Immunotyping of tumor-infiltrating lymphocytes in triple-negative breast cancer and genetic characterization

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Abstract. Tumor-infiltrating lymphocytes (TILs) reflect the host immune response against cancer cells. Immunomodulators have been recently suggested as a novel therapeutic strategy against triple-negative breast cancer (TNBC). However, the TIL profile in TNBC has not been thoroughly investigated. In the present study, the percentage, immunophenotype and genetic profiles of TILs in pre-surgical tumor samples of patients with TNBC were evaluated prior to neoadjuvant chemotherapy (NAC). Patients diagnosed with breast cancer at Hospital San José TecSalud were consecutively and prospectively enrolled in the present study between August 2011 and August 2015. The pathological response to NAC was evaluated using the de Miller-Payne and MD Anderson Cancer Center system. TIL percentage (low, intermediate, and high) was evaluated using special hematoxylin-eosin staining on the core needle biopsies. The immunophenotype of TILs was assessed by immunohistochemistry (IHC) for $CD3^+$, $CD4^+$ and $CD8^+$. In addition, the gene expression profile of *CD3, CD4, CD8, CD20, CD45,* forkhead box P3, interleukin 6, programmed cell death 1 and CD274 molecule was assessed in all patients. A total of 26 samples from patients with TNBC prior to NAC were included in the present study. TILs were low in 30.7%, intermediate in 38.4% and elevated in 30.7% of tumors. $CD3^+$ and $CD4^+$ counts were associated with the pathological response to NAC (P=0.04). Finally, an overexpression pattern of *CD3, CD4, CD8, CD45* and *CD20* genes was observed in patients with a partial or complete pathological response. The present results demonstrated that TILs may predict the pathological response to NAC in patients with TNBC. Furthermore, a more accurate association was identified between the high expression levels of *CD3, CD4, CD8, CD45* and *CD20* genes and partial and complete pathological response, compared with the association between high expression and IHC alone.

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Abbreviations: TILs, tumor-infiltrating lymphocytes; TNBC, triple-negative breast cancer; HER2/neu, human epidermal growth factor receptor 2; pCR, complete pathological responses; pPR, partial pathological response; pNR, no pathological response; DFS, disease-free survival; NAC, neoadjuvant chemotherapy; RMA, robust microarray analysis; FDR, false discovery rate method; CDR3, complementarity-determining-region 3; AJCC, American Joint Committee on Cancer; H&E, hematoxylin-eosin

Key words: tumor-infiltrating lymphocytes, breast cancer, triple-negative breast cancer

Introduction

Triple-negative breast cancer (TNBC) accounts for 10-20% of all types of breast cancer, and by definition, the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu) has to be absent. TNBC, histologically a high-grade neoplasia,

exhibits an aggressive biological behavior and usually relapses during the first 3 years of disease (1-3). Previous studies have evaluated the effectiveness of chemotherapy in TNBC, such as neoadjuvant therapy, and have demonstrated that these tumor types can be very chemosensitive, with 35-50% of the tumor types able to achieve high rates of pathological complete responses (pCR) (4-6).

Tumor-infiltrating lymphocytes (TILs) reflect the local immune response against tumor growth and metastasis. The interaction between the different T lymphocyte subtypes serves an important role in the immune response of breast cancer (7-9). The majority of TILs in solid tumors are $CD3^+$, which includes $CD4^+$ helper cells (Th1 and Th2 subtypes), $CD4^+$ regulatory T-cells and $CD8^+$ cytotoxic T lymphocytes. High $CD3^+$ cell density has been reported to be associated with a favorable outcome in oropharyngeal cancer, and a low $CD3^+$ count has been shown to predict a shorter disease-free survival (DFS) in colon and cervical cancer (10,11). In general, the high number of $CD8^+$ TILs has been associated with an increased DFS and overall survival (12,13).

Previous studies have suggested that an increased percentage of TILs in breast cancer is correlated with an improved prognosis. Loi *et al* (14) analyzed TILs in a murine model of residual molecularly characterized TNBCs following neoadjuvant chemotherapy (NAC), and it was concluded that genetic or transcriptomic alterations in Ras/MAPK signaling were significantly associated with a low TIL percentage.

In addition, in patients with TNBC, a high T lymphocyte percentage has been associated with pathological complete responses (pCR), increased DFS and improved overall survival (15-17). However, molecular analyses, such as the expression profiles of genes regulating TILs, have not been thoroughly conducted in the clinical context.

The aim of the present study was to analyze the percentage, immunophenotype, and molecular gene expression of TILs in patients with TNBC tumors.

Materials and methods

Sample collection. A total of 96 tumor samples from the Department of Pathology of Hospital San José TecSalud (Monterrey, Mexico) were collected for the present study. IRB approval was obtained from the Ethics Committee for Research at Tecnológico de Monterrey, and the National Bioethics Commission (code id: CONBIOETICA19CE10 0820130520), and also was granted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to tumor sample collection.

Tumor tissue from patients with breast cancer was prospectively collected at the Breast Cancer Unit between August 2011 and August 2015. Demographic, familiar, clinical, tumor grade, lymph node status, ER, PR, and HER-2/neu data were recorded.

Tumor samples were collected using ultrasound-guided core needle biopsies. Tumor cylinders (4-6) were obtained from each patient for hematoxylin-eosin (H&E) staining and immunohistochemistry prior to NAC. Tumor sampling for microarray studies was performed as previously described (18).

NAC. Prior to standard NAC, the center of the breast tumor was marked by a charcoal suspension injection. If the tumor was palpable, the injection was performed by a breast surgeon. The injection in non-palpable tumors was performed by a radiologist under ultrasound guidance. The charcoal suspension was used to localize the tumor following NAC for pathological analysis.

The NAC regimen consisted of doxorubicin i.v. ($\geq$60 mg/m^2) + cyclophosphamide IV (600 mg/m^2) every 3 weeks for 4 doses, followed by paclitaxel 80 mg/m^2 by i.v. infusion for 1 h weekly for 12 doses.

Pathological analysis. The histological grade of the core needle biopsies was obtained prior to neoadjuvant therapy, using the Bloom-Richardson score (19). The stage of breast cancer was determined according to the American Joint Committee on Cancer (AJCC) (20). The assessment of the percentage of TILs was performed as per the recommendations of the International TIL Working Group 2014 in breast cancer (21). The patients were divided into three categories according to TIL percentage: 1-19%, low (20); <49%, intermediate; and ≥50%, high (21).

All biopsy specimens were fixed in 10% neutral buffered formalin between 6-24 h and embedded in paraffin at room temperature. Tissue sections (4-μm thick) were obtained on coated glass slides prior to NAC treatment and stained with H&E. The H&E stain was performed by deparaffinizing sections, washing twice with xylene for 10 min each. The sections were subsequently rehydrated twice using a descending alcohol gradient (100, 95 and 70%). Next, the sections were briefly washed in distilled water, stained in Harris hematoxylin solution for 8 min, washed in running tap water for 5 min, treated with 1% acid alcohol for 30 sec and washed with running tap water for 1 min at room temperature. Lastly, the sections were treated with 0.2% ammonia water for 30 sec, rinsed in 95% alcohol (10 dips) and counterstained in eosin-phloxine solution for 30-60 sec at room temperature. The stromal component of TILs was evaluated within the borders of the invasive carcinoma using a magnification of x200 (ocular, x10; objective, X20). The following areas were excluded from the study: Tumoral borders; areas around the *in situ* carcinoma; normal breast lobules; and tumor areas exhibiting artifacts; necrosis and hyalinization. The cancer immunophenotype experiments were performed on the core needle biopsies, prior to neoadjuvant therapy. The evaluation of the pathological response following NAC was determined using the de Miller-Payne and MD Anderson Cancer Center (Residual Cancer Burden) system (22).

Immunohistochemistry. ER, PR, HER-2, Ki67, $CD3^+$, $CD4^+$ and $CD8^+$ IHC analysis were performed with a Ventana BenchMarck GX® autostainer (Roche Diagnostics). Sections of 5-mm were obtained, paraffin slides were deparaffinized using 2 changes of xylene for 10 min each and hydrated using graded alcohol and distilled water (2 changes of 100% ethanol, 2 changes of 95% ethanol and 2 changes of distilled water) for 10 min each at room temperature (15-25˚C). Heat-induced epitope retrieval with citrate buffer was performed. Slides were then cooled and rinsed with distilled water, rinsed in tris buffered saline with 20 ml of Tween-20 for 5 min. Slides were then rinsed with 3% hydrogen peroxide, followed by a rinse with a wash buffer and covered with 300 μl of protein block for 5 min at room temperature. Slides were treated with Dako;

Table I. ER, PR, and Her-2 immunohistochemistry antibodies.

	Receptor type		
Antibodies	ER	PR	Her-2
Clones	SPI (Rabbit monoclonal primary antibody)	1E2 Rabbit monoclonal primary antibody)	4B5 Rabbit monoclonal primary antibody)
Supplier	Roche	Roche	Roche
Dilutions	The antibody is diluted in 1:500 M Tris-HCl with 2% carrier protein, and 0.10% ProClin 300, a preservative.	The antibody is diluted in 1:500 M Tris-HCl with 2% carrier protein, and 0.1% ProClin 300, a preservative.	The antibody is diluted in 1:500 M Tris buffered saline, 0.01 M EDTA, 0.05% Brij-35 with 0.3% carrier protein and 0.05% sodium azide, a preservative.
Thresholds	Nuclear positivity >1%	Nuclear positivity >1%	Overexpression must meet threshold criteria for intensity of staining (≥2 on a scale of 0-3+) and percent positive tumor cells (>10%). Staining must also localize to the cellular membrane.
Guidelines	ASCO/CAP	ASCO/CAP	ASCO/CAP

ER, estrogen receptor; PR, progesterone receptor; Her-2, human epidermal growth factor receptor 2; ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists.

Agilent Technologies Inc. monoclonal primary antibodies to HER2/Neu (1:500; Clone 4B5 for 32 min at 37˚C), ER (1:500; Clone SP1), PR (1:500; Clone 1E2), Ki67 (1:100; Clone 30-9), $CD3^+$ (1:500; clone 2GV6), $CD4^+$ (1:500; clone SP35) and $CD8^+$ (1:500; clone SP57). Except for HER2/Neu all the other primary antibodies were incubated for 16 min at 37˚C. Slides were then rinsed with wash buffer and secondary antibody Dako Agilent Technologies Inc. Envision labeled polymer HRP anti-rabbit (1:100; cat. no. K4002) was applied for 5 min at 15-25˚C. After the secondary reagent, DAB was applied for 10 min and the slides were rinsed with distilled water. Counterstaining was done with hematoxylin for 3 min at room temperature and slides were washed in tap water at room temperature. Slides were then blued in ammonia water, rinsed in tap water, dehydrated in graded alcohol (95 and 100% ethanol), cleared in xylene (2 changes) for 10 min each at room temperature and coverslipped for microscopic examination. The immunohistochemistry were performed following the American Society of Clinical Oncology/College of American Pathologists guidelines (Tables I and II) (23).

Microscopic evaluation of $CD3^+$, $CD4^+$ and $CD8^+$ TILs. TIL-positive immunohistochemical scoring was performed by 2 breast pathologists from Hospital San José TecSalud, Tecnológico de Monterrey (Monterrey, Mexico). $CD3^+$, $CD4^+$ and $CD8^+$ TILs were counted in 5 randomly selected high-power fields using a Carl Zeiss® Axio Lab A1 light microscope (magnification, x40), from which the average was calculated. TIL count in the stroma was rated as follows: + (1-25 cells); ++ (26-50 cells); and +++ (≥51 cells); as previously described (24). TIL cases with <25 cells were considered as 'low TIL count', and those with >25 cells (++, +++) as 'high TIL count' (Fig. 1) (24).

RNA extraction. RNA extraction of tissue biopsies was performed according to the manufacturer's instructions, using RNeasy Fibrous Tissue Mini kit (Qiagen, Inc.), which is used to obtain high-quality RNA in small tissue biopsies. The RNA quality was assessed by capillary electrophoresis using the Experion™ Automated Electrophoresis Station (Bio-Rad Laboratories, Inc.). The mean RNA Quality index was 7.84 (range, 6.1-9.8). The RNA concentration was determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Inc.). The mean RNA concentration of the tested samples was 269.02 ng/μl, (range, 38.30-999.13 ng/μl). If several core tumor samples were collected during the procedure, 2-3 core samples were used to isolated total RNA and another 2-3 for the immunohistochemistry (IHC) assay.

Microarray analysis. Gene expression analysis was performed using frozen fresh tissue tumor samples. The tumor tissue samples from the biopsies were immediately treated with an RNase inhibitor preserver solution (RNAlater; Thermo Fisher Scientific, Inc.) (25). Selected RNAs were used for microarray hybridization and gene expression analysis, which were conducted using the GeneChip 3'IVT Express kit (Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. A recommended quantity of 100 ng total RNA input was used for each sample. Poly-A controls (Thermo Fisher Scientific, Inc.), which are exogenous positive controls that monitor the entire target preparation, were used for all samples. The hybridization mixture was prepared and applied to the GeneChip Human Genome U133 Plus 2.0 array (Thermo Fisher Scientific, Inc.), measuring >43,000 transcripts that represented >20,000 human genes. Washing and scanning processes were performed in the Fluidics Station 400 and GeneChip Scanner 3000 7G, respectively. The preliminary

Table II. $CD3^+$, $CD4^+$, and $CD8^+$ immunohistochemistry antibodies.

	Antigen type		
Antibodies	$CD3^+$	$CD4^+$	$CD8^+$
Clones	2GV6 Rabbit monoclonal primary antibody	SP35 Rabbit monoclonal primary antibody)	SP57 Rabbit monoclonal primary antibody)
Supplier	Roche	Roche	Roche
Dilutions	The antibody is diluted in 1:500 M Tris-HCI with 1% carrier protein and ProClin 300, a preservative.	The antibody is diluted in 1:500 M Tris-HCL with 1% carrier protein and 0.10% ProClin 300, a preservative.	The antibody is diluted in 1:500 M Tris-HCL with 1% carrier protein and a preservative
Thresholds	The cellular staining pattern anti-CD3 (2GV6) antibody is membranous and/or cytoplasmic.	The cellular staining pattern anti-CD4 (SP35) is membranous.	The staining pattern anti-CD8 (SP57) is membranous.

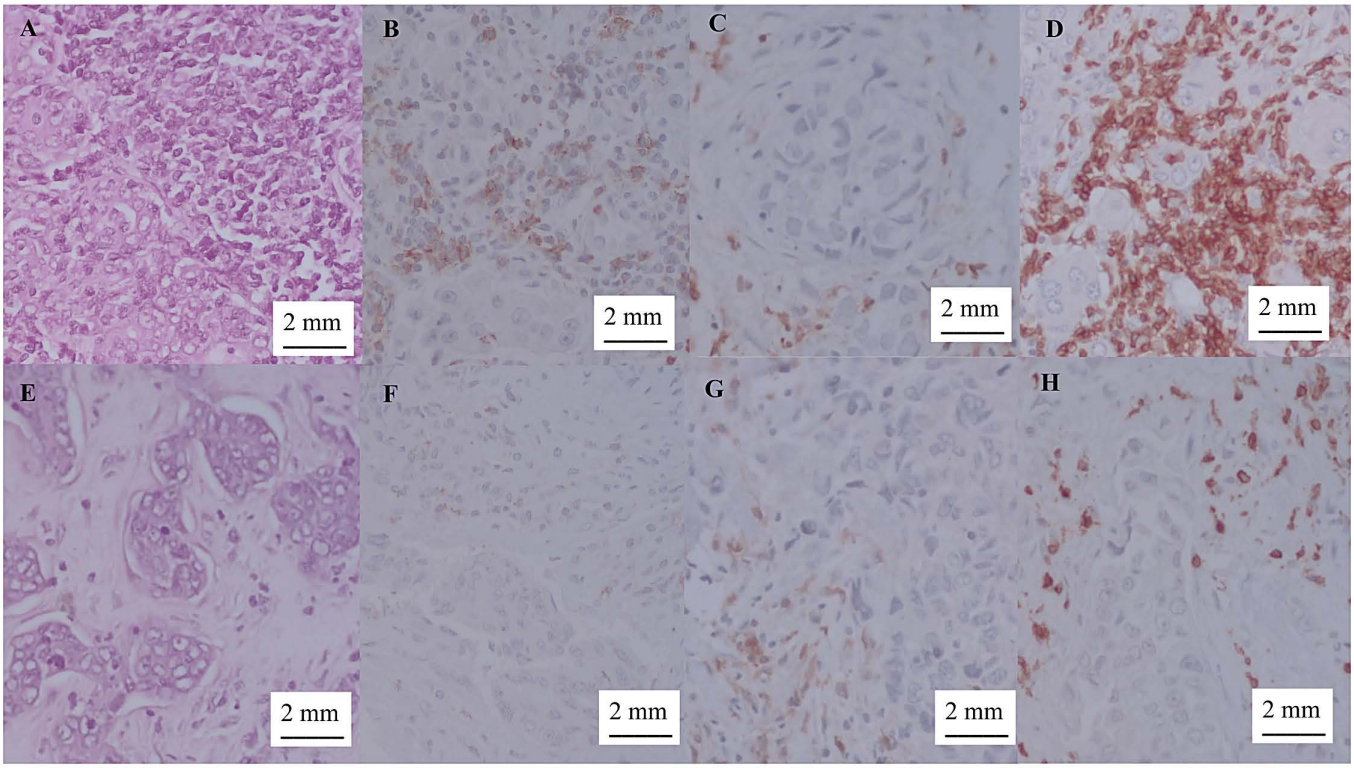

Figure 1. *Microscopic evaluation of $CD3^+$, $CD4^+$ and $CD8^+$ TILs*. (A) High TILs. (B) High $CD4^+$ count. (C) High $CD8^+$ count. (D) High $CD3^+$ count. (E) Low TILs. (F) Low $CD4^+$ count. (G) Low $CD8^+$ count. (H) Low $CD3^+$ count. Magnification, x10. TILs, tumor infiltrating lymphocytes.

data analysis was completed using the Affymetrix Micorarray Suite software version 5.0.0.032 (Thermo Fisher Scientific, Inc.).

Microarray data processing. Normalization was performed using Robust Microarray Analysis (RMA) and quantile normalization. One sample exhibited poor quality and was removed from the analysis. The probes whose mean expression (log scale) was <4 (in a logarithmic scale resulting from RMA) were removed. A gene expression signature was performed by t- and Kolmogorov tests with a multiple comparison correction using the false discovery rate (FDR method (26). Probes with significant P-values in both tests (FDRt and Kolmogorov test <0.05%) were considered positive. Analyses were performed in R software [R Core Team. (2014)] (27).

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene network. Interaction analysis of the selected genes was performed using GO (28,29) and KEGG (30-32) databases through the online tool STRING: Functional protein association networks version 11.0 (33). STRING analysis demonstrated functional enrichments of selected genes: GO analysis of Biological Process, Molecular Function, SCellular Component and KEEG Pathways. The combined score was computed by combining the probabilities

Table III. Clinicopathological characteristics of the patient cohort (n=26).

Characteristics	n	%
Age at diagnosis, years		
18-39	1	5
40-59	15	57
>60	10	38
Sex		
Female	26	100
Male	0	0
Sample collection method		
Core needle biopsy	26	100
Treatment		
NAC	26	100
Radiotherapy	22	85
Tumoral grade		
1	0	0
2	5	20
3	21	80
Lymphovascular invasion		
Present	19	73
Absent	7	27
Lymph node status		
Positive	8	30.7
Negative	18	69
Metastasis		
Yes	1	3.4
No	25	96.6
Pathological Stage		
I	5	19.2
II	15	57.6
III	4	15.3
IV	2	7.6

NAC, neoadjuvant chemotherapy.

from the different evidence channels and corrected for the probability of randomly observing an interaction (34).

Statistical analysis. Categorical variables are presented as whole numbers and percentages, while continuous variables are described using the median and interquartile range. The χ^2 test was used to evaluate the association between pathological response and TILs. Descriptive and association analysis was performed using GraphPad Prism 6 for Windows version 6.01 software (GraphPad Software Inc.). Due to the small sample size and lack of power, specific association methods (exlogistic and firthlogit) were used.

Results

Demographics and clinical characteristics. A total of 96 tumor samples were prospectively collected at the Breast Cancer Unit of Hospital San José TecSalud between August 2011 and August 2015. All patients were female. A total of 26/96 samples were diagnosed as TNBC. These 26 patients with TNBC comprised the study population.

The median age was 49-years old (range, 43-56 years); 80% of the tumors were poorly differentiated (grade 3) and 20% moderately differentiated (grade 2). Lymphovascular invasion was present in 18 (73%) patients and ipsilateral lymph node metastasis in 8 (30.7%) patients. Only 1 (3.4%) patient had metastatic disease upon presentation.

Tumor staging according to the AJCC revealed that 5 patients had (19.2%) stage I, 15 (57.6%) stage II, 4 (15.3%) stage III and 2 (7.6%) stage IV disease. All patients received standard NAC. The clinicopathological characteristics of the cohort are summarized in Table III.

Pathological response. The pathological response to NAC was assessed, and it was identified that 4 (15.3%) patients had pNR, 16 (61.5%) pPR and 6 (23%) pCR. TIL assessment demonstrated low, intermediate, and high TIL counts in 8 (30.7%), 10 (38.4%), and 8 (30.7%) patients, respectively. The CD3 count was high in 22 (85.6%) and low in 4 (15.3%) of the specimens. Notably, the CD4 count was low in 22 (85.6%) and high in 4 (15.3%) specimens. The CD8 count was low in 21 (80.7%) and high in 5 (19.2%) samples. The CD counts determined by microscopy were associated with the number of lymphoid cells; CD3 showed a higher TIL count of lymphoid cells (>25 lymphoid cells), meanwhile CD4 and CD8 were associated with a low TIL count (<25 lymphoid cells). With regard to pCR cases, 50% had a high number of TILs, 16% an intermediate number, and 33% a low number. With regard to the patients with pPR, 25% had a high number of TILs, 50% an intermediate number and 25% a low number. Finally, none of the patients with pNR exhibited a high number of TILs, while 25% of patients with pNR had an intermediate number and 75% pNR a low number (Table IV).

Immunohistochemistry, gene expression and analysis results. Using immunohistochemistry, CD3 and CD4 counts were significantly associated with pPR and pCR (P=0.04), but CD8 was not associated with pathological response (P=0.75) (Table V). On the contrary, microarray analysis presented in a heat map demonstrated that CD3, CD4 and CD8 were significantly associated to pathological response (P<0.05; Fig. 2). This difference may be due to the heat map being an average of a global analysis that includes all pathological responses (pCR, pPR, and pNR) (35). In addition, microarray analysis determines the RNA messenger protein expression whereas IHC only analyzes the protein expression (36). In addition, most pCR cases had a high CD3 count (83.3%). No additional associations between TILs and other clinicopathological parameters were identified in the study cohort.

Gene expression analysis of selected genes [*CD3, CD4. CD8, CD20, CD45*, forkhead box P3 (*FOXP3*), interleukin 6, programmed cell death 1 and CD274 molecule] showed that *CD3, CD4, CD8, CD45* and *CD20* exhibited a high expression in the patients with pPR and pCR, whereas a low expression of the aforementioned genes was observed in patients with pNR (Fig. 2).

Table IV. Association between TIL count (including CD3, CD4 and CD8 count) and pathological response.

Disease presentation	n	%	CD3		CD4		CD8	
			Low (n=4)	High (n=22)	Low (n=22)	High (n=4)	Low (n=21)	High (n=5)
Pathological response								
Complete response	6	23	16.7	83.3	100	0	100	0
Partial response	16	61.5	6.2	93.8	87.5	12.5	75	25
No response	4	15.3	50	50	50	50	75	25
TILs								
High	8	30.7	100	0	25	75	50	50
Intermediate	10	38.4	20	80	100	0	90	10
Low	8	30.7	25	75	75	25	100	0

TILs, tumor infiltrating lymphocytes.

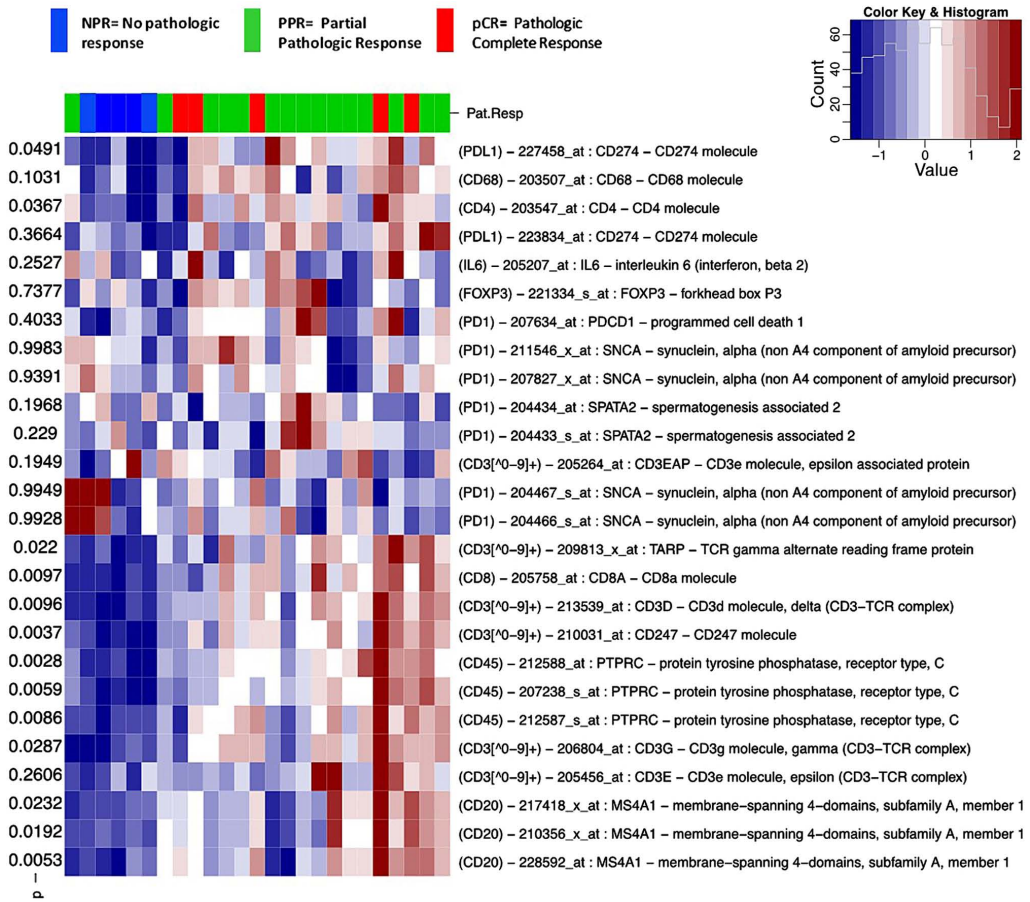

Figure 2. *Heat map of immunity-associated genes with, P-values and color key*. Heatmap of differential gene expression of selected genes (*PDL1, CD68, CD4, IL6, FOXP3, PD1, CD3, CD8, CD3, CD45* and *CD20*) associated with immunity. P-values ranging from 0.002-0.998 are presented on the left side of the heat map and demonstrate the association of pathological response and selected genes. The histogram in the color key in columns presents the low gene expression from 0 to -1 in blue and high gene expression from 1-2 in red. nPR, no pathologic response; pPR, partial pathologic response; pCR, pathologic complete response.

Gene interaction. GO analysis showed that the predicted target genes exhibited a stronger interaction among *CD68, IL6, FOXP3, PTPRC, CD274, CD4, CD8A, PDCD1, CD3G, CD3D, CD247* and *CD3E*, a weaker interaction with *KRT20, MS4A1* and *CD3EAP*, and no interaction with *SPATA2* and *SNCA*. A total of 14 genes were involved in immune response, 7 in T-cell receptor signaling pathway, 9 in lymphocyte activation, 8 in T-cell activation, and 7 in T-cell differentiation (background gene, 131-1560; FDR, 2.90E-9-3.07E-10; Fig. 3A). Simultaneously, the KEGG database demonstrated

Table V. Association between TILs and pathological response.

Variable	No pathological response	Pathological response	χ^2
CD3 TILs			
Low	2	2	0.04
High	2	20	
CD4 TILs			
Low	2	20	0.04
High	2	2	
CD8 TILs			
Low	3	18	0.75
High	1	4	

TILs, tumor infiltrating lymphocytes.

8 genes that were involved in the T-cell receptor signaling pathway, 7 genes involved in hematopoietic cell lineage and TH17 cell differentiation, and 5 genes involved in primary immunodeficiency and Th1/Th2 cell differentiation (background gene 88-99; FDR, 1.33 E-7-7.39E-13; Fig. 3B).

Discussion

In the present study, the percentage of stromal TILS on core needle biopsies of TNBC tumors, as well as their immunophenotype prior to NAC, were evaluated. Moreover, the association between TILs and pathological responses was examined. Even though the pathological response of tumors to NAC has been previously associated with a high percentage of TILs (37,38), these have usually been classified into only two categories, low and high (24). The present study differs from previous studies in that it divided TILs into three groups, to obtain a clearer understanding of their association with the pathological response to NAC. Furthermore, prior studies performed TIL counts using H&E staining, whereas, the count in the present study was performed prior to staining. Notably, the results demonstrated that patients with TNBC with pCR had an increased $CD3^+$ TIL population without increased $CD4^+$ and $CD8^+$ TILs.

It is noteworthy that the vast majority of studies performed in this field have included HER2/neu amplified cases, which allowed for increased statistical power, but lacked homogeneity within their sample cohort (15-18). On the contrary, the present study used a purely TNBC cohort, thus achieving a homogenous group, in which the majority of patients with pCR exhibited a high percentage of TILs, consistent with prior studies (4,5). Similarly, a high $CD3^+$ expression was observed in the majority of pCR (83.3%) and pPR (93.7%) cases. These data were consistent with the study by Rathore *et al* (39), who reported an association between high $CD3^+$ expression and improved survival, which has also been observed in cervical and epithelial ovarian cancer (11,40). Clinically, the density of $CD3^+$ expression could be routinely measured to predict pathological response in patients with TNBC.

Even though high $CD4^+$ and $CD8^+$ density have been consistently associated with improved overall survival (12,13,18), in the present cohort, $CD4^+$ and $CD8^+$ were identified to be associated with a low TIL count in a patient with pCR. This discrepancy may be due to the demographic data of the patient and the size of the cohort; larger multicentric cohorts are required to explore this association further.

Kim *et al* (41) reported a decreased $CD8^+$ count in breast cancer with lymph node metastasis, high proliferation rate and advanced tumor stage. However, in the present cohort, no association was observed between TIL count and vascular invasion or lymph node metastasis. Furthermore, the majority of the patients included in the present study were diagnosed with early-stage breast cancer (76.8% in stage I and II), which could explain the absence of a correlation between TILs and advance tumor stages herein. Nonetheless, a high percentage of patients exhibited a high TIL count and $CD3^+$ lymphocytes, which has been described previously (24,37).

Notably, patients with pCR and pPR exhibited high gene expression levels of *CD3, CD4, CD8, CD45* and *CD20*. Conversely, patients with pNR exhibited a limited expression of these genes, suggesting that there is limited participation of both B (CD20) and T lymphocytes (CD3, CD4 and CD8) in the antitumor cell response. This immune activity inevitably affects the pathological response to NAC.

Levy *et al* (42) reported an immune signature of T-cell infiltration in breast tumors through an exome study. They described that exome reads mapping to the complementarity-determining-region 3 (CDR3) of mature T-cell receptor β can be used as an immune DNA signature. Exomes from the CDR3 fraction of breast cancers in The Cancer Genome Atlas were used to study $Her2^+$ patients. Improved survival was associated with increased TIL fraction, tumor purity, as well as adaptive immunity gene expression signatures in the $Her2^+$ population. However, these differences were not observed in patients with TNBC (42). In addition, gene profiling was not performed.

The immune signature of metastatic breast cancer has been studied (41), in which the $Her2^+$ expression and previous taxane treatment were positively correlated with a high expression of 9 genes associated with immune checkpoints: *PDCD1 (PD-1); CD274 (PD-L1); CD276 (B7-H3); CTLA-4; IDO1; LAG3; VTCN1; HAVCR2;* and *TNFRSF4* (*OX40*) (41). Importantly, these genes have interactions with each other.

Finally, quantitative immunofluorescence assay has been performed to measure the stromal expression of CD3, CD8 and CD20. It was identified that CD20 score predicted pCR independently of age, size, nuclear grade, nodal status, ER, PR, HER2 and Ki-67. CD3, CD8, and pathologist estimation did not demonstrate an association with pathological response (18).

Even though the aforementioned studies have performed important immune analyses, there is an absence of literature examining immunophenotypic signatures in the context of gene expression profiling of TILs in breast cancer. To the best of our knowledge, the present study was the first to report an association between immunophenotype and gene expression profiling in patients with TNBC. This provides a new promising method for assessing pathological response in TNBC.

The present study was not without limitations. RT-qPCR experiments could not be performed to verify the results of the

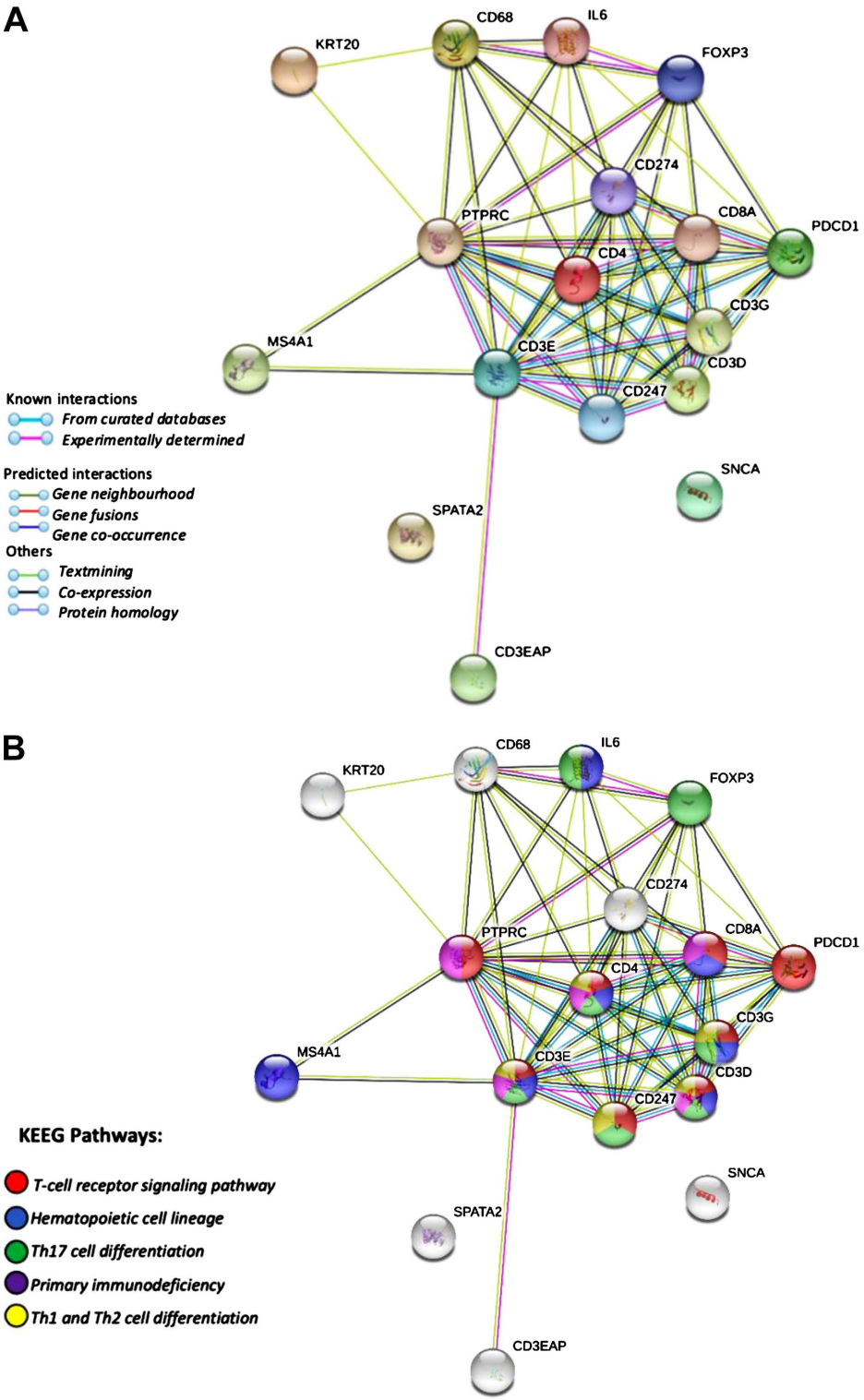

Figure 3. Gene ontology terms and KEGG pathways. Predicted target genes (*CD68*, *IL6*, *FOXP3*, *PTPRC*, *CD274*, *CD4*, *CD8A*, *PDCD1*, *CD3G*, *CD3D*, *CD247*, *CD3E*, *KRT20*, *MS4A1*, *CD3EAP*, *SPATA2* and *SNCA*) and PPI networks of target genes. (A) STRING PPI network of co-expressed and interacting genes. The clusters of 17 genes represent proteins. The colored nodes represent proteins, white nodes represent second-shell interactions, filled nodes for 3D known or predicted structures and edges represent protein-protein associations that did not necessarily need to bind physically to each other. (B) KEGG pathways associated with the proteins identified and reported in the STRING analysis. Red represents the T-cell receptor pathway, blue represents the hematopoietic cell lineage, green represents Th17 cell differentiation, purple represents primary immunodeficiency, yellow represents Th1 and Th2 cell differentiation. KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interactions network.

GO and KEGG analyses, due to lack of tissue. However, our previous study reported a gene expression signature for TNBC in a previous study (25). Even though the sample size was relatively small, the present study included a cohort comprised solely of patients with TNBC. However, larger multicentric studies are required to expand and confirm the present results.

In conclusion, the results of the present study demonstrated that TILs may predict the pathological response to NAC in

patients with TNBC. In addition, the results identified a more accurate association between the high expression levels of *CD3, CD4, CD8, CD45* and *CD20* genes and pPR and pCR, compared with the association between the high expression of those genes and immunohistochemistry alone.

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Availability of data and materials

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

Authors' contributions

GSGM, revise the manuscript for intellectual content, and gave final approval for the version to published. GSGM, GMF, CALG, AD and SSF made contributions to the acquisition, collection, interpretation of data and writing of the manuscript. HDA, JVE, VTA, ROL and SCH made contributions to the data analysis and funding. EEZ, MAAJ, CVG, and OPC made contributions for the analysis and collection of data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

IRB approval was obtained from the Ethics Committee of Research at Tecnológico de Monterrey and the National Bioethics Commission (code id: CONBIOETICA19CE100820130520), and was also granted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to tumor sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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