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Review article

Recent advances in *HER2* positive breast cancer epigenetics: Susceptibility and therapeutic strategies

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ABSTRACT

HER2 amplification/overexpression accounts for aggressive clinical features of *HER2* positive breast cancer. Epigenetic changes including DNA methylation, histone modifications and ncRNAs/miRNAs are associated with regulation of DNA chromatin and specifically, gene transcription. Hence, these produce eminent effects upon proto-oncogenes, tumor-suppressors and key cancer-regulatory signaling pathways. Understanding of epigenomic regulation of *HER2* overexpression and signaling may help uncover the unmatchable physiology of *HER2* gene/protein. Moreover, this may also aid in resolving the major issue of resistance-development towards *HER2* targeted agents (trastuzumab and lapatinib), since epigenetic alterations are important therapeutic markers and modulate the response towards *HER2* targeted therapy. Therefore, in this review the information regarding various epigenetic markers implicated in *HER2* positive breast cancer susceptibility and therapeutic-strategies has been compiled.

Contents

1. Introduction

Human epidermal growth factor receptor 2 (*HER2*) is a tyrosine

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kinase receptor protein, belonging to epidermal growth factor receptor (EGFR) family. The other three members of EGFR family are-HER1/EGFR, HER3 and HER4 [1,2]. *HER2* acts as major predictive marker in breast cancer [3]. 15–30% of breast cancer cases are associated with *HER2* overexpression and are referred to as *HER2* positive [3]. *HER2* is 185 kDa and 1255 amino-acid long protein and is also known as ErBb2/c-ErBb2/neu/p185. *HER2*/ErBb2/neu oncogene is located on chromosomal position 17q12 [3–7]. In most of the breast cancer cases, *HER2* overexpression occurs due to *HER2* gene amplification [8,9]. Polysomy 17 has been suggested as an alternative mechanism for *HER2* overexpression [10]. Genetic variations in *HER2* gene and many other genes have also been reported to be associated with *HER2* positive breast cancer susceptibility as well as modulation of response towards *HER2* targeted therapy, elaborated upon by us in our recent review [11].

HER2 overexpression prompts homodimerisation or heterodimerization of *HER2* with other EGFR family members, which accelerates the events like cell proliferation, cell-cycle degradation and apoptosis inhibition by activation of various cell-signaling pathways [1,3,5,6]. *HER2* positive breast cancers have aggressive histopathological and clinical features such as high histologic grade [12], increased potential of metastization to brain and viscera and increased resistance towards anti-*HER2* agents (trastuzumab and lapatinib) [8].

Epigenetic changes that do not produce any change in DNA sequence, are eminent over genetic changes in carcinogenesis [13,14]. Epigenetic modifications result from altered DNA methylation and/or post-translational modification of chromatin specially, histone modifications.

In addition, it is also evident that epigenome can be modulated by a variety of environmental factors. Evidence is also emerging for the important role played by non-coding RNAs (ncRNAs) and protein interactions in the management of epigenome [15]. This review has been compiled with an aim to focus on epigenetic modifications, especially DNA methylation, histone modifications and ncRNAs in relation to *HER2* positive breast cancer with focus on susceptibility and therapeutic-strategies. Various epigenetic alterations affecting *HER2* positive breast cancer have been shown in (Fig. 1).

2. Epigenetics in *HER2* positive breast cancer susceptibility

Epigenetic alterations have their key impact upon *HER2* overexpression, pathogenesis, histopathology, *HER2* induced cell-signaling, hence, concomitantly affecting the *HER2* positive breast cancer susceptibility.

2.1. DNA methylation

DNA methylation is the most common epigenetic modification of mammalian DNA, which involves the enzymatic transfer of a methyl group to the 5th position of cytosine residues in CpG dinucleotides [16]. DNA methylation is known to be involved in altered gene expression in various cancers, including breast cancer and its subtypes [17,18]. DNA hypermethylation has been extensively studied in triple-negative breast cancers. This epigenomic dysregulation has been reported in many genes including cadherin-1 (CDH1), carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), cystatin-M (CST6), estrogen receptor 1 (ESR1), G protein subunit alpha 11 (GNA11), mucin 1 (MUC1), myeloblastosis proto-oncogene (MYB), sodium channel epithelial 1 alpha subunit (SCNN1A), trefoil factor 3 (TFF3), breast cancer 1 (BRCA1) and Kelch-like ECH-associated protein 1 (Keap1) in triple-negative breast cancer [19–21]. A strong association between frequent CpG island (CGI) methylation and *HER2* amplification in human breast cancers has also been identified [22]. On the basis of frequency and quantitative levels of CGI methylation, the process of DNA methylation is categorized as hypomethylation and hypermethylation. DNA hypomethylation results into proto-oncogene and reactivation of transposable elements, loss of gene imprinting [23]; whereas DNA hypermethylation leads to suppression of tumor-suppressor and DNA-repair genes [24]. For many genes aberrant DNA methylation pattern has been observed in *HER2* positive breast cancers, as well. These genes showing aberrant methylation patterns in *HER2* positive breast cancer mainly regulate cell-proliferation, differentiation and signaling. All these genes along with their respective functions have been summed up in (Table 1).

Hypermethylation of genes like RAR-β2, RASSF1A, APC, DLEC1, GRIN2B, HOXA1, HOXA10, IGF2, MT1G, RUNX3, SCGB3A1, SFRP1, SFRP4, TMEFF2, MINT31, CDH-13, HSD17B4, PGR, MYOD1, GSTP1, SLC25A43, VIM, PTPRO and LRH-1 genes has been reported in *HER2* positive breast tumors [18,75–81]. Most of the studies have reported hypermethylation of genes involved in various carcinogenic pathways. However, a single study carried out by Park et al. has shown Alu and LINE-1 hypomethylation in *HER2*-enriched breast cancer. This supports the probable association of these hypomethylated genes with chromosomal instability of *HER2*-enriched subtype [85].

With recent advancements in genomic technologies like Genome-Wide Association Studies (GWAS), microarray approach, a clearer picture of genes being hyper-/hypomethylated in *HER2* positive breast cancer has emerged. Bediaga et al. analyzed breast cancer samples for DNA methylation using microarray approach and reported a strong association of *HER2* overexpressing breast cancer with hypermethylated genes include NPY, FGF2, HS3ST2, RASSF1, and Let-7a [82]. Lindqvist et al. conducted genome-wide methylation study on 17 *HER2* positive breast cancer patients. An altered methylation profile of 69 biomarker genes associated with multicellular development, differentiation and transcription was observed. Overrepresentation of homeobox family genes was found including DBX1, NKX2-6, SIX6 genes that were not reported in association with cancer, previously. PI3K and Wnt signaling pathway related genes were among other affected genes. This study also reported for the first time an altered methylation pattern in *HER2* gene itself and other genes, AKT3, HK1 and PFKP. The 450K array findings were confirmed for six top candidate genes (AKR1B1, INA, FOXC2, NEUROD1, CDKL2, IRF4) by analyzing them for DNA methylation by PCR technique. Exploration of these genes may provide further insights into the biology of *HER2* positive breast cancer [83]. Yamaguchi et al. reported heavy aberrant methylation of RASSF1 among 15 of the 24 *HER2*-positive breast cancers. Moreover, aberrant methylation patterns of WNT/β-catenin signaling pathway regulatory genes (DKK3 and SFRP1) and p53 pathway regulatory gene IGFBP7 were observed among all 24 *HER2* positive breast cancer samples analyzed [84]. DKK3 down-regulation has been associated with cancer progression [86], whereas IGFBP7 is capable of cell growth inhibition and apoptosis induction [70].

2.2. Histone modifications

Histone modifications can influence gene regulation and carcinogenesis by dysregulating chromatin structure. Histones are basic proteins which serve in packaging of DNA onto chromosomes. The covalent post translational modifications to histone proteins include methylation, phosphorylation, acetylation, ubiquitylation, proline isomerization, ADP ribosylation and sumoylation [87]. These modifications are controlled by a group of enzymes including histone acetyltransferases (HATs) and deacetylases (HDACs),

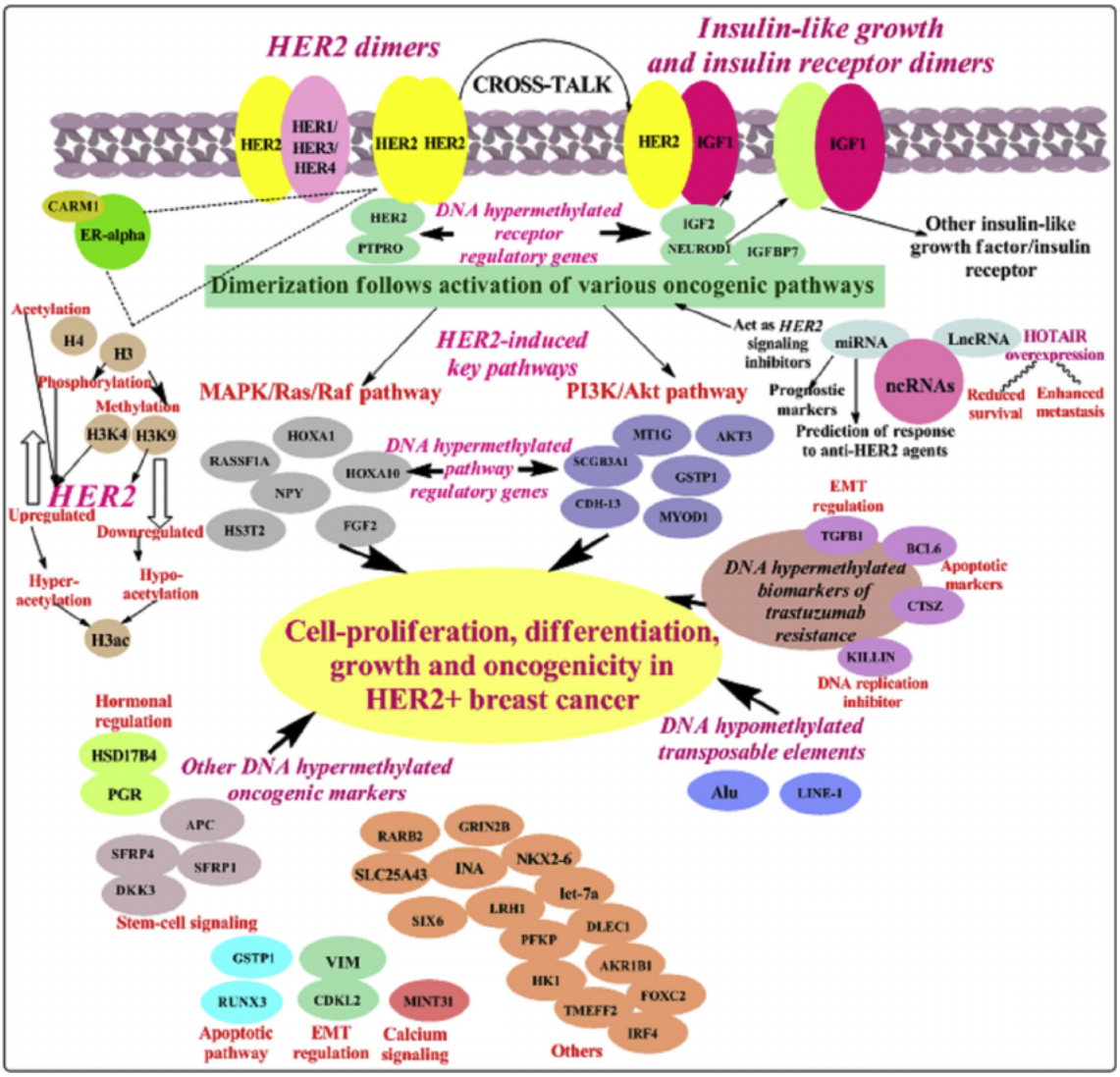

Fig. 1. Various epigenetic markers involved in *HER2* positive breast cancer. Major epigenetic modifications incriminated in *HER2* positive breast cancer: DNA methylation, histone modifications and non-coding RNAs. These markers mainly have their impact on cell-signaling and proliferation. Histone modulations regulate *HER2* gene chromatin. Specifically, ncRNAs impair the major proteins associated with *HER2* pathway, hence, produce effects upon susceptibility, as well as, therapy. (*Refer text for details*).

methyltransferases (HMTs) and demethylases (HDMs), kinases, phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligase and proteases. These modifications are reversible and govern the structural prestige of chromatin. Histone modifications involve in different biological processes like transcriptional activation or inactivation, chromosome packaging and DNA damage or repair [88,89]. In spite of the role of histone modifications in transcriptional gene regulation, there are fewer but more elaborate studies that demonstrate the role of histone modifications in *HER2* overexpression. Mainly phosphorylation, acetylation and methylation of histones have been found to be incriminated in *HER2* positive breast cancer [90–93]. *HER2* expression is regulated by estrogen receptor-alpha (ERα) [94], by interacting with histone-modulating enzymes. ERα extensively regulates the epigenetic modifications of histones, which has been reviewed critically by Mann et al. [95]. On the other side, estrogen signaling has the ability to potentiate various kinase signaling cascades including *HER2* signaling which, in turn, cause the histone modulations. Hence, either ERα-induced histone modifications can influence *HER2* expression or ERα-induced *HER2* signaling can generate histone modifications [95].

Mishra et al. reported the enhanced levels of acetylated and phosphorylated histone H3 and acetylated histone H4 in *HER2* overexpressing breast cancer cells. In addition, among *HER2* overexpressing cells, decreased recruitment of histone deacetylase was reported. Treatment with histone deacetylase inhibitor upregulated the association of acetylated histone H4 with *HER2* promoter and *HER2* expression. On the other hand, the association of histone deacetylase-1 and -2 was reduced on treatment with histone deacetylase inhibitor. Moreover, the association of phosphorylated histone H3 with *HER2* promoter and *HER2* expression was stimulated by tumor promoters 12-*o*-tetradecanoylphorbol-13-acetate and okadaic acid. Thus, histone acetylation and histone phosphorylation are the regulatory epigenetic alterations targeting *HER2* gene chromatin. Besides, the enhanced levels of chromatin-remodeling components in the neighbourhood of *HER2* promoter probably follows non-genomic mechanism of *HER2* overexpression in human breast cancer [90].

Certain histone modulations are of great importance as these have been found to repress *HER2*. Falahi et al. transduced *HER2* overexpressing cancer cells with *HER2* targeting zinc finger protein

Table 1
Abberantly-methylated genes in *HER2* positive breast cancer. Representing the role of each gene.

Gene	Description	Function
RARβ2[a]	Retinoic acid receptor β2	Binds retinoic acid that aids cell signaling in embryonic morphogenesis [25]
RASSF1A[a]	Ras association domain-containing protein 1	Negatively regulates the (Mitogen-activated protein kinase) MAPK/Ras/ERK pathway, blocks cell-cycle progression [26,27]
APC[a]	Adenomatous polyposis coli	Regulates stability and nuclear export of β-catenin, a transcriptional co-activator of Wnt/ β-catenin stem-cell signaling pathway [28]
DLEC1[a]	Deleted in lung And esophageal Cancer 1	Suppression of colony formation in some cancer cell lines and reduced tumorigenesis [29]
GRIN2B[a]	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	Encodes for N-Methyl D-Aspartate receptor that plays role in synaptic transmission [30]
HOXA1[a]	Homeobox A1	Transcription factor that controls cell proliferation and differentiation, acts through MAPK pathway to stimulate oncogenicity [31,32]
HOXA10[a]	Homeobox A10	Transcription factor that regulates the expression of tumor-suppressor p53 gene, stimulates cell-invasion by acting on MAPK pathway [33,34]
IGF2[a]	Insulin-like growth factor 2	Acts through IGF2 receptor pathway to regulate cell growth and tumor suppression [35,36].
MT1G[a]	Metallothionein 1G	Functions as tumor-suppressor by modulating phosphoinositide-3-kinase (PI3K/Akt) pathway [37]
RUNX3[a]	Runt related transcription factor 3	Tumor suppression, regulates apoptosis by targeting B-cell lymphoma 2 (Bcl-2) [38]
SCGB3A1[a]	Secretoglobin, family 3A, member 1	Methylation activates PI3K/Akt pathway [39].
SFRP1[a]	Secreted frizzled related protein 1	Tumor suppressor, represses Wnt/β-catenin signaling pathway [40,41]
SFRP4[a]	Secreted frizzled related protein 4	Inhibition of Wnt/β-catenin signaling, interferes with cell-migration [42]
TMEFF2[a]	Transmembrane protein with EGF like and two follistatin like domains 2	Functions as tumor-suppressor in many cancers [43]
MINT31[a]	Methylation in gastric non-invasive neoplasia 31	Modulates calcium signaling which, in turn, affects cell-proliferation [44]
CDH-13[a]	H-Cadherin	Gene inactivation results in aberrant PI3K/Akt signaling [45]
HSD17B4[a]	Type 4 17-β-hydroxysteroid dehydrogenase	Hormonal regulation [18]
PGR[a]	Progesterone receptor	Hormonal regulation [18]
MYOD1[a]	Myogenic differentiation 1	Mutations lead to alteration in PI3K/Akt pathway components [46]
GSTP1[a]	Glutathione S-transferase P	Cellular detoxification enzyme that acts on apoptotic pathway; prognostic marker in breast cancer [47]
SLC25A43[a]	Solute carrier family 25 member 43	Mitochondrial protein that regulates cell-cycle [48]
VIM[a]	Vimentin	Regulates EMT in breast cancer [49]
PTPRO[a]	Protein tyrosine phosphatase, receptor type O	Suppresses *HER2*-driven carcinogenesis by dephosphorylation and endosomal internalization of *HER2* [50]
NPY[a]	Neuropeptide Y	Regulates MAPK pathway activation [51]; inhibits estrogen-induced proliferation in breast cancer [52]
FGF2[a]	Fibroblast growth factor 2	Induces cell-cycle arrest in MAPK-driven tumor cells [53]
HS3ST2[a]	Heparan sulphate -glucosamine 3- sulphotransferase 2	Regulates cell invasiveness *via* MAPK pathway [54]
Let-7a[a]	Lethal-7a	Involved in the regulation of oncogenic pathways in numerous types of tumors [55]
DBX1[a]	Developing brain homeobox 1	Role in patterning the central nervous system during embryogenesis [56]
NKX2-6[a]	NK2 homeobox 6	Oligodendriocyte differentiation [57]
SIX6[a]	Sine oculis homeobox homolog 6	Ocular development [58]
HER2[a]	Human epidermal growth factor receptor 2	*HER2* amplification/overexpression leads to enhanced cell-signaling and confers development of *HER2* positive breast cancer [3]
AKT3[a]	Serine/threonine kinase 3	Putative oncogenic marker [59,60]
HK1[a]	Hexokinase 1	Oncogenic marker [61]
PFKP[a]	Phosphofructokinase, platelet type	Regulates glycolytic activity in breast cancer [62]
AKR1B1[a]	Aldo-keto reductase family 1 member B	Catalyzes the reduction of a number of aldehydes; found to be having enhanced expression and activity in human breast cancers [63]
INA[a]	Internexin neuronal intermediate filament protein alpha	Involved in the morphogenesis of neurons [64]
FOXC2[a]	Forkhead box C2	Plays role in the vascular tissue development [65]
NEUROD1[a]	Neuronal differentiation 1	Regulates expression of the insulin gene [66]
CDKL2[a]	Cyclin dependent kinase like 2	Promotes epithelial-mesenchymal transition (EMT) and breast cancer [67]
IRF4[a]	Interferon regulatory Factor 4	Regulation of interferons; cell-growth [68]
DKK3[a]	Dickkopf WNT signaling pathway inhibitor 3	Involved in embryonic development through its interactions with the Wnt/β-catenin signaling pathway and may function as tumor suppressor gene [69]
IGFBP7[a]	Insulin like growth factor binding protein 7	Cell-growth inhibition and apoptosis induction [70,71]
LRHI[a]	Liver receptor homologue 1	Controls cell-proliferation by regulating cyclin-dependent kinases [72]
Alu[b]	Arthrobacter luteus	Transposable elements that regulate transcription [73]
LINE-1[b]	Long interspersed nuclear element-1	Gene regulation and carcinogenesis [74]

[a] Hypermethylated gene [18,75–84].
[b] Hypomethylated gene [85].

(ZFP) fused to histone methyltransferases (G9a, SUV39-H1)/super KRAB domain (SKD). These cancer cells were evaluated for epigenetic changes. *HER2*-ZFP fused to G9a was found to induce histone modification, histone H3 lysine 9 dimethylated (H3K9me2). Induction of H3K9me2 caused *HER2* repression. Transduction with ZFP fused to SKD caused *HER2* downregulation and efficient removal of histone acetylation mark, histone H3 acetylated (H3ac) leading to hypoacetylation. The results of this study indicated that H3K9 methylation was responsible for *HER2* downregulation and hypoacetylation effect was a consequence of *HER2* downregulation. These epigenetic alterations sufficiently inhibited cellular metabolism and clonogenicity [91].

Histone methylation may not always be associated with *HER2* repression, but *HER2* overexpression as well. The process seems to be site-dependent such as histone H3 methylation. Unlike, the association of H3K9 methylation with *HER2* downregulation [91],

Table 2
miRNA dysregulation in *HER2* positive breast cancer. Representing upregulation/downregulation with *HER2* overexpression.

Expression	miRNAs
Upregulated	miR-520d, miR-376b [99]; miR-146a-5p [100]; miR-375 [101]; miR-21 [102,103]; miR-155 [104,105]; miR-4728-3p [106]; miR-10b, miR-19a [103]; miR-210 [107]
Downregulated	let-7f, let-7g, miR-107, miR-10b, miR-126, miR-154, miR-195 [108]; miR-181c [99]; miR-200f [109]; miR-200b, has-miR-200b [110]; miR-181d and miR-195-5p [100]; miR-122 [101]; miR-125b [111]; let-7c [112]; miR-744 [113]

H3K4 methylation has been associated with *HER2* overexpression [92]. Mungamuri et al. confirmed that non-amplified *HER2* overexpressing breast cancers attain epigenetic mark, histone H3 lysine 4 trimethylated (H3K4me3) on the *HER2* promoter. On the contrary, *HER2* amplified tumors attain histone H3 lysine 9 acetylated (H3K9ac) mark, which in turn, depends on the acquisition of H3K4me3 mark. These findings indicate that *HER2* amplification and overexpression are epigenetically linked. *HER2* overexpression is correlated with H3K4me3 mark on the *HER2* promoter and WD repeat domain 5 (Wdr5) is needed for enhancement of H3K4me3. Hence, targeting Wdr5 resulted in decreased *HER2* overexpression [92]. Consistent with results of studies conducted by Mungamuri et al. [92], Li et al. also demonstrated the acquisition of H3K4me3 mark on the *HER2* promoter in *HER2* overexpressing breast carcinomas. In this study, enrichment of histone H3 lysine 27 acetylation (H3K27ac) mark was also identified in the *HER2* overexpressing cell lines [93].

Histone H3 methylation is aided by co-activator arginine methyltransferase1 (CARM1) that is an ER cofactor. CARM1 is basically embroiled in chromatin remodeling and, hence, gene regulation through histone H3 methylation. Davis et al. found that higher levels of both nuclear and cytoplasmic CARM1 are associated with *HER2* positive breast tumors. However, as a consequence of *HER2* upregulation, CARM1 preferentially localizes itself to the nucleus. In spite of the fact that CARM1 is an ER cofactor, its expression levels didn't rely upon ER. Therefore, this study signifies the association of CARM1 with *HER2* positive as well as ER negative status [96].

2.3. ncRNAs/miRNAs

2.3.1. Acting as prognostic markers

Scientists have also ventured into the field of ncRNAs to explore their role in *HER2* positive breast carcinogenesis. Their efforts have characterized several miRNAs that are associated with *HER2* positive breast cancer. microRNAs (miRNAs) are naturally occurring small regulatory ncRNAs approximately 21–25 nucleotides long, which function as guide molecules in messenger RNA silencing and cause destabilization or inhibition of translation [97]. Decreased mean miRNA expression has been reported in *HER2* positive tumors [98]. miRNAs are either upregulated or downregulated in *HER2* positive breast cancers. These have been enlisted in (Table 2).

In particular, miRNA dysregulation caught the attention of researchers who were trying to speculate the mechanism of *HER2* overexpession, when a more direct and clearer study carried out by Persson and colleagues came into picture. Persson et al. found that miR-4728 encoded within intron of *HER2* gene is overexpressed in tumors and cell lines showing *HER2* amplification. Based on this study, Persson et al. hypothesized that miR-4728 could explain *HER2* overexpression [106]. Persson et al. indicated that downstream targets of *HER2* activation *i.e.*, mitogen-activated protein kinase1 (MAPKI) and son of sevenless1 (SOS1) were among the predicted targets of miR-4728-3p. Hence, this study speculates that miR-4728-3p is a constituent of a negative feedback loop that regulates *HER2* function, establishing a direct correlation between miRNA and *HER2* overexpression [106].

miRNA-200 family (miR-200f) members have been characterized as important prognostic markers in *HER2* positive breast cancer. Castilla et al. found lowered miRNA-200 family (miR-200f) expression in *HER2* positive tumors [109]. Moreover, Castilla and colleagues depicted that miR-200f could be an ideal biomarker to judge the extent of occurrence of epithelial to mesenchymal transition (EMT) in breast cancer [109,114]. EMT and its contrary process, mesenchymal to epithelial transition (MET) are the rudimentary phenomena implicated in carcinogenesis [115,116]. miRNA dysregulation may cause impairment of immune and EMT pathways, collaboratively. Oncostatin-M has been identified as an important marker in mesenchymal-like breast cancer cells that mediates EMT and is associated with *HER2* negative breast cancer [117]. Studies suggested that miR-200f targets genes controlling motility and invasion and hence, combat EMT [118]. Emphatically, miR-200f has been found to be epigenetically regulated to exert modulatory effect upon EMT-MET process [119]. Another study performed by Wee et al. depicted the association between miR-200f member (miR-200b) and *HER2* positive breast cancer along with the epigenetic mechanism underlying miR-200b expression. Wee et al. determined that promoter hypermethylation of miR-200b precursor (hsa-mir-200b) is associated with *HER2* positive status and found an inverse association between hsa-mir-200b methylation and miR-200b expression [110].

Circulating miRNAs have also been explored in relation to *HER2* overexpression. Wu et al. found that enhanced miR-375 expression and diminished miR-122 expression were associated with *HER2* overexpressing tumors [101].

HER2 positive breast cancer remains associated with aggressive clinicopathological properties such as higher cancer grade, advanced stage, metastasis, reduced survival [12,120]. Geneticists have also reported correlation between miRNA overexpression and clinicopathology of *HER2* positive breast cancer. Enhanced expression of miR-21 was found to be associated with large tumor size, advanced stage, higher carcinoma grade and reduced overall survival in *HER2* positive breast cancer [102]. Song et al. found higher miR-155 levels in advanced stage *HER2* positive breast cancer patients with lymph node metastasis [104]. Nassar et al. also confirmed the association between enhanced miR-155 levels and *HER2* overexpression. Significant overexpression of miR-155 was found in PR negative, *HER2* positive women whose age at time of diagnosis was more than 40 years [105]. Jung et al. identified the correlation of higher levels of circulating miR-210 with tumor presence, and lymph node metastases [107]. Anfossi et al. found enhanced levels of miR-21 in the serum of patients with non-metastatic *HER2* positive breast cancer. High expression levels of serum miR-19a and miR-10b were present in patients with metastatic *HER2* positive invasive breast cancer [103]. Ferracin et al. characterized erythropoietin (EPO) and its receptor (EPOR) as true targets of miR-125b. Further, downregulation of miR-125b in metastatic breast cancers and a significant positive association between EPOR and *HER2* levels was also observed. This indicates EPO/EPOR and *HER2* coregulation in breast cancer and the association of miR125-b with clinically relevant cancer features [111]. In a study carried out by Bailey et al., miR-125b was found to directly target *HER2* [112].

In addition, a study has also reported the involvement of long ncRNA in *HER2* positive breast cancer. Su et al. have reported the overexpression of long ncRNA Hox-antisense intragenic RNA (HOTAIR) in *HER2* overexpressing breast cancer patients [121]. HOTAIR accounts for enhanced chances of metastasis and reduced survival [122].

2.3.2. Inhibition of HER2 positive breast cancer by directly targeting HER2 and/or by interruption of HER2 signaling

Either homodimerisation or heterodimerisation activates *HER2*. *HER2* homodimerisation doesn't require activation by ligand. It happens only when there is *HER2* overexpression (Brennan et al., 2000). *HER2* heterodimerisation takes place in the presence of ligand of dimerisation partner of *HER2*. Despite being devoid of ligand, *HER2* remains the most compatible as dimerisation partner in comparison to other members of EGFR family. It is known to account for most potent cell-signaling through activation of various downstream signaling events such as phosphoinositide-3-kinase (PI3K/Akt) pathway, mitogen-activated protein kinase (MAPK/Ras/ERK) pathway and phospholipaseC-γ (PLCγ) pathway [3]. The most potent *HER2*-HER3 heterodimer inflicts the most efficient signaling and acts through PI3K pathway. The role of *HER2* in cell-signaling has already been reviewed by us [11].

Various miRNA act as *HER2* and/or *HER2*-induced signaling inhibitors Key miRNAs that directly inhibit or lower *HER2* expression are: miR-125a/b, miR-491-5p, miR-634, miR-637, miR-342-5p, miR-34a, miR-199b-5p, miR-205-5p, miR-489, hsa-miR-125a [113,123e128]. Among these, miR-125a/b also inhibited HER3 mRNA expression and miR-205-5p also inhibited HER1 mRNA expression [123,126]. miR-552, miR-541, miR-193a-5p, miR-453, miR-134, miR-498, miR-331-3p, miR-548d-3p and miR-559 act as regulators of translational *HER2* mRNA expression [113,129].

Some miRNAs do not directly target *HER2*, but target its dimerisation partners (HER1 and HER3) and/or the proteins mediating *HER2* signaling/*HER2* positive breast carcinogenesis. miR-450b-3p, miR-149, miR-148b, miR-326 and miR-520a-3p inhibited HER3 expression, further causing impairment in EGFR family signaling [130]. Importance of HER3 expression or suppression lies in the fact of its accountability for the most potent signaling as *HER2*/HER3 heterodimers and activation of PI3K/Akt pathway [11,131]. miR-125a/b and miR-199b-5p caused inhibition of *HER2* downstream signaling pathways (MAPK/Ras/ERK1/2 and PI3K/Akt) and *HER2* positive breast cancer cell growth [123,125]. miR-342-5p specifically inhibited *HER2* positive breast cancer cell growth [113]. miR-221 inhibited expression of p27 and PTEN genes and hence, promotes *HER2* positive metastatic breast cancer [132,133]. The miRNAs involved in regulation/inhibition of *HER2* expression and/or cell-signaling along with the mechanism (if known) have been summarized in (Table 3).

3. Epigenetics in *HER2* targeted therapeutics

Among the members of EGFR family, HER1 and *HER2* are mainly involved in the pathogenesis of various cancers [134]. Resistance towards therapies targeting HER1 has been reported that arises as a result of HER1 cross-interactions with other genetic markers, extensively reviewed by Nigro et al. [135]. Likewise the resistance towards anti-HER1 agents, the problem of resistance towards anti-*HER2* agents due to cross-talk with genetic markers has also been well-documented [11]. In addition to the genetic markers, epigenetic markers are also implicated in the modulation of response towards *HER2* targeted therapies.

3.1. DNA methylation

Disease-relapse as a result of development of resistance against trastuzumab remains the major obstacle in therapeutics targeting *HER2* positive breast cancer [136]. Palomeras et al. found that the methylation profile was variable among the trastuzumab-resistant SKTR and trastuzumab-sensitive SKBR3 cells. In SKTR cells, four hypermethylated genes: transforming-growth factor β1 (TGFβ1), B-cell lymphoma 6 (BCL6), p53-regulated DNA replication inhibitor (KILLIN) and cathepsin Z (CTSZ), were selected and analyzed by polymerase chain reaction (PCR) that indicated the downregulated expression of these genes. Further, the SKTR cells were given treatment with demethylation agent resulting into regain of expression of all four genes. The restored expression levels of these genes were concordant with those of SKBR3 cells. Hence, hypermethylated genes: TGFβ1, BCL6, KILLIN and CTSZ present themselves as predictive biomarkers of trastuzumab resistance in *HER2* positive breast cancer patients [137].

As already discussed, DNA hypermethylation has been correlated with HER2 amplification [22]. Aberrant DNA hypermethylation patterns are observed because of overactivity of DNA methyltransferases (DNMTs). Hence, DNMTs have been developed as therapeutic targets in cancer. Dou et al. facilitated the specific delivery of siRNA-targeting DNMT1 and/or DNMT3b (siDNMTs) into *HER2* expressing breast tumor cells. This caused silencing of DNMTs and hence, promoting demethylation of RASSF1A tumor suppressor gene promoter. Demethylation of RASSF1A led to re-expression of RASSF1A, trigerring the suppression of tumor cell proliferation. Thus, this study suggests that delivery of siDNMTs might prove as fruitful strategy to treat *HER2* positive breast cancer [138]. Besides, DNMT inhibitors, azacytidine and decitabine have also been utilized to target tumor cells. DNMT inhibitors can produce highly synergistic effects, when given in combination with traditional chemotherapeutics *i.e.* doxorubicin, 5-fluorouracil and oxiplatin [139]. However, these have not been explored particularly in *HER2* positive breast cancer.

3.2. Histone modifications

HDAC inhibitors hold an important place in therapeutic strategies targeting epigenetic markers, on the basis of aberrant HDAC activity documented across various tumors. Fuino et al. reported the downregulation of *HER2* by HDAC inhibitor dacinostat/LAQ824. Besides, dacinostat also increased the sensitivity of breast cancer cells towards trastuzumab by potentiating trastuzumab-mediated apoptosis [140]. In a study carried out by Bali and colleagues, activity of another HDAC inhibitor vorinostat/suberoylanilide hydroxamic acid (SAHA) against *HER2* amplified breast cancer cells has also been reported [141]. Activity of HDAC inhibitor, OSU-HDAC42 against *HER2* positive breast cancer *via* heat-shock protein 90 (HSP90) acetylation-mediated *HER2* downregulation was observed by Weng and co-workers [142]. In a study performed by Finn et al., panobinostat/LBH589 induced cell-death and also, boosted the antitumor activity of trastuzumab against *HER2* amplified cell lines [143]. Huang et al. identified that HDAC inhibitor entinostat/SNDX-275 can resolve the critical issue of trastuzumab resistance, as increased efficacy of trastuzumab in presence of entinostat was reported in *HER2* overexpressing breast cancer cells [144]. Wang et al. reported that entinostat caused significant upregulation of miR-125a, miR-125b, and miR-205 which, in turn, target *HER2* and/or HER3 [145]. Thus, HDAC inhibitors represent putative *HER2* targeting molecules. Moreover, entinostat has been found to enhance efficacy of *HER2* targeting small molecular tyrosine kinase inhibitor, lapatinib in *HER2* overexpressing breast cancer cells. Lee and coworkers have propounded that entinostat

Table 3
miRNAs acting as inhibitors of *HER2* expression/signaling. Indicating the role of miRNAs.

miRNA	Role	Reference
miR-125a/b	Suppression of *HER2*/HER3 by targeting 3⁰-UTR region of *HER2*/HER3 mRNA, impairment of *HER2*/HER3 mediated signaling involving inhibition of ERK1/2 pathway and Akt phosphorylation, inhibition of anchorage-dependent cell growth, reduction in cell migration and invasion in *HER2* overexpressing SKBR3 cells	[123]
miR-491-5p, miR-634, miR-637 and miR-342-5p	Inhibition of *HER2*	[113]
miR-552, miR-541, miR-193a-5p, miR-453, miR-134, miR-498 and miR-331-3p	Regulation of *HER2* by directly targeting 3⁰-UTR region of *HER2* mRNA	[113]
miR-342-5p	miRNA expression gets downregulated that is responsible for specific inhibition of *HER2* positive cancer cell growth	[113]
miR-221	Regulates expression of cyclin-dependent kinase inhibitor 1B (CDKNIB/p27) and PTEN genes and promotes metastatic *HER2* positive breast cancer	[132,133]
miR-34a	miR-34a overexpression resulted in decreased *HER2* expression, acts by targeting *HER2* directly and hence, inhibited breast cancer cell invasion and growth *in vitro*	[124]
miR-199b-5p	Directly targeted *HER2* to hamper its expression, inhibited *HER2* downstream signaling pathways (MAPK/Ras/ERK1/2 and PI3K/Akt pathways) and further restrained clonogenicity and migration in *HER2* positive breast cancer cells	[125]
miR-205-5p	Highly expressed in breast cancer stem cells, cause repression of HER1/*HER2*	[126]
miR-489	miR overexpression resulted in decreased *HER2* levels and Akt activation. significantly reduced the tumor forming ability of *HER2* positive cells	[127]
miR-548d-3p and miR-559	Directly target *HER2* by cooperatively regulating translational *HER2* mRNA expression	[129]
miR-125a	Precursor hsa-miR-125a targets -UTR of *HER2* mRNA. Variant rs12976445 (C/T and C/C) of hsa-miR-125a sequence is associated with reduced levels of mature miR-125a and *HER2* overexpression in breast tumor samples	[128]
miR-450b-3p	Inhibited HER3 mRNA expression by directly targeting 3⁰ UTR of HER3 mRNA, hence causing impairment of HER family signaling	[130]
miR-149, miR-148b, miR-326 and miR-520a-3p	Caused HER3 downregulation and cell-signaling impairment, particularly affecting HRG-induced activation of the PI3K-Akt pathway	[131]

enhances the lapatinib efficacy through Forkhead Box O3 (FOXO3) mediated bisindolylmaleimide-based protein kinase C inhibitor (Bim1) expression [146]. These studies indicate that HDAC inhibitors can be a part of adjuvant therapeutics that can help to synergize the impact of anti *HER2* agents.

On the basis of previous studies, HDAC inhibitors (vorinostat, entinostat and panobinostat) were taken to clinical evaluation. Vorinostat in combination with lapatinib showed antitumor activity and is tolerable in intensely pre-treated advanced solid tumors [147]. In phase 1/2 study (NCT00258349), a combination of vorinostat and trastuzumab was evaluated for treating patients with metastatic or locally recurrent *HER2* positive metastatic breast cancer and 22.22% of serious adverse events in phase 2 were recorded [148]. Vorinostat combined with trastuzumab failed to reverse the trastuzumab resistance in patients with *HER2* positive metastatic or locally recurrent metastatic breast cancer [149]. A phase 1/2 clinical study (NCT00574587) is ongoing, evaluating vorinostat in combination with trastuzumab and chemotherapy against locally advanced *HER2* positive breast cancer [150]. Entinostat in combination with lapatinib and trastuzumab is still under phase 1 evaluation (NCT01434303) for the treatment of locally recurrent or metastatic *HER2* positive breast cancer patients initially treated with trastuzumab only [151]. A Phase 1/2 study (NCT00567879) that was planned to evaluate panobinostat and trastuzumab in *HER2* positive metastatic breast cancer who progressed on or after trastuzumab, was terminated due to lacking clinical benefit [152]. A phase 1 trial (NCT00788931), evaluating panobinostat in combination with trastuzumab and paclitaxel in adult *HER2* positive metastatic breast cancer has been completed, but the data remains unpublished [153].

3.3. ncRNAs/miRNAs

miRNAs are induced upon trastuzumab/lapatinib treatment, mediate the antiproliferative effects of *HER2* targeted agents and/or act as biomarkers for sensitivity towards them. These include: mi-26a, miR-30b, miR-194, miR-16, miR-210, miRNA-450b-3p and miR-205 [107,130,154e157]. The roles of these miRNAs along with their respective targets and mechanisms (if known) have been summed up in (Table 4).

Trastuzumab is an effective targeted therapy for *HER2* positive breast cancer. Most of the patients develop resistance against it that represents the major drawback associated with this therapy [136]. To avoid limitations of trastuzumab, lapatinib has been developed as the *HER2* targeted agent. It is an approved small-molecule *HER2* tyrosine kinase inhibitor. However, the issue of resistance development has emerged here, also [158]. miRNAs act through different mechanistic pathways and confer resistance to *HER2* targeted agents that have been tabulated in (Table 5).

Resistance towards targeted therapy may occur due to various factors such as *HER2* cross-talk with other receptors (HER1, HER3, HER4 and IGF1R), masking of *HER2* by CD44 and formation of spliced *HER2* protein. PI3K/Akt pathway is the major pathway activated during this course of resistance development [136].

To improve survival in patients receiving neoadjuvant chemotherapy, pathological complete response (pCR) comprises an effective predictive marker. In a study carried out by Ohzawa et al., 40 *HER2* positive breast cancer patients who underwent neoadjuvant chemotherapy with trastuzumab were sorted into pCR and non-pCR groups. Differential miRNA expression was observed between these pCR and non-pCR groups. Among the 40 patients analyzed, the downregulated miRNAs identified were: miR-106b-3p, miR-1180, miR-1238-5p, miR-142-5p, miR-150-5p, miR-181c-5p, miR-182-5p, miR-200a-5p, miR-218-5p, miR-3609, miR-362-5p, miR-3620-3p, miR-4418, miR-4506, miR-4657, miR-505-3p and miR-505-5p. Some miRNAs were also upregulated including miR-210, miR-31-3p, miR-449a and miR-449b-5p. Many of these miRNAs were found to be associated with carcinogenesis, prognostic features, outcome and chemoresistance in breast cancer [162].

4. Discussion and future perspectives

In the past few years, *HER2* overexpressing breast cancer has

Table 4
miRNAs acting as biomarkers for sensitivity towards anti-*HER2* agents. Representing respective mechanism involved.

miRNA	Role	Reference
miR-26a	Induced by trastuzumab causing arrest of G1 phase of cell-cycle in *HER2* positive breast cancer cells	[154]
miR-30b	Induced by trastuzumab causing G1 arrest and induction of apoptosis in *HER2* positive breast cancer cells, reduces expression of cell-cycle regulatory gene, cyclin E2 (CCNE2)	[154]
miR-194	Upon trastuzumab treatment mediates downregulation of cytoskeletal protein talin2 to exert cell migration inhibitory effects	[155]
miR-16	Mediates anti-proliferative effects of trastuzumab and lapatinib by targeting cyclin J and far upstream element-binding protein 1 (FUBP1)	[156]
miR-210	Biomarker of trastuzumab sensitivity	[107]
miR-450b-3p	Targets 3$^{\prime}$-UTR of HER3 mRNA; inhibits clonogenic potential and increases sensitivity to trastuzumab in SKBR3 cells	[130]
miR-205	Inhibits the colonogenic potential and increases sensitivity to lapatinib by targeting HER3 and further, preventing PI3K/Akt pathway activation	[157]

Table 5
miRNAs acting as biomarkers for resistance towards anti-*HER2* agents. Representation of respective mechanism involved.

miRNA	Role	Reference
miR-221	Confers resistance to trastuzumab by targeting phosphatase and tensin homologue (PTEN) gene	[133]
miR-375	Overexpression of miR-375 restored the sensitivity of cells to trastuzumab	[133]
miR-520c-3p, miR-520-5p, miR-587-5p	Caused reversal of trastuzumab resistance by significantly decreasing stem-cell marker, CD44 expression	[159]
miR-21	Confers resistance to trastuzumab by targeting PTEN gene and triggering nuclear factor- κβ (NF-κβ) mediated signaling (through interleukin-6 (IL-6) and transcription factors activation) that further activates PI3K pathway	[160]
miR-542-3p	Knockdown results in the resistance to trastuzumab *via* PI3-Akt pathway activation and decreased cell apoptosis	[161]

become a matter of concern since, 15–30% of breast cancers are labelled as *HER2* positive. Contributory factors in *HER2* over-expression mainly include *HER2* gene amplification/polysomy 17. Alterations in *HER2* gene as well as other genes have been well explored with an aim to unravel the mechanistic pathway of *HER2* overexpression. However the dominating role of epigenetics over genetics in cancer can't be neglected. Epigenetic alterations like aberrant DNA methylation patterns, histone modifications and ncRNA/miRNAs have been associated with *HER2* overexpression. Hypermethylation of genes involved in cell signaling and hypo-methylation of transposable elements, have been found in *HER2* positive breast cancer. Histone modifications including acetylation, phosphorylation and methylation regulate *HER2* gene chromatin. ncRNAs/miRNAs play significant role in epigenome management. These mainly regulate *HER2* induced cell signaling and/or act as prognostic markers in *HER2* positive breast cancer. Since, epigenetic alterations regulate *HER2* signaling, these present themselves as putative therapeutic markers. In addition epigenetic markers are also involved in modulation of response towards targeted therapy.

In context of the role of epigenetic modifications in cancer, therapeutic strategies against these markers have been developed. DNMT inhibitors (azacytidine and decitabine) are known to produce synergistic effects in tumor cells when given along with traditional chemotherapeutic agents. However, their scope is limited to myelodysplastic syndrome. This may be attributed to the limitations like poor bioavailability, high toxicity associated with these agents [163]. This might be the reason for not exploring DNMT inhibitors in *HER2* positive breast cancer. In that case, siDNMTs may help to combat the effect of DNMTs. However, problems with administration of siDNMTs in humans may arise. So, appropriate agents targeting DNMTs need to be developed against *HER2* positive breast cancer. Among the various histone modulations, histone deacetylation has been extensively developed as therapeutic target in cancer through design and development of HDAC inhibitors. Much data has emerged in favour of the antitumor activity of these agents. As a consequence, these agents have reached the clinical evaluation. From the available clinical data, it is quite evident that these agents in combination with trastuzumab seem to be ineffective. The result of evaluation of these agents either as monotherapeutic agents or in combination with lapatinib/chemotherapy plus trastuzumab and/or lapatinib, is awaited. Clinical trials, NCT01118975 and NCT00777335 those were pre-planned to evaluate vorinostat plus lapatinib and panobinostat alone, respectively in *HER2* positive breast cancer were terminated. The reasons behind the termination of clinical studies, NCT01118975 and NCT00777335 were loss of sponsorship and low recruitment rate of patients, respectively [164, 165]. To establish the clinical status of these agents, these studies should be re-initiated.

Till date, mainly HDAC inhibitors have remained under focus for development as agents against *HER2* positive breast cancer. The development of HMT inhibitors remains to be instigated, yet. The indirect inhibition of histone methyltransferase, polycomb repressive complex (PRC) 2 member enhancer of zeste homolog 2 (EZH2) *via* S-adenosylhomocysteine (AdoHcy) hydrolase inhibitor (AHI) 3-deazaneplanocin A is well-documented. EZH2 is associated with histone modulatory mark H3K27me3 which, in turn, correlates with *HER2* positive breast cancer. In breast cancer models, the synergistic interactions of AHIs with HDAC inhibition and *HER2* targeted therapy, has also been reported [166]. Hence, this study lays the foundation for the development of HMT inhibitors for treatment of *HER2* positive breast cancer. miRNAs have also been found to act as modulatory markers for the targeted therapy in *HER2* positive breast cancer. Therefore, there is a need for antisense oligonucleotides targeting miRNAs in *HER2* positive breast cancer.

Epigenetics represents an emerging arena that needs to be exploited for resolving mechanism of *HER2* overexpression, as well as, for the management of treatment strategies in *HER2* positive breast cancer.

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Competing interests

The authors declare that they have no conflict of interest.

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