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IFN-γ: a beneficial immune marker in triple-negative breast cancer

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Abstract: Cancer immunotherapy has shown promising results in triple-negative breast cancer (TNBC), however, only a small proportion of patients benefit from the immune checkpoint inhibitors (ICI). To explore robust biomarkers to predict the prognosis of TNBC patients is in great need. In this study, we first established an immune risk scoring signature with the Cancer Genome Atlas (TCGA) database. The GO and KEGG enrichment were used for cluster analysis. The KM curve was performed for prognosis. The differentially expressed immune genes were obtained for univariate and multivariate Cox and Lasso regression analysis, and 18 independent immune genes associated with prognosis were obtained to establish an immune risk score signature (IRSS). The Kaplan-Meier results showed that the prognosis of the high-risk group was significantly favorable than that of the low-risk group ($P < 0.001$; HR = 3.06, 95% CI = 2.16-4.32). The 18 targeted immune genes were enriched in JAK-STAT signaling pathway and IFNG was the most focus gene in TNBC compared with Luminal A/B and HER2 subtypes. Furthermore, TNBC patients with high level of both IFNG and checkpoints (CD274, PD-1, CTLA-4 and TIGHT) would have longer survival and IFNG was positively relevant to various checkpoints, especially PD-1/PD-L1. Taken together, our results indicate that dispite of the complicated effects of IFNG in immune microenvironment, IFNG is associated with better prognosis in TNBC and may act as a biomarker guiding the ICI therapy.

1. Introduction

Triple-negative breast cancer (TNBC) is a heterogeneous breast cancer subtype, accounting for 12%-17% of all breast cancers[1]. A high rate of distant recurrence and poor prognosis are main characteristics of TNBC, in which conventional therapies such as radiotherapy and chemotherapy have limited efficacy. As for the lack of specific receptors, targeted therapy is not applicable for TNBC[2]. Evidence indicates that immunotherapy especially checkpoint blockade (ICB) has been groundbreaking in expanding treatment options for multiple solid tumors, while less than one-third

of patients have a vigorous response due to different TNBC subtypes and tumor heterogeneity[3-5]. To identify certain patients responding to ICB is of great significance to improve efficacy and reduce economic burden. Therefore, it is urgent to find robust biomarkers to predict the prognosis of TNBC patients and provide potential therapeutic targets. Here we used the Cancer Genome Atlas (TCGA) database to explore the correlation between immune mechanisms and the occurrence of TNBC and established a novel risk score signature based on immune genes to effectively predict the outcome of TNBC patients.

2. Methods

2.1. Data Collection and Processing

The datasets of breast cancer (TCGA-BRCA) and lung adenocarcinoma (TCGA-LUAD), including their gene expression profiles, clinic information and survival information, were downloaded from the TCGA database[6]. From the Gene List module of the Immunology Database and Analysis Portal (ImmPort) database[7], the list of totaling 2483 immune-related genes was downloaded. Breast Cancer Gene-Expression Miner (bc-GenExMiner v4.8, http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Accueil.php?js=1) was used to detect the hub gene expression, molecular correlation and prognosis in different phenotypes of breast cancer (BRCA)[8].

2.2. Differential Expression Analysis

Based on the expression of genes in BRCA, we first performed a differential expression analysis to identify genes differentially expressed in normal and tumor groups[9]. Briefly, differentially expressed genes (DEGs) were obtained using the "limma" software package in R. Among them, $Log_2|FC| > 1.5$ and false discovery rate (FDR) < 0.25 were the criteria. "ggplot2," "Cairo," and "ggrepel" packages in the R were used to plot volcanoes to visualize the DEGs.

2.3. Establishment of Immune Risk Scoring Signature (IRSS) for Prognosis

A total of 1793 immune genes were expressed in BRCA and intersected with DEGs to obtain differentially expressed immune genes (DEIGs). Subsequently, DEIGs were used in univariate Cox regression analysis to identify significant prognosis-related immune genes, followed by Least absolute shrinkage and selection operator (LASSO) regression analysis to obtain independent prognostic genes. Multivariate Cox regression analysis was conducted to obtain regression coefficients for independent prognostic factors. Finally, an immune risk score signature (IRSS) was established based on the multivariate Cox regression coefficient beta value.

By calculating the risk score for each sample of TCGA-BRCA, patients were divided into low- and high-risk groups using the median as the cut-off value. Furthermore, visualization of the Kaplan-Meier (KM) curve was utilized to compare overall survival (OS) between the two groups by the log-rank test. The area under the receiver operating characteristic (ROC) curve (AUC) was adopted for analyzing the prognostic predictive value of IRSS in patients with BRCA.

2.4. Immune Risk Score Signature Combined With Clinicopathological Information

We screened for prognostic predictive factors, including clinical characteristics and established IRSS[10]. Specifically, the univariate Cox proportional hazard model was employed to analyze the correlation between IRSS and OS, and the multivariate Cox regression analysis was used to evaluate whether the established IRSS could serve as an independent prognostic predictor. Further, to comprehensively assess patient survival, we constructed a nomogram integrating distinct clinicopathological information, including age, tumor stage, PAM50 and IRSS, using the "rms"

package. Additionally, the concordance index (C-index) was used to evaluate the predictive accuracy of the nomogram. Similarly, the decision curve analysis (DCA) of 2, 3, and 5 years was calculated to evaluate whether the synthetic nomogram established by us is suitable for clinical application. The x-axis represents the percentage of the threshold probability, and the y-axis represents the net income.

2.5. Validation of IRSS

To assess the general applicability of the signature, we selected the TCGA-LUAD (n = 598) to further validate the established model due to the anatomical and histological similarities. The risk scores of each patient in the LUAD cohort were calculated and ranked using the formula of the IRSS established in TCGA-BRCA. LUAD samples that had been sorted by scores were divided into high- and low-risk groups according to the cutoff values obtained in the TCGA-BRCA cohort. KM curves were used for comparison of the survival differences between the two groups, and ROC curves were used to assess the accuracy of the signature prediction.

2.6. Statistical analysis

For data with normal distribution, results were presented as the mean ± SD. Unpaired t-test or ANOVA was used for analyzing statistical assessments. For data do not accord with normal distribution, results were present with median (IQR) and wilcoxon test was utilized for analysis. Statistically significant difference was considered when $P < 0.05$.

3. Results

3.1. Differential prognostic immune genes in BRCA

First, we performed a differential analysis between normal and tumor groups and obtained genes significantly associated with BRCA. Results showed that a total of 6487 DEGs were identified (**Figure 1A**). To explore whether immune genes could be used as effective biomarkers to indicate the prognosis of BRCA, we selected immunerelated genes from the DEGs for further analysis. The Venn diagram (**Figure 1B**) showed that 325 DEIGs were screened from the overlap of immune genes and DEGs. To find DEIGs with prognostic value, the 325 DEIGs were intersected with prognostic genes in BRCA, and 42 genes were finally selected for the establishment of IRSS.

3.2. Construction and prognostic value of IRSS

The LASSO regression analysis was performed to screen variables with 42 prognostic DEIGs. Combining the results of **Figure 1C, D**, it was considered that the model fit the best when the penalty coefficient was 18, and the corresponding 18 immune genes were selected into the model (**Figure 1E**). Additionally we verified the model reliability and the KM curve showed that the prognosis of the high-risk group was worse than that of the low-risk group (**Figure 2A**). ROC curves were employed to assess the accuracy of established models for predicting OS in patients with BRCA. As shown in **Figure 2B**, the AUC values of 2, 3, and 5 years were 0.724, 0.747, and 0.690, respectively, indicating the robustness and accuracy of the model in predicting patient prognosis. To determine whether the established IRSS has prognostic significance, we further performed univariate and multivariate Cox regression analysis (**Table 1**). Univariate Cox regression analysis showed that risk score, age, stage, PAM50 subtype classification and IRSS risk score were prognostic predictors in TGGA-BRCA.

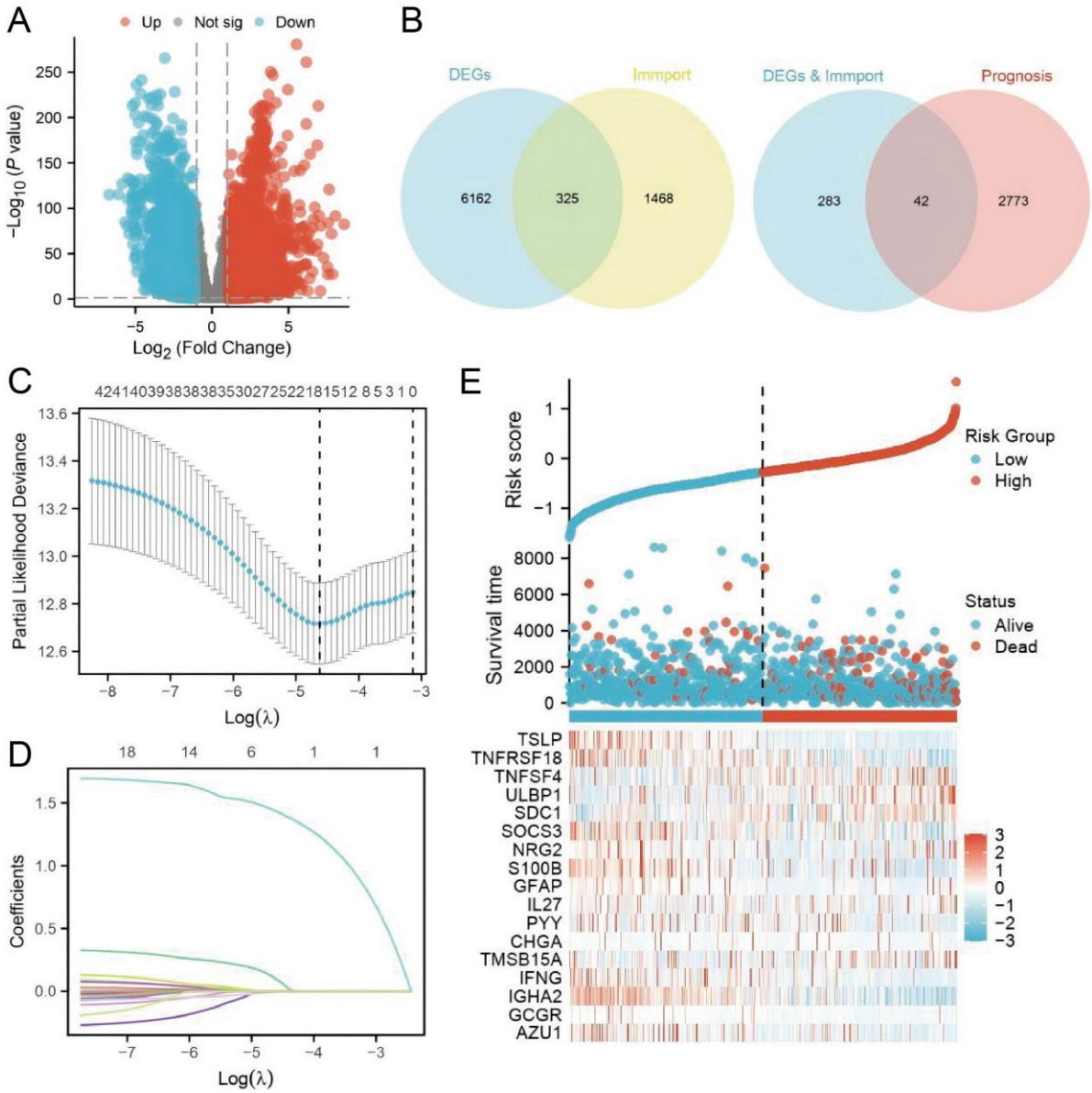
Nomograms, which simplify statistical prediction models to single numerical estimates of event probabilities tailored to individual patient profiles, are widely used for prognostic assessment of

tumors[11]. Therefore, we established a nomogram containing multiple clinicopathological characteristics as well as IRSS. As shown in **Figure 2C**, scores for each variable could be calculated and combined to comprehensively predict the prognosis of patients with BRCA. Consistent with this result, the DCA figure (**Figure 2D**) also proved that the nomogram combined with various clinical features has better clinical application value. To verify the general applicability of the IRSS, the data of the TCGA-LUAD cohort with similar tissue and anatomical characteristics was downloaded as the validation cohort and investigated in **Supplemental Figure 1**. Taken together, the established IRSS had good applicability and could not only predict the prognosis of BRCA.

Table 1: Univariate/multivariate Cox regression analysis of clinicopathological features of BRCA associated with OS.

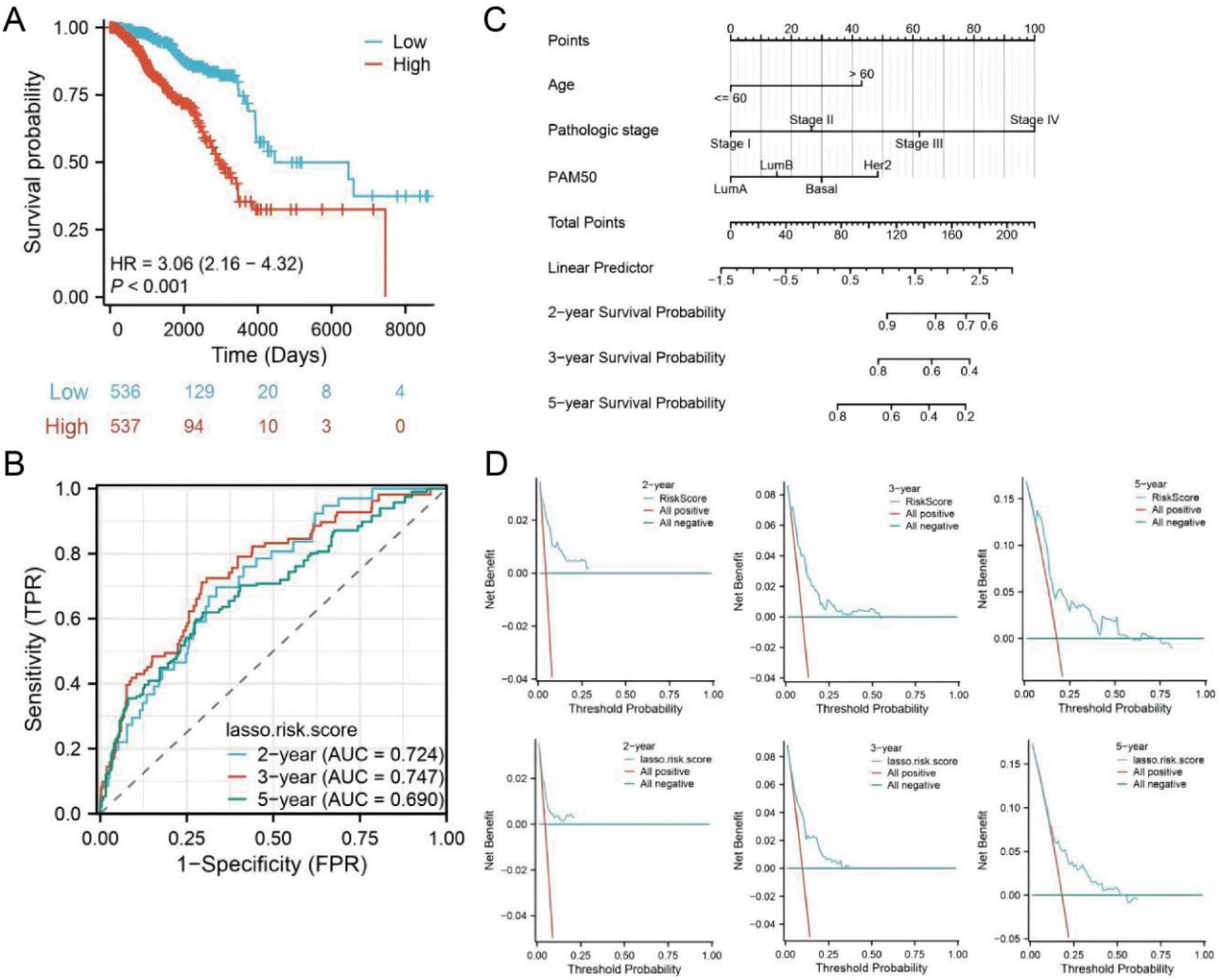
Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age	1,086		**< 0.001**		
<= 60	603	Reference		Reference	
> 60	483	2.024 (1.46 - 2.79)	**< 0.001**	2.510 (1.74 - 3.60)	**< 0.001**
Pathologic stage	1,062		**< 0.001**		
Stage I	181	Reference		Reference	
Stage II	619	1.702 (0.98 - 2.93)	0.055	1.767 (0.98 - 3.16)	0.055
Stage III	244	2.935 (1.64 - 5.22)	**< 0.001**	3.767 (2.03 - 6.96)	**< 0.001**
Stage IV	18	11.637 (5.58 - 24.25)	**< 0.001**	8.471 (3.82 - 18.77)	**< 0.001**
PAM50	1,046		**0.012**		
LumA	563	Reference		Reference	
LumB	206	1.664 (1.08 - 2.54)	**0.019**	1.383 (0.88 - 2.16)	0.157
Her2	82	2.270 (1.33 - 3.87)	**0.003**	2.811 (1.61 - 4.88)	**< 0.001**
Basal	195	1.290 (0.83 - 1.98)	0.250	1.896 (1.19 - 3.01)	**0.007**
IRSS score	1073		**< 0.001**		**< 0.001**
Low	536	Reference		Reference	

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
High	537	3.06 (2.16 - 4.32)	< **0.001**	2.58 (1.98 -3.32)	< **0.001**



(A) Volcano plot of 6487 differentially expressed genes. (B) Venn diagram of the intersection of DEGs and immune genes. (C) Ten-time cross-validation for tuning parameter selection in the LASSO model. (D) LASSO coefficient profiles. (E) The risk score, survival status, and heat map of 18 immune genes in patients with BRCA.

Figure 1: Establishment of IRSS Signature.



(A) Kaplan-Meier curves show that OS was significantly different between the high- and low-risk groups in TCGA-BRCA. (B) The signature is shown by the time-dependent ROC curve for predicting 2, 3, and 5-year survival. (C) 2-, 3-, and 5-year nomogram for predicting OS of BRCA. (D) Decision curve analysis for the evaluation of the net benefits of IRSS (below) and nomogram (upper) at 2, 3, and 5 years.

Figure 2: Evaluation of IRSS signatures and establishment and evaluation of nomograms.

3.3. GO and KEGG Analysis of 18 targeted immune genes

To explore the potential association between gene expression and immunity in 18 targeted immune genes selected in figure 1, we performed GO and KEGG enrichment pathway analysis and the results showed that these genes were enriched in various immune-related pathways including chemokine production and T cell activation, especially regulating JAK-STAT signaling pathway (**Figure 3**). Furthermore, we found IFNG, SOCS3, TNFRSF18 and TSLP were the hub genes involved in JAK-STAT signaling pathway.

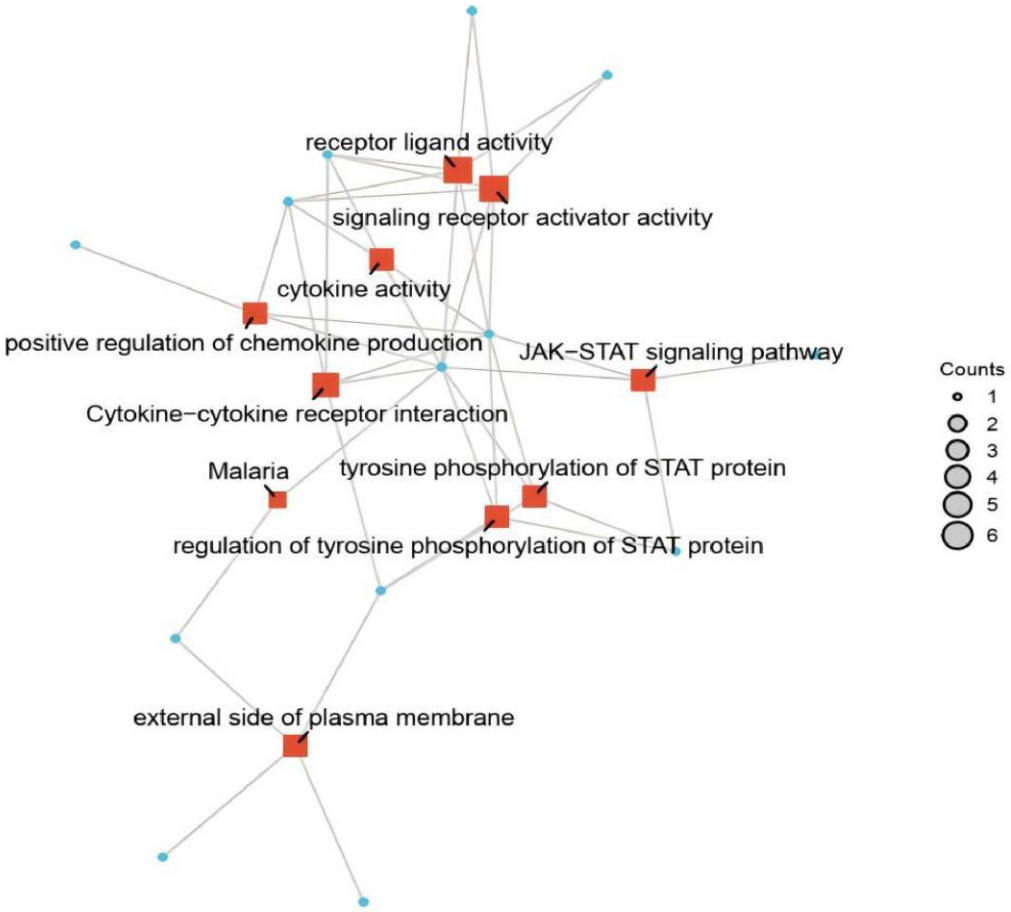
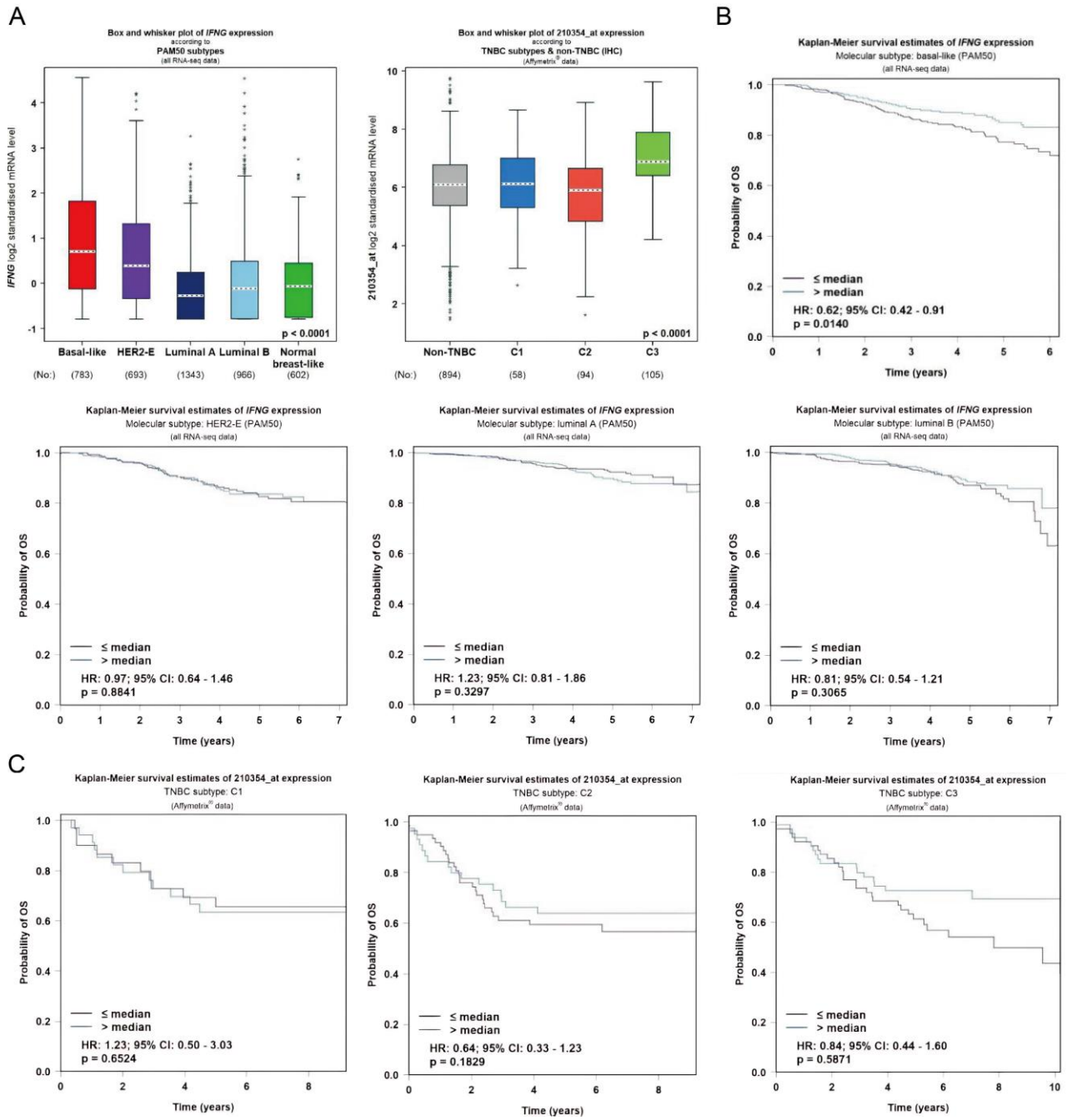


Figure 3: The enrichment analysis of 18 immune genes by GO and KEGG.

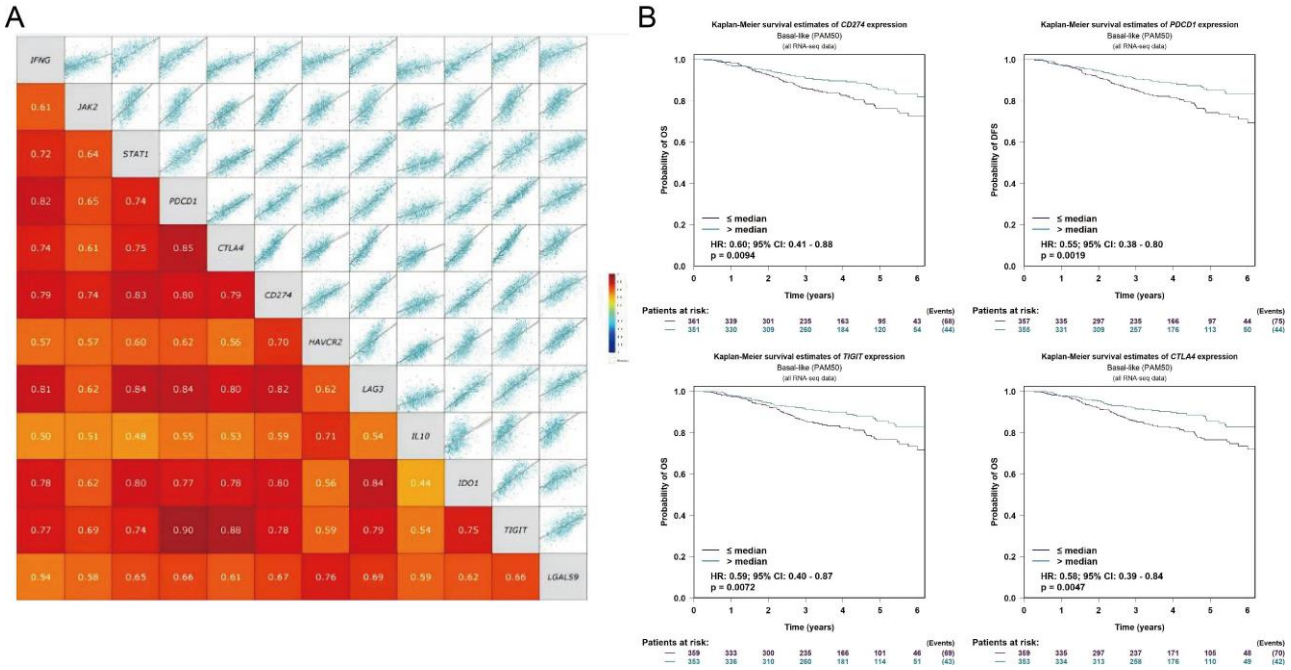
3.4. The clinical value of IFNG in triple negative breast cancer (TNBC)

The application of immune-checkpoint inhibitors (ICI) is promising to raise the survival rate of TNBC and thus the in-depth research on immune regulatory mechanisms will improve the effectiveness of ICI. We analyzed the level of IFNG, SOCS3, TNFRSF18 and TSLP in subtypes of BRCA and found pronounced IFNG expression in TNBC (**Figure 4A**) rather than SOCS3, TNFRSF18 and TSLP (**Supplemental Figure 2**). According to the Affymetrix™ data[12], TNBC was further split into three subtypes based on principal component analysis: C1 (luminal androgen receptor), C2 (basal-like tumours infiltrated by immune suppressive cells) and C3 (basal-like tumours associated with high tumour infiltrating lymphocytes and plasma cells, tertiary lymphoid structures and upregulation of immune checkpoints). It also showed that IFNG was markedly elevated in C3 TNBC (**Figure 4A**), suggesting that IFNG may facilitate immune cell infiltration and immune checkpoints in TNBC. Moreover, the survival analysis was performed in different pathological subtypes of BRCA (**Figure 4B**) and the C1-C3 subtypes (**Figure 4C**). The results showed that high level of IFNG may indicate better prognosis in BRCA especially in the basal-like subtype. However, no significance was seen between low and high IFNG groups in C1 to C3 subtype in the prognostic curve inspite of prolonged survival in the C3 subtype. Furthermore, it showed that IFNG had strong positive correlation with various immune checkpoints (**Figure 5A**), especially CD274, PD-1, CTLA-4 and TIGHT, which are statistically significant for prognosis in TNBC (**Figure 5B**).



(A) The expression of INFG in different BRCA subtypes (left) and TNBC subtypes (right). (B) The KM curves for IFNG in different BRCA subtypes. (C) The KM curves for IFNG in different TNBC subtypes.

Figure 4: The prognostic value of PFNG in different BRCA subtypes.



(A) The analysis of Breast Cancer Gene-Expression Miner analysis on the correlation of IFNG and various immune checkpoints in TNBC. (B) The KM curves for immune checkpoints in TNBC.

Figure 5: The correlation of IFNG and various immune checkpoints.

4. Discussion

Recent studies have indicated the potential role of IFNG in immunotherapy[13], however, the opposing effects of IFNG in terms of regulating immune function but also driving T cell exhaustion through PD-L1 makes it hard to be a prognostic marker[14-17]. Joseph L Benci et al.[18] reported that while inhibiting tumor IFNG signaling decreases interferon-stimulated genes (ISGs) in cancer cells, it increases ISGs in immune cells by enhancing IFNG produced by exhausted T cells (T_{ex}). It follows that in tumors with favorable antigenicity, these T_{ex} mediate rejection, and otherwise in tumors with neoantigen or MHC-I loss, T_{ex} instead utilize IFNG to drive maturation of innate immune cells. In this study, although we can't further elaborate the mechanism of IFNG in immune response, the big data analysis could tell that the high IFNG is a better prognostic factor of TNBC, which may become a biomarker of the immunomodulatory subtype.

In this study, we first established and validated an immune risk scoring signature for prognosis of all breast cancer and found 18 targeted immune genes, which are enriched in JAK-STAT signaling pathway. Among the 18 targeted genes, IFNG is closely related to JAK-STAT signaling pathway and is the only pronounced gene in TNBC compared with Luminal A/B and HER2 subypes. Furthermore, to our surprise, patients with high level of both IFNG and checkpoints (CD274, PD-1, CTLA-4 and TIGHT) would have longer survival and IFNG is positively relevant to various checkpoints, especially PD-1/PD-L1. This result is contrary to our knowledge that the high expression of checkpoints indicate more T cell exhaustion and stronger immunosuppression resulting in a short survival time.18 We also analyzed the relationship of IFNG and prognosis in pan-cancers (**Supplemental figure 3**). It showed that among 33 types of tumors, high IFNG may indicate increased risk in 6 tumors (THYM, LGG, UVM, KIRP, PAAD, KIRC) and decreased risk in 6 tumors (HNSC, BLCA, OV, CESC, BRCA, SKCM).

Taken together, our results indicate that based on the immune score prediction model, IFNG is associated with better prognosis in TNBC and may act as a biomarker guiding the ICB therapy. Further studies are needed to elucidate the underlying mechanism of IFNG in TNBC.

Acknowledgements

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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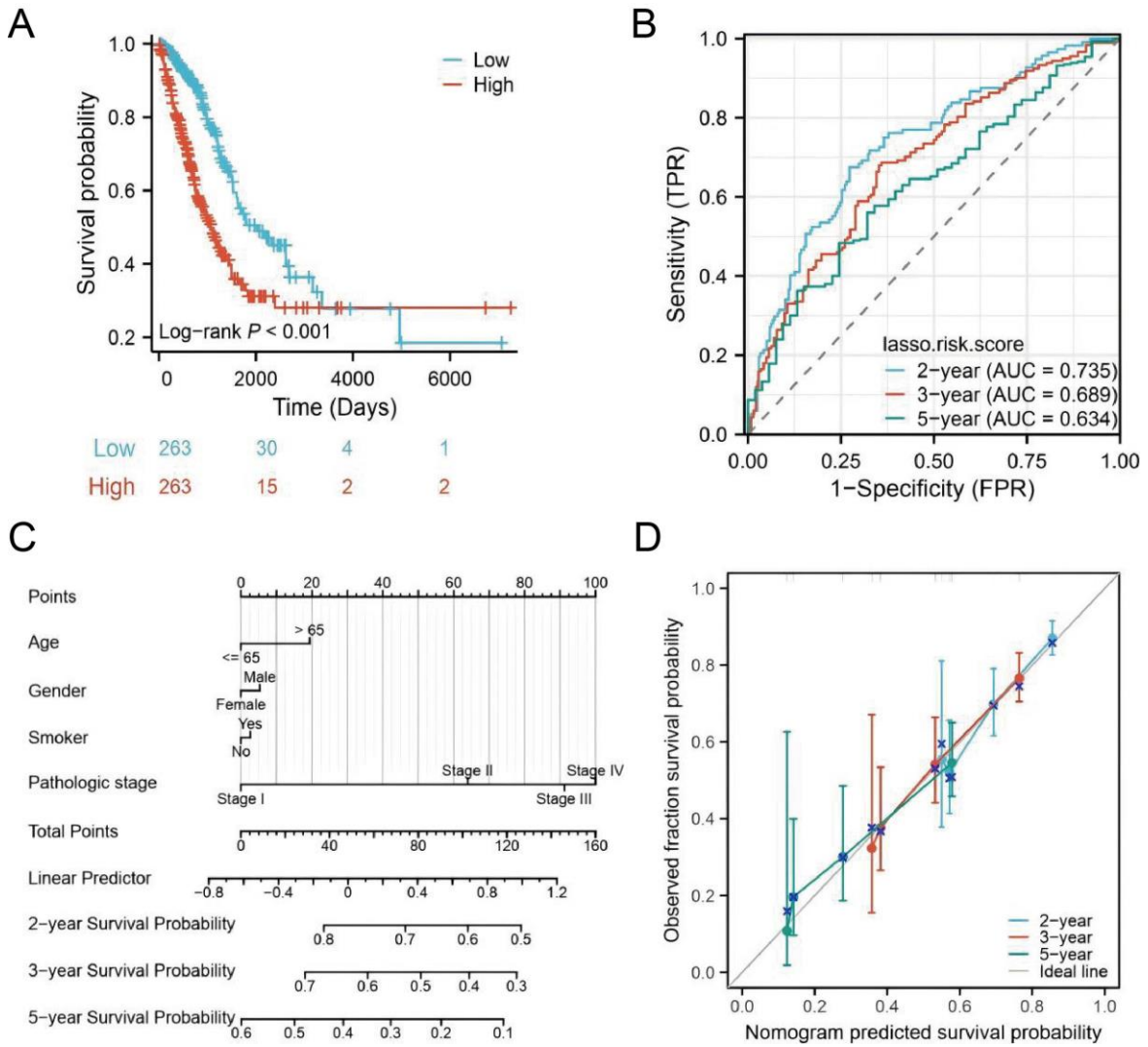
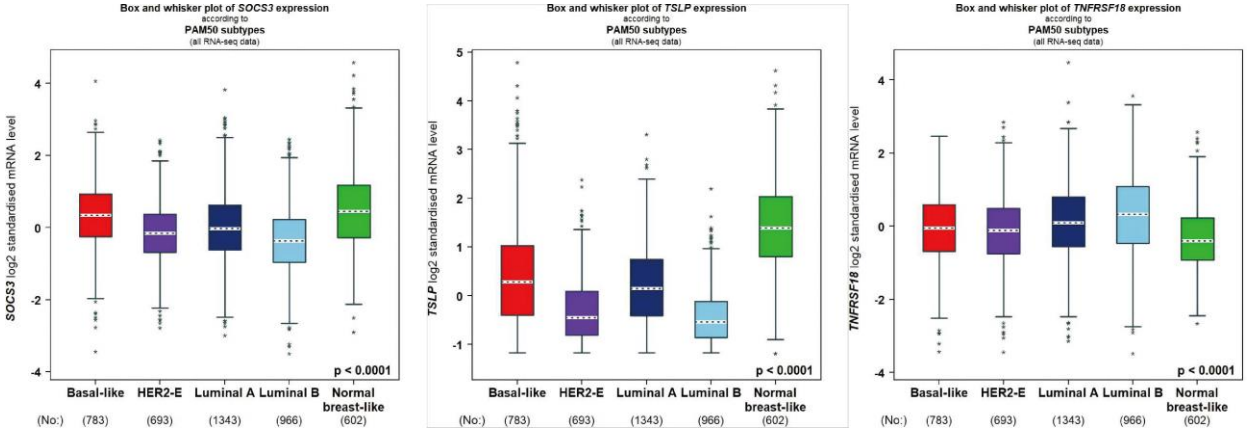
Appendix

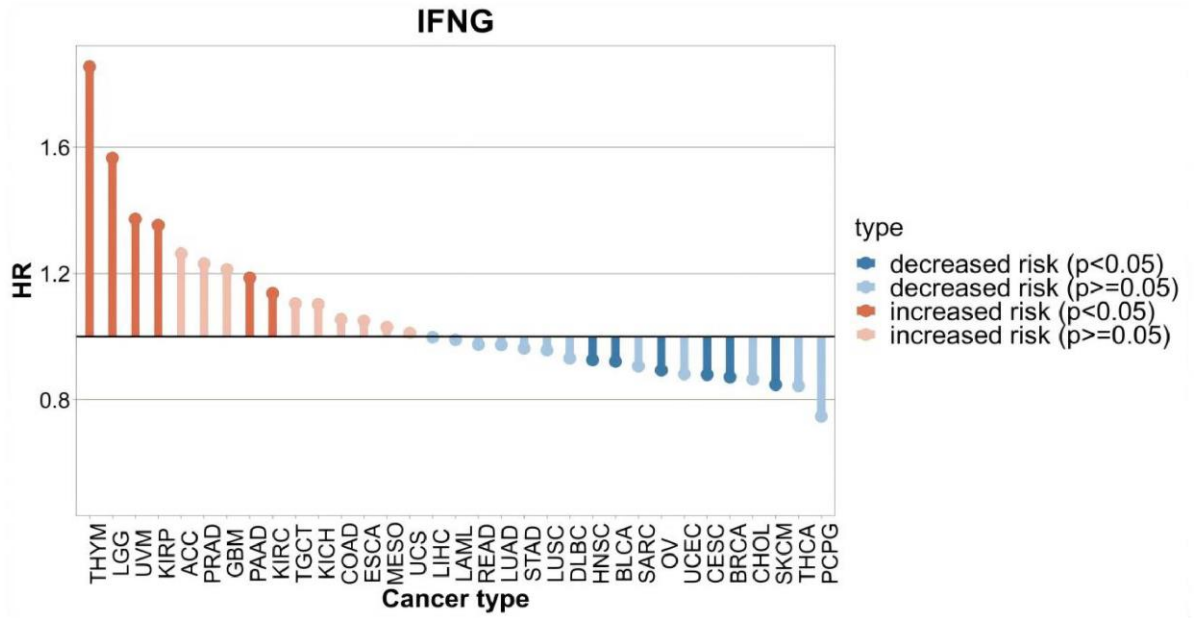


(A) Kaplan-Meier curves show that OS in the low-risk was significantly higher than in the high-risk group. (B) Time-dependent ROC curve analysis of the IRSS at 2, 3, and 5 years. (C) 2-, 3-, and 5-year nomogram for predicting OS of LUAD. (D) The Calibration curve of the nomogram for predicting OS rate at 2, 3, and 5 years.

Supplemental Figure 1: Validation of IRSS signature with TCGA-LUAD.



Supplemental Figure 2: The expression of SOCS3, TNFRSF18 and TSLP in different BRCA subtypes.



Supplemental Figure 3: The risk prediction of IFNG in pan-cancers by Tumor Immune Single-cell Hub.