Neoantigens elicit T cell responses in breast cancer

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Abstract

Neoantigens are tumor-specific antigens that arise from non-synonymous mutations in tumor cells. However, their effect on the immune responses in tumor microenvironment are still unclear in breast cancer.

We performed whole exome and RNA sequencing of 31 fresh breast cancer tissues and neoantigen prediction on the non-synonymous single nucleotide variants (nsSNVs) among exonic mutations. Neoantigen profiles were determined by predictive HLA binding affinity (IC50<500nM) and mRNA expression with a read count≥1. We evaluated the association between neoantigen load and expression levels of immune-related genes. Moreover, using primary tumor cells established from a breast cancer patient with malignant ascites, we tried to induce cytotoxic T lymphocytes (CTLs) by co-culturing neoantigen peptide-pulsed dendritic cells (DCs) and autologous peripheral lymphocytes. The functions of CTLs were examined by cytotoxicity and IFN-γ ELISpot assay.

. Neoantigen load ranged from 6 to 440 (mean, 95) and was positively correlated with the total number of nsSNVs. Although no associations between neoantigen load and mRNA expressions of T cell markers were observed, but the co-culture of neoantigen-pulsed DCs and lymphocytes successfully induced CTLs *ex vivo*.

These results suggest that neoantigen analysis may show utility in developing strategies to elicit T cell responses.

Background

Treatment of breast cancer has advanced significantly in recent decades, owing to the development of new drug therapies [1, 2]. However, many breast cancer patients still die, especially from drug resistance [3, 4]. Therefore, the development of new therapeutic strategies for breast cancer is critical.

Recently, immune checkpoint inhibitors (ICIs) have shown anti-tumor efficacy in many cancer types [5–7]. Although the response rate to ICIs is limited and ICIs have been associated with immune-related adverse events, the response to ICIs is sometimes durable [8, 9]. Notably, the existence of tumor antigen-specific T lymphocytes is an important factor for ICIs to show effectiveness [10, 11]. Among tumor antigens, peptides containing amino acid substitutions caused by somatic non-synonymous mutations present in tumor cells but not in non-tumor cells are called neoantigens.

A higher number of neoantigens is expected to elicit a stronger immune reaction in the tumor microenvironment. A recent study of multiple cancer types including breast cancer showed that increased numbers of neoantigens are associated with higher *CD8A* expression [12]. Another study showed that the number of neoantigens has a positive correlation with cytolytic activity, which is based on transcription levels of *GZMA* and *PRF1*, in breast cancer [13]. In contrast, other studies showed a negative impact of

high neoantigen load on immune response in breast cancer [14, 15]. Therefore, the effect of neoantigen burden on the immune response in breast cancer is still controversial.

Current research has focused on developing treatments targeting neoantigens [16, 17]. A recent study reported the remarkable effect of adaptive transfer of neoantigen specific T cells in a hormone positive metastatic breast cancer patient [18]. Another study demonstrated that neoantigens can induce antitumor immunity in xenograft models [19]. However, studies on the therapeutic application of neoantigens in breast cancer are still limited and further evidence is required.

In the current study, we performed neoantigen profiling in fresh tumor tissues from 31 patients with breast cancer and examined the correlation between neoantigen load and the immune environment in breast cancer. We also examined whether dendritic cells (DCs) pulsed with the *in silico* predicted neoantigen peptides were able to induce tumor-specific T lymphocytes in an *ex vivo* model.

Results

Mutational landscape of breast cancer patients

To perform neoantigen prediction, we first detected tumor-specific gene mutations by whole exome sequencing of 31 fresh breast cancer tissues and paired PBMCs. The clinical characteristics of the 31 breast cancer patients are shown in **Table 1**. We performed neoantigen prediction only on nsSNVs among the exonic mutations. A total of 1976 nsSNV mutations were found in the samples from 31 patients, and nsSNVs accounted for approximately 62.4% of the total exonic mutations (Fig. 1a) and showed a strong linear correlation with total exonic mutations ($r = 0.99$, $p < 0.0001$, Fig. 1b). The top three most frequent genes with nsSNVs were *TP53* (13 cases), *PIK3CA* (five cases), and *TTN* (five cases), and five identical paired nsSNVs were identified (**Table 2**).

Neoantigen landscape of breast cancer patients

We created neoantigen profiles by predicting the binding affinity of possible 8-11mer amino acid sequences generated by each nsSNV to patient-specific HLA-class I molecules. The number of neoantigen peptides with binding affinity IC50 < 500 nM ranged from 9 to 834 (mean 192) and showed a linear correlation with the number of nsSNVs ($r = 0.78$, $p < 0.0001$, Fig. 2a). Considering the importance of endogenous neoantigen peptides to elicit the immune reaction *in vivo*, we added the mRNA read count covering the mutated site to this criteria with read count ≥ 1. We refer to the number of neoantigens detected by these criteria as “neoantigen load.” The neoantigen load ranged from 6 to 440 (mean 95) and also showed a high linear correlation with the number of nsSNVs ($r = 0.725$, $p < 0.0001$, Fig. 2b). The neoantigen load was significantly higher in TNBC cases (n = 13; median = 150, range: 9–440) than non-TNBC cases (n = 18; median = 51, range: 6–196) ($p < 0.05$, Fig. 2c).

Association between the number of neoantigen peptides and immune-related gene expression

To investigate the effect of neoantigen load on the immune environment in breast cancer, the expression of immune-related genes was analyzed by RNAseq using the same sample that had been subjected to neoantigen analysis. There was no significant association between mRNA expression levels of T cell markers *CD8a*, *CD4*, and *TCRB* and the total number of nsSNVs and neoantigen load (**Additional file 1**). *GZMA*, *GZMB* and *PRF1*, which encode T cell activation markers, also did not show significant correlations (**Additional file 1**).

Induction of CTLs by synthesized neoantigen peptides

Another application of neoantigen profiles is for therapeutic uses such as cancer vaccines. Therefore, we examined whether CTLs could be induced by co-culturing lymphocytes with autologous DCs pulsed with the predicted neoantigen peptides. First, we performed neoantigen analysis of primary tumor cells established from ascites fluid of a patient with TNBC. We synthesized 10 of the mutant peptides from the neoantigen profiles that showed high affinity for HLA-A02:06 and mRNA expression with read count ≥ 2 (**Table 3**). We also synthesized one wild-type peptide as a control. After co-culturing autologous peripheral lymphocytes with synthesized peptide-pulsed mDCs for 48 h, we quantified IFNγ-producing lymphocytes using ELISpot assays. Although the results did not show statistical significance, lymphocytes pulsed with two peptides (peptide 3 and peptide 9) showed more than 50% higher response compared with the control with no peptide pulsation (Fig. 3a). We performed the same experiments using various concentrations of the two peptides and confirmed that the IFNγ-producing reaction increased depending on the concentration (Fig. 3b). From these results, we speculated that naïve T lymphocytes capable of reacting with peptides 3 and 9 were already present in the peripheral lymphocytes. We then cultured peripheral lymphocytes with mDCs pulsed with these two peptides under low concentrations of recombinant human IL-2 for 4 weeks to induce CTLs. CTLs generated by co-culture with peptide 9-pulsed mDCs showed significantly higher cytotoxicity than the wild-type peptide control in calcein releasing assay ($p < 0.01$, Fig. 3c). The same CTLs also showed significantly higher IFNγ-producing ability against the autologous tumor cells than the wild-type control ($p < 0.05$, Fig. 3d).

Discussion

We conducted neoantigen profiling of 31 breast cancers using an *in silico* prediction pipeline and analyzed the correlation of neoantigen load with immune-related gene expression. We also performed *ex vivo* CTL induction experiments using patient-derived TNBC primary tumor cells and autologous PBMCs along with the predicted neoantigen peptides. In this study, we demonstrated the following findings: 1) the neoantigen profiles were highly unique among individuals; 2) the neoantigen load showed a linear correlation with the total number of nsSNVs; 3) the neoantigen load was not correlated with immune-related gene expression; and 4) DCs pulsed with synthetic peptides predicted from our neoantigen analysis successfully induced CTLs *ex vivo.*

In silico neoantigen prediction analysis has recently been widely performed for many cancers, and this approach is considered as a potential useful tool for clinical parameters or immunotherapies [30, 31]. Previous studies showed that 8-11mer peptides with high affinity to HLA-class I are considered to elicit

CD8 + CTLs, which are the most effective cells in eliminating cancer [32]. Here we performed neoantigen profiling of 31 breast cancer specimens based on the binding affinity of possible 8-11mer peptides harboring substituted amino acids altered by nsSNVs to HLA-class I molecules and mRNA expression level of mutated genes. We succeeded in detecting neoantigens in all cases, indicating the good feasibility of neoantigen analysis in breast cancer regardless of the specimen sites. Among the total 1976 nsSNVs detected in the 31 cases, only five pairs of identical nsSNVs were observed, although commonly mutated genes such as *TP53*, *PIK3CA*, and *TTN* were found. Accordingly, the neoantigen profiles were highly unique among all cases. This is consistent with a previous report showing that the majority of neoantigens are specific to the individual [33]. This result indicates neoantigen profiling will be important in developing therapies that are highly personalized.

Although there are no common criteria for predicting neoantigens, the binding affinity of IC50 < 500 nM to HLA molecules is widely used [34, 35]. In this study, we used two criteria: binding affinity IC50 < 500 nM and RNAseq data with one or more read count containing the mutated position. Although how many levels of RNA are required to elicit T cell reactions is unclear, one study showed that a single peptide-MHC complex can induce cytotoxic T cells, indicating that a very low expression level may be sufficient for T cell activation [36]. Therefore, we consider that even only one read count of RNAseq can be a marker for a neoantigen peptide to elicit T cell response. In this study, we referred to the number of neoantigen peptides predicted by not only binding affinity to HLA class I molecules but also RNA expression as “neoantigen load.” Both neoantigen load and the numbers of neoantigens predicted only by binding affinity to HLA class I molecules were positively correlated with the total number of nsSNVs. This is consistent with a previous study of TCGA-based neoantigen analysis in breast cancers [15, 34]. This result suggests that tumor mutation burden (TMB) simply reflects an increased opportunity of gaining neoantigens and that TMB can be a surrogate marker for neoantigen load. In the analysis by subgroup, we found that the neoantigen load was higher in TNBC cases than non-TNBC cases, which was consistent with previous reports [15, 34]. Although the mechanism underlying the higher neoantigen load in TNBC compared with non-TNBC has not been elucidated, one possible cause is that compared with luminal type breast cancer, which has specific tumorigenic factors such as signaling pathway via hormone receptor, TNBC requires a higher rate of mutations to gain tumor driving mutations.

In contrast to our expectation, neoantigen load did not show positive correlations with mRNA expressions of T cell markers or T cell activated markers. Consistent with our result, another report showed an inverse association between immune metagene expression and TMB or neoantigen load in TNBC and the HER-2 positive type [14]. Another report also showed that lower TMB and neoantigen load were associated with high immune infiltration in TNBC [15]. Moreover, this trend is reported in multiple tumor types [37]. One reason for the inability of higher neoantigen load to elicit immune-related gene expression may be the complexity of immunity in the tumor microenvironment, which comprises multiple immune cell populations and cytokines [38, 39]. Even if neoantigens are endogenously expressed and immune response occurs, the existence of immune suppressive factors such as regulatory T cells, myeloid derived suppressor cells, and immune suppressive cytokines may counteract the immunogenicity of neoantigens. Another reason may be the neoantigen heterogeneity in breast cancers [40, 41]. Several studies

demonstrated that tumor heterogeneity correlates with reduced immune response [15, 42]. Collectively, although neoantigens themselves induce immune response, other multiple factors in the tumor microenvironment may cancel the immunogenicity of neoantigens.

The relationship between neoantigen load and tumor infiltrating lymphocytes (TILs) is a very interesting research area [43]. However, in about half of the cases in the current study, we were unable to evaluate TILs because of the limited biopsy specimens that were available and prioritized for neoantigen analysis. TIL evaluation from limited samples suggested there may be no significant correlation with TILs and neoantigen load (data not shown). Consistent with this result, a recent study showed no correlation between the number of nsSNVs and stromal TILs within each subtype of breast cancers [44]. We believe that the presence of neoantigen-specific TILs is more important than just the number of TILs or neoantigen load, and further analyses are required to address this issue.

In addition to the use of neoantigen profiles as a clinical immune parameter, there is great interest in its therapeutic use, such as for neoantigen vaccines and adaptive immunotherapy of neoantigen-specific CTLs [16, 17]. Using autologous tumor cells, monocyte-derived DCs, peripheral lymphocyte, and the synthetic neoantigen peptides, we were able to successfully induce CTLs *ex vivo*. Importantly, the result of CTLs to release IFN-γ against autologous tumor cells indicated that the neoepitope predicted by our pipeline was expressed on the surface of tumor cells in the context of HLA class I molecules. Although we recognize the limitations of a single preclinical model, this result demonstrated the theoretical rationale for clinical application of neoantigen peptides.

Several limitations in this study should be stated. First, we predicted neoantigens only for each nonsynonymous SNV depending on the binding affinity for HLA-class I. As INDELs including gene fusions are reported to be good resources of highly immunogenic neoantigens [45, 46], and neoantigens with high affinity for HLA-class II are reported to have a key role in the anti-tumor response [47, 48], these factors should be included in predicting neoantigens in future studies. Second, the proportion of tumor cells in the specimen probably affected the neoantigen load, because mutation was called for mRNA read count relative to that of normal sequences. In the future, a neoantigen analysis system that takes into account the tumor purity of specimens should be developed. Third, the number of specimens was small. As breast cancers are reported to consist of multiple cancer subtypes [49], a study should ideally be performed based on subtypes, and a large number of specimens is needed for these analyses. Finally, we did not check whether the predicted neoantigen peptides were expressed on the tumor surface with MHC class I molecules and elicit specific CTLs in the tumor microenvironment. In pursuing the therapeutic application of neoantigen peptides, this may be an important direction to explore.

Conclusions

We performed neoantigen analysis using fresh tumor specimens from patients with breast cancer. The neoantigen load was not positively correlated with immune-related gene expression. However, synthesized neoantigen peptide-pulsed DCs induced CTLs from peripheral lymphocytes *ex vivo*.

Collectively, these results indicate that endogenous neoantigens in the tumor microenvironment in breast cancer may be insufficient to elicit an immune response in the tumor microenvironment. Therefore, inducing neoantigen-specific immune responses outside the tumor microenvironment by adoptive neoantigen-specific CTLs or neoantigen-pulsed DC vaccines may represent alternative strategies to make use of neoantigen prediction analysis.

Methods

All methods in this study were approved by the Ethical Committees from the Kyushu University Hospital (No.128) and Fukuoka General Cancer Clinics (FGCC-EC001), based on the Act on Securement of Safety on Regenerative Medicine in Japan.

This study complies with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Breast cancer patients and samples

This study included 31 patients with histologically confirmed breast cancer from Kyushu University and Fukuoka General Cancer Clinic. Fresh breast cancer specimens were obtained immediately after biopsies or tumor resection and placed into microtubes containing the RNA stabilization solution RNAlater (Thermo Fisher Scientific Inc., Tokyo, Japan). Peripheral blood mononuclear cells (PBMCs) were obtained using a leukapheresis procedure (Haemonetics, MA, USA) performed according to the manufacturer's instructions. The leukapheresis product was diluted with RPMI-1640 (Kojin-Bio Inc., Saitama, Japan) for isolation by Ficoll-Hypaque. After isolation, the cells were washed three times with RPMI-1640, and the obtained PBMCs were cryopreserved at − 80ºC. Tumor specimens and PBMCs were obtained at various time points from patients after diagnosis (as shown in Table 1).

Establishment of primary breast cancer cells from a patient with chemo-refractory metastatic triple negative breast cancer (TNBC)

Pleural effusion was obtained from a 33-year-old woman with chemo-refractory TNBC who was diagnosed and treated at Kyushu University. The cytology of the pleural effusion revealed a number of tumor cells. The cells were first cultured in DMEM/F12 (Life Technologies, Grand Island, NY, USA). After colonies were formed, they were cultured in DMEM/F12 supplemented with 10% foetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and cryopreserved at − 80ºC. We previously reported the phenotype of the established primary breast cancer cells (MUK-BC1) in another study [20].

Whole-exome and RNA sequencing (RNAseq)

Genomic DNAs and total RNAs were extracted from fresh tumors in RNAlater using the AllPrep DNA/RNA mini kit (Qiagen Inc., Venlo, Netherlands), according to the manufacturer's instructions. Control genomic DNAs were extracted from patient-matched PBMCs. Whole-exome libraries were prepared from 200–3000 ng of genomic DNAs using the SureSelect Human All Exon V6 kit (Agilent Technologies Inc., Santa

Clara, CA, USA), according to the manufacturer's instructions. RNAseq libraries were prepared using the TruSeq Stranded mRNA Library Prep kit (Illumina Inc., San Diego, CA, USA). The prepared whole-exome and RNAseq libraries were sequenced by a HiSeq 4000 or NovaSeq 6000 sequencer (Illumina). To analyze RNAseq data, we used Cufflinks software [21], and FPKM (fragments per kilobase of transcript per million mapped reads) values were calculated for immune-related gene expression analysis.

Read mapping and variant calling

Sequence alignment and mutation calling were performed as described previously [22]. For whole-exome sequencing, the sequence reads were mapped to the human reference genome GRCh37/hg19 using Burrows–Wheeler Aligner (BWA, v0.7.10) [23]. The possible PCR duplicates, read pairs with a mapping quality of < 30 and mismatches more than 5% were excluded. For RNAseq, the sequence reads were mapped to the human reference genome GRCh37/hg19 using STAR (v2.4.0a) [24]. Somatic variants were called using Fisher's exact test-based methods according to the following parameters as described previously [22]: (i) base quality of ≥ 15; (ii) sequence depth of ≥ 10; (iii) variant depth of ≥ 4; (iv) variant frequency in tumor of ≥ 10%; (v) variant frequency in normal samples of < 2%; and (vi) Fisher *P*-value of < 0.05. SNVs, indels, and splicing region were annotated based on ANNOVAR [25]. We classified the somatic mutations as follows: (i) non-synonymous SNVs (nsSNVs); (ii) splicing; (iii) stop gain; (iv) stop loss; (v) insertion/deletion (INDEL); and (vi) synonymous SNVs (sSNVs). The splicing mutation indicates a single nucleotide variation occurred at the position of the splicing acceptor of the donor site.

We next used RNAseq data of tumors to examine gene expression. The actual counts of sequence read covering the somatic mutated position were used to confirm mRNA expression from mutated genes [26]. This strategy was used, as if the FPKM value was used, the mRNA expression level would be calculated from the sum of mutated genes and non-mutated genes. The total RNAseq read counts were over 20,000,000 per specimen.

Prediction of HLA class I binding peptides

Based on whole-exome sequencing data from the normal DNAs of PBMCs, HLA class I genotypes (HLA-A, HLA-B, HLA-C) were determined by OptiType algorithm [27]. We used only nsSNVs derived possible peptides for the prediction of the HLA genotype restricted neoantigens, because the number of INDELs (and fusion, if detected by RNAseq) was much lower than that of nsSNVs and also because our neoantigen prediction algorithm for INDEL/Fusion is under development and not yet validated [22]. Briefly, we examined all possible 8- to 11-mer peptides harboring each substituted amino acid by applying filtering with the predicted binding affinity to HLA-A, -B, and -C of IC50 < 500 nM using NetMHCv3.4 and NetMHCpanv2.8 software [28, 29].

In the selection of neoantigen epitopes, we also considered the mRNA expression level of mutated genes by counting RNAseq reads containing the nsSNVs.

Generation of DCs from PBMCs

DCs were generated from the cryopreserved PBMCs. PBMCs were thawed and cultured in 6-well plates (BD FALCON) in RPMI-1640 for 30 min. After removing the floating cells and washing the plate with RPMI-1640, adherent cells were cultured in complete DC medium containing GM-CSF (Primmune Inc., Kobe, Japan) and interleukin-4 (Primmune) in RPMI-1640. On day 6, the cells were stimulated with a maturation cytokine cocktail containing TNF-α (PeproTech Inc., NJ, USA) and interferon-α (Dainippon Pharma, Osaka, Japan) for 18 h. Morphological changes were monitored by light microscopy and cells were examined by flow cytometric analysis. DCs that expressed high levels of HLA class I, HLA-DR, CD40, and costimulatory molecules (CD86) with negative expression of CD14 (data not shown) were used for further experiments and referred to as matured DCs (mDCs).

Generation of neoantigen peptide-pulsed cytotoxic T lymphocytes (CTLs) ex vivo

After pulsing mDCs with the indicated synthesized neoantigen peptides (Table 3, closed square) for 4 h, we co-cultured peptide-pulsed mDCs with peripheral lymphocytes obtained by excluding plastic adherent monocytes from PBMCs. RPMI-1640 containing 10% healthy human serum and 40 U/ml recombinant human IL-2 (Primmune Inc. Kobe, Japan) was used as culture medium. We exchanged the medium once every 3 to 4 days. After 2 weeks of co-culture, we harvested the lymphocytes and co-cultured them with newly generated peptide-pulsed mDCs for another 2 weeks in the same culture condition as in the first co-culture. We harvested the lymphocytes and used them as CTLs for further experiments.

ELISpot assay

ELISpot assay was performed using the Human IFN-γ ELISpot^{plus} kit (MABTECH, Cincinnati, OH, USA) according to the manufacturer's instructions. Briefly, 96-well plates with nitrocellulose membranes (Millipore, Molshelm, France) pre-coated with primary anti-IFN-γ antibody were pretreated with RPMI medium containing 10% autologous serum at 4 °C overnight. A total of 1×10^4 autologous immature DCs were added to each well along with DC maturation cocktail, and cells were incubated overnight. Synthesized neoantigen peptide (final concentration at 5, 50, and 100 μg/ml) was added to each well and cells were incubated for 4 h. After three washes with RPMI medium, 1.0×10^5 or 1.5×10^5 autologous peripheral lymphocytes were added to each well and cells were incubated for 48 h. After three washes in PBS, secondary antibody was added to each well and cells were incubated for 2 h. The plates were incubated with HRP-reagent and stained with TNB (MABTECH). Spots were captured and analyzed by an automated ELISpot reader 08 classic (AID GmbH, Strassberg, Germany), and positivity of the neoantigen-specific T cell response was quantitatively defined as specific spots.

We also performed ELISpot assay to examine the IFN-γ releasing ability of CTLs against autologous tumor. Briefly, target tumor cells (5×10^3 per well) were seeded in ELISpot 96-well plates prepared as stated above and cultured overnight. CTLs were then added to the wells (5×10^4 per well), and the cells were incubated for 4 h at 37ºC, followed by the same procedure to detect IFN-γ spots as stated above.

Calcein-release cytotoxicity assay and cell imaging

Target tumor cells (5×10^3 per well) were seeded in a 96-well flat bottom plate and cultured overnight. Cells were then incubated at 37ºC for 1 h in the presence of calcein-AM. CTLs were then added to the wells (5×10^4 per well), and the cells were incubated with the tumor cells for 4 h at 37ºC. Images were captured using a conventional fluorescence microscope (IX81; Olympus, Tokyo, Japan) equipped with a color CCD camera (DPI72; Olympus) and objective lens (LUC plan FLN; Olympus). All procedures were performed at 20–25ºC. Images were analyzed using Lumina Vision software. The cytotoxity percentage was calculated as : (control fluorescence – sample fluorescence) / control x 100 %.

Statistical analysis

Pearson correlation (r) was used for correlation analysis between all parameters examined. Statistical analysis was performed using GraphPad Prism Version 8.3.0 (GraphPad software, La Jolla, CA, USA). A *p* value of < 0.05 was considered to be statistically significant.

Abbreviations

CTLs: cytotoxic T lymphocytes, DC: dendritic cells, FPKM: fragments per kilobase of transcript per million mapped reads, ICIs: immune checkpoint inhibitors, INDEL: insertion/deletion, nsSNV: nonsynonymous single nucleotide variant, PBMC: peripheral blood mononuclear cell, sSNV: synonymous single nucleotide variant, TILs: tumor infiltrating lymphocytes, TMB: tumor mutation burden, TNBC: triple negative breast cancer.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee from the Kyushu University Hospital (No. 128) and Fukuoka General Cancer Clinics (FGCC-EC001).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the first or corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TM and MK designed the study, analyzed the data, and wrote the manuscript. TM, MK, MK, and MY contributed to acquisition of breast cancer specimens and data collection. PYY, SY, JHP and KK developed and/or contributed to the computational neoantigen prediction analysis. TM and MU conducted the *ex vivo* experiment. TM, MK, PYY, SY, JHP, KK, YO, YN, TM and MN provided material support and study supervision and were major contributors in reviewing and editing the manuscript. All authors reviewed and approved the final manuscript.

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Tables

Due to technical limitations, table 1, 2, 3 is only available as a download in the Supplemental Files section.

Figures

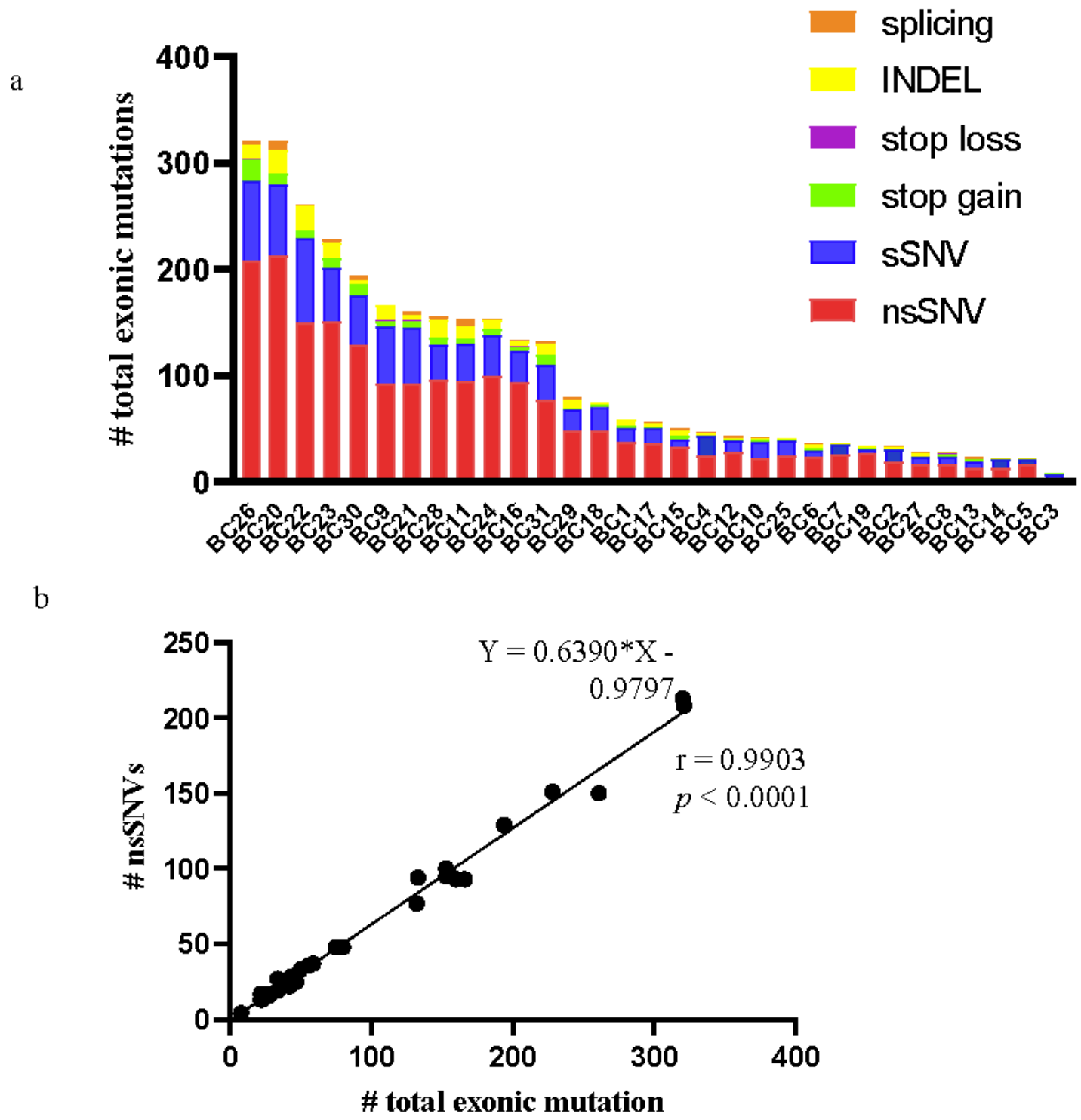

Figure 1

Exonic mutation landscape in breast cancer determined by whole exome sequencing of tumor tissues and peripheral blood mononuclear cells. a The numbers of total exonic mutations and mutation types in the 31 breast cancer patients. b The number of total exonic mutations is highly correlated with the number of nsSNVs. INDEL: insertion/deletion; sSNV: synonymous single nucleotide variant; nsSNV nonsynonymous single nucleotide variant.

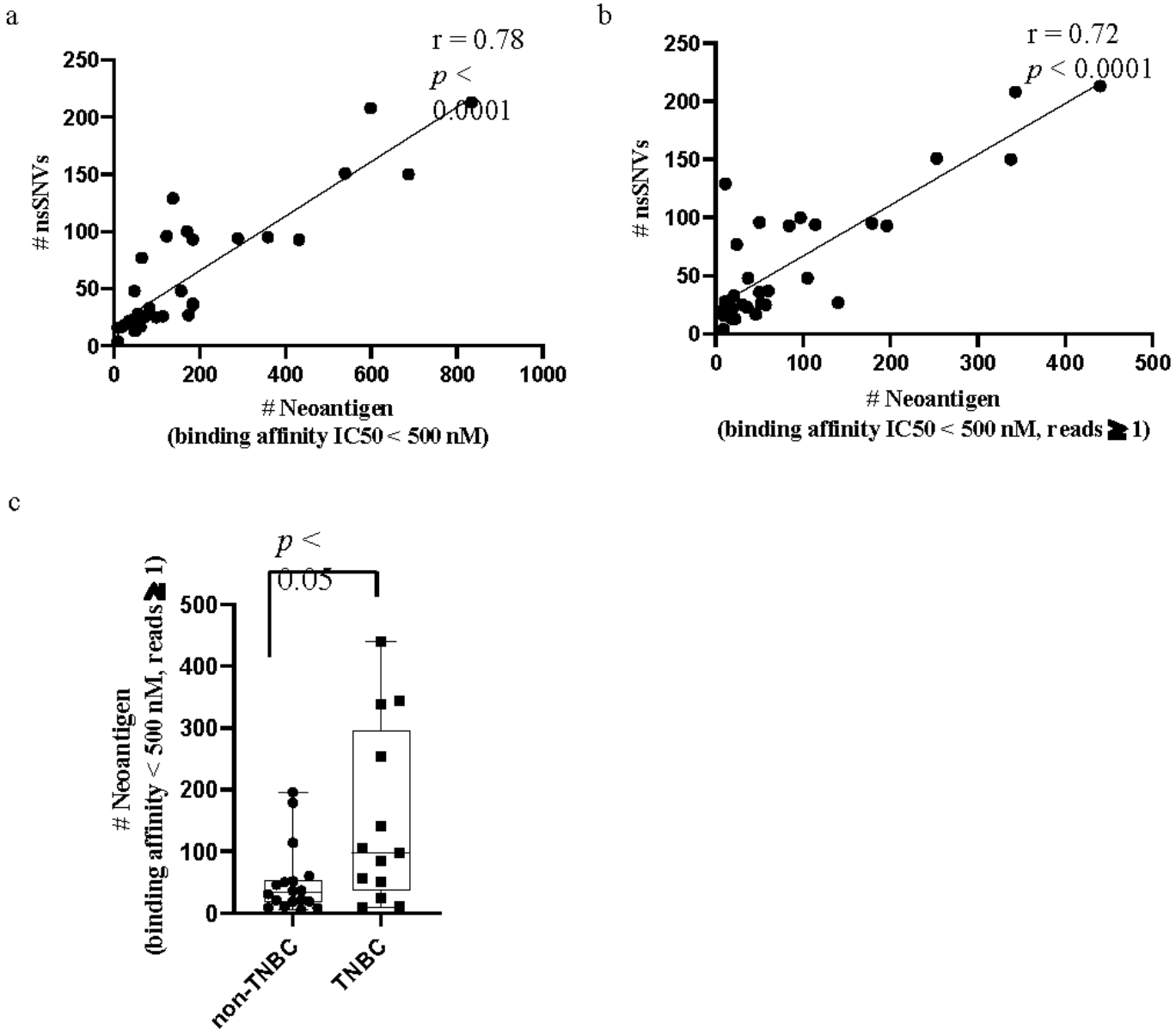

Figure 2

Neoantigen landscape in breast cancer determined by computational HLA class I binding assay and RNA sequencing. a The number of predicted neoantigens (IC50 < 500 nM) is highly correlated with the number of nsSNV (r = 0.78, p < 0.0001). b The number of predicted neoantigens (IC50 < 500 nM, read count ≥ 1) is highly correlated with the number of nsSNVs (r = 0.72, p < 0.0001). c. The number of predicted neoantigens (IC50 < 500 nM, read count ≥ 1) in TNBC is statistically higher than that in non-TNBC. TNBC: triple negative breast cancer.

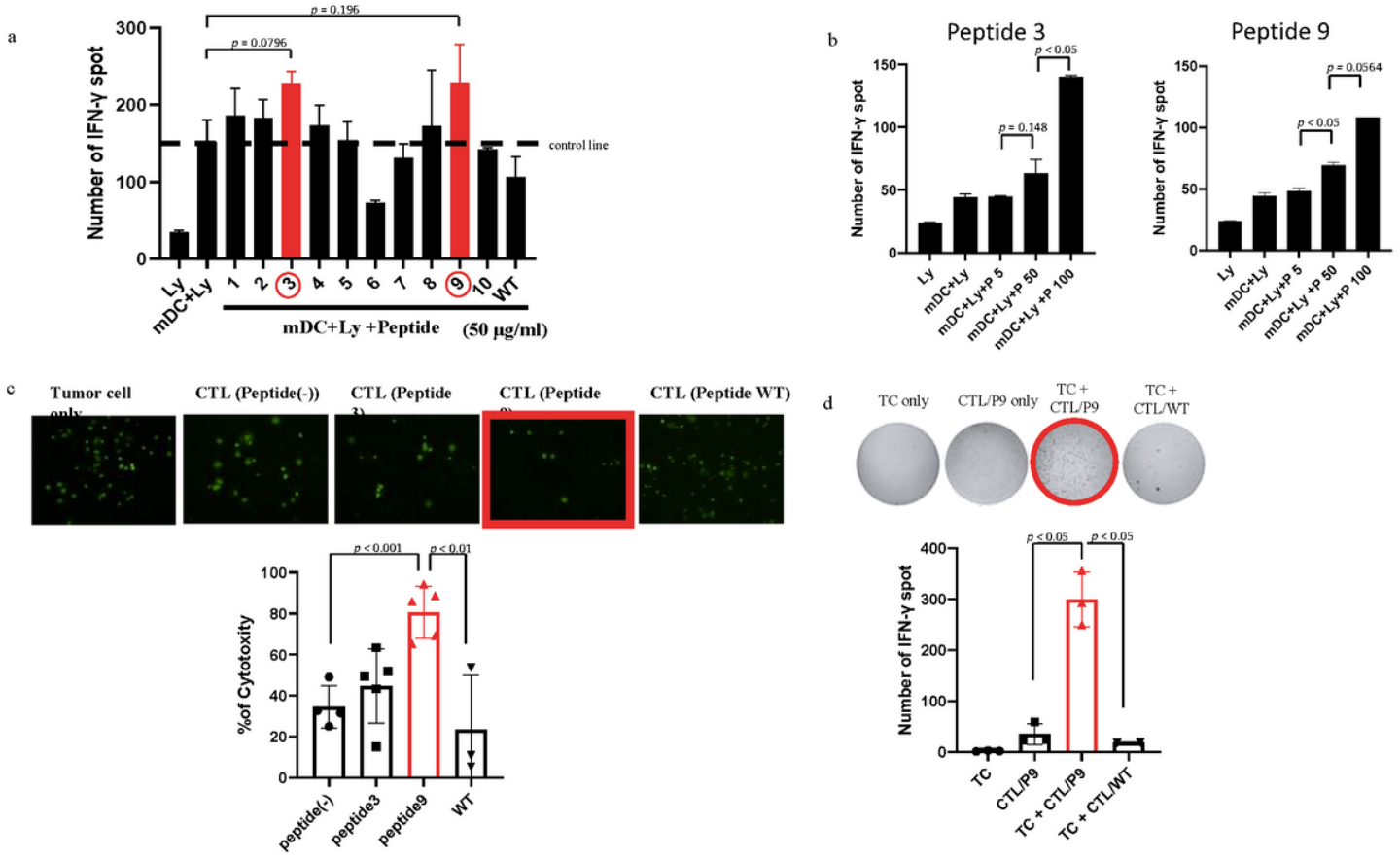

Figure 3

Response of cytotoxic T lymphocytes stimulated with the neoantigen peptide-pulsed matured dendritic cells (mDCs). a IFN-γ ELISpot response by peripheral blood lymphocytes stimulated with neoantigen peptide-pulsed mDCs. The final concentration of peptide was 50 μg/ml. The mean number of IFN-γ ELISpot ± s.e.m. is shown (n = 2). b Dose-dependent increase in IFN-γ ELISpot response by peripheral blood lymphocytes stimulated with peptide 3- or peptide 9-pulsed mDCs. The final concentrations of peptides were 5, 50, and 100 μg/ml. The mean number of IFN-γ ELISpot ± s.e.m. is shown (n = 2). c Cytotoxicity assay of CTLs induced by 4 weeks of co-culture with neoantigen-pulsed mDCs. Numbers of calcein-positive living tumor cells were significantly decreased when co-cultured with CTLs stimulated with peptide 9-pulsed DCs. The mean % of cytotoxity ± s.e.m. is shown (n = 3-5). d IFN-γ releasing responses by peptide 9-pulsed mDC stimulated CTLs against autologous tumor cells. Ly: lymphocyte; mDC, matured dendritic cell; TC, tumor cell; CTL/P9, CTLs induced by peptide 9-pulsed DCs; CTL/WT, CTLs induced by wild-type peptide-pulsed DCs. The mean number of IFN-γ ELISpot ± s.e.m. is shown (n = 2-3).

Supplementary Files

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