several drug-metabolizing enzymes, especially members of the cytochrome P450 family, together with drug transporters increase the polarity of the drugs during phase I (247, 248). In the following phase II, the polarity of the drugs is further increased by conjugation reactions (249, 250). Finally, in phase III the resulting drug metabolites are exported by transmembrane transporter like ATP-binding cassette (ABC) proteins and solute carrier (SLC) transport proteins (251–254).

The vaults are known to contribute to drug resistance by transporting drugs away from their intracellular targets and vaults are involved in drug sequestration (187). The vRNAs hvg-1 and hvg-2 that are present in the vaults (**Table 2**) interact with drugs *via* specific binding sites (188). In agreement with their role in regard to drug resistance, the number of vaults is increased in cancer patients who developed resistance under chemotherapy (187). In addition, the vRNAs are producing several small RNAs among them is svRNAb which downregulates the key enzyme in drug metabolism CYP3A4 and accounts so for multidrug resistance in GI cancers (186).

Furthermore, lncRNA H19 was identified as another non-coding RNA involved in drug resistance. The oncogenic potential of lncRNA H19 was demonstrated in different tumor types (e.g., liver and esophageal cancer) and overexpression of lncRNA H19 was observed in parallel with upregulation of the membrane glycoprotein p95 in multidrug-resistant tumors (36, 37). In liver tumor cells, resistant to doxorubicin, etoposide, paclitaxel, and vincristine lncRNA H19 expression was increased (36). LncRNA H19 participates in the regulation of *MDR1* gene (also known as *ABCB1* gene) expression and modulates the drug transport out of the cell (36). *In vitro* models of hepatocellular carcinoma suggest that lncRNA H19 can alter *MDR1* promoter methylation and, in doing so, increases the transcription of P-glycoprotein (36).

Similarly, in gastric cancer, MDR-related and upregulated lncRNA (lncRNA MRUL) acts as an enhancer for transcription of P-glycoprotein (MDR1) (69) increasing the number of

transmembrane transporters on the tumor cell membrane and fosters the drug export (69). As we described above, different non-coding RNAs can merge onto the same pathway: this is the case of lncRNA AK022798 whose expression is induced by NOTCH-1 overexpression during gastric cancer progression (28). LncRNA AK022798 in turn upregulates the expression of P-glycoprotein and is responsible for increased cisplatin resistance in gastric cancer patients (28). Similarly, in cisplatin and 5-fluorouracil-resistant gastric cancer patients the expression of lncRNA plasmacytoma variant translocation 1 (PVT-1) and lncRNA ANRIL (antisense to CDKN2B locus) are also increased and these non-coding RNAs promote MDR1 upregulation and drug resistance (29, 30).

Non-coding RNA dysregulation is tissue specific, indeed Wnt-β-catenin pathway activation triggers the expression of a different lncRNA, colorectal cancer-associated lncRNA (CCAL). The effect on phenotype is the same as in other cancers given CCAL in turn upregulates P-glycoprotein expression and causing chemotherapy resistance (34).

Additional to the regulation *via* lncRNAs ABC transporter expression levels are also controlled by miRNAs (255, 256).

In colon cancer, P-glycoprotein expression was found to be directly deregulated at posttranscriptional level by binding of miR-145 to the 3′-UTR of the *MDR1* gene transcript (122). Downregulation of miR-145 results in increased ABCB1 protein level (122). Analogously miR-297 binds to the 3′-UTR of ABCC2 mRNA and supresses the expression of ABCC2 transporter (166). In chemoresistant colorectal carcinoma, miR-297 is often downregulated and consequently ABCC2 is expressed on a higher level compared to the surrounding colon tissue (166). Interestingly, *in vitro* and *in vivo* models suggest that resistance to vincristine and oxaliplatin could be overcome by restoring miR-297 expression in therapy-resistant cells (166). Virtually expression of all the transporters can be affected by microRNA dysregulation; ABCB5 transporter is highly expressed in colon cancer cell lines with downregulated miR-522 expression and renders these cells resistant to doxorubicin treatment (182). miR-522 binds to the ABCB5 mRNA 3′-UTR and overexpression of miR-522 reverse chemoresistance to doxorubicin (182). Similarly, 5-fluorouracil resistance in microsatellite instable colon cancer [caused by deregulated miR-21 or miR-155 (124, 138) as mentioned in detail later] can be enhanced by downregulation of miR-23a resulting in higher expression of the direct target ABCF1 (156).

Similar examples exist across the board: in gastric cancer for example, downregulation of miR-508-5p was identified as a reason for multidrug resistance (180). miR-508-5p represses the expression of P-glycoprotein and the transcription factor zinc ribbon domain-containing 1 (ZNRD1) that is an important factor for *MDR1* gene translation (180). Loss of miR-508-5p decreased drug sensitivity in gastric cancer *in vitro* and *in vivo*, whereas ectopic expression of miR-508-5p overcomes drug resistance (180).

In pancreatic cancer cell lines, expression of the transporter ABCC1 is controlled by miR-1291 binding to the 3′-UTR (118). miR-1291 is often downregulated in pancreatic cancer resulting in an increased expression of ABCC1 that finally leads to higher efflux rate of toxic substances (257, 258). This is the reason for resistance to many chemotherapeutics, such as anthracyclines (e.g., doxorubicin), platinum derivates, and the folate antagonist methotrexate (257, 258). Another transporter, called ATP7A (ATPase Cu^{2+} transporting alpha polypeptide), is upregulated in *in vitro* models of resistant pancreatic tumors due to decreased expression of miR-374b (174) and increased ATP7A protein expression is at least partially responsible for cisplatin resistance in pancreatic cancer model systems (174).

Downregulation of miR-122 in liver tumors results in high expression of ABC transporter proteins and causes increased drug export of doxorubicin in liver cancer patients (114). Similarly, ABCB1 transporter expression is upregulated in hepatocellular cancer cells when the posttranscriptional regulator miR-223 is downregulated and the result is again resistance to doxorubicin treatment (259).

Downregulation of microRNAs let-7g and let-7i results in increased expression of ABCC10 that in turn is responsible for resistance to cisplatin therapy in esophageal cancer patients (102).

An important barrier for oral anticancer drugs is represented by intestinal epithelial cells of the GI tract (256, 260). The absorption of most nutrient components as well as drugs is related to a variety of influx transporters such as members of the SLC transporter family (256). The expression pattern of the SLC transporter varied according to the differentiation status of intestinal epithelial cells which is controlled by microRNAs (261). Therefore, changes in the expression level of microRNAs have most probably an important influence on the drug uptake rate (261). Up to now the role of microRNAs for the expression level of SLC transporter have been studied only in cell culture models for colon carcinoma, liver, pancreatic, and gastric tumors (115, 183). In colon cancer cells, expression of miR-92b reduces the amount of SLC15A and SLC15A1 transporter resulting in decreased drug absorption (183). In the context of liver and pancreatic tumors miR-29a, miR-29b, and miR-124 target SLC16A1 and reduce the expression of this transporter (115). Recently, it was shown that miR-939 targets direct SLC34A2 in gastric cancer (184). In 5-fluorouracil-resistant gastric cancer, miR-939 is downregulated and results in increased expression level of SLC34A2. The transport protein SLC34A2 acts as mediator of miR-939 and activates the Ras/MEK/extracellular signal-regulated kinase (ERK) pathway which is known to be deregulated often in cancer and to cause resistance to chemotherapy (184). In *in vitro* models of gastric cancer, overexpression of miR-939 strongly decreased MEK1/2 phosphorylation as well as Raf-1 level, whereas SLC34A2 restoration rescued these effects (184).

Also for some drug-metabolizing enzymes posttranscriptional regulations by miRNAs have been proven (256, 262, 263). Due to their pivotal role in maintaining chemical and functional homeostasis of cells, cytochrome P450 enzymes are strictly controlled. Under physiological conditions, cytochrome P450 enzymes are involved in the regulation of endogenous molecules like bile acids and steroids and under pathological conditions in the case of chemotherapy these enzymes are important in regard to drug metabolism. Deregulated expression of cytochrome P450 enzymes is linked to drug resistance and therapy failure (264).

For example, miR-378 targets mRNA coding for CYP2E1 and reduces the expression level of CYP2E1 protein in cell culture

models of liver tumors (175, 265). In liver cancer patients, CYP2E1 expression is increased while miR-378 is downregulated (175, 265). Also, a direct regulation of CYP1B1 by miR-27b was demonstrated in hepatocellular cancer cell lines (164). Decreased expression of miR-27b results in high expression level of CYP1B1 and renders by this liver tumor resistant to docetaxel treatment (164).

In pancreatic cancer cells, overexpression of miR-27b leads to downregulation of CYP3A4 protein and results in drug resistance to cyclophosphamide because CYP3A4 is necessary for drug activation (165). MicroRNA-based regulation of enzymes involved in phase II reactions are less analyzed but nevertheless, in the context of esophageal cancer, regulation of glutathione S-transferase P1 (GSTP1) was found to be regulated by miR-133a (121). Reduced expression of the tumor suppressor miR-133a resulted in increased level of GSTP1 protein (121). In phase II detoxification reactions—including inactivation of platinum derivates and alkylating reagents—GSTP1 catalyses the addition of glutathione to the drug activated during phase I reactions with electrophiles (249, 250).

A more specific influence of non-coding RNAs on drug metabolism was demonstrated for 5-fluorouracil in liver and colon tumors (163, 178). Dihydropyrimidine dehydrogenase, an important enzyme in 5-fluorouracil metabolism, is repressed by miR-494 in colon tumors and by miR-27a as well as miR-27b in liver cancer (163, 178). The fact that the translation of one and the same enzyme in two different tissues is under the control of different miRNAs underlines the tissue-specific regulation and fine-tuning of protein expression that is exerted by miRNAs.

In liver cancer, the translation of two of the most important targets of chemotherapeutic agents, dihydrofolate reductase and thymidylate synthase, are repressed by upregulation of miR-215 (148). Reduced expression of dihydrofolate reductase and thymidylate synthase leads to the development of insensitivity to doxorubicin treatment (148).

Thymidylate synthase is the target of 5-fluoruracil therapy and this enzyme is downregulated by increased expression of miR-192 and miR-215 in colon cancer patients (129). In this case, altered microRNA expression results in down-modulation of the drug target and leads to therapy failure. In addition, miR-192 and miR-215 alter the cell-cycle control at multiple levels and prevent progression into the S-phase leading to 5-fluorouracil resistance (129).

A similar case was observed in pancreatic tumors where ribonucleotide reductase regulatory subunit M2 (RRM2) the target of gemcitabine is under direct control of miR-211 and let-7a (101, 147). Decreased expression of miR-211 and let-7a results in higher RRM2 protein level and renders the tumors resistant to gemcitabine (101, 147).

Deregulation of Cell-Cycle, DNA Repair Pathways and Alteration in Death Pathways

Impaired cell cycle regulation and alteration of cell death pathways are common causes of drug resistance (243, 266). Increased cell cycle progression and reduced cell death rate lead to accumulation of mutations and uncontrolled cell proliferation, a hallmark of tumor cells (267). Errors in the DNA-damage response program pathways [nuclear excision repair (NER), base excision repair (BER), and DNA mismatch repair (MMR)] play an important role in cancer progression and chemoresistance (268–271). A complex interaction interplay exists between non-coding RNAs and the DNA-damage pathways: on one hand the DNA-damage pathway induces the expression of several non-coding RNAs especially of microRNAs and on the other hand non-coding RNAs regulate directly the expression of several genes involved in DNA-damage pathway. This interaction is cell type specific and dependent on the intensity and nature of DNA damage (272–276).

LncRNA HOX transcript antisense RNA (HOTAIR) is highly expressed in a broad variety of solid tumors including liver, colorectal, pancreatic, and GI stromal tumors (39, 40, 277). LncRNA HOTAIR reprograms chromatin organization together with the polycomb repressive complex PRC2 (40). Upregulation of lncRNA HOTAIR results in higher expression level of members of the PRC2 complex (SUZ12, EZH2, and H3K27me3) (40). Therefore, increased lncRNA HOTAIR expression is associated with a genome-wide reprogramming *via* PRC2 mediated epigenetic silencing of chromatin (40). In addition, lncRNA HOTAIR downregulates cyclin-dependent kinase inhibitor 1 [p21(WAF/CIP1)] (41) causing the loss of an important regulator of the G_1 and S phase progression (38, 278, 279). Due to the fact that p21(WAF/CIP1) represents a major target of p53 activity DNA damage in lncRNA HOTAIR expressing tumor cells don't go into cell cycle arrest and this promote cisplatin resistance (38, 41, 278, 279).

In esophageal, gastric, colorectal, and hepatocellular cancer as well as cholangiocarcinomas, lncRNA taurine-upregulated gene 1 (TUG1) is involved in causing resistance to chemotherapy (79–85). In tumor tissue, lncRNA TUG1 is upregulated and promotes cell growth by increased transcription of the *Bcl-2* gene and epigenetic silencing of cyclin-dependent protein kinase inhibitors (p15, p16, p21, p27, and p57) and proapoptotic genes (caspase-3, caspase-9, and Bax) (79–85). Therefore, lncRNA TUG1 is an excellent example for the fact that non-coding RNAs target simultaneously the expression of different genes; beside increasing the expression level of the antiapoptotic protein Bcl-2, expression of key players in the caspase-mediated apoptosis pathway are inhibited together with different cyclin-dependent protein kinase inhibitors. This results in decreasing the G0/G1 arrest during cell cycle and reduces the apoptosis rate of the tumor cells. Most probably lncRNA TUG1 has also a role in the EMT (83, 85) that increases resistance to drug treatments further as outlined in detail below.

Also, the lncRNA promoter of CDKN1A antisense DNA damage-activated RNA (PANDAR) is often deregulated in different GI tumors like gastric, colorectal, and hepatocellular cancer as well as cholangiocarcinoma (71–74). In all these tumors, upregulation of lncRNA PANDAR results in increased proliferation rate and reduced apoptosis (71–74). LncRNA PANDAR interacts with the transcription factor NF-YA, an important regulator for transcription of proapoptotic genes (70). This interaction between lncRNA PANDAR and NF-YA results in decreased expression of proapoptotic genes and eventually leads to drug resistance (71–74).

LncRNA urothelial carcinoma associated 1 (UCA1) mediates resistance to doxorubicin treatment in gastric cancer (94). In *in vitro* systems, knockdown of lncRNA UCA1 overcomes the doxorubicin resistance due to an increased expression of PARP and reduced expression of Bcl-2 resulting in higher apoptosis rate (94).

Furthermore, it was shown that lncRNA UCA1 sequesters miR-204-5p in colorectal cancer and reduces the level of this microRNA in cancer cells (90). The consequence is enhanced cell proliferation and 5-fluorouracil resistance (90).

Another example of non-coding RNAs influencing cell-cycle is lncRNA adriamycin resistance associated (ARA) (31, 32). LncRNA ARA was found to be overexpressed in doxorubicin-resistant liver cancer cell lines compared to the parental cell lines (31). Downregulation of lncRNA ARA results in cell-cycle arrest in G2/M phase, suppressed proliferation, increased apoptotic cell death and, as expected, a reduced resistance against doxorubicin (31, 32). Furthermore, lncRNA ARA is involved in the regulation of multiple signaling pathways including the MAPK-pathway (31, 32). Beside lncRNA ARA the lncRNA upregulated in hepatocellular carcinoma (URHC) is found among the most upregulated lncRNAs in hepatocellular carcinoma. One target of lncRNA URHC is the tumor-suppressor ZAK (97). Downregulation of ZAK *via* lncRNA URHC results in increased cell proliferation and inhibits apoptosis (97).

In pancreatic cancer, lncRNA HOXA transcript at the distal tip (HOTTIP) upregulates the homeobox-transcription factor HOX13 resulting in deregulation of the cell cycle as well as gemcitabine resistance (49, 50).

Downregulation of lncRNA LOC285194 in esophageal cancer results in resistance to chemoradiotherapy (radiation in combination with platinum- or paclitaxel-based chemotherapy) by influencing cell-cycle progression and non-apoptotic cell death pathway *via* regulating VEGF receptor 1 (60).

In contrast, lncRNA metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1) is strongly overexpressed in esophageal tumor tissue and binds miR-107 and miR-217 (62, 63). miR-107 and miR-217 decoy translates in reduced activity of the ATM-CHK2 signaling pathway leading to reduced cell-cycle arrest and cell death as response to DNA damage (61, 63) and overexpression of the transcription factor B-Myb—an important regulator for G1/S and G2/M cell-cycle progression and cell survival (62, 63).

In addition, several microRNAs have been identified as regulators for cell cycle progression and induction of cell death pathways. Therefore, deregulated microRNA expression pattern is often a reason for drug resistance in GI tumors.

Colorectal cancers with upregulated mir-203 are resistant to oxaliplatin (136). Failure of oxaliplatin therapy is caused by miR-203 mediated downregulation of the important mediator protein for DNA damage response ATM (136). As reaction to DNA damage, ATM induces the expression of DNA repair proteins, interrupts the cell cycle, and induces cell death in the case of extended DNA damage (280). Oxaliplatin resistance can also be caused by upregulation of miR-503-5p in colorectal cancer (179). Increased expression of miR-503-5p results in downregulation of the apoptotic protein p53 upregulated modulator of apoptosis (PUMA) and leads to resistance to oxaliplatin-induced apoptosis (179). In colon cancer tissues, downregulation of miR-320 is linked to resistance to 5-fluorouracil therapy (169). Among the targets for miR-320 is the transcription factor SOX4 which is involved in inhibition of p53-mediated apoptosis as well as the cell cycle regulators FOXM1 and FOXQ1 both known to have oncogenic potential (169, 170).

In colorectal cancer cells, miR-21 overexpression results in inhibition of the MMR proteins MSH2 and MSH6, two important proteins for DNA damage recognition and repair (138). Inhibition of MSH2 and MSH6 leads to reduced G2/M cell-cycle arrest caused by 5-fluorouracil induced DNA damage and lower apoptosis rate *in vitro* and *in vivo* (138). Therefore, miR-21 overexpression reduces the therapeutic efficacy of 5-fluorouracil-based chemotherapy in colorectal cancer treatment (138). Furthermore, it was proven that the core mismatch repair proteins MSH2, MSH6, and MLH1 are also downregulated by miR-155 potentially contributing to drug resistance (124). According to another study, 5-fluorouracil resistance in colorectal cancer cells can also be mediated by increased expression of miR-31 causing cell cycle deregulation and reduced apoptosis rate (167, 168). Efficacy of 5-fluorouracil treatment in colorectal cancer patients can also be limited due to upregulation of antiapoptotic proteins like X-linked inhibitor of apoptosis (XIAP) and ubiquitin-conjugating enzyme E2N (UBE2N) as a consequence of decreased miR-96 expression (185) or due to upregulation of the antiapoptotic proteins Bcl-2, Bcl-2-like protein 11 (BIM), or Bcl-2-like protein 2 (Bcl2L2) by reduced expression of miR-129, miR-10b, or miR-195, respectively (106, 117, 131). In other colon cancer studies, reduced expression levels of miR-365, miR-1915, and miR-34a have been described as reason for increased expression of BCL-2 (128, 172, 173).

Increased Bcl-2 expression has been identified as a reason for resistance to 5-fluorouracil in other GI tumors, too, but the posttranscriptional regulation of mRNA coding for Bcl-2 is under the control of different miRNAs; e.g., in gastric cancer diminished expression of miR-204 is the reason (281). According to another study upregulation of Bcl-2 is caused by lower miR-15b and miR-16 expression level and leads to drug resistance in gastric cancer cells due to reduced apoptosis (125). miR-25 overexpression was related to cisplatin resistance in gastric cancer cells (160). miR-25 targets directly mRNAs coding for tumor suppressors like FOXO3a, ERBB2, and F-box/WD repeat-containing protein 7 (FBXW7) (157–160). All these proteins are involved in cell cycle regulation and apoptosis (160, 282, 283). Upregulation of miR-223 targets FBXW7 and leads to cell-cycle deregulation and cisplatin resistance in gastric tumors (154). Furthermore, upregulation of miR-103/107 results in decreased expression of caveolin-1 in gastric cancer cells (109). The tumor suppressor caveolin-1 is a counter regulator for the Ras-p42/p44 MAP kinase pathway and due to the downregulation by miR-103/107 increased activity of the Ras-p42/44 Map kinase pathway results in increased cell cycle progression and reduced cell death (107, 108). In gastric cancer, increased cell cycle progression is also caused by increased expression of miR-215 resulting in reduced expression of the tumor suppressor retinoblastoma 1, an important cell cycle regulator (149, 150). Upregulation of miR-106a targets FAS and inhibits the extrinsic apoptotic pathway in gastric cancer (110, 111).

In turn, reduced amount of FAS leads to increased cell proliferation, reduced apoptosis rate, and drug resistance (110, 111).

Overexpression of miR-21 inhibits cell cycle arrest resulting in increased cell proliferation, reduced apoptotic rate, gemcitabine, and 5-fluorouracil resistance in pancreatic cancer (284–286). Similarly, in other pancreatic cancer studies, miR-21 overexpression results in reduced level of PTEN and Bcl-2 leading to activation of AKT-mTOR pathway, reduced apoptosis, and resistance against gemcitabine treatment (140, 141). Increased expression of miR-214 represses directly ING4 in pancreatic tumor (287). This impairs cell-cycle arrest, DNA repair as well as apoptosis and results in resistance to gemcitabine treatment (287). The expression of the important proapoptotic protein BIM is reduced by miR-17-5p in pancreatic cancer and results in decreased apoptotic rate leading to resistance to gemcitabine treatment (127). Therapy failure is also caused by the repression of a tumor suppressor network involved in cell cycle and apoptosis regulation composed of PDCD4, BTG2 and NEDD4L by the combined action of miR-21, miR-23a, and miR-27a (145, 146). Furthermore, overexpression of miR-1246 results in decreased expression of cyclin-G2 and impairs the cell cycle regulation resulting in resistance to gemcitabine (116). Recently, miR-1307 was identified to be responsible for FOLFIRINOX resistance in pancreatic cancer (120). miR-1307 is upregulated in *in vitro* models of FOLFIRINOX-resistant pancreatic cancer as well as in patient derived material compared to the surrounding tissue (120). Reduced apoptosis rate and an extended acceptance of DNA damage seem to be the consequence of higher miR-1307 expression (120).

In hepatocellular carcinoma, the liver specific miR-122 is downregulated and as consequence the expression of the target gene *CCNG1* is increased (113). High level of cyclin G1 protein is found in several human tumors and results in reduced cell cycle control in the G2/M phase and modulation of p53 activity (113, 114). This results in reduced DNA-repair and diminished apoptotic rate (113, 114). As already mentioned above, ABC transporter proteins are highly expressed in liver tumors due to the missing posttranscriptional regulator miR-122 (114). All these effects caused by miR-122 downregulation promote doxorubicin resistance in liver cancer patients (113, 114). Another reason for doxorubicin resistance in liver cancer is based on reduced expression of miR-26b (161). Among the miR-26b targets in liver are the NF-κB activating proteins TAB 3 and TAK1 (161, 162). Therefore, a reduced expression of miR-26b results in increased activation of NF-κB and promotes drug resistance (161, 162). Also, downregulation of miR-101 is described as reason for resistance to doxorubicin in hepatocellular carcinoma (105). The antiapoptotic protein Mcl-1 is among the targets of miR-101 and high levels of Mcl-1 renders liver tumor cells resistant to doxorubicin treatment (105). Furthermore, doxorubicin treatment failure in liver cancer patients has been connected to downregulation of miR-199a-3p (133). Besides targeting mTOR and c-Met, miR-199a-3p influences cell cycle regulation (133). Decreased miR-199a-3p level results in downregulation of the G1-checkpoint CDK inhibitors p21 (CDKN1A) and p27 (CDKN1B) and abrogate the G1 arrest following damage to DNA (132, 133). In another study, downregulation of the G1 inhibitor CDKN1A in hepatocellular carcinoma was linked to upregulation of miR-519d (181). Consequently the apoptotic rate is reduced due to downregulated miR-199a-3p as well as upregulated miR-519d expression (133, 181).

Another important tumor suppressor protein involved in resistance to anticancer drugs is PTEN because it is a main regulator for PI3K-AKT-mTOR pathway which is often hyperactivated in cancer and is one of the drivers for tumor growth and survival (288, 289). PTEN itself is regulated by different microRNAs in different GI tumors, e.g., by miR-21 in liver and gastric cancer, miR-22 in p53-mutated colon cancer and mir-17-5p in colorectal cancer (126, 142–144, 151). In all cases, upregulation of microRNAs results in decreased PTEN level in the tumor cell and subsequent activation of AKT-mTOR pathways resulting in resistance to cisplatin (gastric cancer), paclitaxel (p53-mutated colon tumor), and FOLFOX (colorectal cancer) (126, 142–144, 151). Downregulation of PTEN due to overexpression of miR-19a and miR-19b in gastric cancer results in multi-drug resistance (134).

Furthermore, mTOR is an important regulator under physiological as well as pathological conditions. In p53 mutant colorectal cancer, mTOR is downregulated by miR-338-3p and results in resistance to 5-fluorouracil treatment (171). Indeed, inhibition of miR-338-3p in cell culture models restored sensitivity to 5-fluorouracil (171) likely due to increased autophagy and reduced apoptosis following decrease in mTOR expression (171, 290).

Autophagy is a further mechanism for chemoresistance (51, 291–293). In liver cancer, upregulation of lncRNA HULC activates autophagy by increasing the expression of ubiquitin-specific peptidase 22 (USP22) which in turn prevents the ubiquitin-mediated degradation of silent information regulator 1 (SIRT1) by removing the conjugated polyubiquitin chains from SIRT1 (51). Autophagy causes resistance to oxaliplatin, 5-fluorouracil and epitubicin treatments in liver tumors (51). In addition, lncRNA HULC downregulates the expression of microRNAs that target directly the 3′-UTR of USP22 (miR-6825-5p, miR-6845-5p, and miR-6886-3p) in liver cancer cells and prevents by this inhibition of USP22 at translational level (51).

LncRNA MALAT-1 is highly expressed in gastric cancer cells resistant to 5-fluoruracil and cis-platin, respectively, compared to parental gastric cancer cells (67). LncRNA MALAT-1 quenches miR-23b-3p and subsequently increases the expression of ATG12, an important regulator of autophagy (67).

In oxaliplatin-resistant colon cancer, miR-409-3p is downregulated so that the direct target Beclin-1 is expressed and induces autophagy (176). Overexpression of miR-409-3p results in low autophagic activity and overcomes oxaliplatin resistance in model systems of colon cancer (176).

Induction of EMT

Drug resistance can be caused by EMT (294, 295). Several EMT-related signaling pathways are well known to be involved in mediating drug resistance in tumors (22, 295–297). Cells undergoing EMT have several features in common with cancer stem cells (e.g., increased drug efflux pumps and antiapoptotic effects) and furthermore EMT is instrumental for generation and maintenance of cancer stem cells (22, 295, 297).

The lncRNA plasmacytoma variant translocation 1 (PVT1) has been found to be elevated in nearly all GI tumors including gastric,

esophageal, pancreatic, colon, and liver cancers (75–77, 298). Increased expression of lncRNA PVT1 results in EMT and drug resistance (75–77).

The tumor suppressor lncRNA LEIGC prevents normal cells to undergo EMT. Therefore, the reduced expression of lncRNA LEIGC in gastric cancer fosters EMT and results in resistance to 5-fluorouracil treatment (54, 55).

Upregulation of lncRNA HULC has been correlated to induce EMT and suppressed apoptosis in gastric tumors, leading to cisplatin resistance (52, 53).

Increased expression of lncRNA-activated by TGF-β (lncRNA-ATB) in liver cancer results in competition with members of the miR-200 family for binding sites in the 3′-UTR of mRNAs coding for the transcription factors ZEB1 and ZEB2 (33). In turn, high expression of ZEB1 and ZEB2 causes EMT and increased drug resistance (33).

In pancreatic cancer, the lncRNA MALAT-1 is a regulator of EMT (64, 65). In addition, the lncRNA MALAT-1 suppress G2/M cell cycle arrest and apoptosis leading to resistance to gemcitabine treatment (65). As demonstrated by this example, the same lncRNA can induce resistance to chemotherapy by regulating different mechanisms at the same time.

Induction of EMT and resistance to gemcitabine treatment in pancreatic cancer cells can also be caused by miR-223 overexpression (153). Inhibition of miR-223 restored the sensitivity of pancreatic cancer cell lines to gemcitabine treatment (153). Similarly, gemcitabine resistance in pancreatic cancer can also be caused by downregulation of microRNAs as demonstrated for miR-200 (miR-200a, miR-200b, and miR-200c) and let-7 family resulting in EMT (100, 135).

In colon cancer cells, downregulation of miR-147 results in EMT and increases the phosphorylation rate of AKT (123). Beside the activation of the PI3K-AKT pathway, the lower expression level of miR-147 also activates the TGF-β pathway and eventually leads to resistance to gefitinib treatment (123). Increased expression of miR-224 in colon cancer tissue was identified as another reason for resistance to 5-fluorouracil treatment. Increased miR-224 expression translates in increasing phosphorylation rate of ERK and AKT, resulting in activation of both pathways (155). In addition, miR-224 seems to activate also EGFR dependent- and NF-κB-signaling pathway leading to EMT (155).

Cancer Cell Stemness

A further reason for drug resistance is the presence of cancer stem cells. Cancer stem cells are well known for being refractory to chemotherapies and therefore cause therapy failure and tumor recurrence or progression (299–305). Once again non-coding RNAs especially lncRNAs and microRNAs are involved in sustaining the cancer stem cell niche (95, 306–309).

The lncRNA urothelial carcinoma associated 1 [identical with lncRNA CUDR (cancer upregulated drug resistant)] is strongly expressed in different tumors; among these, gastric, hepatocellular, pancreatic, colorectal cancers, and esophageal squamous cell carcinoma (94–96, 310–314). LncRNA UCA1 binds to several microRNAs in different tumors (e.g., miR-216b in liver cancer, miR-204 in esophageal and colon cancer, miR-27b in gastric cancer) and influences entire transcriptional programs as well as response toward therapy (90, 92, 312, 314, 315). Well-established upregulated targets of lncRNA UCA1 are members of the Wnt-β-catenin signaling pathway, several transcription factors and cell division regulators (87, 93). For stem cells, the Wnt-β-catenin pathway is of pivotal importance for cell self-renewal and mediating drug resistance (316, 317). Overexpression of lncRNA UCA1 results in resistance to cancer treatments with tamoxifen, 5-fluorouracil, gemcitabine, cisplatinum, doxorubicin, imatinib, and tyrosine-kinase inhibitors targeting EGFR (90, 94, 96, 314).

Silencing of lncRNA UCA1 in *in vitro* and *in vivo* systems proved the oncogenic role of lncRNA UCA1 in gastric cancer (94, 96). Reduced expression level of lncRNA UCA1 results in reduced proliferation rate, increased apoptosis rate and overcomes the resistance to doxorubicin (94, 96). Furthermore, lncRNA UCA1 is a direct regulator of the PI3K-AKT-mTOR pathway (96) which is often found to be deregulated in human cancers and is known to contribute to chemoresistance of cancer cells (318, 319). In another study, overexpression of lncRNA UCA1 was shown to cause reduced miR-27 expression causing diminished apoptosis of gastric cancer cells due to increased Bcl-2 protein level in combination with reduced cleaved caspase-3 (92). This results in multidrug resistance of gastric tumors (92).

Overexpression of lncRNA UCA1 is also a reason for chemoresistance against 5-fluorouracil treatment in colon cancer (90). LncRNA UCA1 causes resistance by binding miR-204-5p and consequently upregulating the expression of its target genes Bcl-2, RAB22A, and CREB1 (90). miR-21 was identified as an important player in regard to failure of 5-fluorouracil therapy in colon cancer patients (139). miR-21 is able to increase the number of undifferentiated cancer stem cells during 5-fluorouracil treatment and contributes by this to therapy failure (139).

In liver cancer, lncRNA UCA1 contributes to chemotherapy resistance and malignant transformation of hepatocyte-stem cells (88, 93, 95, 320–322). LncRNA UCA1 increases directly the transcription rate of the oncogene c-myc well known to be involved in drug resistance as well as in activating stem-cell like properties in hepatocarcinoma (86, 89, 323–325). Furthermore, lncRNA UCA1 also induces the expression of lncRNA HULC (highly upregulated in liver cancer) in liver cancer and lncRNA HULC in turn stimulates the activity of the Wnt-β-catenin pathway (88). In addition, lncRNA UCA1 forms a complex with the cell-cycle regulator cyclin-D which enhances the expression of lncRNA H19 by inhibiting the methylation of the lncRNA H19 promoter (89, 95). High level of lncRNA H19 induces the telomerase activity and enhances the length of telomere thereby supporting the stem cell properties (35, 89, 326). Another effect of lncRNA UCA1 is the enhanced phosphorylation of the tumor suppressor retinoblastoma protein 1 (RB1). RB1 phosphorylation results in increased cell cycle progression and in interaction of the phosphorylated retinoblastoma protein 1 with the SET1A complex. Such interaction catalyses the transcription-activating methylation of histone H3 lysine-4 on several gene promoters including telomeric repeat-binding factor 2 promoter an important component for the telomerase extension process (91, 320).

In liver cancer as well as in pancreatic, gastric, esophageal, and colon cancers a critical role in inducing the transformation of stem cells into cancer stem cell has been demonstrated

for lncRNA HOTAIR (45, 95, 327–331). LncRNA HOTAIR is a strong activator for expression of *OCT4*, *RNF51*, *CD44*, and *CD133* genes—all these proteins are involved in reprogramming the gene network to acquire cancer stem cell properties (46, 47). LncRNA HOTAIR expression causes resistance against cisplatin and doxorubicin treatment in liver cancer model systems (332) and renders gastric tumors resistant to cisplatin therapy by binding miR-126 and activating the PI3K-AKT-mTOR pathway (48). In the context of several GI cancer stem cells, it has been shown that lncRNA HOTAIR downregulates the expression of histone methyltransferase SETD2 and reduces the phosphorylation rate of SETD2 resulting in reduced trimethylation of histone H3 lysine-36 on several gene promoter, e.g., Wnt inhibitory factor-1 (WIF-1) (44, 45, 331, 333). Reduced WIF-1 expression leads to activation and increased signaling through the Wnt-β-catenin pathway (44, 45). Furthermore, the modulated chromatin organization account for a reduced efficiency of the mismatch repair system and damaged DNA can escape from corrections leading to microsatellite instability (MSI) and altered expression of cell cycle regulators as well as reduced apoptosis (124, 327, 331, 334, 335). In addition, lncRNA HOTAIR induces accumulation of replication errors by hindering the complex formation of MSH2 with MSH6; one essential dimer for DNA mismatch recognition and repair (42, 43, 124, 138, 336).

In pancreatic cancer, the oncogenic lncRNA MALAT-1 contributes to the expression of the cancer stem cell marker CD133, CD44, CD24, and aldehyde-dehydrogenase (65, 66, 337). In addition, the expression of the core pluripotent factors OCT4, NANOG, and SOX2 are also under the control of lncRNA MALAT-1 (66). LncRNA long intergenic ncRNA regulator of reprogramming (linc-ROR) inhibits the expression of p53 and activates by this the transcription factor ZEB1 in pancreatic cancer (56). ZEB1 in turn suppress the expression of the miR-200 family that leads to maintenance of pancreatic cancer stemness and induces EMT known to be responsible for paclitaxel resistance in pancreatic cancer patients (56, 57). Downregulation of miR-205 results in increased expression of stem cell markers OKT3, OKT8, and CD44 in pancreatic cancer tissue and is linked to gemcitabine resistance (137). Re-expression of miR-205 is able to overcome the gemcitabine resistance in pancreatic cancer model systems (137).

The lncRNA-34a mediates an increase in self-renewal of colon cancer stem cells and induce Wnt as well as NOTCH signaling pathways *via* sequester miR-34a expression (98, 99).

In hepatocellular carcinoma, the lncRNA is involved in regulating core pluripotent factors (OCT-4, NANOG, SOX2) necessary for the stem cell like phenotype and causes resistance to chemotherapy (59). LncRNA linc-ROR competes with miR-145 for the same binding sites present in the mRNAs coding for OCT-4, NANOG, and SOX2 (58). Presence of lncRNA linc-ROR prevents the binding of miR-145 to the mRNA of the core pluripotent factors resulting in translation of these mRNAs and maintains the stem cell phenotype (58). Furthermore, the expression of CD133, another cancer stem cell marker, is directly induced by lncRNA linc-ROR (59).

miR-130b is connected to cancer stem cells growth in liver tumors (119). Increased expression of miR-130b targets directly the mRNA coding for tumor protein 53-induced nuclear protein 1 and reduces the expression level of the corresponding protein (119). Furthermore, high level of miR-130b renders liver tumor cells resistant to doxorubicin treatment (119). Another reason for doxorubicin resistance in liver cancer patients is downregulation of the tumor suppressor miR-101 resulting in increased protein expression of enhancer of zeste homolog 2 (EZH2) (103, 104). EZH2 is a histone-lysine N-methyltransferase enzyme that silence Wnt-pathway antagonists and other tumor suppressor genes on the transcriptional level by histone methylation (338). Overexpression of EZH2 is positively correlated with increased Wnt-β-catenin signaling (338).

miR-221 is over-expressed in 5-fluorouracil-resistant esophageal tumors (152). The mechanisms of resistance is mediated *via* downregulation of the direct target dickkopf-related protein 2 (DDK2) and subsequent activation of the Wnt-β-catenin pathway (152). Furthermore, increased miR-221 expression fosters EMT and facilitates the formation of tumor stem cells (152).

In colon cancer stem cells, miR-451 was found to be downregulated compared to colon cancer cells (177). Reduced level of miR-451 seems to be essential for the self-renewal of colon cancer stem cells (177). In addition, expression of ABCB1 transporter is increased in colon cancer stem cells due to lack of miR-451 posttranscriptional downregulation resulting in resistance to irinotecan treatment (177).

miR-1182 is often downregulated in gastric cancer tissue (112). One direct target of miR-1182 is telomerase reverse transcriptase (hTERT), an enzyme that is involved in controlling the length of telomere. Overexpression of hTERT due to missing transcriptional regulation by miR-1182, results in cell immortality and stem-cell property of gastric cancer cells (112).

Targeted Therapies and Drug Resistance

For GI cancer several targeted therapies exist (**Table 3**) (339–345). They are used alone or in combination with chemotherapy. Unfortunately in most cases the patients develop resistance also against these targeted therapies and the above outlined general principles of drug resistance based on non-coding

TABLE 3 | Approved targeted therapies for GI cancer.

GI cancer	Drug	Target
Gastric cancer	Trastuzumab	HER2
	Ramucirumab	VEGFR-2
	Pembrolizumab	PD-1
Hepatocellular cancer	Sorafenib	RAF, VEGFR-2, VEGFR-3, PDGFR, c-KIT
Colon cancer	Cetuximab, panitumumab	EGFR
	Bevacizumab	VEGF
	Regorafenib	VEGFR-1, VEGFR-2, VEGFR-3, BRAF, c-KIT, RET, PDGFR
Colon cancer with MSI-H	Pembrolizumab	PD-1

HER2, human epidermal growth factor receptor 2; VEGFR, vascular endothelial growth factor receptor; PD-1, programmed cell death protein-1; RAF, rapidly accelerated fibrosarcoma; PDGFR, platelet-derived growth factor receptor; c-KIT, SCFR, mast/stem cell growth factor receptor; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; RET, rearranged during transfection; MSI-H, microsatellite instability-high.

RNA dysregulation are involved. Beside that non-coding RNAs interfering with the targeted protein itself or (up-)regulating the targeted signal pathway are involved in drug resistance (342). Furthermore, therapy failure can be related to activation of alternative signal pathways by non-coding RNAs (68, 342).

Recently, it was demonstrated that resistance to cetuximab in colon cancer patients and in *in vitro* 3-D-cell culture models can be caused by overexpression of lncRNA MIR100HG (68). Two microRNAs, miR-100, and miR-125b, are generated from lncRNA MIR100HG and these microRNAs downregulate in a concerted way five negative regulators of the Wnt/β-catenin pathway resulting in increased Wnt signaling (68). This kind of cetuximab resistance can be overcome by inhibition of Wnt signaling, underscoring the potential clinical relevance of the interactions between EGFR and Wnt/β-catenin pathways (68). Increased mir-125b expression is also correlated with trastuzumab resistance in HER2-positive gastric cancer patients but up to now the molecular basis for this resistance is unclear (346). Sorafenib resistance in hepatocellular carcinoma is caused by lncRNA TUC338 (78). RAS protein activator like-1 (RASAL-1) is a direct target of lncRNA TUC338 and high expression of lncRNA TUC338 inhibits the RASAL-1 expression resulting in activation of RAS-signaling (78). According to another *in vitro* study, reduced expression of miR-193b leads to higher expression of the antiapoptotic protein Mcl-1 and renders hepatocellular carcinoma cells resistant to sorafenib treatment (130).

Non-Coding RNAs as Potential Biomarkers of Resistance and Novel Therapeutics: Promises and Hurdles

Our review summarizes most of the current evidence supporting the role of non-coding RNAs in resistance to chemotherapy and targeted agents. It is likely that, in the near future, given the promising and exciting results obtained with the use of immunotherapy in gastroesophageal (347) and colorectal cancer (348, 349), new data will emerge on the already known regulation of PD-1, PD-L1, and CTLA-4 by non-coding RNAs and response to nivolumab and pembrolizumab (350–352).

The contribution of non-cording RNAs in resistance mechanisms to a broad range of anticancer treatments makes their use as biomarkers or novel therapeutics quite promising but several challenges remain.

Given microRNAs and, to a lesser extent, other non-coding RNAs can be reliably detected in tissues and biofluids such as plasma, serum, and urine, it is tempting to hypothesize the use of non-coding RNA based tools to predict and monitor resistance to anticancer treatments. Few studies have already tested the validity of microRNAs as biomarkers of response to anticancer treatment in other cancers such as prostate (353), chronic lymphocytic leukemia (354), and sarcomas (355). In colorectal cancer, we (356) and others (357–359) have tested the contribution of a single nucleotide polymorphism (SNP) in the binding site of let-7 in the *KRAS* 3'UTR in predicting benefit from anti-EGFR treatment with conflicting results across different trials. Despite the good reproducibility of the assay, the predictive value of the test was not confirmed in all trials likely due to use of cetuximab in different context (neoadjuvant, adjuvant and metastatic colorectal cancer, respectively). Similarly the analysis of a SNP in miR-608 led to contradicting results in patients treated with neoadjuvant or adjuvant chemo- and radiochemotherapy in colon and rectal cancers highlighting some of the challenges in validating data obtained in retrospective series (360–363). Tissue (cancer *versus* stroma) and organ (colon *versus* rectum) specificity in non-coding RNA expression might represent potential explanations for different findings obtained in some of these studies. Beside SNPs, expression of microRNAs can be detected in fresh frozen or formalin fixed paraffin embedded tissues and serve as potential biomarker of sensitivity or resistance to treatment. Robust data have emerged from the retrospective analysis of a prospective phase III clinical trial (364). In this study, *KRAS* wild-type patients were classified based on high or low miR-31-3p expression: patients with high expression were resistant to cetuximab while patient with low expression had good and durable responses which translated in survival benefit. The miR-31 expression cutoff for the classification into high or low expression was predefined in the above study. However, one of the key challenges in validating these interesting findings will be design of a clinically approved assay that can accurately assign patients into one of these two categories. In this prospective, the use of different sources of material (i.e., primary colorectal cancer *versus* metastasis) might result in different basal expression of the microRNA and as such different scoring. Source of material and choice of reference controls represent important obstacles that might bias the definition of a threshold for high or low expression of microRNAs in tissues and biofluids. MicroRNAs can be detected in plasma, serum and urine samples and have been used for early detection and prognostic purposes in GI cancer (365–367). The use of digital droplet approaches allows the quantitative detection of copies of the microRNA of interest based on the starting volume of biofluids and, potentially overcomes or at least mitigates, the issues related to the normalization of data against reference controls, making the definition of cutoff easier to standardize. One study has reported the potential role of miR-126 in predicting and tracking response to chemotherapy and anti-VEGF treatment in colorectal cancer (368) and, with the advent of digital quantitative technologies, more studies are expected.

In consideration of their role in cancer initiation, progression and resistance to treatment, non-coding RNAs and among them microRNAs have been proposed as potential therapeutics (369). A large body of pre-clinical evidence is available on the use of anti-microRNAs or molecules re-expressing microRNAs alone or in combination with other agents in order to increase efficacy and prevent or revert drug resistance (370). Inhibition of microRNAs has been tested in clinical trials in the context of HCV infection (371, 372) and in mesothelioma (373). These trials highlighted a huge potential for microRNA-based therapeutics but at the same time pinpointed some of the criticalities in further clinical development of such approaches. miR-122 inhibition led to durable viral load reduction in both HCV trials and was associated with manageable side effects. Similarly, in mesothelioma patients treated with miR-16-loaded minicells the disease control rate was satisfactory and the toxicity profile acceptable warranting further investigations. Overall in both

approaches the risk of off-target effects represent the main hurdle to be taken into account: indeed miR-122 inhibition has been associated with risk of developing liver cancer in preclinical models (374) and, similarly, overexpression of miR-16 might lead to uncontrolled cardiac effects as proven in the phase I trial (373). These effects might be increased in combination studies in which anti-microRNAs or microRNA-conjugates are delivered together with chemotherapy leading to cumulative side effects. Therefore, a robust understanding of the biology underpinning microRNA deregulation in physiology and pathological conditions in order to implement effort that can minimize the risk of serious adverse events hampering the clinical development of microRNA-based strategies.

CONCLUSION

Non-coding RNAs especially lncRNAs and microRNAs are important mediators for drug resistance. They function in an organ and tissue specific manner and through different molecular mechanisms. One non-coding RNA always have several targets and in the end deregulation of one non-coding RNA alters the expression level of several proteins in a tissue specific way. For example, in the case of miR-374b more than 700 genes have been identified as direct target in pancreatic tissue (174). Drug resistance is a dynamic process caused by several cell and non-cell autonomous mechanisms. Given non-coding RNAs can simultaneously control several cancer-associated pathways, non-coding RNA dysregulation plays a crucial role in treatment resistance. Future studies will continue to shed insights in the fine interplay among lncRNA, microRNA and their target genes and might provide opportunities for more effective strategies to prevent or overcome resistance. In the interim, given non-coding RNAs and especially microRNAs can be tested in tissues and biofluids in a rapid, cost/effective and robust way. More investigational studies should explore their utility to monitor and forecast treatment response and resistance in order to personalize treatments and improve patient's outcomes.

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NV and JCH: idea, conception, and writing the review.

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Circular RNA Signature Predicts Gemcitabine Resistance of Pancreatic Ductal Adenocarcinoma

Feng Shao[1,2†], Mei Huang[2†], Futao Meng[2] and Qiang Huang[1,2]*

[1] Medical College, Shandong University, Jinan, China, [2] Anhui Province Key Laboratory of Hepatopancreatobiliary Surgery, Department of General Surgery, Anhui Provincial Hospital, Hefei, China

Gemcitabine resistance is currently the main problem of chemotherapy for advanced pancreatic cancer patients. The resistance is thought to be caused by altered drug metabolism or reduced apoptosis of cancer cells. However, the underlying mechanism of Gemcitabine resistance in pancreatic cancer remains unclear. In this study, we established Gemcitabine resistant PANC-1 (PANC-1-GR) cell lines and compared the circular RNAs (circRNAs) profiles between PANC-1 cells and PANC-1-GR cells by RNA sequencing. Differentially expressed circRNAs were demonstrated using scatter plot and cluster heatmap analysis. Gene ontology and pathway analysis were performed to systemically map the genes which are functionally associated to those differentially expressed circRNAs identified from our data. The expression of the differentially expressed circRNAs picked up by RNAseq in PANC-1-GR cells was further validated by qRT-PCR and two circRNAs were eventually identified as the most distinct targets. Consistently, by analyzing plasma samples form pancreatic ductal adenocarcinoma (PDAC) patients, the two circRNAs showed more significant expression in the Gemcitabine non-responsive patients than the responsive ones. In addition, we found that silencing of the two circRNAs could restore the sensitivity of PANC-1-GR cells to Gemcitabine treatment, while over-expression of them could increase the resistance of normal PANC-1 and MIA PACA-2 cells, suggesting that they might serve as drug targets for Gemcitabine resistance. Furthermore, the miRNA interaction networks were also explored based on the correlation analysis of the target microRNAs of these two circRNAs. In conclusion, we successfully established new PANC-1-GR cells, systemically characterized the circRNA and miRNA profiles, and identified two circRNAs as novel biomarkers and potential therapeutic targets for Gemcitabine non-responsive PDAC patients.

Keywords: pancreatic ductal adenocarcinoma, gemcitabine resistance, circular RNA, serum marker, diagnosis

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***Correspondence:**
Qiang Huang
ahslyyhq2016@163.com

† These authors have contributed equally to this work.

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INTRODUCTION

Pancreatic cancer is one of the most malignant cancers with very poor prognosis. The incidence of pancreatic cancer has been increasing over the past 20 years. 85% of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) (Siegel et al., 2017). Although great progress has been made in surgery and other treatments for PDAC, the 5-year survival rate of PDAC is still less than 4%, while

the median survival of PDAC patients is only 5–6 months. Since most PDAC patients are diagnosed at an advanced stage, 80% of patients with PDAC is unable to undergo surgical resection treatment. Chemotherapy has become an essential treatment for advanced PDAC (Saung and Zheng, 2017). Gemcitabine is currently a first-line drug of chemotherapy treatment for PDAC patients. However, it is well known that only very few PDAC patients are able to maintain a lasting sensitivity to Gemcitabine-based chemotherapy (Amrutkar and Gladhaug, 2017). The drug resistance has already become the main reason for poor performance of Gemcitabine in the current treatment. Therefore, screening of Gemcitabine resistance related biological markers and improvement of Gemcitabine sensitivity are the main challenge of PDAC research.

Accumulating evidence has shown that circular RNAs (circRNAs), one of endogenous non-coding RNAs, play a key regulatory role in the cellular physiological process and the cancer biological process (Memczak et al., 2013). It is reported that circRNAs act as miRNA sponge which absorb miRNAs and then regulate the expression of miRNA targeted genes (Hansen et al., 2013a,b; Han et al., 2017). Most recently, circRNAs have been shown to be closely associated with various human diseases, such as cancer, cardiovascular disease and neurodegenerative diseases (Westholm et al., 2014; Wang et al., 2016; Chen et al., 2017a; Kristensen et al., 2017). In addition, the inherent stability of circRNAs conferred by the circular structure, allows them to be enriched in the exosomes and stably present in plasma, saliva and other peripheral tissues, which renders them potential diagnostic molecular markers for various diseases (Chen et al., 2017b; Wang et al., 2017).

In this study, we established a new Gemcitabine-resistant cell line (PANC-1-GR) from a pancreatic cancer cell line, PANC-1. The circRNAs expression profile of PANC-1-GR cells was systematically explored comparing with parental PANC-1 cells by RNA sequencing. Our results showed that circRNAs expression profiles are very different between PANC-1 cells and PANC-1-GR cells. The characterization of circRNAs and miRNAs in these cell lines and patient samples led to the identification of two novel circRNAs biomarkers and potential drug targets.

MATERIALS AND METHODS

Patient Samples

This study was approved by the Institutional Ethics Review Board of Anhui Provincial Hospital and was conducted according to the Ethical Guidelines for Human Genome/Gene Research issued by the Chinese Government. The plasma samples of PDAC patients were prospectively collected from 40 patients of Anhui Provincial Hospital from January 2015 to June 2016. All of the patients were histologically or cytologically confirmed as pancreatic ductal adenocarcinoma and received Gemcitabine-based chemotherapy. Twenty of these patients were found to be Gemcitabine non-responsive, as they meet the following definition: progression during or <6 months of previous Gemcitabine treatment including adjuvant therapy.

The peripheral blood (5 ml) was collected in ethylenediaminetetraacetic acid tube, centrifuged at 3000 g for 10 min to harvest plasma, and stored at liquid nitrogen. Patients consent forms were duly signed by the patients according to the regulation of ethical guidelines issued by Anhui provincial hospital.

Cell Lines

PANC-1 cells were obtained from Shanghai Cell Bank. Cells were cultured in RPMI1640 supplemented in 10% fetal bovine serum (FBS) with 100 U/ml penicillin–100 g/ml streptomycin within a humidified incubator containing 5% CO_2 at 37°C.

The cell line which was resistant to Gemcitabine was generated by selection under increasing gradient of Gemcitabine in our lab. The initial concentration of Gemcitabine in the cell culture medium was 0.1 μg/ml. The concentration increased when the survival cells entered the logarithmic growth phase. After 40 weeks of continuous Gemcitabine induced culture, the final concentration of Gemcitabine in the resistant cell culture medium reach up to 200 μg/ml. The Gemcitabine resistant cell line was named PANC-1-GR. The PANC-1-GR cell line was cultured in periodically added 10 μg/ml Gemcitabine to maintain cell resistance.

Cell Viability and Proliferation Assays

MTT assay was performed to determine cell viability of PANC-1 and PANC-1-GR cell lines under Gemcitabine treatment and then indirectly reflected cell sensitivity to Gemcitabine. Cells seeded in 96-well plates, at a density of 5×10^4 cells per well, were given Gemcitabine treatment at different concentrations for 72 h. 100 μl of MTT solution (500 μg/ml) was added to each well and after its conversion to a soluble formazan, cell viability was measured by spectrophotometric absorbance at 570 nm.

Cell proliferation was monitored with xCelligence system. PANC-1 and PANC-1-GR cells were seeded in a 96-well electronic microtiter plate (E-Plate), incubated at 37°C with 5% CO_2, and then monitored on the RTCA System at 30-min time interval for up to 100 hours. The electronic readout of cell-sensor impedance is displayed in real-time as cell index (CI), which directly influenced by cell attachment, spreading, or cell proliferation. The cell index is presented as mean ± SD from three independent wells (calculated by xCELLigence) (Stefanowicz-Hajduk et al., 2016).

RNA Extraction and Quality Control

Total RNA was isolated from tumor tissues and plasma samples using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), according to the manufacturer's protocol. RNA integrity was assessed using standard denaturing agarose gel electrophoresis. The total RNA from each specimen was quantified and quality assurance was provided by NanoDrop ND-1000 spectrophotometer (NanaDrop, Wilmington, DE, United States).

TABLE 1 | Top 10 most differentially expressed circRNAs between PANC-1 and PANC-1-GR.

circRNA	chrom	txStart	txEnd	Strand	CircBase ID	Gene Name	Regulation
1	chr14	21825356	21829372	–	hsa_circ_0000522	SUPT16H	Up
2	chr4	77045802	77065626	–	hsa_circ_0070033	NUP54	Down
3	chr1	15537793	155385714	–	hsa_circ_0008161	ASH1L	Down
4	chrX	154736558	154766779	–	hsa_circ_0006355	TMLHE	Down
5	chr4	174305801	174325101	+	Novel	SCRG1	Up
6	chr19	47421744	47440665	+	hsa_circ_0000943	ARHGAP35	Up
7	chr1	169947225	170001116	–	Novel	KIFAP3	Up
8	chr14	101402108	101464448	+	Novel	SNORD114-1	Up
9	chr4	52729602	52780244	+	Novel	DCUN1D4	Up
10	chr6	29901994	29911250	+	Novel	HLA-G	Up

Transcriptome High-Throughput Sequencing and Subsequent Bioinformatics Analysis

Transcriptome high-throughput sequencing and subsequent bioinformatics analysis were performed by Cloud-Seq Biotech (Shanghai, China). The RNA sequencing data had been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO). The GEO accession number is GSE110580[1]. The scatter plot and cluster heatmap are visualization methods used for assessing the circRNA expression variation. The differentially expressed circRNA between PANC-1 and Gemcitabine resistant PANC-1-GR cells were analyzed by edgeR package in R. Differentially expressed circRNAs with statistical significance (fold changes $\geq$1.5 and $p < 0.05$) between groups were identified using fold change cut-off or volcano plot filtering, respectively. The Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool for KEEG pathway enrichment analysis and Gene Ontology[2], were applied to determine the roles that these differentially expressed circRNAs played in GO terms of biological pathways (Huang da et al., 2009). The circRNA/microRNA interaction was predicted using Arraystar's home-made miRNA target prediction software based on TargetScan and miRanda. The circRNA-miRNA network was constructed and visualized using Cytoscape v3.5.1 (Shannon et al., 2003).

Quantitative Reverse Transcription-Polymerase Chain Reaction Validation Assay

Total RNA samples were reverse-transcribed into cDNA with a random primer using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The expression of circRNAs was measured using quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix (Takara, Tokyo, Japan) in a ViiA 7 Real-time PCR System (Applied Biosystems Inc., Foster City, CA, United States). The sequences of the divergent primers for the detection of the 10 circular RNAs by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were shown in **Table 2**. The RNA levels were normalized to human GAPDH. The expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method.

All of the quantitative PCR reactions were conducted in triplicate. The appearance of a single-peak in the melt-curve suggested the specificity of the PCR products.

Silencing and Over-Expression of circRNA

siRNA sequences for chr14:101402109-101464448+ including: siRNA-1: CUUAAUUGUGGGCUCACAU; siRNA-2: CCUAUAGCUGUGGUAUAAC. siRNA sequences for chr4:52729603-52780244+ including: siRNA-1: CAUAGUAAUAGACGAAUUGA; siRNA-2: UGAAUUCUUAGAAGUUAAAG.

siRNAs were synthesized by GeneChem (Shanghai, China).

The pCD-ciR plasmids was used to carry the circular framework of chr14:101402109-101464448+ and chr4:52729603-52780244+. The primers for chr14:101402109-101464448+ are F: 5′-ATAAGTCTACTTTTCTTCCACGTAA-3′

R: 5′-TTATASTGACATTCTCCTTACTCTGA-3′. The primers for chr4:52729603-52780244+ are F: 5′-CAGCTGAACTCTCATCTCTCAACAC-3′

R:5′-CCTTCCAGAAGTTGGCCTCTTAAAC-3′.

Annexin V-FITC Cell Apoptosis Assay

Cells were harvested for Annexin V-Propidium Iodide (PI) staining after 24 h with 0.1 ug/ml gemcitabine treatment. Cells were analyzed by the FACS Calibur (BD Biosciences). Annexin V-FITC$^+$ PI-cells were considered as apoptosis cells.

TUNEL Assay

Cells were exposed to 0.1 ug/ml gemcitabine for 24 h, washed with PBS, and fixed in cold 4% PFA for 30 min, followed by incubation in 0.1% Triton X-100 in PBS for 2 min on ice. After washing twice in PBS, cells were incubated in working solutions from a One-Step TUNEL apoptosis assay kit (Beyotime Biotechnology).

Statistical Analysis

All experimental data were analyzed using SPSS software (version 22.0; IBM, Armonk, NY, United States) and GraghPad Prism 5.0 (GraphPad Software, La Jolla, CA, United States). The expression

[1] www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110580

[2] https://david.ncifcrf.gov/

TABLE 2 | Primers used for qRT-PCR analysis of circular RNA and mRNA levels.

CircRNA Name	Primer sequences, 5′-3′	Tm (°C)	PS (bp)
hsa_circ_0000522	F: ACTTTGAGCGGGTCCAGTTT	61.05	195
	R: TCTGAAGGTTCAAGGCTGGT	59.84	
hsa_circ_0070033	F: GCCAAAATTGCACAATACAAGA	60.01	198
	R: TTGTGCCAAAACCAGTACCA	60	
hsa_circ_0008161	F: TTGGCTTAGTTGGATCCTCTG	59.32	198
	R: TTTTCCCTTGGGATGAGAGA	59.6	
hsa_circ_0006355	F: AGGCACCTGAGGAATTTGAA	59.67	195
	R: TCCTTTCTCCTGCCACATTC	60.2	
chr4:174305802-174325101+	F: GCTGTTTCACAGACACAAGCA	60.09	161
	R: CCCACGTTACTGAGCACAAA	59.76	
hsa_circ_0000943	F: GACAGAAACCAAAGCCCAAA	60.09	197
	R: TGGTCACTGTTCACCACCTC	59.55	
chr1:169947226-170001116-	F: ACCAGATGGTTTTCCACCAA	60.21	176
	R: CTTTGTTGCTTTCCTCATTGC	59.87	
chr14:101402109-101464448+	F: CAGGATGGGTAGACCAGAGC	59.68	182
	R: TACCCCACGGATCTAACTGC	59.96	
chr4:52729603-52780244+	F: TGGCATTTCTAGTCCCTTTTT	57.88	184
	R: TGCCAGTGTTGAGAGATGAGA	59.56	
chr6:29901995-29911250+	F: AAGGATTACATCGCCCTGAA	59.53	198
	R: GTCCCTGGTACAGGTGTGCT	60.03	
GAPDH	F: GGCCTCCAAGGAGTAAGACC	60.07	122
	R: AGGGGAGATTCAGTGTGGTG	59.96	

Tm, melting temperature; PS, product size; bp, base pairs; F, Forward; R, Reverse

level of each circRNA was represented as fold-change using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Comparison of PANC-1 and PANC-1-GR Cell Lines

A new PANC-1-GR cell line was derived from PANC-1 cell line by selecting under Gemcitabine gradients as shown in Section "Materials and Methods." The drug resistance of this new cell line was confirmed by culturing with Gemcitabine. After incubation with different concentrations of Gemcitabine (0, 0.1, 0.5, 2, 10, 20, 40, 80,120, 160, 200 μg/ml) for 72 h, cell viability was assessed by MTT assay. As shown in cell survival curves in **Figure 1A**, the 50% inhibition concentrations (IC_{50}) of Gemcitabine to PANC-1 and PANC-G cells were 0.06 ± 0.003 μg/ml and 56.2 ± 2.16 μg/ml, respectively. Cell proliferating ability of PANC-1 and PANC-1-GR cell lines was further monitored with xCelligence system. Cell growth data showed that PANC-1-GR cells proliferated slower than PANC-1 cells (**Figure 1B**), which may be due to some cell cycle regulatory molecules differentially expressed between parental and resistant lines. And we will investigate the relevant cell cycle regulatory mechanisms in further study. We also determined the expression of the multidrug efflux pump MDR1, which is commonly observed to be upregulated in various drugs resistance cancer cells and also has been shown to cause gemcitabine resistance in pancreatic cancer cells. It was demonstrated that MDR1 expression was up-regulated in PANC-1-GR cells. (Supplementary Figure S1) The results confirmed that a PANC-1-GR cell line was successfully established for subsequent circRNAs profiling.

Characterization of circRNAs Profiles in PANC-1 and PANC-1-GR Cell Lines

To screen circRNAs which could be involved in Gemcitabine resistance in PDAC, we analyzed and compared circRNAs expression in PANC-1 cells and PANC-1-GR cells using transcriptome high-throughput sequencing analysis. Total RNAs were isolated from PANC-1 and PANC-1-GR cell lines and analyzed by RNA sequencing. Differential gene expression analysis between PANC-1 and PANC-1-GR cells revealed 126 circRNAs whose expression was significantly different in these two cell lines (fold change $\geq$2.0, $p \leq 0.05$), with 68 of them up-regulated and 58 down-regulated in PANC-1-GR cells compared to PANC-1 cells (**Figure 2**).

CircRNAs Gene Symbols and Pathway Analysis

Recent studies have shown that circRNAs are derived from the exons or introns of their parental genes and may regulate the expression of the parental genes (Lasda and Parker, 2014). Based on evaluation of the parental genes attribute in the biological process, cellular components and molecular functions and pathways, we conducted GO and pathway analysis for circRNAs to speculate their potential functions. The lower the p value was, the more significant

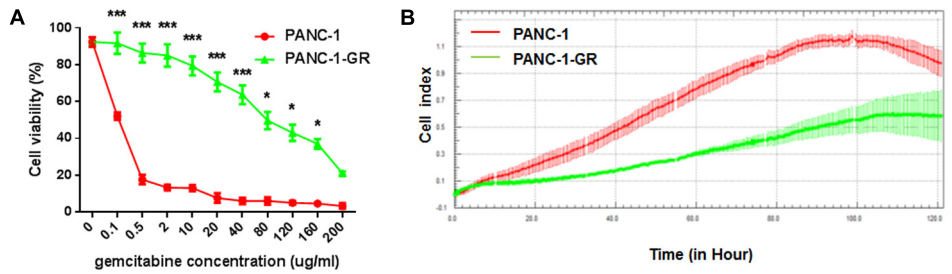

FIGURE 1 | Comparison of PANC-1 and PANC-1-GR cells. **(A)** Gemcitabine cytotoxicity to PANC-1 and PANC-1-GR cells. Cells were incubated continuously with different concentrations of Gemcitabine for 72 h and the cell viability was determined by MTT assay. **(B)** Proliferation assay of PANC-1 and PANC-1-GR cell lines. Data were collected from three independent cultures. Shown are mean values ± standard deviation. ***$p < 0.001$,*$p < 0.05$.

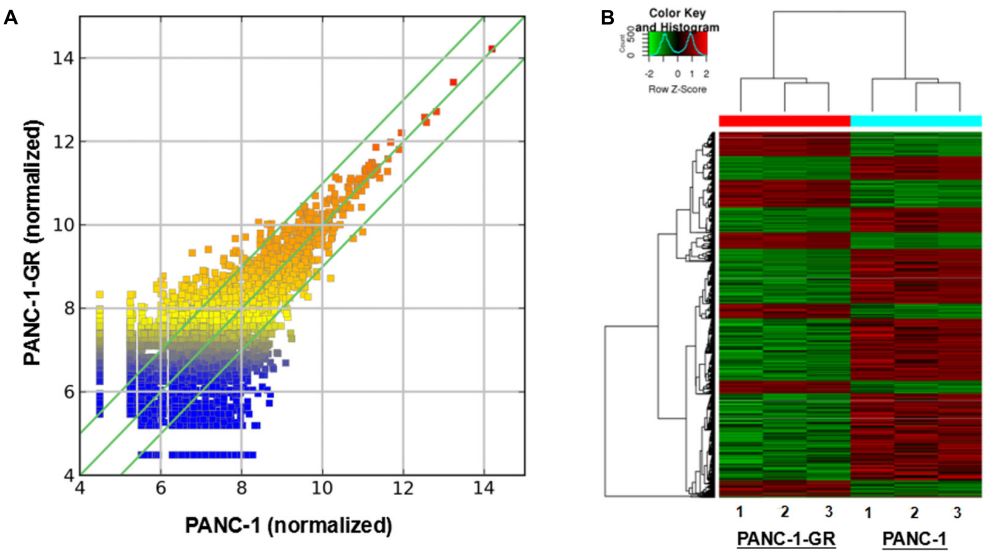

FIGURE 2 | circRNA expression profile of PANC-1-GR cells versus parental PANC-1 cells. **(A)** The scatter plot shows the circRNA expression variation between the parental PANC-1 and PANC-1-GR cell lines. The values of X and Y axes in the scatter plot are the averaged normalized signal values of groups of samples (log2 scaled). The green lines are fold change lines. The circRNAs above the top green line and below the bottom green line indicated more than 1.5-fold change of circRNAs between the two groups of samples. **(B)** Clustered heatmap of the differentially expressed circRNAs in three paired PANC-1 and PANC-1-GR cell lines. Rows represent circRNAs while columns represent cell lines. The circRNAs were classified according to the Pearson correlation.

the correlation was ($p < 0.05$ is recommended). We found that the most significantly enriched GO term in the biological process was the positive regulation of tolerance induction (GO:0002645, $P = 0.0005$) (**Figure 3A**); the most significantly enriched GO term in the cellular component was protein complex (GO:0043234, $P = 0.0001$) (**Figure 3B**); the most significantly enriched GO term in the molecular function was K63-linked polyubiquitin binding (GO:0070530, $P = 0.0016$) (**Figure 3C**). Among the significantly related eight pathways, ErbB signaling pathway and VEGF signaling pathway were previously reported to be involved in the progression of PDAC (**Figure 3D**).

Quantitative PCR Validation in Cell Lines

To verify the sequencing results, the top 10 most differentially expressed circRNAs in PANC-1 and PANC-1-GR cells including seven up-regulated circRNAs (hsa_circ_0000522, hsa_circ_0070033, hsa_circ_0000943, chr1:169947226-170001116-, chr14:101402109-101464448+, chr4:52729603-52780244+, and chr6:29901995-29911250+) and three down-regulated circRNAs (hsa_circ_0070033, hsa_circ_0008161, and hsa_circ_0006355) (**Table 1**) were further confirmed by qRT-RCR. The qRT-PCR data showed that although the trend of expression patterns of these 10 circRNAs were consistent with the sequencing results, among these circRNAs, only two of them (chr14:101402109-101464448+, chr4:52729603-52780244+) were found to be the most significantly up-regulated in PANC-1-GR cell line (**Figure 4**).

Expression of circRNAs in Plasma of PDAC Patients

Subsequently, we verified the expression levels of these two most significant circRNAs (chr14:101402109-101464448+,

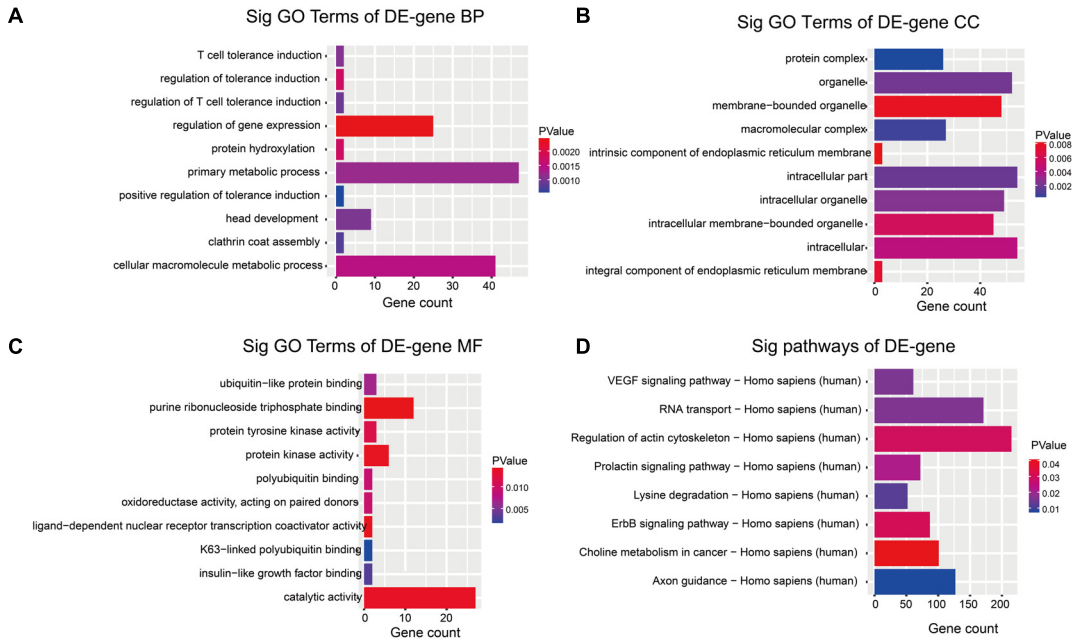

FIGURE 3 | GO enrichment and pathway analysis for dysregulated circRNAs gene symbols. **(A)** Most significantly enriched GO [–log10 (*P* value)] terms of circRNAs gene symbols according to biological process. **(B)** Most significantly enriched [–log10 (*P* value)] GO terms of circRNAs gene symbols according to cellular component. **(C)** Most significantly enriched [–log10 (*P* value)] GO terms of circRNAs gene symbols according to molecular function. **(D)** The bar plot shows the top 10 enrichment score [–log10 (*P* value)] of the significantly enriched pathways.

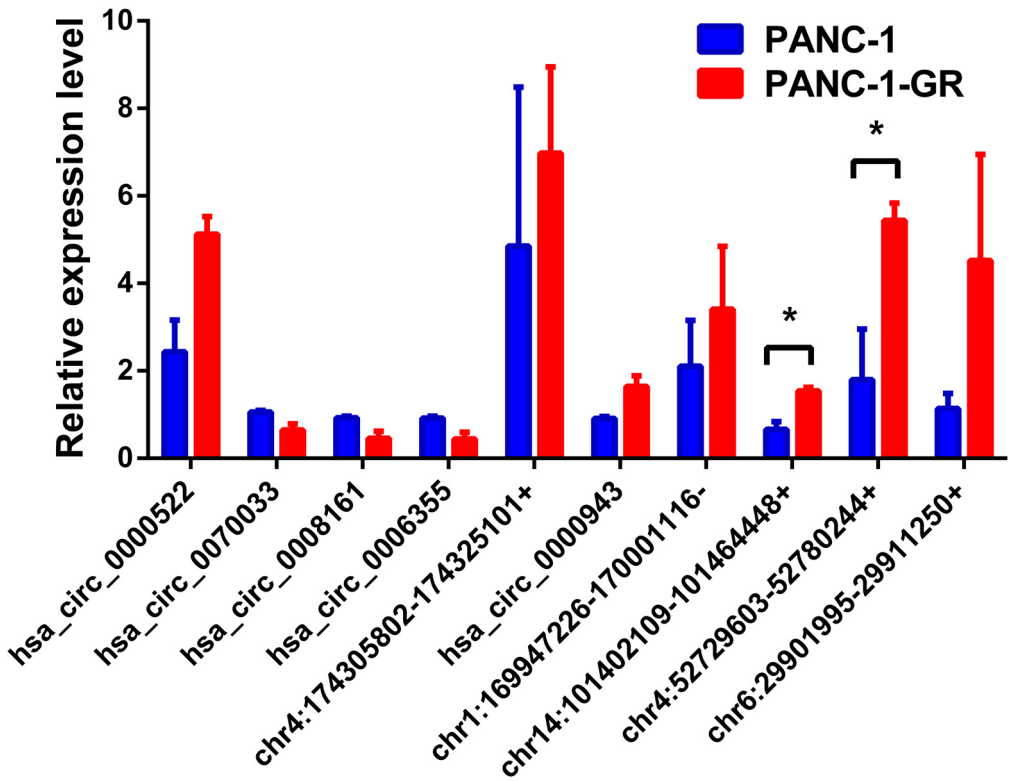

FIGURE 4 | Validation of the top 10 dysregulated circRNAs by quantitative RT-PCR analysis. The top 10 most differentially expressed circRNAs were validated by qRT-PCR in PANC-1 and PANC-1-GR cell lines. The results are presented as mean ± SEM. $^{*}p < 0.05$.

chr4:52729603-52780244+) in plasma of PDAC patients who received Gemcitabine-based chemotherapy via qRT-PCR. In addition, hsa_circ_0008161, which was not significantly regulated as in qRT-PCR data, was used as a negative control. The Gemcitabine-treated PDAC patients were divided into responsive and non-responsive groups according to the effect of Gemcitabine treatment. The clinical characteristics of the patients were shown in **Table 3**. Consistent to RNA sequencing and qRT-PCR data from PANC-1-GR cells, chr14:101402109-101464448+ and chr4:52729603-52780244+ were found significantly up-regulated in non-responsive group ($p < 0.001$), while there was no significant difference in hsa_circ_0008161 (**Figure 5**).

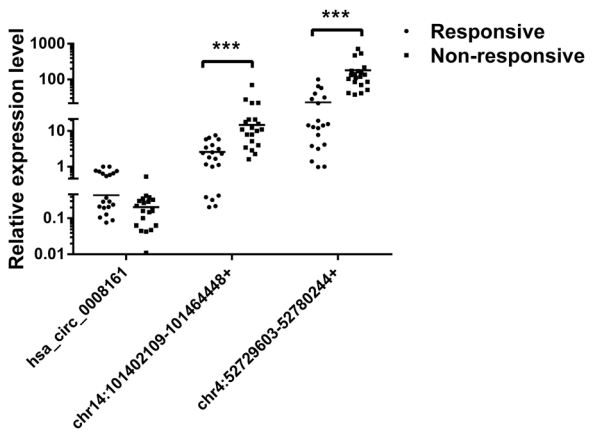

FIGURE 5 | The expression levels of circRNAs in plasma of PDAC patients. PDAC patients who had received chemotherapy were divided into responsive and non-responsive groups according to the effect of Gemcitabine treatment. Plasma from these patients were collected and analyzed by qRT-PCR for three circRNAs, $n = 20$. Data are presented as fold changes of expression levels to GAPDH. $^{***}p < 0.001$.

Network of circRNAs and the Predicted Binding miRNAs

To better explore and understand the upstream and downstream miRNAs associated to the two circRNAs, we analyzed the potential binding miRNAs for the two circRNAs by sequence analysis with TargetScan. A tree diagram of circRNAs and their potential binding miRNAs is generated in **Figure 6A**. Based on circRNA/miRNA interaction network, chr14:101402109-101464448+ and chr4:52729603-52780244+ were predicted to be able to bind a spectrum of miRNAs with known functions, suggesting their potential roles in Gemcitabine resistance of PDAC. We selected three potential target miRNAs from the tree diagram, including miR-19a-3p, miR-138-5p, and miR-145-5p, which may bind both of the two circRNAs, and compare the expression of them between PANC-1 and PANC-1-GR cells. It was found that miR-19a-3p and miR-145-5p were down-regulated in PANC-1-GR cells, while miR-138-5p expression did not change significantly (**Figure 6B**). We also analyzed the miR-145-5p expression of plasma of PDAC patients who received gemcitabine treatment. It was found miR-145-5p was down-regulated in non-responsive group, compared with responsive group (**Figure 6C**).

TABLE 3 | Clinical characteristics of the patients.

Clinicopathologic factors	*n*	Responsive	Non-responsive	*P*-value
Age				0.519
<60 years	24	11	13	
≥60 years	16	9	7	
Sex				0.256
Male	31	14	17	
Female	9	6	3	
Tumor location				0.376
Head	34	18	16	
Body/tail	6	2	4	
Serum CA19-9				0.633
≤37 U/mL	5	3	2	
>37 U/mL	35	17	18	
Number of metastatic lesions				0.376
1	6	2	4	
≥2	34	18	16	
Karnofsky performance status score				0.248
100	7	4	3	
90	8	3	5	
80	19	10	9	
70	3	2	1	
50–60	3	1	2	

Silencing of circRNAs Enhances Gemcitabine Sensitivity of PANC-1-GR

To evaluate the functions of these two new circRNAs in Gemcitabine resistance, we applied small RNA interference (siRNAs) to silence the expression of chr14:101402109-101464448+ and chr4:52729603-52780244+ in PANC-1-GR cells. Two siRNAs were designed to target the backsplice sequence of each circRNA, respectively. A non-specific control siRNA sequence was also used as the negative control. After transfection, siRNA1 of chr14:101402109-101464448+ and siRNA2 of chr4:52729603-52780244+, dramatically inhibited the expression of chr14:101402109-101464448+ and chr4:52729603-52780244+, respectively, in PANC-1-GR cells (**Figure 7A**), which were further used for transfection in cytotoxicity assay. It was demonstrated that after silencing of chr14:101402109-101464448+ or chr4:52729603-52780244+, PANC-1-GR cells restored sensitivity to Gemcitabine (**Figure 7B**). Annexin V staining apoptosis assay also demonstrated that siRNA group had more Annexin V positive apoptosis cells after cells were cultured with 0.1 ug/ml gemcitabine for 24 h (**Figure 7C**). These results suggested that the two circRNAs may serve as potential therapeutic targets for Gemcitabine resistance in PDAC.

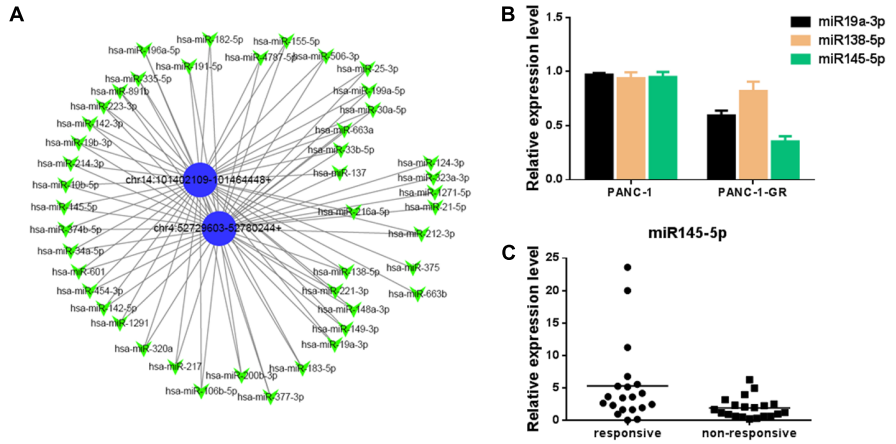

FIGURE 6 | Network of circular RNAs and the predicted binding miRNAs. **(A)**The two circRNAs biomarkers were annotated in detail according to the circRNA/miRNA interaction information by Cytoscape. **(B)** miR19a-3p, miR138-5p and miR145-5p expression in PANC-1 and PANC-1-GR cells. **(C)** miR145-5p expression in plasma of PDAC patients who received gemcitabine treatment, including responsive and non-responsive groups.

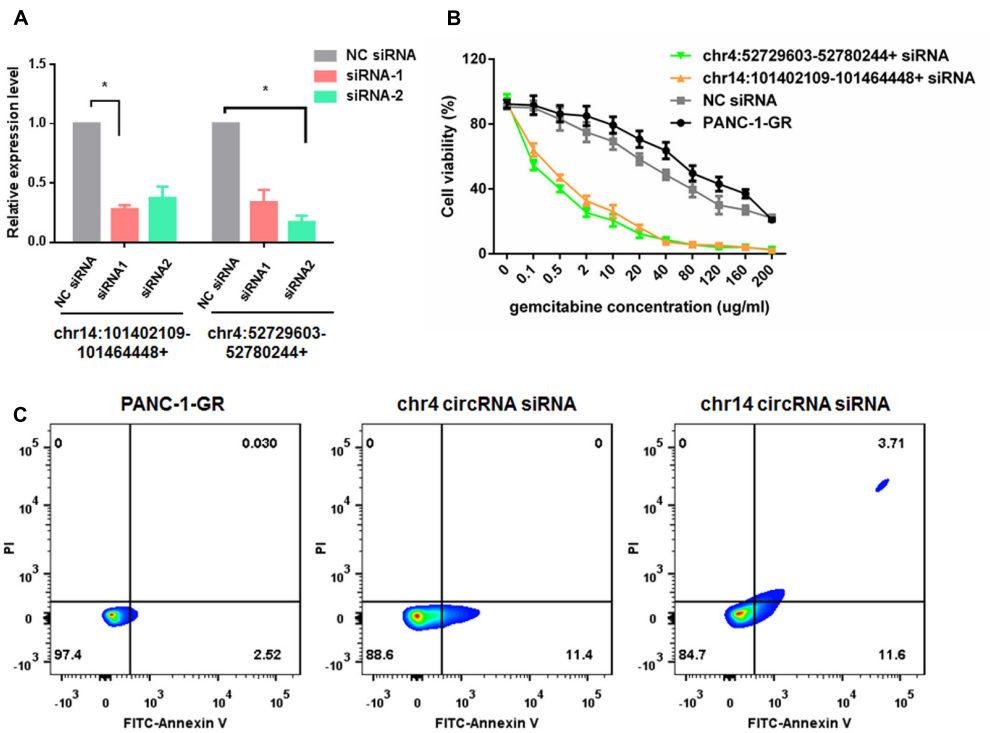

FIGURE 7 | Silencing of circRNAs enhances Gemcitabine sensitivity of PANC-1-GR cell lines. **(A)** qRT-PCR analysis of circRNA expression level after siRNAs transfection. NC siRNA: negative control siRNA. **(B)** Cell viability of PANC-1-GR cells after transfection with siRNAs. Cells were incubated with different concentrations of Gemcitabine for 72 h and then cell viability was determined by MTT assay. $*p < 0.05$. **(C)** Annexin V-FITC staining assay was performed for PANC-1-GR cells and siRNA tranfected PANC-1-GR cells.

Overexpression of circRNAs Enhances Gemcitabine Resistance of PANC-1 and MIA PACA-2 Cells

We also overexpressed the circRNAs in parental PANC-1 and MIA PACA-2 cells. pCD-ciR plasmids was used to carry the circular framework of chr14:101402109-101464448+ and chr4:52729603-52780244+. After transfection and overexpression of chr14:101402109-101464448+ and chr4:52729603-52780244+ in PANC-1 cells, PANC-1 cells showed more resistant to gemcitabine, with less Annexin V positive cells after 0.1 ug/ml gemcitabine treatment for 24 h (**Figure 8A** and Supplementary Figure S2). The same result can be observed

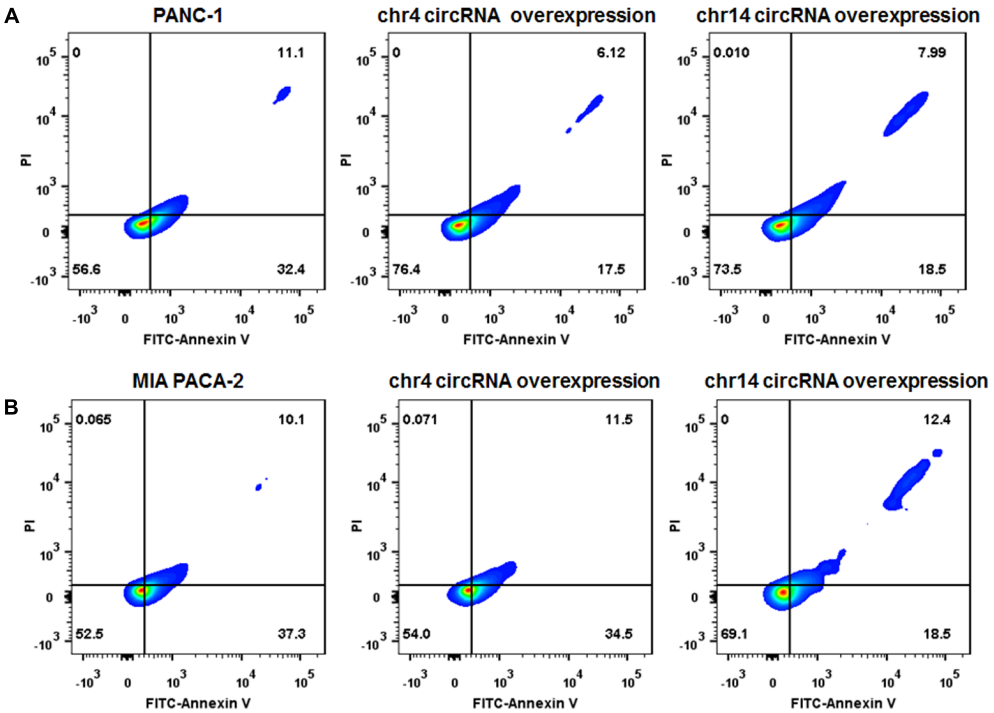

FIGURE 8 | Over-expression of circRNAs enhances Gemcitabine resistance of PANC-1 and MIA PACA-2 cells. **(A)** Annexin V-FITC staining assay was performed for PANC-1 cells, chr4 circRNA and chr14 circRNA tranfected PANC-1 cells. Cells were incubated with 0.1 ug/ml of Gemcitabine for 24 h. **(B)** Annexin V-FITC staining assay was performed for MIA PACA-2 cells, chr4 circRNA tranfected and chr14 circRNA tranfected MIA PACA-2 cells. Cells were incubated with 0.1 ug/ml of Gemcitabine for 24 h.

in MIA PACA-2 cells, when tranfected with chr14:101402109-101464448+ circular framework (**Figure 8B** and Supplementary Figure S2). However, only overexpression of chr14:101402109-101464448+ was able to exert some resistant phenotype to Gemcitabine in MIA PACa-2 while chr4:52729603-52780244+ was not. The downstream regulatory function of chr4:52729603-52780244+ may be compensated or antagonized by other regulatory mechanisms in MIA PACA-2 cells. We also analyzed miR-145 expression in parental PANC-1 and MIA PACA-2 cells when they overexpressed chr4:52729603-52780244+ and chr14:101402109-101464448+. It was found that miR-145 expression decreased to different extents, when chr4:52729603-52780244+ and chr14:101402109-101464448+ overexpressed in parental PANC-1 and MIA PACA-2 cells. (Supplementary Figure S3) It further suggests that miR145 may be involved in gemcitabine resistance by circRNA-miRNA interaction.

DISCUSSION

In this study, we developed Gemcitabine resistant pancreatic cancer cell line PANC-1-GR as a research tool to investigate Gemcitabine resistance. Subsequently, we compared the differences of circRNAs expression profile between PANC-1 and PANC-1-GR cell lines using RNA sequencing analysis. From the sequencing data, it was found that there were 68 up-regulated circRNAs and 58 down-regulated circRNAs which are possibly related to Gemcitabine resistance in PANC-1-GR. Upon validating the top 10 dys-regulated circRNAs using qRT-PCR in these cell lines, it is interesting to see that RNA sequencing data was quite consistent with qRT-PCR and combining both, we were able to identify two most distinctly expressed circRNAs from PANC-1-GR cells when compared to PANC-1 cells. It is even more exciting to confirm that these two markers are also consistently found to be highly expressed in the plasma from Gemcitabine non-responsive PDAC patients but not in Gemcitabine responsive ones. Our study demonstrated that the two circRNAs may be functionally involved in generating Gemcitabine resistance as the silencing of them can restore the sensitivity of PANC-1-GR cells to Gemcitabine.

Besides, GO and pathway analysis of circRNAs patent genes were investigated. GO enrichment analysis revealed that some gene symbols were involved in the regulation of biological process, cellular component and molecular function. Among the GO terms found in this study, "primary metabolic process" and "insulin-like growth factor binding" may play important roles in the drug resistance of PANC-1 cells via drug metabolic or cell proliferation pathway (Ireland et al., 2016; Chen et al., 2017c). Meanwhile, "ErbB signaling pathway" has been reported to be involved in Gemcitabine resistance of pancreatic cancer (Skrypek et al., 2015) and "VEGF signaling pathway" has been reported to be involved in the progression of pancreatic cancer, which may contribute to cell drug resistance (Zhou et al., 2016; Zhu et al., 2016). These findings indicated that in

addition to the two biomarkers identified in this study, there could be more circRNAs involved in the Gemcitabine resistance of PANC-1-GR cells. Recent studies have demonstrated that circRNAs could regulate gene expression as miRNA sponges or potent competitive endogenous RNA (ceRNA) molecules (Qu et al., 2017). Given that miRNAs play important roles in the Gemcitabine resistance of pancreatic cancer (Amponsah et al., 2017; Chaudhary et al., 2017; Xiong et al., 2017), some circRNAs could likely be involved in Gemcitabine resistance via interacting with miRNAs. We found that the majority of circRNAs contained one or more miRNA binding sites based on the sequences analysis. Since we identify the two circRNAs markers in this study, preparing more information about miRNA networks of these two circRNAs is meaningful as it may lead to a better understanding of their upstream and downstream miRNA targets which could serve as potential markers and drug targets. The association of miRNAs with PDAC indicated that circRNAs may have a regulatory role in PDAC. For example, among the founded potential circRNA/miRNA interactions, chr4:52729603-52780244+ is potentially able to bind miR124-3p, which has been reported playing a critical role in Gemcitabine resistance of pancreatic cancer (Li et al., 2016). MiR-145, which may bind to both chr14:101402109-101464448+ and chr4:52729603-52780244+, was also known to be associated with the resistance of pancreatic cancer cells to Gemcitabine (Zhuang et al., 2017). It was found that MiR-145-5p was down-regulated in both PANC-1-GR cells and plasma of non-responsive patients. Certainly, future studies are required to clarify the underlying mechanism of these circRNAs-miRNA interactions in Gemcitabine resistance of pancreatic cancer.

In conclusion, our study provides a new research tool, PANC-1-GR cell line and based on this tool, new insights into Gemcitabine resistance in pancreatic cancer treatment by identifying two novel circRNAs biomarkers and drug targets. Future investigation should be followed up focusing on the exploration of underlying mechanism of the two circRNAs and their associated networks. We hope this work would help to accelerate the development of novel therapeutic strategies targeting Gemcitabine-based chemotherapy of PDAC patients.

AUTHOR CONTRIBUTIONS

FS and MH carried out the experiments and drafted the initial manuscript. FM contributed to the literature search and bioinformatic analysis. QH reviewed the statistics and contributed to critical revisions. All authors reviewed and approved the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00584/full#supplementary-material

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REVIEW
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Circular RNAs in Cancer – Lessons Learned From microRNAs

*Mihnea Dragomir[1,2,3] and George A. Calin[1,4]**

[1] Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [2] Research Center for Functional Genomics, Biomedicine and Translational Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania, [3] Department of Surgery, Fundeni Clinical Hospital, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, [4] Center for RNA Interference and Non-Coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

Circular RNAs (circRNA) are RNA molecules built from fragments of linear pre-messenger RNAs and other linear RNA species through a process termed "back-splicing" in which the 3′ and 5′ ends are joined together giving rise to a covalently uninterrupted loop. circRNAs are not new members of the RNA world; they were first discovered in the early 1990s. The novelty is their abundance in the mammalian cells, as recently thousands of circRNAs were discovered and annotated. The biogenesis of circRNAs is a partially characterized process, regulated by three different mechanisms: exon skipping, intron pairing, and RNA-binding proteins. On the other hand, the function of circRNAs remains largely unknown and only a handful of singular reports describe in detail the biological roles of some circular transcripts. In a very short period of time, numerous circRNAs were associated with various cancer types and were also identified in bodily fluids with the potential of being disease-specific biomarkers. In this review, we briefly describe the biogenesis and function of circRNAs and present the circular transcripts that were more than once reported in literature to be associated with cancer. Finally, we point out some of the difficulties encountered in the study of circRNAs in cancer, as we consider that taking these into account could accelerate and improve our understanding of the biologic and translational use of circRNAs in human diseases.

Keywords: circular RNA, microRNA, non-coding RNA, cancer, biomarker

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****Correspondence:***
George A. Calin
gcalin@mdanderson.org

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GENERALITIES ABOUT CIRCULAR RNA

Circular RNAs (circRNAs) are among the last addition to the ever-growing world of non-coding RNA (ncRNA) molecules. CircRNAs are built from the exons of linear pre-messenger RNAs (mRNAs) through a process termed "back-splicing" in which the 3′ and 5′ ends of the exon are joined together giving rise to a covalently uninterrupted loop. This classical definition of circRNA is challenged by numerous exceptions making this transcript so hard to characterize.

First, circRNAs are not new members of the ncRNA world. CircRNAs were first discovered in the 1990s when Nigro et al. observed that the exons of the tumor suppressor DCC after being spliced are joined in a different order than their genomic sequence. The upstream 5′ exons were moving downstream of 3′ exons where they were binding in a non-sequential manner, resulting in circular isoforms of DCC. They termed this novel RNA transcripts as scrambled exons (1). Two years later, it was discovered that in adult mice, the Sry gene is expressed as a circular transcript and not as a linear mRNA, which plays a crucial role in the determination of the sex during embryogenesis. The circular Sry does not bind polysomes and most probably is not translated

(2). In the next decades, some anecdotal accounts describing the circularization of endogenous RNA followed (3–6), but none were convincing enough to change the perception of the scientific world regarding this topic. The breakthrough came in 2012, when Salzman et al. underlined the abundance of circRNA species in the mammalian cells, discovering and annotating thousands of circular transcripts (7).

Second, circRNAs can originate from several types of RNA molecules. Not only exons are susceptible to circularization, but also introns, long non-coding RNAs (lncRNAs), antisense transcripts, or intergenic regions. Additionally, circRNAs originating from coding regions can be assembled from multiple exons and sometimes they also include intronic regions (8) (**Figure 1**). A possible generalization regarding the source of circRNA is that most of them originate from two or three pre-mRNA exons that exceed the average length and that the flanking introns are likewise unusually long (9).

Third, some circRNAs are translated into proteins (10, 11). The first circRNA which was described to associate with polysomes and to translate is circ-ZNF609 (10). Therefore, one can ask if all circRNAs are in fact ncRNA transcripts.

In conclusion, this new class of transcripts cannot be yet very precisely defined. CircRNAs can be perceived as transcripts that mainly originate from the coding region of the DNA, which occasionally prove to have a translational potential, but usually are non-coding.

BIOGENESIS OF circRNA

The unanswered questions regarding circRNA biogenesis does not concern the mechanism which is mostly explained, but the factors that regulate the formation of the circRNA loop. Very intriguing, circRNAs are expressed differently in various cell types of an organism and during the stages of ontogenesis, but from a phylogenetic point of view, a similar expression level between the same cell types is conserved (12). Hence, the synthesis of circRNAs seems to be a regulated process. Additionally, the relationship between the expression of an mRNA and circRNA originating from the same pre-mRNA is not linear and is not predictable. There are several publications revealing that some circRNAs are more abundantly expressed than their associated linear mRNA isoform (8, 13). Therefore, circRNAs are most probably not transcriptional noise and most likely have a biological function.

The term that summarizes the circRNA biogenesis is back-splicing, in which the head-to-tail splice junctions are joined together and form a circular transcript. But what makes this non-canonical splicing possible? There are three known processes that can lead to back-splicing (**Figure 2**).

The first mechanism that promotes back-splicing is exon skipping. A pre-mRNA is spliced and two transcripts are generated: a mRNA from which one or more exons are missing, and a lariat containing the skipped exons which makes the circularization possible. The exon lariat is one more time spliced (process termed intralariat splicing) and two other molecules are produced: a circRNA and an intron lariat (4). In order to confirm this mechanism, Jeck et al. researched the association of circRNAs and exon skipping transcripts. For 45% of tested circRNAs, they identified a corresponding exon skipping mRNA (9). Furthermore, these observations are supported by another group which found a linear correlation between circularization and the exon skipping phenomenon (14).

A second mechanism that leads to the circularization of exons is intron pairing: the two introns that flank the exon/exons of the nascent circRNA have a complementary structure able to bind to each other. The pairing of the flanking introns brings the

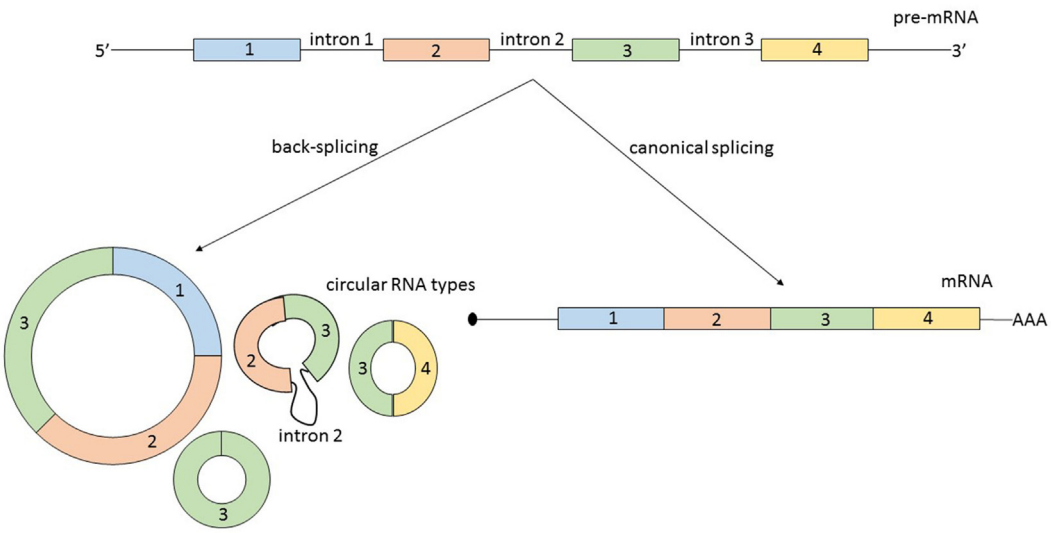

FIGURE 1 | From the same linear pre-mRNA, multiple types of circular RNA (circRNAs) can be generated via back-splicing, a form of alternative splicing. CircRNAs can be composed by one or more exons and some circular transcripts are containing also intronic segments. As a generalization: circRNAs contain two or three exons, exceeding the average length, and the flanking introns are likewise longer. The relationship between the expression level of a mature messenger RNA (mRNA) and circRNA originating from the same pre-mRNA is not always correlated and is not predictable.

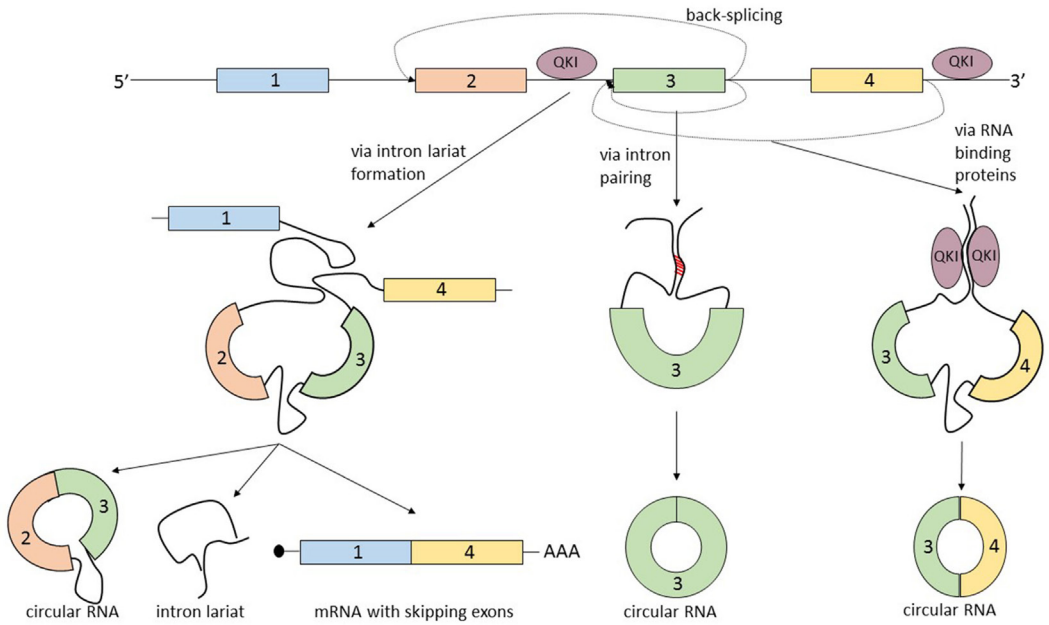

FIGURE 2 | The biogenesis of circular RNAs (circRNAs) is summarized by the term "back-splicing." Until now, three different mechanisms that promote this process were described. The first mechanism is characterized by exon skipping and intron lariat formation. In order to bring distant exons together (jumping exons), the introns build a circular lariat. The lariat formation makes the circularization of the "skipped" exons possible. This process leads to the formation of three different types of RNA molecules: circRNA, intron lariat, and mRNA with skipping exons. In the second mechanism, the circularization of the exon/exons is promoted by the complementary pairing of the flanking introns. A common hallmark of the introns that are prone to pair is the inverted ALU repeats. The third mechanism is controlled by RNA-binding proteins, which bind the neighboring introns of the future circular exon and dimerize, creating an RNA loop. The most studied proteins able to induce circularization are Quaking (QKI) and Muscleblind (MBL).

splice sites close to each other creating a secondary structure that makes back-splicing possible (15–18). Computational analysis revealed that a common feature of this flanking introns are the inverted ALU repeats (9). This feature of circRNA makes it possible to predict the sites of circularization by using bioinformatic methods.

The third mechanism of circRNA formation is directed by RNA-binding proteins (RBP). The simplest example of this model of biogenesis is the interaction between the RBP Quaking (QKI) and the flanking introns of the forming circRNA. QKI proteins bind to each of the flanking introns and dimerize creating a bridge between them. This process leads to the formation of a closed RNA loop allowing back-splicing. This mechanism was demonstrated in the case of the SMARCA5 gene, where only in the presence of QKI, a circular transcript can be generated (19). A similar mechanism was described by Ashwal-Fluss et al., which showed that the biosynthesis of the circRNA, circMbl, is controlled by the Muscleblind protein (MBL). High levels of MBL bind to its own pre-mRNA and determine the back-splicing of it, leading to the inhibition of the canonical splicing and decreasing the levels of MBL, and increasing the levels of circMBL. The authors describe the circRNA biogenesis as a process that competes with canonical splicing, and as a post-transcriptional regulator of protein synthesis (20). It is not yet clear how broad the RBP mechanism of generating circRNA is and in which way it can be used to create computational predictions regarding the exons prone to circularization. There is an additional mechanism that describes the biogenesis of circRNA which actually combines two of the previous described mechanisms: intron pairing and RBP. The RBP ADAR is responsible for A-to-I RNA editing and was recently linked to the circRNA biogenesis. Because it was well known that ADAR interacts with double strand ALU repeats (21, 22), Ivanov et al. hypothesized that the ADAR enzyme by decreasing the complementary of the inverted ALU repeats of introns can lead to a downregulation of the synthesis of circRNA. These observations were confirmed by knockingdown ADAR *in vitro*. The authors detected a two-fold upregulation of 84 circRNA and the downregulation of the corresponding linear transcripts (23).

Most probably the biogenesis of circRNA is not yet fully deciphered and the above described mechanisms represent only a limited view. Ebbesen et al. consider that the three mechanisms of circRNA biogenesis can be overlapping processes and what we currently know are three perspectives on the same phenomenon (24). Future research is needed, because our understanding of the biogenesis of circRNA will enable the development of computational tools that can predict the genomic sights susceptible to circularization.

FUNCTION OF circRNA

Research regarding the function of circRNAs is still limited and challenging. Conservation of a transcript between species is

always a powerful argument that supports the functionality of a molecule. Even the conservation of circRNA is disputed. The first study that affirms the conservation of circRNA mentions that there are hundreds of analogous transcripts in the brain of mouse and humans but no details exist about the similarity of the primary structure of the molecules (7). Another study that compares the neuronal circRNAs in the brain of mice and humans (separated by about 80 million years of evolution) reports that 4,522 circRNAs out of 15,849 are highly conserved between the two species (12). By comparing the number of human genes that produce circRNAs to murine genes that code circular loops, it was detected an overlap of 22%, but only 69 circular molecules share the same start and stop points (9). Guo et al. compared the same species and observed that if a mouse gene can code for a circular transcript, then in 66% of the cases the ortholog human gene can also code circRNAs. Of these genes, only one-third shared the same splicing sites for circRNA in humans and mice. This discovery led to the conclusion that the pre-mRNA fragments that give rise to circRNAs do not have a higher degree of conservation than their neighboring exons (25). Additionally, another research group studied the conservation of circRNA from the brain of pig and mice and discovered that the splice site of circularized exon loops matches between the two species at a proportion of 20.4% (26). The results between studies are divergent and the cause is easily identifiable: the conservation is higher if comparing the expression of circRNA between the same organs or tissues (i.e., brain), and if comparing the conservation of highly expressed circRNAs.

A second argument for the functionality of circRNAs is that these transcripts are expressed in a cell-specific manner and have a determined subcellular localization. Regarding their abundance, circRNAs have the highest level in brain and this feature is highly conserved (12, 26–28). In regard to their diversity, Maass et al. analyzed the expression of circRNAs in 20 different human tissues and discovered 5,225 transcripts, with the highest expression of circRNAs in platelets (3,324 circRNAs, out of which 2,339 unique to this cell type) (8). Recently, circRNAs were discovered also in bodily fluids with the potential of being disease-related biomarkers. The first account of circRNAs in the extracellular environment described over 400 different circRNAs in the saliva of healthy donors (29). Subsequently, circRNAs were detected in whole blood; over 2,400 circRNAs were discovered, which had expression levels similar to neuronal tissues (30). The world of circulating circRNAs was further characterized: circRNAs are abundant in exosomes and can be used to diagnose colorectal cancer (CRC). Compared to healthy controls, exosomes from CRC patients contain 257 new species of circRNAs, while other 67 circRNAs are missing (31). It appears that a precise mechanism controls the sorting and exporting of circRNAs into circulation via exosomes exists and needs to be revealed.

A third indirect argument of the functionality of circRNAs is their high stability compared to other RNA species. It was reported that a circRNA has a half-life of approximately 48 h (9), probably due to the resistance to RNA exonuclease. The half-life of a circRNA is two to four times longer than a mRNA. This difference in kinetics makes it difficult to interpret the relationship between the two transcripts (circRNA and corresponding mRNA) that originate from the same pre-mRNA.

Concrete information of the function of circRNA is scarce. Data about the mechanism of some individual circRNAs are available. But in all cases, the properties that confer functional potential cannot be extrapolated to other circRNAs. Simultaneously, two articles reported that the circRNA, antisense to cerebellar degeneration-related protein 1 transcript (CDR1as) has more than 70 binding sites for miR-7. MiR-7, associated with Argonaute proteins, binds to CDR1as, but the RISC complex does not degrade the circRNA (32, 33). CDR1as, a circRNA abundant in the brain of mammals (34), seems to act as a sponge for miR-7, which in the presence of CDR1as is strongly suppressed (**Figure 3A**). *In vivo*, overexpression of CDR1as leads to a phenotype characterized by impaired midbrain development, comparable to miR-7 knockdown models (32). Similarly, SRY, one of the first described circRNAs (2), has 16 binding sites for miR-38, also appearing to be an endogenous sponge (33). In order to check if other circRNAs also have sponging potential, Guo et al analyzed the interaction of 7,112 circRNAs with 87 microRNA (miRNA) families. The authors concluded that CDR1as which contains 71 binding sites for miR-7 is an exception and the next best miRNA sponge is circRNA ZNF91 which has 24 binding sites for miR-23 (25).

Recently, two papers proved that circRNAs can be translated into proteins (**Figure 3B**). Circ-ZNF609 has the ability to associate with polysomes and because of its primary structure containing "start" and "stop" codons, this transcript is translated in a cap-independent manner (10). One of the papers also confirmed that several circRNAs, *in vitro* and *in vivo*, associate with translating ribosomes and produce small proteins in a cap-independent way. Furthermore, the authors present the necessary characteristics of a circRNA to be translated: frequently contain the start codon of the host gene, are longer than other circRNAs, and contain a stop codon highly conserved and located near the splice site. The translated proteins originating from circular transcripts are long enough and contain a full protein domain, suggesting functional potential (11). The activity of the proteins encoded by these circRNAs is unknown and remains to be elucidated.

Several research papers report that circRNAs associate with RBP (**Figure 3C**). Maybe the best known example is that of circMBL. The mature MBL protein not only binds to the introns flanking the circularized exon but also interacts directly with the exons. The interaction with the exon does not affect the biogenesis of circRNA and the authors assume that circMBL can sponge excessive MBL and play a role in subcellular delivery of the mature protein (20). In another paper, it was proven that circRNAs form cytoplasmic complexes with proteins. 12 circRNAs were tested, and all of them can build complexes with proteins. A more detailed analysis using RNA-seq and immunoprecipitation showed that the RBP, insulin-like growth factor 2-binding protein 3 (IGF2BP3) can interact with three distinct circRNAs (CDYL, NFATC3, and ANKRD17) (35). These functional details open more questions than provide answers: (1) Why do circRNAs bind to proteins? (2) Are all circRNAs able to interact with RBP? and (3) What characteristics should a circRNA have to bind to a specific type of protein? Hentze and Preiss provide

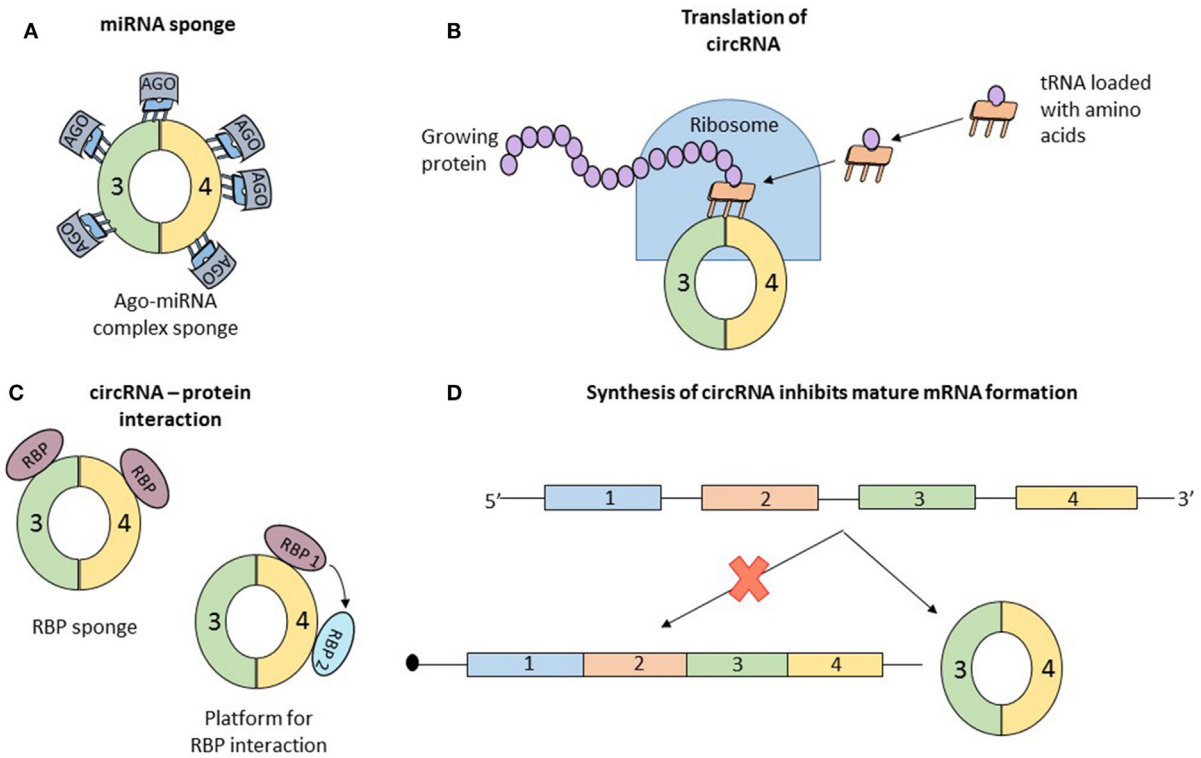

FIGURE 3 | Data about the function of circular RNAs (circRNAs) is limited; until now, only four roles of circRNAs were studied in detail, and the observations regarding the function cannot be generalized to all circular transcripts. **(A)** The most studied function of circRNAs is microRNA (miRNA) sponging. By sequestering miRNA molecules that downregulate mRNAs, circRNAs indirectly increase the expression of mRNAs. Initially proposed as potent miRNA sponges which are able to bind to dozens of short RNAs, circRNAs are considered now to have the same number of interaction sites with miRNAs as other molecules. **(B)** Recently, two papers reported that circRNAs can associate with ribosomes and be translated into short proteins, long enough to contain a functional domain. **(C)** Similar to miRNA sponging, circRNAs also bind to proteins. The exact function of this interaction is not clear and numerous speculations exist: circRNA are a transport vehicle for proteins; circRNAs are sequestering proteins; circRNAs are a platform for protein interactions; or circRNAs bind to proteins and induce allosteric changes, regulating their function. **(D)** Finally, simply the formation of circRNAs can be perceived as a function of circular transcripts. It was reported that synthesis of a circRNA from a pre-mRNA competes with the formation of a linear, functional mRNA. Therefore, circRNA formation is another form of post-translational regulation.

some hypothetical answers for these questions but experimental confirmation is still missing. The two authors propose multiple scenarios: (a) circRNAs are vehicles for RNP, delivering these molecules in a specific subcellular localization; (b) similar to the circRNA–miRNA interaction, circRNAs can be sponges for RNP, sequestering the proteins which will be unable to perform their functions; (c) circRNAs can be a platform for multiple RBPs, which can interact with each other by binding to a circularized transcript; and (d) circRNAs can bind RBPs and induce allosteric changes, regulating the function of the proteins (36). Like in the case of circRNAs acting as miRNA sponge also the hypothesis that circRNAs can interact with RBPs is disputed. You et al. predicted the capacity of circRNA to bind RBP and concluded that circular transcripts possess a lower affinity to bind RBPs than coding regions and 3′UTR of linear transcripts (28). Future research is necessary to clarify the role of the interaction of circRNAs with RBPs.

Finally, the circRNAs may not have a function, but simply the assembly of the circular transcripts can be perceived as a translational brake, a different type of post-transcriptional regulation (**Figure 3D**). Every time a circRNA is synthesized, the formation of the mature linear mRNA is impeded, the two transcripts (mRNA and circRNA) compete for their production. The already well-known example of muscleblind gene is a model of this phenomenon. Conditioned by the presence of the mature MBL protein, the pre-mRNA of MBL can be spliced canonical or back-spliced. If the MBL protein is abundant, it binds to the flanking introns of exon 2 of the pre-mRNA and induces its circularization generating a circRNA; if the expression of MBL is low, canonical splicing takes place and a mature mRNA is synthesized, which will be transcribed into MBL proteins. This process is highly conserved, from flies to humans, and can be perceived like an auto-feedback mechanism that controls the expression level of MBL. Exogenous MBL overexpression leads to an increased circularization of the second exon, whereas a low level of MBL induces canonical splicing (20). But once again, this process remains singular and may be an exception.

CANCER AND circRNA

The same enthusiasm observed after the discovery of the association of miRNA with cancer (37) is happening now with

circRNA. In a very short period of time, numerous circRNAs were associated with various solid and hematologic malignancies. Complete reviews of the circRNAs down-/upregulated in cancers can be found elsewhere (38–40). We included in this review only circRNAs that were associated with the same type of cancer/different types of cancer in at least two publications (**Table 1**). Only a negligible number of circRNAs fulfill these conditions. By including circRNAs that were linked with multiple types of

TABLE 1 | CircRNAs that were reported by two or more studies to be associated with the same/different types of cancers.

CircRNA alias(circRNA)	Host gene	Up/down	Tumor type	Function and phenotype	Reference
Hsa_circ_0022383 (hsa_circRNA_100833)	FADS2	Down	BCC	Potentially anti-tumorigenic role, by sponging different miRNAs	(42)
		Down	CSCC		(41)
Hsa_circ_0022392 (hsa_circRNA_100834)	FDAS2	Down	BCC		(42)
		Down	CSCC		(41)
hsa_circ_0001946 (CDR1as/ciRS-7)	CDR1	Up	HCC	Potentially pro-tumorigenic role, by sponging miR-7. Knockdown of CDR1as inhibits cell proliferation and invasion	(44)
		No change	HCC	Uncertain role, potential miR-7 sponge	(45)
		Up	CRC	Potentially pro-tumorigenic role, miR-7 sponge, positively regulating EGFR and IGF-1R. Knockdown of CDR1as suppresses cell invasion and proliferation	(46)
		Up	CRC	Potentially pro-tumorigenic, miR-7 sponge, leading to the activation EGFR/RAF1/MAPK pathway. *In vitro* and *in vivo* CDR1as overexpression increases the oncogenic phenotype	(47)
		Down	Glioma	Potentially anti-tumorigenic role, CDR1as is a target of miR-671-5p, and overexpressing miR-671-5p leads to downregulation of CDR1as, increasing cellular migration and proliferation	(48)
(Cir-ITCH)	ITCH	Down	ESCC	Potentially anti-tumorigenic role, by sponging miR-7 positively regulates ITCH, an inhibitor of WNT/beta-catenin. Overexpression of circ-ITCH leads *in vitro* and *in vivo* to a less aggressive phenotype	(49)
		Down	CRC		(50)
		Down	Lung cancer		(51)
		Down	HCC	Contains multiple SNPs which can modify the susceptibility to HCC	(52)
		Down	Bladder cancer	Potentially anti-tumorigenic role, by sponging miR-17 and miR-224 positively regulates p21 and PTEN. Overexpressing circ-ITCH *in vivo* leads to inhibition of metastasis	(53)
(Circ-Foxo3)	FOXO3	Down	*In vitro* and *in vivo* breast cancer models	Potentially anti-tumorigenic role, increases the expression of its corresponding transcript, Foxo3 by sponging several miRNAs. Circ-Foxo3 has an inhibitory effect on tumor growth *in vivo*	(54)
		Down	*In vitro* breast cancer models	Potentially anti-tumorigenic, builds a ternary complex with CDF2 and p21 which inhibits cell cycle progression, decreasing cell proliferation	(55)
		Down	Breast	Potentially anti-tumorigenic, blocks the interaction of MDM2 with Foxo3 and decreases the degradation of this protein, inducing cell apoptosis	(56)
Hsa_circ_0001649	SHPRH	Down	HCC	Potentially anti-tumorigenic, the low expression correlates with the tumor size and occurrence of tumor embolus	(57)
		Down	GC[a]	Potentially anti-tumorigenic, correlates with the histopathological differentiation level	(58)
		Down	CCA	Potentially anti-tumorigenic, inhibits proliferation, invasion, migration and apoptosis	(59)
		Down	CRC[a]	Potentially anti-tumorigenic, correlates with the histopathological differentiation level	(60)
Hsa_circ_0000284 (circHIPK3/bladder cancer-related circular RNA-2)	HIPK3	Up	HCC	Potentially pro-tumorigenic role, by sponging miR-124, it leads to increased cell proliferation	(61)
		Up	HCC	Possible pro-tumorigenic potential, by sponging miR-124, circHIPK3 indirectly induces proliferation and migration	(62)
		Down	Bladder cancer	Potentially anti-tumorigenic role, by sponging miR-558 circHIPK3 inhibits cancer growth and metastasis *in vivo*	(63)
		Down	Bladder cancer	Possible anti-tumorigenic role by sponging miR-124. High levels of circHIPK3 associate with better survival	(64)

(*Continued*)

TABLE 1 | Continued

CircRNA alias(circRNA)	Host gene	Up/down	Tumor type	Function and phenotype	Reference
(Hsa_circ_001569)	ABCC1	Up	CRC	Potentially pro-tumorigenic role, by sponging miR-145 it induces proliferation and invasion	(65)
		Up	HCC	Potentially pro-tumorigenic role correlates with TNM stage and differentiation grade. *In vitro* and *in vivo* silencing of the circRNA suppresses tumor growth	(66)
Hsa_circ_0002768 (CircMYLK)	MYLK	Up	Bladder cancer	Potentially pro-tumorigenic role by sponging miR-29a (*in silico* prediction)	(67)
		Up	Bladder cancer	Potentially pro-tumorigenic role, by sponging miR-29. Up-regulation of the circRNA induces epithelial-mesenchymal transition *in vitro* and promotes metastasis *in vivo*	(68)
(CircPVT1)	PVT1	Up	GC	Potentially pro-tumorigenic role, by sponging miR-125 family. *In vitro* silencing of circPVT1 inhibits tumor cell proliferation	(69)
		Up	AML	Potentially pro-tumorigenic role and associates with 8q24 chromosome amplicons	(70)
		Up	HNSCC	Potentially pro-tumorigenic by sponging miR-497-5p. Mutated p53 enhances the expression of circPVT1 and increases cell proliferation, migration and colony formation	(71)
hsa_circ_0075825	LINC00340	Up	BCC	Potentially pro-tumorigenic role	(42)
		Up	GC[a]	Potentially pro-tumorigenic role	(72)
circRNA_100269	LPHN2	Down	GC	Potentially anti-tumorigenic role, possible predictive tool for early recurrence after surgery	(73)
		Down	GC	Potentially anti-tumorigenic role, by sponging miR-630 inhibits tumor growth *in vitro*	(74)

We adopted the circBase name (circRNA alias) and other additional names used by researchers for these transcripts. [a]circRNAs that were found deregulated in serum/plasma—potential non-invasive biomarker, all other were detected in tumor tissues vs normal.

cancer, we observe a lack of specificity of a circRNA for a given type of malignancy.

Circular RNAs were linked to dermatologic malignancies. By performing microarray and confirming the data by qRT-PCR, Sand et al. discovered 143 up- and 179 downregulated circular transcripts in tissue samples of cutaneous squamous cell carcinoma (CSCC) compared to controls. The top 2 up- and downregulated circRNAs were hsa_circ_0070933, hsa_circ_0070934 and hsa_circ_0022392, hsa_circ_0022383, respectively (41). By performing the same analysis, the same research group reported that the top two downregulated circRNAs in CSCC have also a lower expression level in basal cell carcinoma (BCC) compared to normal tissue (42). To our knowledge, only one circRNA was linked to melanoma, the circular isoform of the lncRNA ANRIL, circANRIL. It was observed that numerous isoforms of circANRIL are present in the cytoplasm of melanoma cell lines, while the linear lncANRIL is abundant in the nucleus (43).

Most of the cancer related circRNAs reported by now are associated with malignancies of the gastrointestinal tract. Yu et al. detected CDR1as to be upregulated in hepatocellular carcinoma (HCC) compared to normal adjacent tissue and that the expression of miR-7 anticorrelates with that of CDR1as. *In vitro* experiments showed that CDR1as is an oncogene by sponging miR-7 and increasing cancer cell proliferation and invasion potential (44). More recently, another group analyzed the level of CDR1as in HCC tissue versus paired neighboring normal tissue and observed that the circular transcript has the tendency for a lower expression in malignant tissue. Curiously, a high expression of CDR1as was associated with microvascular invasion and acted as a miR-7 sponge (45). The two publications contradict to some extent one another; thus, supplementary research is needed to elucidate the real function of CDR1as in HCC, if any function exists. Furthermore, other two publications reported that CDR1as is upregulated in CRC tissue compared to paired healthy mucosa, and also both papers show independently that a high level of this circular transcript is associated with poor overall survival. Mechanistically, the overexpressed CDRas1 sponges the tumor suppressor miR-7 and the downstream target of miR-7 – EGFR – is upregulated (46, 47). CDR1as was also associated with glioblastoma, since tumor biopsies had a lower level of CDR1as compared to biopsies from normal controls. The expression of CDR1as negatively correlates with that of miR-671-5p in glioblastoma. Because this miRNA can degrade CDR1as, it can be hypothesized that miR-671-5p directly controls the expression of a circular transcript in cancer (48).

Cir-ITCH is another well-known circRNA already linked with five types of cancer. Three papers reported that cir-ITCH is downregulated in esophagus squamous cell carcinoma (ESCC), CRC, and lung cancer. The proposed mechanism of action was similar in all three articles: low levels of cir-ITCH are not able to sponge miR-7 and miR-214 which will target ITCH, an inhibitor of WNT/beta-catenin pathway. Cir-ITCH positively regulates the level of its host gene (49–51). In HCC, cir-ITCH seems similarly to be downregulated and low cir-ITCH is an independent prognostic factor. Moreover, cir-ITCH displays six different single-nucleotide polymorphism (SNPs) regions which can change the susceptibility to HCC (52). Recently, low cir-ITCH was associated with

bladder cancer, and cir-ITCH plays a tumor suppressing role by directly binding miR-17 and miR-224, which if sponged lead to the inhibition of PTEN and p21 (53).

By using *in vitro* and *in vivo* models of breast cancer it has been shown that the circ-Foxo3 has a tumor suppressor potential. The authors demonstrated that high levels of circ-Foxo3 and Foxo3 pseudogene can sponge multiple miRNAs that target the mRNA Foxo3, which is transcribed in a protein with anti-neoplastic effect (54). The same study group additionally showed that circ-Foxo3 has a tumor suppressor function, independently of controlling the expression level of its host gene. Circ-Foxo3 can build together with p21 and CDK2, a complex that blocks the cell cycle progression by inhibiting the interaction of CDK2 with cyclin A and cyclin E (55). In a third paper, the authors confirmed that circ-Foxo3 is downregulated in breast tumor samples compared to benign mammary lesions. Circ-Foxo3 is additionally able to block the interaction of MDM2 with Foxo3 and rise the expression level of Foxo3, increasing the apoptosis of tumor cells (56).

Hsa_circ_0001649 is a circular transcript linked to multiple types of gastrointestinal tract cancers. In HCC, it was shown that the expression of circ_0001649 is downregulated in tumor tissue compared to paired healthy tissue (57). In gastric cancer (GC), hsa_circ_0001649 is downregulated in tumor tissue, consequently leading to expression drops in serum of GC patients after surgery, being a potential recurrence biomarker (58). The same circRNA is downregulated in cholangiocarcinoma (CCA) tissue and regulates cell proliferation, apoptosis, migration, and invasion (59). Ultimately, this circRNA is downregulated in CRC tumor tissue and in serum of CRC patients (60). Very curiously, no *in vitro* or *in silico* data exists regarding the molecular mechanism of hsa_circ_0001649, a transcript that seems to be implicated in multiple cancer types.

CircHIPK3 is the circular transcript formed from the second exon of HIPK3, a tumor suppressor gene with protein kinase activity. In HCC, circHIPK3 is overexpressed and sponges 9 different miRNAs displaying 18 miRNA interacting binding sites. Knockdown of circHIPK3, but not of HIPK3, leads to an inhibition of cellular proliferation, proving that the function of the circRNA is independent from that of the linear transcript (61). In a more recent study, it was confirmed that circHIPK3 is upregulated in HCC tissue compared to normal tissue and the level of the circular transcript anticorrelates with miR-124. The sponging of miR-124 by circHIPK3 leads to the overexpression of aquaporin 3, a transmembrane channel with tumorigenic function (62). On the contrary, circHIPK3 was reported to be downregulated in bladder cancer tissue compared to normal adjacent epithelium. Mechanistically, a low expression level of circHIPK3 cannot efficiently sponge miR-558, a miRNA which promotes angiogenesis by targeting heparanase. *In vivo* overexpression of the circRNA inhibits cell growth and lung metastasis (63). Furthermore, in a fourth study, it has been demonstrated that low levels of circHIPK3 are associated with low rate of progression free survival in bladder cancer, confirming the results of a previous research group (64). Hence, it is possible to hypothesize that circHIPK3 plays opposite roles in HCC and bladder cancer, being an oncogene and a tumor suppressor gene, respectively.

CircRNA_001569 was identified as an oncogene in CRC and HCC. In CRC, Xie et al. found that circ_001569 is upregulated and directly correlates to the T stage, N stage, M stage, and histopathological differentiation grade. The authors demonstrated that the circRNA regulates the proliferation and invasion of neoplastic cells by sponging miR-145 – a miRNA that downregulates multiple oncogenes: E2F5, BAG4, and FMNL2 (65). In HCC, the same circ_001569 is overexpressed and also correlates with the TNM stage and tumor differentiation grade, but data regarding the function of the circRNA were not provided (66).

CircMYLK is an additional circular transcript linked to bladder cancer. Huang et al. discovered circMYLK overexpressed in bladder cancer, and by using *in silico* methods, predicted that this circRNA with the lncRNA H19 could sponge miR-29a-3p leading to the overexpression of DNMT3B, VEGFA, and ITGB1 (67). In a subsequent study, the same research group confirmed their *in silico* data: circMYLK is overexpressed in bladder cancer and the expression level correlates with progression level. Additionally, circMYLK can directly bind to miR-29a-3p and control the expression level of VEGFA (68).

CircPVT1, due to the amplification of its DNA locus, is overexpressed in GC. The expression of circPVT1 and its equivalent not circularized RNA, PVT1, are inversely correlated. The association of high circular transcript and low lncRNA is an accurate overall survival prognosis marker in GC. The function of circPVT1 in GC is described only partially: this circRNA sponges the members of the tumor suppressor miR-125 family (69). Afterward, it was discovered that the same amplification of the 8q24 region containing the PVT1 gene leads to the overexpression of circPVT1 and in a lesser amount of the lncRNA PVT1 in acute myeloid leukemia (AML) patients with cytogenetic abnormalities. The phenotype of 8q24 amplification in AML is known, but remains unclear how circPVT1 contributes to this genomic alteration (70). A third article confirms the oncogenic potential of circPVT1: the circularized transcript is high in head and neck squamous cell carcinoma (HNSCC), especially in patients with TP53 mutations, and has the ability to sponge miR-497-5p (71).

One of the top overexpressed circRNAs in BCC (42), has_circ_0075825, was also found by RT-droplet digital PCR to have a high expression level in the plasma of GC patients (72).

In stage III GC, it was observed that 46 different circRNAs are deregulated, the top downregulated circular transcript being circRNA_100269 (73). In a subsequent paper from the same research group, it was confirmed that circRNA_100269 and its corresponding mRNA, LPHN2 are downregulated in GC. CircRNA_100269 has an anti-neoplastic activity by directly targeting miR-630, but further details are still lacking regarding the downstream targets of miR-630 (74).

UNDERSTANDING circRNAs LESSONS LEARNED FROM THE STUDY OF microRNAs

Shortly after the discovery of miRNAs implication in cancer, these transcripts were divided in oncomiRs and tumor suppressor

miRNAs. Later, a more in depth research made it clear that a miRNA, which has the ability to target hundreds of different mRNAs, is too versatile to be classified as "good" or "bad." The role of a miRNA in tumorigenesis is largely context dependent (i.e., depending on genomic features of the tumor). The same miRNA can be an oncogene in some tumor types and a tumor suppressor gene in other cancers. A similar tendency to divide circRNAs in oncogenes and tumor suppressor genes can be now remarked. We presented in this review only circRNAs which are reported by more than one paper. Already two circRNAs, CDRas1 and circHIPK3, were defined to have tumor suppressor potential by some researchers and oncogenic potential by others (44–48, 61–64).

There are hundreds of papers defining circRNAs as cancer biomarkers or tools that can aid the stratification and predict the outcomes of cancer. Already in some cases, the same circRNA was proposed by different groups as a diagnostic tool for multiple types of cancers. We selected for this review only the circRNAs which were linked more than once with neoplastic disease. Twelve different circRNAs fulfill this condition and nine of these are linked to more than one cancer type. Only three circRNAs are, until now, cancer type specific (**Table 1**). Each of these three specific circRNAs is supported by publications that originate from the same research group and have common authors. The example that best depicts the lack of specificity from our list is cir-ITCH, a circRNA associated with five different types of cancers: ESCC, CRC, lung cancer, HCC, and bladder cancer. This low level of specificity reminds us of the miRNA world, where the same miRNA is "specific" for multiple non-neoplastic and neoplastic diseases. This raises the question of how could these non-specific molecules find their way to the clinic. Two possible interpretations can be given to these observations: (a) circRNAs are a common driving mechanism of oncogenesis or (b) circRNAs are a common byproduct/end-product of oncogenesis. A very important aspect of future research is to determine where circRNAs are localized in the molecular pathogenic pathway.

The discovery of circRNAs in serum/plasma and their abundant presence in blood cells should be treated with caution. CircRNAs could be potential non-invasive biomarkers, and same pitfalls already proven for circulating miRNAs could be also true for circRNAs. Relatively late it has been proven that 58% of serum/plasma circulating miRNAs, which were proposed as cancer-specific biomarkers, are highly expressed in sub-populations of blood cells and only reflect the level of the circulating cells (75). The same can be true with circRNAs, since it was described that circRNAs are abundant in blood cells, predominantly in platelets and erythrocytes (8); hence, the high level of circRNAs from serum/plasma echoing the number of cells. Therefore, a precise characterization of the abundance of circRNAs in different blood cells, a good description of the in serum/plasma transportation mechanism, and the employment of methods that can predict the origin of the circulating circRNAs should be considered as preliminary steps before proposing that circRNA can solve the problem of "liquid biopsy." Additionally, important steps like the pre-analytical and analytical processes, which can be the cause of supplementary errors and data misinterpretation, should be considered and analyzed before claiming that a circRNA is a circulating cancer specific biomarker.

The majority of the papers providing evidence that circRNAs play a role in cancer offer only limited information about their function. Most of the studies suggest that circRNAs fulfill their function by miRNA sponging. The concept of miRNA sponging became attractive after the competitive endogenous RNA (ceRNA) hypothesis was proposed: ncRNAs interfere with mRNA translation by binding (sponging) miRNAs that were supposed to complementary target the mRNA (76). A number of theoretical papers mathematically modeled this interaction (77, 78) and additional experimental data (79) showed this crosstalk is impossible because of the very low abundance of binding sites harbored by lncRNA or mRNA. The discovery of CDR1as with its repetitive structure and over 70 binding sites for the same miRNA relaunched the hypothesis. However, following *in silico* and experimental studies showed that no other circRNA has this inhibitory potential (25, 28). Therefore, a circRNA that usually is not abundant and has a limited number of interaction sites for a miRNA could not be a potent miRNA sponge and its function remains largely unknown. Moreover, the lack of a clear function of circRNAs reminds us of the fact that only a part of miRNAs are abundant enough in a tissue to truly exert a post-translational regulation of mRNA: miRNAs act more as buffers that maintain the translation in a state of equilibrium (80). Despite this, numerous papers present a dysregulated miRNA as the main mechanism of a pathogenic chain. Therefore, a better characterization of the function of circRNAs is necessary before truly demonstrating their implication in cancer. Moreover, it is important to realize that circRNAs, like miRNAs, are part of a complex molecular network. In order to systematically study their function it is necessary to assess their relationship not only with the linear mRNAs that arise from the common pre-mRNAs, but also the miRNAs and RBPs with which the circular transcript interacts and furthermore their downstream targets.

In conclusion, a basic understanding of the biology (i.e., biogenesis, function, localization, conservation) of circRNAs is necessary before trying to find a clinical application for these new molecules.

AUTHOR CONTRIBUTIONS

Conception and design (MD); Provision of study materials (MD, GC); Collection and assembly of data (MD); Manuscript writing: (MD, GC); Figure Design (MD); Final approval of manuscript: (MD, GC).

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Corrigendum: Circular RNAs in Cancer – Lessons Learned From microRNAs

Mihnea Dragomir [1,2,3] *and George A. Calin* [1,4]*

[1] Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [2] Research Center for Functional Genomics, Biomedicine and Translational Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania, [3] Department of Surgery, Fundeni Clinical Hospital, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, [4] Center for RNA Interference and Non-Coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

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***Correspondence:**
George A. Calin
gcalin@mdanderson.org

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In the original article, the details for reference (70) were incorrect. The In Press version of the article was cited instead of the final published article. The full reference appears below. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

The original article has been updated.

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