BIOMARKERS

original reports abstract

Circulating T-Cell Repertoires Correlate With the Tumor Response in Patients With Breast Cancer Receiving Neoadjuvant Chemotherapy

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PURPOSE Neoadjuvant chemotherapy (NAC) has been widely used in patients with breast cancer to minish tumor burden and increase resection rate of cancer. T-cell repertoire has been believed to be able to monitor antitumor immune responses. This study aimed to explore the dynamic change of T-cell repertoire and its clinical value in evaluating the tumor response in patients with breast cancer receiving NAC.

MATERIALS AND METHODS Ninety-four patients who underwent NAC before surgery were recruited, and peripheral blood samples were collected at multiple time points during NAC. High-throughput T-cell receptor (TCR)-β sequencing was used to characterize the T-cell repertoire of every sample and analyzed the changes in circulating T-cell repertoire during NAC.

RESULTS We found that the diversity of TCR repertoires was associated with age and clinical stage of the patients with breast cancer. The distribution of Vβ and Jβ genes in TCR repertoires was skewed in patients with human epidermal growth factor receptor 2–positive (HER2+) breast cancer. Vβ20.1 and Vβ30 expression levels before NAC correlate with tumor response after all cycles of NAC in HER2– and HER2+ patients, respectively. Some CDR3 motifs that correlated with clinical response in either HER2+ or HER2– patients were identified. Besides, TCR repertoire evolved during NAC and the diversity of TCR repertoire decreased more after two cycles of NAC in patients with good tumor response after all cycles of NAC ($P = .0061$).

CONCLUSION Our results demonstrated that TCR repertoire correlated with the characteristics of the tumor, such as the expression status of HER2. Moreover, some characteristics of TCR repertoires that correlated with clinical response were identified and they might provide useful information to tailor therapeutic regimens at the early cycle of NAC.

This study identified T-cell repertoires that correlated with clinical response in patients with breast cancer.

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INTRODUCTION

Neoadjuvant chemotherapy (NAC) is mainly used to treat patients with locally advanced or inoperable cancer. Patients who are responsive to NAC may achieve tumor shrinkage, stage reduction, and even pathologic complete remission (pCR), thereby increasing the chance of surgical resection.[1-4] NAC has been widely used as standard treatment for locally advanced breast cancer, and its application has been extended to operable early breast cancer to increase the chance for breast-conserving surgery.[5-7]

Clinical studies have demonstrated a significant improvement in the survival rate of patients with pCR after NAC.[8-10] However, only 7%-50% of patients with breast cancer achieve pCR after NAC,[10-12] and some other patients even develop tumor progression during NAC.[13] For these non-pCR patients, NAC does not result in enough therapeutic effects and even causes adverse effects. Therefore, predicting the eventual therapeutic response of patients with breast cancer after early cycles of NAC is an important clinical issue. If there are certain factors indicating a poor therapeutic response, NAC can be terminated in a timely manner to avoid more serious side effects and disease progression. To date, unfortunately, an efficient way is yet missing to effectively evaluate the efficacy of NAC at an early stage.

T cells play a key role in antitumor immune responses.[14-17] T-cell receptor (TCR) high-throughput sequencing (TCR-HTS) can detect the sequence of the TCR repertoire and comprehensively assess the T-cell clonal composition and frequency. TCR repertoire analyses on the basis of TCR-HTS help us explore the relationship between T cells and diseases. Our previous research reports and many studies have characterized the signatures of TCR repertoires and demonstrated their diagnostic and prognostic value in

ASSOCIATED CONTENT

Data Sharing Statement

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Only 7%-50% of patients with breast cancer achieved pathologic complete remission after neoadjuvant chemotherapy (NAC); we therefore predict that the eventual therapeutic response at early cycles of NAC is important. This study aimed to explore the TCR repertoire differences at early cycles of NAC between patients who either achieved or did not eventually achieve pathologic complete remission.

Knowledge Generated

Two T Cell receptor beta variable genes and the diversity of TCR repertoire were independent risk factors that affect the clinical response of NAC. Specific clones of CDR3 motifs were identified and correlated with clinical response in human epidermal growth factor receptor 2–positive (HER2+) and HER2– cohorts, respectively.

Relevance

In this study, several TCR repertoire characteristics were identified, were correlated with clinical response after NAC, and might provide timely information to tailor therapeutic regimens at early cycles.

infectious diseases,[18-20] autoimmune diseases,[21-23] and tumors.[24-29] However, there are no published studies that analyze the circulating TCR repertoire in patients with breast cancer receiving NAC to predict the efficacy.

We previously collected $CD8^+$ T cells from peripheral blood at multiple time points during chemotherapy and monitored the $CD8^+$ TCRβ repertoire in seven patients with advanced breast cancer. The results indicated that the $CD8^+$ TCRβ repertoire evolved during chemotherapy and correlated with clinical responses to chemotherapy,[26] which suggests that TCRβ repertoire analysis on the basis of peripheral blood T-cell TCR-HTS might also have the potential to predict the therapeutic effects of NAC in patients with breast cancer.

MATERIALS AND METHODS

Sample Collection

This study was approved by the ethics committee of the Affiliated Foshan Hospital of Sun Yat-Sen University. All patients were recruited for this study from January 2018 to May 2019. All of them were confirmed as having breast cancer by pathologic diagnosis and accepted eight cycles of NAC (the therapy regimen for every patient is listed in the Data Supplement). All patients had an evaluation of the tumor size by physical examination and imaging (mammography and ultrasound and/or breast magnetic resonance imaging) before every cycle of NAC. Depending on the surgeon, they underwent mastectomy or breast-conserving surgery plus radiation therapy and axillary dissection. Patients without residual invasive cancer in the breast and nodes were considered to have pCR. The Miller-Payne score was used to reflect the proportion of tumor cells in pathologic sections.[30-32] Peripheral blood mononuclear cells were isolated by density gradient centrifugation, lysed with TRIzol reagent, and frozen at –80°C.

High-Throughput Sequencing of TCRβ Chains

We extracted RNA from the sample using the total RNA Kit of Omega Bio-tek. Then, the RNA was reverse transcribed using the SMARTer PCR cDNA Synthesis Kit (Clontech, Mountain View, CA). A forward universal primer and a reverse constant region–specific primer (5′-AACACSTTKTTCAGGTCCT-3′) were used to amplify the TCRβ fragments. The polymerase chain reaction (PCR) conditions are as follows: 94°C for 3 minutes, followed by 35 cycles of denaturing at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. The PCR products were purified using 2% agarose gel electrophoresis and a gel extraction kit (QIAGEN, Hilden, Germany). Beijing Genomics Institution sequence adaptors were ligated to construct libraries, which were then sequenced on the BGI2000 platform.

Bioinformatics Analysis of TCRβ Repertoire Data

The sequencing data were stored in FASTQ format and were analyzed by an in-house developed pipeline: TCR_one_step (available for download from GitHub). A brief summary of the pipeline is as follows. First, we filtered the low-quality sequences. Second, we aligned the sequences of TCRβ reference genes by BLAT (-stepSize = 5, -minIdentity = 0, and -minScore = 0).[33,34] The reference sequences were downloaded from the IMGT/GENE database.[35-37] If the V, J, and C genes were all identified in a sequence, we translated them into an amino acid (aa) sequence. The aa sequences without a terminator were selected as the productive TCR sequences. Finally, the sequences that started with cysteine and ended with the FGXG motif [C…FGXG] were defined as CDR3 sequences.[33,38]

Shannon's entropy index (H) and Simpson index (SI) were used to estimate the diversity of TCR repertoire.[33] The Morisita-Horn (MH) similarity index was used to estimate the similarity of two different TCRβ repertoires.[39-41] It was calculated on the basis of the number of shared sequences between the two samples and the contribution of the shared sequences to each repertoire, and it ranged from 0 to 1.

Statistical Analysis

Comparisons between groups were conducted by the chi-square test, *t* test, paired t test, or Mann-Whitney U tests if appropriate. The correlations of TCR repertoire with clinicopathologic characteristics were assessed using the Pearson χ^2 test. The receiver operating characteristic curve was constructed to illustrate the predictive ability of the TCR V genes. All *P* values were two-sided, and all analyses were performed using SPSS 20.0 and GraphPad Prism version 5.1.

Ethics Statement

This study was approved by the ethics committee of the Affiliated Foshan Hospital of Sun Yat-Sen University. All the participants provided written informed consent, and all the experiments were conducted in accordance with the guidelines of the Declaration of Helsinki.

RESULTS

The Clinical Characteristics of Patients and Sequencing Data

A total of 94 patients with breast cancer were enrolled in the study. Their clinical characteristics are listed in Table 1 and the Data Supplement. The median age of these patients was 46.5 years (26-71 years). Sixty-two of them had stage I and II disease, and 32 patients had stage III and IV disease according to the 7th American Joint Committee on Cancer staging system. According to the protein expression of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2), and Ki-67,[42] the patients were classified as having luminal A subtype (n = 1), luminal B subtype (n = 73), HER2 subtype (n = 10), and basal-like subtype disease (n = 10). After NAC, 45 patients (47.9%) showed pathologic complete response (pCR) after NAC.

A total of 478,033,204 productive sequences were obtained from 244 blood samples of the patients (Data Supplement). The average count of unique clonotype per sample was 143,756. Sixty-five Vβ genes and 13 Jβ genes were identified in our data. Vβ5.1 (average of 15.58%), Vβ20.1 (12.80%), Vβ12.4 (9.41%), Vβ29.1 (6.75%), and Vβ7.2 (5.78%) were the most frequent Vβ genes, and Jβ2.1 (18.99%), Jβ2.7 (18.04%), Jβ2.3 (11.69%), Jβ2.5 (10.94%), and Jβ1.1 (10.38%) were the most frequent Jβ genes, consistent with previous studies.[39,43,44]

The Diversity of TCR Repertoires was Associated With Age and Clinical Stage of Patients With Breast Cancer

We first calculated the H and SI to evaluate the diversity of TCR repertoire in each sample. Next, we found that the diversity of T-cell repertoires was inversely correlated with age (Figs 1A and 1B), which was consistent with previous studies.[45,46] Then, we compared the diversity indexes of patients with different clinicopathologic features and found that the SI indexes of the patients with advanced-stage (III and IV) disease were significantly lower than those with early-stage disease (Fig 1D). There was no difference among different molecular subtype groups (Figs 1E and 1F).

The Distribution of Vβ and Jβ Genes in TCR Repertoires was Influenced by the Tumor HER2 Expression Status

As HER2 gene has been known to influence the immunologic profiles,[47,48] we divided the patients into HER2– subgroup and HER2+ subgroup. The HER2+ subgroup consisted of 44 patients, whereas the HER2– subgroup consisted of 50 patients. The H index and SI index of T-cell repertoires in the

TABLE 1. Clinical Characteristics

Characteristic	No.
Median age in years (range)	46.5 (26-71)
Sex	
Female	94
Male	0
TNM stage	
I and II	62
III and IV	32
Biologic subtype	
Basal-like	10
HER2	10
Luminal B	73
Luminal A	1
ER expressed in tumor	
Positive	61
Negative	33
PR expressed in tumor	
Positive	70
Negative	24
HER2 expressed in tumor	
Positive	44
Negative	50
Ki67 expressed in tumor, %	
≥ 30	69
< 30	25
Clinical response	
pCR	45
Non-pCR	49
Miller-Payne score	
5	8
4	9
3	19
2	12
1	46

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; pCR, pathologic complete remission; PR, progesterone receptor.

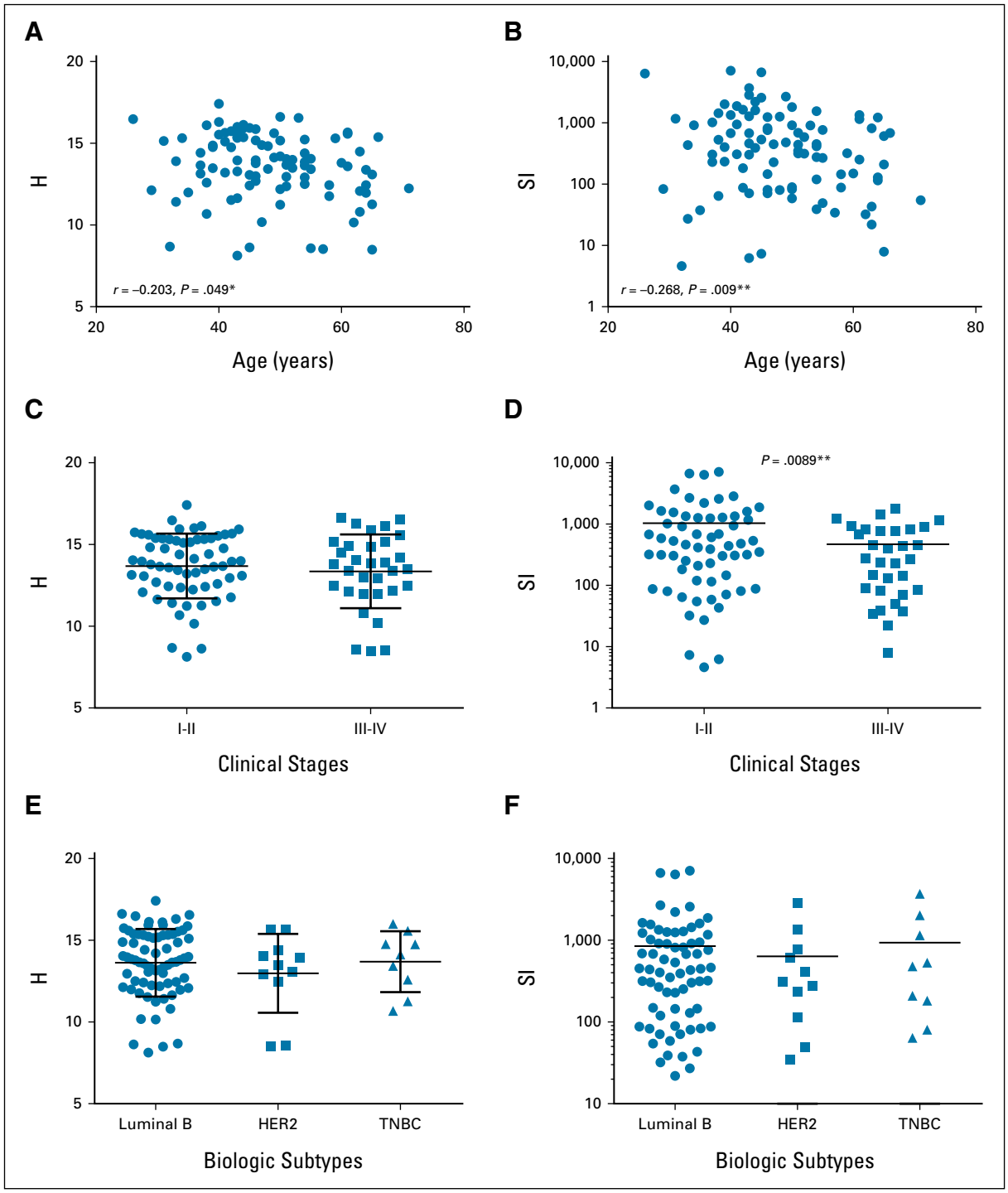

FIG 1. The diversity indexes of the peripheral blood TCRβ repertoire correlated with clinical characteristics: the H and SI in (A and B) different age groups, (C and D) clinical stages, and (E and F) biologic subtypes. *$P < .05$ and **$P < .01$. H, Shannon index; HER2, human epidermal growth factor receptor 2; ns, not significant; SI, Simpson index; TCR, T-cell receptor; TNBC, triple negative breast cancer.

HER2+ subgroup were slightly lower than those in the HER2– subgroup (Figs 2A and 2B). We also calculated the H index of the VJ combinations (H_{VJ}) for each sample and found that the H_{VJ} index in the HER2+ subgroup was significantly lower (Fig 2C). Next, we investigated the Vβ and Jβ genes present in HER2+ and HER2– patients. The usage frequencies of the Vβ7.4 (P = .018) and Vβ5.3 (P = .023) genes were significantly lower in the HER2+ subgroup (Fig 3A). By contrast, no differences in Jβ genes were identified. These results indicate that HER2 expression in tumor might influence the distribution of V and J genes in TCR repertoires of peripheral blood.

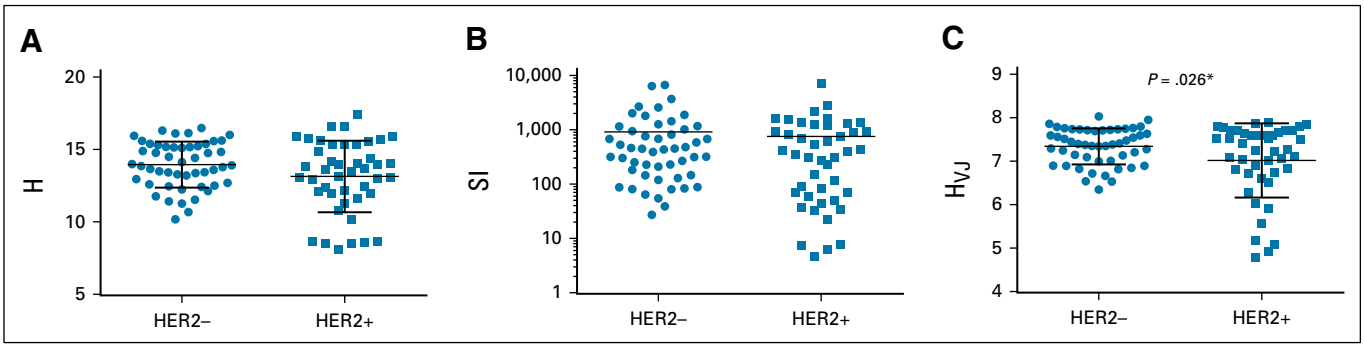

FIG 2. The diversity indexes of the peripheral blood TCRβ repertoire in HER2– and HER2+ patients: (A) H index, (B) SI index, and (C) H_{VJ}. *$P < .05$. H, Shannon index; HER2, human epidermal growth factor receptor 2; H_{VJ}, H index of the VJ combinations; ns, not significant; SI, Simpson index; TCR, T-cell receptor.

The Usage of V Genes in TCRβ Repertoire Before Therapy Correlated With Clinical Response

The usage of trastuzumab improved the outcomes of patients with HER2+. In this study, the pCR rate and Miller-Payne score were significantly higher in the HER2+ subgroup (pCR rate: 16 of 50 *v* 29 of 44, $P = .001$; Miller-Payne score: 3.42 ± 1.40 *v* 4.32 ± 1.14, $P = .001$, mean ± SD). To avoid the effects of trastuzumab, we investigate the correlation between TCRβ repertoire and outcomes in the HER2+ subgroup and HER2– subgroup, respectively. Vβ20.1 (for HER2– patients, $P = .0085$) and Vβ30 (for HER2+ patients, $P = .009$) were significantly higher in the pCR group (Fig 3B) and positively correlated with the Miller-Payne score (Vβ20.1: $P = .0211$, $R = 0.325$; Vβ30: $P = .0096$, $R = 0.391$; Spearman rank correlation). Moreover, multivariable logistic regression analysis showed that the frequencies of Vβ20.1 and Vβ30 were independent risk factors that may affect the clinical response of NAC in HER2– and HER2+ patients.

Given the fact that TCRs with similar CDR3 sequences might recognize the same antigenic peptides, we identified CDR3 motifs on the basis of the similarities in their CDR3 aa sequences using the CD-HIT program (90% quantile). We compared the specific clones of CDR3 sequences between pCR and non-pCR patients in the blood drew before or at early stage of NAC HER2+ and HER2– cohorts. The results showed that in HER2+ patients, some sequences are present more often in pCR patients, such as CASSWGLXTDTQYF in the blood of 12 of 29 pCR patients vs 0 of 15 non-pCR patients ($P = .003387$) and CASSDRDRXYSEQYF in 10 of 29 pCR and 0 of 15 non-pCR patients ($P = .009185$; Fig 4A). Similarly, in HER2– patients, sequences such as CASSLGXXXNEQFF (pCR: 6 of 16, non-pCR: 0 of 34, $P = .000504$), CSVARQTNTEAF (pCR: 7 of 16, non-pCR: 0 of 34, $P = .000115$), and CASSPTXATGTNYGYT (pCR: 7 of 16, non-pCR: 0 of 34, $P = .000115$) are found only in the blood of pCR patients (Fig 4B). We therefore believe that these sequences may help predict efficacy of patients with breast cancer in the early stage of NAC and tailor therapeutic regimens.

The TCR Repertoire Evolved During NAC and Correlated With Tumor Response

We investigated the TCR repertoire across multiple cycles of NAC from 69 patients (Data Supplement). The diversity indexes gradually decreased during NAC (Figs 5A and 5B). Moreover, after two cycles of NAC, H indexes of TCR repertoire decreased more in the patients with good tumor response, which indicated stronger immune response (Fig 5D). These results suggest that the obvious changes in diversity of T-cell repertoires during NAC may provide useful information for evaluating the tumor response. We also calculated the MH

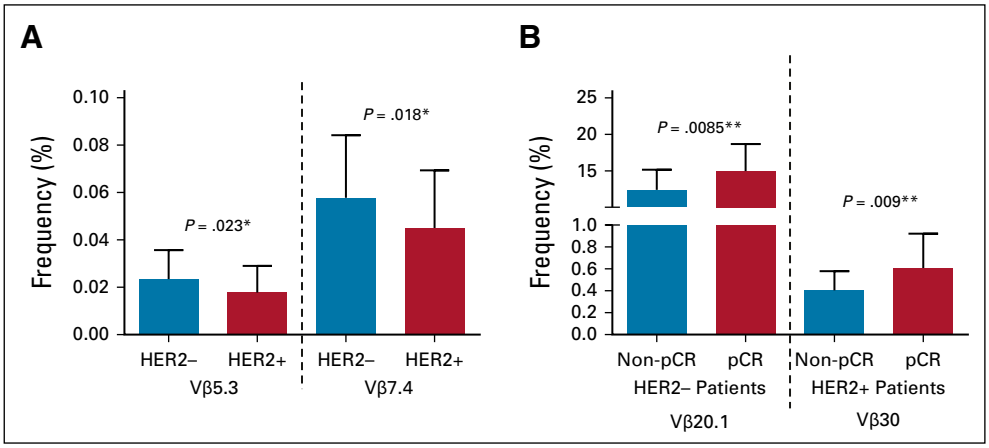

FIG 3. Comparison of Vβ gene usage in blood samples from different patient subgroups: (A) Vβ genes with a significant difference between HER2+ and HER2– patients and (B) between non-pCR and pCR groups in HER2+ and HER2– patients, respectively. *$P < .05$ and **$P < .01$. HER2, human epidermal growth factor receptor 2; pCR, pathologic complete remission.

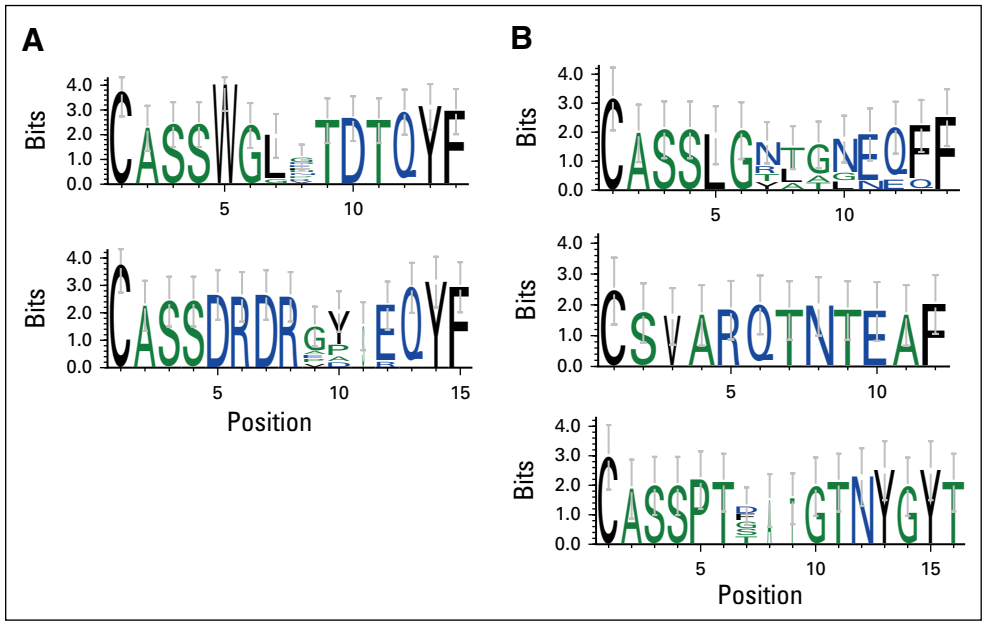

FIG 4. Sequence logo figures of the CDR3 motifs associated with patients with (A) HER2+ and (B) HER2– breast cancer who had pCR. Each logo consists of stacks of symbols, with one stack for each amino acid in the sequence. The overall height of the stack indicates the degree of sequence conservation at that position, whereas the height of the symbols within the stack indicates the relative frequency of each amino acid at that position. The width of the stack is proportional to the fraction of valid symbols at that position. HER2, human epidermal growth factor receptor 2; pCR, pathologic complete remission.

similarity indexes of TCRβ repertoires between the baseline and a certain cycle of NAC. As we expected, the TCR repertoire changed more and more during NAC (Fig 5C).

DISCUSSION

RNA-based TCR repertoires are subjected to the differences in abundances of distinct TCR clonotypes. Because of that, the sequence data from genomic DNA-based approaches are considered to be more accurate. In the latest study by Barennes et al,[49] seven RACE-PCR methods to capture T cell receptor alpha chain and nine RACE-PCR or multiplex-PCR methods to capture T cell receptor beta chain were tested. The results showed that the RACE-3 kit to capture T cell receptor beta chain showed high performance in replicability, reliability, and sensitivity. Our previous studies with the RACE-3 method also showed similar results.[25,27] In the current study, we used the RACE-3 method as continuation of our previous work.

As reported, the TCR diversity was associated with age.[45,46] In our study, the young patients with breast cancer showed higher TCR diversity than older patients and the patients with early stage have higher TCR diversity than those with advanced stage. Moreover, there was no significant difference in age between early and advanced stage patients. These results indicated that the age and the tumor have a superimposed effect on T-cell repertoires.

HER2 was expressed in several types of cancer cells, and it was regarded as a tumor-associated antigen.[50] Studies reported that HER2 could be found in peripheral blood and recognized by circulating T cells.[51,52] Our data showed that the distribution of Vβ and Jβ genes is different between HER2+/– patients. This result confirmed that the TCR repertoire was influenced by the HER2 expression status of tumor. Given that in HER2+ patients, the T-cell response against HER2 was activated,[48] this study may provide information to help reveal the correlation between HER2 and TCR repertoire.

During NAC, the cancer cells and tumor-derived antigens were released. This could enhance the susceptibility of tumor cells to T-cell immune attack.[53] Therefore, investigating the T-cell repertoires could reflect the release of tumor-derived antigens. Our results show that the TCR repertoire changed more and more during NAC and the diversity of TCR repertoire decreased more (indicates stronger immune response) in patients with good clinical response.

In our opinion, this study had two limitations. First, to monitor multiple time points of the patients during NAC, the samples needed to be collected over a long time, which made recruitment difficult, and some samples were censored. Second, this study failed to collect survival information because of the long survival period of patients with breast cancer, so we used pCR and Miller-Payne score to evaluate the clinical response.

In conclusion, this study explored the dynamic change of circulating TCRβ repertoire and its clinical value in patients with breast cancer receiving NAC. Our results demonstrated that TCR repertoire correlated with the characteristics of the tumor, such as the expression status of HER2. Moreover, some TCR repertoires that correlated with clinical response were identified and they might provide timely information to tailor therapeutic regimens at early cycles of NAC.

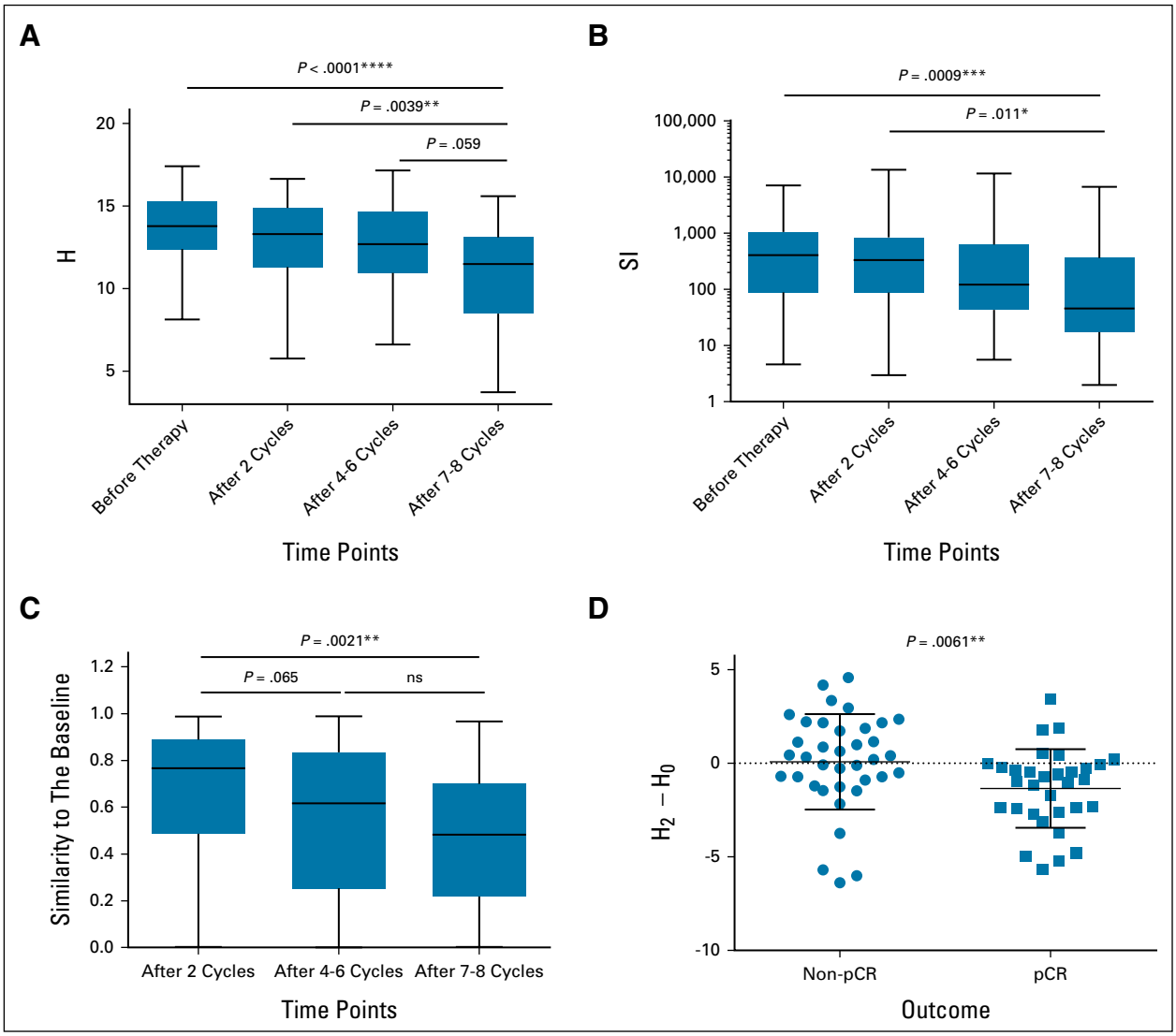

FIG 5. The changes in TCRβ repertoire during NAC correlated with clinical response: (A-C) the H index, SI index, and the similarity to the baseline at multiple time points during NAC and (D and E) the changes in H index and SI index after two cycles of NAC in pCR and non-pCR patients. $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$. H, Shannon index; NAC, neoadjuvant chemotherapy; pCR, pathologic complete remission; SI, Simpson index; TCR, T-cell receptor.

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EQUAL CONTRIBUTION

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DATA SHARING STATEMENT

The raw sequence data of the TCRβ repertoire and the source code of our own TCR sequence bioinformatical analysis tool are available on request.

AUTHOR CONTRIBUTIONS

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Data analysis and interpretation: Zhanwen Guan, Yabin Jin, Wei Luo
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Chunlin Wang
Employment: Chapter Diagnostics
Leadership: Chapter Diagnostics
Stock and Other Ownership Interests: iRepertoire, Chapter Diagnostics

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