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Investigation of the Role of DNA Methylation in the Expression of *ERBB2* in Human Myocardium

Adolfo Quiñones-Lombraña,

Department of Pharmaceutical Sciences, The State University of New York at Buffalo, Buffalo, NY 14260, USA

Rachael Hageman Blair, and

Department of Biostatistics, School of Public Health and Health Professions, The State University of New York at Buffalo, Buffalo, New York, 14260, USA

Javier G. Blanco*

Department of Pharmaceutical Sciences, The State University of New York at Buffalo, Buffalo, NY 14260, USA

Abstract

The *ERBB2* gene encodes a transmembrane tyrosine kinase receptor that belongs to the epidermal growth factor receptor (EGFR) family. *ERBB2* plays a pivotal role during heart development and is essential for normal cardiac function, particularly during episodes of cardiac stress. The monoclonal antibody drug trastuzumab is used for the therapy of breast cancers that overexpress *ERBB2*. The clinical use of trastuzumab is limited by the development of cardiotoxicity in some patients. Inter-individual differences in the expression of *ERBB2* in cardiac tissue may impact the risk of cardiotoxicity. In this study, we examined whether DNA methylation status in the proximal promoter region of *ERBB2* is associated to variable *ERBB2* mRNA and *ERBB2* protein expression in human myocardium. Complementary studies with *ERBB2* gene reporter constructs and chromatin immunoprecipitation suggest that differential methylation in specific CpG sites modify the binding of Sp1 to the promoter of *ERBB2*. DNA methylation in the *ERBB2* locus may contribute to the variable expression of *ERBB2* in human myocardium.

Keywords

ERBB2; DNA methylation; Trastuzumab; Human myocardium; Specificity protein 1

*Corresponding author at: Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, 470 Kapoor Hall, Buffalo, NY 14214 – 8033, USA, Phone: 716-645-4820, Fax: 716-829-6569, jgblanco@buffalo.edu.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

1. Introduction

ERBB2 (Erb-B2 Receptor Tyrosine Kinase 2) encodes a member of the epidermal growth factor family of receptor tyrosine kinases. *ERBB2* is part of several cell surface receptor complexes that regulate cell survival and proliferation along multiple pathways including the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Moasser, 2007). Although *ERBB2* does not bind ligands, it does form heterodimers with other *ERBB* receptors to enhance and diversify ligand-induced receptor signaling. For example, *ERBB2* forms heterodimers with *ERBB4* in the myocardium (Wadugu and Kuhn, 2012). *ERBB2* plays a pivotal role in heart development and is required for cardiomyocyte proliferation during the embryonic and neonatal stages (Lee et al., 1995; Olson and Srivastava, 1996; Negro et al., 2004). *ERBB2* continues to be expressed in adult heart tissue, specifically in the surface of cardiomyocytes where it participates in interactions with neuregulins derived from endocardial and endothelial cells (Zhao et al., 1998; D'Uva et al., 2015). *ERBB2* signaling is essential for normal cardiac function including contraction as well as for the control of responses to cellular stressors like ischemia or cardiotoxic drugs (Zhao et al., 1998; Sanchez-Soria and Camenisch, 2010; D'Uva et al., 2015; Rochette et al., 2015; Vermeulen et al., 2016).

The monoclonal antibody drug trastuzumab (Herceptin™) is used for the treatment of breast cancers that overexpress *ERBB2* (Klapper et al., 2000; Vu and Claret, 2012). The clinical use of trastuzumab is hampered by the development of cardiotoxicity in some patients. A systematic review of randomized clinical trials showed that the risk of heart toxicity is five times higher in women receiving trastuzumab in comparison to women receiving standard therapy alone (Moja et al., 2012). It is unclear why some women develop cardiotoxicity after treatment with trastuzumab-containing regimens whereas others identically treated do not (Martin et al., 2009; Moja et al., 2012). In ventricular cardiomyocytes, the inhibition of *ERBB2* signaling by trastuzumab interferes with multiple downstream pathways associated with cellular survival (Rochette et al., 2015). In this context, it is licit to hypothesize that inter-individual differences in the cardiac expression of *ERBB2* would impact the fraction of trastuzumab that binds to cardiac tissue, and consequently affect the risk of cardiotoxicity (Ewer and Yeh, 2006). Although the elucidation of the molecular bases that control the expression of *ERBB2* in cardiac cells has been the subject of intense investigation, there is paucity of information in regards to the potential role of DNA methylation status during the control of *ERBB2* gene expression in heart tissue (Holzmann et al., 1992; Hattori et al., 2001). Thus, the goal of this study was to examine whether DNA methylation status in the *ERBB2* locus influences gene expression in a collection of human myocardium samples. Complementary functional studies were performed to determine the effect of DNA methylation on the transcriptional regulation of *ERBB2*.

2. Material and methods

2.1. Human heart samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Heart samples (n = 33) were procured from The National Disease Research Interchange (NDRI, funded by the National Center for Research Resources), and The

Cooperative Human Tissue Network (CHTN, funded by the National Cancer Institute). Relevant diagnoses (Supplemental table 1) were obtained from anonymous medical histories. The postmortem to tissue recovery interval was 10 h. Heart samples (2 – 20 g, myocardium, left ventricle only) were frozen immediately after recovery and stored in liquid nitrogen until further processing. DNA and RNA was isolated from myocardial tissue by following standardized procedures as described (Gonzalez-Covarrubias et al., 2009; Kalabus et al., 2012). For the analysis of ERBB2 protein expression, myocardial tissue was homogenized in phosphate buffer supplemented with protease inhibitor (Thermo Fisher Scientific, Waltham, MA) using zirconium oxide beads and a Bullet Blender homogenizer (Next Advance, Averill Park, NY). The resulting homogenates were centrifuged at 8000 rpm for 5 minutes at 4°C, and the supernatants were collected for ERBB2 protein analysis. Total protein concentration was determined with the bicinchoninic acid assay kit (Thermo Fisher Scientific) as per the manufacturer's protocol.

2.2. Quantification of ERBB2 mRNA expression

Total RNA was isolated from myocardium samples with Trizol reagent (Thermo Fisher Scientific). *ERBB2* mRNA expression was analyzed by quantitative real time polymerase chain reaction with specific primers (*ERBB2*_F: 5'-CCTTCCTGCAGGATATCCAG-3'; *ERBB2*_R: 5'-CAAGATCTCTGTGAGGCTTCG-3') following the MIQE guidelines (Bustin et al., 2009). Total RNA (25 ng) was reverse transcribed and amplified with the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Venlo, The Netherlands). *ERBB2* and *ACTB* (reference gene, *ACTB*_F: 5'-GGACTTCGAGCAAGAGATGG-3', and *ACTB*_R: 5'-AGCACTGTGTTGGCGTACAG-3') were amplified in parallel in an iQ5 thermal cycler (Bio-Rad) with the following cycling parameters: 50°C for 30 min (reverse transcription), 95°C for 10 min (Taq DNA polymerase activation), 40 cycles of 95°C for 15 s (denaturation), 56°C for 30 s (annealing) and 72°C for 30 s (extension). Calibration curves were prepared to analyze linearity ($r^2 = 0.99$) and PCR efficiency for *ERBB2* (efficiency: 110%) and *ACTB* (efficiency: 101%) amplifications. For each sample, the averaged Ct values for *ERBB2* were normalized against the averaged Ct values for *ACTB* using the dCt method (Schmittgen and Livak, 2008). The expression of *ERBB2* mRNA in individual heart samples was expressed relative to the averaged expression of *ERBB2* mRNA in all samples, which was assigned an arbitrary value of 1.0.

2.3. Quantitative DNA methylation analysis of ERBB2

Targeted methylation analysis was performed with a Sequenom MassARRAY EpiTYPER (Sequenom, San Diego, CA) at the Genomics Shared Resource, Roswell Park Cancer Institute (Buffalo, NY). Briefly, genomic DNA from myocardial tissue was bisulfite-converted using an EZ Bisulfite conversion kit according to the manufacturer's instructions (Zymo Research, Irvine CA). Bisulfite-converted primers were designed with Sequenom's primer design algorithm (<http://www.epidesigner.com>). PCR amplification products were treated with shrimp alkaline phosphatase and T-cleavage transcription RNase A cocktail. Cleavage products and appropriate genomic DNA standards (0, 25, 50, 100 % methylated) were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Methylation data analysis was performed with Sequenom's proprietary software and MS Excel (Microsoft Office; Microsoft, Redmond, WA).

2.4. Quantification of ERBB2 protein expression

ERBB2 protein content in homogenates of myocardium was measured with the human ERBB2 ELISA kit (Abcam, Cambridge, MA) as per the manufacturer's instructions.

2.5. Cell culture

Lung carcinoma-derived A549 cells (American Type Culture Collection, Manassas, VA) were routinely cultured in T75 flasks using F12K (Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (Thermo Fisher Scientific), and 100 µg/ml streptomycin (Thermo Fisher Scientific). Cultures were grown and maintained at low passage numbers ($n < 12$) using standard incubation conditions at 37°C, 5% CO₂, and 95% relative humidity.

2.6. Reagents

Mithramycin A, NADPH, monobasic potassium phosphate, dibasic potassium phosphate, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich. Mithramycin A solutions were prepared with PBS. Control treatments included equal volumes of PBS vehicle.

2.7. ERBB2 reporter constructs and site-directed mutagenesis

CpG-free pCpGL basic and pCpGL-CMV/EF1 vectors were kindly donated by Dr. Michael Rehli (University Hospital Regensburg, Regensburg, Germany) (Klug and Rehli, 2006). A 499 base pairs (bp) DNA fragment from human *ERBB2* (−1 to −499 bp upstream the translation initiation codon A₊₁TG) was amplified by PCR with the following primers: *ERBB2*_{499f}, 5'-GGGTGTTAAGAGTGGCAGCC-3', and *ERBB2*_r, 5'-GGTGCTCACTGCGGCTCC-3'. The fragment was ligated into the BamHI site of the pCpGL basic vector. Deletions were produced by PCR with the following forward primers: *ERBB2*_{413f}, 5'-CGAGGAAAAGTGTGAGAACG-3'; *ERBB2*_{274f}, 5'-GAGCTGGGAGCGCGCTTG-3'; *ERBB2*_{192f}, 5'-GTTGTGAAGCTGAGATTCCCC-3'; *ERBB2*_{109f}, 5'-CCCTTTACTGCGCCGCGC-3'; *ERBB2*_{55f}, 5'-GCGCCCTCCCAGCCGGG-3'. All PCR products were cloned into the BamHI site of the pCpGL basic vector. Sp1 consensus binding sequences were mutated with the QuikChange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA). The following primers were used for site-directed mutagenesis (mutated bases are underlined): Sp1.1_f, 5'-TACTGCGCCGCGCAAACGCCCCACCCC-3'; Sp1.1_r, 5'-GGGGTGGGGGCGGTTTGCGCGCGCAGTA-3'; Sp1.2_f, 5'-CGCAGCACCCCGCAAACGCGCCCTCCCA-3'; Sp1.2_r, 5'-TGGGAGGGCGCGGTTTGCGGGGTGCTGCG-3'. All constructs were verified by DNA sequencing.

2.8. In vitro methylation of ERBB2 reporter constructs

The −499*ERBB2*-pCpGL reporter plasmid was methylated using the CpG methyltransferases M.SssI or HpaII (New England Biolabs, Massachusetts, USA). The extent of methylation was determined by bisulfite sequencing PCR. Briefly, plasmid DNA was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research). A 513 bp region of the −499*ERBB2*-

pCpGL reporter plasmid containing the CpG sites of interest was amplified by PCR with the following primers: *ERBB2*_{methf}: 5'-GGAGGGGGCAGAGTTATTAGTTTTT-3'; *ERBB2*_{methr}: 5'-AAACCTTTCTTAATATTCTTAACATCCTC-3'. PCR product were cloned into pCR 2.1- TOPO vectors (Invitrogen, Carlsbad, CA), and DNA methylation was verified by DNA sequencing.

2.9. Transfections

Twenty-four hours prior to transfections, cells were plated in 96-well plates. Cells were transfected with the specific *ERBB2* luciferase reporter construct or the backbone vector (100 ng) plus the internal control plasmid pRL-TK (10 ng) using the Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific). In co-transfection assays, cells were transfected with 100 ng of *ERBB2* reporter constructs, 10 ng of pRL-TK, and 150 ng of Sp1 expression vector or empty pCMV6-XL6 plasmid (OriGene, Rockville, MD). Twenty-four hours post transfection, cultures were washed once with PBS; cells were lysed in freshly diluted passive lysis buffer (100 µl/well, Promega) by incubating the plates at room temperature on a shaker at 200 rpm for 60 min. Luciferase reporter gene activities were determined with the Dual-Luciferase Reporter Assay System (Promega) as per manufacturer's instructions. Light intensity was measured in a Synergy HT luminometer equipped with proprietary software for data analysis (BioTek, Winooski, VT). Corrected firefly luciferase activities were normalized to renilla luciferase activities and expressed relative to the averaged activity of the ₋₄₉₉*ERBB2* construct which was assigned an arbitrary value of 100.

2.10. Cellular viability

The viability of mithramycin A treated A549 cells was assessed by recording the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich). Briefly, cells were plated in 96 well plates and treated with mithramycin A at different concentrations for 24 h. Then, 20 µl of MTT solution (5 mg/ml) were added to each well followed by incubation at 37 °C for 4 h. After incubation, the medium was removed and 100 µl of DMSO were added into each well. The plate was gently rotated on an orbital shaker for 10 min to dissolve the MTT precipitate. The absorbance at 570 nm was recorded in a Synergy HT luminometer (BioTek). Cellular viability was expressed as percentages relative to control incubations.

2.11. Chromatin immunoprecipitation

Assays were performed using the ChIP-IT High Sensitivity kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, A549 cells were cross-linked with 1% formaldehyde at room temperature for 5 min, repeatedly washed with ice-cold PBS, and lysed using a Dounce homogenizer followed by centrifugation. Chromatin was fragmented using sonication. A fraction of the mixture of protein-DNA complex was used as "input DNA". Sheared chromatin (15 µg) was then incubated overnight at 4°C with 5 µg of anti-Sp1 antibody (sc-14027-X, Santa Cruz Biotechnology, Dallas, TX) or normal rabbit IgG. Immuno-precipitated DNA was eluted using protein G agarose beads, then the cross-linking was reversed and the DNA was purified. A 109-bp fragment of the *ERBB2* proximal promoter region was amplified by PCR with the primers *ERBB2*_{109f} and *ERBB2*_R. PCR

products were electrophoresed on a 2% agarose gel stained with SYBR Safe (Thermo Fisher Scientific) for visualization.

2.12. Data analysis

The presence of transcription factor binding motifs in the *ERBB2* promoter region was examined with a combination of algorithms including PhysBinder (<http://bioit.dmbr.ugent.be/physbinder/index.php>) and the JASPAR core database (<http://jaspar.genereg.net/>) (Sandelin et al., 2004; Broos et al., 2013). Statistics were computed with Excel 2013 (Microsoft Office; Microsoft, Redmond, WA) and GraphPad Prism version 4.03 (GraphPad Software Inc., La Jolla, CA). The Kolmogorov - Smirnov test was used to analyze the normality of datasets. The Student's t test was used to compare group means. Spearman's rank correlation coefficient was used for the analysis of non-normally distributed data. Data are expressed as the mean $\pm$ standard deviation (SD). Differences between means were considered significant at $p < 0.05$.

Multiple regression models were fit for *ERBB2* normalized mRNA expression and ERBB2 protein expression using the covariates: Age, Sex (14 males and 16 females), Ethnicity, and six methylation sites and regions, defined as, r1: (−99 to −88 bp), r2: −74 bp, r3: (−64 to −55 bp), r4: −43 bp, r5: −33 bp, and r6: −19 bp. Specifically, the full model was represented as:

$$Y \sim \beta_0 + \beta_1 \times \text{Age} + \beta_2 \times \text{Sex} + \beta_3 \times \text{Ethnicity} + \beta_4 \times r1 + \beta_5 \times r2 + \dots + \beta_9 \times r6 + \varepsilon,$$

where the β_j for $j = 0, 1, \dots, 9$ are regression coefficients and the error term is $\varepsilon \sim N(0, 1)$. Models of this type were fit separately for *ERBB2* mRNA expression and ERBB2 protein levels. Ethnicity was stratified into White/European ($n = 21$) and non-White/European ($n = 7$) for regressions. There were five samples with missing values for ethnicity, which were taken out of the modeling when ethnicity was used as a covariate; one of which also had missing methylation data. There were also three observations that were missing for ERBB2 protein expression, which were eliminated from regression modeling. Methylation regions were transformed to a $\log(1 + x)$ scale for the modeling. From the full model, predictors were sequentially eliminated using hypothesis tests on the individual regression coefficients that show no significance ($p > 0.05$), with the exception of age, which was retained in all models.

3. Results

3.1. ERBB2 methylation status in human myocardium

First, the extent of DNA methylation in selected regions of the *ERBB2* locus was analyzed in 33 samples of myocardial DNA. DNA methylation analysis was focused on a 1 kb region centered at the translation initiation codon ($A_{+1}TG$) of *ERBB2* (Fig. 1A). Table 1 lists the most differentially methylated CpG sites in the *ERBB2* locus. The −19 to −160 bp region contained differentially methylated CpG sites exhibiting relatively high levels of DNA methylation in some samples. For example, three samples of myocardial DNA had highly methylated CpG sites ($>50\%$) in this region (Fig. 1B). Next, the expression of *ERBB2* mRNA and ERBB2 protein was examined in the samples of human myocardium. The

relative expression of *ERBB2* mRNA varied by 56 fold (range: 0.1 – 5.6 relative fold. Fig. 2A) and the expression of ERBB2 protein varied by 3 fold (range: 0.2 – 0.6 relative fold. Fig. 2B). There was no significant correlation between relative *ERBB2* mRNA expression and ERBB2 protein content in the samples of myocardium ($r_s = 0.289$, $p = 0.121$. Fig. 2C). There were significant negative associations between cardiac DNA methylation status at individual CpG sites within the –19 to –99 bp region of *ERBB2* and the myocardial expression of *ERBB2* mRNA (Fig. 2D).

Multiple linear regression for *ERBB2* mRNA expression were fit and the most parsimonious model was extracted (Table 2) with an adjusted R-squared = 0.3535. Differentially methylated regions in *ERBB2* were found to have negative associations with *ERBB2* mRNA expression at a suggestive ($p < 0.10$) and significant ($p < 0.05$) levels. Specifically, the suggestive regions are –99 to –88 bp ($p = 0.0859$) and –64 to –55 bp ($p = 0.0505$). The –74 bp CpG site was significant at $p = 0.0116$. Multiple linear regression models were also fit to explore associations between methylated CpG sites in *ERBB2* and ERBB2 protein content in myocardium. Table 3 summarizes the most parsimonious model (adjusted R-squared = 0.33). The model revealed a suggestive region at –99 to –88 bp ($p = 0.0912$), which was also found in the multiple regression model for *ERBB2* mRNA expression. A significant CpG site was also found at –43 bp ($p = 0.0426$).

3.2. Impact of DNA methylation on ERBB2 promoter activity

A fragment of *ERBB2* corresponding to the differentially methylated region in myocardial DNA (i.e. –499 to –1 bp) was cloned into a CpG-free luciferase reporter to perform gene reporter studies in A549 cells (Kawano et al., 2009). The $_{-499}ERBB2$ -pCpGL and $_{-413}ERBB2$ -pCpGL reporter constructs displayed similar promoter activities. Deletion of the –499 to –274 bp fragment led to a significant decrease in the activity of the *ERBB2* promoter; this region contains binding sites for the transcription factors AP2 and Sp1 (Fig. 3A) (Chen and Gill, 1994). To examine the impact of DNA methylation on *ERBB2* promoter activity, the $_{-499}ERBB2$ -pCpGL reporter construct was methylated using the methyltransferases SssI and HpaII. SssI methylates all CpG sites while HpaII methylates CpG sites within the CCGG sequence. Proper methylation of the inserts was confirmed by bisulfite sequencing. *ERBB2* promoter methylation with SssI led to an 86% decrease in *ERBB2* promoter activity in comparison with the unmethylated construct (Student's t-test $p < 0.001$, Fig. 3B). Methylation of the *ERBB2* promoter with HpaII methyltransferase also led to a significant decrease in *ERBB2* promoter activity (61%, Student's t-test $p < 0.001$. Fig. 3B).

3.3. Impact of DNA methylation on the regulation of ERBB2 promoter activity by Sp1

In addition to the functional Sp1 site identified by Chen and Gill (i.e. Sp1 site X), the *ERBB2* proximal promoter has two Sp1 motifs (A and B) with the consensus binding sequence CGCCC (Chen and Gill, 1994). Sp1 sites A and B are located in the differentially methylated –19 to –99 bp region (Fig. 2D). To verify the impact of Sp1 on the activity of the *ERBB2* promoter, cells transfected with the $_{-499}ERBB2$ -pCpGL reporter construct were treated with a non-toxic concentration of mithramycin A (Supplemental table 3). Mithramycin A binds to DNA GC boxes and inhibits the binding of Sp1 to its corresponding

transcriptional motifs (Blume et al., 1991). Treatment with mithramycin A (100 μ M) decreased the activity of the *ERBB2* promoter by 77% in comparison to control incubations (Student's t-test $p < 0.001$, Fig. 4A). Next, similar experiments were performed by co-transfecting a Sp1 expression construct. Co-transfection of the Sp1 expression construct led to a 64% increase in the activity of the *ERBB2* promoter (Student's t-test $p < 0.001$, Fig. 4A).

The potential contributions of Sp1 binding sites A and B were further examined by performing site directed mutagenesis. Gene reporter assays showed that mutation of the binding cores of Sp1 sites A and B led to significant decreases (Sp1 site A: 64% decrease, Student's t-test $p < 0.001$; Sp1 site B: 28% decrease, $p < 0.001$) in *ERBB2* promoter activity in comparison to non-mutated constructs (Fig. 4B). The interaction between Sp1 and the proximal promoter region of *ERBB2* was confirmed with a chromatin immunoprecipitation assay (Fig. 4C).

To investigate the effect of *ERBB2* promoter methylation on the binding of Sp1, gene reporter assays were performed in cells co-transfected with differentially methylated $_{-499}$ *ERBB2*-pCpGL reporter constructs and the Sp1 expression construct. Bisulfite sequencing showed that SssI methylated CpG sites in Sp1 binding sites A and B whereas HpaII methylated a single CpG site (CpG -88) located in Sp1 site A. The exogenous expression of Sp1 significantly enhanced the activity of the HpaII methylated *ERBB2* promoter in comparison to the vector control (95%, Student's t-test $p = 0.001$, Fig. 5). Methylation of the three Sp1 sites (i.e. Sp1X, Sp1 A and Sp1 B) in the *ERBB2* promoter abrogated the induction of promoter activity by the exogenous expression of Sp1 (Fig 5).

4. Discussion

The results from this study suggest that DNA methylation status in the -19 to -160 bp region of *ERBB2* impacts gene expression in human myocardium. The expression of *ERBB2* mRNA and ERBB2 protein in human myocardium is variable (Figs 1 and 2). Linear regression suggest that a fraction of the variation in *ERBB2* mRNA expression can be explained by differential methylation in specific CpG sites within the *ERBB2* locus (e.g. CpG sites -99 to -98: ~17% of variation, and CpG sites -43, -33 and -19: ~13% of variation, Fig. 2). Multiple linear regression lend further support to the notion that increased methylation in the proximal promoter region of *ERBB2* is associated with a decrease in *ERBB2* mRNA expression (Table 2). Results from multiple regression also suggest that differential methylation impacts myocardial ERBB2 protein expression (Table 3).

Analysis of the *ERBB2* promoter region showed two putative Sp1 binding sites (A and B) in the -19 to -99 bp region. It is known that interactions between Sp1 and certain CpG islands contribute to the control of gene expression (Clark et al., 1997). Furthermore, methylation of the CpG dinucleotide within the canonical Sp1 binding site (5'-GGGCGG-3') inhibits the expression of several Sp1 target genes (Mancini et al., 1999; Zelko et al., 2010; Mamrut et al., 2013). Thus, it is possible that differential CpG methylation in the -19 to -99 bp region may impact the binding of Sp1, and consequently, the expression of *ERBB2* mRNA in myocardium. To assess how DNA methylation may influence the expression of *ERBB2*,

gene reporter experiments were performed using a CpG-less luciferase reporter construct (Klug and Rehli, 2006). The experiments with mutated $_{-499}ERBB2$ -pCpGL promoter constructs suggested that Sp1 sites A and B contribute to *ERBB2* promoter activity (Figs. 3 and 4). Furthermore, the results from methylation experiments with SssI and HpaII lend further support to the notion that differential CpG methylation in Sp1 sites A and B impacts promoter activity (Fig. 5).

We propose that *ERBB2* gene promoter methylation may alter the dynamics of cardiac ERBB2 expression within the context of therapy with trastuzumab. In cells with low basal expression levels of ERBB2, the treatment with trastuzumab results in intracellular clearance of the ERBB2-trastuzumab membrane complexes followed by an overall decrease in ERBB2 receptor levels due to lysosomal degradation and negligible recycling (Ram et al., 2014). In cardiomyocytes, the expression of ERBB2 is relatively low in comparison to other cell types (Fuchs et al., 2003). Thus, it is possible to speculate that myocardial cells with the “high *ERBB2* methylation-low *ERBB2* expression signature” would exhibit diminished protein translation and expression of ERBB2 after repeated exposure to trastuzumab in comparison to cells with low *ERBB2* methylation levels. Of note, typical chemotherapeutic regimens for ERBB2 positive breast cancers include the serial administration of trastuzumab for up to one year (weekly doses of 2 – 6 mg/kg) plus the administration of cytotoxic drugs such as doxorubicin and cyclophosphamide. Notably, an imaging study with 111 Indium-trastuzumab showed that the administration of doxorubicin induced the myocardial expression of ERBB2 in up to 50% of the patients (de Korte et al., 2007). Therefore, it would be interesting to explore whether the “high *ERBB2* methylation-low *ERBB2* expression signature” impacts the intracellular dynamics of ERBB2 and ERBB2-trastuzumab complexes in the context of chemotherapy with doxorubicin.

In the breast cancer setting, the cardiotoxic potential of trastuzumab increases with the administration of anthracyclines (e.g. doxorubicin) (Onitilo et al., 2014). Recent studies have identified multiple associations between variants in germline DNA (e.g. *SLC28A3* rs7853758, *RARG* rs2229774, *CELF4* rs1786814) and the risk for anthracycline-related cardiotoxicity (Aminkeng et al., 2016). The impact of non-synonymous polymorphic variants in *ERBB2* (e.g. rs1058808 and rs1136201) on the risk of trastuzumab cardiotoxicity has also been investigated (Beauclair et al., 2007; Stanton et al., 2015). It is possible that a combination of genetic and epigenetic factors may contribute to the risk of cardiotoxicity in patients exposed to the trastuzumab-anthracycline combination. Whereas the interrogation of polymorphic genetic variants for the prediction of drug toxicity can be performed by using DNA isolated from accessible locations (e.g. oral cavity, peripheral blood), the examination of epigenetic signatures such as DNA methylation in *ERBB2* would require cardiac DNA obtained from a tissue biopsy; this requirement hampers the potential clinical utility of epigenetic profiling for the prediction of drug toxicity. In an attempt to tackle this limitation, we examined methylation profiles in the *ERBB2* locus in a subset of 11 paired DNA samples isolated from myocardial tissue and peripheral white blood cells (WBCs), respectively. There were significant associations between *ERBB2* methylation statuses in paired samples of myocardial DNA vs white blood cells DNA (Supplemental Fig. 1). For example, the CpG site –398 located outside the –19 to –99 bp region showed variable levels of methylation in DNA from WBCs and myocardium (CpG –398; WBCs: 19 – 53%,

myocardium: 0 – 23%). Similarly, CpG site –33, located inside the –99 to –19 bp regulatory region exhibited variable levels of DNA methylation (CpG –33; WBCs: 0 – 54%, myocardium: 0 – 7%) (Supplemental Fig. 2). Although limited by the small sample size, this pilot finding suggests that *ERBB2* methylation analysis in DNA isolated from white blood cells may be a surrogate to infer *ERBB2* methylation status in myocardial tissue DNA from the same individual.

The findings from this study suggest that *ERBB2* methylation status in myocardial tissue contributes to a fraction of the variability in cardiac *ERBB2* expression. This study provides a foundation to test whether differential DNA methylation status in the proximal promoter region of *ERBB2* impacts on the cardiotoxic potential of trastuzumab by modifying the intracellular dynamics of ERBB2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACTB	beta-actin
AP2	activating protein 2
CELF4	CUGBP Elav-like family member 4
ChIP	Chromatin Immunoprecipitation
DMSO	Dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAPK	mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase

RARG	Retinoic Acid Receptor Gamma
SLC28A3	solute carrier family 28 member 3
Sp1	specificity protein 1
WBCs	white blood cells

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Highlights

- The proximal promoter region of *ERBB2* contains differentially methylated CpG sites in DNA samples isolated from human myocardium.
- *ERBB2* mRNA expression in myocardium is variable, and a fraction of the variation can be explained by differential methylation in specific CpG sites within the *ERBB2* locus.
- Differential methylation in specific Sp1 sites within the proximal promoter region of *ERBB2* impacts promoter activity.

Fig. 1. DNA methylation analysis of *ERBB2* in human myocardium

(A) Schematic representation of the -500 to +500 bp region of *ERBB2*. Vertical boxes represent CpG sites. Potential binding sites for transcription factors AP2, YY1, and Sp1 are represented. (B) Quantitative DNA methylation analysis of the *ERBB2* locus in myocardium samples. Points depict methylation (%) of individual samples versus the location of CpG sites in *ERBB2*.

Fig. 2. *ERBB2* expression in human myocardium
(A) *ERBB2* mRNA expression. (B) *ERBB2* protein content. Each symbol depicts the average of individual samples. Horizontal lines indicate group means. Insets show the corresponding calibration curves. Samples were analyzed in triplicates. (C) Linear regression analysis of *ERBB2* mRNA expression versus *ERBB2* protein expression in myocardial samples. (D) Linear regression analysis of *ERBB2* methylation levels at distinct CpG regions versus *ERBB2* mRNA expression.

Fig. 3. Reporter gene activities of human *ERBB2* promoter constructs

(A) Luciferase activities of progressive *ERBB2* deletion constructs in A549 cells (see text for details). Normalized luciferase activities were expressed relative to the values from the $_{-499}ERBB2$ -pCpGL construct, which was assigned an arbitrary value of 100. (B) Effect of DNA methylation status on the luciferase activity of the $_{-499}ERBB2$ -pCpGL construct. CpG sites methylated by HpaII or SssI methyltransferase are indicated. Data represent the mean $\pm$ standard deviation of three independent experiments. *** $p < 0.001$ (Student's t-test).

Fig. 4. Sp1 interactions with human *ERBB2* promoter constructs

(A) Luciferase activities of the $-499ERBB2$ -pCpGL construct in A549 cells treated with 100 nM mithramycin A or cotransfected with the Sp1 expression vector for 24 hours. Normalized luciferase activities were expressed relative to the values from the control treatment, which was assigned an arbitrary value of 100. (B) Luciferase activities of mutated *ERBB2* constructs (see text for details). For each mutated construct, normalized luciferase activities were expressed relative to the values from the $-499ERBB2$ -pCpGL construct, which was assigned an arbitrary value of 100. (C) Chromatin immunoprecipitation (ChIP) analysis of Sp1 binding to the *ERBB2* promoter. Data represent the mean $\pm$ standard deviation of three independent experiments. *** $p < 0.001$ (Student's t-test).

Fig. 5. Effect of DNA methylation on the binding of Sp1 to *ERBB2* promoter constructs
 Effect of Sp1 overexpression on the luciferase activity of differentially methylated $_{-499}ERBB2$ -pCpGL constructs. CpG sites methylated by HpaII or SssI methyltransferase are shown. Data represent the mean $\pm$ standard deviation of three independent experiments. *** $p < 0.001$ (Student's t-test).

Table 1*ERBB2* methylation status at individual CpG nucleotides

CpG position	Methylation range (%)
-160	0 – 79
-138	0 – 95
-130 to -126	5 – 96
-114	0 – 95
-99 to -88	0 – 100
-74	3 – 100
-64 to -55	0 – 96
-43	0 – 100
-33	0 – 78
-19	0 – 81

Table 2

Results of multiple regression analysis for *ERBB2* mRNA expression

	Beta Est.	Std. Error	t-stat	P-value	Significance
Intercept	4.1529	1.2129	3.4239	0.0023	***
Age	-0.0266	0.015	-1.7711	0.0898	*
reg. -99 to -88	-1.0718	0.5973	-1.7943	0.0859	*
reg. -74	-1.7033	0.6211	-2.7424	0.0116	**
reg. -64 to -55	1.2439	0.6027	2.0639	0.0505	*

Significance codes are as follows:

- *** for $p < 0.001$,
- ** for $p < 0.01$,
- * $p < 0.05$,
- $p < 0.1$

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Table 3

Results of multiple regression analysis for ERBB2 protein expression

	Beta Est.	Std. Error	t-stat	P-value	Significance
Intercept	0.3275	0.1009	3.2455	0.0037	***
Age	-0.0001	0.0014	-0.1223	0.9038	
reg. -99 to -88	0.0645	0.0365	1.7665	0.0912	*
reg. -43	-0.0696	0.0324	-2.1529	0.0426	**

Significance codes are as follows:

- *** for $p < 0.001$,
- ** for $p < 0.01$,
- * $p < 0.05$,
- $p < 0.1$