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Original Article

Increased intratumor heterogeneity, angiogenesis and epithelial to mesenchymal transition pathways in metaplastic breast cancer

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Abstract: Metaplastic breast cancer (MBC) constitutes a rare but unique histologic entity with poor prognosis. We hypothesized that MBC possesses unique genetic profile and tumor immune microenvironment. MBC cases were identified from a total of 10827 breast cancer entries in the Cancer Genome Atlas Data Set (TCGA) and the AACR-GENIE (Genomics Evidence Neoplasia Information Exchange) cohorts. Tumor infiltrated immune cells were estimated by xCell. Baseline clinical characteristics were compared, and gene set enrichment analysis (GSEA) was performed. MBC comprised 0.66% of the cohorts (1.2% of TCGA and 0.6% of GENIE). MBC cases were predominantly triple-negative (TNBC) (8 (61.5%) vs 151 (14.4%), P<0.001), and high Nottingham histological grade (8 (61.5%) vs 222 (21.1%), P=0.02) compared to non-MBC in the TCGA cohort. Increased infiltration of M1 macrophages (P=0.012), dendritic cells (P<0.001) and eosinophils (P=0.036) was noted in the MBC cohort however there was no difference in cytolytic activity (P=0.806), CD4 memory (P=0.297) or CD8 T-cells (P=0.864). Tumor mutation burden was lower in the MBC compared to the non-MBC, median: 0.4 vs 1.6/Mb in the TCGA-TNBC cohort (P=0.67) and 3.0 vs 4.0/Mb (P=0.1) in the GENIE-cohort. MBC had increased intratumor heterogeneity (P<0.001), macrophage regulation (P=0.008) and TGF-beta response (P<0.001). Disease-specific survival was decreased in MBC (P=0.018). Angiogenesis and epithelial-to-mesenchymal transition pathways were enriched in triple-negative MBC by GSEA (P=0.004 and P<0.001, respectively). Our results suggest that high intratumor heterogeneity, enriched angiogenesis and EMT pathway expression represent possible mechanisms leading to worse disease-specific survival found in metaplastic breast cancer.

Keywords: Metaplastic breast cancer, immune microenvironment, intratumor heterogeneity, M2 macrophages

Introduction

Metaplastic breast cancer is a distinct but rare pathologic subtype characterized by an aggressive tumor biology, comprising 0.25-1% of all invasive breast cancers [1, 2]. Some of the attributes that explain its aggressiveness include the resistance to conventional systemic therapies and the more common hematogenous spread as opposed to lymphatic dissemination seen with invasive ductal carcinoma. Furthermore, it poses a challenge for the pathologists due to its significant heterogeneity containing squamous cells and/or mesenchymal tissue that can be markedly atypical or bland, resembling fibromatosis. A recent, matched analysis from our institution compared metaplastic breast cancer with triple negative breast cancer cases and found worse disease-free and overall survival for the metaplastic subtype [3]. The heterogeneity in phenotype poses a question regarding underlying

genomic and transcriptomic profiles that are not well defined.

The genomic and transcriptomic analysis of metaplastic breast cancer subtypes is not completely elucidated in the literature with some studies reporting high *PD-L1* gene amplification as opposed to a low rate in others [4, 5]. A Surveillance, Epidemiology, and End Results (SEER database) study of metaplastic breast cancer has shown that human epidermal growth factor receptor 2 (HER2) status was associated with improved survival and postulated engagement of the innate and adaptive immune systems related to the HER2-antibodies [6]. However, the exact immune cell composition and expression has not been investigated.

We hypothesized that there is a unique transcriptomic profile that explains the phenotype of metaplastic breast cancer using an *in silico* translational research approach.

Materials and methods

Patient cohort

The Cancer Genome Atlas Data Set (TCGA) breast cancer cohort [7] and the American Association of Cancer Research (AACR) Genomics Evidence Neoplasia Information Exchange (GENIE) project were utilized to obtain clinicopathologic and genomic data through the cBioportal as previously described [8-14]. A total of 10827 breast cancer cases, 1064 from TCGA and 9763 from AACR-GENIE, were analyzed. Metaplastic breast cancer was identified by the pathological description in each dataset. Institutional review board approval was waived as both TCGA and AACR-GENIE cohorts are publicly accessible, de-identified databases.

Immune cell composition and gene set enrichment analysis (GSEA)

We used an online computational algorithm, xCell [15, 16], to estimate the immune cell composition based on gene expression data, as previously described [9-12, 14, 17-26]. Gene set enrichment analysis (GSEA) [27] was conducted using publicly available software provided by the Broad Institute [22-24, 28-36]. False discovery rate (FDR) of less than 0.25 was used to define statistical significance as recommended by the Broad Institute [27]. Comparison between the non-metaplastic breast cancer group and the metaplastic group as well as a subgroup analysis for the triple-negative subtype was performed using the Hallmark gene set collection of The Molecular Signatures Database (MSigDB) [37] similar to prior work by our group [11, 30-32, 34, 38-43].

Statistical analysis

Statistical analysis of the group comparison was calculated by one-way ANOVA or Fisher's exact test. Survival analysis was performed using Kaplan-Meier plots with log-rank test. All statistical analyses were performed using R software v. 4.0.1.

Results

Metaplastic breast cancer clinicopathologic characteristics

Out of 10827 breast cancer cases, 72 (0.66%) were identified to be metaplastic carcinomas, 13/1064 (1.2%) and 59/9763 (0.6%) in the TCGA and GENIE cohorts, respectively. In the TCGA cohort, estrogen receptor (ER) status was positive in 3 (23.1%) vs 776 (73.8%), (P<0.001), progesterone receptor (PR) status was positive in 2 (15.4%) vs 671 (63.8%), (P< 0.001), human epidermal growth factor receptor 2 (HER2) status was positive in 1 (7.7%) vs 174 (16.6%), (P=0.48) in the metaplastic versus non-metaplastic group (**Table 1**).

Multidimensional scaling plot designed based on pairwise genetic distances among the different PAM50 (based on the 50-gene classifier) intrinsic subtypes [44] including metaplastic breast cancer demonstrated clustering of the metaplastic cases between the HER-2 and the basal subtypes (**Figure 1**). The majority of metaplastic tumors were triple negative (by immunohistochemistry) compared with the other subtypes, 8 (61.5%) vs 151 (14.4%), respectively (P<0.001). The distribution of metaplastic vs non-metaplastic breast cancer cases based on American Joint Committee on Cancer (AJCC) Cancer Staging 8th edition [45] was: 2 (15.4%) vs 271 (25.8%) Stage I, 6 (46.2%) vs 594 (57.8%) Stage II, 3 (23.1%) vs 239 (22.7%) Stage III, 0 vs 18 (1.7%) Stage IV, respectively (P=1). Node positive disease was

Table 1. Clinicopathologic data from the TCGA clinical data resource (TCGA-CDR) of the metaplastic and non-metaplastic cohorts

	Metaplastic n=13	Non-metaplastic n=1051	*P* value
ER positive	3 (23.1)	776 (73.8)	<0.001
PR positive	2 (15.4)	671 (63.8)	<0.001
HER2 positive	1 (7.7)	174 (16.6)	0.481
Triple-negative	8 (61.5)	151 (14.4)	<0.001
T stage			
T1	2 (15.4)	271 (25.8)	0.134
T2	6 (46.2)	607 (57.8)	
T3	4 (30.8)	133 (12.7)	
T4	1 (7.7)	37 (3.5)	
Node positive	2 (15.4)	538 (51.2)	0.01
AJCC stage			
Stage I	2 (15.4)	176 (16.7)	1.00
Stage II	8 (61.5)	594 (56.5)	
Stage III	3 (23.1)	239 (22.7)	
Stage IV	0	18 (1.7)	
Tumor grade			
Grade 1	0	76 (7.2)	
Grade 2	1 (7.7)	259 (24.6)	0.021
Grade 3	8 (61.5)	222 (21.1)	
Mitotic score			
1	1 (7.7)	246 (23.4)	
2	2 (15.4)	149 (14.2)	0.037
3	6 (46.2)	162 (15.4)	
Nuclear score			
1	0	33 (3.1)	
2	0	262 (24.9)	0.008
3	9 (69.2)	262 (24.9)	
Tubular score			
1	0	24 (2.3)	
2	0	110 (10.5)	0.379
3	9 (69.2)	423 (40.2)	

noted in 2 (15.4%) metaplastic cases vs 538 (51.2%) non-metaplastic cases, P=0.01. There were 0 vs 76 (7.2%) Nottingham histological grade I, 1 (7.7%) vs 259 (24.6%) grade II, 8 (61.5%) vs 222 (21.1%) grade III in the metaplastic vs non-metaplastic groups respectively, P=0.02.

Metaplastic breast cancer has increased infiltration of dendritic cells, eosinophils, M1 macrophages and regulatory T-cells

There was no difference in lymphocyte infiltration in metaplastic breast cancer, including CD8 T-cells (P=0.864), CD4 memory T-cells (P=0.297), type 1 helper T-cells (P=0.485), type 2 helper T-cells (P=0.149), gamma delta T-cells (P=0.844) and B-cells (P=0.902), except for regulatory T-cells (P=0.001). In terms of innate immunity, M1 macrophages (P=0.012), dendritic cells (P<0.001) and eosinophils (P=0.036) were highly infiltrated in the metaplastic group, however, cytolytic activity, that depicts the overall cell killing activity by immune cells, was comparable between the two groups (P=0.806). **Figure 2** depicts the complete results in boxplots.

Metaplastic breast cancer has increased leukocyte fraction, macrophage regulation, TGF-beta response and intratumor heterogeneity compared to other subtypes

Thorsson et al. published a comprehensive list of pre-calculated values of many computational algorithms of all the patients in TCGA [46]. Among the algorithms are leukocyte fraction, tumor infiltrating lymphocyte (TIL) regional fraction, macrophage regulation, lymphocyte infiltration signature, interferon (IFN)-gamma response, TGF-beta response, silent and non-silent mutation rate, single-nucleotide variation and insertion and deletion neoantigens, intratumor heterogeneity, and cell proliferation scores. When metaplastic was compared with others, there was a statistically significant higher proportion of leucocyte fraction (P=0.004), TGF-beta response (P<0.001), more macrophage regulation (P=0.008) and higher intratumor heterogeneity (P<0.001) in the metaplastic group. There was no difference in lymphocyte infiltration signature score (P=0.605), tissue-infiltrating lymphocytes (TIL) regional fraction (P=0.971), silent and non-silent mutation rates (P=0.597 and P=0.654, respectively) or proliferation scores (P=0.165) (**Figure 3**).

Metaplastic breast cancer was associated with worse disease-specific survival (DSS), but not progression-free survival (PFS), disease-free survival (DFS), or overall survival (OS)

In a comparison between the metaplastic and non-metaplastic breast cancer in the TCGA cohort there was no difference in progression-free survival (P=0.237), disease-free survival (P=0.616) or overall survival (P=0.111). However, the metaplastic breast cancer was as-

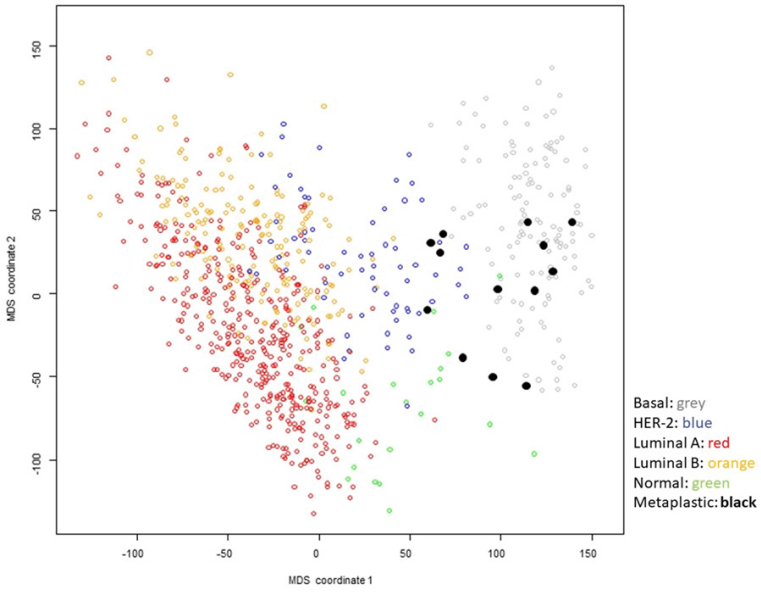


Figure 1. Multidimensional scaling plots (MDS) obtained on pairwise genetic distances between the different PAM50 intrinsic subtypes including metaplastic breast cancer in TCGA cohort. Open circles are color coded with PAM50 subtypes as the following; Basal: grey, HER2: blue, Luminal A: red, Luminal B: orange, Normal: green. Closed black circles represent metaplastic breast cancer.

sociated with worse disease-specific survival (P=0.018) (**Figure 4**).

Metaplastic breast cancer has lower mutation burden and frequent p53 mutations

In the GENIE cohort, the median mutation count for metaplastic subtypes was 3 vs 4 for non-metaplastic subtypes (P=0.1) and 0.4 vs 1.6/Mb in the TCGA-TNBC cohort (P=0.67). p53 mutations were more frequently observed in metaplastic tumors, 37/59 (55.9%) vs 3805/9763 (38%) in the GENIE cohort and 5/8 (62.5%) vs 348/1059 (32.9%) in the TCGA cohort (**Figure 5**).

Despite the fact that we used a massively larger cohort, we observed similar mutations as previously reported, albeit at a higher frequency. No new MBC-specific mutations were identified.

Significant enrichment of angiogenesis-related and epithelial-mesenchymal transition gene sets in metaplastic triple-negative breast cancer

GSEA analysis revealed enrichment in angiogenesis (Normalized Enrichment Score (NES) =1.81, False discovery rate (FDR) =0.14, P=0.004) and epithelial-mesenchymal transition gene sets (NES=1.88, FDR=0.017, P<0.001) for metaplastic triple-negative versus non-metaplastic triple-negative breast cancer (**Figure 6**).

In a subset analysis of the triple-negative metaplastic subgroup, intratumor heterogeneity (P<0.001) as well as TGF-beta response (P=0.04) remained high. However, the lymphocyte infiltration score was decreased (P=0.04) and there was decreased expression of CD4 memory T-cells (P=0.034) in the metaplastic triple-negative subgroup. Finally, there was increased number of fibroblasts (P<0.001), lymphatic endothelial cells (P=0.019) and microvascular endothelial cells (P=0.018) in metaplastic triple-negative breast cancer, which is in agreement with enhanced angiogenesis (**Figure 7**).

Discussion

Amongst 10827 breast cancer cases in GENIE and TCGA cohorts, 72 (0.66%) cases were identified by pathology to be metaplastic carcinomas. Similar to prior reports in the literature, metaplastic breast cancer was predominantly ER, PR negative, node-negative and predominantly high-grade [1, 2, 47]. Computational analysis showed higher infiltration of regulatory T-cells, M1 macrophages, dendritic cells and eosinophils in the metaplastic tumors while the cytolytic activity was not statistically different. There was no difference in mutation rate or neoantigens, but higher intratumor heterogeneity and higher *TP53* mutation rate was associated with metaplastic tumors. Epithelial-mesenchymal transition and angiogenesis gene sets were enriched in the MBC cohort as well as signaling gene sets such as androgen, estrogen response and *TGF*-beta.

Similar to prior studies, the majority of metaplastic cases were triple negative compared to

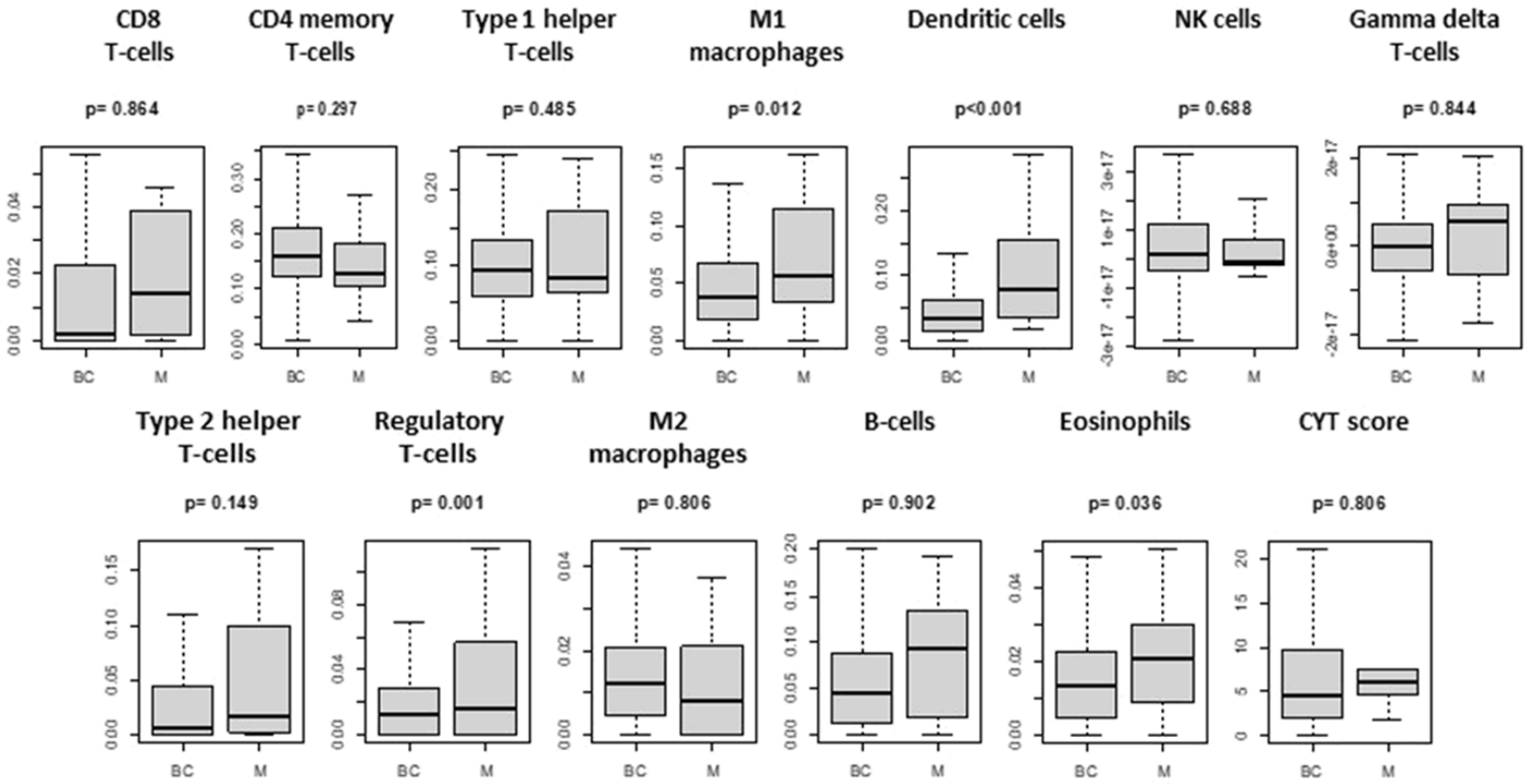


Figure 2. Tukey boxplots of fractions of immune cells and cytolytic activity (CYT) in metaplastic (M) versus non-metaplastic (BC) breast cancer using xCell algorithm in TCGA cohort. Y-axis shows the fraction of cells. Boxes depict medians and interquartile ranges. Depicted *P*-values were calculated using one-way ANOVA.

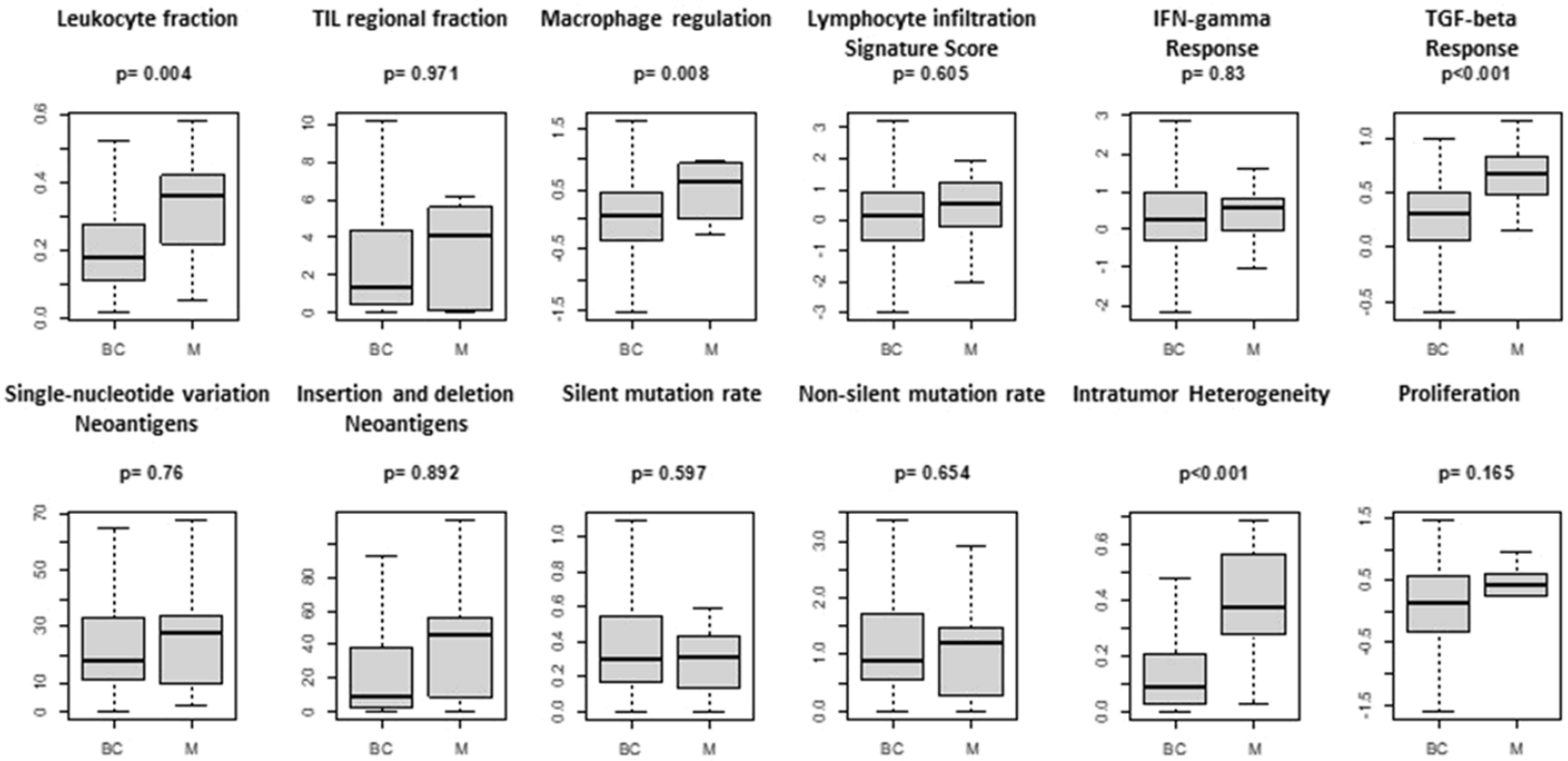


Figure 3. Association between metaplastic (M) versus non-metaplastic (BC) breast cancer and immune cell fraction and function scores, neoantigen expressions, mutation rates, intratumor heterogeneity and proliferation score in the TCGA cohort. Each score was previously calculated on every patient in TCGA by Thorsson et al. (22). One-way ANOVA test was used to calculate the *P* values.

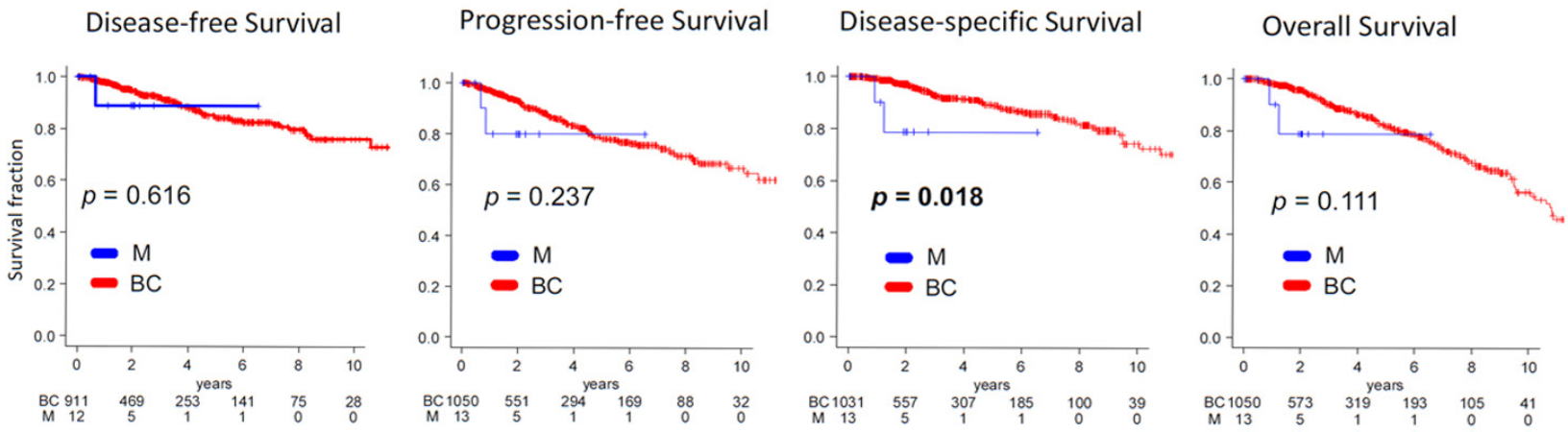


Figure 4. Survival curves of the metaplastic (M) versus non-metaplastic (BC) breast cancer in the TCGA cohort. Kaplan-Meier survival plots comparing patients with M (blue line) and BC (red line) along with log-rank test *P*-values are shown for disease-free survival, progression-free survival, disease-specific survival, and overall survival.

All breast cancer cases (N = 9763)			Metaplastic breast cancer cases (N = 59)		
Gene	Mutation number	Frequency	Gene	Mutation number	Frequency
TP53	3805	38%	*TP53*	37	55.90%
PIK3CA	3946	35.50%	*PIK3CA*	21	33.90%
CDH1	1105	11.10%	*HRAS*	6	10.20%
GATA3	1030	11.60%	*PTEN*	5	6.80%
MAP3K1	1005	8.30%	*PIK3R1*	5	8.30%
KMT2C	723	9.20%	*TERT*	4	12.50%
PTEN	609	5.60%	*RUNX1*	4	8.30%
ARID1A	570	6%	*NF1*	4	11.10%
KMT2D	539	5.70%	*KDM6A*	3	8.60%
ESR1	478	7.10%	*ZFHX3*	2	9.50%

The mutation number reflects the mean number of mutations in all samples. Frequency is the number of samples with mutations over the number of samples tested for each gene. The percentages do not add up to 100% as some samples have several mutations and there are samples that were not tested for all genes listed.

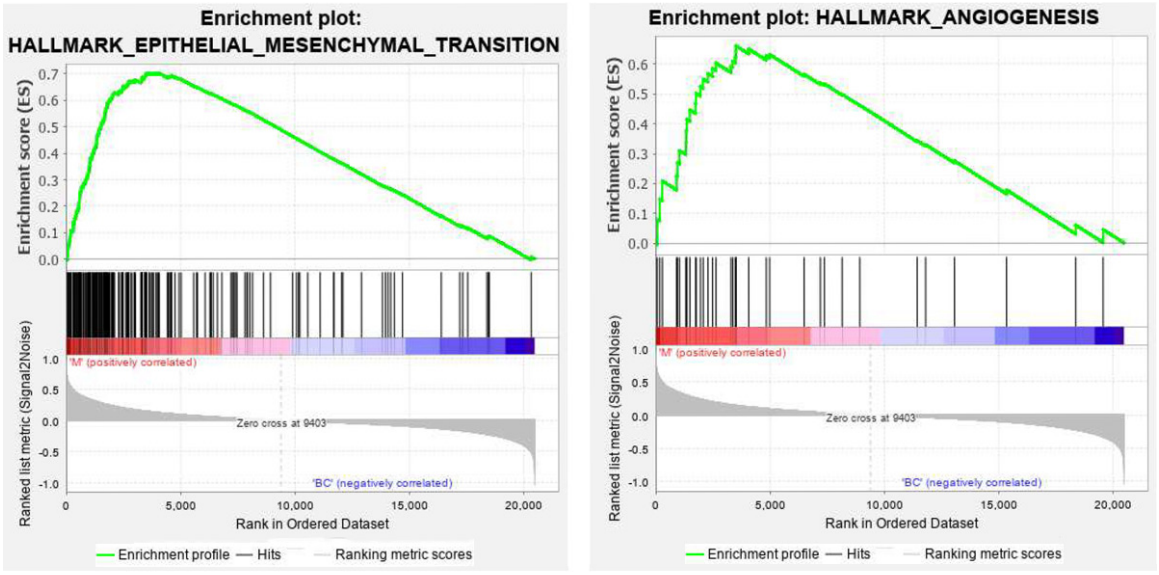
Figure 5. Mutation distribution in the entire breast cancer cohort and the metaplastic subgroup in GENIE cohort.

non-metaplastic and were noted to have a basal-like phenotype [2, 6]. *TP53* mutations were noted frequently in the metaplastic group [48-51]. However, there is great variability in the extent of lymphocyte infiltration and the immune cell composition in metaplastic breast cancer. In a recent study of 75 metaplastic cases, PD-L1 (Programmed death-ligand 1) expression was detected more frequently in metaplastic tumors (46%) compared to the other subtypes by immunohistochemistry [4]. Another case report has documented a dramatic response with pembrolizumab and nab-paclitaxel in a stage IV metaplastic breast cancer [52]. In our study, there was increased expression of the leukocyte fraction in the metaplastic subgroup however there was no statistical significance in terms of the expression of tissue-infiltrating regional fraction or the lymphocyte infiltration signature score. This might be related to the low tumor mutational burden as shown in a recent genomic profiling analysis of 192 MBC tumors, where tumor-infiltrating lymphocytes were more commonly observed in high mutational burden MBC [5].

Our findings provide a possible explanation of the chemotherapy resistance in MBC since there is no difference in the expression of cell proliferation gene-sets as seen in other cancer types such as pancreatic adenocarcinoma [53]. Interestingly, in our cohort, triple negative metaplastic breast tumors were noted to have enriched expression of angiogenesis gene-sets as well as epithelial-to-mesenchymal transition pathways. Mutations in the mTOR pathway have been shown to be rare in triple negative MBC [54]. Despite the low tumor mutational load, MBC cases carried multiple mutations including the *PIK3CA* 33.9%, *PTEN* 6.8%, *NF1* 11.1% and *TERT* 12.5% similar to prior reports [4, 49, 54]. In a recent genomic and transcriptomic analysis of 17 metaplastic breast carcinomas, Piscuoglio et al. demonstrated the significant variability of mutations present as well as differences at the genetic level that characterize histologically distinct subgroups of metaplastic breast cancer [54]. The authors also conclude that it is unlikely that a single genetic alteration, notwithstanding the prevalence of loss of *PTEN* expression or mutations affecting *PIK3CA*, could define any of the defined metaplastic subtypes.

A large percentage of breast cancer cases have been known to possess a “cold” tumor microenvironment and not being responsive to novel immunotherapy agents. With the exception of regulatory T-cells, MBC cases did not show any difference in lymphocyte infiltration compared to non-MBC cases. However, there was significant activation of the innate immune system with a higher infiltration of M1 macrophages, dendritic cells and eosinophils. Plasmacytoid dendritic cells have shown affinity to the tumor microenvironment and contribute to an immunosuppressive response [55]. TGF-beta activation is also important to mediate immunosuppression, further impeding antitumoral effects.

The current study has several limitations including the small number of metaplastic breast cancer cases analyzed as well as the absence of details regarding the particular histopathologic subtype within the metaplastic group. Recent data suggests significant heterogeneity amongst different metaplastic tumors that is reflected on the World Health Organization (WHO) classification [49, 54]. Therefore, even though our data represents a subset from large validated datasets, extrapolations might be limited. There is limited information on the immune composition of metaplastic breast cancer and our data is a significant contribution towards better understanding of the immunopathology of this subtype. Given the lack of *CTLA-4* in the cohort analyzed, we are



NAME	NES	NOM p-val	FDR q-val
HALLMARK_ANGIOGENESIS	1.816252	0.004098	0.143654
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.882927	0	0.172542

Figure 6. Hallmark gene sets with significant enrichment to metaplastic breast cancer in TCGA cohort. Gene set enrichment (GSEA) plots along with normalized enrichment score (NES) and false discovery rate (FDR) are shown for epithelial-mesenchymal transition and angiogenesis gene sets. The statistical significance of GSEA was determined by FDR<0.25.

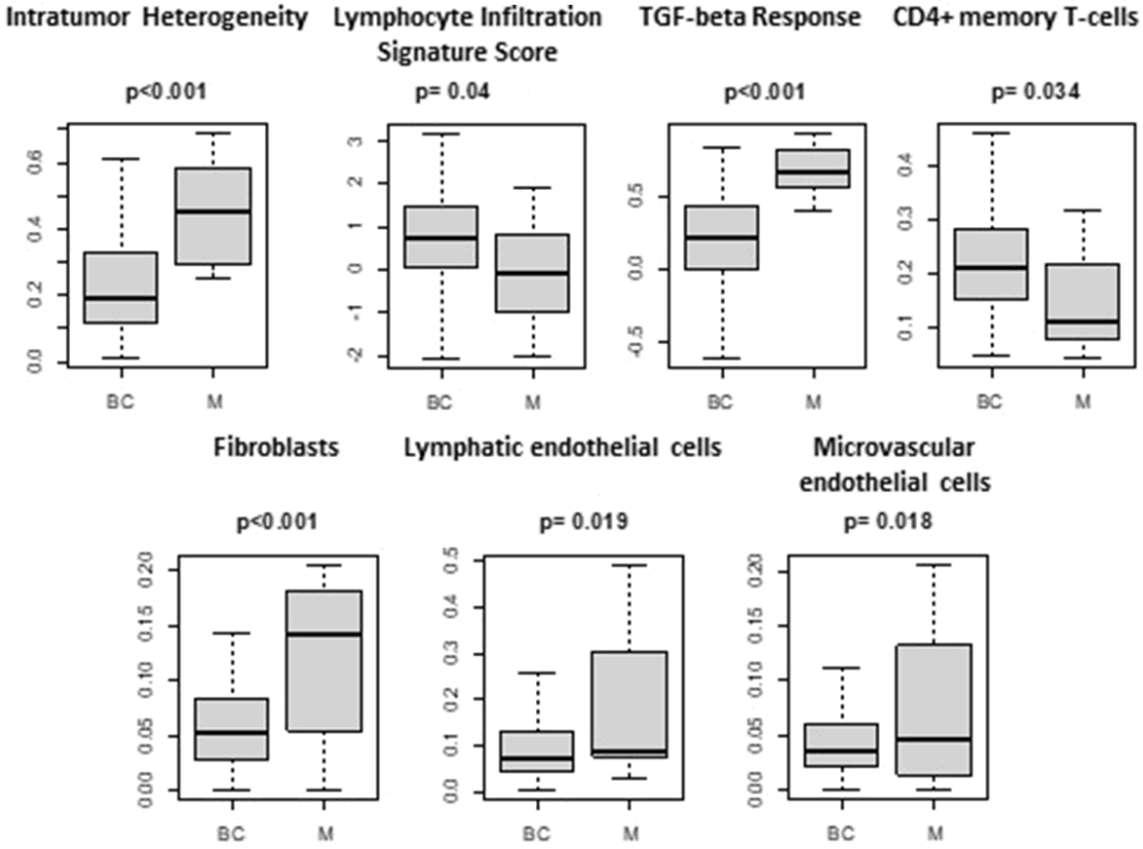


Figure 7. Association between metaplastic triple-negative (M) versus non-metaplastic triple-negative (BC) breast cancer and immune cell fraction and function scores, neoantigen expressions, mutation rates, intratumor heterogeneity, lymphocyte infiltration, TGF-beta response, CDD4 memory T cells, fibroblasts, lymphatic endothelial cells, and microvascular endothelial cells in the TCGA cohort. Each score was previously calculated on every patient in TCGA by Thorsson et al. (22). One-way ANOVA test was used to calculate the *P* values.

awaiting the results of the phase II DART study (NCT02834013) that is actively enrolling participants with rare tumors, including MBC to receive nivolumab and ipilimumab (anti-*CTLA-4* antibody).

Despite its rarity, metaplastic breast cancer represents a significant challenge in the multidisciplinary treatment given its preponderance for triple-negative, higher grade tumors. In this computational analysis, we demonstrated a significant infiltration of eosinophils, dendritic cells, M1 macrophages and regulatory T-cells in MBC. However, cytolytic score, CD8 T-cell and type 1 helper T-cell expression were no different between metaplastic and non-metaplastic subtypes. Furthermore, significant intratumor heterogeneity and TGF-beta response was noted in the metaplastic cohort. When we analyzed the triple-negative metaplastic subgroup, intratumor heterogeneity and TGF-beta response was noted to be significantly higher, which aligns with a previous report by Lien HC et al. [56]. We demonstrated that MBC significantly enriched Hallmark EMT gene set, composed of 200 EMT-related genes, which validate the findings by Zhang Y et al. that several EMT markers were highly expressed in MBC [57]. Moreover, MBC enriched angiogenesis, but not cell proliferation-related gene sets.

MBC is associated with enrichment of EMT pathways as well as angiogenesis gene sets, but the lack of enrichment in cell-proliferation gene-sets renders it less sensitive to traditional cytotoxic chemotherapy [50, 56]. Furthermore, the low tumor mutational burden and the inconsistent PD-L1 expression make it challenging to treat with immunotherapy despite some interesting case reports in the literature. These factors highlight potential mechanisms rendering MBC resistant to chemotherapy and provide insight into its tumor microenvironment.

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Disclosure of conflict of interest

None.

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