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MicroRNA and HER2-overexpressing Cancer

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Abstract: The discovery of microRNAs (miRNAs) has opened up new avenues for studying cancer at the molecular level, featuring a post-genomic era of biomedical research. These non-coding regulatory RNA molecules of ~22 nucleotides have emerged as important cancer biomarkers, effectors, and targets. In this review, we focus on the dysregulated biogenesis and function of miRNAs in cancers with an overexpression of the proto-oncogene HER2. Many of the studies reviewed here were carried out in breast cancer, where HER2 overexpression has been extensively studied and HER2-targeted therapy practiced for more than a decade. MiRNA signatures that can be used to classify tumors with different HER2 status have been reported but little consensus can be established among various studies, emphasizing the needs for additional well-controlled profiling approaches and meta-analyses in large and well-balanced patient cohorts. We further discuss three aspects of microRNA dysregulation in or contribution to HER2-associated malignancies or therapies: (a) miRNAs that are up- or down-regulated by HER2 and mediate the downstream signaling of HER2; (b) miRNAs that suppress the expression of HER2 or a factor in HER2 receptor complexes, such as HER3; and (c) miRNAs that affect responses to anti-HER2 therapies. The regulatory mechanisms are elaborated using mainly examples of miR-205, miR-125, and miR-21. Understanding the regulation and function of miRNAs in HER2-overexpressing tumors shall shed new light on the pathogenic mechanisms of microRNAs and the HER2 proto-oncogene in cancer, as well as on individualized or combinatorial anti-HER2 therapies.

Keywords: Breast cancer, cancer, epidermal growth factor receptor 2, miR-21, miR-125, miR-205.

INTRODUCTION

MicroRNA AND CANCER

The discovery of microRNAs (miRNAs) in *C. elegans* [1] has initiated an ever-growing interest in the biogenesis and physiological functions of regulatory small RNAs. MiRNAs are naturally-occurring non-coding small RNA molecules of 21–25 nucleotides (nt) that form partially complementary base pairs within the 3' untranslated regions (UTRs) of protein-encoding mRNAs, resulting in mRNA destabilization and/or translational inhibition [1, 2]. MiRNAs have been identified in a wide variety of multicellular organisms, and many of them are highly conserved across species. The biogenesis of miRNA is controlled at both transcriptional and post-transcriptional levels. In general, the primary miRNA transcript (pri-miRNA) is first processed by the RNase III Drosha into a ~65–80 nt precursor with a hairpin structure (pre-miRNA), which is transported into the cytoplasm and further processed by another RNase Dicer [3-5]. Drosha-independent processing has been reported for some intronic miRNAs, which use the splicing machinery to generate pre-miRNAs [6]. Likewise, some miRNAs bypass Dicer processing by using the RNase activity of Argonaute-2 (Ago2) [7]. MiRNA expression is spatially and temporally regulated in different tissues and during development and differentiation [8, 9]. Dysregulation of miRNAs is linked to various human diseases, especially cancers [10, 11]. More than 50% of all miRNA genes are located at chromosome fragile sites or within deletion/amplification regions associated with human cancers [12]. MiRNAs display regulatory functions in almost all vital cellular events, such as development, differentiation, cell proliferation, and apoptosis. In animals, each miRNA can regulate hundreds of target mRNAs [13], simultaneously affecting several cellular pathways or even exerting genome-wide regulation. Through targeting genes that control tissue homeostasis, miRNAs can function as *bona fide* tumor suppressors or oncogenes, and are involved in the initiation and progression of various cancers [10]. Moreover, several miRNAs may function as a group to synergistically regulate one or multiple genes involved in a critical pathway or biological function, leading to a rapid and effective control of cell behaviors [14].

Altered miRNA expression in cancer is first demonstrated in chronic lymphocytic leukaemia [15], and subsequently in cancers of the breast, lung, colon, thyroid, liver, and pancreas [10, 11]. The unique miRNA expression patterns in various human cancers have shown promise as tissue-based markers for cancer classification and prognosis [10, 12]. Compared to the protein-coding mRNAs, which are generally long, unstable, and large in numbers (~30,000 mRNA genes in humans), miRNAs are short, relatively stable, and of a smaller number (about 800–1000 miRNA genes in humans), allowing large-scale evaluation with higher efficiency and lower cost. In addition, circulating

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miRNAs have recently been detected in serum, plasma, or whole blood specimens from cancer patients [16-19]. Using PCR or microarray assays, deregulated miRNAs are readily detectable in the circulation, where they show associations with various types of cancer [20-23]. A recent study using deep sequencing to profile circulating small RNAs in early stage breast cancer patients has discovered circulating miRNA patterns associated with tumors' histopathological profiles, including the status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2; also known as ErbB2) [19]. These recent findings strongly support the clinical applications of miRNAs as tissue and blood based biomarkers for cancer detection, classification, and outcome prediction.

Whereas certain miRNAs can be upregulated by oncogenic transcription factors, a global downregulation of miRNAs is often seen in cancer. The global downregulation may be due to mutations or reduced expression of genes encoding the miRNA processing machinery (reviewed in [24]). Expression of individual miRNAs can be regulated at the transcriptional level or at a post-transcriptional step. Several pathways, including DEAD-box helicases p68 (DDX5) and p72 (DDX17), transforming growth factor β/bone morphogenic protein (TGFβ/BMP)/SMAD, p53, or ERα, have been shown to regulate protein factors involved in miRNA maturation. Moreover, miRNAs may be regulated by RNA-binding proteins that interact with cis-regulatory sequences within pri- or pre-miRNAs, such as in the case of let-7 regulation by Lin28. The multi-level regulation of miRNA biogenesis, especially those of the same family or co-transcribed into the same primary transcript, is thought to be necessary for highly specific and fine-tuned expression of miRNAs (reviewed in [25]).

MiRNA-mediated gene regulation is involved in almost every aspect of cancer biology. For example, miR-155 and miR-210 that are upregulated in breast cancer [11, 26] regulate critical cancer genes. MiR-155 is upregulated by the TGFβ/SMAD4-mediated promoter activation, and contributes to TGFβ-induced epithelial-mesenchymal transition (EMT) and tight junction dissolution through targeting RhoA [27]. MiR-155 also promotes proliferation through targeting the suppressor of cytokine signaling 1 (SOCS1) [28], and affects cell survival and chemosensitivity through targeting the forkhead box O3 (FOXO3a) [29]. The hypoxia-inducible miR-210 augments c-MYC oncogenic signaling through targeting the MAX binding protein (MNT), a known c-MYC antagonist [30], and may also regulate DNA repair and genetic instability through targeting RAD52 [31]. MiR-145 and miR-10b are down-regulated in breast cancer [11, 26]. MiR-145 suppresses proliferation and metastasis through downregulating ERα, Rhotekin, and Mucin 1 [32-34]. MiR-10b is a target of the Twist transcription factor, and inhibits the expression of homeobox D10 (HOXD10) [35] and T lymphoma invasion and metastasis 1 (TIAM1) [36]. Recent studies have revealed a critical role of miRNAs in regulating cancer stem cells. Three miRNA clusters – miR-200c-141, miR-200b-200a-429, and miR-183-96-182 – are down-regulated in purified breast cancer stem cells, leading to de-repressed expression of their target genes with functions in self-renewal, tumor formation, and embryogenic development [37].

PROTO-ONCOGENE HER2 AND CANCER

HER2 (ErbB2), together with the epidermal growth factor receptor (EGFR, ErbB1), HER3 (ErbB3), and HER4 (ErbB4), constitute the ErbB family of transmembrane receptor tyrosine kinases. Ligand binding to the ectodomains of EGFR, HER3, and HER4 results in the formation of catalytically active homo- and hetero-dimers to which HER2, an "orphan receptor" that does not bind to any ligand but exhibits constantly activated conformation, is recruited as a preferred partner [38-40]. HER2 has been recognized as an amplifier of the ErbB signaling network through binding to a large repertoire of phosphotyrosine-binding proteins [41], as well as increasing ligand binding affinity and/or ErbB receptor recycling and stability [38, 42-44]. Activation of the ErbB network triggers the receptor autophosphorylation of C-terminal tyrosines and recruitment to these sites of cytoplasmic signal transducers that regulate vital cellular processes such as proliferation, differentiation, adhesion, migration, and survival. Transducers of an ErbB signal, including Ras/MAPK, PI3K/AKT, JAK/STAT, PAK/JNK, Src, PLC-γ1, and SAPK [45], are heavily involved in cell transformation and cancer.

HER2 gene amplification and/or protein overexpression has been reported in human cancers of the breast, ovarian, lung, gastric, colon, pancreas, and endometrium [46]. HER2 abnormality has a high prevalence (~22%) in breast cancer, where overexpression of HER2 is associated with a higher histological tumor grade, increased cell proliferation, cell motility, tumor invasiveness, metastases and angiogenesis, decreased apoptosis, and a poor overall prognosis [47, 48]. HER2-overexpressing breast cancer cell lines and human tumors exhibit constitutive HER2 phosphorylation and activation [49, 50]. Overexpression of HER2 is thought to cause enhanced signaling and delayed inactivation of the ErbB network as a preferred dimerization partner and signal amplifier [46], and can induce the colony-forming efficiency and tumorigenicity of normal human mammary epithelial cells [51]. Transgenic mice carrying rat homolog of the *HER2/ErbB2* proto-oncogene *neu*, driven by mouse mammary tumor virus (MMTV) promoter/enhancer, spontaneously develop focal mammary tumors followed by secondary metastatic tumors in the lung [52, 53]. These early *in vitro* and *in vivo* studies indicate that HER2 is a potent oncogene in mammary gland and a causative factor for breast cancer.

Trastuzumab (Herceptin), a humanized IgG_1 that binds to HER2 ectodomain, is the first approved therapy for treating HER2-overexpressing breast cancers [54, 55]. It has been shown to induce tumor regressions in 12–35% of heavily pretreated metastatic breast cancers with HER2 overexpression [56-58]. In patients with HER2-positive early stage breast cancer, addition of trastuzumab to adjuvant chemotherapy has been associated with significant benefits in survival and locoregional/distant recurrence, but a worse outcome in central nervous system (CNS) recurrence [59]. This may be explained by the difficulty for systemically administered trastuzumab to cross the blood-brain barrier

(BBB), resulting in cancer cell migration to the "sanctuary" brain niche escaping therapy. The antitumor effect of trastuzumab is mediated by several mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC) [60], induction of HER2 endocytosis and degradation [61], inhibition of the generation of a constitutively active truncated HER2 fragment by protease cleavage [62], and inhibition of HER2 downstream signal transduction pathways [63]. Despite the benefits associated with trastuzumab, a significant fraction of HER2-overexpressing tumors do not respond to trastuzumab, and many tumors that initially respond eventually relapse due to *de novo* or acquired therapeutic resistance. Resistance to trastuzumab may be related to activation of the IGF-I receptor [64], EGFR [65], or the PI3K/AKT pathway [66, 67]. Lapatinib (Tykerb), a small-molecule tyrosine kinase inhibitor that targets both HER2 and EGFR, has recently been approved for the treatment of HER2-amplified metastatic breast cancer [68]. In addition to its inhibitory effect on trastuzumab-resistant breast cancer and the synergistic effect when combined with trastuzumab, the ability of lapatinib to penetrate CNS may alleviate tumor recurrence in the CNS [69]. It is further recognized that combinations of agents targeting the HER2 network or other pathways synergizing with HER2 may be beneficial for efficient treatment of HER2-overexpressing breast cancer by overcoming drug resistance (reviewed in [70]).

The role of HER2 as an important biomarker, key driver of tumorigenesis, and therapeutic target has also emerged in other cancers, such as gastric cancer [71]. In the recent open-label, international, phase 3, randomized ToGA (Trastuzumab for Gastric Cancer) trial involving 594 patients with HER2-overexpressing advanced gastric or gastro-oesophageal junction cancer, addition of trastuzumab to chemotherapy significantly improves overall survival compared with chemotherapy alone [72]. In pre-clinical studies, trastuzumab and lapatinib individually suppress the growth of HER2-amplified gastric cancer cell lines [73, 74] and exhibit a synergistic effect when combined together [75]. Mutations in the HER2 tyrosine kinases domain, although much less frequent than HER2 amplification/overexpression, have also been reported firstly in non-small cell lung cancer [76, 77], and later in gastric, colorectal, and breast cancer [78]. These mutants of HER2 confer ligand-independent constitutive activation of HER2-containing ErbB dimers and potent tumorigenicity of normal lung and mammary epithelial cells, but they remain sensitive to HER2-targeted therapies [79].

It is clear that HER2 has a well documented causative role in various human cancers and is a validated target for effective cancer treatment. We next review the importance of miRNAs as additional mediators of HER2 signaling or modulators of therapeutic responses to anti-HER2 drugs.

ASSOCIATION BETWEEN MIRNAS AND HER2-OVEREXPRESSING TUMORS

Microarray-based expression profiling of miRNAs in breast cancer has been carried out to identify miRNAs associated with various clinicopathological characteristics [11, 80, 81]. Using 20 stage I–II breast cancer biopsies including 13 cases of HER2 overexpression, Mattie *et al.* identified 43 miRNAs that are significantly lower in HER2$^+$ as compared to HER2$^-$ tumors (Table **1**), many of them are significantly higher in ER$^+$ tumors. It appears that there is a general suppression of the miRNA machinery in HER2$^+$ tumors perhaps due to a function of HER2 overexpression, which we will discuss later. Among those miRNAs inversely correlated with HER2 overexpression, let-7f, let-7g, miR-107, miR-10b, miR-126, miR-154 and miR-195 are more restrictedly specific to HER2 status [81]. Another study by Lowery *et al.* revealed a signature of five miRNAs (miR-520d, miR-181c, miR-302c, miR-376b, and miR-30e-3p) that can accurately predict HER2 status in early stage breast tumors [80]. Among them, increased expression of miR-520d and miR-376b and decreased expression of miR-181c are closely associated with HER2$^+$ tumors (Table **1**), whereas miR-302c and miR-30e-3p show weaker correlation to HER2 status. The same study also identified several

Table 1. MiRNAs associated with HER2-overexpressing breast tumors.

Tissue	Study	Higher in HER2$^+$	Lower in HER2$^+$
Tumor	Mattie *et al.* (2006)	Not detected	let-7a/b/c/f/g/I; miR-100/103/106a/107/ 10b/125a/125b/126/126*/141/143/145/ 150/154/15a/16/191/195/19b/205/21/22/ 221/222/224/24/26a/26b/27a/27b/29a/ 30b/30c/330/342/95/99a
Tumor	Lowery *et al.* (2009)	miR-342/376b/520d	miR-181c
Tumor	Wee *et al.* (2012)	Not detected	miR-200b†
Tumor	Lee *et al.* (2011)	miR-21	Not detected
Tumor	Persson *et al.* (2011)	miR-4728	Not detected
Serum	Wu *et al.* (2012)	miR-184/196a/200a/200b/ 200c/205/375/429/96	miR-122/125b-2*/1228*/193b*/217/30a/ 30a*/320b/320c/34a/483*/503/885/885*
Tg mouse tumor	Zhu *et al.* (2011)	mmu-let-7d/e/f; mmu-miR-130b/185/193/ 469/539/684/691/98	Not detected

†Predicted downregulation based on promoter hypermethylation.

additional miRNAs whose levels differentiate between HER2^{+} and HER2^{-} tumors, including miR-30b, miR-217, miR-363, miR-383, miR-377, miR-130a, miR-422a, and miR-342. However, they are not independent markers and are therefore not included in the HER2-classifying signature [80]. Of note, miR-376b, miR-377, and miR-342 are localized to Ch14q32, suggesting a possible mode of co-regulation. It remains to be investigated whether the dysregulation of these HER2-associated miRNAs is related to chromosome duplication/deletion, or whether their expression is dysregulated at the transcriptional and/or post-transcriptional levels.

By using integrated analyses of miRNA expression, mRNA expression, and genomic changes in primary breast tumors, Blenkiron *et al.* identified a number of miRNAs differentially expressed among tumors of various molecular subtypes. None of the miRNAs, however, shows a strong correlation with HER2 status, possibly due to the small number of HER2^{+} tumors analyzed ($N = 5$) [82]. Their study suggests that, while changes in the expression of some miRNAs are likely due to genomic loss or gain, most miRNA changes are perhaps regulated through transcription or other miRNA biogenesis steps. Indeed, it has been shown that down-regulations of the components of miRNA machinery are associated with breast and some other cancers. For example, Dicer1 expression is lower in the HER2^{+}, basal-like and luminal B type breast tumors, and the Dicer1 expression has predictive value for metastasis-free survival [82, 83], whereas Ago2 expression is higher in these more aggressive tumor subtypes [82]. Downregulation of Drosha has also been associated with overexpression and gene amplification of HER2 [84]. Whether these observations in HER2-overexpressing tumors are related to a general suppression of the miRNAs processed by Drosha and Dicer, and the consequent dominance of miRNAs that can bypass Drosha or Dicer by using alternative machineries such as the RNA splicing complex or Ago2 [6, 7], are intriguing topics for future investigation. It is interesting to note that a global analysis of miRNA levels has detected decreases in mean miRNA expression in the HER2^{+} tumors [82].

A major mechanism for regulating individual miRNAs is transcriptional activation or inhibition through the action of transcription factors or promoter methylation. A recent study has mapped and examined the promoters of breast cancer associated miRNAs for their status of methylation, and found an inverse correlation between promoter methylation of the hsa-mir-200b cluster and miR-200b expression in breast tumors [85]. Hypermethylation of hsa-mir-200b promoter is associated with HER2 positivity [85], suggesting suppressed gene transcription of miR-200b in HER2-overexpressing tumors. Genome-wide interrogation of the DNA binding events by transcription factors using chromatin immunoprecipitation (ChIP) in combination with next-generation sequencing technologies (ChIP-Seq) or microarrays (ChIP-on-chip) is expected to provide global-level understanding of the promoter regulations of miRNA genes, as it did for protein-coding genes. This has been recently employed to identify miRNA promoters regulated by ERα, ERβ and TCF4 in cancer cell lines [86-88]. In addition, genome-wide binding patterns of RNA Polymerase II have been used to identify promoters of miRNAs actively transcribed [89]. Applications of these approaches in clinical samples will provide new insights into the dynamic functions of various transcription factors in miRNA deregulation in different types and subtypes of cancer and at different stages of cancer progression.

Perhaps the most direct relationship between HER2 and miRNA is demonstrated by the recent discovery of a new miRNA located in an intron of the *HER2* gene [90]. This miRNA, miR-4728, together with four additional new miRNA candidates, are mapped to the 17q12 region whose amplification is frequent in breast cancer and causes HER2 overexpression. Overexpression of miR-4728-3p is detected in breast cancer cell lines with *HER2* amplification and in HER2^{+} tumors [90]. Although there has not yet been any further studies on the target genes or functions of these newly identified miRNAs, sequence-based target prediction for miR-4728-3p suggests a role of this miRNA in the negative feedback loop that fine-tunes HER2 signaling through targeting MAPK1 and SOS1, two important effectors in HER2-initiated signaling cascade [90].

Circulating miRNAs have also been associated with HER2-overexpressing tumors. Using comprehensive *de novo* sequencing, our colleagues have profiled small RNAs in the pre-treatment sera of early stage breast cancer patients, and identified sets of miRNAs that are associated with various clinicopathological parameters and clinical outcome [19]. Among the circulating miRNAs differentially presenting in patients with HER2^{+} and HER2^{-} tumors, higher levels of miR-375 and lower levels of miR-122 are associated with positive HER2 status and an optimal response to neoadjuvant chemotherapy. Two miRNA clusters, miR-200b-200a-429 and miR-200c-141, are also associated with positive HER2 status and negative ER status, and with inflammatory breast cancer. This study, together with the body of literature reviewed above, demonstrate the feasibility of classifying and even sub-classifying HER2-overexpressing tumors using validated miRNA signatures in tumor tissue or blood. Those miRNAs clinically associated with HER2 overexpression may play important roles in mediating the oncogenic function of HER2 and/or response to anti-HER2 therapies, which we will discuss by using examples in the following section.

The MMTV-HER2/neu transgenic mouse tumor model has also been examined by global miRNA profiling [91]. By comparing to normal mouse mammary glands from pregnant mice and tumors driven by other oncogenes, miRNAs specifically expressed in MMTV-HER2/neu tumors are determined to be mmu-let-7d/7e/7f and mmu-miR-193, -185, -130b, -98, -691, -684, -469, and -539 (Table **1**). Most of these miRNAs are shared with other mammary tumor models (*e.g.*, c-Myc, H-Ras, Wnt1) that are also classified as the luminal tumor subtype (in contrast to basal subtype), whereas mmu-miR-193 is the only miRNA specific to the HER2/neu model. Integrated mRNA profiling has further identified groups of the predicted target genes that exhibit inverse correlation with their corresponding miRNA regulator [91]. These mouse miRNAs characteristically expressed in HER2/neu-driven mouse mammary tumors, as well as their target genes, may provide insights into the role

of their human counterparts in mediating HER2-directed tumorigenesis in future investigations.

It has been recently proposed that certain cancers may exhibit a dependency on the expression of a single oncomiR, causing an addiction to the oncomiR [92], which may be used to efficiently target the malignancy, similar to the rationale of current therapies targeting addictive oncogenes such as HER2. Identifying miRNAs clinically associated with a group of tumors (*e.g.*, HER2-overexpressing) may therefore reveal valid targets for anti-miRNA therapies as potential single-agent or combinatory treatment.

MECHANISMS OF MIRNA ACTION IN HER2 PATHWAY AND TRASTUZUMAB RESPONSE

The mechanism that links miRNAs and HER2 can be multifaceted. We here discuss it from three angles: (a) HER2 regulates miRNAs and in turns regulates downstream genes, (b) miRNAs regulate HER2 or its partner proteins, and (c) miRNAs modulate the response to anti-HER2 therapy (Fig. **1**). We will discuss these regulations by focusing on a few better studied miRNAs, such as miR-205, miR-21, and miR-125 (Table **2, 3**, & **4**).

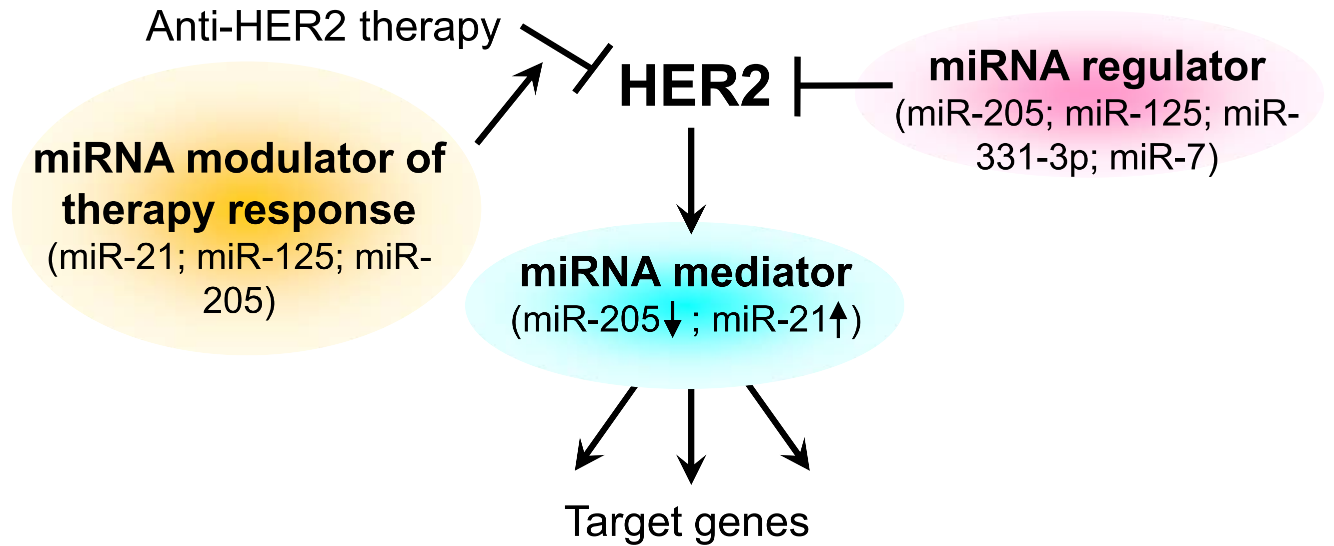

Fig. (1). The three modes of miRNA action in HER2-overexpressing tumors summarized in this review. (**a**) up- (e.g., miR-21) or down-regulated (e.g., miR-205) by HER2 and mediate HER2 downstream signaling; (**b**) directly target HER2 (e.g., miR-125 and miR-331-3p) or the HER2 receptor complex (e.g., miR-205 and miR-7); and (**c**) modulate cancer response to anti-HER2 agents (e.g., miR-21).

Table 2. MiRNAs that are regulated by HER2 and mediate HER2 signaling.

miRNA	Regulation by HER2	Validated Target Genes with a Potential Role In cancer
miR-205	Downregulated	ERBB3; VEGF-A; E2F1; LAMC1; ZEB1; SHIP2
miR-21	Upregulated	PDCD4; PTEN; TPM1; SERPINB5; BCL2; SPRY1/2; RECK; TIMP3; ANP32A; SMARCA4; JAG1; MSH2

Table 3. MiRNAs that regulate HER2 receptor complex.

miRNA	Validated Targets in HER2 Complex
miR-205	HER3
miR-125	HER2; HER3
miR-331-3p	HER2
miR-7	EGFR

Table 4. MiRNAs that modulate cancer response to anti-HER2 therapy.

miRNA	Effect on Anti-HER2 Therapy Response
miR-205	Sensitize
miR-125	Sensitize
miR-21	Insensitize

a. Mediator of HER2 Signaling

1. MiR-205

A lower expression of miR-205 is associated with HER2$^+$ breast tumors [81] and vascular invasion in breast cancer [11]. Overexpressing HER2 down-regulates miR-205 and confers MCF10A breast epithelial cells an anchorage-independent transformed phenotype, and forced expression of miR-205 suppresses this HER2-induced phenotype, suggesting that downregulation of miR-205 in HER2-transformed cells is essential for HER2-induced tumorigenesis [93]. Using *in situ* hybridization, miR-205 is shown to exclusively express in the myoepithelial/basal cell compartment in normal mammary glands [94]. However, miR-205 positively associates with E-cadherin and tumors of ductal morphology, where miR-205 predicts survival independence of grade and tumor size [95]. Both studies documented frequent reduction or loss of miR-205 in tumor specimens or breast cancer cell lines [94, 95]. Although the differential expression of miR-205 in breast tumors may simply reflect the origin of cancer cells (*i.e.,* myoepithelial/basal vs. luminal), additional studies strongly suggest an active role of miR-205 in cancer progression.

MiR-205 and the miR-200 family are downregulated during EMT and cooperatively regulate expression of the E-cadherin transcriptional repressors ZEB1 and SIP1 [96]. In invasive bladder tumors, promoters of miR-205 and miR-200 are silenced through hypermethylation, and both associate with Twist1, which may function as a transcriptional repressor of these miRNAs [97]. MiR-205 is also a direct target of the tumor suppressive transcription factor p53, and decreased expression of miR-205 is common in triple-negative breast cancer cells that are frequently p53 deficient [98]. It appears that p53 inactivation, EMT induction, and promoter methylation may all be involved in the downregulation of miR-205 in HER2-overexpressing tumors.

Although the miR-205 level is lower in tumors expressing HER2 [81], the circulating miR-205 level appears to be higher in HER2$^+$ breast cancer patients [19]. It appears that miR-205 could be secreted from the tumor cells into the extracellular environment (and circulation) as a way to communicate with other cells or as a way to reduce its intracellular level in tumor cells. This hypothesis, which may be extended to other circulating miRNAs, will need further investigation.

MiR-205 down-regulates in prostate cancer cells a number of genes involved in DNA replication and mitotic cell cycle progression [99]. This indicates that miR-205 plays an important role in cell cycle regulation. MiR-205 also down-regulates HER3, cell cycle regulator E2F1, extracellular matrix component LAMC1, angiogenesis inducer VEGF-A, and migration regulator SHIP2 [98, 100-102]. Expression of these genes is de-repressed in HER2-overexpressing tumors with reduced miR-205 and the low expression of these genes likely contributes to the tumor growth and metastasis. Indeed, re-expression of miR-205 in breast cancer cells strongly inhibits cell proliferation, cell cycle progression, and clonogenesis *in vitro*, and inhibits tumor growth *in vivo* [98].

2. MiR-21

The well-studied oncomiR miR-21 is also a mediator of HER2 signaling. In a study of 109 primary breast tumors, high miR-21 expression is associated with larger tumor size, higher tumor stage and grade, poor patient survival, and positive HER2 status and negative ER status [103]. Overexpression of HER2 in normal or cancer cells with low endogenous HER2 expression significantly induces miR-21 [104, 105]. MiR-21 induction by HER2 is blocked by ERK1/2 inhibitors, but not by AKT inhibitors [104]. Expression of miR-21 is directly regulated by transcriptional factors AP-1 [106], STAT3 [107], and ETS-1 [104]. All these transcriptional factors may contribute to HER2-induced miR-21 expression, as disruption of ETS-1, an ERK-dependent transcriptional factor, only partially blocks the miR-21-induction by HER2 [104].

Through up-regulating miR-21, HER2 overexpression results in downregulation of the programmed cell death 4 gene (PDCD4), a direct target of miR-21 and a potent inhibitor for breast cancer cell invasion [104]. Other tumor suppressor genes targeted by miR-21 include PTEN, tropomyosin 1, maspin, Bcl-2, Sprouty 1/2, RECK, TIMP3, ANP32A, SMARCA4, JAG1, and MSH2 [105, 108-117]. These miR-21 targets likely participate in the various aspects of HER2 action in cancer cell survival, invasion, and drug response.

b. Regulator of HER2 Receptor Complex

1. MiR-205

In addition to its role as a mediator of HER2 signaling, miR-205 also serves as a potent regulator of HER2 receptor complexes through directly targeting HER3 [100, 101]. Although kinase-defective, HER3 can be phosphorylated by HER2 or EGFR [45] and provide six binding sites for the regulatory subunit of PI3K [118]. In HER2-overexpressing breast cancer cells, HER3 is constitutively phosphorylated and associated with HER2 [50, 66]. Downregulation of HER3 or mutagenesis of its PI3K binding sites results in inhibition of ErbB receptor-dependent transformation [119, 120]. Moreover, HER3 expression is the highest in luminal mammary cells and loss of HER3 shifts gene expression patterns toward a mammary basal cell/stem cell signature [121]. This HER3 property may be linked to the myoepithelial/basal-specific localization of miR-205 observed by Sempere *et al.* [94]. Restoration of miR-205 expression inhibits both HER3 expression and AKT phosphorylation as well as suppresses the clonogenic potential in HER2-overexpressing cells [93, 100], which is consistent with the role of HER3 in activating the PI3K/AKT pathway through dimerization with HER2. These results also suggest that downregulation of miR-205 by HER2 [93] is essential for HER2-induced tumorigenesis, and restoration of miR-205 may alleviate HER2 malignancy.

2. MiR-125

Another miRNA that directly targets HER2 complexes is miR-125/*lin-4*. The *C. elegans* miRNA *lin-4* was the first miRNA to be identified [1]. In humans, the *lin-4* family members are known as miR-125. Expression of miR-125 is

highly stage-specific during development, due to its critical role as a regulatory switch in stem cell differentiation [122].

The miR-125 family (miR-125a and miR-125b) is implicated in human cancers. MiR-125b (miR-125b-5p) and to a less extent miR-125b-2* (miR-125b-2-3p) are downregulated in breast, lung, colon, liver, prostate, and ovarian cancer, and lymphoma [11, 26]. Breast cancer with HER2 gene amplification is significantly downregulated in miR-125a and miR-125b as compared to clinically matched HER2$^-$ breast cancer [81]. In gastric cancer, lower miR-125a expression is observed in high HER2 expressing tumors and associated with increased tumor size, tumor invasion, liver metastasis, and poor prognosis; miR-125 also serves as an independent prognostic factor for survival [123].

MiR-125a and miR-125b share the same seed sequence, and both target HER2 and HER3 [124]. The dual targeting may result in more efficient suppression of cell signaling initiated by HER2:HER3 heterodimers. Overexpression of either miR-125a or miR-125b inhibits the signaling by AKT and ERK, as well as reduces cell growth, migration, and invasion capacities of HER2-overexpressing breast cancer cells. MiR-125 overexpression has marginal effects on non-tumor cells that do not overexpress HER2 [124], suggesting miR-125a/b exerts their tumor suppressive function through the HER2 pathway**.**

In addition to targeting HER2 and HER3, miR-125b also targets c-Raf, an important proliferative and survival factor frequently activated in cancer [125]. Furthermore, miR-125b is linked to several aspects of chemoresistance: miR-125b is upregulated in Taxol-resistant breast cancer cell lines [126] and upon cisplatin treatment in leukemia cells [127]. It contributes to drug resistance through targeting the pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) [126].

3. MiR-331-3p and MiR-7

Expression of HER2 is also reportedly down-regulated by miR-331-3p. Two binding sites for miR-331-3p have been validated in the 3'UTR of HER2 transcripts. Expression of miR-331-3p in prostate cancer cells causes reduction in HER2 expression and inhibition of the PI3K/AKT pathway downstream of HER2 [128]. The level of miR-331-3p is decreased in HER2-overexpressing prostate cancer tissue as compared to that in adjacent normal tissue [128]. These reports suggest a role of miR-331-3p down-expression in HER2-overexpressing tumors.

Another HER2 dimerization partner, EGFR, is regulated by miR-7 through two miRNA binding sites in the 3'UTR of EGFR transcripts [129]. In various cancer cell lines, expression of miR-7 down-regulates EGFR and induces cell cycle arrest and cell death [129]. Whether reduced expression of miR-7 is responsible for EGFR overexpression in tumors without *EGFR* gene amplification is an important question remained to be addressed.

c. Modulator of Responses to Anti-HER2 Therapy

1. MiR-205 and MiR-125

MiR-205, down-regulated by HER2 and targeting HER3, increases the responsiveness of HER2-overexpressing breast cancer to gefitinib and lapatinib, both are tyrosine kinase inhibitors of EGFR and HER2 [100]. These augmentation effects are similar to those obtained by HER3 knockdown. This is consistent with the notion that HER3 can be downregulated by miR-205. In HER2-overexpressing gastric cancer cell line, expression of miR-125a significantly enhances the inhibitory effect of trastuzumab [123], suggesting an effective strategy to optimize the beneficial effects of anti-HER2 therapy.

2. MiR-21

MiR-21 has also been linked to resistance to anti-HER2 therapy. MiR-21 inhibits apoptosis through targeting the tumor suppressors phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) [111, 130]. High expression of miR-21 in cancer cells may directly contribute to suppressed cell killing during cancer therapy. In HER2$^+$ breast cancer cell lines that have acquired trastuzumab resistance upon long-term exposure to the antibody, further elevation of miR-21 expression accompanied by PTEN reduction is observed. Overexpression of miR-21 induces trastuzumab resistance in drug-sensitive breast cancer cells, whereas blockade of miR-21 with antisense oligonucleotides re-sensitizes the resistant cells to the inhibitory effect of trastuzumab [131]. In clinic, higher miR-21 levels in pre-treatment HER2$^+$ breast tumors are associated with poor trastuzumab response in patients receiving neoadjuvant therapy, and miR-21 expression is further elevated following trastuzumab therapy in both resistant and sensitive tumors [131].

The data collectively suggest that miR-21 may serve as a predictor for tumor response to trastuzumab, and that targeting miR-21 may be a valid strategy to inhibit intrinsic and acquired trastuzumab resistance. The ability of miR-21 to inhibit apoptosis implies its general role in modulating cancer response to various therapeutic agents, such as chemotherapy drugs. Indeed, it has been shown that miR-21 induces resistance to doxorubicin in HER2$^-$ breast cancer cells through downregulation of PTEN [132]. Since trastuzumab is currently used in combination with chemotherapy drugs to treat HER2-overexpressing tumors, miR-21-induced multi-drug resistance may attribute some of the failed cancer treatments. Because PTEN is an essential mediator of this miR-21 function, it would be of interest to further define the miR-21 effects in tumors carrying genetic deletions or loss-of-function mutations of *PTEN* [133]. To overcome drug resistance by miR-21 intervention shall improve the beneficial effects of anti-HER2 therapy.

3. MiR-26 and MiR-30

In a study to identify miRNAs whose expression is altered by trastuzumab treatment in HER2$^+$ breast cancer cell lines, miR-26a and miR-30b are identified as trastuzumab-inducible miRNAs. Expression of miR-26a and miR-30b induces cell growth suppression, cell cycle arrest, and apoptosis in breast cancer cells, suggesting a role of these two miRNAs in mediating the effects of trastuzumab [134]. Whether the intrinsic levels of miR-26a and miR-30b determine trastuzumab response in HER2-overesxpressing tumors remains unclear but is of high clinical relevance.

CONCLUSION

Increasing evidence demonstrates that miRNAs are indeed a new group of independent or auxiliary biomarkers for cancer diagnosis, classification, and prognosis. The documented roles of certain miRNAs as oncomiRs or tumor suppressors further depict their participation in the complex tumorigenic network with protein effectors and signaling pathways. As HER2 overexpression and anti-HER2 therapy become increasingly prevalent in breast, gastric, and other cancers, understanding the roles of miRNAs in HER2-overexpressing cancer is of great importance. On the one hand, miRNA markers detected in the tumor or blood may further classify HER2-overexpressing tumors into subgroups, which may have different miRNA-mediated tumorigenic events and may respond differently to various therapies. On the other hand, identifying and targeting addictive oncomiRs or miRNAs that mediate HER2 signaling or anti-HER2 therapeutic responses will improve the management of HER2-overexpressing cancer. Investigations into these aspects using integrated analysis of miRNAs, mRNAs, and genes, as well as clinical validations using large patient cohorts and mechanistic studies using *in vitro* and *in vivo* models, shall shed new light on miRNA-mediated functions in HER2-overexpressing cancer.

CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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