Title

Single-cell RNA-seq data reveals TNBC tumor heterogeneity through characterizing subclone compositions and proportions

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Abstract

Understanding subclonal architecture and their biological functions poses one of the key challenges to deeply portray and investigative the cause of triple-negative breast cancer (TNBC). Here we combine single-cell and bulk sequencing data to analyze tumor heterogeneity through characterizing subclone compositions and proportions. Based on sing-cell RNA-seq data (GSE118389) we identified five distinct cell subpopulations and characterized their biological functions based on their gene markers. It is worth noting that C1 and C2 relate to immune functions, while C5 relates to programmed cell death. Then based on subclonal basis gene expression matrix, we applied CBS deconvolution algorithm on TCGA tissue RNA-seq data, and found that patients with low and high C1 proportions have different immune microenvironment; and high C5 proportions would led to poor survival outcome, *p*-value and HR [95%CI] for five years overall survival in TCGA dataset were 0.0326 and 1.664 [1.038-2.667], and in GSE96058 dataset were 0.0158 and 2.557 [1.160-5.636]. Collectively, our analysis reveals the both intra-tumor and inter-tumor heterogeneity and their association with subclonal microenvironment in TNBC (subclone compositions and proportions), and uncovers the organic combination of subclones dictating poor outcomes in this disease.

Keywords:
triple-negative breast cancer, subclone, single-cell RNA-seq, deconvolution, survival analysis

Abbreviations:
TNBC, triple-negative breast cancer; HPCA, Human Primary Cells Atlas; CBS, Cibersort; TCGA, The Cancer Genome Atlas.

1. Introduction

Triple-negative breast cancer (TNBC) is one special type of breast cancer which does not express the genes for estrogen receptor (ER), progesterone receptor (PR) and Her2, and always accompanied with weaker treatment effect and worse clinical outcome (Lehmann et al., 2011; Li et al., 2015; Liu et al., 2018; Marra et al., 2019). Most triple-negative breast cancers have a basal-like genetic pattern (ER-/PR-/Her2-), and It has been reported that 56–85% of TNBCs are basal-like breast cancers (Lachapelle and Foulkes, 2011; Li et al., 2015; Prat et al., 2013). What's more, TNBC is a disease with heterogeneity not only inter-tumor but also intra-tumor, and represents various clinically and biologically distinct subgroups that have not been clearly defined yet (Cho et al., 2011; Karaayvaz et al., 2018; Mills et al., 2018; Montagna et al., 2013).

Inter-tumor heterogeneity denotes that patients suffering from one same cancer but have a great difference on clinical outcome and factors that can cause this phenomenon are various, such as gene expression, mutation events, methylation pattern and so on (Cusnir and Cavalcante, 2012; Nault, 2014; Wright et al., 2017). And nowadays, tumor microenvironment has arisen more interests which generally means non-cancerous cells present in and around a tumor (Buoncervello et al., 2019; Ferrone and Whiteside, 2007; Zheng and Gao, 2019), and especially, tumor immune microenvironment which focuses on immune cells has become one research hotspot (Bao et al., 2019; Jiang et al., 2019; Zheng et al., 2019). On the other hand, intra-tumor heterogeneity denotes that some subpopulations of cancer cells which differ in their genetic patterns, phenotypic characteristics or cell type composition within a given tumor tissue (Cresswell et al., 2016; Stanta and Bonin, 2018; Wright et al., 2017). Each subpopulation of cancer cells would build up a distinct subclone and if get a growth advantage, that subclonal population can expand over time and inhibit proliferation of normal cells (Giessler et al., 2017; Newcomb et al., 1978). Hence, how to identify that more malignant subclone from tumor tissue would be the key to understand intra-tumor heterogeneity.

Meanwhile, deep sequencing techniques are experiencing in high-speed development and the appearance of single-cell sequencing technologies transfers researchers' perspective from traditional bulk tissue samples to single cells (Aran et al., 2019; Chu et al., 2016; Schelker et al., 2017; Zong, 2017). Armed with that technique, researchers can analyze every single cell on features of somatic mutations, copy number variations, gene expression, methylation and so on, then mine deeper differences among distinguish cell populations (Chen et al., 2016; Garvin et al., 2015; Hui et al., 2018; Poirion et al., 2018). For example, Olivier et al. developed a linear modeling framework, SSrGE, combined with single-cell sequencing to link eeSNVs associated with gene expression, and further identified different subpopulation and linkage of genotype-phenotype relationship (Poirion et al., 2018). Anna et al. developed a novel method: Topographic Single Cell Sequencing (TSCS) to measure genomic copy number profiles of single

tumor cells, and applied it to 10 early-stage breast cancer patients. And they found that multiple clones crossed the basement membrane that brings out the invasion in Ductal carcinoma in situ (Casasent et al., 2018).

There are only a small number of studies have characterized the intra-tumor heterogeneity of TNBC through single-cell RNA-seq revealing the distinguish patterns that can reflects evolution of copy number variations during tumor progression. While these findings still focus on signature gene markers of cell subpopulations (Aarts et al., 2001; Arienti et al., 2017; Karaayvaz et al., 2018), but in our study we mainly analyze subclonal proportion in tissue bulks. Based on the deconvolution algorithm (Jin et al., 2018; Newman et al., 2015; Zeng et al., 2019), we connected single-cell level with bulk-tissue level by estimating subclones' proportion in tumor tissue then reveal genetic features, biological functions and clinical behaviors of TNBC subclones. And in result, we found that distinct subclone has its special function such as "regulation of T cell activation" in C1, "regulation of ARF GTPase activity" in C3, "programmed cell death" in C5 and so on.

2. Materials and Methods

2.1 Data source

We got the TNBC single-cell RNA-seq data GSE118389 including 1534 cells from GEO database (https://www.ncbi.nlm.nih.gov/geo/) and obtained TCGA data of 113 TNBC patients from GDAC FIREHOSE database (http://gdac.broadinstitute.org) including gene expression, copy number variation, somatic gene mutation data and corresponding clinical information [version: 2016_01_28]. What's more, to further verify our result, we also downloaded an external dataset GSE96058 which contained RNA-seq data of 135 TNBC patient and corresponding clinical information.

2.2 Cell type classification

Based on the Human Primary Cells Atlas (HPCA) cell expression dataset stored in R package "SingleR" which was named as "hpca.rda", we used differential expressing gene markers (stored as "hpca$de.genes") between each two cell type and corresponding expressing data to classify single cells' cell type (Mabbott et al., 2013). For one unknown single cell and two cell types, we used euclidean distance to determine that cell is more likely to which cell type; then used Bubbling Sort method to traverse all of the main cell types for identifying the best match cell type for that unknown cell.

2.3 Subclone identification in TNBC

To identify tumor subclones in TNBC cells, we inferred copy number variation (CNV) from RNA-seq gene counts data by using the R package "inferCNV" (https://github.com/broadinstitute/inferCNV) and clustered cells by K-means method. For "inferCNV" part, we used GETx (https://gtexportal.org/home/) samples as the reference normal samples and set default arguments but only sd_amplifier changed as 2. And for cell clustering part, we estimated the best cluster number by function from R package "cluster" with default arguments, then clustered 1000 times randomly (set the seed from 1 to 1000) to definite the final seed which has the lowest tot.withinss and the largest betweenness. Combined with the final seed and the best cluster number, we applied "kmeans" function on cell CNV data with default arguments to distribute every cell to its closest cluster.

2.4 Subclonal gene expression marker identification

After clustering, we got several subclones within TNBC cells which have significant differences on gene copy number. Based on gene TPM values provided by GSE118389, we identified gene expressing markers for each subclone. Firstly, we filtered genes by Analysis of Variance ("anova" function) with threshold of adjusted *p*-value < 0.1 ("p.adjust" function, BH method); then applied Wilcoxon Rank Sum Test ("wilcox.test", alternative = "greater") on that highly variable genes, cluster by cluster, to determine cluster-specific gene markers (adjusted *p*-value < 0.05 and Fold Change value > 2). And we used DAVID (https://david-d.ncifcrf.gov/) to annotate subclonal biologic function based on their marker genes. Furthermore, we constructed subclone basis matrix based on the above cluster-specific gene markers and their expression patterns (threshold is mean value).

2.5 Subclonal proportion estimating in bulk tissue

Based on the basis matrix constructed from heterogeneous subclones, we used R package "EpiDISH" to deconvolute bulk gene expressing data (TPM value) to subclones' proportion by CBS deconvolution algorithm. Then we performed the survival analysis on the subclonal proportions, we fitted Cox proportional hazards regression models and *p*-values are obtained from log-rank tests.

3. Results

3.1 Subclone within malignant TNBC tumor blocks

Tumor subclones usually tend to have different mutation patterns and copy number distributions, resulting in a difficult task in the identification of relative tumor subclone-specific clusters. Hence, we used "inferCNV" methods to estimate single-cell CNV profiles from RNA-seq read counts through comparing with GTEx (https://www.gtexportal.org/home/) normal breast tissues which was used as reference. After that, we applied k-means clustering methods on that inferred CNV data which were log2-transformed and distinct subclones within 1534 single cells. At first, we used "clusGap" function with default default arguments to estimate the number of clusters and we found split to 5 representative clusters was most ideal for our data. Then to determine the relatively optimal seed, we clustered 1000 times (set seed from 1 to 1000) and compared tot.withinss (the total within-cluster sum of squared errors) and betweenness generated from each time. Finally we set 34 as the best seed which has the lowest tot.withinss and largest betweenness. Combined with the above number of clusters and seed, we finally distributed every single cell to its closest group in 5 clusters (Figure 1A). What's more, we also identified 767 malignant cells in those cells which have accumulated sum of absolute CNV (log2-transformed) over median values. Then to verify the classification result, we compared tumor scores transformed from ESTIMATEScore [(max(x) – x) / (max(x) - min(x))] in those cells with normal cells and found they have significant Wilcoxon Rank Sum *p*-value < 2.2e-16 (Figure 1B, 1C) (Schelker et al., 2017).

After that, we identified distinct cell populations within the tumors by comparing with reference HPCA dataset (Human Primary Cells Atlas) as described in Methods. We used 153 primary cell types to identify our single cells, and 29 main cell types were matched. And we found 26.01% single cells and 35.3% malignant cells (Figure 3F) are more likely to be induced pluripotent stem (iPS) cells that maybe indicated most cancer single cells have pluripotent potentiality (Malchenko

et al., 2010; Werbowetski-Ogilvie and Bhatia, 2008). Meanwhile, 21.71% cells were similar to neutrophil may hinted that tumor tissue could recruit neutrophil and cause inflammatory response. While except for the above two type, none of the rest cell types' proportion was more than 10%. On the other hand, for each cell cluster, we also statistics cell types within and found that both C1 and C5 have more than 40% cells were iPS cells, and in C2 and C3 iPS cells' proportion also the highest (28.40% in C2 and 24.43% in C3), but only C4 have 58.75% cells were Neutrophils (Figure 3).

Considerate on that gene expression profile data is more widely used and easier to get, hence, we characterized the above 5 subclones on gene expression data and construct a basis matrix to deconvolute on TCGA bulk tissues (Figure 2E). From cell to tissue, we transformed TCGA bulk gene expressing matrix to subclone proportion matrix, and through this way we reduced the dimension from tens of thousands genes to only just five subclonal proportions. Then based on subclone proportion, we can reveal how every subclone influence tumor development and what's role they played in by analyzing the changes in subclonal composition among patients.

3.2 Heterogeneous subclones play different roles

With further research, we found that every subclone plays important but entirely different roles, and the organic combination of those subclones makes tumor so complicated and uncontrollable. And we hypothesized that inter-tumor heterogeneity, in some instance, was affected by intra-tumor heterogeneity, especially the compositions of subclones.

3.2.1 C1 and C2 associated with immunity

Based on highly expressed gene markers identified above, we annotated GO BP terms for C1 and C2 clusters by DAVID and found that two subclones may tightly associated with immune-related function.

For C1 subclone, we got one GO term "regulation of T cell activation" indicated C1 subclone may closely related to T cells (Figure 2A). To verify the relevance between them, we estimated both subclone and immune microenvironment in TCGA TNBC patients by CBS deconvolution algorithm provided in "EpiDISH" package, and then used Wilcox Rank Sum Test to check the proportion difference of 22 special immune cells (CIBERSORT LM22 dataset) between high and low (threshold was mean value) C1 proportion (Newman et al., 2015). We observed that CD4 T cells, Treg cells, Macrophages and mast cells have significant difference (with Wilcox Rank Sum Test *p*-values = 1.2846e-5, 0.0024, 5.1568e-07 and 0.0036, Figure 4A) which may indicated that C1 subclone may influence immune-related function via changing T cell microenvironment. What's more, we also checked whether patients with high and low C1 proportion have different immune ability. We used "EASTIMATE" package to calculate immuneScore for each TNBC sample and found that high C1 proportion patients have significant higher scores than low C1 proportion (Wilcox Rank Sum Test, greater alternative, *p*-value = 0.0478, Figure 4B).

As for C2 subclone which was related to GO term "lymphocyte mediated immunity" and "immunoglobulin mediated immune response" (Figure 2B), while we observed that there was no significant difference between high and low proportion patients in immune microenvironment and immune ability. But very interestingly, we found that high C2 proportion patients have higher Tumor Mutational Burden (TMB) than low C2 proportion (Fold Change was 2.110048 and Wilcox Rank Sum Test *p*-value was 0.01027, Figure 4E), showing that high C2 proportion patients may have benefits from immuno-oncology (IO) therapies (Allgauer et al., 2018; Boumber, 2018; Romero, 2019; Wu et al., 2009).

Furthermore, we also compared TMB among four main IHC breast cancer subtypes (including ER+, HER2+, ER+ / HER2+ and TNBC), and we found that both HER2+ and TNBC have more higher TMB than the other two, and TNBC compared with ER+ and ER+ / HER2+ were all significant (Liu et al., 2018; Marra et al., 2019) (Wilcox Rank Sum Test *p*-value were 7.841e-13 and 2.525e-4, Figure 4G).

3.2.2 C3 may relate to metastasis

Different from others, C3 subclone has none significant highly expressed genes, so we used significant down-expressed genes to characterize its biological functions and roles in tumor development.

We used DAVID to annotated GO BP terms, and found that C3 subclone related to “regulation of ARF GTPase activity” and “regulation of ARF protein signal transduction” (Figure 2C). While some researchers proposed that ARF GTPase-activating proteins may play an important role in tumor metastasis (Campa and Randazzo, 2008; Hongu et al., 2016). On the other hand, we also found that C3 proportion abnormal rise in stage III and IV patients, maybe it plays an important role in conversion from stage II to III and IV, especially maybe associated with tumor metastasis (Figure 4C). To support that, we collected micrometastasis information and first non-lymph node metastasis anatomic sites of TNBC patients, and tested whether high C3 proportion interrelated with tumor metastasis.

We used one-side Wilcoxon Rank Sum Test to check whether patients with high C3 proportion really have higher probability to suffer from tumor metastasis, but we got *p*-value was 0.542. Hence, we thought that there is not one subclone plays a role in tumor metastasis process but subclonal cooperation drives metastasis. Then we compared the subclonal distributions between metastatic patients and non-metastatic patients, we found that, in metastatic patients, C1 and C3 raise, C2 and C5 declined, only C4 were stable (Figure 4D). Hence we hypothesis that C1 / C3 were risk factors and C2 / C5 were protection factor, then we constructed a risk score to divide samples into high or low risk of metastasis (threshold is MAD), and we verified low risk score generally related to low risk of metastasis (Cumulative Hypergeometric Test *p*-value was 0.059, Table 2).

$$\text{Risk Score} = \text{coef}_{C1} \times \text{prop}_{C1} + \text{coef}_{C3} \times \text{prop}_{C3} - \text{coef}_{C2} \times \text{prop}_{C2} - \text{coef}_{C5} \times \text{prop}_{C5}. \quad (1)$$

And in TCGA dataset:

$$\text{Risk Score} = 0.1251 \times \text{prop}_{C1} + 0.1377 \times \text{prop}_{C3} - 0.1401 \times \text{prop}_{C2} - 0.463 \times \text{prop}_{C5}. \quad (2)$$

Where coef were coefficients got from cox proportional hazards regression model of subclonal proportions for 5-years TNBC patients, and prop were the proportions of subclones.

3.2.3 C4 may be constructed by neutrophils

When we annotated functions for differential expression genes in C4, we found that there is no GO term or KEGG pathway enriched in neither up- nor down-regulated gene sets. But we found that a great part of cells in C4 (44%) have a similar expression pattern with neutrophils (Figure 3D), while C1, C2, C3 and C5 all have a large part of cells more likely to be iPS cells (Figure 3A, 3B, 3C, 3E).

What’s more, to determine genes which were differential mutated in one subclone, we applied Fisher's Exact Test on TNBC patients’ mutation details to filter genes which have different mutation frequency between high and low subclone proportion (mean threshold, Table 1). And only differential mutated genes in C4 can be annotated and them enriched in one of the most popular tumor-related pathway - “ErbB signaling pathway” (hsa04012 in KEGG), including

ERBB4 and PIK3CA which are key genes in that pathway (Cizkova et al., 2012; Hoque et al., 2010; Lau et al., 2014).

3.2.4 C5 can influence patients' survival outcome

Functional annotation of differental expressed genes showed that C5 was tightly related to "programmed cell death" and "apoptosis" (FIgure 2D), and proved the cancer hallmark "resisting cell death".(Hanahan and Weinberg, 2011) Interestingly, we also found that different stage patients in TCGA TNBC have nearly same level of C5 proportion, and that subclone kept presenting in all stages (Figure 4C). Hence, we assumed that C5 may be the key subclone in TNBC tumors which can make tumor malignant through resisting programmed cell death then induce patients' poorer survival outcome.

What's more, C5 also was the only one subclone that its proportion can influence patients' survival outcome and the TSNE plot of tumor cells distribution showed that in C5 most of cells are malignant. Based on the result of overall survival analysis, threshold is 0.2 (over 0.2 means high risk),we found that in TCGA 103 TNBC patients, C5's log-rank test *p*-value and HR [95%CI] were 0.0326 and 1.664 [1.038-2.667] (5 years, Figure 5A), 0.06 and 1.664 [0.988-2.414] (10 years, Figure 5B); in GSE96058 133 TNBC patients were 0.0158 and 2.557 [1.160-5.636] (5 years, Figure 5C), 0.04 and 2.557 [1.018-4.612] (10 years, Figure 5D). What's more, whatever 5 years or 10 years, the results of univariate and multivariate Cox proportional hazard regression analysis both proved that C5 proportion was an independent risk factor, and its HR and 95%CI in multivariate model were 1.573 [0.9714 - 2.547] and 1.504 [0.9489 - 2.384] (Table 3).

Taken all together, we found that five subclones all played very important role in tumor development and progression, but on the other hand they also are distinctive with each other. To further verify our hypothesis that: intra-tumor heterogeneity in TNBC embedded in the diversity of the above five subclones, then further brings about inter-tumor heterogeneity reflected in different subcloneal composition, we divided TCGA TNBC patients into TNBCtype6 subtypes which have been used wildly (Lehmann et al., 2011). And we found that those six subtypes really have very different subclone composition (Figure 4G), and especially in IM subtype C1 and C2 are enriched markedly which were proofed related to immune functions.

4. Discussion

Single-cell RNA-seq has offered unprecedented opportunity for deeply understanding the origin, evolution and heterogeneity of cancer, but also brought the challenge in how to associate single-cell genotype with tissue phenotype. Hence, we developed a systematic pipeline for providing a novel insight to understand the subclonal architecture and their biological functions of TNBC through the integration of TNBC scRNA-seq data and deconvolution algorithm. We characterized the inner diversity among TNBC single cells on CNV level and gene expression level, then identified five heterogeneous subpopulations. Combined with HPCA reference cell type markers collected in "singleR" package, we identified potentially cell type for every single cells based on gene expression similarity and we found that a great number of cells are more likely to be iPS cells which have highly differentiation pluripotency. After that, based on "inferCNV" method we identified five distinguish cell subpopulations and characterized the biologic functions or their special roles played in cancer progressing. After in-depth and detailed analysis, we found that C1,C2,C4 were related to immune functions, C3 may potential connected with tumor

metastasis and C5 might the key subclone in TNBC.

Especially, C1 is tightly associated with the GO term “regulation of T cell activation” and the result of immune microenvironment also shows that C1 can influence the distribution of CD4 T cells, Treg cells, Macrophages and mast cells. And considering on the relationship between C2 and TMB and C4 is enriched in neutrophils, we guess TNBC maybe one special subtype in breast cancer can benefits from immuno-oncology (IO) therapies. On the other hand, our analyze on tumor metastasis shows that not only C3 but also C1, C2 and C5 play important role in that and indicates that metastasis is not the result of single factor but caused by a series of factors. Most importantly, we detect one key subclone C5 which can influence patients’ survival outcome, high C5 proportion means poor outcome. Whatever its enriched GO term “programmed cell death” and its stable distribution in patients with different stage all indicate that C5 may be the hotspot to understand TNBC.

4.1 Conclusion

Taken together, we presented a new thought to analysis tumor heterogeneity and found every subclone plays important but entirely different roles in TNBC and single-cell combined with deconvolution algorithm may be one powerful weapon to understand that deadly cancer type. Additionally, the result of survival analysis applied on C5 let us believe that C5 can be one biomarker for diagnosis and treatment of TNBC. Undoubtedly, future studies will uncover additional features and reveal novel cell subpopulations to understand intra-tumor heterogeneity in tumor progression. Through deconvolution transformed we link single cells and bulk tissue together, and our study substantiates subclonal microenvironment can reveal intra-tumor heterogeneity and influence intra-tumor heterogeneity.

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China [Grant Nos. 31871336, 61573122, 31501078, 61801150]. Wu lien-the youth science fund project of Harbin medical university [Grant Nos. WLD-QN1407]. The Department of Heilongjiang Province (Grant Nos. 12541415), the Heilongjiang Natural Science Fund Project (Grant Nos. LH2019C087).

Competing interests

The authors declare no conflict of interest.

Author contributions

Chaohan Xu, Weida Wang and Jinyuan Xu conceived and designed the study; Weida Wang, Bo Zhang, Lei Yu, Li zhang and Peng Xia read and collected details from the published articles; Qian Song, Jie Wu, Shuyuan Wang, and Bo Zhang made the tables; Chaohan Xu and Weida Wang wrote the paper; Yun Xiao and Chaohan Xu revised the manuscript for important intellectual content. all authors have read and approved the final version prior to publication.

Figure legends

Figure 1. Subclone identification based on single-cell CNV and malignant cells classification. **A** T-SNE plot for 1534 single-cells and their distributions in five subclones. **B** Bar plot depicting the proportions of five subclones in patients. **C** T-SNE plot for 1534 single-cells and their distributions in six patients. **D** Box plots representing the distribution of tumor scores between malignant cells and normal cells.

Figure 2. Subclonal GO terms enrichment and heatmap for gene markers expression. A - D pie plot depicting the enriched top 10 Go terms in cluster 1, 2, 3 and 5. **E** heatmap for gene markers expression in five subclones. **F** differential mutated genes in cluster 4.

Figure 3. Cell types identification and their distributions in five subclones. A - E depicting the cell type proportions in five subclones. **F** representing the proportions in malignant cells.

Figure 4. Analyses focus on cluster 1, 2 and 3. **A** depicting differential immune cell types between patients with high and low C1 proportion. **B** boxplot for ImmuneScore between patients with high and low C1 proportion. **C** representing the distribution of five subclones in different stage patients. **D** representing the distribution of five subclones in metastatic and non- Metastatic patients. **E** boxplot for TMB between patients with high and low C2 proportion. **F** representing the distribution of five subclones in TNBCtype6. **G** boxplot for TMB among ER+, ER+/HER2+, HER+ and TNBC patients.

Figure 5. Survival analysis focues on cluster 5. **A-B** K-M plot (5, 10 years overall survival) for cluster 5 proportions in TCGA 103 TNBC patients and threshold is 0.2. **C-D** K-M plot (5, 10 years overall survival) for cluster 5 proportions in GSE96058 133 TNBC patients and threshold is 0.2.

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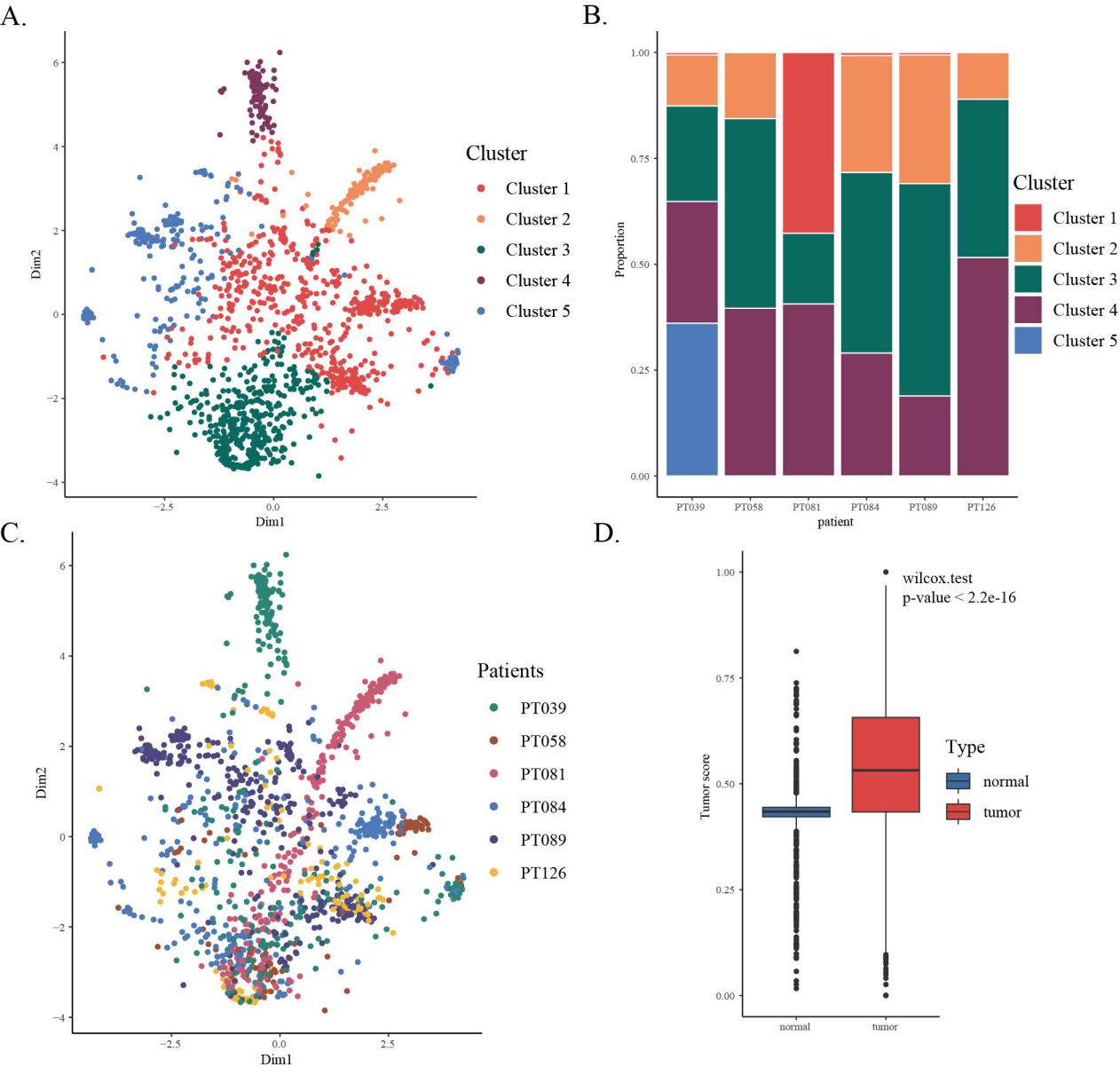
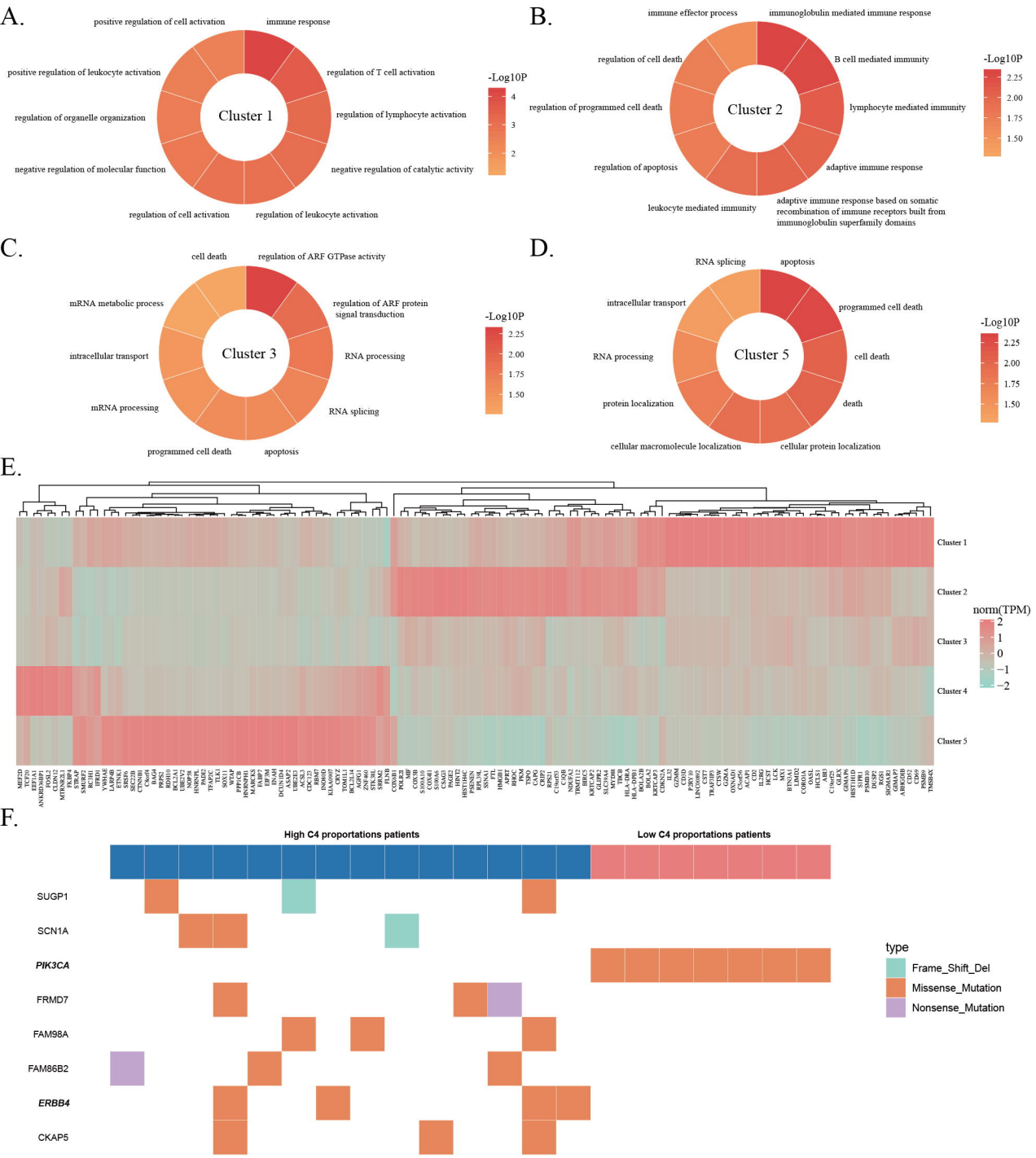
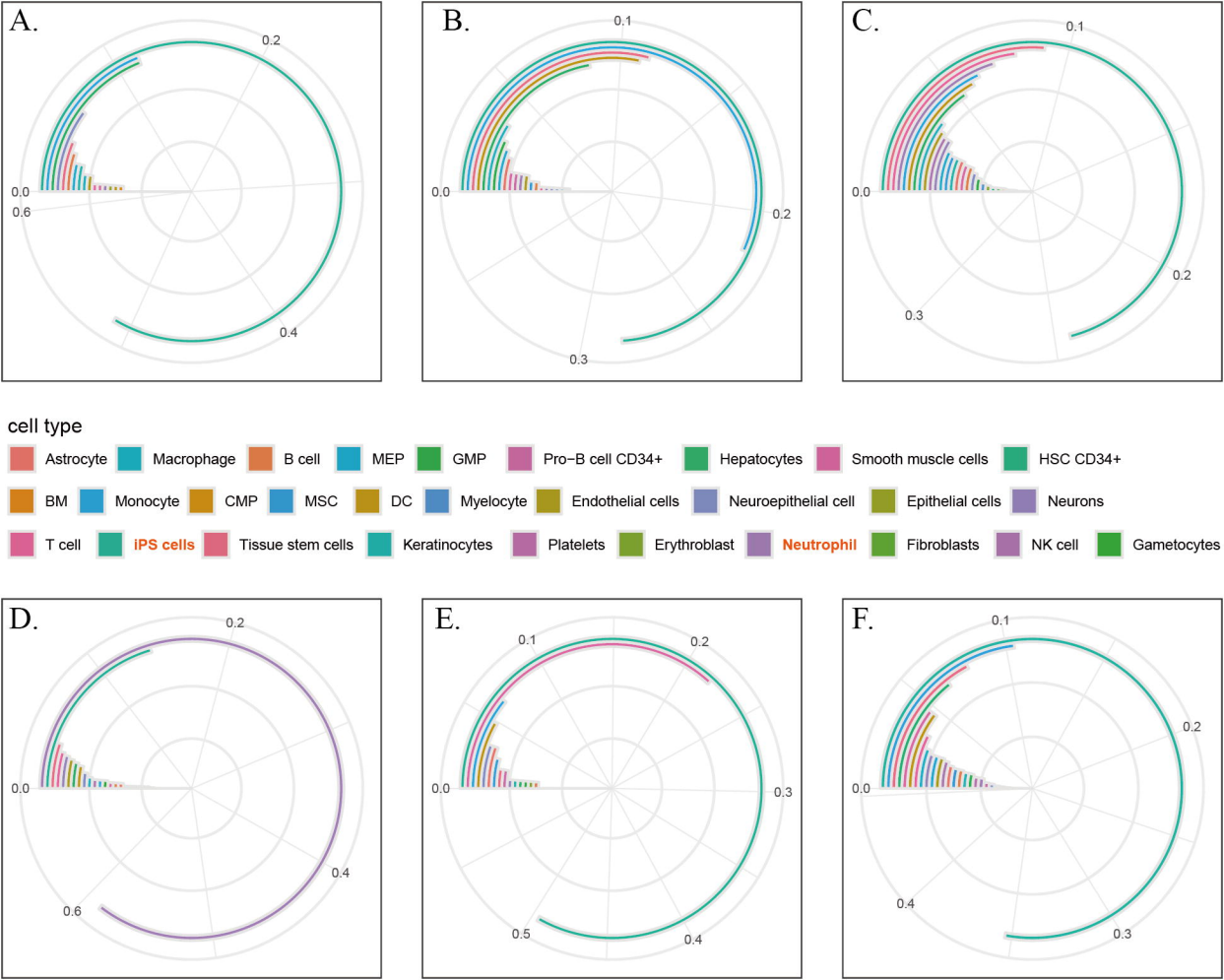
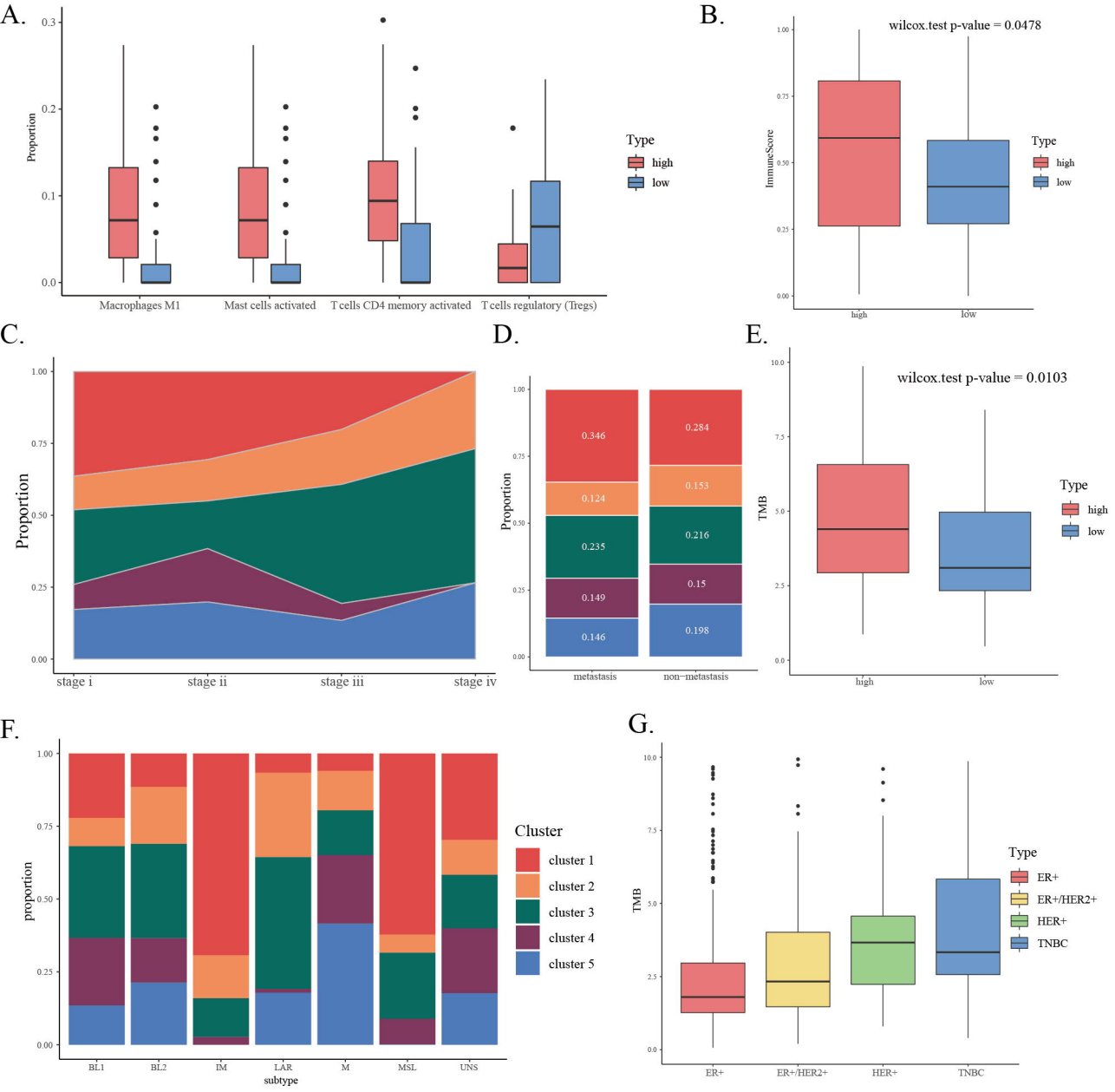
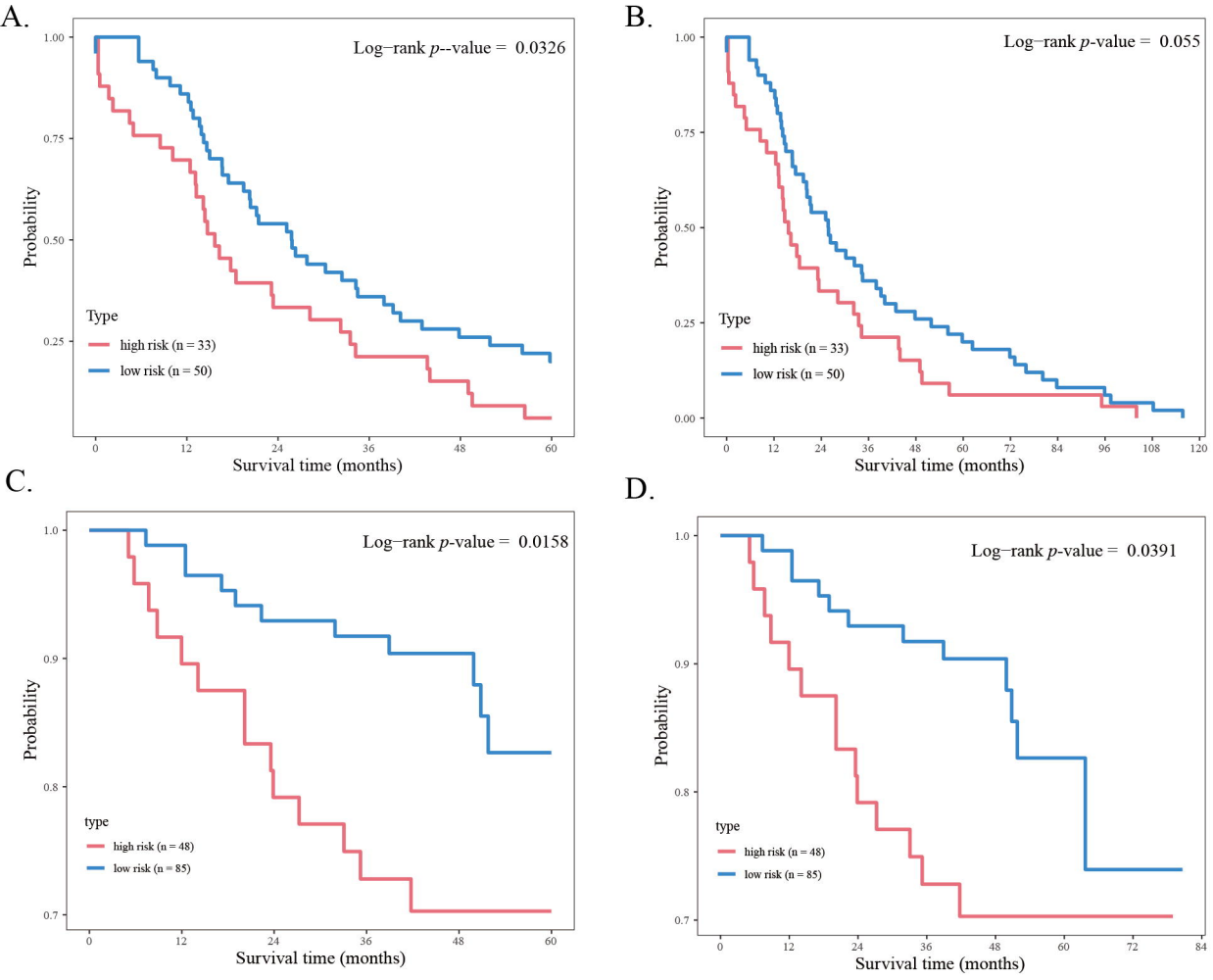

A.
Cluster
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Dim1
Dim2
B.
Cluster
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Proportion
patient
PT039
PT058
PT081
PT084
PT089
PT126
C.
Patients
PT039
PT058
PT081
PT084
PT089
PT126
Dim1
Dim2
D.
wilcox.test
p-value < 2.2e-16
Type
normal
tumor
Tumor score
normal
tumor


A.
Cluster 1
positive regulation of cell activation
immune response
regulation of T cell activation
regulation of lymphocyte activation
negative regulation of catalytic activity
regulation of leukocyte activation
regulation of cell activation
negative regulation of molecular function
regulation of organelle organization
positive regulation of leukocyte activation
-Log10P
4
3
2
B.
Cluster 2
immune effector process
immunoglobulin mediated immune response
B cell mediated immunity
lymphocyte mediated immunity
adaptive immune response
adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains
leukocyte mediated immunity
regulation of apoptosis
regulation of programmed cell death
regulation of cell death
-Log10P
2.25
2.00
1.75
1.50
C.
Cluster 3
cell death
regulation of ARF GTPase activity
regulation of ARF protein signal transduction
RNA processing
RNA splicing
apoptosis
programmed cell death
mRNA processing
intracellular transport
mRNA metabolic process
-Log10P
2.25
2.00
1.75
1.50
D.
Cluster 5
RNA splicing
apoptosis
programmed cell death
cell death
death
cellular protein localization
cellular macromolecule localization
protein localization
RNA processing
intracellular transport
-Log10P
2.25
2.00
1.75
1.50
E.
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
norm(TPM)
2
1
0
−1
−2
F.
High C4 proportions patients
Low C4 proportions patients
SUGP1
SCN1A
PIK3CA
FRMD7
FAM98A
FAM86B2
ERBB4
CKAP5
type
Frame_Shift_Del
Missense_Mutation
Nonsense_Mutation


A.
0.2
0.0
0.6
0.4
B.
0.1
0.0
0.2
0.3
C.
0.1
0.0
0.2
0.3
cell type
Astrocyte
Macrophage
B cell
MEP
GMP
Pro−B cell CD34+
Hepatocytes
Smooth muscle cells
HSC CD34+
BM
Monocyte
CMP
MSC
DC
Myelocyte
Endothelial cells
Neuroepithelial cell
Epithelial cells
Neurons
T cell
iPS cells
Tissue stem cells
Keratinocytes
Platelets
Erythroblast
Neutrophil
Fibroblasts
NK cell
Gametocytes
D.
0.2
0.0
0.4
0.6
E.
0.1
0.2
0.0
0.3
0.5
0.4
F.
0.1
0.2
0.0
0.4
0.3


A.
Proportion
0.3
0.2
0.1
0.0
Macrophages M1
Mast cells activated
T cells CD4 memory activated
T cells regulatory (Tregs)
Type
high
low
B.
wilcox.test p-value = 0.0478
ImmuneScore
1.00
0.75
0.50
0.25
0.00
high
low
Type
high
low
C.
Proportion
1.00
0.75
0.50
0.25
0.00
stage i
stage ii
stage iii
stage iv
D.
Proportion
1.00
0.75
0.50
0.25
0.00
0.346
0.124
0.235
0.149
0.146
0.284
0.153
0.216
0.15
0.198
metastasis
non−metastasis
E.
wilcox.test p-value = 0.0103
TMB
10.0
7.5
5.0
2.5
0.0
high
low
Type
high
low
F.
proportion
1.00
0.75
0.50
0.25
0.00
BL1
BL2
IM
LAR
M
MSL
UNS
subtype
Cluster
cluster 1
cluster 2
cluster 3
cluster 4
cluster 5
G.
TMB
10.0
7.5
5.0
2.5
0.0
ER+
ER+/HER2+
HER+
TNBC
Type
ER+
ER+/HER2+
HER+
TNBC


A.
Log−rank p--value = 0.0326
Probability
Survival time (months)
Type
high risk (n = 33)
low risk (n = 50)
B.
Log−rank p-value = 0.055
Probability
Survival time (months)
Type
high risk (n = 33)
low risk (n = 50)
C.
Log−rank p-value = 0.0158
Probability
Survival time (months)
type
high risk (n = 48)
low risk (n = 85)
D.
Log−rank p-value = 0.0391
Probability
Survival time (months)
type
high risk (n = 48)
low risk (n = 85)