DNMT1 has prognostic values in HER2-positive breast cancer

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Abstract

Background:

Interleukin-6 (IL-6) was found to induce aberrant methylation in critical genes involved in insulin signaling and angiogenesis in humans, presumably due to protein stabilization of DNA methyltransferases. Whether IL-6 and DNMT1 impact breast cancer (BC) prognosis remains unknown.

Methods

TIMER2.0 web server was used for comprehensive analysis from TCGA. Associations between DNMT1 and IL-6 in tumor immune microenvironment was explored via single cell sequencing (SCS) from TISCH. IL-6 and DNMT1 expressions were investigated in tissue microarray of our own cohort (n = 285) as well as in BC cell-lines. Invasion activity was compared between high and low IL-6/DNMT expressing BC cell-lines treated with/without IL-6 antibody.

Results

DNMT1 mRNA was significantly higher in the BC tissues (p < 0.001) with a mutation rate of 1.16%. A positive correlation between IL-6 and DNMT1 protein levels was found in tissue array. Increased IL-6 mRNA did not appear to be a good prognostic marker for overall survival in HER2 + BC patients whereas higher DNMT1 mRNA was a good prognostic marker for poor overall survival in HER2 + BC patients. Among different BC subtypes in our cohort, hormone receptor negative (HR-) /HER2 positive (HER2+) patients had the poorest survival (n = 43). Cox regression indicated that IL-6, and DNMT1 are independent prognostic factors in HR-/HER2 + BC patients. DNMT1 expressed in malignant cells, also in innate and adaptive immune cells including macrophages, CD4(+)T and CD8(+)T cells, whereas IL-6 was only found in malignant cells. HER2 + MDA-MB-453 (high IL-6/high DNMT1) exhibited higher invasiveness compared to HER2 + SKBR3 (low IL-6/low DNMT1). IL-6 (10 ng/ml) significantly promoted the invasiveness in SKBR3 whereas IL-6 antibody (10 µg/ml) significantly suppressed the invasiveness of MDA-MB-453.

Conclusions

DNMT1 overexpression could be responsible for HR-/HER2 + BC progression in tumor immune microenvironment. We suggest that IL-6 inhibition in combination with anti-HER2 therapy is a potential therapeutic strategy for treating DNMT1-overexpressing HER2-positive BC patients.

Introduction

For female cancers, the total 50% of all new diagnoses are those originated from breast, lung, and colorectal tissues; among them breast cancer accounts for 30%, lung cancer accounts for 12%, and
colorectal cancer accounts for 8% [1]. The death rate of female breast cancer has declined from its peak by 40% between 1989 and 2017 [1]. However, the reductions slowed down for female breast and colorectal cancers over the past decade. The incidence rates of breast cancer increased by approximately 0.3% per year since 2004, presumably attributed to the decreased fertility rate and the increased obesity prevalence [2].

The discordant expression of Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) in breast tumors has different clinical outcomes [3]. The expression of ER is observed in 70–80% of breast tumors and PR is expressed in approximately 60–70% of breast tumors [4, 5]. Overexpression of oncoprotein HER2 is present in approximately 20–25% of primary breast tumor that was determined by immunohistochemistry (IHC) staining. Approximately 10–20% of the ambiguous cases by IHC were validated as HER2-amplified by fluorescence in situ hybridization (FISH) [6].

The St Gallen International Breast Cancer Conference Expert Panel has endorsed four classification of breast cancer molecular subtypes: luminal A (ER + and/or PR+), luminal B (ER + and/or PR+/HER2+), HER2-positive (ER-, PR-, HER2 overexpression), and basal-like triple-negative breast cancer (TNBC) (ER- and PR-/HER2-) since 2013. Among the breast cancer molecular subtypes, luminal A was the most frequently diagnosed molecular subtype (59.0%), and usually has the greatest survival, whereas triple-negative had the poorest survival from the Ontario Cancer Registry [7]. On the other hand, Her2/neu positive cases had presented the worst 5-year disease-free survival and overall survival in Southern China [8]. In assessing breast cancer survival, the molecular subtype, along with age, stage at diagnosis and comorbidity are critical. The survival of hormone-receptor-negative cancers lags behind that for hormone-receptor-positive cancers [9]. Previously we demonstrated that protein expression of metadherin (MTDH) and interleukin-10 (IL-10) are independent predictors of worse prognosis, especially in ER-Negative or PR-Negative breast cancer patients [10], suggesting that different molecular markers may apply for different subtypes of breast cancer. The pleiotropic cytokine interleukin-6 (IL-6) is a key player in systemic inflammation that regulates both the inflammatory response and tissue metabolism during acute stimulations. Its systemic levels being a biomarker for tumor burden, physical in-activity, and impaired metabolism, while local intratumoral IL-6 signaling was found to be critical in controlling breast cancer cell growth, metastasis, and self-renewal of cancer stem cells [11]. IL-6 expression is associated with poor prognosis and anti-IL-6 has therapeutic potential for estrogen receptor-α (ERα)-positive breast cancers [12].

DNA hypermethylation is a common early event in carcinogenesis, which is typically mediated by DNA methyltransferases (DNMTs) [13, 14]. Inactivation of tumor suppressor genes, often results from epigenetic silencing associated with promoter hypermethylation, is central to the development of human cancer [13, 14]. Silencing DNA methyltransferase 1 (DNMT1) inhibits proliferation, metastasis and invasion in esophageal squamous cell carcinoma (ESCC) [15]. The protein expression of DNMT1 were also increased after IL-6 induction and further regulated epithelial-mesenchymal transition (EMT) in normal prostate epithelial cells [16].
In vitro deletion studies discovered that DNMT1 and DNMT3b cooperatively maintain DNA methylation and gene silencing in human cancer cells; and such methylation is essential for optimal tumor proliferation [14]. Deletion of DNMT1 and DNMT3b resulted in loss of insulin-like growth factor II (IGF2) imprinting [17]. Dysregulation of the expression and function of insulin receptor (IR) and insulin-like growth factors (IGFs) and receptor (IGF-1R) signaling pathway is present in many tumors including breast cancers. Aberrant IR/IGF-1R signaling and their downstream signaling effectors drive breast cancer initiation and progression, often in a subtype-dependent manner [17]. Animal models suggested a highly specific role of the insulin receptor in breast cancer progression [18].

In human umbilical vein endothelial cells (HUVECs), IL-6 treatment reduced DNMT1 and DNMT3B but not DNMT3A protein levels. IL-6 also resulted in promoter hypo- and hypermethylation of genes associated with insulin signaling and angiogenesis, presumably due to protein stabilization of DNA methyltransferases [19]. These findings suggested a possible causal link between IL-6-induced changes in DNA methylation that may alter expression of critical genes involved in insulin signaling and angiogenesis in humans. Optimal methyl donor balance and methylation are essential for metabolic reprogramming and cancer prevention and antifolate has been a treatment for cell proliferative diseases [20] including cancer. We recently discovered that knocking down methylene tetrahydrofolate reductase assists cell defense against folate depletion induced chromosome segregation and uracil misincorporation in the DNA, shedding light on the potential regulatory mechanism by which folate and methylation balance modulates the risk for tumorigenesis [21]. Methionine cycle enzymes methionine adenosyltransferases (MATs) catalyze the formation of S-adenosylmethionine (SAM), the principal biological methyl donor, whereas glycine N-methyltransferase (GNMT) utilizes SAM for sarcosine formation. Our previous studies showed that GNMT is critical for of hepatic methyl group balance homeostasis and DNA methylation [22], for hepatic folate-dependent homocysteine remethylation [23], as well as for cellular defense against DNA damage in the liver [24]. Downregulation of methionine cycle genes MAT1A and GNMT enriches protein-associated translation process and worsens hepatocellular carcinoma prognosis [23]. Our recent study also revealed that expression of GNMT protein is downregulated in breast tumors [24]; and that MAT2A localization is independently prognostic for breast cancer patients [25]. These studies demonstrated that perturbation in methylation balance is involved in tumorigenesis including breast cancer development. In this study, we present that overexpression of IL-6 and DNMT1 coincide a highly positive correlation and worse prognosis in HER2-overexpressing breast cancer patients.

Materials And Methods

Patients

This single cancer center cohort study enrolled women diagnosed with breast cancer and treated in Changhua Show-Chwan Memorial Hospital Cancer Center between March 2011 to January 2017. All pathologic reports in this project were histopathologically confirmed by two independently experienced pathologists according to breast cancer diagnostic criteria.
All clinical characteristics, molecular types, and survival data were recorded in the cloud of the cancer registry system of Changhua Show-Chwan Memorial Hospital that is anonymously linked to the Taiwan Cancer Registry (http://www.iacr.com.fr/). The variables included age, gender, tumor size, N (lymph nodes), m (metastasis), stage, ER, PR, HER2 status, Ki67, date of operation, diagnosis, death. Survival data was annotated to be the time from the date of primary surgery to the date of death. The diagnosis parameters and clinical outcomes were recruited until patient death or loss to follow-up. This project was in accordance with Good Clinical Practice and also approved by the Ethics Committee of the Institutional Review Board (IRB) of Show-Chwan Memorial Hospital (IRB No. 1060407).

**Immunohistochemistry and scoring**

For each breast cancer patient, tumor specimens as well the paired normal specimens were carefully sliced and fabricated in the tissue microarray. IHC was performed using primary antibodies specifically against IL-6 (GenTex, GTX110527, Alton Pkwy Irvine, CA 92606 USA) and DNMT1 antibody (GenTex GTX116011), and secondary antibodies were then applied the manufacturer’s suggestion of those primary antibodies. IHC image evaluation and protocol used to determine score have been previously described [10].

**Web Server Survival Analysis**

The correlation of expression of IL-6 and DNMT1 mRNA in breast cancer patients was calculated using Pearson’s correlation using data at Timer2.0 at http://timer.cistrome.org/. The associations between DNMT1, IL-6 and tumor immune microenvironment were explored via the Tumor Immune Single-cell Hub (TISCH), a single cell RNA sequencing (scRNA-seq) database focusing on tumor microenvironment. TISCH provides detailed cell-type annotation at the single-cell level, enabling the exploration of TME across different cancer types at http://tisch.comp-genomics.org.

**Cell culture**

The human breast cancer cells, MDA-MB-453 and SKRB3 (ATCC, Gaithersburg, MD, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (HyClone, Logan, UT, USA) and maintained in a humidified incubator with 5% CO2 at 37°C.

**Western blot analysis**

Proteins from breast cancer cells were extracted and separated by 10% SDS–PAGE before transferring to a nitrocellulose membrane, which was subsequently exposed to the appropriate the mixture of the primary antibody and its diluted buffer (1:1000) before detection using the horseradish peroxidase conjugated secondary anti-mouse or anti-rabbit antibody. All the nitrocellulose membranes were added Enhanced chemiluminescence (ECL) (Millipore, Darmstadt, Germany), and then visualized using the enhanced plus chemiluminescence assay kit (EMD Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Primary and secondary antibodies used as follows: IL-6 (GTX110527; GeneTex), GAPDH (GTX100118; GeneTex), DNMT1 (GTX116011; GeneTex), and HRP-conjugated polyclonal
secondary antibody (1:5,000; GTX213110-01/GTX213111-01; GeneTex). Protein expression levels were quantified by ImageJ software as described previously [26].

**Transwell invasion assay**

For invasion assay, SKRB3 cells were treated with 10 ng/mL human recombinant IL-6; and MDA-MB-453 cells were treated 10 µg/mL anti-IL-6 monoclonal antibody, the referenced concentration as previously described [10]. The cell invasion was conducted using Matrigel invasion chambers with a pore size of 8 µm (Costar; Corning Life Sciences, Cambridge, MA, USA) as previously described [26]. In brief, a total of 4×10^4 MDA-MB-453 of SKRB3 in serum-free medium were seeded in each well of the upper chamber, and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc. USA) was used as a chemoattractant in the bottom well. After incubation for 24 h at 37°C, the non-invasive cells on the upper surface of the membrane were removed, and the invasive cells on the bottom side were fixed in 100% methanol at room temperature, stained with 1% crystal violet, and counted using a microscope under ×200 magnification with five fields of view per cells [26].

**Statistical analysis**

Chi-squared tests were used to examine the associations between IL-6 and DNMT1 protein expression with the clinical and pathological parameters; and Pearson's correlation was performed to examine the association between IL-6 and DNMT1 protein expressions. The overall survival curves were drawn by the Kaplan-Meier model and compared using a log–rank test. Additionally, Cox's proportional hazards regression model was used to analyze the associations between age, stage, IL-6, and DNMT1, and p < 0.05 was considered statistically significant. SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was applied to all statistical analyses.

**Results**

**DNMT1 mRNA expression in TCGA datasets from TIMER2.0**

We performed a pan-cancer analysis of DNMT1 expression, and found that DNMT1 was upregulated in several different tumor types from web platform TIMER2.0 (http://timer.cistrome.org/) for The Cancer Genome Atlas (TCGA) (Fig. 1A). Specifically, transcription level of DNMT1 was significantly higher in the breast invasive carcinoma (BRCA) tissues (n = 1093) than that in normal tissues (n = 112, p < 0.001) (Fig. 1A). TIMER2.0 displays a bar plot showing somatic mutation frequency of the gene for each TCGA cancer type, and DNMT1 mutation rate was 1.16% (12/1026) (Fig. 1B).

**The association between DNMT1 and tumor immune microenvironment in breast invasive carcinoma (BRCA)**

We first explored the correlation between DNMT1 and IL-6 mRNA expression in breast invasive carcinoma (BRCA). Analysis of the TGCA database found a strong correlation between the two (n = 1100, P < 0.0001; Fig. 1C). IL-6 did not appear to be a good prognostic marker for overall survival in HER2+ BC patients.
On the other hand, HER2 + BC patients with higher DNMT1 mRNA expression had poor overall survival (Fig. 1E).

To further explore the roles of DNMT1 and IL-6 during immunologic events of BRCA using single cell sequencing data via TISCH, we discovered that DNMT1 but not IL-6 were expressed in malignant cell cluster in a dataset (BRCA_GSE143423) without chemotherapy, immunotherapy, or targeted therapy (Fig. 2A). Data from the other dataset (BRCA_GSE114727_10X) without chemotherapy, immunotherapy, or targeted therapy showed that DNMT1 was positively correlated with specific types of immune cells, including CD8(+) T cells, CD4(+) T cells, regular T cells, proliferating T cells, and macrophages, whereas IL-6 was not detected in any innate or adaptive immune cells (Fig. 2B). Furthermore, DNMT1 was positively correlated with specific types of innate immune cell infiltration, including M0 macrophages, M1 macrophages, and M2 macrophages (Fig. 2C-2E), and also found in the adaptive immune cells (CD4 T cells, CD8 T cells, regulatory T cells (Tregs), and T helper cells (Fig. 2F-2I).

**IL-6 and DNMT1 protein expression in breast cancer tissues**

Specimens of 285 breast cancer patients from our cohort study were further examined for the expression of IL-6 and DNMT1 proteins were investigated in breast tumor tissues. The clinicopathological data are presented in Table 1, and representative IHC staining for each molecule is shown in Fig. 3A-D. Pearson's correlation analysis revealed that IL-6 was positively correlated with DNMT1 protein level in breast tumor tissues ($r = 0.53$, $P < 0.001$, $n = 285$, Fig. 3E). The immunohistochemically ER negative expression was significantly observed in 60% (49/82) patients with lower DNMT1 expression ($P = 0.040$, Table 1).

**Identification of IL-6 and DNMT1 expression signature for survival**

We examined the correlation between the IL-6 and DNMT1 expression levels and determined the correlations between clinical parameters, IL-6 and DNMT1 in the five-year relative survival rate in 285 patients. Kaplan–Meier analysis were conducted to examine associations between stage, age, expression level of ER, PR, HER2, Ki67, IL-6, DNMT1 and the survival rate in 285 patients. The results revealed that shorter overall survival in breast cancer patients were significantly associated with late stage (stage III and stage IV) ($p < 0.001$, Fig. 4A), aged 65 and over ($p < 0.001$, Fig. 4B), ER-negative type ($p = 0.002$, Fig. 4C), and PR-negative type ($p = 0.003$, Fig. 4D).

In our tissue microarray samples, the HER2 and Ki67 expression showed weaker associations with breast cancer survival rate compared to stage, age, ER or PR status (Fig. 4E and 4F, $P = 0.115$ and $P = 0.225$, respectively), and IL-6 and DNMT1 expression was associated with a trend of decreased breast cancer survival rate compared with tumors that stained low for IL-6 and DNMT1 (Fig. 4G and 4H, $P = 0.064$ and $P = 0.370$, respectively). As for the clinicopathologic characteristics of our cohort study, 53.7% (153/285) were found luminal A subtype, 18.2% (52/285) were luminal B subtype, 15.1% (43/285) were HER2 subtype, and 13.0% (37/285) were TNBC-subtype (Table 1). Among the HR+ subtypes, patients with luminal B subtype had less survival rate than luminal A subtype; and among the HR- subtypes, HER2
positive patients showed lower survival rate than TNBC (P = 0.037, Fig. 4I). As the HR-/HER2 positive patients had poorest survival in our cohort, we further investigated the potential roles of DNMT1 and IL6 in BRCA prognosis in patients of this subtype.

**Protein expression pattern of IL-6 and DNMT1 is the independent prognostic factors for HER2- positive breast cancer outcomes**

Kaplan–Meier survival analysis showed that in HR- patients, HER2 expression was associated with a trend of decreased breast cancer survival rate compared with tumors that stained negative for HER2 (Fig. 4I, P = 0.037). HER2 expression was negatively correlated with DNMT1 expression in our patients (P = 0.065, Table 1). Herein, we further performed stratified analysis to evaluate the prognostic significance of IL-6 and DNMT1 expression in HER2- positive breast cancer patients. The results revealed that high IL-6 expression in the tumorous tissues was strongly and significantly associated with poorer survival in HER2-positive breast cancer patients (P = 0.032, Fig. 5A); and high DNMT1 expression in the tumorous tissues was also strongly and significantly associated with poorer survival in HER2-positive breast cancer patients (P = 0.043, Fig. 5B). Furthermore, breast cancer patients with high expressions of IL-6 and DNMT1 had the worse survival rate in HER2-positive (P = 0.032, Fig. 5C). In addition, multivariate logistic-regression analysis indicated that the high expression of IL-6, DNMT1, and IL-6 and DNMT1 significantly correlated with poorer survival (hazard ratio = 5.94, 95%CI = 1.12–31.63, p = 0.037 for IL-6; hazard ratio = 5.87, 95%CI = 1.36–25.38, p = 0.018 for DNMT1; hazard ratio = 5.87, 95%CI = 1.36–25.38, p = 0.018 for IL-6 and DNMT1) (Fig. 5D-5F). Taken together, HER2-positive breast cancer patients with concurrent high IL-6 and DNMT1 expressions in the tumorous tissues had poorest prognosis that is independent of age, ER, PR, Ki67, and tumor-node-metastasis (TNM) stage.

**Expression of IL-6 and DNMT1 is associated with invasive ability in HER2-positive cells**

As HER2-positive breast cancer patients with concurrent high IL-6 and DNMT1 expressions had poorest prognosis, we used HER2 positive breast cancer cell-lines to examine the relationships between IL-6 and DNMT1 expression pattern with their invasive abilities. The basal levels of IL-6 and DNMT1 protein expression were compared between two HER2 positive breast cancer cell lines, MDA-MB-453 and SKBR3. IL-6 and DNMT1 levels were higher in MDA-MB-453 compared to those in the SKBR3 using western blotting (Fig. 5F).

To examine whether IL-6 can increase invasiveness in low IL6 expressing cells, the effect of IL-6 was examined in the low IL-6 expressing SKBR3 cells. Exogenous IL-6 (10 ng/ml) treatment significantly promoted SKBR3 cell invasion (P = 0.002, Fig. 5G), indicating IL-6 can promote cell invasion in low IL-6 expressing breast cancer cells. To determine whether the observed higher IL-6 expression may account for the invasion ability in MDA-MB 453, high IL-6 expressing MDA-MB-453 cells were treated with IL-6
antibody (10 µg/ml); and a significant decrease in cell invasion was observed using the Boyden chamber assay (P = 0.012, Fig. 5G).

**Discussion**

In combination of public RNA-seq data, human tissue array from our own cohort, and cell model experiments, the present study demonstrated novel findings that HER2-positive breast cancer patients with concurrent high IL-6 and DNMT1 expressions in the tumorous tissues had poorest prognosis, independent of age, ER, PR, Ki67, and tumor-node-metastasis (TNM) stages. Using *in vitro* models of breast cancer cells, we also compared the expression pattern of IL-6 and DNMT1, and discovered that HER2-positive breast cancer cell lines with elevated IL-6 and DNMT1 exhibited increased invasiveness. Addition of exogenous IL-6 significantly increased the invasiveness of low IL-6 expressing HER2-positive breast cancer cells. Furthermore, inhibition of IL-6 by IL-6 monoclonal antibody significantly suppressed the invasiveness of IL-6 overexpressing HER2-positive breast cancer cells. These results provided potential mechanisms by which IL-6 and DNMT overexpression may intertwine with one another and contribute to the poor prognosis of HER2-positive breast cancer patients.

Oncoprotein HER-2 is commonly (20–25%) overexpressed in invasive breast cancers and has been associated with aggressive tumor phenotypes and reduced survival rate. Although trastuzumab in combination with chemotherapy is currently considered one of the most effective therapies in treating breast cancer, many HER-amplified breast cancer patients cannot benefit from this human HER2 monoclonal antibody therapy due to drug resistance [27]. The potential mechanisms for such drug resistance include: mutations that prevent trastuzumab from binding to HER2, upregulation of HER2 downstream signaling pathways mediated from other HER receptors, and failure to trigger an immune-mediated mechanism to kill cancer cells [27, 28, 29, 30, 31]. Additionally, the presence of primary and acquired resistance to trastuzumab treatment involves the escape from antibody-dependent cell-mediated cyto-toxicity such as the inhibition of cell death through the phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway; expression of other TKRS and proteins in the cellular membrane; crosstalk between estrogen receptor and HER2 pathways; and intrinsic alterations in HER2. The cancer cell can escape trastuzumab via ER activity in ER-positive/HER2-positive cells that decreases trastuzumab binds to Fragment crystallizable region (Fc) receptors on immune response cells by the FcRIIa-158 valine (V)/phenylalanine (F), FcRIIa-131 histidine (H)/arginine (R), and FcRIIb-232 isoleucine (I)/threonine (T) polymorphisms [31]. The alternate pathways of trastuzumab resistance include: loss function of the tumor suppressor phosphatase and tensin homolog (PTEN) gene, enriched Akt signaling; and insulin-like growth factor 1 (IGFR1) receptor signaling leads to decreased sensitivity to trastuzumab [31, 32, 33, 34]. Upregulation of PD-L1 through engagement of immune effector cells has also been proposed as a potential mechanism of trastuzumab resistance [35]. These previous studies highlighted the significance of immune-mediated mechanism(s) and a potential role of IGFR1 in trastuzumab resistance. Secretory factors released by the tumor cells into the microenvironment such as IL-6 have been found to confer resistance towards cancer therapies. IL-6 overexpression has been reported in many types of tumors including breast cancer; and IL-6 is one of the
major cytokines in the tumor and immune cell microenvironment [36]. High IL-6 levels in the tumor microenvironment can promote tumorigenesis via signaling pathways of apoptosis, survival, proliferation, angiogenesis, invasiveness, metastasis, and metabolism [36]. IL-6 can protect cancer cells from therapy-induced DNA damage, oxidative stress and apoptosis by facilitating the cell repair and induction of NF-κB and STAT3 signaling pathways [37, 38]. IL-6 also mediates HER2-driven breast tumor growth via activation of the STAT3 pathway which activates MMP3, MMP10, and MMP12 [39]. Our current study suggests that upregulation of IL-6 proteins may attenuate prognosis of HER2-positive breast cancer patients by increasing HER2-positive cancer cell invasion.

So far, the impact of DNMT1 on biological functions of tumors is still controversial, and therapeutic approaches targeting DNMT1 are still under exploration [40]. Oncogenic role of DNMT1 in pancreatic cancer indicated that methylated transcription repression of suppressor of cytokine signaling 3 (SOCS3) mediated by IL-6/STAT3 signaling via DNMT1 promotes tumor growth and metastasis [41]. In lung cancer cells, IL-6 induced the expression of DNMT1 that may result in hypermethylation of p53 and p21, and the enrichment of lung cancer stem-like properties [42]. IL-6 treatment of colon cancer cells resulted in an increase in DNMT1 expression that increased the methylation of promoter regions of genes associated with tumor suppression (plasminogen activator inhibitor-1, PAI-1, mammary serpin, Maspin); adhesion (interferon regulatory factor 7, IRF-7), and apoptosis resistance (Interleukin 4, IL-4) [43], implying inflammation-associated colon tumorigenesis.

DNA hypermethylation is a common early event in carcinogenesis, which is typically mediated by DNMTs [12, 13]. The silencing of tumor-suppressor genes by shutting down of their promoter regions is a key event in human cancer development [12, 13]. Silencing DNMT1 inhibits proliferation, metastasis and invasion in esophageal squamous cell carcinoma (ESCC) [14]. In prostate epithelial cells, the protein expression and methyl-transferase activity of DNMT1 can be increased by IL-6; and DNMT1 also regulates IL-6-induced epithelial mesenchymal transition (EMT) [14]. IL-6 enhanced phosphorylation of the DNMT1 nuclear localization signal by PKB/AKT kinase results in DNMT1 nuclear translocation [44, 45]. Previous studies demonstrated that chronic inflammation may perturb the balance of cellular DNMT1 distributions, through phosphorylation of the DNMT1 nuclear localization signal (NLS) via activating PKB/AKT kinase. Such evidences revealed a potential mechanism by which IL6 may modulate DNA methylation status [46, 47]. Interestingly, DNMT1 can downregulate FOXO3a expression and promote breast cancer stem cell properties and progression by inducing FOXM1/SOX2 signaling [48, 49].

IL-6 was found to impact protein stabilization of DNA methyltransferases and alter DNA promoter methylation of genes associated with insulin signaling and angiogenesis [16]. It was reported that IL-6 induced promoter-specific DNA methylation that reduced DNMT1 and DNMT3B protein levels in human umbilical vein endothelial cells (HUVECs) [16]. IL-6 resulted in promoter hypo- and hypermethylation of genes associated with insulin signaling and angiogenesis, presumably due to protein stabilization of DNMTs [16].
In breast cancer cells, DNMT1 promotes estrogen related receptor α (ERRα) stability which in turn couples DNMT1 transcription with that of the methionine cycle and S-adenosylmethionine synthesis to drive methylation of tumor suppressor gene, interferon regulatory factor-4 (IRF4) [46]. DNMT1 represents an epigenetic target for TNBC cells destruction that causes to facilitate demethylation of estrogen receptor 1 (ESR1), p53, U-rich element RNA-binding protein 1 (AUF1), and LIM homeobox 1 (ISL1) and inhibits their metastatic and aggressive phenotypes [48]. DNMT1-ISL1 axis is a necessary for cancer stem cell population maintenance and tumorigenesis [48]. A previous study showed that protein and mRNA expression levels of DNMT1 were negatively correlated with ERα expression [46], and we found that protein expression level of DNMT1 was positively correlated with ERα expression that potentially due to the difference of the proportion of HER2-positive patients (Table 1). DNMT1 expression is differentially expressed by molecular subtype and stromal histological type, and DNMT1 was highly expressed in TNBC [47]. These studies suggested a possible causal link between IL-6-induced changes in DNA methylation that may alter expression of critical genes involved in insulin signaling and angiogenesis in humans. Deletion of Dnmt1 in double-positive thymocytes impaired activation-induced proliferation in the mouse model, indicating that DNMT1 and DNA methylation are required for the proper expression of certain genes that define fate and determine function in T cells [50]. Single cell sequencing data from TISCH database of breast cancer T cells revealed a tissue-resident memory subset associated with improved prognosis [51]. Tissue-resident memory T cells in breast cancer control and immunotherapy responses [52]. CD39(+)PD-1(+)CD8(+) T cells mediate metastatic dormancy in breast cancer [53]. These studies and our present study suggest that DNMT1 may serve as an additional therapeutic target for breast cancer, and future studies on how DNMT may modulate breast cancer immune function.

Taken together, these studies demonstrated a reciprocal regulation by IL-6 and DNMT1 in tumor progression. We predicted that IL-6 could interact with DNMT1 between DNA methyltransferase-associated protein (DMAP) binding domain and replication foci domain (RFD) domain by iFrag (http://sbi.imim.es/iFrag) [54]. We postulated IL-6 promote HER2-positive cell malignancy via directly interacting with DNMT1 protein that will cause DNMT1 dislocation that DNMT1 protein expressed in cytoplasmic fractions of the breast tumor.

We showed for the first time, there was a positive correlation between IL-6 and DNMT1 protein expression and that predicts the prognosis in HER2-positive breast cancer. We also observed this association of invasion ability and the expression of IL-6 and DNMT1 in the two HER2-positive breast cancer cell-lines treated with IL-6 antibody or recombinant IL-6 protein (Fig. 5). Our results suggest that IL-6 inhibition in combination with anti-HER2 therapy could be a potentially effective regimen for both trastuzumab-sensitive and -resistant breast cancers. Exploring single cell RNA-seq datasets revealed a potential role of DNMT1 in tumor immune microenvironment and suggest that DNMT1 may serve as an additional therapeutic target for breast cancer.

Conclusions
Although the introduction of monoclonal antibodies, tyrosine kinase inhibitors, and antibody-drug conjugates directed against HER2 impressively improved patient prognosis in the last two decades, HER2-positive metastatic breast cancer remains a tricky disease that due to the acquired resistance to anti-HER2 therapies. The present study demonstrated a novel strategy that used the expression of IL-6 and DNMT1 for HER2-positive breast cancer prognosis. In vitro studies found that HER2-positive breast cancer cells with a higher expression of IL-6 and DNMT1 were more invasive; and a decrease of invasiveness was found in highly invasive HER2-positive breast cancer cells treated with anti-IL-6 monoclonal antibody. We suggest that anti-IL-6 monoclonal antibody therapy decreased the incidence of cancer cells-related invasion thus may be useful in the treatment of HER2-positive cancer patients. Moreover, DNMT1 is involved in breast cancer tumor immune microenvironment that may serve as a potential therapeutic target for breast cancer.

Abbreviations

BC: breast cancer; IL-6: interleukin-6; DNMT1: DNA methyltransferase 1; SCS: single cell sequencing; TCGA: The Cancer Genome Atlas Program; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; ER: estrogen receptor; HR: hormone receptor; IHC: immunohistochemistry; TNBC: triple-negative breast cancer; MTDH: metadherin; IL-10: interleukin-10; ESCC: esophageal squamous cell carcinoma; EMT: epithelial-mesenchymal transition; IGF2: insulin-like growth factor II; IR: insulin receptor; IGFs: insulin-like growth factors; IGF-1R: insulin-like growth factors; HUVECs: human umbilical vein endothelial cell; MATs: methionine adenosyltransferases; GNMT: glycine N-methyltransferase; CT: computed tomography; TISCH: the Tumor Immune Single-cell Hub; scRNA-seq: single cell RNA sequencing; PTEN: phosphatase and tensin homolog.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Institutional Review Board of Show-Chwan Memorial Hospital (IRB no. 1060407).

Consent for publication

Not applicable.

Availability of data and material

The dataset and materials presented in this investigation is available by request from the corresponding author.

Competing interests

The authors declare that they have no competing interests.
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Author Contributions


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Not applicable.

References


Table

**Table 1.** Relationship of clinical parameters with IL-6 and DNMT1 protein expression in breast cancer patients (n=285)
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>IL-6</th>
<th>DNMT1</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Low (N=142)</td>
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<tr>
<td><strong>Age</strong></td>
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<tr>
<td>&lt;65</td>
<td>234</td>
<td>115 (49)</td>
</tr>
<tr>
<td>≥65</td>
<td>51</td>
<td>27 (53)</td>
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<tr>
<td><strong>Stage</strong></td>
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<tr>
<td>I, II</td>
<td>229</td>
<td>114 (50)</td>
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<tr>
<td>III, IV</td>
<td>56</td>
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<td><strong>ER</strong></td>
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<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
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<td>102 (50)</td>
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<td><strong>PR</strong></td>
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<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>172</td>
<td>87 (51)</td>
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<td><strong>HER2</strong></td>
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<td>Negative</td>
<td>190</td>
<td>93 (49)</td>
</tr>
<tr>
<td>Positive</td>
<td>95</td>
<td>49 (52)</td>
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<td><strong>Ki67</strong></td>
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<td>34 (54)</td>
</tr>
<tr>
<td>Positive</td>
<td>222</td>
<td>108 (49)</td>
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Data are shown as numbers (%). P-values were calculated by Chi-squared test.

**Figures**
High DNMT1 expression was observed in breast invasive carcinoma (BRCA) and DNMT1 mRNA is associated with IL-6 in TCGA datasets. (A) mRNA expression of DNMT1 in human cancers. (B) Somatic mutation rate of DNMT1 for each cancer type. (C) The correlation between DNMT1 and IL-6 mRNA expressions in breast invasive carcinoma patients. (D) IL-6 did not appear to be a good prognostic marker.
for overall survival in HER2+ BC patients. (E) HER2+ BC patients with higher DNMT1 mRNA expression had poor overall survival.

Figure 2

The association between DNMT1, IL-6, and tumor immune microenvironment explored via single cell sequencing data from TISCH database. (A) DNMT1 and IL-6 pattern in the BRCA_GSE143423 dataset.
DNMT1 and IL-6 expression pattern shown in malignant cell cluster. (B) DNMT1 expression pattern in the BRCA_GSE114727_10X dataset showed that DNMT1 expressed in adaptive immune cell cluster, but IL-6 was not detected. (C) The correlations between DNMT1 expression and infiltrations of M0 macrophages in breast invasive carcinoma (BRCA) analyzed via TIMER2.0 database. (D) The correlations between DNMT1 expression and infiltrations of M1 macrophages in BRCA analyzed via TIMER2.0 database. (E) The correlations between DNMT1 expression and infiltrations of M2 macrophages in BRCA analyzed via TIMER2.0 database. (F) The correlations between DNMT1 expression and infiltrations of CD4 T cells in BRCA analyzed via TIMER2.0 database. (G) The correlations between DNMT1 expression and infiltrations of CD8 T cells in BRCA analyzed via TIMER2.0 database. (H) The correlations between DNMT1 expression and infiltrations of regulatory T (Treg) cells in BRCA analyzed via TIMER2.0 database. (I) The correlations between DNMT1 expression and infiltrations of T helper cells in BRCA analyzed via TIMER2.0 database.
Figure 3

IL-6 and DNMT1 immunohistochemical staining in breast cancer tissues (×200) using a set of serial sections of each tumor specimen. (A) Low IL-6 staining in breast cancer tissues; (B) High IL-6 staining in breast cancer tissues; (C) Low DNMT1 staining in breast cancer tissues; (D) High DNMT1 staining in breast cancer tissues. (E) Pearson’s correlations between IL-6 and DNMT1 IHC score.
Figure 4

Kaplan–Meier analysis of stage, age, ER, PR, HER2, Ki67, IL-6, and DNMT1 in breast cancer patients. (A) Overall survival estimates for stage. (B) Overall survival estimates for age. (C) Overall survival estimates for ER expression. (D) Overall survival estimates for PR expression. (E) Overall survival estimates for HER2 expression. (F) Overall survival estimates for Ki67 expression. (G) Overall survival estimates for IL-6 expression. (H) Overall survival estimates for DNMT1 expression. (I) Kaplan-Meier curve of overall survival of four breast cancer types from tissue array cohort. HR: hormone receptor. TNBC: triple-negative breast cancer.
Figure 5

(A)-(C) Kaplan-Meier analysis of IL6 and DNMT1 on overall survival of HR negative and HER2 positive breast cancer patients. (D)-(E) Cox regression analysis for the influence of age, stage, PR, Ki67, IL-6, and DNMT1 on overall survival in HR negative HER2-positive breast cancer patients. Multivariate logistic-regression analysis indicated that the high expression of IL-6, DNMT1, and IL-6 and DNMT1 significantly correlated with poorer survival. (F) Cellular protein expression pattern of IL-6 and DNMT1, and the
invasiveness in HR negative HER2-positive breast cancer cell-lines. Protein lysates were prepared from the MDA-MB-453 and SKBR3 breast cancer cell lines and protein expression levels were analyzed by western blotting using specific antibodies against DNMT1, IL-6, and GAPDH. (G) Invasion assay in high IL-6 expressing cell MDA-MB-453 that was treated with or without anti-IL-6 antibody (10 μg/ml). Invasion assay in low IL-6 expressing SKBR3 breast cancer cell treated with or without recombinant IL-6 protein (10 ng/ml).