Bioinformatics analysis of immune infiltration patterns in breast cancer for identification of prognostic markers

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Abstract

Background/purpose Cancer immunotherapy has revolutionized the clinical treatment of several tumors. Immune infiltration has been found to be closely related to clinical prognosis, but it shows limited activity in breast cancer (BC). Therefore, this study aimed to explore the infiltration pattern of immune cells in BC, and to find potential prognostic markers and new therapeutic targets.Patients and methods We downloaded the immune genome data of BC from the Cancer Genome Atlas (TCGA), and analyzed the tumor- infiltrating immune cells (TIICs) in BC for the first time using the CIBERSORT algorithm. The aim of this study was to assess the proportions of 22 immune cell subsets in BC and examine the correlation between each TIIC and overall survival (OS) as well as clinical characteristics.Results The results indicated that: (1) there was a significant difference between the immune infiltration spectrum of cancerous and adjacent tissues, with M2 macrophages, M0 macrophages, and CD4 + T cells being highly expressed in BC; (2) CD8 + T cells were positively correlated with activated CD4 + memory T cells and negatively correlated with M0 macrophages, and M2 macrophages was inversely correlated with M1 macrophages, T cells regulatory, T cells CD8; (3) T cells, macrophages and BC TNM stage, age, clinical stage were correlated ($P < 0.05$); and (4) high expression of M2 macrophage markers could be an independent biomarker of poor prognosis and a potential therapeutic target for BC.Conclusion This study provides a new research method for the systematic study of immune cells in the BC tumor microenvironment, and provides theoretical guidance for further experiments to verify M2 macrophages and T cell subsets as a potential target for immunotherapy and prognosis.

Background

Breast cancer (BC) is one of the most prevalent malignancies in women worldwide and the second leading cause of cancer-related deaths worldwide.[1, 2] With the advancement of treatments, the survival rate has improved; however, due to BC's invasive characteristics, most patients are diagnosed at an advanced stage, which limits the potential for timely diagnosis and treatment, resulting in poor survival and prognosis. BC is a recognized heterogeneous disease, and there is still a lack of biomarkers to help select personalized treatment options, especially for immunotherapy. Recent advances in immuno-oncology have provided researchers with new insights into the role of innate or adaptive immune cells in regulating tumor progression. Tumor-infiltrating immune cells (TIICs) are closely related to clinical outcomes and are likely to be used as a drug targets to improve patient survival.[3, 4] However, the role of immune cells in the tumor-associated microenvironment remains unclear. Therefore, it is urgent to find new specific TIICs for improving the diagnosis and treatment, and reducing the risk of death, in BC.

Tumor immune cell infiltration refers to the TIICs from the blood to the tumor tissue and start to play its role, which can be separated from the tumor tissue. By combinating a large number of genomic profiles from the public database and the CIBERSORT algorithm can accurately reveal the relative levels of molecular markers of different TIIC subgroups, overcoming the shortcomings of traditional immunohistochemical methods.[5] CIBERSORT assess immune cell subsets using gene expression data profiled from tissues such as bulk tumours[6] and enabling large-scale analysis of RNA mixtures for

cellular biomarkers and therapeutic targets. Studies have shown that CIBERSORT has outperformed other available methods in resolving closely related cell subsets, unknown mixture content, and noise[7], and has been proved reliable for derivation of cell proportions within a complex microarray data[8]. The development of computer-aided and second-generation gene sequencing has injected new vitality into tumor research. The dual role of TIICs in both supporting host defense and promoting tumor progression is well documented.[9-11] In addition, a large number of *in vitro* studies have shown that TIICs can promote tumor invasion and metastasis through a variety of mechanisms.[12, 13] However, it is still difficult to determine the factors that directly affect TIICs and regulate their ability to inhibit or promote tumor progression, which increases the difficulty in determining the clinical significance of TIICs.

BC is a highly heterogeneous immune disease. Studies have shown that its low immunogenicity and strong immunosuppressive tumor microenvironment (TME) limit the benefits of immunotherapy, such as checkpoint inhibitor (CPI) immunotherapy, against the adaptive immune system.[14, 15] The TME can support the growth and development of BC, promoting invasion/metastasis and insensitivity to drug therapy. CPI immunotherapy is a treatment method that improves the body's anti-tumor immune response by co-suppression or co-stimulation to regulate T cell activity, and shows significant clinical effect.[16, 17] With the development of immune checkpoint therapy, the distribution of TIICs in tumors has become the focus of research. The type, density, quantity, and localization of TIICs – including T cells, B cells, monocytes, macrophages, neutrophils, and mast cells – in BC are closely related to the size of the primary tumor, lymph node metastasis, molecular classification, and prognosis. Immunohistochemistry analyses have demonstrated that CD8+ CTLs, known to contribute to tumor clearance, are associated with good prognosis.[18] CD4+ follicular helper cells can also serve as a prognostic marker in BC.[19, 20] These studies reveal the bright future of TIICs in BC.

The Cancer Genome Atlas (TCGA) is currently the largest database of cancer genetic information and has profoundly illuminated the genomic landscape of human malignancy. Genomic and transcriptional data can be used to study the TIICs. The TIICs can also be characterized by the expression of genetic markers as well as by T cell receptor (TCR) and B cell receptor (BCR) expression profiles in order to identify the immune targets of neoantigens. These targets can provide valuable information and with high predictive value for a variety of cancer types. With the discovery that antibodies against CTLA-4, PD-1, and PD-L1 have good therapeutic effect in a variety of malignant tumors,[14] immunotherapy has become the most promising treatment for the radical cure of malignant tumors. However, only a few patients respond to this treatment. Understanding the genes, transcription factors, and cell surface receptors that constitute the unique properties of each immune cell is of great significance for understanding the complex interactions in the TIICs.

In our study, we analyzed the immune microenvironment of BC by means of immunogenomics using the TCGA database, and quantified 22 TIIC subsets from the BC immune response using the CIBERSORT method. The relationships between 22 TIICs and overall survival (OS), clinical stage, pathological classification (TNM stage), and age were also investigated. Our study explored the complex relationship

between the heterogeneity of TIICs and disease progression in BC and looked for potential new targets for BC immunotherapy.

Results

2.1 Characteristics of study subjects

The BC transcriptome profiling data and the corresponding clinical information were obtained using the R bioconductor package TCGABiolinks. A total of 1223 samples were investigated, which included 1110 tumor samples and 113 normal samples. By integrating gene expression quantification data, we obtained the mRNA expression matrix of BC and used the Ensembl online database to annotate mRNAs. Subsequently, we normalized the BC mRNA data using the R “limma” package.

2.2 The composition of infiltrating immune cells in BC

Thanks to the development of science and technology, the immune infiltration of BC has been gradually revealed in TCGA database and verified by experimental PCR, but little is known about these low-abundance cell subsets. Using the CIBERSORT algorithm, we first investigated the difference between immune infiltration in cancer and adjacent tissues in 22 immune cell subsets. The CIBERSORT algorithm can provide information about relative proportions among TIIC subpopulations. The greater the proportion of immune cells identified, the smaller the P-value. In this study, 625 tumor samples and 16 paracancerous samples were selected for further analysis based on CIBERSORT P-values < 0.01.

2.3 The landscape of immune infiltration in BC

The following results were obtained by studying the overall proportion of TIICs in 641 samples with CIBERSORT P-values < 0.01. Figures 1a and 1b show the proportion of each TIIC subtype in each sample. The bar plot in Figure 1a shows that the TIICs that account for the top three proportions in all samples were: M2 macrophages, M0 macrophages, and resting memory $CD4^+$ T cells. The heat map in Figure 1b shows that tumor and normal samples were clearly separated into two distinct groups and that M2 macrophages, M0 macrophages, and resting memory $CD4^+$ T cells have strong heterogeneity. In order to explore the correlation among 22 TIICs in BC, we used the “corrplot” package of R to draw the correlation heat map of 22 TIICs. As shown in Figure 2, the proportions of different TIIC subpopulations were weakly-to-moderately correlated. $CD8^+$ T cells were positively correlated with activated memory $CD4^+$ T cells, and negatively correlated with M0 macrophages. The correlation coefficients were 0.42 and -0.47, respectively. In addition, as shown in Figure 3, there were 7 TIICs that showed different infiltration patterns when comparing tumor and adjacent normal tissue in BC ($P<0.01$): activated memory $CD4^+$ T cells, T follicular helper cells, regulatory T cells (Tregs), M0 macrophages, M1 macrophages, M2 macrophages, and resting mast cells. In summary, the above results consistently showed that abnormal immune infiltration and its heterogeneity were of great significance in BC.

2.4 Survival evaluation for the TIICs in BC

K-M and log-rank test were used to determine the relationship between the 22 TIICs and the OS of BC patients, with a cut-off threshold of $P < 0.05$. As shown in Figure 4, 2 TIICs were found to be linked to overall survival (OS): patients with higher levels of M2 macrophages and activated mast cells had a shorter OS. Using the univariate Cox proportional hazards regression method, we found that 2 TIICs (M2 macrophages and neutrophils) were closely related to survival in BC patients, $P<0.05$ (Figure 5a). The multivariate Cox proportional hazard regression method confirmed that M2 macrophages were an independent prognostic factor for BC (P=1.79E-06).

Figure 5b shows the survival difference between the high-risk group and the low-risk group ($P = 0.001$). The 3 and 5-year survival correlation of the M2 macrophages signature was analyzed by ROC and area under curve (AUC) was computed to assess the discriminatory capacity of TIIC signature (Figures 5c & 5d). The AUC of the M2 macrophages signature was 0.72 and 0.696 indicating its utility as a prognostic model for predicting the survival status of BC. Significantly, we found that M2 macrophages were also shown to be connected to OS in K-M (log-rank test) and Cox regression analysis.

2.5 Relationship between TIIC subsets and clinical characteristics

We downloaded the BC clinical data from TCGA and sorted out the relevant information – survival state, survival time, TNM stage, age, and clinical stages – using R software. Next, we assessed the correlation between 22 TIICs and each clinical trait (Figure 6). We found that TIICs associated with tumors (T) include resting dendritic cells, resting memory $CD4^+$ T cells, M1 macrophages, $CD8^+$ T cells, activated NK cells, plasma cells, and M0 macrophages; those associated with lymph node (N) include monocytes, naïve $CD4^+$ T cells, M0 macrophages, and activated memory $CD4^+$ T cells; those associated with metastasis (M) include activated memory $CD4^+$ T cells, activated NK cells, monocytes, and M1 macrophages; those associated with clinical stages include monocytes, resting mast cells, resting dendritic cells, and activated memory $CD4^+$ T cells; those associated with age include M2 macrophages, neutrophils, naïve B cells, gamma delta T cells, monocytes, resting memory $CD4^+$ T cells, and resting mast cells. This reveals that TIICs not only affect prognosis, but also play an important role in the development and progression of BC.

Discussion

This study describes the distribution of tumor-infiltrating immune cells in BC based on gene expression data in the TCGA cohort, which clearly confirmed that immune infiltration was indeed involved in the occurrence of breast cancer. Compared with other treatment methods, cellular immunotherapy has the advantages of fewer side effects, stronger targeting, and synergistic effects with other treatments. Because of these advantages, immunotherapy has great potential to become the leading tumor therapeutic modality.[21] However, targeting TIICs has only achieved good results in the treatment of melanoma. Melanoma has high expression of tumor neoantigens and a high mutational load, and its immune characteristics are quite different from those of other solid tumors. Although a large number of clinical studies have been carried out on immunotherapy for BC and other solid tumors, the reported

clinical response rates are not very satisfactory, fluctuating between 10% and 30%.[22] Therefore, an in-depth understanding of the immune status of the BC tumor microenvironment, to explore the function and distribution of TIICs in the tumor microenvironment, could be useful for improving immunotherapy.

BC has a high susceptibility and malignancy in women. Traditional surgical resection treatment has limited effect due to frequent distant metastasis and invasion, while chemotherapy and radiotherapy are very harmful to normal body tissues due to a lack of targeted treatment. Therefore, it is urgent to develop more promising treatment strategies. Cancer immunotherapy emerged as a breakthrough cancer treatment strategy[23] and provide promising results for this aggressive cancer. Immune system-related biomarkers have important prognostic significance in many tumor types, including BC, and there are many ongoing clinical studies involving BC patients and various immunotherapy strategies.[24] The most mature immunotherapy revolves around programmed cell death 1 (PD-1) receptor and its ligands, PD-L1 and CTLA-4. It has been found that TIICs play an important role in host defense mechanisms during BC adjuvant and neoadjuvant therapies.[25] Although a growing number of studies have shown that TIICs have great potential to predict clinical outcomes and treatment response, studies on the impact of single TIICs on cancer prognosis have been lacking. It is worth noting that the study of the infiltration pattern of tumor immune cells based on a large number of genomic profiles from the public database and the discussion of the potential mechanism of the interaction of TIICs are further studies on genomics, which can bring the greatest possibility for the clinical cure of tumors and providing new insights into immunotherapy for BC.

The results of this study showed significant heterogeneity of TIICs within the tumor. According to the bar plot and heat map, M2 macrophages, M0 macrophages, resting memory $CD4^+$ T cells, etc. were highly expressed in BC. Moreover, Figure 3 shows the statistical significance of the differential expression of the molecular markers of these three TIICs when comparing tumors and adjacent tissues, and the results were consistent. Through the correlation analysis of 22 TIICs, we found that $CD8^+$ T cells were positively correlated with activated memory $CD4^+$ T cells, and negatively correlated with M0 macrophages. In order to further explore the relationship between these TIICs and the prognosis of BC, we performed survival and Cox regression analysis. The results showed that M2 macrophages and mast cell activation were related to prognosis, and M2 macrophages could be used as an independent prognostic marker for BC. In-depth analysis of the relationship between 22 TIICs and clinical characteristics revealed a complex correlation between macrophages and $CD4^+$ T cells and clinical results. Therefore, we concluded that macrophages, especially M2 macrophages, play an important role in regulating the occurrence, development, and prognosis of BC.

Macrophages have innate heterogeneity, which is closely related to the unique function of their local microenvironment,[26] and demonstrate plasticity of functions in the pathological processes of various diseases, including infectious diseases, autoimmune diseases, and tumor progression.[26, 27] Macrophages are conventionally subdivided into three phenotypes: naïve (; also called M0), pro-inflammatory (M1), and anti-inflammatory (M2).[28] Macrophages act as immune sentries to maintain

the integrity of tissues and can sense, react, and quickly adapt to their local environment[29, 30] in order to maintain their tolerance and resist invading pathogens. [31] However, when the host is challenged for a long time (e.g., by long-term exposure to irritating particles or infection), macrophages may play a harmful role, leading to a low-grade inflammatory state that can lead to disease progression and even cancer.[32, 33] Many macrophage-centered treatment approaches are considered to be potential cancer treatments, including strategies to limit their infiltration or to exert their anti-tumor effects. In the vast majority of cancers, macrophages exhibit a global immunosuppressive phenotype, which is characterized by low levels of inflammatory molecules and increased transcriptional activity of alternatively activated M2 macrophages.[34] In animal models, drugs that inhibit key signaling molecules of M2 macrophage polarization (such as IL-4, STAT3, or PI3- kinase) successfully limit the immunosuppressive function of tumor-associated macrophages (TAMs) and shrink tumors.[35, 36] However, M2 macrophages can also promote tumor angiogenesis, cancer cell migration/invasion, immunosuppression, and matrix remodeling.[37, 38] Our study confirmed and extended the results of previous studies; we found that M2 macrophages are a potential independent prognostic factor for BC, high expression of M2 macrophage markers is associated with poor prognosis.

In addition, it was found that high levels of CD8+ T cell in cancer cell nests heighten neoadjuvant chemotherapy sensitivity in breast cancers,[39-41] and Ali et al reported that CD8 T cells were associated with reduced risk of death in patients with either ER-negative or ER-positive/HER2-positive disease.[42] The interleukin-17 A (IL-17A), a proinflammatory cytokine produced by activated CD4+ memory T cells has been found with a protective role in the development of breast cancer.[43, 44] Furthermore, mast cells (MCs) are a major source of pro-tumorigenic and antitumorigenic molecules, thus, their role on breast cancer is controversial.[45] Some scholars believe that mast cell infiltration suggests a good prognosis of breast cancer.[46, 47] On the other hand, MCs as a proliferating factor, can stimulate the development of breast cancer and is related to the adverse reactions of neoadjuvant chemotherapy for breast cancer,[48, 49] which is consistent with the poor prognosis of high expression MCs found in our study. A large number of studies have shown that TIICs has prognostic value independent of many established prognostic signs, and single TIICs have the ability to predict clinical outcomes. These findings provide a reference for BC immunotherapy; however, owing to the complexity of this topic, further studies are required to validate our findings.

Conclusion

In conclusion, we have combined, for the first time, the reliable CIBERSORT algorithm with large-scale genomic data to analyze the relationship between 22 TIICs and clinical outcomes of BC. We found that M2 macrophages could be used as a potential prognostic marker and could be a new drug target for BC. Our study lays the foundation for further studies on the prognostic value of different subsets of TIICs.

Materials And Methods

5.1 Data acquisition

Individual BC RNA sequencing (RNA-seq) data (level 3) and the corresponding clinical data were obtained from TCGA database.[50] Exclusion criteria were as follows: (1) histological diagnosis negating BC; (2) presence of a malignancy other than BC; and (3) lack of complete clinical data. The HTSeq - FPKM data were downloaded and sorted by the DESeq package of R, and the data were normalized using the "limma" package. Clinical data collected included age, TNM stage, clinical stage, survival status, survival time, etc. This study was conducted in compliance with the publication guidelines provided by TCGA, and the data obtained from TCGA did not require approval from an ethics committee.

5.2 Evaluation of tumor-infiltrating immune cells

CIBERSORT (http://cibersort.stanford.edu/) was used to assess the relative proportions of 22 types of invasive immune cells in the standardized gene expression data.[51] CIBERSORT is a deconvolution method for complex tissues, especially human leukocyte subsets, based on linear support vector regression (SVR) from gene expression profiles. It uses a set of barcode gene expression values (a "signature matrix" of 547 genes) to characterize immune cell composition. CIBERSORT enables large-scale analysis of RNA mixtures to identify cellular biomarkers and therapeutic targets. Through this approach, we distinguished 22 human hematopoietic cell phenotypes from BC genes, including seven T cell types, three B cell types, NK cells, and a variety of myeloid subsets.

5.3 Filtration and visualization of the tumor-infiltrating immune cell matrix

The P-value obtained by CIBERSORT reflects the proportion of samples composed of immune cells and non-immune cells, and the proportion of immune cells is inversely related to the P-value. In our study, $P < 0.01$ was used as the filtration standard. The steps for visualizing the infiltrating immune cell matrix are as follows: (1) R software and the "pheatmap" package were used to draw bar plots and heat maps of the proportion of various immune cells in each sample. 2) The "corrplot" package of R was used to draw the correlation heat map between 22 TIICs. (3) Comparison of the number of immune cells between tumor and paracancerous cells was performed using the R "vioplot" package to draw a violin plot.

5.4 Survival analysis and establishment of prognosis model

Samples with CIBERSORT $P < 0.05$ were included in the main survival analysis. Combining the clinical data of BC patients, the Kaplan-Meier (K-M) survival curves of 22 TIICs were plotted with the "survival" package of R. $P < 0.05$ was regarded as significant. In addition, 22 TIICs were assessed via univariate Cox proportional hazards regression analysis to obtain prognostic values ($P < 0.05$). Independent prognostic factors of BC were determined by multivariable Cox regression analysis, and each prognostic TIIC was weighted according to its estimated regression coefficient to establish a risk-scoring formula. The risk score of each patient was evaluated, and the corresponding median risk score was used as the cutoff value to divide the patients into high-risk or low-risk groups. The "survival ROC" package in R was used to construct the time-dependent receiver operating characteristic (ROC) curves for 3 and 5 years, and to measure the risk prediction rate of specific TIICs between the two groups. K-M and log-rank tests were used to calculate the survival difference between the low-risk and high-risk groups.

5.5 Statistical analysis between tumor-infiltrating immune cells and clinical characteristics

In order to evaluate the correlation between TIICs and clinical characteristics, we extracted the clinical data of each sample, including survival status, survival time, local invasion, lymph node metastasis, distant metastasis, age, and stage. The correlation between clinical traits and 22 TIICs was evaluated. All data were analyzed by R-studio 3.3.3. When $P<0.05$, the TIIC was considered to be correlated with a given clinical trait.

List Of Abbreviations

BC: breast cancer; TCGA: The Cancer Genome Atlas; TIICs: Tumor- infiltrating immune cells; OS: overall survival; TME: tumor microenvironment; CPI: checkpoint inhibitor ; PD-1: programmed death 1 receptor; K-M: kaplan-meier; ROC: receiver operating characteristic ; AUC: area under curve.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The author reports no conflicts of interest in this work.

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Author contributions

Yan Yao and Changgang Sun was involved in the concept and design of the study. Yan Yao drafted the manuscript. All authors were involved in acquisition, analysis, and interpretation of the data, revised the manuscript, and approved the final version.

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References

1. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2018**. *CA Cancer J Clin* 2018, **68**(1):7-30.
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J: **Cancer statistics in China, 2015**. *CA Cancer J Clin* 2016, **66**(2):115-132.
3. Gupta S, Vanderbilt CM, Cotzia P, Arias-Stella JA, 3rd, Chang JC, Zehir A, Benayed R, Nafa K, Razavi P, Hyman DM *et al*: **Next-Generation Sequencing-Based Assessment of JAK2, PD-L1, and PD-L2 Copy Number Alterations at 9p24.1 in Breast Cancer: Potential Implications for Clinical Management**. *The Journal of molecular diagnostics : JMD* 2019, **21**(2):307-317.
4. Shibutani M, Maeda K, Nagahara H, Fukuoka T, Iseki Y, Matsutani S, Kashiwagi S, Tanaka H, Hirakawa K, Ohira M: **Tumor-infiltrating Lymphocytes Predict the Chemotherapeutic Outcomes in Patients with Stage IV Colorectal Cancer**. *In vivo (Athens, Greece)* 2018, **32**(1):151-158.
5. Ali HR, Chlon L, Pharoah PD, Markowetz F, Caldas C: **Patterns of Immune Infiltration in Breast Cancer and Their Clinical Implications: A Gene-Expression-Based Retrospective Study**. *PLoS medicine* 2016, **13**(12):e1002194.
6. AM N, CL L, MR G, AJ G, W F, Y X, CD H, M D, AA A: **Robust enumeration of cell subsets from tissue expression profiles**. *Nature methods* 2015, **12**(5):453-457.
7. Chen B, Khodadoust MS, Liu CL, Newman AM, Alizadeh AA: **Profiling Tumor Infiltrating Immune Cells with CIBERSORT**. *Methods in molecular biology (Clifton, NJ)* 2018, **1711**:243-259.
8. HR A, L C, PD P, F M, C C: **Patterns of Immune Infiltration in Breast Cancer and Their Clinical Implications: A Gene-Expression-Based Retrospective Study**. *PLoS medicine* 2016, **13**(12):e1002194.
9. Milne K, Alexander C, Webb JR, Sun W, Dillon K, Kalloger SE, Gilks CB, Clarke B, Kobel M, Nelson BH: **Absolute lymphocyte count is associated with survival in ovarian cancer independent of tumor-infiltrating lymphocytes**. *Journal of translational medicine* 2012, **10**:33.
10. Chen LJ, Zheng X, Shen YP, Zhu YB, Li Q, Chen J, Xia R, Zhou SM, Wu CP, Zhang XG *et al*: **Higher numbers of T-bet(+) intratumoral lymphoid cells correlate with better survival in gastric cancer**. *Cancer immunology, immunotherapy : CII* 2013, **62**(3):553-561.
11. MacLennan GT, Eisenberg R, Fleshman RL, Taylor JM, Fu P, Resnick MI, Gupta S: **The influence of chronic inflammation in prostatic carcinogenesis: a 5-year followup study**. *The Journal of urology* 2006, **176**(3):1012-1016.
12. Pawelek JM, Chakraborty AK: **The cancer cell–leukocyte fusion theory of metastasis**. *Advances in cancer research* 2008, **101**:397-444.
13. Diakos CI, Charles KA, McMillan DC, Clarke SJ: **Cancer-related inflammation and treatment effectiveness**. *The Lancet Oncology* 2014, **15**(11):e493-503.
14. Esteva FJ, Hubbard-Lucey VM, Tang J, Pusztai L: **Immunotherapy and targeted therapy combinations in metastatic breast cancer**. *The Lancet Oncology* 2019, **20**(3):e175-e186.

15. de la Cruz-Merino L, Palazon-Carrion N, Henao-Carrasco F, Nogales-Fernandez E, Alamo-de la Gala M, Vallejo-Benitez A, Chiesa M, Sanchez-Margalet V: **New horizons in breast cancer: the promise of immunotherapy**. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* 2019, **21**(2):117-125.
16. Lote H, Cafferkey C, Chau I: **PD-1 and PD-L1 blockade in gastrointestinal malignancies**. *Cancer treatment reviews* 2015, **41**(10):893-903.
17. Davidson M, Okines AF, Starling N: **Current and Future Therapies for Advanced Gastric Cancer**. *Clinical colorectal cancer* 2015, **14**(4):239-250.
18. de Melo Gagliato D, Cortes J, Curigliano G, Loi S, Denkert C, Perez-Garcia J, Holgado E: **Tumor-infiltrating lymphocytes in Breast Cancer and implications for clinical practice**. *Biochimica et biophysica acta Reviews on cancer* 2017, **1868**(2):527-537.
19. Zhu S, Lin J, Qiao G, Wang X, Xu Y: **Tim-3 identifies exhausted follicular helper T cells in breast cancer patients**. *Immunobiology* 2016, **221**(9):986-993.
20. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, Ravoet M, Le Buanec H, Sibille C, Manfouo-Foutsop G *et al*: **CD4(+) follicular helper T cell infiltration predicts breast cancer survival**. *The Journal of clinical investigation* 2013, **123**(7):2873-2892.
21. Goff SL, Smith FO, Klapper JA, Sherry R, Wunderlich JR, Steinberg SM, White D, Rosenberg SA, Dudley ME, Yang JC: **Tumor infiltrating lymphocyte therapy for metastatic melanoma: analysis of tumors resected for TIL**. *Journal of immunotherapy (Hagerstown, Md : 1997)* 2010, **33**(8):840-847.
22. Manjili MH, Payne KK: **Prospects in cancer immunotherapy: treating advanced stage disease or preventing tumor recurrence?** *Discovery medicine* 2015, **19**(107):427-431.
23. **Breakthrough of the year 2013. Notable developments**. *Science (New York, NY)* 2013, **342**(6165):1435-1441.
24. Voutsadakis IA: **Immune Blockade Inhibition in Breast Cancer**. *Anticancer research* 2016, **36**(11):5607-5622.
25. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, Budczies J, Huober J, Klauschen F, Furlanetto J *et al*: **Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy**. *The Lancet Oncology* 2018, **19**(1):40-50.
26. Bashir S, Sharma Y, Elahi A, Khan F: **Macrophage polarization: the link between inflammation and related diseases**. *Inflammation research : official journal of the European Histamine Research Society [et al]* 2016, **65**(1):1-11.
27. Al Faraj A, Shaik AS, Alnafea M: **Intrapulmonary administration of bone-marrow derived M1/M2 macrophages to enhance the resolution of LPS-induced lung inflammation: noninvasive monitoring using free-breathing MR and CT imaging protocols**. *BMC medical imaging* 2015, **15**:16.
28. Nahrendorf M, Swirski FK: **Abandoning M1/M2 for a Network Model of Macrophage Function**. *Circulation research* 2016, **119**(3):414-417.

29. Glass CK, Natoli G: **Molecular control of activation and priming in macrophages**. *Nature immunology* 2016, **17**(1):26-33.
30. Koelwyn GJ, Corr EM, Erbay E, Moore KJ: **Regulation of macrophage immunometabolism in atherosclerosis**. *Nature immunology* 2018, **19**(6):526-537.
31. Wynn TA, Chawla A, Pollard JW: **Macrophage biology in development, homeostasis and disease**. *Nature* 2013, **496**(7446):445-455.
32. Grivennikov SI, Greten FR, Karin M: **Immunity, inflammation, and cancer**. *Cell* 2010, **140**(6):883-899.
33. Conway EM, Pikor LA, Kung SH, Hamilton MJ, Lam S, Lam WL, Bennewith KL: **Macrophages, Inflammation, and Lung Cancer**. *American journal of respiratory and critical care medicine* 2016, **193**(2):116-130.
34. Yang M, McKay D, Pollard JW, Lewis CE: **Diverse Functions of Macrophages in Different Tumor Microenvironments**. *Cancer research* 2018, **78**(19):5492-5503.
35. Sun L, Chen B, Jiang R, Li J, Wang B: **Resveratrol inhibits lung cancer growth by suppressing M2-like polarization of tumor associated macrophages**. *Cellular immunology* 2017, **311**:86-93.
36. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, Woo G, Nguyen AV, Figueiredo CC, Foubert P *et al*: **PI3Kgamma is a molecular switch that controls immune suppression**. *Nature* 2016, **539**(7629):437-442.
37. Santoni M, Romagnoli E, Saladino T, Foghini L, Guarino S, Capponi M, Giannini M, Cognigni PD, Ferrara G, Battelli N: **Triple negative breast cancer: Key role of Tumor-Associated Macrophages in regulating the activity of anti-PD-1/PD-L1 agents**. *Biochimica et biophysica acta Reviews on cancer* 2018, **1869**(1):78-84.
38. Goswami KK, Ghosh T, Ghosh S, Sarkar M, Bose A, Baral R: **Tumor promoting role of anti-tumor macrophages in tumor microenvironment**. *Cellular immunology* 2017, **316**:1-10.
39. Lee HJ, Seo JY, Ahn JH, Ahn SH, Gong G: **Tumor-associated lymphocytes predict response to neoadjuvant chemotherapy in breast cancer patients**. *Journal of breast cancer* 2013, **16**(1):32-39.
40. Seo AN, Lee HJ, Kim EJ, Kim HJ, Jang MH, Lee HE, Kim YJ, Kim JH, Park SY: **Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer**. *British journal of cancer* 2013, **109**(10):2705-2713.
41. Yamaguchi R, Tanaka M, Yano A, Tse GM, Yamaguchi M, Koura K, Kanomata N, Kawaguchi A, Akiba J, Naito Y *et al*: **Tumor-infiltrating lymphocytes are important pathologic predictors for neoadjuvant chemotherapy in patients with breast cancer**. *Hum Pathol* 2012, **43**(10):1688-1694.
42. Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA *et al*: **Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients**. *Ann Oncol* 2014, **25**(8):1536-1543.
43. Wang L, Jiang Y, Zhang Y, Wang Y, Huang S, Wang Z, Tian B, Yang Y, Jiang W, Pang D: **Association analysis of IL-17A and IL-17F polymorphisms in Chinese Han women with breast cancer**. *PloS one* 2012, **7**(3):e34400.

44. Kim G, Khanal P, Lim SC, Yun HJ, Ahn SG, Ki SH, Choi HS: **Interleukin-17 induces AP-1 activity and cellular transformation via upregulation of tumor progression locus 2 activity**. *Carcinogenesis* 2013, **34**(2):341-350.
45. Varricchi G, Galdiero MR, Loffredo S, Marone G, Iannone R, Marone G, Granata F: **Are Mast Cells MASTers in Cancer?** *Frontiers in immunology* 2017, **8**:424.
46. Glajcar A, Szpor J, Pacek A, Tyrak KE, Chan F, Streb J, Hodorowicz-Zaniewska D, Okon K: **The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment**. *Virchows Archiv : an international journal of pathology* 2017, **470**(5):505-515.
47. Plotkin JD, Elias MG, Fereydouni M, Daniels-Wells TR, Dellinger AL, Penichet ML, Kepley CL: **Human Mast Cells From Adipose Tissue Target and Induce Apoptosis of Breast Cancer Cells**. *Frontiers in immunology* 2019, **10**:138.
48. SM R, A R, S B, H J, S Z, L W, V G, CW H, MT T, JM R *et al*: **Poor Response to Neoadjuvant Chemotherapy Correlates with Mast Cell Infiltration in Inflammatory Breast Cancer**. *Cancer immunology research* 2019, **7**(6):1025-1035.
49. Carpenco E, Ceausu RA, Cimpean AM, Gaje PN, Saptefrati L, Fulga V, David V, Raica M: **Mast Cells as an Indicator and Prognostic Marker in Molecular Subtypes of Breast Cancer**. *In vivo (Athens, Greece)* 2019, **33**(3):743-748.
50. Wei L, Jin Z, Yang S, Xu Y, Zhu Y, Ji Y: **TCGA-assembler 2: software pipeline for retrieval and processing of TCGA/CPTAC data**. *Bioinformatics* 2018, **34**(9):1615-1617.
51. Newman AM, Liu CL, Green MR, Feng W, Xu Y, Hoang CD, Diehn M, Alizadeh AA, Gentles AJ: **Robust enumeration of cell subsets from tissue expression profiles**. *Nature methods* 2015, **12**(5):453-457.

Figures

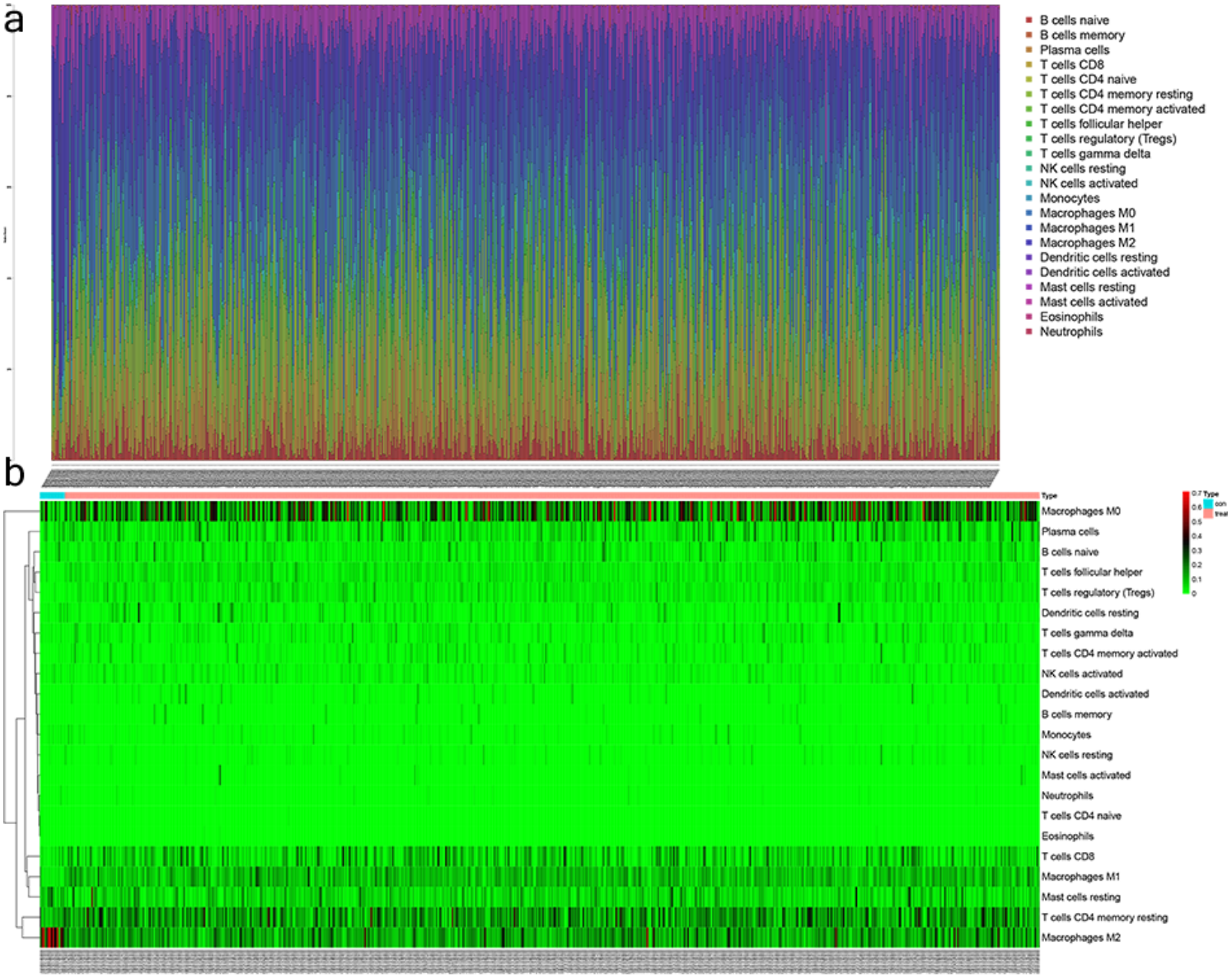

Figure 1

The landscape of immune infiltration of breast cancer in the TCGA cohort. (a) The differences between immune infiltration in cancer and paracancerous tissue, X-axis represents the samples, Y-axis denotes the distribution of 22 TIICs in each sample. (b) A heat map of the proportions of 22 immune cell subsets. The X-axis shows the clustering information of samples which were divided into two major clusters, Y-axis denotes the type of 22 TIICs. TIICs: tumor-infiltrating immune cells.

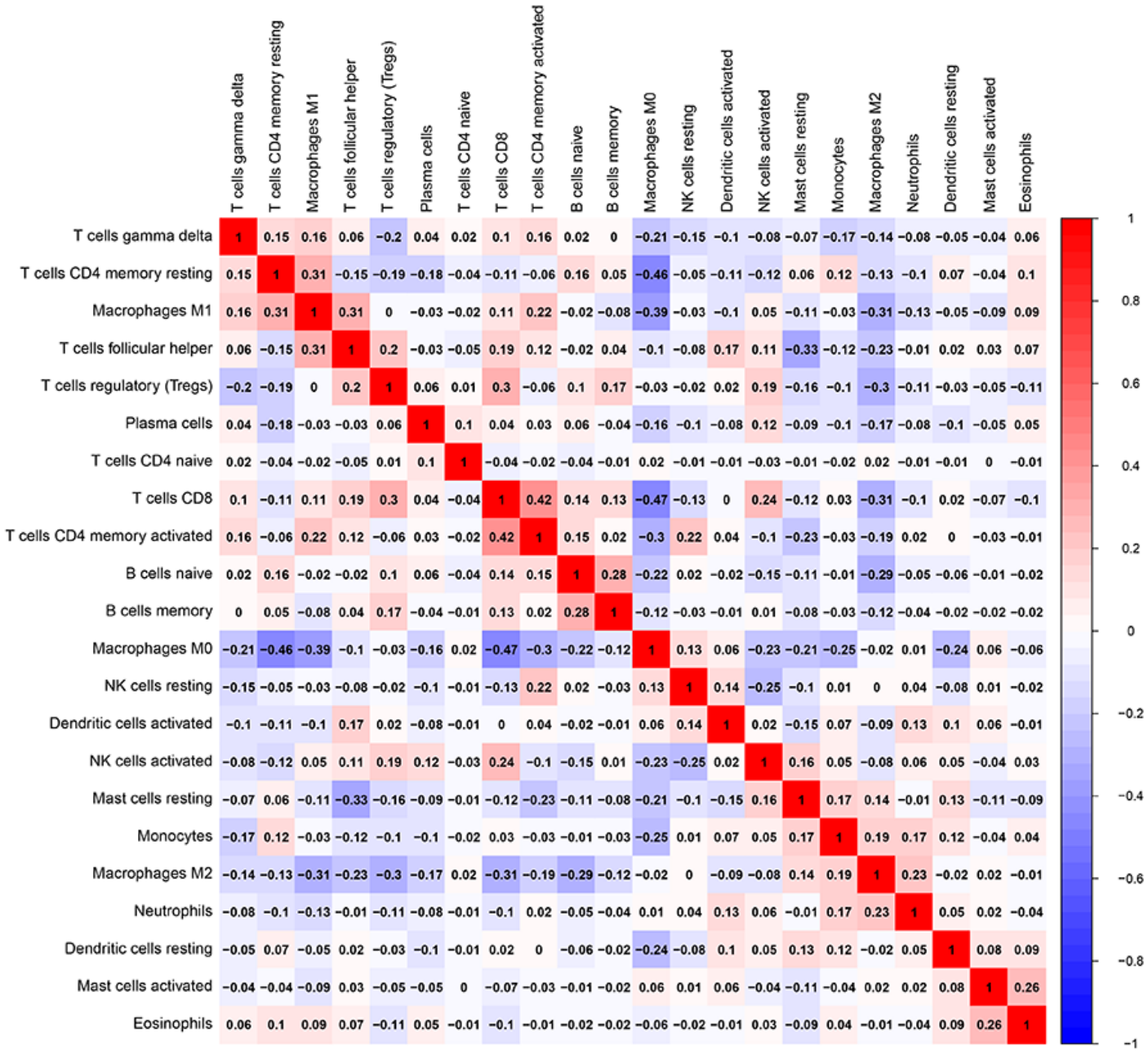

Figure 2

Correlation matrix heat map of the proportions of all 22 immune cell proportions and immune cytolytic activity in TCGA cohort. Variables have been ordered by average linkage clustering.

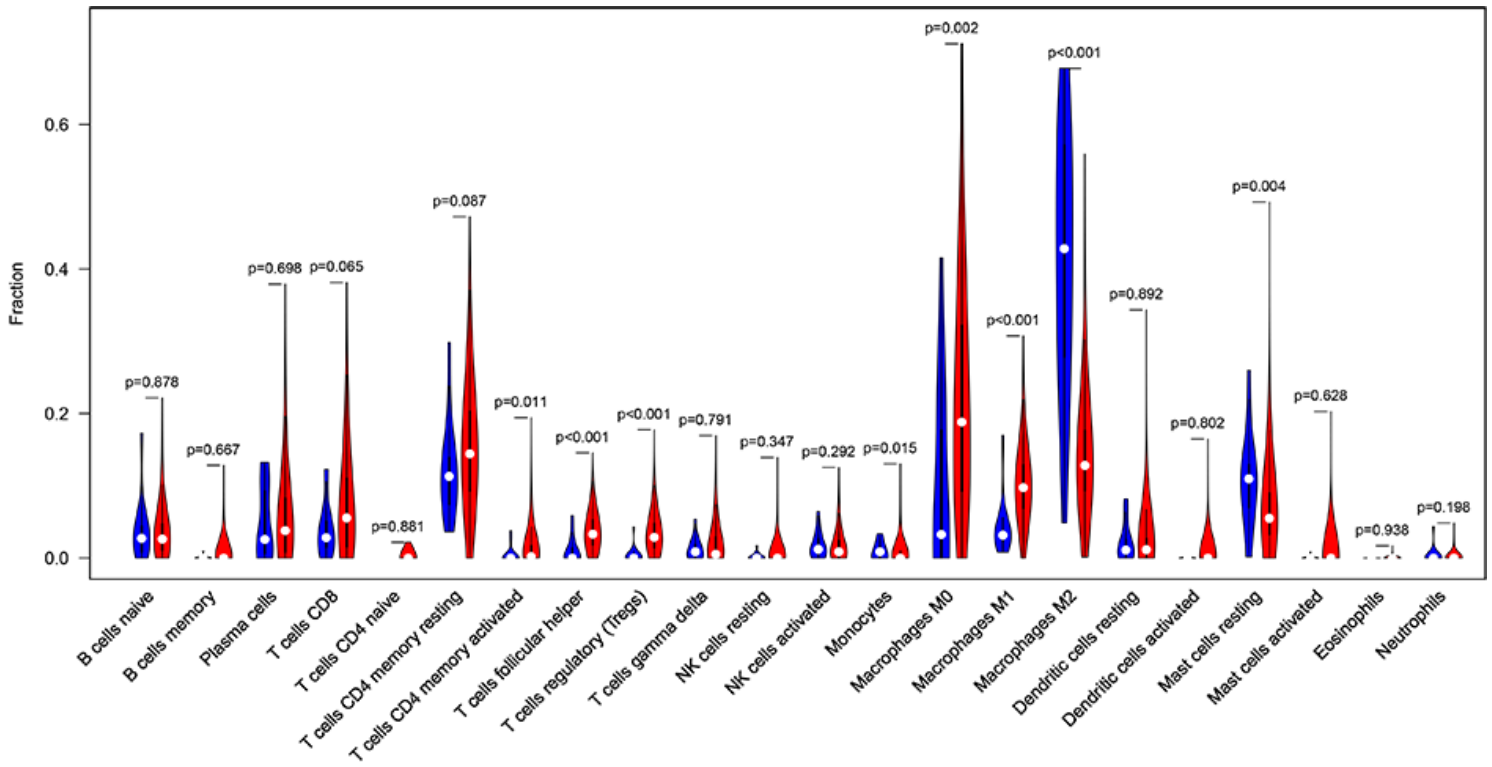

Figure 3

Violin plot diagram visualizing the expression of infiltrated immune cell markers. Blue represents the proportion of marker expression in normal tissues, red represents the proportion of marker expression in tumor tissues, and white points represent the median value of marker expression. The difference between the groups was statistically significant with P < 0.05.

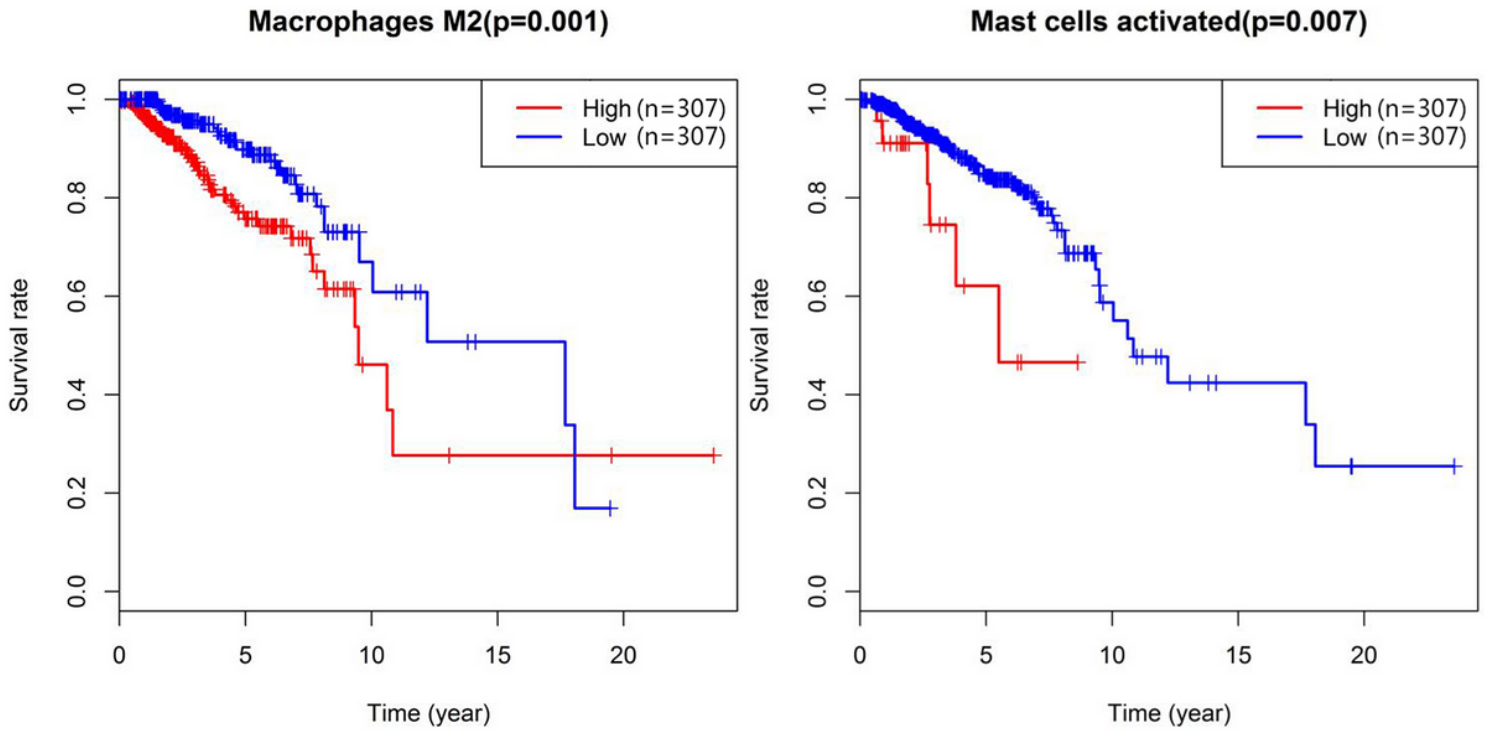

Figure 4

Kaplan - Meier (K-M) curves of overall survival. High infltration of M2 macrophages and mast cells activated were associated with overall poor survival in patients with breast cancer (p<0.05).

a

Variables	TIICs	HR	z	pvalue
Univariate analysis				
	Macrophages M2	38.98	4.74	2.18E-06
	Neutrophils	9.53	3.22	1.29E-03
Multivariate analysis				
	Macrophages M2	58.30	4.73	1.79E-06

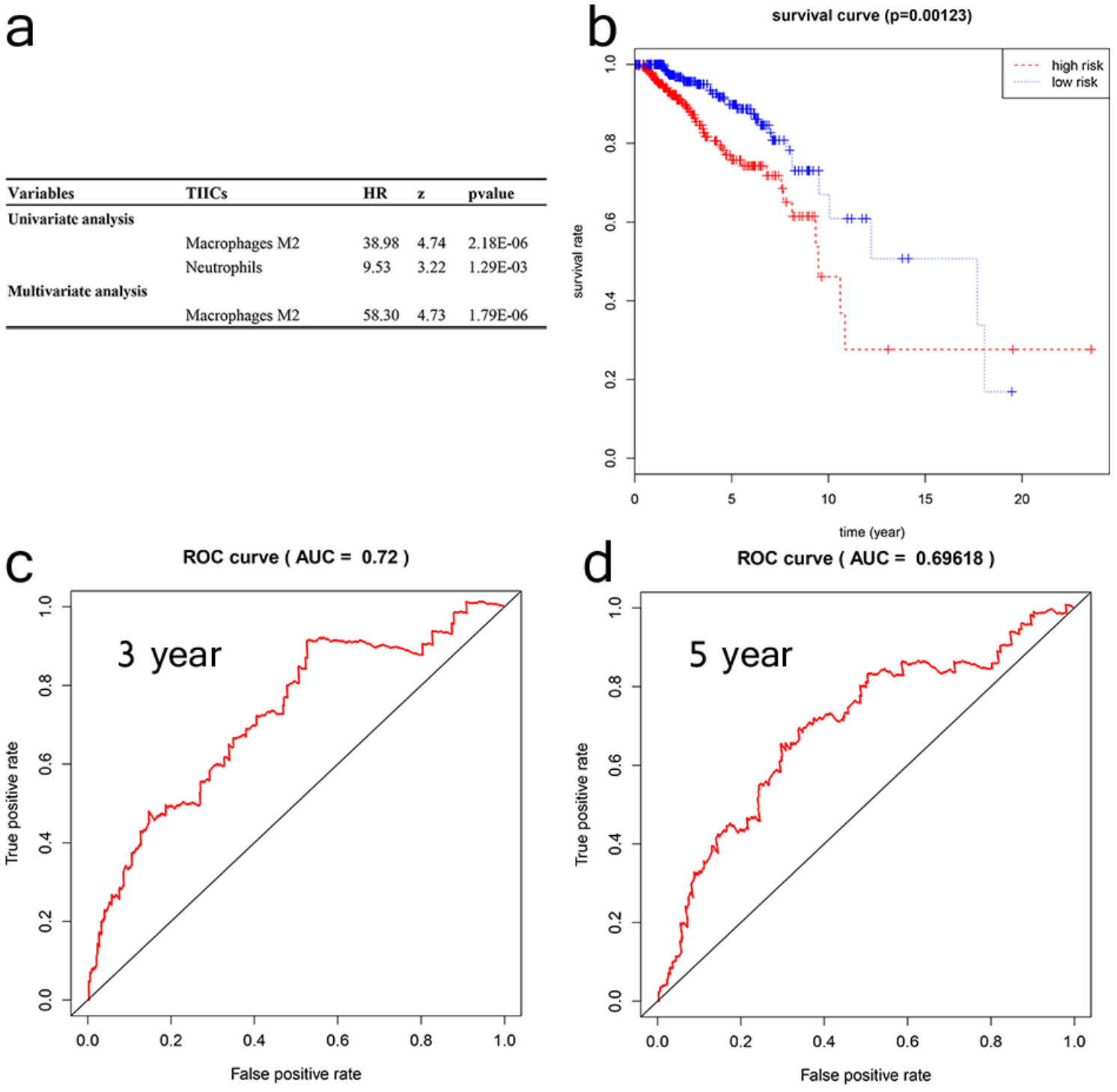

Figure 5

Construction of a survival risk-scoring system based on 22 tumor-infiltrating immune cell signatures. (a) The HR and P-value in the Cox model. (b) The survival curve of patients with high-risk and low-risk. (c-d)

The ROC curves for 3 and 5 years with AUC values. HR: hazard ratio; ROC: receiver operating characteristic ; AUC: area under curve

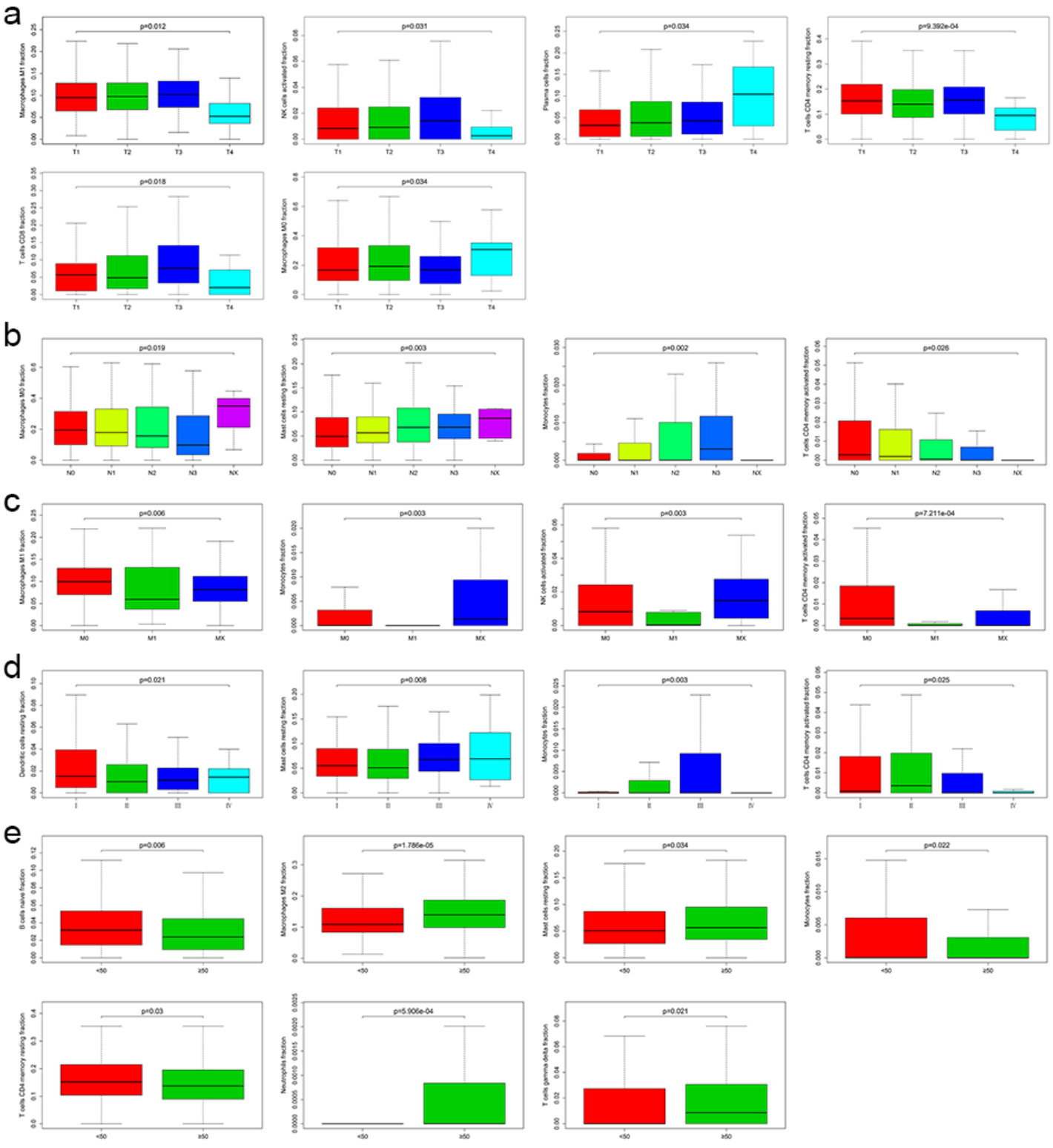

Figure 6

The correlation between tumor-infiltrating immune cells and clinical characteristics (a: Clinical stage; b: Tumor; c: Lymph Node; d: Metastasis; E: Age).