Chapter 9

MicroRNAs and Cancer Drug Resistance

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Abstract

The discovery of small regulatory noncoding RNAs revolutionized our thinking on gene regulation. The class of microRNAs (miRs), a group of small noncoding RNAs (20–22 nt in length) that bind imperfectly to the 3′-untranslated region of target mRNA, has been insistently implicated in several pathological conditions including cancer. Indeed, major hallmarks of cancer, such as cell differentiation, cell proliferation, cell cycle, cell survival, and cell invasion, has been described as being regulated by miRs. Recent studies have also implicated miRs in cancer drug resistance. Regardless of the several studies done until now, drug resistance still is a burden for cancer therapy and patients' outcome, often resulting in more aggressive tumors that tend to metastasize to distant organs. Hence, with this review, we aim to summarize the miRs that influence molecular pathways that are involved in cancer drug resistance, such as drug metabolism, drug influx/efflux, DNA damage response (DDR), epithelial-to-mesenchymal transition (EMT), and cancer stem cells.

Key words MicroRNA, Drug resistance, Noncoding RNAs, Cancer

1 Introduction

MicroRNAs (miRs) were discovered by Victor Ambros and colleagues [1] in 1993, who observed that the *C. elegans lin-4* gene coded for a pair of small RNAs with antisense complementary to multiple sites on the 3′-UTR of *lin-14* gene. This small RNA substantially reduced the amount of LIN-14 protein without noticeably changing the level of *lin-14* mRNA. This landmark study showed that small RNAs possessed regulatory functions and soon the presence of other regulatory RNAs (e.g., *let-7*) was observed in other species namely humans [2]. This group of regulatory RNAs was called microRNAs (miRs) [3], an evolutionary conserved class of small RNAs that was found to control many developmental and cellular processes in eukaryotic organisms. The latest version (June 2014) of the miRBase database (miRbase 21) listed 24 521 miRs loci from 206 species, processed to produce 30,424 mature miR products. Of these, 1881 sequences belonged to the human genome [4].

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MiRs posttranscriptionally modulate gene expression by binding to their target mRNAs. miRs can be intergenic or intragenic and are produced from endogenous hairpin transcripts named pri-miR. Then, the nuclear Drosha/DGCR8 heterodimer cleaves pri-miR hairpin stem, producing the pre-miR (60–100 nucleotides) which is exported to the cytoplasm by Exportin5 and RAN-GTP. The pre-miR is then processed by the RNAse III endonuclease Dicer and its TRBP (HIV transactivating response RNA-binding protein) partner, releasing a duplex with 22–25 nucleotides. This duplex associates with the Argonaute protein forming a RNA-induced silencing complex (RISC). The mature miR stays in the complex and the passenger strand is degraded. The RISC complex is the functional complex that will interact with mRNA and trigger the regulatory effect [5]. Due to their small size, miRs are capable of binding to several regions in the 3′-UTR region of several mRNAs and in turn mRNAs can be targeted by several miRs. Consequently there is a biological redundancy in gene regulation executed by miRs. Thus, their action is extremely broad and their involvement in gene expression and cellular phenotype is well established. Although miR binding sites have also been found in 5′-UTR and in the coding sequences of mRNAs [6], they preferentially interact with seed-matching sequences in the 3′-UTR of mRNA. Several studies have shown that miRs could regulate cell differentiation [7–9], cell proliferation [10, 11], cell cycle [12, 13], cell survival [14, 15], and cell invasion [16–18]. Therefore, any misexpression of miRs can lead to altered cell phenotypes and consequently cancer initiation and progression [19]. Many miRs are located at fragile sites on chromosomes known for having common alterations (i.e., amplification, deletion, and rearrangements) in cancer [20]. MiRs that inhibit translation of proto-oncogenes are considered tumor suppressor miRs, and are usually downregulated in cancer. Other miRs are upregulated in cancer and may act as oncogenic miRs by downregulating tumor suppressor genes [21]. Recent studies have highlighted the intratumoral heterogeneity in expression of miRs [22]. This might explain the different miR expression profiles described by several groups for the same types of cancer and underlines the importance in analyzing numerous sample locations of the primary tumor in order to obtain an accurate profile of miR expression.

As stated in previous chapters, drug resistance is frequently classified into two broad types: intrinsic and acquired. Intrinsic drug resistance is not essentially a genetic attribute of the cancer cells, but can be defined as preexisting to the therapeutical challenge endowing the cancer cell with competence to survive treatment, thus rendering therapy potentially ineffective from the beginning. More often than not, intrinsic resistance could be conceived as the result of the pharmacogenetic/pharmacogenomic configuration of the host of the tumor. On the other hand, acquired

drug resistance is developed during therapy and usually due to adaptive processes, such as compensatory signaling pathways, drug inactivation, increased expression of drug target, alterations in drug targets, increased expression of drug efflux pumps, cell death inhibition, epigenetic phenomena, tumor microenvironment, and DNA damage response and repair augmentation [23–26]. Drug resistance usually results in a more aggressive tumor and cancer cells often tend to metastasize to distant organs.

Within the molecular complexity of the cancer cells and their readily capacity to change the circuitry of molecular regulation, the discovery of miRs and their roles in gene expression quickly led to studies that assessed the influence of miRs in drug resistance. As a consequence, many groups have focused on the role of these small regulatory RNAs in the development of cancer drug resistance. Several studies have shown that drug resistance can also be influenced by miRs, since they can regulate drug resistance-related genes, alter drug targets, change drug concentrations, influence therapeutic-induced cell death, regulate angiogenesis, and be involved in the development of tumor stem cells.

2 MicroRNAs in Cancer Drug Resistance

As stated above, miRs have been linked to several hallmarks of cancer in tumor cells. Differential expression of miRs in tumor cells before treatment has been associated with response to chemotherapy, while changes in miR expression have been observed in cancer cells following treatment. Table 1 summarizes the studies that showed a regulation of drug resistance by miRs. The table is divided into the main categories of drug resistance pathways and the respective regulator miR. Thus, we elaborate on miRs influencing on drug metabolism, drug transporters, DNA repair, epithelial to mesenchymal transition (EMT), and cancer stem cells. Recent studies have attempted to identify single nucleotide polymorphisms either in miR loci or target loci and correlate their presence with altered therapeutic response [27, 28].

2.1 Drug Metabolism

Drug metabolism is a complex pathway of xenobiotic detoxification that involves multiple proteins, and can be divided in three main phases: modification, conjugation, and excretion. Xenobiotics are foreign compounds (such as drugs) that are not normally produced or expected to be present in an organism. Concerted actions of drug-metabolizing enzymes (DME) and drug transporters lead primarily to an increase in the polarity of xenobiotics, called Phase I reactions, followed by conjugation reactions (Phase II reactions) that increase their polarity but block the reactivity of polar groups introduced in Phase I reactions. Thereafter the transmembrane transport of the resulting metabolites is performed by membrane transporter proteins, essentially ABC transporters (Phase III reactions).

Table 1
Pathways of drug resistance regulated by miRs (*NS* not specified)

Target gene	microRNA	Type of cancer/established cell line	Drug	Reference
Drug metabolism				
CYP1B1	miR-27b	Human uterine cervix adenocarcinoma cell line HeLa; Human breast adenocarcinoma cell line MCF-7; Human embryonic kidney cell line HEK293; Human leukemic T-cell line Jurkat; Breast cancerous and adjacent noncancerous tissue	NS	Tsuchiya et al. [30]
CYP2E1	miR-378	Human embryonic kidney cell line HEK293	NS	Mohri et al. [37]
CYP3A4	miR-27b	Human pancreas cancer PANC1; Human colon carcinoma LS-180; Human embryonic kidney cell line HEK293	Cyclophosphamide	Pan et al. [38]
SULT1A1	miR-631	Human breast cancer cell lines ZR75-1 and MCF7; Human mammary epithelial cell line MCF10A; US Cooperative Human Tissue Network under an Institutional Review Board (IRB)-approved protocol	Actinomycin D	Yu et al. [41]
GSTP1	miR-133a	Human head and neck Squamous Cell Carcinoma (SCC); Human esophageal SCC and bladder cell lines	cisplatin and carboplatin	Moriya et al. [42]
Drug transport				
ABCB1	miR-451	Human breast adenocarcinoma cell line MCF-7	Doxorubicin	Kovalchuk et al. [52]; Zhu et al. [56]
ABCB1	miR-200c	Breast cancerous tissue; Human breast adenocarcinoma cell line MCF-7	Doxorubicin	Chen et al. [53]
ABCB1	miR-298	Human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231	Doxorubicin	Bao et al. [54]
ABCB1	miR-27a	Human breast adenocarcinoma cell line MCF-7	Doxorubicin	Zhu et al. [56]
ABCB1	miR-145	Human colon carcinoma cell line Caco-2; Human embryonic kidney cell line HEK293	NS	Ikemura et al. [57]
ABCB1	miR-381	Human chronic myelogenous leukemia cell line K562	Adriamycin	Xu et al. [58]
ABCB1	miR-495	Human chronic myelogenous leukemia cell line K562	Adriamycin	Xu et al. [58]
ABCG2	miR-181a	Human breast adenocarcinoma cell line MCF-7	Mitoxantrone	Jiao et al. [63]
ABCG2	miR-328	Human breast adenocarcinoma cell line MCF-7	Mitoxantrone	Pan et al. [64]
ABCG2	miR-487a	Human breast adenocarcinoma cell line MCF-7	Mitoxantrone	Ma et al. [65]
ABCG2	miR-519c	Human embryonic kidney HEK293; Human breast adenocarcinoma cell line MCF-7	Mitoxantrone	Li et al. [66]
ABCG2	miR-328	Human embryonic kidney HEK293; Human breast adenocarcinoma cell line MCF-7	Mitoxantrone	Li et al. [66]

ABCC1	miR-326	Normal breast and breast tumor tissues; Human breast adenocarcinoma cell line MCF-7	VP-16 and doxorubicin	Liang et al. [69]
ABCC1	miR-345	Human breast adenocarcinoma cell line MCF-7	Cisplatin	Pogribny et al. [68]
ABCC1	miR-7	Human breast adenocarcinoma cell line MCF-7	Cisplatin	Pogribny et al. [68]
ABCC1	miR-1291	Human pancreatic carcinoma cell line PANC-1; Human small lung cancer cell line H69; Human embryonic kidney cell line HEK293	Doxorubicin	Pan et al. [70]
ABCC2	miR-297	Human ileocecal colorectal adenocarcinoma cell line HCT-8 and HCT-116; Colorectal cancerous and adjacent noncancerous tissue	oxaliplatin and vincristine	Xu et al. [72]
SLC15A1	miR-92b	Human colon carcinoma cell line Caco-2-BBE	NS	Dalmasso et al. [74]
SLC16A1	miR-29a	Hepatoma cell line mhAT3F; Pancreatic beta cell line MIN6; Human embryonic kidney cell line HEK293	NS	Pullen et al. [75]
SLC16A1	miR-29b	Hepatoma cell line mhAT3F; Pancreatic beta cell line MIN6; Human embryonic kidney cell line HEK293	NS	Pullen et al. [75]
SLC16A1	miR-124	Hepatoma cell line mhAT3F; Pancreatic beta cell line MIN6; Human embryonic kidney cell line HEK293	NS	Pullen et al. [75]
DNA repair				
RAS	let-7 family	Human non-small-cell lung cancer cells cell line A549		Weidhaas et al. [93]
ERCC1	miR-138	Human non-small-cell lung cancer cells cell line A549	Cisplatin	Wang et al. [95]
MSH2	miR-21	Human Dukes' type C, colorectal adenocarcinoma cell lines Colo-320 DM and SW620; Human colorectal adenocarcinoma cell line HCT-116; Human Dukes' type B, colorectal adenocarcinoma cell line SW480, Human colon carcinoma cell line RKO	5-fluorouracil	Valeri et al. [96]
MSH6	miR-21	Human Dukes' type C, colorectal adenocarcinoma cell lines Colo-320 DM and SW620; Human colorectal adenocarcinoma cell line HCT-116; Human Dukes' type B, colorectal adenocarcinoma cell line SW480, Human colon carcinoma cell line RKO	5-fluorouracil	Valeri et al. [96]
REV1	miR-96	Human Bone Osteosarcoma Epithelial Cell line U2OS; Human uterine cervix adenocarcinoma cell line HeLa; Human breast cancer cell line HCC1937; Human breast adenocarcinoma cell line MDA-MB-231	Cisplatin; PARP inhibitor AZD2281	Wang et al. [97]
RAD51	miR-96	Human Bone Osteosarcoma Epithelial Cell line U2OS; Human uterine cervix adenocarcinoma cell line HeLa; Human breast cancer cell line HCC1937; Human breast adenocarcinoma cell line MDA-MB-231	Cisplatin; PARP inhibitor AZD2281	Wang et al. [97]
RAD51	miR-155	Human breast adenocarcinoma cell line MCF-7; triple-negative breast cancer tissue	NS	Gasparini et al. [92]

(continued)

Table 1
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Target gene	microRNA	Type of cancer/established cell line	Drug	Reference
BRCA1	miR-182	Human acute promyelocytic leukemia cell line HL60; Human chronic myelogenous leukemia cell line K562; Human breast adenocarcinoma cell line MCF-7	PARP inhibitor	Moskwa et al. [100]
BRCA1	miR-146a	Breast cancer tissue	NS	Garcia et al. [101]
	miRNA-146-5p	Breast cancer tissue	NS	Garcia et al. [101]
BRCA1	miR-193a-5p	Human Mammary Epithelial progenitor Cell line HMEpC ; Human Small Airway Epithelial progenitor Cell line HSAEpC; H226; H460; Human breast cancer cell lines MDA-MB-231, MDA-MB-157, and SK-BR-3	Cisplatin	van Jaarsveld et al. [88]
BRCA1	miR-296-5p	Human Mammary Epithelial progenitor Cell line HMEpC; Human Small Airway Epithelial progenitor Cell line HSAEpC; H226; H460; Human breast cancer cell lines MDA-MB-231, MDA-MB-157, and SK-BR-3	Cisplatin; doxorubicin and paclitaxel	van Jaarsveld et al. [88]
BRCA1	miR-183	Human Mammary Epithelial progenitor Cell line HMEpC; Human Small Airway Epithelial progenitor Cell line HSAEpC; H226; H460; Human breast cancer cell lines MDA-MB-231, MDA-MB-157, and SK-BR-3		van Jaarsveld et al. [88]
BRCA1	miR-16	HSAEpCs	Cisplatin and doxorubicin	van Jaarsveld et al. [88]
EMT				
	miR-200c	Human breast adenocarcinoma cell line MCF-7	Doxorubicin	Chen et al. [105]
	miR-200b	Human breast adenocarcinoma cell line MCF-7 and resistant derivates	4-hydroxytamoxifen, fulvestrant	Manavalan et al. [106]
	miR-200c	Human breast adenocarcinoma cell line MCF-7 and resistant derivates	4-hydroxytamoxifen, fulvestrant	Manavalan et al. [106]
	miR-200c	Human breast cancer cell line SKBr-3	trastuzumab	Bai et al. [107]
MIG6	miR-200c	Several human cancer cell lines		Izumchenko et al. [108]

MAGI2	miR-134/miR-487b/miR-655 cluster	Human lung adenocarcinoma cell lines A549, LC2/ad, PC3, PC9, RERF-LCKJ, RERF-LCMS, PC14, and ABC-1	Gefitinib	Kitamura et al. [109]
	miR-147	Human colon cancer cell line HCT116 and SW480; Human lung cancer cell line A549	Gefitinib	Lee et al. [110]
SMAD3	miR-489	Human breast adenocarcinoma cell line MCF-7	Doxorubicin	Jiang et al. [111]
Fbw7	miR-223	The human pancreatic cancer cells AsPC-1 and PANC-1	Gemcitabine	Ma et al. [112]
Stem cells				
Nanog/Oct4	let-7a	Human head and neck cancer tissues	Cisplatin	Yu et al. [125]
Oct4 and Sox2	miR-145	Glioblastoma	Temozolomide	Yang et al. [126]
TP53INP1	miR-130b	Human liver tumor and adjacent non-tumor tissue	Doxorubicin	Ma et al. [127]
p53–Nanog	miR-214	Human ovarian cancer A2780, OV2008, OV8, and SKOV3	Cisplatin and doxorubicin	Xu et al. [128]
ABCB1	miR-451	Human colon carcinoma cell lines DLD1, HT29, LS513, SW620, LoVo, and RKO; Colorectal cancer tissue	Irinotecan	Bitarte et al. [129]
	miR-302	Human head and neck squamous cell carcinoma (HNSCC) cell line HSC-3	Cisplatin	Bourguignon et al. [130]

Although extensive studies have been performed on transcriptional regulation of the DMEs, there is a lack of understanding of their posttranscriptional regulation [29]. Recent studies have shown that miRs also control the expression of some DME [30–32]. However few studies have shown a direct involvement of miRs and DME with drug resistance. One of the key players of the Phase I (modification) are cytochrome P450 (CYP) enzymes that catalyze oxidation reactions of the xenobiotics and occasionally reduction reactions [33]. More than 90 % of the reactions involved in the metabolism of all chemicals, whether general chemicals, natural, physiological compounds, and drugs, are catalyzed by P450s [34]. Three-fourths of the human CYP reactions can be accounted for by a set of five CYPs: 1A2, 2C9, 2C19, 2D6, and 3A4, with the largest fraction of the CYP reactions being catalyzed by CYP 3A enzymes. The importance of CYP 3A4 in metabolic reactions of drugs varies from 13 % for general chemicals to 27 % for drugs [34]. Therefore the regulation of DMEs is crucial to drug efficacy and may be related to drug failure or drug resistance.

Tsuchiya et al. [30] showed a direct association of miR-27b and CYP1B1 in breast cancer. The authors not only validated CYP1B1 as a mirR-27b target in cell lines but also showed that in tissue samples there is an inverse correlation between miR-27b expression and CYP1B1 protein expression. Indeed, the authors showed that miR-27b decreased in expression along the group staining of CYP1B1 by immunohistochemistry, being more expressed in the weak staining group and less expressed in the strong staining group. CYP1B1 is highly expressed in estrogen target tissues, and catalyzes the metabolic activation of various pro-carcinogens and the 4-hydroxylation of 17β-estradiol, and is also abundant in cancerous tissues. However, the authors did not show an association with drug resistance. Nevertheless, since deactivation of 4-hydroxy-tamoxifen, a biotransformation product of tamoxifen that has 100-fold increased affinity to estrogen receptors then tamoxifen itself, occurs via CYP1B1 [35], the increased expression of CYP1B1 in breast cancer cells could augment the resistance to tamoxifen, a widely used drug in breast cancer treatment.

CYP2E1 is the fourth most abundant isoform (approximately 7 % of total P450 protein) after CYP3A4 (30 % of total P450), CYP2C (20 % of total P450), and CYP1A2 (approximately 13 % of total P450). CYP2E1 catalyzes the metabolism of numerous low-molecular-weight xenobiotics, including organic solvents (e.g., ethanol, acetone, carbon tetrachloride, chloroform, vinyl chloride, glycerol, hexane, and toluene), and several procarcinogens, such as *N*-nitrosodimethylamine and *N*-nitrosomethylethylamine. Interestingly, the ectopic expression of CYP2E1 induced ROS generation, affected autophagy, and inhibited migration in breast cancer cells, thus potentially being involved in breast cancer metastasis [36]. Mohri et al. identified a possible miR-responsive

element (MRE378) in the 3′-UTR of human CYP2E1 mRNA, and luciferase assays using HEK293 cells confirmed that miR-378 functionally recognized this region [37]. The overexpression of miR-378 significantly decreased the CYP2E1 protein level and enzyme activity in cells expressing CYP2E1 including 3′-UTR, but not in the cells expressing CYP2E1 excluding 3′-UTR, indicating that the 3′-UTR plays a role in the miR-378-dependent repression. However, the presence of miR-378 did not facilitate the degradation of the CYP2E1 mRNA. Therefore, according to the authors, the downregulation of CYP2E1 by miR-378 would mainly be due to the translational repression, not mRNA degradation. Additionally the relationship between the expression levels of miR-378, CYP2E1 mRNA and protein as well as enzyme activity was assessed using a panel of 25 human livers. CYP2E1 protein levels were significantly correlated with the enzymatic activities but were inversely correlated with CYP2E1 mRNA levels, while miR-378 levels showed a significant inverse correlation with the CYP2E1 protein levels [37]. In another study, Pan et al. [38] showed that miR-27b interacts with the 3′-UTR of CYP3A4, thus regulating its expression. Moreover an overexpression of miR-27b in PANC1 Human pancreas cancer cells led to a lower sensitivity to cyclophosphamide, indicating that miR-27b can alter CYP3A4-catalyzed drug activation, and consequently impact on drug response and resistance.

Regarding Phase II reactions even fewer studies have linked miR-mediated regulation and drug resistance. One example is the sulfotransferase isoform 1A1 (SULT1A1), a member of the sulfotransferase (SULT) family of phase II detoxification enzymes that catalyze the transfer of the sulfonyl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to nucleophilic groups of a variety of xenobiotic and endogenous compounds, thus increasing their solubility and excretion [39]. SULT1A1 is the most highly expressed SULT in the liver. Several therapeutic agents, including 4-hydroxytamoxifen, are substrates for SULT1A1, and variability in the activity levels of the enzyme can markedly influence the efficacy of these drugs and consequently drug resistance [40]. Interestingly, a common single nucleotide polymorphism (SNP) in the coding region of SULT1A1, several proximal promoter SNPs, and copy number variation (CNV) are associated with altered enzymatic activity, but these variants do not fully account for the observed variation of SULT1A1 activity in human populations. Thus, Yu et al. [41] looked for SNPs in the 3′-UTR region of this gene. In silico analyses predicted that the 973C→T SNP would influence the binding of miR-631 to the SULT1A1 3′-UTR. Accordingly, in vitro luciferase reporter assays and overexpression of miR inhibitors in ZR75-1, MCF7, and MCF10A breast cell lines confirmed that SULT1A1 is a direct target of miR-631 [41].

Finally, Moriya et al. [42] found that miR-133a was a potential regulator of GSTP1. Transfection of miR-133a repressed GSTP1 expression at both mRNA and protein levels in several different cell lines. The functional significance of miR-133a was investigated using head and neck Squamous Cell Carcinoma (SCC), esophageal SCC, and bladder cell lines, and the authors showed that restoration of miR-133a expression inhibited cancer cell proliferation, invasion, and migration, suggesting that miR-133a may function as a tumor suppressor. GSTP1 is a member of the GST enzyme superfamily, and catalyzes the conjugation of electrophiles to glutathione in phase II detoxification reactions, including platinum drugs such as cisplatin and carboplatin [43]. GSTP1 has several critical roles in both normal and neoplastic cells, including phase II xenobiotic metabolism, stress responses, signaling, and apoptosis. Overexpression of GSTP1 has been observed in many types of cancer and in human tumor cell lines either inherently or made resistant to chemotherapy drugs, including cisplatin and various alkylating agents [44]. For example, GSTP1 knockdown selectively influenced cisplatin and carboplatin chemosensitivity; cell cycle progression was unaffected, but cell invasion and migration was significantly reduced [45]. The reduced expression of miR-133a may thus lead to an increased expression of GSTP1, contributing to drug resistance.

In spite of these results, miR-dependent regulation of expression in DMEs does not seem to be the most important mode of regulation as few miR-binding regions are found in the 3′-UTR of DME genes. Furthermore, the miR binding sites described for most of the DMEs are poorly conserved, leading one to speculate that other forms of regulation are more important.

2.2 Drug Transport

Drug transport through cell membranes is a critical step in allowing access of pharmacologic agents to intracellular targets. The involvement of drug transport is probably amongst the most studied mechanisms in cancer drug resistance [46]. Multidrug resistance (MDR) is frequently linked to overexpression of one or more of drug transport proteins present in the cytoplasmic membrane. The ABC transporters have an important cellular role in the efflux and influx of several substrates necessary to the cell and also in the efflux of toxic endogenous molecules and xenobiotics (See chapters by Mitra, Viverios, and Gromicho, in this book). Up to now, 49 different ABC transporters were identified and classified in seven families from ABCA through ABCG [47, 48]. The relevance of miRs in regulating the expression of ABC transporters has been recently reviewed [31, 49].

One of the most well-known ABC transporters is ABCB1, also known as MDR1 or P-gp transporter. In chemotherapeutic-resistant cancer cell lines, ABCB1 is often observed to be upregulated. The increased expression of ABCB1 leads to an increased

resistance of several chemotherapeutics, such as taxanes (e.g., paclitaxel and docetaxel), epipodophyllotoxins derivates (e.g., etoposide and teniposide), anthracyclines (e.g., doxorubicin), antibiotics (e.g., actinomycin D), vinca alkaloids (e.g., vinblastine and vincristine), and tyrosine kinase inhibitors (e.g., imatinib and erlotinib) [47, 50]. To date, several authors have published data about misexpression of miRs and ABCB1 [51–54]. Kovalchuck and colleagues [51] showed that the *ABCB1* gene is highly expressed in the MCF-7/DOX breast tumor cell lines resistant to doxorubicin when compared with wild type MCF-7. Conversely, miR-451 expression is undetected, showing a negative correlation between ABCB1 and miR-451 expression. These authors then showed that miR-451 targets the *ABCB1* 3′-UTR regulatory region which consequently leads to a depletion of the drug transporter and increased sensitivity to doxorubicin. Transfection of miR-451 reestablished the sensitivity of the MCF-7/DOX cells to doxorubicin. Similarly, Chen and colleagues [52] showed the same pattern but with miR-200c. The authors also showed a correlation of miR-200c with poor response to neoadjuvant chemotherapeutics using breast cancer tissues. Low expression of miR-200c leads to poor neoadjuvant therapeutic outcomes. However, they did not follow *ABCB1* gene and protein expression in the patients. Although published studies suggest a decreased expression of miR-451 correlated with higher expression of ABCB1 in drug resistant cells [51, 55], in a human ovarian cancer cell line, and its multidrug resistant counterpart, as well as in a human cervix carcinoma cell line and its multidrug resistant variant, expressions of miR-27a and miR-451 were upregulated in multidrug resistant cells as compared with their parental lines, downregulating expression of the *ABCB1* gene [56]. These results seem to point that the involvement of specific miRs in drug resistance should be cautiously taken at the moment, since the results could depend on various factors, including the cell lines under study. Bao et al. [53] used a different breast tumor cell line, MDA-MB-231, to show that miR-298 regulates *ABCB1* gene expression and increases resistance to doxorubicin. Remarkably, the authors also showed that the miR processing is altered in the resistant cell lines, due to the fact that DICER is weakly expressed and higher levels of miR-298 precursor was detected instead of mature form. Other authors also demonstrated a regulation of ABCB1 by miR-145 [57] in intestinal epithelial cells, and mir-381 and miR-495 in leukemia K562 cells resistant to adriamycin (K562/ADM cells) [58]. In this last study, functional analysis indicated that restoring expression of miR-381 or miR-495 in K562/ADM cells was correlated with reduced expression of the *ABCB1* gene and its protein product and increased drug uptake by the cells [58].

ABCG2 is another ABC transporter that, in normal tissues, functions as a defense mechanism against toxins and xenobiotics,

with expression in the gut, bile canaliculi, placenta, blood–testis and blood–brain barriers. ABCG2 recognizes and transports a variety of chemotherapeutic drugs out of cancer cells, thereby resulting in reduced drug concentration, and subsequent drug resistance. Consequently ABCG2 plays a critical role in the development of MDR in breast cancer [59]. Increased ABCG2 expression has been found in breast cancer cells that exhibit resistance to mitoxantrone (MX), topotecan, and 7-ethyl-10-hydroxycamptothecin (SN-38) [60]. Upregulation of ABCG2 also confers resistance to tamoxifen in breast cancer cells [61]. In addition, ABCG2 expression correlates with chemotherapeutic response to anthracycline in patients with breast cancer [62]. Jiao et al. [63], performed microarray analysis to determine the differential expression patterns of miRs that target ABCG2 between the MX resistant breast cancer cell line MCF-7/MX and its parental MX sensitive cell line MCF-7. MiR-181a was found to be the most significantly downregulated miRNA in MCF-7/MX cells. Overexpression of miR-181a downregulated ABCG2 expression, and sensitized MX-resistant MCF-7/MX cells to MX. Moreover, in a nude mouse xenograft model, intratumoral injection of miR-181a mimics inhibited ABCG2 expression, and enhanced the antitumor activity of MX. Other authors have shown that ABCG2 is regulated by other miRs, including miR-328 [64] and 487a [65], and can influence MX resistance. miR-519c and miR-328 were also described as ABCG2 regulators and Li et al. [66] showed intracellular accumulation of MX in cells lacking ABCG2 expression. Interestingly, the authors also showed differences in expression of this miRs in stem-like ABCG2^{+} cells and their ABCG2^{-} counterparts. Thus, further investigation of miR regulation in stem cells may provide new insights into chemoresistance.

Another well-known ABC transporter is ABCC1, also known as MRP1. The main subtracts of ABCC1 are vincristine and etoposide and ABCC1 also confers resistance to anthracyclines (doxorubicin, daunorubicin, epirubicin), mitoxantrone, flutamide, and methotrexate. Curiously, many drugs are only transported in the presence of glutathione [67]. Regarding ABCC1, three reports were published showing a regulation by miRs [68–70]. Pogribny and colleagues [68] revealed that miR-345 and miR-7 increases sensitivity to cisplatin through a negative correlation with ABCC1. For that, the authors used a MCF-7 cell line resistant to cisplatin which expresses high levels of ABCC1 and lower levels of miR-345 and miR-7. Liang et al. [69] showed that miR-326 represses ABCC1 expression and sensitizes VP-16 resistant MCF-7 cells to VP-16 and doxorubicin. Pan et al. [70] reported that miR-1291 targets the 3'UTR of ABCC1 and consequently regulates its expression. This has impact in drug disposition and consequently in drug resistance. Interestingly, miR-1291 was described by these authors as being originated from a small nucleolar RNA, SNORA34.

ABCC2, also known as MRP2, and ABCC1 share a 49 % amino acid identity. As ABCC1, this efflux pump needs the presence of glutathione and can transport methotrexate, cisplatin, irinotecan, paclitaxel, and vincristine. ABCC2 is expressed in some solid tumors from the kidney, colon, breast, lung, ovary, and as well as in cells from patients with acute myelogenous leukemia [71]. Regarding ABCC2, to our knowledge, only one article has been published associating miR misexpression and ABCC2. Xu et al. [72] showed that miR-297 targets the 3′ UTR region of ABCC2 transcripts and consequently downregulates its expression. They also showed an inverse correlation between both molecules in colorectal carcinoma cell lines. Moreover, cell lines resistant to oxaliplatin and vincristine were sensitized when miR-297-mimics were transfected into these cells, *in vitro* and *in vivo*.

Intestinal epithelial cells are responsible for the absorption of most cancer drugs, and they express a variety of influx transporters specific for drugs, amino acids, peptides, organic anions, organic cations, and other nutrients. Peptide transporter 1 (PEPT1/SLC15A1), organic cation/carnitine transporter 2 (SLC22A5), organic anion transporting polypeptide 2B1 (SLCO2B1), and monocarboxylate transporter 1 (MCT1/SLC16A1) are expressed at the brush-border membrane, whereas organic cation transporter 1 (SLC22A1) is mainly expressed at the basolateral membrane in the small intestine [31]. Recent studies have indicated that the regional differences in the expression of these transporters are dependent on the differentiation of intestinal epithelial cells [73]. Hence, misexpression of miRs could have a marked impact on absorption of cancer drugs. There are a limited number of reports on the SLC transporters regulated by miRNAs (Table 1). Dalmasso et al. [74] showed for the first time that SLC15A1 is regulated by a miR, namely miR-92b, causing diminished influx activity. Moreover, it suppresses bacterial peptide-induced proinflammatory responses in intestinal epithelial cells by inhibiting SLC15A. Pullen et al. [75] showed that miR-29a, miR-29b, and miR-124 can target SLC16A1, resulting in decreased expression at the protein level. The authors also refer that this regulation mechanism is not the main regulator but complements other transcriptional mechanisms and mutations that alter SLC16A1 expression.

2.3 DNA Repair

DNA damage by endogenous or exogenous agents elicits a powerful cellular response called the DNA Damage Response (DDR), which call up concerted molecular pathways to detect, repair, induce cell cycle arrest to allow repair, or in cases of high numbers of DNA lesions or irreparable damage, apoptosis, or cellular senescence (permanent cell cycle arrest) [76–79]. In the past few years evidence has accumulated that drug resistance is also linked to alterations in these pathways [26, 80–85]. The DDR pathways include DNA tolerance mechanisms by error-prone polymerases,

the direct reversal of lesions, essentially de-alkylation of alkylated bases by O^6-methyl-guanine-DNA methyltransferase (*MGMT*), alkylation repair homolog 2 (*ALKBH2*) and alkylation repair homolog 3 (*ALKBH3*); nucleotide excision repair (NER); base excision repair (BER); mismatch repair (MMR); and the double strand break repair by homologous recombination (HR) and non-homologous end joining (NHEJ) [86, 87]. Besides these signaling cascades, the DDR also elicits the induction of several noncoding RNAs, including miRs. A large number of miRs are transcriptionally induced upon DNA damage and the level of induction is variable depending on cell type and the nature and the intensity of DNA damage and time after DNA damage [88–93]. Conversely many miRs target DDR genes, thus controlling feed-back and feed-forward loops to fine-tune the response (for a review see refs. 88, 94, 95). Wouters et al. found that 74 (52 %) mammalian DNA repair and DNA damage checkpoint genes contain conserved microRNA target sites predicted in their 3′-UTR by the algorithms Targetscan, Miranda, or both [95].

One of the first indications that implicated miR-mediated regulation of the DDR was knockdown of the miR biogenesis pathway (Dicer and Ago2), which resulted in increased sensitivity to UV and altered cell cycle after UV damage [90]. Following this study many reports have shown that different DNA damaging agents induce different patterns of miR expression [95]. Thus it is conceivable that alterations in miRs are involved in tumor response to anticancer agents.

A few examples indicate indeed that misexpression of miR is associated with drug responsiveness [96, 97]: members of the let-7 family of miRs are rapidly downregulated upon ionizing radiation in A549 lung cancer cells. Interestingly, the let-7 family of miRs regulates expression of oncogenes, such as RAS, and is specifically downregulated in many cancer subtypes. Low levels of let-7 predict a poor outcome in lung cancer. Overexpression of the let-7 family leads to radiosensitization in vitro of lung cancer cells and in vivo in a *Caenorhabditis elegans* model of radiation-induced cell death, whereas decreasing their levels causes radioresistance. In *C. elegans*, this was shown to occur partly through control of the proto-oncogene homologue let-60/RAS and genes in the DNA damage response pathway [96].

In another example, miR-138 was shown to target the ERCC1 gene, involved in NER, and to increase the sensitivity of A549/DDP cells to cisplatin in vitro and augmented apoptosis, suggesting that miR-138 could play an important role in the development of cisplatin resistance [98].

Valeri et al. [99] showed that MMR proteins MSH2 and MSH6 are inhibited by miR-21 overexpression causing a reduction in 5-fluorouracil (5-FU) induced G2/M damage arrest and apoptosis, in vitro. Moreover, xenograft studies demonstrate that miR-21 overexpression reduced the therapeutic efficacy of 5-FU.

REV1, an error-prone Y-family DNA polymerase required for translesion synthesis across interstrand crosslinks, was validated as a target of miR-96. Overexpression of miR-96 promoted cellular hypersensitivity to cisplatin in vitro and in vivo and enhanced sensitivity to the PARP inhibitor AZD2281. This miR also targets RAD51, a recombinase that promotes HR repair of double strand breaks (DSBs) and interstrand DNA crosslink (ICLs) [100]. RAD51 is also targeted by miR-155 in human breast cancer cells and affects the cellular response to ionizing radiation (IR). Due to this interaction, the efficiency of HR repair is reduced and sensitivity to IR augmented in vitro and in vivo. Indeed, overexpression of miR-155 was related with low levels of RAD51 and with better overall survival of patients with triple-negative breast cancers (TNBC) [101]. This emphasizes the possibility of how personalized therapy in TNBC patients could be used, knowing the miR-155 levels.

BRCA1 is an important component of the DDR pathway. BRCA1 encodes a nuclear phosphoprotein and primarily functions to maintain genomic stability via critical roles in DNA repair, cell cycle checkpoint control, transcriptional regulation, apoptosis, and mRNA splicing [102]. Mutations in BRCA1 are associated with an increased risk of developing breast and ovarian cancer. BRCA1 is also a target of miRNA-182 [103], indeed, the authors showed that high expression of this miR in multiple breast tumor cell lines influences BRCA1 levels and sensitivity to PARP1 inhibition. MiRNA-146a and miRNA-146-5p also bind to the same site in the 3′-UTR of BRCA1 and downregulate its expression. In breast tumors, levels of these miRs are inversely correlated with that of the BRCA1 protein and these miRs are overexpressed in triple negative breast cancers, a common type of breast cancer in women with BRCA1 mutations [104].

In another study, although the authors did not show specific targets, miR-296-5p and miR-193a-3p overexpression induced resistance to cisplatin, whereas miR-183 overexpression induced sensitivity. This study was done in breast cancer cells and also showed that miR-296-5p overexpression led to doxorubicin and paclitaxel resistance. These authors also examined whether overexpression of miR-16, miR-21, and miR-382 in Human Small Airway Epithelial progenitor (HSAEpCs) cells could modulate chemotherapy sensitivity. Thus, they found that miR-382 and miR-21 had no effect in resistance, while miR-16 promoted sensitivity to cisplatin and doxorubicin [91].

2.4 Epithelial to Mesenchymal Transition

Metastasis is the ultimate cause of death in most cancer patients. The growth of cancer cells at distant organs of a different tissue requires complex processes of detaching from the original tissue; invasion through the basement membrane; movement in the bloodstream or lymphatic system; and anchorage in other organs. The initial process is called epithelial-to-mesenchymal transition

(EMT) and is characterized by a phenotypic change of the tumor cells from cell–cell adhesion and polarity to motility, invasiveness, and some of the features of stem cells. This process not only enable the spread of the tumor cells but also their anchorage in distant organs, since tumor cells that undergo EMT can reverse this characteristic acquiring the epithelial phenotype again, in a process called mesenchymal-to-epithelial transition (MET). In EMT, cells lose the expression of E-cadherin and gain the expression of vimentin, N-cadherin, and fibronectin, markers of mesenchymal phenotype. Presumably, EMT is sustained by transient molecular changes and not by permanent genetic alterations. Indeed, the reversible nature of EMT must be associated with reversible epigenetic mechanisms, which allows stable but reversible modifications that do not directly affect the DNA primary sequence [105–107].

MiRs, as posttranscriptional regulators, are good candidates as EMT regulators and, as with epigenetic mechanisms, do not affect the DNA primary sequence and can press tumor cells to acquire an EMT phenotype in the tumor microenvironment. The most studied case is the miR-200 family that targets at least two transcriptional repressors of E-cadherin, ZEB1, and ZEB2.

It is known that the sensitivity to some cancer drugs like etoposide, taxol, and epidermal growth factor receptor inhibitors is increased with restoration of E-cadherin expression. Chen et al. [108] showed that miR-200c increases drug sensitivity of breast cancer cells to doxorubicin through the E-cadherin-mediated upregulation of PTEN. Similarly, Manavalan et al. [109] showed that an increased expression of miR-200b and miR-200c enhances the sensitivity to growth inhibition by 4-hydroxytamoxifen (4-OHT) and fulvestrant in breast cancer cells. Although it is known that miR-200 family regulates EMT through ZEB1 and E-cadherin, the real mechanism through which the miR-200 family regulates drug resistance is not known, and thus further studies are necessary to understand these phenomena. In order to answer this question, Bai et al. [110] published interesting data about miR-200c and feedback circuits of miR-200c/ZEB1 and miR-200c/ZNF217/TGF-β/ZEB1. The authors showed that these circuits contribute to trastuzumab resistance and metastasis of breast cancers. Interestingly, this feedback circuits might be related with reverse EMT in metastasis formation, since ZEB1 can inhibit miR-200c expression. The authors also showed that low levels of miR-200c activate the TGF-β signaling pathway and consequently trastuzumab resistance in breast cancer cells. Indeed, restoring miR-200c was sufficient to resensitize cells to trastuzumab and reverse the mesenchymal phenotype by inhibiting TGF-β signaling and ZEB1 expression. Similarly, Izumchenko et al. [111] reported that a high MIG6 expression and a suppression of miR-200c expression is a consequence of TGF-β-induced EMT and a signature for resistance to erlotinib.

Kitamura and colleagues [112] also showed, in lung adenocarcinoma, the importance of TGF-β signaling in drug resistance and EMT, namely, they showed that miR-134/miR-487b/miR-655 cluster promotes the EMT through TGF-β signaling and induces resistance to gefitinib by directly targeting MAGI2, whose suppression is encompassed by loss of PTEN stability [112].

Another example is the overexpression of miR-147, which alone induced reversal of EMT and consequently reversal of the native drug resistance of the colon cancer cell line HCT116 to gefitinib. Although the specific mechanism of action of miR-147 is still unknown, the authors found that miR-147 significantly upregulates CDH1 and represses ZEB1, known EMT markers, and inhibited TGF-β1 expression and also repressed Akt phosphorylation, leading to gefitinib sensitivity [113]. Jiang et al. [114] reported that miR-489 is underexpressed in a MCF7 breast cancer cell line resistant to doxorubicin, a cell line that shows mesenchymal phenotype. On the contrary, SMAD3, involved in TGF-β-induced EMT, is overexpressed in the same cell line. Ectopic expression of mir-489 not only reversed mesenchymal features, as well as sensitized the breast cell line to doxorubicin, through inhibition of SMAD3. No matter what miR and the respective target might be deregulated, all these studies show a point in common that is TGF-β signaling. This enhances the importance of TGF-β signaling in EMT and the regulation of EMT influenced drug resistance by miRs. miR-223 was also associated with drug resistance and EMT in pancreatic cancer. miR-223 is upregulated in gemcitabine resistant pancreatic cancer cells, thus acting as an oncogene, most probably, through inhibition of Fbw7 which consequently overexpresses Notch-1. The authors also showed that by inhibiting miR-223, pancreatic cancer cells were sensitized to gemcitabine [115].

2.5 Cancer Stem Cells and Drug Resistance

Somatic stem cells are typically slowly cycling cells capable of self-renewing mitotic divisions in which one or both of the daughter cells are faithful reproductions of the parent stem cell. The experimental observation that certain minority subpopulations of primary human acute myeloid leukemias (AMLs) could propagate the disease in immunodeficient mouse hosts at higher frequencies than the bulk populations of leukemic cells, led to the basis of what was later called the stem cell hypothesis. These cells made up the so-called side population (SP) cells, described as a subset of cells highly expressing ABC transporters and exhibiting cancer stem cell (CSC)-like phenotypes. Initially they were isolated by fluorescence-activated cell sorting (FACS) techniques based on Hoechst 33342 efflux. The SP cells were first isolated from the hematopoietic system but were then identified in normal tissues and several solid tumors.

Although it is accepted that most tumors arise from a single mutated cell, i.e., their origin is monoclonal, the tumor itself is a

sum of several types of cells, due to the heterogeneity derived from a continuous evolution of the primitive cancer cell. Not all of these cells will display characteristics of cancer cells, such as metastization or unlimited replication potential. Operationally, (CSC) make up subpopulations of neoplastic cells within a tumor that have an elevated ability to seed new tumors upon experimental implantation in appropriate animal hosts [116]. They share many of the features of normal stem cells, including the capacity for self-renewal and differentiation, although their ability to differentiate into more than a few cell types has not been unequivocally proven, besides leukemias [117]. Although CSCs have been well characterized in hematological malignancies, their existence in other tissues has been much debated (for a review see Ref. [118]). Over the past few years CSC have been identified using stem cell specific markers in several solid tumors including breast, brain, colon, prostate, and pancreatic cancer [119–122]. It is often difficult to strictly define CSCs by associating them with traits beyond their tumor-initiating capability [118, 123]. Moreover, the possible existence of CSCs within tumors is intimately linked to tumor heterogeneity and tumor dedifferentiation. Nevertheless, several miRs have been shown to regulate stemness, or what we consider as properties of tumor-initiating and maintaining cancer cells, of different cancer types.

Recent studies showed differential expression of certain miRs between CSC and their differentiated counterparts [6, 124, 125], suggesting that miRs could also be involved in the regulation of CSC. For example, miR-200c and miR-34 have been shown to regulate CSC properties by targeting Bmi1 and downregulating Bcl2 and Notch, respectively [125, 126]. Additionally, miR-134, miR-296, and miR-470 modulate embryonic stem cell differentiation by suppressing the expression of the stem cell transcription factors Nanog, Oct4, and Sox2 [6]. Therefore miRs may impact on cancer drug resistance and several miRs have been reported to regulate stem cell properties and drug resistance concomitantly [127].

Yu et al. showed that let-7a expression was significantly decreased and Nanog/Oct4 expression was increased in head and neck cancer (HNC) tissues as compared to adjacent normal cells [128]. HNC–ALDH1+ cells displayed a decreased level of let-7a than HNC–ALDH1− cells. The overexpression of let-7a in vitro and in vivo showed that the self-renewal, resistance to cisplatin, and tumor initiation properties were significantly suppressed in let7a-overexpressing HNC–ALDH1+ cells, suggesting that the resistance of HNC–ALDH1+ cells to chemotherapy is partially due to the preferential activation of let-7a miRNA gene expression.

In another study, expression of miR-145, a tumor-suppressive miR, was shown to be inversely correlated with the levels of Oct4 and Sox2 in glioblastoma-CD133+ (GBM-CD133+) cells and malignant glioma specimens [129]. CD133 is a putative CSC

marker in glioblastomas. The authors subsequently showed that miR-145 negatively regulates GBM tumorigenesis by targeting Oct4 and Sox2 in GBM-CD133$^+$ cells. miR-145 delivery to GBM-CD133$^+$ cells using polyurethane-short branch polyethylenimine (PU-PEI) significantly inhibited their tumorigenic and CSC-like abilities and facilitated their differentiation into CD133$^-$-non-CSCs. Moreover, PU-PEI-miR145-treated GBM-CD133$^+$ cells suppressed the expression of stemness (Nanog, c-Myc, and Bmi-1), drug-resistance (ABCG2, ABCC5, ABCB1), and anti-apoptotic genes (Bcl-2, Bcl-xL) and increased the sensitivity of the cells to radiation and temozolomide. The in vivo delivery of PU-PEI-miR145 alone significantly suppressed tumorigenesis with stemness, and synergistically improved the survival rate when used with radiotherapy and temozolomide in orthotopic GBM-CD133$^+$-transplanted immunocompromised mice [129].

Some miRs possess the ability to promote the generation of CSC by downregulating tumor suppressors. In hepatocellular carcinoma, miR-130b was shown to be associated with CSC growth that leads to worse overall survival and more frequent recurrence of cancer in patients. The increased miR-130b occurs in parallel with the reduction of tumor protein 53-induced nuclear protein 1, a known miR-130b target. Moreover, cells transfected with miR-130b presented a higher resistance to doxorubicin [130].

Similarly, other studies have revealed a regulation of stem cell properties through stem cell factors, including the p53–Nanog axis. For example, Xu et al. [131] showed that miR-214 regulates ovarian cancer cell stemness and chemoresistance towards cisplatin and doxorubicin treatment by targeting p53–Nanog, and expression of p53 abrogated miR-214-induced ovarian CSC properties.

Bitarte et al. [132] prepared colonospheres with CSCs properties from different colon carcinoma cells, and after performing miR profiling observed that miR-451 was downregulated in colonospheres versus parental cells. Expression of miR-451 caused a decrease in self-renewal, tumorigenicity, and chemoresistance to irinotecan, through a downregulation of the ABCB1 transporter.

Bourguignon et al. [133] observed that human head and neck squamous cell carcinoma (HNSCC) derived HSC-3 cells contain a subpopulation of (CSCs) characterized by high levels of CD44v3 and aldehyde dehydrogenase-1 (ALDH1) expression. These tumor cells also expressed stem cell markers (Oct4, Sox2, and Nanog) and displayed the hallmark CSC properties of self-renewal/clonal formation and the ability to generate heterogeneous cell populations. Hyaluronan (HA) activation of CD44v3 (an HA receptor) lead to nuclear accumulation of oncogenic transcription factors (Nanog, Oct4, Sox2), and CSCs in HNSCC display upregulated miR-302 expression which, in turn, upregulates several survival proteins responsible for clonal formation, self-renewal, and cisplatin resistance. MiR-302 is controlled by an upstream promoter containing

Oct4-Sox2-Nanog binding sites, while stimulation of miR-302 expression by HA-CD44 is Oct4-Sox2-Nanog-dependent in HNSCC-specific CSCs. This process results in suppression of several epigenetic regulators (AOF1/AOF2 and DNMT1) and the upregulation of several survival proteins (cIAP-1, cIAP-2, and XIAP) leading to self-renewal, clonal formation, and cisplatin resistance [133].

Several of these studies have used cell lines in vitro that express stem cell markers; however, one must keep in mind that these cell lines have vastly altered karyotypes (e.g., several translocations, insertions, and deletions) that will obviously alter their biological behavior. Therefore, caution must be exercised in interpreting the results described.

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