

Single-cell RNA sequencing reveals novel gene expression signatures of trastuzumab treatment in HER2+ breast cancer

A pilot study

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Abstract

Human epidermal growth factor receptor 2-positive (HER2+) breast cancer accounts for ~20% of invasive breast cancers and is associated with poor prognostics. The recent outcome of HER2+ breast cancer treatment has been vastly improved owing to the application of antibody-targeted therapies. Trastuzumab (Herceptin) is a monoclonal antibody designed to target HER2+ breast cancer cells. In addition to improved survival in the adjuvant treatment of HER2+ breast cancer, trastuzumab treatment has also been associated with cardiotoxicity side effect. However, the molecular mechanisms of trastuzumab action and trastuzumab-mediated cardiotoxicity are still not fully understood. Previous research utilized bulk transcriptomics analysis to study the underlining mechanisms, which relied on averaging molecular signals from bulk tumor samples and might have overlooked key expression features within breast cancer tumor. In contrast to previous research, we compared the single cancer cell level transcriptome profile between trastuzumab-treated and nontreated patients to reveal a more in-depth transcriptome profile. A total of 461 significantly differential expressed genes were identified, including previously defined and novel gene expression signatures. In addition, we found that trastuzumab-enhanced *MGP* gene expression could be used as prognostics marker for longer patient survival in breast invasive carcinoma patients, and validated our finding using TCGA (The Cancer Genome Atlas) breast cancer dataset. Moreover, our study revealed a 48-gene expression signature that is associated with cell death of cardiomyocytes, which could be used as early biomarkers for trastuzumab-mediated cardiotoxicity. This work is the first study to look at single cell level transcriptome profile of trastuzumab-treated patients, providing a new understanding of the molecular mechanism(s) of trastuzumab action and trastuzumab-induced cardiotoxicity side effects.

Abbreviations: DEGs = differential expressed genes, HER2 = human epidermal growth factor 2, RNA-Seq = RNA sequencing, scRNA-Seq = single-cell RNA sequencing, TPM = transcript per million, Tsne = t-distributed stochastic neighbor embedding analysis.

Keywords: HER2+ breast cancer, single-cell RNA-Seq, TCGA, transcriptome profile, trastuzumab, trastuzumab-mediated cardiotoxicity

1. Introduction

Breast cancer is the most common cancer diagnosed in women worldwide.^[1] The human epidermal growth factor receptor 2-positive (HER2+) breast cancer accounts for ~20% of invasive breast cancers, which overexpresses the HER2 and is correlated

with poor prognosis.^[2] In the recent years, the outcome of HER2 + breast cancer treatment has been improved largely due to the application of targeted therapies.^[3] Trastuzumab (Herceptin; F. Hoffmann-La Roche, Basel, Switzerland) is an anti-HER2 monoclonal antibody that explicitly directed against the HER2 extracellular domain.^[4] Trastuzumab-based therapies have brought significant clinical benefits to HER2+ breast cancer patients including improved responsive rate, responsive duration, and survival rate.^[5] Improved clinical outcomes have been associated with induction of cell cycle arrest and apoptosis, PIK3t pathway inhibition, and antigen-dependent cellular cytotoxicity, in addition to the direct inhibition of HER2.^[4] Apart from its clinical benefits, trastuzumab can also induce cardiotoxicity,^[6] which can cause “left ventricular dysfunction, arrhythmias, hypertension, disabling cardiac failure, cardiomyopathy, and cardiac death” according to the medical package insert. The cardiotoxicity-caused discontinuation rate of trastuzumab treatment ranges between 6% and 31% in multiple randomized adjuvant trials and observational studies.^[7] Trastuzumab-induced cardiotoxicity is thought to be related with the neuregulin (NRG) ERBB pathway.^[8] However, the complete mechanism of trastuzumab action and trastuzumab-mediated cardiotoxicity is still yet to be illuminated.

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A detailed gene profile of trastuzumab is very essential to fully understand trastuzumab's mechanism of action and can provide new target/biomarkers for trastuzumab-mediated cardiotoxicity. Although a handful of studies have investigated the global gene expression pattern under trastuzumab treatment,^[9,10] these studies applied conventional molecular profiling methods such as microarray or bulk RNA sequencing (bulk RNA-Seq), which relied on averaging molecular phenotypes from a large population of cells. Because of the growing evidence showing heterogeneity and infiltration within breast cancer tumor,^[11] measuring the average values of gene expression might ignore the differences within breast cancer cell population that may be crucial for cancer progression and response to target therapies. Therefore, conventional molecular profiling methods can provide only partial information of the molecular state under trastuzumab treatment. The recent development of single-cell RNA sequencing (scRNA-Seq) technology has combined the single-cell isolation and RNA-sequencing technologies which enabled the global transcriptome profiling on single-cell level.^[12] scRNA-Seq can provide a higher resolution of cancer cell transcriptome profile comparing with the conventional cell-averaging experiments. Hence, scRNA-Seq could reveal novel biomarkers that might have been overlooked in traditional bulk transcriptome analysis.

In this pilot study, we compared the single cancer cell expression profile between trastuzumab-treated and nontreated HER2+ breast cancer patients. To the best of our knowledge, this is the first research to exam trastuzumab action in single cell level, which could contribute to a better understanding of the trastuzumab-induced therapeutic and cardiotoxicity effects.

2. Materials and methods

2.1. Single-cell RNA-Seq dataset

The scRNA-seq dataset was downloaded from NCBI Gene Expression Omnibus database (accession number GSE75688). This dataset contains single-cell and bulk RNA-seq data from 3 HER2+ patients diagnosed with invasive ductal carcinoma. Among these 3 patients, 1 patient (T1) received adjuvant trastuzumab treatment and other 2 patients (C1, C2) did not receive any treatment before tumor removal. All 3 patients are Asian females diagnosed with T2 or T3 breast cancer. Primary tumor specimens were collected by breast-conserving surgery or total mastectomy and processed for single-cell and bulk RNA sequencing. A total of 74, 48, and 19 carcinoma scRNA-seq samples and 1 bulk RNA-seq samples were retrieved from T1, C1, and C2 patients, respectively. These carcinoma single cells were classified by Chung et al^[11] using chromosomal gene expression patterns and validated by ESTIMATE algorithm.^[13] The trastuzumab-treated patient T1 was excluded by Chung et al^[11] for further transcriptome analysis because their research was focused on classifying nontreated cancer single cells. However, we felt this dataset provided a great opportunity to explore single cell transcriptome profile under trastuzumab treatment, and revisited this dataset. All data in this study were based on previously published studies, and thus, no ethical approval or patient consent was required.

2.2. TCGA dataset

A total of 1112 mRNA expression profiles along with their corresponding clinical data from patients identified with primary breast invasive carcinoma were downloaded from TCGA data

portal.^[14] After filtering out patients with follow-up shorter than 90 days, a total of 1078 patient profiles were used in this study.

2.3. Sequence analysis

The scRNA-Seq and bulk RNA-Seq sequences were aligned to human reference genome GRCh37 by bowtie2 program^[15] using default parameters. Relative gene express as transcript per million (TPM) was qualified using RSEM v1.3.0 (Ensemble 92 gene model).^[16]

2.4. Statistical analysis

Principal component analysis was performed in R using prcomp package.^[17] Unsupervised hierarchical clustering based on heatmap was conducted using ComplexHeatmap package in R. t-distributed stochastic neighbor embedding analysis (tSNE) was performed by the Rtsne package in R.^[18]

We applied the R Seurat package^[19] to identify differential expressed genes (DEGs) in scRNA-Seq samples between treated and nontreated patients. The Seurat package implemented a likelihood ratio test model to identify DEGs between 2 cell groups. Raw *P* value output by Seurat package was adjusted by Bonferroni correction. Genes with adjusted *P* value <.05 and fold change >1.5 were considered as significantly differential expressed.

Survival analysis for TCGA dataset was performed using Kaplan-Meier method by survival package in R.^[20] Patients were divided into high expression, low expression, and median expression groups according to *MGP* gene's expression value. Patients were categorized in high expression group if *MGP* expression was in the fourth quartile, low expression group if in the first quartile, and median expression group if in the second or third quartile. Differences between survival curves were measured by a log-rank test using survdiff package in R.

2.5. Enriched biological pathway analysis

We used Ingenuity Pathway Analysis software (IPA; Qiagen, Valencia, CA) to evaluate the most significant pathways and biological functions involved in trastuzumab treatment. DEGs identified from scRNA-Seq dataset and their expression values were uploaded to IPA for core analysis, which included canonical pathways, upstream regulators, disease and bio functions, and toxic functions analysis. IPA applied a Fisher exact test to identify significantly enriched canonical pathways, and only pathways with *P* value <.05 were further investigated. The IPA "Top Networks" analysis calculated a *P* score (-log₁₀ [*P* value]) based on the fitting of uploaded genes to a list of known biological function. Networks with *P* score >3 were further investigated.

3. Results

3.1. Separation of treated and nontreated scRNA-seq samples

We applied 3 clustering methods to reveal the differences of transcriptome profiles between treated and nontreated scRNA-Seq samples. First, PCA analysis was applied to identify the potential clusters of single cells based on TPM values of detected genes. Our PCA result indicated that the whole variance of single-cell dataset could be well-explained by first 3 principle components (PCs), while PC1 explaining 10.96 of total variance

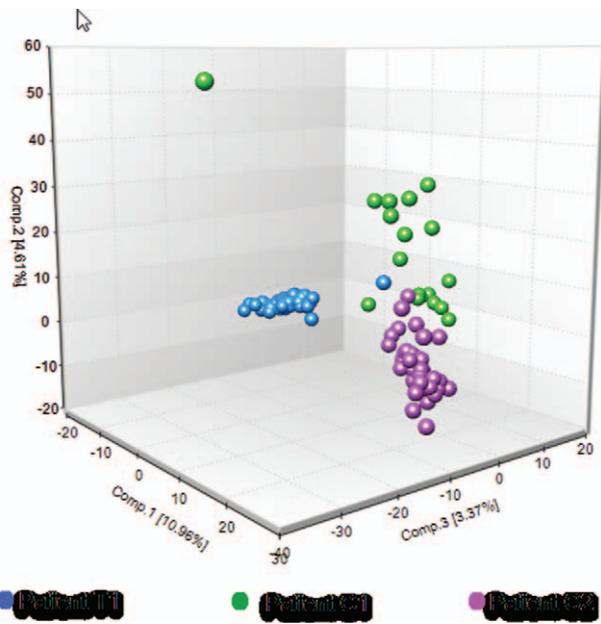


Figure 1. Principal component analysis (PCA) of trastuzumab-treated (T1) versus nontreated (C1, C2) single cancer cells. Unsupervised 3D PCA plot reveals 2 major groups of cells showing the separation of trastuzumab-treated and nontreated single cancer cells. Individual cells are colored blue for patient T1, green for patient C1, and purple for patient C2. The color scheme is maintained throughout the manuscript.

(Fig. 1). The 3D PCA plot also showed that PC1-3 separated the treated versus nontreated single-cell samples into 2 distinguishing clusters. Secondly, we selected the top 1000 highly expressed genes and conducted the unsupervised hierarchical clustering

based on heatmap. The separation of trastuzumab treated and nontreated single cells was also supported by the results of heatmap and dendrogram as the heatmap has been visually separated into 2 distinctive clusters (Fig. 2). Third, separation of single cells was also corroborated by our tSNE analysis as single cells from treated and nontreated patients were clustered into 2 distinguishing groups (Fig. 3A).

We hypothesized that the separation of 2 major single-cell clusters resulted from trastuzumab treatment. However, an alternative hypothesis was that cells were clustered by cell cycle phases. To test these 2 hypotheses, we applied tSNE analysis using a set of cell cycle-related genes^[21] and a set of trastuzumab-responsive genes.^[9] Our results showed that clustering using only cell cycle-related genes was not able to distinct treated or nontreated single cells (Fig. 3B). Clustering based on trastuzumab-responsive genes revealed a grouping that is highly like original grouping (Fig. 3C). This suggests the clustering of single cells was due to trastuzumab treatment, but not different cell cycle phases.

3.2. Identification of DEGs

We identified 461 DEGs between treated and nontreated scRNA-Seq samples (Table 1 and Supplementary Table 1, <http://links.lww.com/MD/D45>). Among these, a total of 303 genes were upregulated and 158 genes were downregulated. Top dysregulated genes were selected as candidate prognostic indicators for further validation using TCGA dataset.

3.3. TCGA survival analysis

Using mRNA profiles and clinical data that retrieved from 1078 patients in TCGA database, we tested whether the top

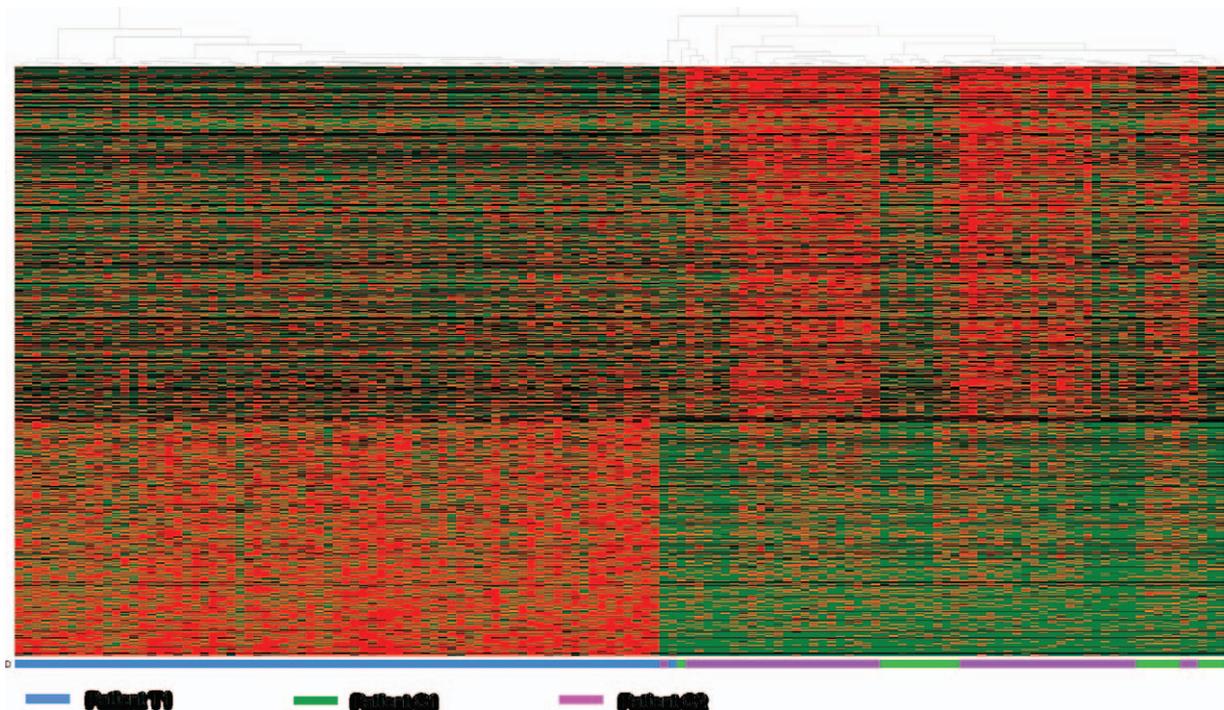


Figure 2. Heat map based hierarchical clustering separates trastuzumab-treated (T1) versus nontreated (C1, C2) single cancer cells. Individual cells are colored blue for patient T1, green for patient C1, and purple for patients C2 on color bar underneath hierarchical clustering heatmap.

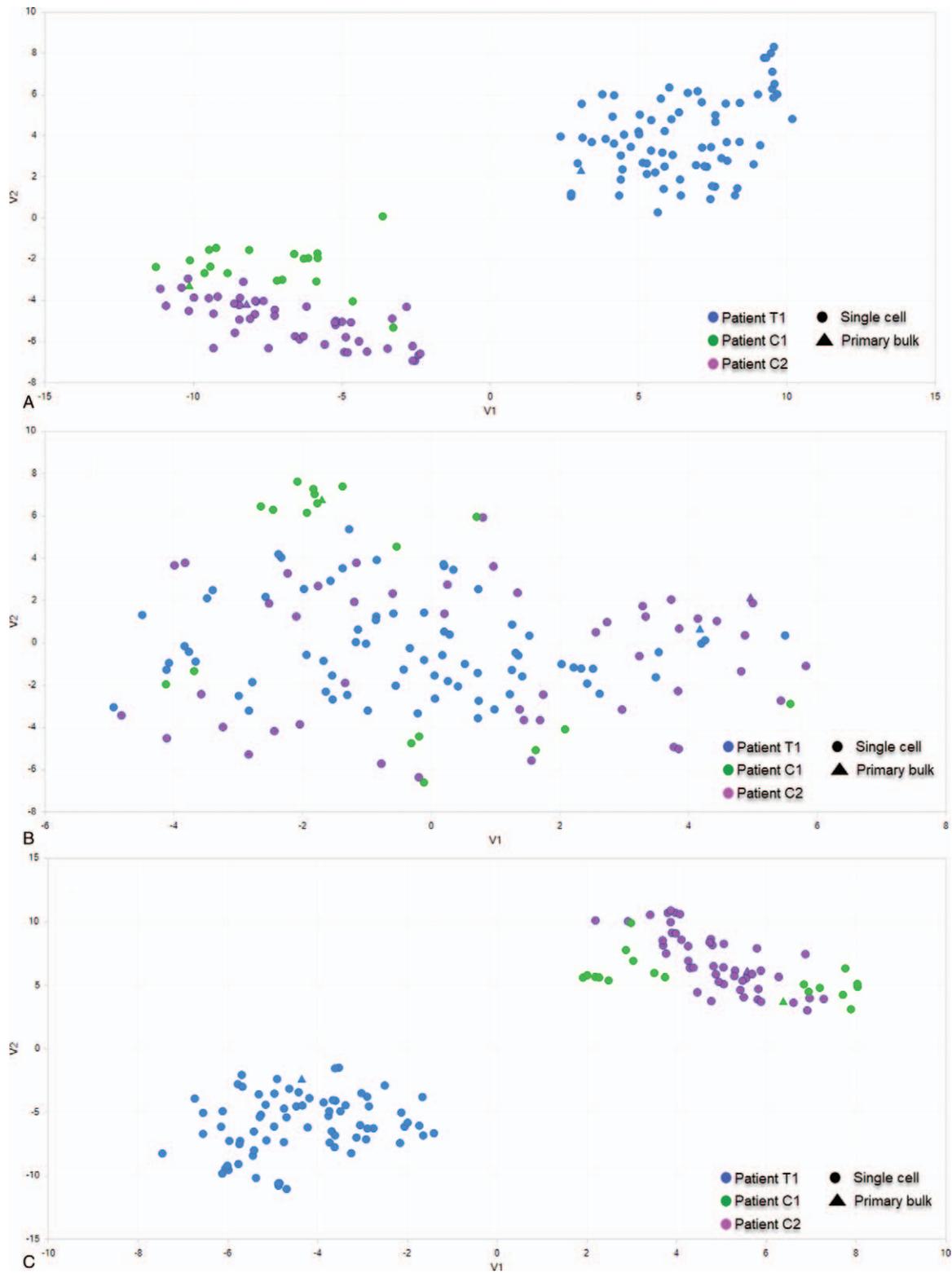


Figure 3. Tsne clustering of trastuzumab-treated (T1) versus nontreated (C1, C2) single cancer cells using different sets of genes. (A) Tsne separates single cancer cells into 2 major groups using top 2000 highly expressed genes. (B) Tsne is not able to separate single cancer cells using a list of cell cycle related genes. (C) Tsne separates single cancer cells into 2 major groups using a list of trastuzumab-responsive genes.

Table 1

Top 10 most significantly upregulated and downregulated genes between trastuzumab treated versus nontreated single cancer cells.

Gene ID	Estimate	Fold change	Raw P	Bonferroni adjusted P
IL-8	-5.2501	-38.0563	3.2383E-16	1.7958E-11
HLA-DRA	-4.8251	-28.3473	3.1197E-17	1.73E-12
CD74	-4.4628	-22.051	2.5143E-21	1.3943E-16
TFF3	-3.3728	-10.3588	1.0686E-08	.0006
HLA-B	-3.3131	-9.9389	6.8568E-25	3.8024E-20
B2M	-3.1411	-8.8219	2.5767E-20	1.4289E-15
LDHB	-3.1302	-8.7553	1.8061E-26	1.0016E-21
CXCL1	-3.117	-8.676	7.4085E-19	4.1083E-14
IFITM3	-3.0724	-8.412	1.6979E-20	9.4158E-16
RGS1	-2.9804	-7.892	5.7703E-18	3.1999E-13
YWHAE	2.6475	6.2657	4.346E-20	2.41E-15
PSMB3	2.7851	6.8927	1.2327E-22	6.8356E-18
CLEC3A	2.8833	7.3782	1.1418E-12	6.3316E-08
CPB1	3.4418	10.866	2.2348E-10	.000012393
SULT1E1	3.5841	11.9929	5.8837E-22	3.2627E-17
RPL23	3.8111	14.036	1.2081E-24	6.6992E-20
SEPP1	3.8631	14.5514	6.7132E-25	3.7227E-20
SCGB2A2	4.5616	23.6138	3.1213E-14	1.7309E-09
PIP	4.7832	27.5358	9.3925E-27	5.2085E-22
DCD	6.3026	78.9347	6.6683E-24	3.6979E-19
MGP	6.4026	84.5995	2.3895E-24	1.3251E-19

Genes with adjusted P value <.05 and fold-change >1.5 were considered as significantly differential expressed.

dysregulated genes in the single cell dataset could be used as prognostic indicators for breast cancer overall survival. We found the expression of *MGP* gene, 1 of the most significantly upregulated gene under trastuzumab treatment, was significantly associated with breast cancer patients survival ($P=.03$). Low *MGP* expression group has significant poorer survival rate comparing with high *MGP* expression or median *MGP* expression group (Fig. 4).

3.4. IPA analysis

Our IPA analysis identified 33 significantly enriched canonical pathways under trastuzumab treatment (Table 2, Supplementary Table 2, <http://links.lww.com/MD/D45>). IPA analysis revealed the top molecular and cellular functions related with DEGs are “cell death and survival,” “cellular movement,” “cellular development,” “cellular growth and proliferation,” and “protein

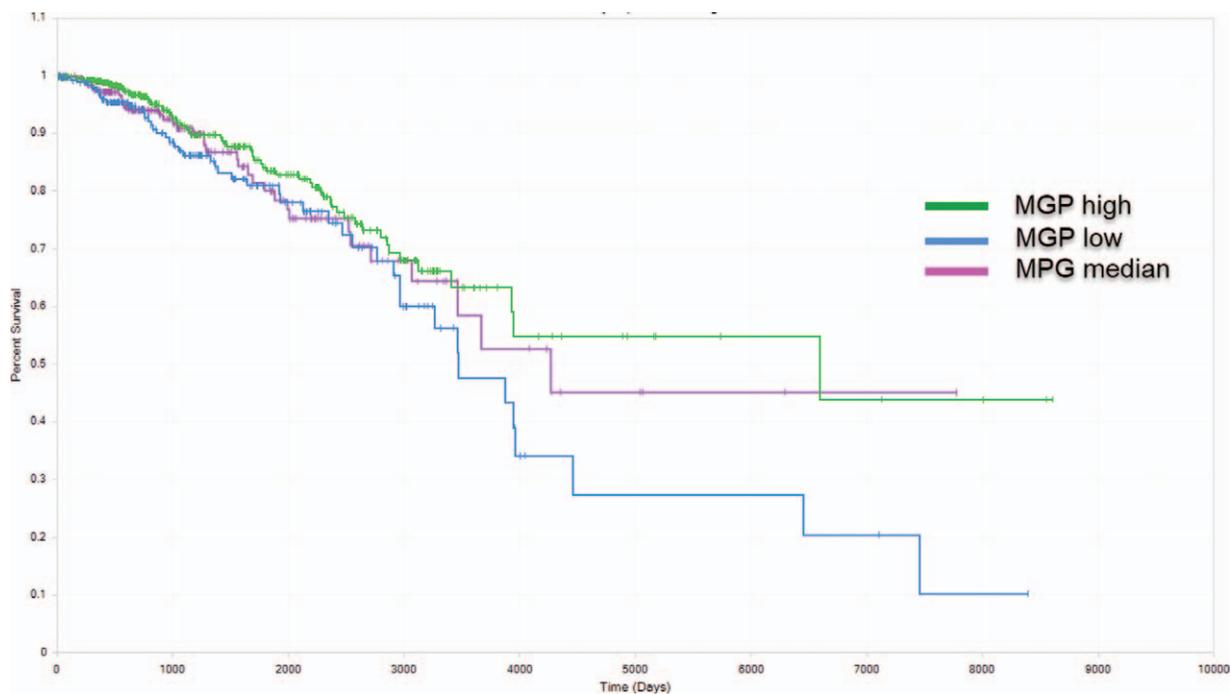


Figure 4. Overall survival curves based on *MGP* expression. Patients from TCGA breast cancer dataset were divided into high expression (green), average expression (purple), and low expression (blue) group according to *MGP* gene’s expression value. *MGP* low expression group was associated with poor overall survival ($P=.03$).

Table 2**Top 10 enriched diseases and biological functions identified by IPA.**

Categories	Diseases or functions annotation	P	Predicted activation state	Activation z-score
Cell death and survival	Cell death	6.11E-47	Increased	2.002
Cell death and survival	Apoptosis	4.03E-39	Increased	2.608
Organismal survival	Morbidity or mortality	5.09E-22	Increased	4.321
Organismal survival	Organismal death	6.43E-22	Increased	4.685
Infectious diseases	Replication of virus	1.23E-19	Increased	2.481
Infectious diseases	Replication of RNA virus	1.19E-17	Increased	2.063
Cardiovascular disease, hematological disease, organismal injury, and abnormalities	Anemia	1.44E-15	Increased	2.047
Infectious diseases	Bacterial infections	1.98E-12	Increased	2.812
Metabolic disease	Amyloidosis	1.45E-10	Increased	2.158
Cell death and survival, organismal injury, and abnormalities	Apoptosis of epithelial cells	7.11E-09	Increased	2.006

Differentially expressed genes, as determined by scRNA-Seq, were further analyzed with Ingenuity Pathway Analysis (IPA) software to outline the most enriched biological functions. The table shows the top 10 enriched terms corresponding to diseases/bio-functions along with the overlapping number of genes in the experimental dataset, *P* value, and activation z-score.

synthesis.” In addition, IPA predicted increased cell death, apoptosis and decreased metabolism of protein, cell movement, and invasion in trastuzumab-treated cancer cells (Supplementary Table 2, <http://links.lww.com/MD/D45>). Interestingly, our IPA analysis also identified 48 DEGs (Supplementary Table 3, <http://links.lww.com/MD/D45>) that were associated with “cell death of cardiomyocytes” ($P = .0003$), which might explain the trastuzumab-induced cardiotoxicity.

4. Discussion

In this study, we investigated the expression pattern of HER2+ breast cancer cells in response to trastuzumab treatment. Using single-cell transcriptomics analysis, we could clearly separate the single cancer cells from trastuzumab-treated or nontreated patients. The separation of cancer cells was well-supported by our PCA, tSNE, and hierarchical clustering results. We further tested the hypothesis that separation of cancer single cells was due to trastuzumab treatment, but not different cell cycle phases. Our tSNE results supported this hypothesis because tSNE clustering using trastuzumab-responsive genes could clearly separate trastuzumab-treated and nontreated cancer cells, whereas cell cycle-related genes cannot.

Many previously defined expression signatures and pathways at bulk tumor level were also captured in our scRNA-Seq analysis. For instance, we found significantly upregulated *CLU* and *SEPP1* gene in trastuzumab-treated patient (T1), which agreed with Necela et al^[22] that *CLU* and *SEPP1* were upregulated in trastuzumab-treated breast cancer cell lines. Interestingly, Necela et al^[22] also observed upregulated *CLU* and *SEPP1* expression in trastuzumab-treated cardiomyocytes. This suggests *CLU* and *SEPP1* could be used as early indicator of trastuzumab-mediated cardiotoxicity. In addition, we found *GATA3* gene, a top predictive gene for trastuzumab response,^[23] was significantly upregulated in T1 patient. Consistently, our IPA analysis revealed enhanced antigen presentation pathway under trastuzumab treatment, which has also been well documented by Kono et al.^[24] The identification of previously defined expression signatures and pathways indicates that traditional bulk transcriptome profiling can still be useful to reveal part of the global gene expression profile. Overlapping gene expression signatures between our study and previous research indicate that our identified DEGs were induced by trastuzumab treatment, and it

further confirmed our hypothesis that separation of single cancer cells was due to trastuzumab treatment.

In addition to previously identified expression signatures, our scRNA-Seq analysis revealed novel expression signatures that might have been overlooked in previous bulk transcriptome analysis. For example, we found 2 chemokine (C-X-C motif) ligands *CXCL1* and *CXCL8* (also known as *IL-8*) among the most significantly downregulated genes under trastuzumab treatment. These 2 chemokines act as key cytokine to expand tumor neovasculature and promote tumor establishment. In lymph node-negative breast cancer, patients with enhanced *CXCL8* expression were associated with poor prognosis of shorter survival time and higher chance of distant metastasis.^[25] Therefore, decreased *CXCL8* expression under trastuzumab treatment could contribute to a better clinical outcome. In addition, we found multiple tumor promoting genes, such as *CIB1* and *IFITM1*, were significantly downregulated in trastuzumab-treated patients. There is increasing evidence showing *CIB1* serve critical roles in cancer cell proliferation, apoptosis, and migration, and can promote tumor growth and angiogenesis.^[26] Another tumor-promoting gene *IFITM1* is required for the progression of colorectal cancer.^[27] The downregulation of these 2 tumor-promoting genes could also explain the trastuzumab-mediated therapeutic effects. To the best of our knowledge, this is first time that the dysregulation of *CXCL1*, *CXCL8*, *CIB1*, and *IFITM1* were associated with in vivo trastuzumab treatment. Our results highlighted new roles of these genes in trastuzumab treatment of HER2+ breast cancer. We expect these novel gene expression signature and the future follow-up studies could contribute to a better understanding of the molecular mechanisms of trastuzumab therapy.

Another important finding of this research is the upregulation of *MGP* expression under trastuzumab treatment and its potential role to serve as prognostic marker. We found *MGP* gene among 1 of the most significantly upregulated genes under trastuzumab treatment and hypothesized that upregulation of *MGP* could contribute to better clinical outcomes. Matrix Gla protein (*MGP*) is a vitamin K2-dependent, Gla-containing protein that acts as a multifunctional inhibitor of normal and abnormal angiogenesis, therefore acting as an endogenous inhibitor of tumor angiogenesis.^[28] *MGP* downregulation was observed in lung cancer^[29] and colorectal adenocarcinoma.^[28] Repression of *MGP* gene was also shown to stimulate breast

cancer cell proliferation and invasiveness.^[30] Using TCGA breast cancer dataset, we confirmed our hypothesis that patients with enhanced *MGP* expression had significantly longer overall survival period than patients with lower *MGP* expression. Consistently, Tuo and Ye^[31] also reported upregulation of *MGP* gene may predict better survival outcomes in ER+ breast cancer patients. This suggests enhanced *MGP* expression could contribute to better clinical outcome and could serve as prognostic marker for breast cancer overall survival.

Apart from our findings in trastuzumab's mechanism of action, we also identified a 48-gene signature that is related to cell death of cardiomyocytes. Molecular mechanisms that can potentially suppress cancer cells growth, proliferation, and survival in both direct and indirect manners are considered as trastuzumab's mechanism of action. Molecular mechanisms that can directly and indirectly lead to cardiotoxicity are considered as trastuzumab-induced cardiotoxicity. Among these 48 genes, 14 genes were upregulated and 34 genes were downregulated under trastuzumab treatment. Several of the upregulated genes such as *SPP1* and *HMOX1* are known to increase the apoptosis of cardiac myocytes.^[32,33] Further, many of the downregulated genes, such as *TIMP1* and *NAMPT*, are known to decrease apoptosis of cardiomyocytes. Other downregulated genes, such as *CXADR*, are essential for cardiomyocyte development.^[34] As predicted by IPA analysis, dysregulation of these genes could increase apoptosis of cardiomyocytes and disrupt the cardiomyocytes development, which could explain trastuzumab-mediated cardiotoxicity. Further validation of this 48-gene signature on cardiomyocytes is still required, which could discover novel therapeutic targets to prevent cardiac side effects or novel biomarkers to monitor trastuzumab-mediated cardiotoxicity.

5. Conclusions

To summarize, this is the first study exploring single cell level transcriptome profile of trastuzumab-treated HER2+ breast cancer patients. Our study offered new insights into the genes and potential biomarkers of trastuzumab-induced therapeutic effects and cardiotoxicity side effects. Gaining better understanding of these novel expression signatures remains a critical area to investigate in the future study. Limitation of this study includes the small number of patients and single cells. Due to the small sample size of this pilot study, analysis of more patient samples and more single cells per patient sample will be required.

Author contributions

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Visualization: Rengen Xu, Haiyan Yuan, Yunning Zhang.

Writing – original draft: Jun Wang, Rengen Xu, Yunning Zhang, Sean Cheng.

Writing – review & editing: Jun Wang, Sean Cheng.

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