SHORT COMMUNICATION

A novel HER2 gene body enhancer contributes to HER2 expression

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The transcriptional regulation of the human epidermal growth factor receptor-2 (HER2) contributes to an enhanced HER2 expression in HER2-positive breast cancers with HER2 gene amplification and HER2-low or HER2-negative breast cancers following radiotherapy or endocrine therapy, and this drives tumorigenesis and the resistance to therapy. Epigenetic mechanisms are critical for transcription regulation, however, such mechanisms in the transcription regulation of HER2 are limited to the involvement of trimethylated histone 3 lysine 4 (H3K4me3) and acetylated histone 3 lysine 9 (H3K9ac) at the HER2 promoter region. Here, we report the identification of a novel enhancer in the HER2 enhancer, which we have termed HER2 gene body enhancer (HGE). The HGE is transcriptionally regulated and that DNA methylation in the HGE region inhibits the transcriptional activity of the HER2 promoters. We also found that TFAP2C, a known regulator of HER2, binds to HGE and is required for its enhancer function and that DNA methylation in the HGE region inhibits the transcriptional activity of the HER2 promoters. The identification of this novel enhancer sheds light on the roles of epigenetic mechanisms in HER2 transcription, in both HER2-positive breast cancer samples and individuals with HER2-low or HER2-negative breast cancers undergoing radiotherapy or endocrine therapy.

INTRODUCTION

Human epidermal growth factor receptor-2 (HER2)/Erb-B2 receptor tyrosine kinase 2 is a member of the erbB-like oncogene family, its overexpression occurs in approximately 20–30% of breast cancers1 and is strongly associated with poor prognosis.2 HER2 has roles in the development of HER2-positive breast cancers3,4 and resistance to therapy in HER2-low or HER2-negative breast cancers, in which HER2 is transcriptionally regulated by radiotherapy or by endocrine therapy.5-7 HER2 gene amplification is a major mechanism for HER2 overexpression, however, higher transcription rate of HER2 per gene copy was also observed in HER2-amplified breast cancer cells,8-10 that is, HER2 mRNA levels are 4- to 8-fold and 64- to 128-fold higher in HER2 overexpressing and HER2-amplified breast cancer cells, respectively, than would be expected from HER2 gene copy numbers.8 A run-on assay showed SKBR3 cells displayed about two fold HER2 transcription rate higher than in BT474 cells.11 Transcription factors such as TFAP212,13 Sp1,14 PBP,15 YY1,16 ETS,17 YB-118 and EGR219 have been shown to positively regulate HER2, whereas MYB,20 FOXP3,21 GATA4,22 PEA3,23 MBP-1,24 NOTCH and RBP-Jk25 have negative effects. Most of these studies focused on the regulation of the originally characterized HER2 promoter (promoter 2),14,26,27 which has the dominant role in the overexpression of HER2 in breast cancers28 despite of the identification of an alternative promoter (promoter 1)29 (Figure 1a). Moreover, intron 1 enhancer when bound by PAX2 mediates transcriptional repression of HER2 by activated ER.5 Although these transcriptional mechanisms can explain HER2 regulation in part, the molecular basis of the increase in HER2 transcription in certain cancers remains unexplained.

Chromatin modifications can greatly influence transcriptional regulation and contribute to cancer development.30 H3K4me3 and H3K9ac, two histone marks typically associated with gene activation, were reported to be critical for inducing HER2 transcription through promoter 2.10 WDR5, a key component of the H3K4me3 methyltransferase complex, is essentially involved in this process.10 However, these mechanisms are common to general transcriptional activation. Thus, additional mechanisms may exist and specifically contribute to HER2 overexpression. We discovered a novel enhancer HER2 gene body enhancer (HGE) in the 3’ gene body of HER2.

The HGE activates promoters 1 and 2 in trans, and hence the TFAP2C-mediated transcriptional induction of HER2 expression. This novel regulatory mechanism of HER2 transcription contributes to the understanding of increased expression of HER2.

RESULTS AND DISCUSSION

Identification of a novel enhancer in HER2 locus

Enhancer interacts with promoter to recruit RNA polymerase II and regulate transcription.31 Chromatin signatures such as DNase I hypersensitivity sites32 and histone modifications, that are, H3K4me1 and H3K27ac can be used to predict putative enhancers.33-35 We took advantage of data from the Encyclopedia of DNA Elements (ENCODE) Consortium36 to search for novel regulatory elements that may contribute to the increased expression of HER2.
Figure 1. Epigenetic demarcation of the HGE. (a) Top: schematic illustration of the locations of the HER2 promoters (1 and 2), two C-terminal transcripts (CTF611 and CTF687), and the HGE region, as well as the exon composition of HER2, based on UCSC gene annotation (GRCh37/hg19). Bottom: enrichment of H3K4me1, H3K4me3, H3K27ac and DNase I hypersensitivity signals, retrieved using the ENCODE Regulation Super-track Settings. Colors representing different cell types are shown in the legend. Chromatin immunoprecipitation (ChIP)-PCR amplicons are shown. NC: sequences to which nonspecific controls were generated. (b) Profile of TFAP2C binding to the HER2 gene in the indicated cell lines, as retrieved from ChIP-seq data (GSE36351). The maximum reads on the y axis represent normalized coverage (reads per million mapped). (c, d) TFAP2C binding and H3K4me1 and H3K27ac modification, in SKBR3 (c) and HCC1954 (d) cells, as assessed by ChIP. Enrichment was interpreted as a percentage of input. Fold change over input normalized to NC is shown. P1, HER2 promoter 1; P2, HER2 promoter 2; EHN1, intron 1 enhancer. Mean ± s.d. was determined for three independent experiments, and the Student’s t-test was used to calculate the significance. *P < 0.05, **P < 0.001.
transcription. With the ENCODE Regulation Super-track Settings, DNase I hypersensitivity sites, H3K4me1 and H3K27ac were found in the previously identified intron 1 enhancer41 (using the annotation of NM_001005862); 5-kb upstream of promoter 2; and in a previously undiscovered region within the 3’ gene body (Figure 1a). The 3’ gene body region starts from intron 19 and ends within intron 22 (based on NM_001289936) (Figure 1a) and, in addition to the above-described features, it contains binding sites for many transcription factors (including POLII, TEAD4, c-MYC and PHF8) identified in K562 cells (Supplementary Figure 1). We named this region as the HGE.

TFAP2C is known to positively regulate HER2 expression as it is required for the HER2 expression in BT474 cells;37 binds to and regulates HER2 promoter 2;29,30 mediates the repression of HER2 by estrogen;38 its expression is positively correlated with HER2 expression in primary breast cancers.37 We analyzed the TFAP2C occupancy data from four cell lines (GSE36351)40 and revealed that TFAP2C is enriched at both promoter 2 and intron 1 enhancer in all cell lines, with stronger enrichments in HER2-amplified SKBR3 and BT474 lines than in HER2-low MCF7 cells (Figure 1b). Importantly, TFAP2C also binds to the HGE in both SKBR3 and BT474 cells, with the stronger occupancy in SKBR3 cells than in BT474 cells (Figure 1b), which supports HGE as a candidate enhancer. Chromatin immunoprecipitation experiments of TFAP2C, H3K4me1 and H3K27ac were performed in SKBR3 cells and TFAP2C occupancies were confirmed at promoters 1 and 2, the intron 1 enhancer and HGE (Figure 1c). H3K27ac is enriched at all four regions, whereas H3K4me1 enrichment was only obvious at HGE (Figure 1c). Chromatin immunoprecipitation experiments in another HER2-amplified breast cancer cell line, HCC1954 (Figure 1d), revealed that TFAP2C was significantly enriched at all four regions, however, its enrichments at both enhancers were dramatically lower than at promoter 2. H3K27ac was significantly enriched at both promoters and intron 1 enhancer, whereas, H3K4me1 was more enriched at promoter 2 compared with a slight increase at promoter 1 and HGE (Figure 1d). These data support the enhancer feature of the HGE and its cell type dependency. Importantly, a chromosome 17-wide binding data of slight increase at promoter 1 and HGE (Figure 1d). These data suggested that TFAP2C is enriched at both promoter 2 and intron 1 enhancer but slightly weaker for promoter 2 (Figure 2c). These data support that the HGE has enhancer function comparable with the intron 1 enhancer.

TFAP2C regulates the enhancer function of HGE
To determine the minimal enhancer element of HGE, we generated a series of deletions including the deletion of exon 20 and intron 20 (T20), the deletion of exon 21 and intron 21 (T21) and the deletion of exon 22 and intron 22 (T22) (Figure 3a, left panel). The transcriptional activities of these constructs were analyzed using the luciferase assay in 293T and SKBR3 cells. The T21 deletion abolished the enhancement of transcriptional activity from promoter 2 in both cell lines, whereas the T20 deletion had no significant effect in either case, and the T22 deletion increased the transcriptional activity of promoter 2 (Figure 3a). These data suggested that exon 21 and intron 21 contain sequences involved in transcriptional activation, and exon 22 and intron 22 contain sequences involved in transcriptional repression. We next searched for the TFAP2C consensus sequence (GCCCTAGGG)43 and identified three closest potential TFAP2C-binding sites (GCCCCAGAG, GCCCTAGGG, GCCCAGGGC) (Figure 3b) located within intron 21. An electrophoretic mobility shift assay using in vitro translated TFAP2C and three oligonucleotide probes corresponding to the three potential TFAP2C-binding sites showed that TFAP2C binds to all three probes (Figure 3b). The specificity of TFAP2C binding was further confirmed by competition of non-labeled oligos and the supershifts when cultured with a specific TFAP2C antibody (Figure 3b). TFAP2C silencing attenuated luciferase activity of the HGE enhancer in both 293T and SKBR3 cells (Figure 3c) and downregulated HER2 protein levels in SKBR3 cells. (Figure 3d). These data support our hypothesis that TFAP2C has an important role in regulating the enhancer function of the HGE.

We next carried out genomic editing using CRISPR-Cas9 system to determine the role of the TFAP2C-binding sites at the HGE in the regulation of HER2 expression. Six single-guide RNAs (gRNA) were designed to mutate or truncate TFAP2C-binding sites (Figure 3b). SKBR3 stable cell lines with the single or combined gRNA(s) were attempted to be established, however, only one stable cell line with gRNA6 was achieved. It is likely that the cell viability of SKBR3 cells depends on HER2 as knockdown HER2 in SKBR3 cells results in growth arrest and apoptosis.44 HER2 protein in this cell line was dramatically downregulated while cleaved PARP was induced (Figure 3e). The genotyping of SKBR3-gRNA6 showed heterogenous genomic compositions, that is, normal HGE region and extended mutations from the gRNA6-targeting site (Supplementary Figure 2). This data suggest that CRISPR-Cas9 system with these gRNAs introduced additional mutations in HER2 gene and may interfere with HER2 mRNA splicing, resulting in decreased HER2 expression and apoptosis of SKBR3 cells. In fact, a recent study show that genomic editing of HER2 gene using CRISPR-Cas9 system produced short truncated HER2 caused by alternative splicing of HER2 gene and inhibits cell proliferation in both SKBR3 and BT474 cells,45 consistent with a recent observation of off-target mutations introduced by CRISPR-Cas9 system.46

Considering the HER2-dependent cell viability of SKBR3 cells, the six gRNAs were transiently transfected to SKBR3 cells for 48 h and downregulation of HER2, phospho-AKT levels at various extents was found without inducing obvious apoptosis (Figure 3f). These data suggest that the gRNAs targeting intron 21 containing three TFAP2C-binding sites and the junction of exon 21 and intron 21 interfere with HER2 expression. We also examined the mRNA levels of TFAP2C and HER2 from 12 unidentifiable HER2-positive breast cancer samples and found positive correlation ($r^2 = 0.6073$) between them (Figure 3g).
DNA methylation within the HGE inhibits the enhancer histone modifications and is inversely correlated with HER2 gene expression in breast cancer samples.

The cell-type-dependent enrichments of TFAP2C, H3K4me1 and H3K27ac in the HGE region (Figures 1c and d) prompted us to investigate the underlying mechanism. DNA methylation is known to prevent TFAP2C from accessing the target promoter, and DNA methylation and certain histone modifications such as H3K4me3 are mutually exclusive. Thus, we hypothesized that DNA methylation within the HGE affects TFAP2C binding and enrichment of the enhancer histone modifications. Analysis of both Methyl 450 K bead array data (ENCODE/HAIB) and Reduced Representation Bisulfite Seq data (ENCODE/HudsonAlpha) showed that DNA hypomethylation in the HGE region is coincident with enrichments of transcription factors and enhancer histone modifications in K562 cells (Supplementary Figure 1). We performed a bisulfite sequencing assay to determine the DNA methylation status of 28 CpG sites within the HGE. The HGE is extensively DNA-methylated in MCF7, BT474, HCC1954, MDA-MB-231, MCF10A and ZR-75-1 cells. It is less methylated in K562 and hypomethylated in SKBR3 cells (Figure 4a). These data support our hypothesis that DNA methylation status is critical for the enrichments of TFAP2C, H3K4me1 and H3K27ac in the HGE region. The minor enrichments of TFAP2C at HGE in both BT474 and HCC1954 cells (Figures 1b and d) likely reflect the existence of minor cell populations possessing hypomethylated HGE or the cells toward complete establishment of DNA methylation during

Figure 2. The HGE enhances transcriptional activity of HER2 promoters. (a) Relative luciferase activities of reporter constructs illustrated at left, normalized to expression of Renilla luciferase from co-transfected pRL-TK-Rluc plasmid. (a) Relative luciferase activity elicited by HER2 promoter 1 (P1), HER2 promoter 2 (P2) and Luciferase (Luc), in 293T, SKBR3 and BT474 cells. # indicates the significance between each group versus pGL3-basic vector. (b) Relative luciferase activities of the pGL3-HGE and pGL3-HGE-promoters in 293T cells. Mean ± s.d. was obtained from three independent experiments. The Student’s t-test was used to calculate the significance between underlined groups. *P < 0.05, **P < 0.001. # indicates the significance between each group versus pGL3-basic vector. (c) Comparison of the enhancement activities of HGE, reverse HGE and intron 1 enhancer by luciferase assay. R-HGE, reverse HGE; EHN1, intron 1 enhancer. Student’s t-test was used to calculate the significance between constructs with R-HGE or EHN1 versus HGE with each promoter separately. No significance was observed between R-HGE and HGE, whereas EHN1 significantly enhance transcriptional activities of the basic vector and HER2 promoter 2 compare with HGE and R-HGE.
cell cycle. We next performed in vitro methylation of the pGL3-promoter constructs with and without HGE, using the CpG methyltransferase M.SssI, and found a strong decrease of enhanced luciferase activity of the methylated pGL3-promoters vector with HGE compared with that without HGE (Supplementary Figure 3).

Furthermore, we performed CRISPR-dCas9-guided specific DNA methylation\(^\text{31}\) at the HGE region in SKBR3 cells. The six gRNAs (Figure 3b) were inserted into pCas9-DNMT3A-EGFP vector and again, stable cell lines using SKBR3 cells were failed to establish. However, we were able to collect cells from gRNA 1 and 6 during the attempt and found that the cells undergo apoptosis (Figure 4b, left panel). Importantly, the HGE region became partially methylated in SKBR3 cells that transfected with pooled gRNAs, validating the target-specific DNA methylation by the dCas9-DNMT3A system and indicating that the apoptosis might be caused at least partially by loss of HER2 (Figure 4c). The dCas9-DNMT3A-gRNA 1 and 6 transient transfected into SKBR3 cells for 48 h markedly downregulated HER2 expression, phospho-AKT, but not that of TFAP2C and no apparent apoptosis was observed (Figure 4b, right panel). These data suggest that the DNA methylation at the
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28 CpG sites of HGE region

During stable selection

Transient transfection

Pearson's r between DNA methylation and HER2 mRNA expression in breast invasive carcinoma

Average of Probes

HER2 mRNA Z-Score
Figure 4. DNA methylation determines the enhancer function of HGE and is inversely correlated with HER2 expression in breast cancers. (a) DNA methylation of the 28 CpG dinucleotides in the HGE region in the HGE region in multiple cell lines, assessed using the bisulfite sequencing. Each circle represents a CpG dinucleotide: open circle, unmethylated site; closed circle, methylated site; half-closed circle, semi-methylated site; circle with an x, nucleotide sites are marked, and TFAP2C consensus nucleotide sites are indicated by arrows. (b) Indicated proteins from SKBR3 cells either transfected with CRISPR-dCas9-DNMT3A-EGFP carrying gRNA 1 or 6 and undergoing stable selection (left) or transiently transfected with the same plasmids for 48 h (right) were assessed by western blotting. (c) DNA methylation status of the HGE region in SKBR3 during the stable selection as assessed by bisulfite sequencing. (d) Correlation (Pearson’s r) of all 47 DNA methylation sites around HER2 gene including HEG regions (red color) with HER2 expression in 839 breast invasive carcinoma samples was analyzed using MethHC. (e) The inverse correlation of average and each individual CpG sites located in HGE with mRNA expression in 839 breast invasive carcinoma samples was analyzed using MethHC. (f) Correlation between TFAP2C, ESRRA, PPARGC1B and HER2 mRNA level in HER2-positive breast cancer samples sub grouped by DNA methylation status of HGE region were plotted. Light and dark dots are designated to hypomethylation and hypermethylation in the HGE region, respectively.

HGE region is important for repression of HER2 expression in SKBR3 cells.

DNA methylation is in general strongly correlated with HER2 expression ($r^2 = 0.5055868$) in breast cancer. Using the methylation database MethHC, we analyzed the correlation of the DNA methylation status of 47 CpG probes and HER2 expression in 839 breast invasive carcinoma samples cataloged in The Cancer Genome Atlas. The general inverse correlation of DNA methylation in HER2 gene body and HER2 mRNA is stronger ($r^2 = 0.48$) compared with that of the promoters ($r^2 = 0.22$ for promoters 1 and 2, respectively) (Supplementary Figure 4). The correlation of the DNA methylation status of all 47 CpG probes with HER2 mRNA expression shows strong inverse correlation ($r$ values ranging from $-0.291$ to $-0.408$) between the DNA methylation of the four HGE CpG sites and HER2 mRNA expression (Figure 4d). Further analysis in HER2-positive breast cancers revealed that the DNA hypomethylation of all four HGE CpG sites is inversely correlated with HER2 mRNA (Figure 4e). In the samples with hypomethylated HGE region, TFAP2C mRNA is positively correlated with HER2 mRNA (Figure 4f). As discussed earlier that ERα also binds to HGE region and regulates HER2 expression in SKBR3 cells, we also found a positive, but weaker association between the mRNA of the coding gene ESRRA and HER2 expression. In contrast, the expression of PPARGC1B does not show any association. These data suggest that the hypomethylation of the HGE region in breast cancers contributes to HER2 expression by gaining the accessibility of transcription factors such as TFAP2C and ERα.

In sum, this study unveiled a novel regulatory mechanism by a 3’ gene body enhancer contributing to the transcriptional regulation of HER2. Further studies are sought to determine the role of this enhancer in the transcriptional upregulation of HER2 in HER2-low or HER2-negative breast cancers that undergo radiotherapy or endocrine therapy.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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