Role of microRNAs in mechanisms of resistance to small molecules tyrosine kinase inhibition therapies in solid tumours

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Running title: MicroRNAs in the resistance to TKI therapies in solid tumours

Abstract

Receptor tyrosine kinases (RTKs) are widely expressed transmembrane proteins that act as receptors for growth factors and other extracellular signalling molecules. Upon extracellular ligand binding, RTKs are responsible for activation of intracellular signalling cascades and as such are involved in a broad variety of cellular functions including differentiation, proliferation, migration, invasion, angiogenesis and survival under physiological as well as pathological conditions. Aberrant RTK expression can lead to benign proliferative conditions as well as various forms of cancer. Indeed, more than 70% of the known oncogene and proto-oncogene transcripts involved in cancer code for RTKs. Consequently, these receptors are broadly studied as targets in treatment of different tumours and a broad variety of small molecule RTK inhibitors (TKIs) are approved for therapy. In most cases, the patients develop resistance against the TKIs within a short time. Mounting lines of evidence point towards a significant role of microRNAs for mediating the resistance to TKIs. MicroRNAs are short (18–22 nucleotides) non-protein coding RNAs that fine tune cell homeostasis by controlling gene expression at the post-transcriptional level. Deregulation of microRNAs is common to many cancers and increasing evidence also exists for an important role of microRNAs for the development of resistance to therapies including – but not limited to – TKIs.

In this review we focus on the role of microRNAs for mediating resistance to small molecule TKIs in solid tumours.

Key points:

- MicroRNA expression patterns are currently used to predict efficiency of different cancer therapies including targeted agents, radio- and chemotherapy.

- MicroRNA expression may allow early prediction of treatment failure and development of resistance. This could result in an early switch of therapies, preventing unnecessary side effects and, eventually, improvement in survival outcomes.

- MicroRNA and receptor tyrosine kinase crosstalk is a common mechanism of resistance to tyrosine kinase inhibitors (TKI). During TKI treatment, microRNAs regulate the expression of receptor tyrosine kinase (RTKs) by increasing or decreasing their expression. On the other hand, RTKs are known to affect the processing of microRNAs.

1. Introduction

Receptor tyrosine kinases (RTKs) are multifunctional transmembrane proteins broadly expressed in human tissues. Binding of their ligands induce dimerization of RTKs and result in structural modifications, which elicit their intracellular enzymatic domain to start exerting its tyrosine kinase activity. By this, an extracellular signal is transferred into an intracellular signal and downstream signalling cascades are activated resulting in expression of genes that control cell survival, proliferation and differentiation [1]. Mutations in genes encoding RTKs and/or abnormal stimulation by the respective growth factor ligands – as well as improper regulation of the RTK downstream signalling cascades – lead to uncontrolled transduction of pro-survival/proliferative signals, eventually leading to malignant cell transformation and subsequently driving cancer progression and spread [2, 3]. It is well established that dysregulated growth factor receptors (e.g. human epidermal growth factor receptors (HER), vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), mast/stem cell growth factor receptors (SCFR/Kit)) as well as their intracellular signalling pathways (e.g. MAP/ERK- and PI3K/PTEN/AKT-pathway) can act either as major oncogenic drivers or as key contributors of the processes leading to different stages of cancer development such as initiation, growth, vascularization, metastatic spread and immune-escape [4-8].

Outstanding progresses in cancer treatment have been made over the past two decades, mainly through the advent of molecular based tailored approaches [9]. Due to the pivotal role of RTKs in the context of several tumours, a broad variety of RTK-inhibitors (TKIs) are already in use for cancer treatment or under investigation in clinical studies [10-15]. These TKIs can be divided into two main groups: i) small molecule inhibitors that affect the cytoplasmatic enzymatic domain of the growth factor receptors or inhibit the mediators of

their signal cascade (Table 1) and ii) monoclonal antibodies (mAbs) that target the extracellular domain of RTKs or their ligands [16, 17].

Many of these agents have dramatically changed the prognosis of neoplastic diseases, successfully prolonging the overall and progression-free survival of patients [18-22]. In addition, the advent of novel high-throughput molecular and genomic techniques has led to the identification of well-defined molecular subtypes of tumours, each of which harbours particular targetable alterations, further optimizing the efficacy of tailored approaches including TKIs [23-26].

However, despite remarkable achievements in improving survival outcomes, resistance to these therapies arises almost unavoidably at some point during the treatment [27]. Thus, a full elucidation of the mechanisms that drive primary and secondary resistance to treatments is needed in order to expand the number of patients that could benefit from said treatments, as well as to prolong the patients' survival.

Based on data collected so far, resistance to RTK-targeted therapies can occur through different mechanisms: 1) mutations in drug binding site which reduce or inhibit the binding of the drug to its RTK target, limiting its inhibitory potential; 2) RTK locus amplifications that generate high numbers of RTK proteins and therefore limit the efficacy of the drug; 3) over-expression of RTK ligands which interfere with the binding of the drug to its target or antagonizes the inhibitory effect of the drug through abnormal stimulation; 4) mutations or locus amplifications of proteins belonging to downstream signal mediators of the drug target, resulting in permanent signal activation and independence from the upstream regulator; 5) activation of alternative signalling pathways able to by-pass the block provided by the drug thus allowing the transduction of pro-cancerous signals; 6) loss of differentiation by epithelial-to-mesenchymal transition (EMT), by which cancer cells lose their epithelial-type properties and acquire a more invasive and drug resistant phenotype [28, 29].

It is well established that microRNAs (miRNAs), a group of short non-coding RNAs (18-22 nucleotides), are not only involved in tumour development and progression but also in response and resistance to anti-tumour agents [30]. Furthermore, strong interaction networks exist between RTKs and miRNAs [31-33]. On the one hand, RTKs are known to affect the processing of miRNAs; e.g. EGFR inhibits the maturation of tumour suppressor miRNAs (like miR-31, miR-192 and miR-193a-5p) [34] and transforming growth factor beta (TGF-β) signalling increases the presence of miR-21, a well-known onco-miRNA [35]. On the other hand, miRNAs are known to regulate the expression of RTKs: the regulation of EGFR by miR-7, miR-128, miR-133b, miR-146a, miR-302b and miR-608 has been extensively characterised [36-38].

In general, miRNAs regulate gene expression by acting on target proteins at the translational level [39]. Each miRNA can have several target mRNAs, and the interaction of miRNA with the target mRNAs results in direct deregulation of different target proteins, thus acting simultaneously in regulation of several cellular pathways. Therefore, variation in miRNA expression can result in reduced mRNA level of the targets and subsequent change in protein levels within the cell [40]. MiRNA expression patterns are tissue-specific [41] and often define the physiological nature of the cell [42]. Numerous publications show that altered mRNA expression in the context of several diseases (e.g. cancer, viral diseases, neurodegenerative disease, immune-related diseases) and some pathological conditions are caused by deregulation of miRNA expression [43-46].

Recently, mounting lines of evidence have shed light on the critical role of miRNAs in mediating resistance to TKI treatments [31] (Table 2).

A number of studies have demonstrated that, under pharmacological influence of TKI therapy, the activity of miRNAs can be modulated by RTKs directly or indirectly towards increasing or lowering the tumour cell sensitivity to the drug [31, 33, 47].

Here, we aim to discuss the currently available evidence for how miRNAs affect drug response in *in-vitro* and *in-vivo* models of solid tumours under treatment with small molecule TKIs.

2. Roles of deregulated microRNAs' expression in therapeutic resistance to TKIs

2.1 Epidermal Growth Factor Receptor (EGFR) TKIs

2.1.1 Gefitinib and Erlotinib

Both gefitinib and erlotinib are indicated for the treatment of EGFR-mutated non-small cell lung carcinoma (NSCLC) [19, 48]. These TKIs target mutated EGFR containing an in-frame deletion in exon 19 as well as the missense mutation L858R in exon 21 [19, 49]. Some non-coding RNAs both of prognostic and predictive relevance have been associated with erlotinib and gefitinib treatment so far.

According to a clinical study, increased miRNA-21 expression is associated with EGFR mutations in non-small cell lung carcinoma (NSCLC), and this was verified in another study using liquid biopsies from patients and *in-vivo* and *in-vitro* models of NSCLC [50, 51]. A well-established effect of up-regulated miRNA-21 in NSCLC is down-regulation of PTEN [51, 52]. PTEN down-regulation in turn results in activation of PI3K/AKT– and ERK-signalling pathways and therefore has a similar effect as PIK3CA activating mutations [52, 53]. Hence, aberrant expression of miRNA-21 might contribute to gefitinib resistance in NSCLC by activation of PI3K/AKT- and ERK-signalling pathways [52]. In a recent report, the human PC9 gefitinib-resistant (PC9R) lung adenocarcinoma cell line was infected with a lentivirus containing a miR-21 inhibitor. Inhibition of miR-21 suppressed PC9R proliferation

and induced cells' apoptosis by up-regulating PTEN and programmed cell death protein 4 (PDCD4) and inhibiting both PI3K and AKT, known downstream actors in the EGFR pathway. Moreover, levels of circulating miR-21 in a cohort of patients was found to be an independent prognostic indicator of acquired resistance to erlotinib, as they were significantly increased in patients who progressed during the TKI treatment [51].

In lung cancer, miR-146a acts as tumour suppressor by inhibiting EGFR down-stream signalling and reducing cell proliferation, as well as stimulating cell apoptosis caused by drugs targeting EGFR [54, 55]. It was established that co-treatment with a miR-146a mimic and gefitinib results in a significant reduction of invasion in resistant lung cancer cells induced by EMT [55]. In different studies based on *in-vitro* models of NSCLC it was demonstrated that members of the miR-200 family also suppress EMT [56-61]. The expression of miR-200c has been extensively evaluated in the mechanisms of response to erlotinib in *in-vitro* systems. MiR-200c acts by negatively regulating the process of EMT, which is inversely correlated with response to erlotinib. EMT is driven by TGFβ1 and a network of transcriptional repressors, including zinc-finger E-box binding factor (ZEB1) [61]. Treating erlotinib-resistant A549 lung adenocarcinoma cells with TGFβ1 induced EMT and increased levels of ZEB1 and neural cadherin (N-cadherin), as well as reducing the level of epithelial cadherin (E-cadherin). On the contrary, the same cells stably over-expressing miR-200c did not show EMT and have reduced expression of ZEB1. These findings indicate a complementary and independent effect of TGFβ1 and miR-200c on EMT promotion when over-expressed [62]. In another *in-vitro* study, expression of miR-147 was found to revert the process of EMT resulting in restored sensitivity of the tumour cells to gefitinib [56-60].

A second crucial pathway for EMT regulation in NSCLC is that of Hedgehog (Hh). A549 cells treated with TGFβ1, in order to generate A549M cells with a mesenchymal phenotype, were subsequently transfected with a small-interfering RNA (siRNA) specific for Hh.

Inhibition of Hh resulted in a sensitization of A549M cells to erlotinib and cis-platinum treatment. Moreover, ectopic expression of members of the miR-200 family (miR-200a, miR-200b and miR-200c) and transfection of let-7 miRNAs (let-7b and let-7c) were able to reverse erlotinib resistance in A549M cells. As a whole, these data suggest a strong link between EMT regulating miRNAs (such as miR-200b and let-7c), TGFβ1 and Hh signalling in the process of EMT-mediated resistance to erlotinib [63].

MiR-200c down-regulation was reported in the case of primary resistance to EGFR-TKIs in *in-vitro* models, and the mechanism of resistance was mediated through hyper-activation of the PI3K/AKT pathway [64]. Moreover, in resistant cell lines, down-regulation of miR-200c resulted in over-expression of the mitogen-inducible gene 6 (MIG6), a negative regulator of EGFR. An elevated ratio of mRNA coding for MIG6/miR-200c expression was associated with erlotinib resistance, while inhibition of TGFβ1 resulted in up-regulation of miR-200c, correspondent down-regulation of MIG6 and, eventually, increased erlotinib sensitivity [61]. However, when miR-200c expression levels were evaluated in NSCLC patients treated with EGFR-TKI, only EGFR wild-type patients with miR-200c over-expression demonstrated longer overall survival, progression-free survival and disease control rate compared with the low miR-200c expressing subgroup. Surprisingly, EGFR-mutated patients with high miR-200c showed a similar but not statistically significant trend [64].

KRAS mutations (which are mutually exclusive with *EGFR* mutation with a prevalence of 15-19%) and *TP53* mutations (which are present in 50-70% of NSCLC patients) are among the aberrations causing erlotinib resistance [65]. The RAS and p53 pathways are regulated in part by let-7b and miR-34a, which act as onco-suppressors. Both miRNAs were found to be down-regulated in NSCLC. Restoration of let-7b and miR-34a sensitized NSCLC cells to erlotinib and, in addition, these two miRNAs had shown a synergistic effect with erlotinib in inhibiting cell proliferation [65]. Furthermore, miR-34a regulates the PI3K/AKT-signalling

pathway as well as the ERK-signalling pathway; additionally, MET has been validated as target of miRNA-34a in *in-vivo* and *in-vitro* models of NSCLC. Therefore, reduced miR-34a expression in NSCLC results in a lower apoptosis rate, higher migration and increased invasion of tumour cells. Furthermore, cell cycle arrest and senescence mediated by miR-34a expression is impaired thus resulting in tumour growth and increased MET activation by its ligand, hepatocyte growth factor (HGF). In addition, reduced miR-130a expression enhances MET receptor expression and activates the downstream pathway. Increased expression of the MET receptor has the same effect as MET gene amplification and overcomes the therapeutic effects of gefitinib treatment [66].

Besides targeting the MET receptor, miR-130a also represses the expression of miR-221 and miR-222. A reduced miR-130a expression results in increased expression of miR-221 and miR-222. MiR-221 and miR-222 in turn both directly inhibit caspase-3, the central caspase in the caspase-cascade [67, 68], thus preventing apoptosis of tumour cells [68]. This shows that even reduced level of miRNAs, as shown for miR-34a and miR-130a, can result in RTKs' downstream pathway deregulation and in gefitinib resistance. Over-expression of MET ligand HGF has been shown to increase MET receptor-mediated signalling and by this cause gefitinib resistance in relevant *in-vivo* models [66]. It has been shown *in-vivo* that the MET-receptor as well as EGFR regulate the expression of miR-103, miR-203, miR-30b, miR-30c, miR-221 and miR-222 following receptor activation. MiR-103 and miR-203 are down-regulated whereas miR-30b, miR-30c, miR-221 and miR-222 are up-regulated by activation of both receptors; this results in inhibition of PTEN expression and down-regulation of the pro-apoptotic protein BIM and apoptotic peptidase activating factor 1 (APAF1), thus stimulating EMT transition. As aforementioned, caspase-3 is down-regulated by miR-221 and miR-222, but caspase-3 is also targeted by miR-30b and miR-30c. The decreased

caspase-3 expression level counteracts the gefitinib-induced apoptosis, thus therapy efficacy is impaired [67].

Gefitinib resistance can be also based on an increased expression of the miR-134/miR-487b/miR-655 cluster, resulting in reduced expression of the membrane-associated-guanylate-kinase-inverted-2 protein (MAGI2) that in turn leads to PTEN down-regulation and activation of PI3K/AKT-signalling pathway [69]. In contrast to this indirect PTEN regulation, a direct regulation of PTEN by miR-214 has been also demonstrated in *in-vitro* NSCLC models [70].

Gefitinib resistance following EMT activation in NSCLC cells can also be caused by miR-23a over-expression. MiR-23a over-expression is triggered by TGF-β and in turn it suppresses E-cadherin expression and favours EMT, resulting in enhanced gefitinib resistance in lung cancer cells [71]. EMT triggers increased expression of the miR-134/miR-487b/miR-655 cluster in NSCLC that causes down-regulation of PTEN [69], as previously mentioned.

A recently described resistance mechanism related to gefitinib treatment and mediated by activation of AXL receptor tyrosine kinase is also based on miRNAs [72, 73]. AXL over-expression in NSCLC is partially caused by EMT activation and thus AXL was evaluated as a potential therapeutic target in patients with acquired resistance to EGFR-TKIs [73, 74]. AXL kinase activation increases the level of miRNA-374a expression and in parallel reduces miR-548b expression, resulting in EMT activation that leads to increased cell migration as well as invasion of tumour cells [72].

Beyond the mechanisms of miRNA mediated deregulation of downstream RTKs signalling, miRNAs have been shown to directly decrease sensitivity to gefitinib therapy by physical interaction with RTKs, and by this mechanism limiting the drug’s efficacy without the mediating resistance mechanism described above. For instance, reduced levels of miR-133b

and miR-128b lead to an increased active amount of EGFR mRNA and protein thus overcoming the effects of gefitinib [36, 75].

Recently, first results of clinical trials for gefitinib-based treatment of patients with recurrence or metastases of advanced nasopharyngeal carcinoma have been published. In nasopharyngeal cancer EGFR is in general highly expressed, but surprisingly patients have different responses to gefitinib treatment; in this setting, miRNAs seem to be the key counterparts of mediating resistance [76-78]. In gefinitib-sensitive models the expression of miRNA-125a-5p was found to be increased by gefitinib treatment in *in-vivo* and *in-vitro* models for nasopharyngeal carcinoma. Increased miR-125a-5p expression results in improved anti-proliferative and pro-apoptotic effects. Therefore miR-125a-5p expression represents a potential marker for gefitinib sensitivity in the context of nasopharyngeal carcinoma [77].

In summary, identification of miRNAs relevant to gefitinb and erlotinib resistance has mostly been based on clinical studies which have been confirmed in relevant *in-vitro* and *in-vivo* models. The role of miR-21 was proved in a clinical study composed of training and validation cohorts and the importance of miR-125a-5p is based on two different clinical phase II studies, both with small patient numbers. Nevertheless, some miRNAs have been identified to be important for mediating resistance to gefitinb and erlotinib only in relevant *in-vitro* and *in-vivo* models, respectively.

2.1.2 Lapatinib

Lapatinib is indicated for the treatment of HER-2 positive advanced breast cancer [79]. Studies based on primary human breast cancer and normal samples demonstrated that resistance to Lapatinib can be caused by deregulated microRNA expression and these findings have been verified in *in-vitro* systems [80, 81].

MiR-205 is down-regulated in breast cancer tissue compared to normal breast tissue and it was shown that miR-205 directly targets HER3, as well as its function as an inhibitor for the activation of the downstream mediator AKT. Following HER2 mediated trans-phosphorylation of HER3, the PI3K/AKT-pathway is activated, thus by-passing lapatinib's effect. Further proof of the essential role of miR-205 in relation to lapatinib treatment was the observation that re-introduction of miR-205 in *in-vitro* models of breast cancer reverted cells sensitive to lapatinib treatment [80]. In another study, miR-630 has been shown to be involved in the resistance to lapatinib [81]. MiR-630 expression is decreased in lapatinib-resistant tumour cells and a lower or induced down-regulated miR-630 expression results in an increased expression of HER2 and EGFR. However, these effects of miR-630 are secondary; the direct miR-630 target is IGF1R, which after phosphorylation leads to cascade phosphorylation and thus activation of HER2 and EGFR. In this case activation of the target counteracts the treatment [81]. Therefore miR-205 and miR-630 might be considered as tumour suppressor miRNAs in the context of breast cells.

In conclusion, the role of miRNAs for resistance to lapatinib treatment is based on patient-derived material and has been analysed further in relevant *in-vitro* models. Both miR-205 and miR-630 play an important role in this process.

2.2 BRAF TKIs

Vemurafenib is the standard treatment for patients with advanced melanoma harbouring V600E mutation [20]. Vemurafenib-induced over-expression of some miRNAs in A375 melanoma cells has been demonstrated, such as miR-211-5p, which is negatively regulated by B-RAF V600E mutation [82]. MiR-211-5p is induced in melanotic melanoma cells, impairing the degradation of tyrosinase (an enzyme involved in melanin pigment biosynthesis) and promoting pigmentation [82]. Pigmentation is a result of B-RAF and MEK

inhibition [82, 83] but it limits vemurafenib activity and results in drug resistance [82-86]. In fact, melanosomes can promote multidrug resistance by sequestering drugs and releasing them outside the cells. For this reason, a novel treatment strategy based on a combination of ERK pathway and pigmentation inhibitors for melanotic melanomas has been proposed [82]. MiR-211-5p is directly involved in melanoma cells' adhesion and invasion, directly targeting NUAK1, an AMP-activated protein kinase-related kinase. Moreover, miR-211-5p is a target of the master regulator microphthalmia-associated transcription factor (MITF). Up-regulation of miR-211-5p promotes adhesion, while inhibition of the same miRNA increases NUAK1 expression and decreases melanoma adhesion [87]. Indeed, up-regulation of miR-211-5p in A375 melanoma cells following vemurafenib exposure was associated with the emergence of drug resistance [88]. MiR-211-5p was detected in extracellular vesicles, released from cancer cells after vemurafenib treatment. Increase of miR-211-5p under treatment and its inclusion in vesicles may explain the mechanism of intercellular resistance caused by vemurafenib treatment [89]. Another common mechanism of resistance is autophagy, a process that maintains cell survival in stressful conditions, including antitumoral treatment [90]. MiR-216 was found to have an important role in alleviating autophagy by directly targeting three key autophagy genes (Beclin-1, UVRAG and ATG5). In this way, ectopic expression of miR-216 increased vemurafenib activity both *in-vitro* and *in-vivo*, while its suppression generated drug resistance [90]. Along with miR-216, miR-7 was studied for its onco-suppressive effects. MiR-7 was found down-regulated in VemR A375 melanoma cells, resistant to vemurafenib, while EGFR, IGF-1R and CRAF were over-expressed. Re-introduction of miR-7 in VemR A375 cells led to reduction in expression of these oncogenes and reverted resistance to vemurafenib [91]. MiR-579-3p acts as an onco-suppressor by targeting the 3'UTR of mRNAs coding for B-RAF and E3 ubiquitin protein ligase MDM2. MiR-579-3p is down-regulated in vemurafenib-resistant melanoma cells and low expression of miR-579-3p correlates with

poor survival in human melanoma samples [92]. Among mechanisms of resistance, prohibitin 1 (PHB) protects melanoma cells from chemotherapy and targeted therapy-induced cell death. PHB is a direct target of miR-195, which acts as a chemosensitizer and increases vemurafenib cytotoxicity [93].

Among up-regulated miRNAs, miR-514a-3p is involved in melanocyte transformation and melanoma growth. MiR-514a-3p directly binds to neurofibromatosis type 1 (NF1) and inhibits its expression. In V600E B-RAF mutant cells treated with vemurafenib, miR-514a-3p was over-expressed, while NF1 expression was suppressed [94].

Taken together, nearly all miRNAs involved in resistance to vemurafenib treatment have been identified in *in-vitro* or *in-vivo* model systems. Up to now only the role of miR-579-3p in vemurafenib resistance has been analysed in a small series of patient derived material.

2.3 mTOR TKIs

Everolimus has multiple indications: in pre-treated advanced renal cell carcinoma [95], advanced ER+ breast cancer [96] and advanced pancreatic neuroendocrine tumours [97].

In breast cancer, the mTOR pathway is regulated by 17α-estradiol (E2) signalling and mediates E2-proliferation and progesterone receptor (PgR) expression [98]. Estrogen receptor positive ($ER\alpha^+$) disease mainly correlates with mTORC2 signalling. By using ER^+ MCF-7 cell lines, over-expressed miR-155 was found to enhance mTORC1 signalling, targeting the AKT/mTOR pathway with concomitant inhibition of mTORC2. MiR-155-mediated mTOR signalling led to dysregulated ERα signalling and repressed PgR expression. When miR-155 over-expressing MCF-7 cells were treated with everolimus and E2, the E2-induced PgR gene expression was restored. Thus, miR-155 induces a crosstalk between mTOR and ER by enhancing mTORC1 and suppressing mTORC2 signalling. Moreover, alterations in the

mTOR cascade in miR-155 over-expressing ER^+ cells can result in the loss of PgR expression without prior growth factor stimulation [98].

In summary, the role of miRNAs in regard to resistance to everolimus is only based on *in-vitro* experiments at this point, and mainly involves miR-155.

2.4 Multikinase TKIs

2.4.1 Sorafenib

Sorafenib is the treatment of choice in advanced hepatocellular carcinoma (HCC) [99]. Changes of expression of several microRNAs have been associated with sorafenib treatment in HCC and it seems that they are involved in acquired resistance against sorafenib [100, 101]. The tumour-suppressor miR-122 is the most-expressed liver-specific microRNA and low levels of miR-122 correlate with poor prognosis and a more aggressive behaviour of the disease, as demonstrated on resected human HCC tissue samples [102]. *In-vitro* miR-122 was found to down-regulate the expression both of disintegrin and metalloproteinase family 10 (ADAM10) and serum response factor (SRF), normally over-expressed in HCC and involved in the mechanism of EMT [102]. Moreover, in HCC cell lines and *in-vivo* experiments, miR-122 acts as a negative regulator of IGF1R, which induces the downstream RAS/RAF/ERK pathway, stimulates cells' proliferation and promotes metastasis. Inhibition of IGF1R activity and block of the angiogenic potential by miR-122 could explain the process of re-sensitization of HCC cells to sorafenib following restoration of miR-122 expression [102, 103].

MiR-193b is down-regulated in HBV^+ HCC cell lines, with concomitant elevation of the anti-apoptotic protein myeloid cell leukemia-1 (MCL-1). Restoration of miR-193b expression led

to an increased anti-tumoral action of sorafenib with a resensitization of HBV^+ HCC cells to the antiangiogenic drug [104].

Also, in HCV^+ HCC cells the IC_{50} of sorafenib was higher compared with uninfected controls in *in-vitro* experiments. In the control cells, miR-193b was highly expressed leading to inhibition of MCL-1 and an increase in sensitivity to sorafenib [105].

MiR-34a was reported to be usually down-regulated in HCC tissues from patients and HCC cell lines with up-regulated expression of the oncogene B-cell lymphoma 2 (BCL-2), being associated with poorer survival [106]. MiR-34a and sorafenib negatively regulate the expression of BCL-2 and Cyclin-D1, and miR-34a over-expression increased sorafenib-induced apoptosis [106, 107].

MiR-193a, a negative regulator of the pro-metastatic factor urokinase-type plasminogen activator (uPA) is down-regulated in HCC. Cell transfection with miR-193a promoted apoptosis and decreased proliferation *in-vitro*, and the combination with sorafenib had a synergic anti-tumour effect [108].

Other miRNAs which were reported to act as chemosensitizers for sorafenib when their expression was restored were miR-486 [109], miR-338-3p [110] and let-7 [111]. In patients who received sorafenib treatment these miRNAs were often down-regulated and involved in treatment failure [109-111]. The down-regulation of miR-486 results *in-vitro* in increased cell proliferation and progression of metastatic sites via up-regulation of claudin-10 (CLDN10) and CITRON Rho interacting kinase [109]. A reduced miR-338-3p or let-7 family expression results in decreased apoptosis in HCC cell lines due to an increased HIF-1α or Bcl-xL expression, respectively [110, 111].

Chemoresistance against sorafenib is also caused by over-expression of the miR-216a/217 cluster, miR-93 and miR-21 in HCC patient-derived material and in HCC cell lines [112-114]. Up-regulation of the miR-216a/217 cluster results in increased EMT as well as in

down-regulation of mothers against decapentaplegic homolog 7 (SMAD7) and PTEN [113]. Consequently, the TGF-β and PI3K/AKT pathway are activated [113]. Enhanced activity of the PI3K/AKT pathway is also caused by down-regulation of PTEN due to an increased miR-21 or miR-93 expression [112, 114]. Furthermore, up-regulation of miR-93 results in decreased abundance of cyclin-dependent kinase inhibitor 1 (CDKN1), and by this mechanism cell cycle arrest is prevented and the rate of apoptosis diminished [114].

Beside its use in HCC, sorafenib is also indicated in advanced and pre-treated renal cell carcinoma (RCC) [115]. In the context of RCC, miR-30a was found to have a role in modulating sorafenib-induced anti-tumour activity in resected human RCC tissue and *in-vitro*. MiR-30a, which is commonly down-regulated in kidney cancer, inhibits the process of autophagy by repressing Beclin-1 [116]. Autophagy is enhanced during chemotherapy treatment in order to promote cell survival and represents a mechanism of tumour resistance and progression under therapy [117]. Treatment with sorafenib induced Beclin-1 up-regulation and activation of autophagy with simultaneous miR-30a suppression. When miR-30a was exogenously restored, Beclin-1 expression was inhibited and sorafenib activity was enhanced. On the contrary, use of an antagomiR-30a increased Beclin-1 expression, promoted autophagy and reduced sorafenib-induced cytotoxicity [116].

Furthermore, Beclin-1 expression is also regulated by miR-200c. MiR-200c is known to regulate the sensitivity of renal carcinoma cells to two TKIs, sorafenib and imatinib. Expression of miR-200c was significantly reduced by promoter hypermethylation in *in-vitro* models treated with sorafenib and imatinib. Moreover, miR-200c restoration reduced the expression of heme oxygenase-1 (HO-1) in cell lines, while treatment with anti-miR-200c up-regulated HO-1 mRNA expression levels [118]. HO-1 is an important enzyme for heme degradation and displays a strong anti-inflammatory and anti-apoptotic effect [118, 119]. HO-1 over-expression in cancer cells promotes proliferation and survival, and induces

angiogenesis. In *in-vitro* experiments it was proven that miR-200c exerts synergistic effects with sorafenib and imatinib on cell proliferation and on autophagy, at least partially by antagonizing Beclin-1 [119].

In conclusion, miRNAs involved in resistance to sorafenib treatment have been identified in different small series of patient derived material, and the role of these miRNAs for mediating resistance to sorafenib treatment have been confirmed in relevant *in-vitro* and *in-vivo* models. Mir-122 expression is correlated with sensitization to sorafenib in HCC, while miR-30a and 200c mainly regulate response to the same TKI in kidney cancer.

2.4.2 Sunitinib

Sunitinib is indicated in the treatment of advanced renal cell carcinoma [21], advanced pancreatic neuroendocrine tumours [120] and gastrointestinal stromal tumours [14]. After an array screening on FFPE tissues of patients presenting extreme phenotypes of sunitinib sensitivity and resistance, 64 microRNAs were found differentially expressed between the two cohorts [121]. Increased levels of miR-942 was verified in another cohort of sunitinib-resistant patients and over-expression of miR-942 was significantly associated with reduced time to progression and overall survival. MiR-942 down-regulates several genes of the Fanconi Anemia/BRCA pathway and transcriptional repression of this pathway was shown to determine a relevant up-regulation of matrix metallopeptidase-9 (MMP-9) and its effector VEGF. It is well established that MMP-9 is produced in large amount from neoplastic cells and, when over-expressed, correlates with poor prognosis and survival. MiR-942 promotes endothelial cell migration and tumour cell resistance to sunitinib most probably through a paracrine mechanism resulting in activation of VEGF signalling pathways and activation of MMP-9 expression [121].

Another study evaluated microRNA expression in blood samples collected before sunitinib treatment and 14 days after treatment [122]. Following evaluation of expression levels of microRNAs by microarray platform, two different prognostic models were built: one for the poor responders (progression before 6 months) and one for prolonged responders (progression after 18 months). Specific to the poor responders were high expression levels of miR-192, miR-193a-3p and miR-501 [122]. These miRNAs are involved in promoting tumour cells' resistance against sunitinib therapy by inducing TGF-ß, resulting in increased angiogenesis as well as up-regulation of the PI3K/AKT-pathway and promoted both cell proliferation and survival via p53 inactivation [123-125].

According to another clinical study, down-regulation of miR-141 is specific to poor responders to sunitinib therapy [126]. MiR-141 is able to modulate the EMT process through the inhibition of transcriptional repressors ZEB-1 and -2. Altered EMT regulation could increase resistance to hypoxia, facilitating refractoriness to sunitinib. Indeed, reintroduction of miR-141 in cell lines promoted the down-regulation of ZEB-2 [126].

A study based on autopsy specimens of patients with renal cell carcinoma (RCC) revealed a significant down-regulation of miR-101 in patients treated with sunitinib [127]. MiR-101 targets and suppresses ubiquitin-like with PHD and ring finger domains 1 (UHRF1) and enhancer of zeste homolog 2 (EZH2), both known as master regulators of epigenetic modifications [127-129]. UHRF1 was found over-expressed in RCC tissues and its knock-down inhibited proliferation, migration and invasion in *in-vitro* assays [127] . Administration of sunitinib led to miR-101 down-regulation and subsequent up-regulation of the oncogenes UHRF1 and EZH2 [127].

In summary, the miRNAs involved in resistance to sunitinib treatment have been identified in the frame of clinical studies composed of training and validation cohorts, or are based on small series of patient derived material. Up to now only a few of these miRNAs have been

confirmed in relevant *in-vitro* models. Among them, miR-942 causes cell resistance to the TKI and favours metastasization.

2.4.3 Imatinib

Imatinib is the treatment of choice for advanced gastrointestinal stromal tumours (GIST) [130]. In imatinib-resistant GIST patients, down-regulation of the tumour suppressor miR-320a results in direct up-regulation of β-catenin and subsequent increased expression of the anti-apoptotic induced myeloid leukemia cell differentiation protein-1 (MCL-1). Therefore miR-320 seems to be involved in imatinib resistance by suppressing apoptotic signal pathways [131]. In another study based on patient specimens miR-125a-5p was found to be involved in mediating imatinib resistance. MiR-125a-5p target genes are functionally associated with anti-apoptosis, cell cycle, signal transduction, protein phosphorylation and cell differentiation [132]. Based on studies with GIST patient derived material it is known that deregulation of genes from these biological processes are associated with imatinib resistance [133, 134]. Up-regulation of miR-301a-3p, miR-1915 and miR-150-3p under imatinib treatment was found to correlate with metastasis formation *in-vivo* [132]. This finding is in accordance with other studies demonstrating an important role for miR-301a-3p and miR-150-3p for establishing metastatic tumour sites [135, 136]. Using cell culture models, miR-218 down-regulation was detected in imatinib-resistant cells, and over-expression of miR-218 reverts cells sensitivity to imatinib treatment. It has been proposed that the PI3K/AKT-pathway might be involved in the outcome of imatinib treatment via miR-218 [137]. Furthermore, by analysing GIST patient derived material, miR-518a-5p was found to be another miRNA that could link imatinib resistance and PI3K/AKT-pathway. MiR-518a-5p was found to be down-regulated in imatinib-resistant GIST patients and PIK3CA was identified as target gene for miR-518a-5p. Reduced expression of miR-518a-5p results in up-

regulation of PIK3CA and affects the cellular response to imatinib, resulting in drug resistance [138].

In conclusion, the role of miRNAs involved in resistance to imatinib is mainly based on different small series of patient derived material. Nevertheless, all deregulated miR-125a-5p target genes have been verified in the frame of a large multicentre phase III clinical studies, and by this the role of miR-125a-5p for resistance to Imatinib treatment has been indirectly proven. In the case of miR-218 the current evidence is restricted to *in-vitro* studies only.

2.4.4 Crizotinib

Crizotinib is indicated in the treatment of NSCLC with EML4-ALK fusion protein [139]. Few reports have investigated the role of microRNAs for the resistance to crizotinib treatment so far. In one study, miR-96 levels were found to be markedly decreased in ALK-expressing cancer cell lines and primary human tumours compared with their normal cellular and tissue counterparts [140]. Furthermore, by targeting ALK expression post-transcriptionally, miR-96 reduced the abundance of phosphorylated ALK target proteins, including AKT, signal transducer and activator of transcription 3 (STAT 3), Jun N-terminal kinases (JNK), and type I insulin-like growth factor receptor (IGF-1R), and down-regulated the transcription factor JunB. These effects were associated with reduced proliferation, colony formation, and migration of ALK-expressing cancer cells [140]. Therefore, the decrease in miR-96 levels could represent a mechanism underlying the aberrant expression of ALK in cancer cells resulting in resistance to crizotinib therapy.

In another *in-vitro* study miRNA-134 was identified as a further RTK-regulated mediator that is also regulated by crizotinib in glioblastoma models [32]. Expression of the tumour-suppressor miRNA-134 correlates inversely with EGFR, PDGFR and MET activation and is mediated via MAPK and the transcription factor KFL4. This creates a feedback loop between

miRNA-134 and the both RTKs. A high expression level of miRNA-134 results in decreased protein level of KRAS and STAT5B, and by this mechanism miRNA-134 contributes to the anti-tumour effects of crizotinib [32].

In summary, the role of miRNAs in regard to crizotinib resistance is not well-examined at this time, and most results are based on in-vitro experiments. Both miR-96 and 134 are associated with resistance to the drug.

2.4.5 Vandetanib

Vandetanib is the standard treatment for patients with locally advanced or metastatic medullary thyroid cancer (MTC) [141]. The mechanism of resistance to vandetanib is associated with miRNA expression. MiR-182 is strongly up-regulated in MTC-derived tumour specimens and cell-lines harbouring RET mutation. MiR-182 is up-regulated after RET-induced translocation of NF-kB into the nucleus and subsequent binding of NF-kB to the miR-182 promoter. MiR-182 targets HES1 (hairy and enhancer of split 1), a protein that represses the Notch inhibitor. Therefore, miR-182 causes down-regulation of Notch1 and promotes migration [142].

In MTC patient-derived tissues more than 60 microRNAs are significantly deregulated in tumour samples compared to surrounding healthy tissue. MiR-375 was identified as the most up-regulated miRNA in a large series of sporadic and hereditary MTCs. MiR-375 targets SEC23A, resulting in decreased cell proliferation and increased sensitivity to vandetanib. For this reason, expression levels of both SEC23A and miR-375 have been proposed as indicators of eligibility for treatment with vandetanib in patients with MTC [143].

In conclusion, the miRNAs involved in resistance to vandetanib treatment have been identified in patient-derived material – miR-182 in small series and miR-375 in a large cohort of patient derived material (sub grouped into training and validation cohort), and the target

genes of these miRNAs as well as their roles for resistance have been verified in relevant *in-vitro* models.

Conclusions

MiRNAs are expressed in a tissue specific manner and they are well established post-transcriptional regulators for gene expression under physiological conditions. Increasing evidence exists for an important role of miRNAs also in the context of pathological conditions, *e.g.* cancer, as well as for the development of resistance to targeted therapies, *e.g.* TKIs. Nevertheless, up to now most data that exists to link miRNAs to resistance to TKIs in a number of solid malignancies is preclinical, and the level of evidence for most of these associations is low. Regardless of the fact that some miRNAs have also been identified in patient-derived material or in the context of clinical studies, a major drawback is the small number of patients included. Only some miRNAs have been convincingly shown to be involved in the resistance to TKIs in larger clinical studies composed of training and validation cohorts and subsequently confirmed in relevant *in-vitro* and *in-vivo* models (e.g. miR-21 in regard to resistance to gefitinb and erlotinib; miR-101 in regard to resistance to sunitinib; miR-375 in regard to resistance to vandetanib).

Notably, several miRNAs either with onco-suppressor or oncogenic activity were found to be able to predict survival of cancer patients on treatment with TKIs. This finding underlines the importance of miRNAs as potential biomarkers [144, 145].

Another noteworthy fact is that some miRNAs can be over-expressed because of TKI treatment and this event leads to the potentiation of the therapeutic effect of the drug [67]. This evidence paved the way to the exploration of the potential applications of miRNAs in clinical practice either as predictive markers or as therapeutic tools.

Over recent years, blood levels of circulating miRNAs from patients with solid tumours have been broadly investigated as potential predictors of response to targeted therapies [146]. Regarding the exploitation of miRNAs within tailored therapeutic strategies, novel anti-cancer agents aimed at restoring cell sensitivity to drugs in resistant models by mimicking the regulatory functions of onco-suppressor miRNAs have been investigated in early clinical trials with promising results [147, 148]. Furthermore, alternative therapeutic approaches targeting oncogenic miRNAs to antagonize cancer cell resistance to treatments have been developed and investigated in pre-clinical settings [147, 149]. It is possible to use miRNA antagonists (anti-miRs, locked-nucleic acids, antagomiRs) for silencing oncogenic miRNAs and by this restore the expression of several genes. By re-introduction of a tumour suppressor miRNA, expression of tumour promoting genes is silenced. According to mouse studies no adverse side effects are observed by the therapeutic delivery of tumour suppressor miRNAs [147].

In conclusion, overcoming resistance to targeted therapies to enhance their efficacy has become an urgent need. There is robust evidence for candidate miRNAs as potentially useful instruments to tackle this major challenge: 1) as predictive tools of response allowing identification of patients with primary resistance to a specific targeted strategy, and those who have developed secondary resistance and are expected to no longer respond; 2) as novel therapeutic targets likely integrating currently available treatments.

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Table 1: Small molecules RTK-inhibitors, their targets and clinical application

Name of drug	Targets	Examples of Clinical Application
Gefinitib/Erlotinib	EGFR exon 19 del; EGFR L858R exon 21 mutation: GAK	Non-small cell lung carcinoma with mutated EGFR [19, 48]
Lapatinib	HER2 EGFR	Breast cancer overexpressing HER2 [79]
Vemurafenib	BRAF V600E	Advanced melanoma V600E mutated [20]
Everolimus	mTOR	Pre-treated advanced renal cell carcinoma [95] Advanced breast cancer ER^+ [96] Advanced pancreatic neuroendocrine tumours [97]
Sorafenib	RAF/MEK/ERK pathway VEGFR PDGFR	Advanced Child-Pugh score A hepatocellular carcinoma [99]
Sunitinib	VEGFR-2 PDGFRα PDGFRß FLT3 CSF-1 RET KIT GCSFR	Advanced renal cell carcinoma [21] Advanced pancreatic neuroendocrine tumours [120] Gastrointestinal stromal tumours [14]
Imatinib	ABL BCR-ABL KIT PDGFRα	Gastrointestinal stromal tumours [130]
Crizotinib	ALK MET ROS-1	Non-small cell lung carcinoma with EML4-ALK fusion protein [139]
Vandetanib	RET	Advanced medullary thyroid cancer [141]

Legend: ABL: Abelson murine leukemia viral oncogene homolg 1; ALK: anaplastic lymphoma kinase; BCR: breakpoint cluster region; CSF-1: colony stimulating factor-1; del: deleted; ER+: estrogen receptor positive; EGFR: epidermal growth factor receptor; EML4: echinoderm microtubule protein like4; ERK: extracellular signal–regulated kinases; FLT3: FMS-like tyrosine kinase 3; GAK: cyclin G-associated kinase; GCSFR: granulocyte colony-stimulating factor receptor; HER2: human epidermal growth factor receptor 2; KIT: mast/stem cell growth factor receptor; MEK: ; mut: mutated; mTOR: mammalian target of rapamycin; PDGFR: platelet-derived growth factor receptor; RET: rearranged during transfection; ROS-1: c-ros oncogene 1 receptor tyrosine-kinase; VEGFR: vascular endothelial growth factor.

Table 2: Mechanisms of miRNA-mediated resistance to RTK-inhibitors

Drug	Disease	Name of miRNA	Up ↑ /Down ↓	Effect causing resistance
Gefitinib/ Erlotinib	**NSCLC**	MiR-21	↑	PTEN ,↓ PDCD4 ↓ [51, 52]
		MiR-146a	↓	EGFR pathway ↑, NF-kB pathway ↑[54, 55]
		MiR-200 family	↓	PI3K/AKT pathway ↑, ZEB-1 ↑ [61]
		Let-7b Let-7c	↓	P53 pathway ↓, RAS ↑, ZEB-1 ↑ [63, 65]
		MiR-34a	↓	P53 pathway ↓, RAS ,↑ PI3K/AKT pathway ↑ , MET receptor ↑ [66]
		MiR-130a	↓	MET receptor ↑, MiR-221 ↑, MiR-222 ↑[66, 67, 129]
		MiR-103 MiR-203	↓	PTEN ↓, BIM ↓, APAF1 ↓ [67]
		MiR-30b MiR-30c MiR-221 MiR-222	↑	PTEN ↓ , BIM ↓, APAF1 ↓ , Caspase-3 ↓ [67]
		MiR-134/487b/655 cluster	↑	PTEN ↓[69]
		MiR-214	↑	PTEN ↓[70]
		MiR-23a	↑	E-cadherin ↓[71]
		MiR-374a	↑	Wnt5a ↓ [72]
		MiR-548b	↓	CCNB1 ↑ [72]
		MiR-133b MiR-128b	↓	EGFR pathway ↑[36, 75]
		MiR-125a-5p	↓	p53 ↓, Her2 ↑ [77]
Lapatinib	**Breast**	MiR-205	↓	HER3 ↑ [80]
		MiR-630	↓	IGF1R ↑ [81]
Vemurafenib	**Mel**	MiR-211-5p	↑	NUAK1 ↑[87]
		MiR-216	↓	Beclin-1 ↓ , UVRAG ↓ , ATG5 ↓[90]
		MiR-7	↓	EGFR ↑, IGF-1R ↑, CRAF ↑[91]
		MiR-579-3p	↓	B-RAF ↑, MDM2 ↑[92]
		MiR-195	↓	PHB ↑[93]
		MiR-514a-3p	↑	NF1 ↓[94]
Everolimus	**Breast**	MiR-155	↑	PI3K/AKT pathway ↑[98]

Sorafenib	**HCC**	MiR-122	↓	ADAM10 ↑, SRF ↑, IGF1R ↑, PDK4 ↑, LDHA ↑, GALNT10 ↑, EGFR ↑ [102, 103]
		MiR-193b	↓	Mcl-1 ↑[104]
		MiR-34a	↓	BCL-2 ↑, Cyclin-D1 ↑[106, 107]
		MiR-193a	↓	uPA ↑ [108]
		MiR-486	↓	CITRON Rho interacting kinase ↑, CLDN10 ↑ [109]
		MiR-338-3p	↓	HIF-1α ↑[110, 111]
		Let-7 family	↓	Bcl-xL ↑ [111]
		MiR216a/217 cluster	↑	PTEN ↓, SMAD7 ↓, TGF-ß ↑[112-114]
		MiR-21	↑	PTEN ↓[112, 114]
		MiR-93	↑	PTEN ↓, CDKN1A ↓[114]
Sorafenib	**RCC**	MiR-30a	↑	Beclin-1 ↓ [116]
		MiR-200c	↓	Beclin-1 ↓, HO-1 ↑[118, 119]
Sunitinib	**RCC**	MiR-942	↑	MMP-9 ↑ [121]
		MiR-192 MiR-193a-5p MiR-501-3p	↑	TGF-ß ↑, AKT ↑[122]
		MiR-141	↓	ZEB-1 ↑, ZEB-2 ↑ [126]
		MiR-101	↓	UHRF1 ↑, EZH2 ↑[127-129]
Imatinib	**GIST**	miR-320	↓	Mcl-1 ↑ [131]
		MiR-125a-5p	↑	Mcl-1 ↑ [132]
		MiR-218 MiR-518a-5p	↓	PIK3CA ↑[138]
Crizotinib	**NSCLC**	MiR-96	↓	ALK ↑ [140]
		MiR-134	↓	EGFR ↑, PDGFR ↑, MET ↑ [32]
Vandetanib	**MTC**	MiR-182	↑	HES1 ↓[142]
		MiR-375	↑	SEC23A ↓[143]

Legend: down: down-regulated; GIST: gastrointestinal stromal tumour; HCC: hepatocellular carcinoma; Mel: melanoma; MiR: microRNA; MTC: medullary thyroid cancer; NSCLC: non-small cell lung carcinoma; RCC: renal cell carcinoma; up: up-regulated.

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