


Establishment and Validation of a Prognostic Immune Signature in Neuroblastoma

Cancer Control
Volume 28: 1–14
© The Author(s) 2021
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: [10.1177/10732748211033751](https://doi.org/10.1177/10732748211033751)
journals.sagepub.com/home/ccx


Yunhu Yu^{1,2,#}, Yu Zeng^{3,#}, Xiangping Xia⁴, Jian-Guo Zhou⁵  and Fang Cao⁴ 

Abstract

Background: Neuroblastoma (NBL) is the most common extracranial solid tumor in childhood, and patients with high-risk neuroblastoma had a relatively poor prognosis despite multimodal treatment. To improve immunotherapy efficacy in neuroblastoma, systematic profiling of the immune landscape in neuroblastoma is an urgent need.

Methods: RNA-seq and according clinical information of neuroblastoma were downloaded from the TARGET database and GEO database (GSE62564). With an immune-related-gene set obtained from the ImmPort database, Immune-related Prognostic Gene Pairs for Neuroblastoma (IPGPN) for overall survival (OS) were established with the TARGET-NBL cohort and then verified with the GEO-NBL cohort. Immune cell infiltration analysis was subsequently performed. The integrated model was established with IPGPN and clinicopathological parameters. Immune cell infiltration was analyzed with the XCELL algorithm. Functional enrichment analysis was performed with clusterProfiler package in R.

Results: Immune-related Prognostic Gene Pairs for Neuroblastoma was successfully established with seven immune-related gene pairs (IGPs) involving 13 unique genes in the training cohort. In the training cohort, IPGPN successfully stratified neuroblastoma patients into a high and low immune-risk groups with different OS (HR=3.92, $P = 2 \times 10^{-8}$) and event-free survival (HR=3.66, $P=2 \times 10^{-8}$). ROC curve analysis confirmed its predictive power. Consistently, high IPGPN also predicted worse OS (HR=1.84, $P = .002$) and EFS in validation cohort (HR=1.38, $P = .06$). Moreover, higher activated dendritic cells, M1 macrophage, Th1 CD4⁺, and Th2 CD4⁺ T cell enrichment were evident in low immune-risk group. Further integrating IPGPN with age and stage demonstrated improved predictive performance than IPGPN alone.

Conclusion: Herein, we presented an immune landscape with IPGPN for prognosis prediction in neuroblastoma, which complements the present understanding of the immune signature in neuroblastoma.

Keywords

neuroblastoma, Immune-related prognostic gene pairs for neuroblastoma, immune-related gene pairs, immune cell infiltration, overall survival, event-free survival

Introduction

Neuroblastoma (NBL) is the most common extracranial solid tumor in childhood, originating from neurons of the sympathetic nervous system,¹ and accounting for 8–10% of pediatric cancers in the USA and Europe.^{2,3} According to age at diagnosis, the extent of disease, tumor subtype, and disease course can vary from spontaneous regression to implacable progression even with intensive multimodality therapy.^{1,4} With precise stratification generally accepted, neuroblastomas can be divided into three major subtypes, subtypes 1, 2A, and 2B, based on genetic features.⁴ While subtype 1 neuroblastoma is associated with favorable features such as young age and lower tumor stage, subtypes 2A and 2B are associated

¹Department of Neurosurgery, the Third Affiliated Hospital of Zunyi Medical University, Zunyi, China

²Clinical Research Center for Neurological Disease, the People's Hospital of HongHuaGang District of ZunYi, Zunyi, China

³Department of Cell Biology, School of Basic Medical Science, Southern Medical University, Guangzhou, China

⁴Department of Cerebrovascular Disease, Affiliated Hospital of Zunyi Medical University, Zunyi, China

⁵Department of Oncology, Second Affiliated Hospital of Zunyi Medical University, Zunyi, China

#Authors contributed equally to this work (Yunhu Yu and Yu Zeng).

Corresponding Author:

Fang Cao, Department of Cerebrovascular disease, Affiliated Hospital of Zunyi Medical University, No.149, Dalian Road, Zunyi 563000, China.
Email: Caof@zmu.edu.cn



Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (<https://creativecommons.org/licenses/by-nc/4.0/>) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE

and Open Access pages (<https://us.sagepub.com/en-us/nam/open-access-at-sage>).

with advanced tumor stage and worse prognosis, with subtype 2B being the most aggressive type.⁵ However, despite the individualized therapeutic strategy brought by a well-defined subset, metastasis occurred in nearly half of the neuroblastoma patients. The five-year event-free survival (EFS) in high-risk neuroblastoma patients remains less than 50%.⁶

Dysregulated tumor immune microenvironment is a critical hallmark of neuroblastoma. The tumor microenvironment is a rather complex composition of surrounding blood vessels, fibroblasts, immune cells, signal molecules, and extracellular matrix,⁷ and interaction between tumor cells and these microenvironment components could significantly modulate the malignant phenotype of tumor cells, including proliferation, cancer cell stemness, and metastasis potential.⁸⁻¹⁰ In contrast, tumor cells could also shape the surrounding microenvironment to form a more suitable tumor niche through angiogenesis promotion.¹¹ Given immune signatures in neuroblastoma, numerous immune molecules, including NF- κ B,¹² tumor necrosis factor (TNF),¹³ and interleukins,¹⁴ have been reported to be associated with tumorigenesis, apoptosis, and chemoresistance in neuroblastoma. Moreover, the mutual interaction between neuroblastoma cells and immune cells, such as tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs), may further contribute to tumor proliferation, metastasis, and adaptive chemoresistance.^{15,16} It has come to the researchers that neuroblastoma could exhibit either immunogenic or non-immunogenic type,¹⁷ but there is still a lack of ample evidence considering the immune landscape in neuroblastoma.

Our team has previously established a prognostic model in neuroblastoma with TARGET-NBL cohort and GEO-NBL cohort (GSE62564),¹⁸ but the immune landscape of neuroblastoma remained unraveled. Herein, in the present study, we established and validated a prognostic immune-signature in neuroblastoma based on RNA sequencing data of totally 644 cases from these two NBL cohorts, which exhibited satisfactory stratification for both overall survival (OS) and event-free survival (EFS). Compared with the prognostic NBL signature reported previously,¹⁸ though the same participant cohorts were used, our prognostic model was established with a different gene set and provided a more specific and comprehensive view focused on the NBL immune landscape, which is not elaborated in the previous publications.

Materials and Methods

RNA-Seq and Clinical Data Curation

The overall workflow was shown in Figure 1. Neuroblastoma RNA-seq data and corresponding clinical data were downloaded from the TARGET database (<https://ocg.cancer.gov/programs/target>) and GEO database (<https://www.ncbi.nlm.nih.gov/geo>, GSE62564).¹⁹ The TARGET neuroblastoma cohort was set as a training cohort (n=152), while the GEO neuroblastoma cohort was selected as a validation cohort (n=492). No significant bias was found between the TARGET training cohort and the GEO validation cohort.

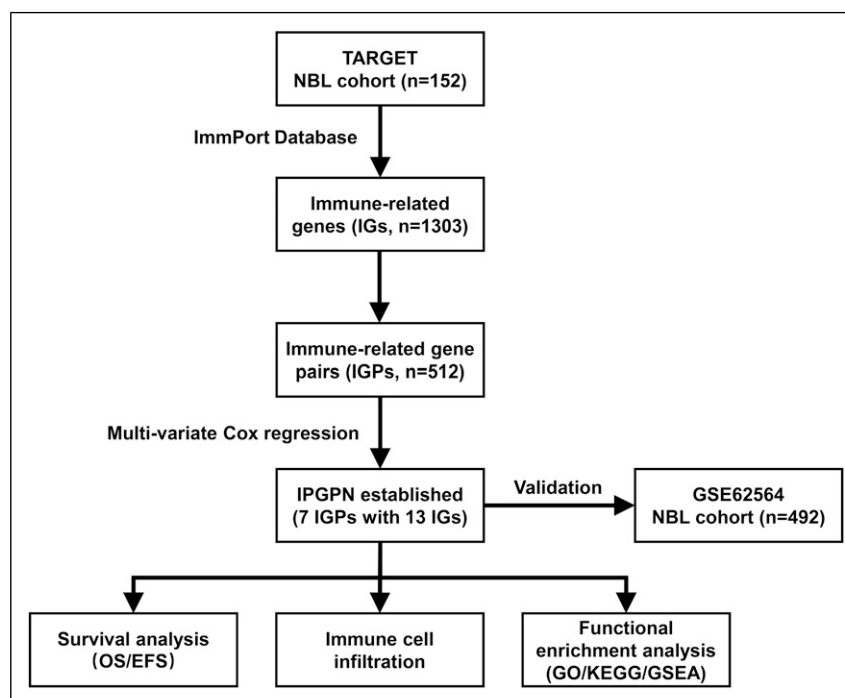


Figure 1. The overall workflow of the study.

Identification of Immune-Related Gene Pairs (IGPs)

Identification of prognostic IGPs was performed as described previously.²⁰ A total of 1811 immune-related genes (IGs) were retrieved from ImmPort database,²¹ all measured in the TARGET and GEO cohort. IGs with relatively high variation, as determined by median absolute deviation (MAD) more than .5, were selected. Each IGP was calculated by pairwise comparison of each sample's gene expression level. Precisely, during a pairwise comparison, the output is 1 if the first gene expression level is higher than the later one, and 0 for other different orders. After removing IGPs with constant ordering, 512 IGPs remained and were pooled into further prognosis prediction to establish IPGPN.

Establishment and Validation of Immune-Related Prognostic Gene Pairs for Neuroblastoma (IPGPN)

The establishment of a prognostic signature based on IGPs was performed as described previously.²⁰ With the remaining IGPs, an immune signature IPGPN was established with the least absolute shrinkage and selection operator (LASSO) regression. To stratify patients into high/low-risk groups, the optimal cut-off value of the IPGPN was determined with time-dependent receiver operating characteristic (ROC) curve analysis,²² at 5 years in the TARGET training cohort for overall survival. The prognostic prediction value for the overall survival of IPGPN was validated in the GEO cohort using the log-rank test. The sensitivity and specificity of IPGPN were also determined using ROC curve analysis.

Immune Cell Infiltration Analysis

To profile immune cell infiltration in different risk groups, Immunedeconv (2.0.3) package in R was used to characterize the immune-cells composition based on tumor gene expression profiles.

Functional Enrichment Analysis

To perform functional enrichment analysis, including KEGG pathway analysis and GSEA, differentially expressed genes (DEGs) were first confirmed with limma package in R and then analyzed with clusterProfiler package in R.

Statistical Analysis

Continuous variables were presented as mean \pm SD, and categorized variables were presented as frequency (n) and proportion (%). The prognostic value for OS and EFS was evaluated by the multivariate Cox regression model in the survival analysis. Kaplan–Meier survival analysis was performed to compare different subgroups. Single, double, and triple asterisks indicate statistical significance, * $P < .05$, ** $P < .01$, and *** $P < .001$, respectively.

Results

Identification and Definition of the IPGPN

A total of 152 neuroblastoma patients from the TARGET database and 492 neuroblastoma patients from GSE62564 were involved in the training cohort and validation cohort, respectively (Table 1). A total of 1303 immune-related genes (IGs) were measured in both training and validation sets. Afterward, 456 236 IGPs with MAD >0 were established based on these IGs. After removing IGPs with constant ordering, 512 IGPs remained and were selected for IPGPN calculation. Through LASSO regression for OS, 7 IGPs with 13 unique IGs were finally pooled into the final model of IPGPN (Table 2).

Table 1. Clinical and Pathological Features of Patients in TARGET-NBL and GSE62564.

	TARGET-NBL (N = 153)	GSE62564 (N = 498)	Total (N = 651)
Gender			
Male	89 (58.17%)	287 (57.63%)	376 (57.76%)
Female	64 (41.83%)	211 (42.37%)	275 (42.24%)
Age at diagnosis in days			
≥ 18 months	124 (81.05%)	193 (38.76%)	317 (48.69%)
<18 months	29 (18.95%)	305 (61.24%)	334 (51.31)
MYCN status			
Amplified	33 (21.57%)	92 (18.47%)	125 (19.20%)
Not amplified	119 (77.78%)	401 (80.52%)	520 (79.88%)
Unknown	1 (.65%)	5 (1.01%)	6 (.92)
Stage			
4	126 (82.35%)	183 (36.75%)	309 (47.47%)
1, 2, 3, 4S	27 (17.65%)	314 (63.05%)	341 (52.38%)
Unknown	—	1 (.20%)	1 (.15)

Table 2. Details of 7 Gene Pairs.

Gene Pair	Gene 1	Full Name	Gene 2	Full Name	Coefficient
ENSG00000163273. ENSG00000169248	NPPC	Natriuretic peptide C	CXCL11	C-X-C motif chemokine ligand 11	.0926
ENSG00000186951. ENSG00000125257	PPARA	Peroxisome proliferator activated receptor alpha	ABCC4	ATP binding cassette subfamily C member 4	-.1456
ENSG00000215788. ENSG00000082684	TNFRSF25	TNF receptor superfamily member 25	SEMA5B	Semaphorin 5B	-.5047
ENSG00000239697. ENSG00000134259	TNFSF12	TNF superfamily member 12	NGF	Nerve growth factor	-.0447
ENSG00000132274. ENSG00000176919	TRIM22	Tripartite motif containing 22	C8G	Complement C8 gamma chain	-.5695
ENSG00000132274. ENSG00000164761	TRIM22	Tripartite motif containing 22	TNFRSF11B	TNF receptor superfamily member 11b	-1.6212
ENSG00000144802. ENSG00000115598	NFKBIZ	NFKB inhibitor zeta	IL1RL2	Interleukin 1 receptor like 2	-.5160

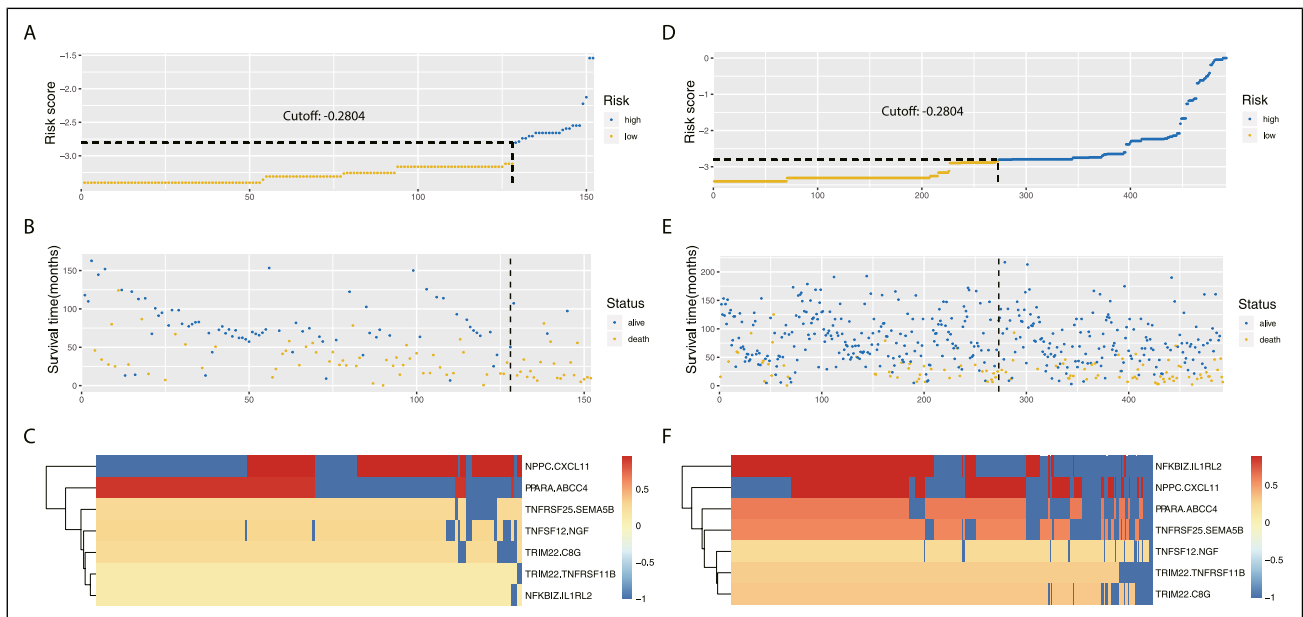


Figure 2. Establishment of IPGPN based on Immune-related Prognostic Gene Pairs. (A-B) The risk score considering IPGPN of each NBL patient in the training and validation cohort, and the cut-off value between high- and low-risk groups. (C-D) Survival status of each NBL patient in the training and validation cohort. (E-F) Heatmap of the seven immune-related gene pairs in the training and validation cohort. Abbreviations: IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma; NBL, neuroblastoma.

IPGPN Exhibited Satisfactory Performance on OS and EFS Prediction

For OS prediction, the optimal cut-off of the IPGPN was set as -2.084 , determined by time-dependent ROC curve analysis (Figure 2). Of note, IPGPN successfully stratifies neuroblastoma patients in the TARGET training cohort into low and high immune risk groups with diametrically different OS in the training set (Figure 3A), in which high IPGPN score predicted an unfavorable prognosis (HR=3.92, 95%CI=2.35-6.53; $P = 2 \times 10^{-8}$). The median OS was 124 (78.4 months to not reach (NR)) and 16 months (11.3 months

to 34 months) in the low-risk group and high-risk group, respectively.

We also interrogate the predictive value of IPGPN on EFS. Intriguingly, the TARGET-NBL cohort was similarly divided into two distinct groups in terms of EFS, and a low IPGPN score is correlated with a better EFS (Figure 4A; HR=3.66, 95%CI 2.25-5.95; $P = 2 \times 10^{-8}$). The median EFS is 35.34 and 9.62 months in the low-risk and high-risk groups, respectively. Subsequently, time-dependent ROC curve analysis was performed to examine both OS and EFS prediction's sensitivity and specificity with IPGPN in the TARGET-NBL training cohort. ROC curve analysis revealed

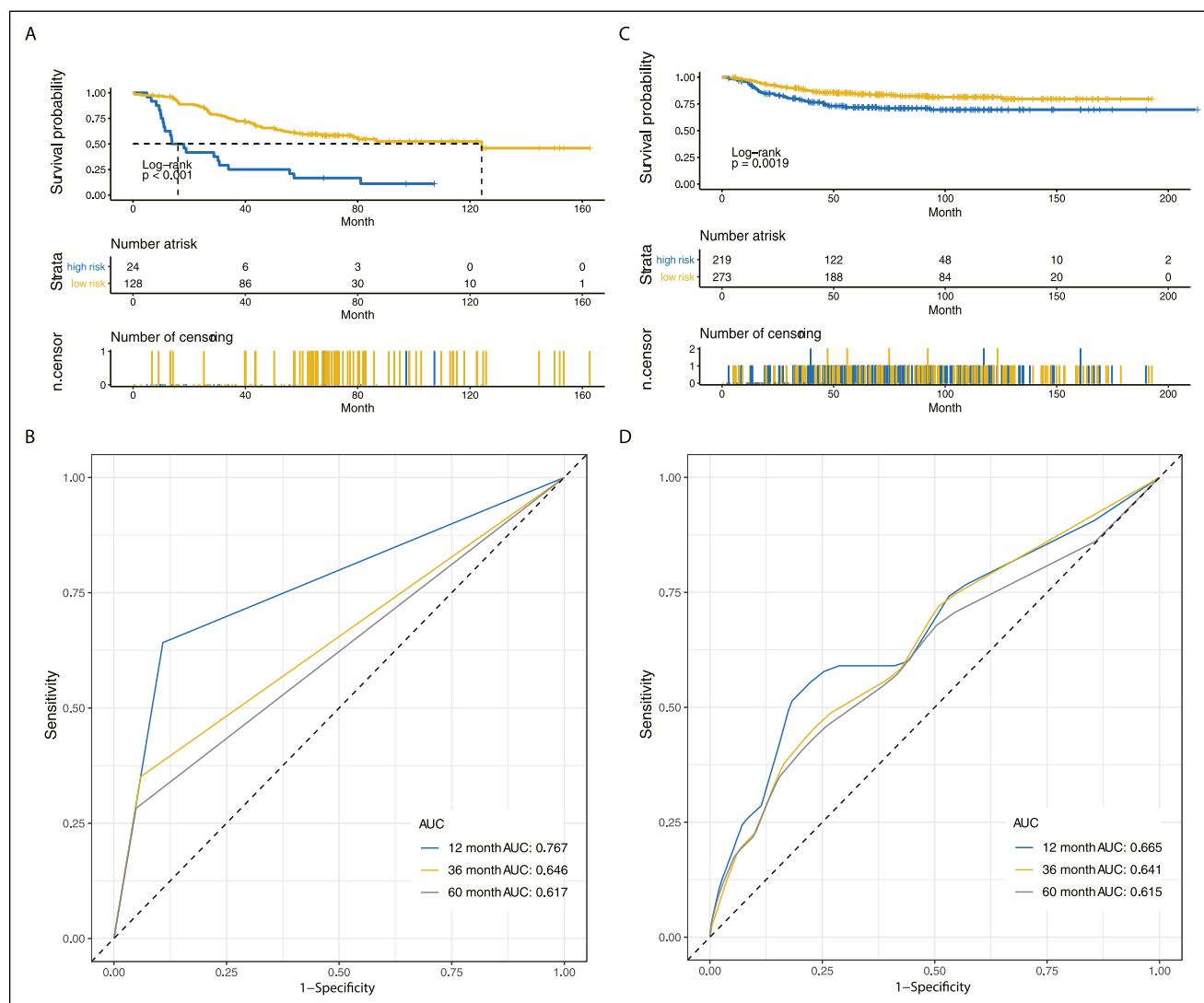


Figure 3. IPGPN is prognostic for OS in NBL patients. (A) OS analysis of NBL patients in the low- and high-risk group in the training cohort. (B) ROC curve demonstrating the prognostic value of IPGPN in predicting OS in the training cohort. (C) OS analysis of NBL patients in the low- and high-risk group in the validation cohort. (D) ROC curve demonstrating the prognostic value of IPGPN in predicting OS in the validation cohort. Abbreviations: IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma; NBL, neuroblastoma; ROC, receiver operating characteristic.

that IPGPN exhibited the best performance for 12-month survival prediction with an AUC value of .82, while IPGPN reached a 36-month AUC value of .68 and a 60-month AUC value of .72 (Figure 3B). A similar result was obtained in terms of EFS (AUC of 12 months is .69, Figure 4B), indicating that IPGPN had satisfactory predictive value for both OS and EFS.

Validation of IPGPN

To further interrogate the predictive value of IPGPN in other populations, we then applied the same formula to the GEO-NBL cohort (GSE62564). Consistently, for OS, IPGPN significantly divided GEO-NBL patients into a low and high-risk group, in which a high IPGPN score predicted an extended OS (Figure 3C;

HR=1.84, 95%CI=1.24-2.71; $P = .002$). In ROC curve analysis, IPGPN had a better AUC for 12-month OS (AUC=.67) compared with those for 36-month (AUC=.64) and 60-month (AUC = .62) (Figure 3D). Considering EFS, the IPGPN also exhibited satisfied stratification in the validation set, while the high immune risk was correlated with an unfavorable EFS (HR=1.52, 95%CI= 1.13-2.03; $P = .005$) (Figure 4C and 4D). Collectively, our results demonstrated that IPGPN was a robust predictor for both OS and EFS across different neuroblastoma populations.

Immune Cell Infiltration Analysis Based on IPGPN

Emerging evidence links increased immune cell infiltration in the tumor microenvironment with tumorigenesis and metastasis.

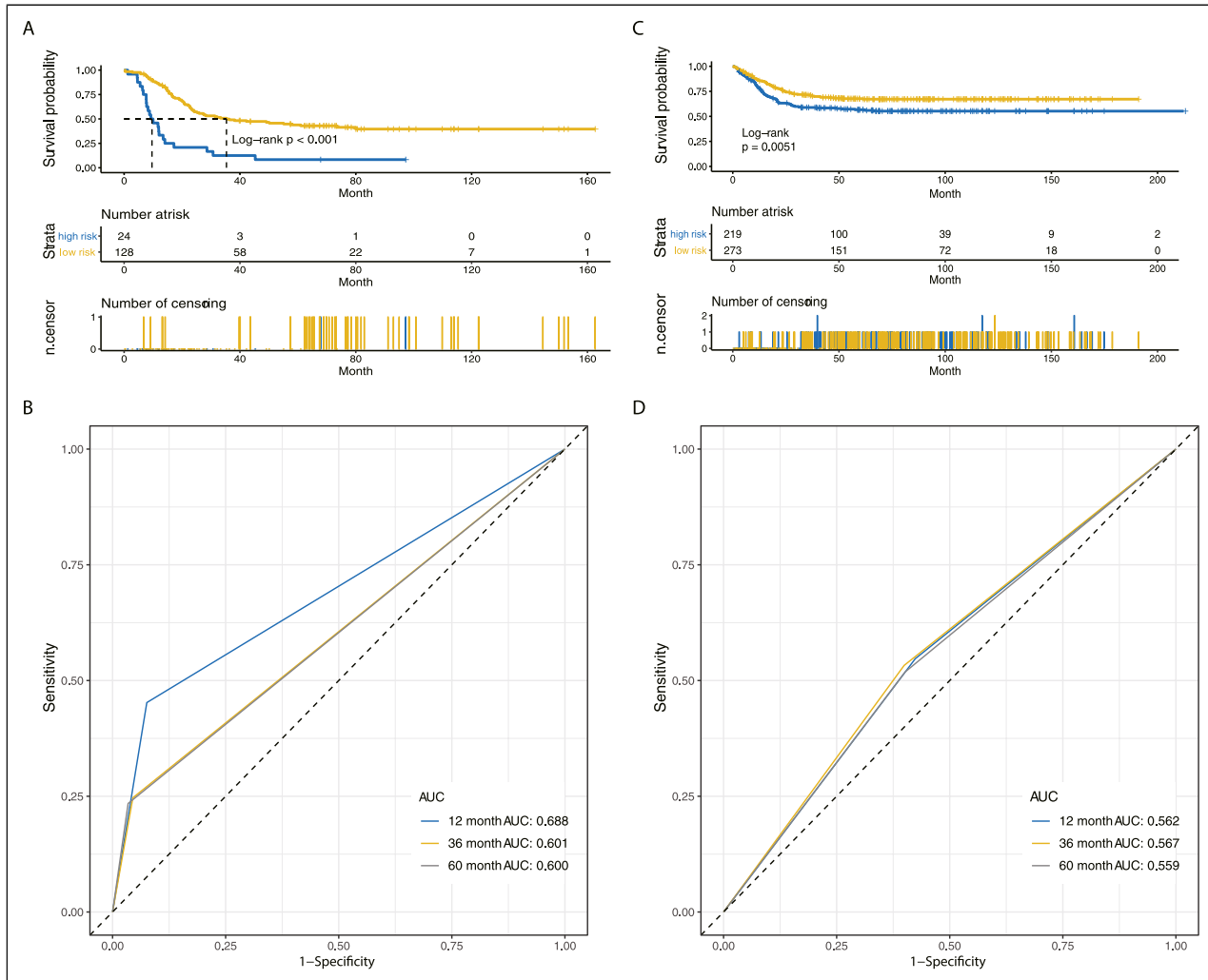


Figure 4. IPGPN is prognostic for EFS in NBL patients. (A) EFS analysis of NBL patients in the low- and high-risk group in the training cohort. (B) ROC curve demonstrating the prognostic value of IPGPN in predicting EFS in the training cohort. (C) EFS analysis of NBL patients in the low- and high-risk group in the validation cohort. (D) ROC curve demonstrating the prognostic value of IPGPN in predicting EFS in the validation cohort. Abbreviations: EFS, event-free survival; IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma; NBL, neuroblastoma; ROC, receiver operating characteristic.

To further profile the infiltration of different immune cells, including macrophages, B cells, and T cells, the XCELL algorithm was applied in both training and validation sets. As shown in Figure 5, activated dendritic cells ($P=7.27 \times 10^{-4}$ and 3.51×10^{-7} in the training and validation cohort, respectively) and M1 macrophage ($P=6.83 \times 10^{-6}$ and 1.61×10^{-9} in the training and validation cohort, respectively) were enriched in the low immune risk group, while Th1 CD4⁺ (no significant difference was observed in the training cohort while $P=4.51 \times 10^{-15}$ in the validation cohort) and Th2 CD4⁺ T cells ($P=.01$ and 5.85×10^{-12} in the training and validation cohort, respectively) were enriched in the high immune risk group, indicating a more active immune response predicted a favorable prognosis.

Integrated Prognostic Signature Combining IPGPN and Clinicopathological Characteristics

To further confirm whether IPGPN could serve as an independent prognostic factor for OS in NBL and potential supplement of clinicopathological characteristics, both uni- and multivariate Cox regression analyses were employed in the training cohort and validation cohort, respectively. In univariate analysis, OS was correlated with age, stage, and IPGPN in the training cohort and was correlated with age, stage, and MYCN in the validation cohort; multivariate analysis confirmed that IPGPN was an independent prognosis predictor in the training cohort, whereas age, stage, and MYCN and IPGPN independently predicted prognosis in the

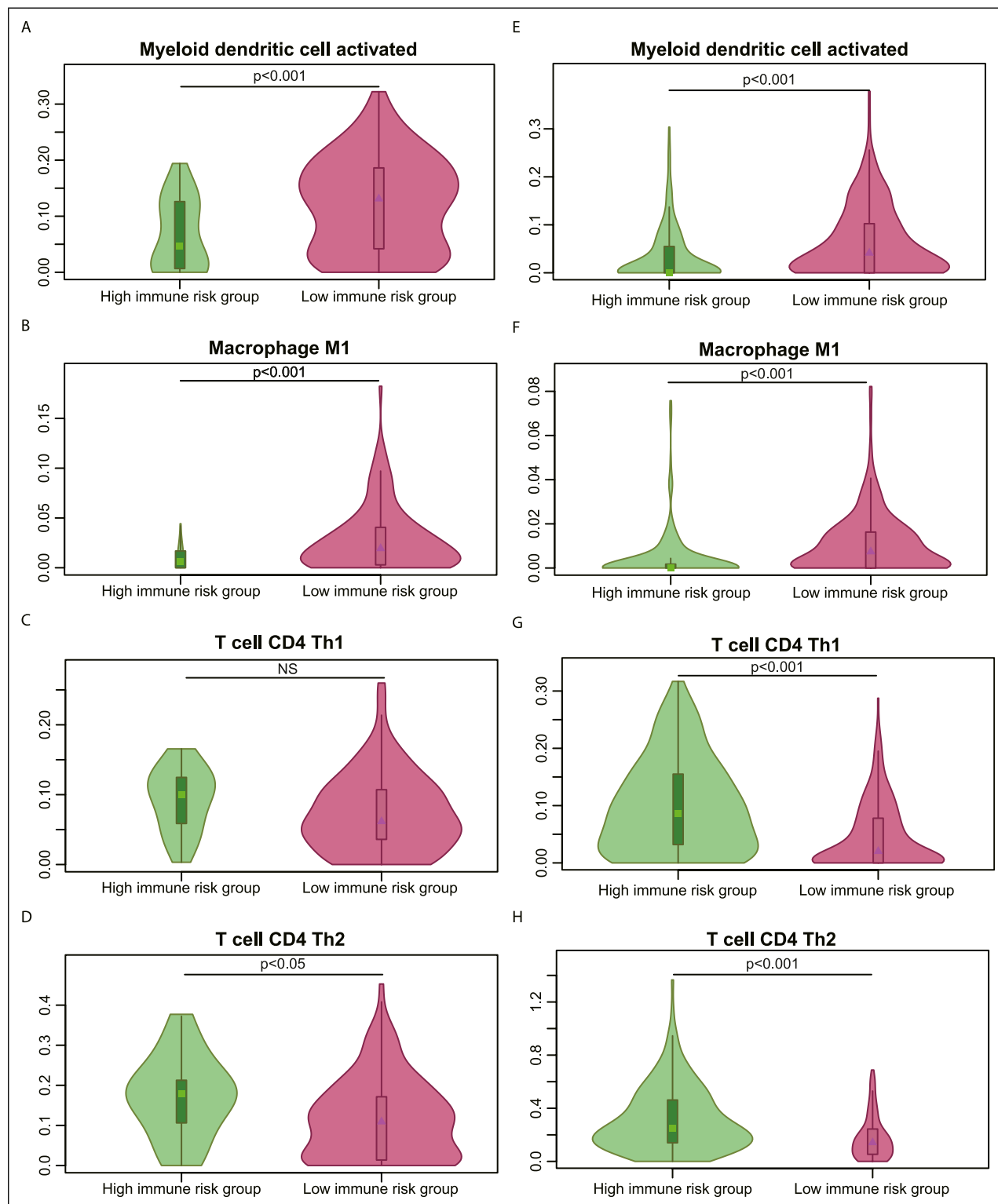


Figure 5. Immune cell infiltration analysis based on Immune-related Prognostic Gene Pairs for Neuroblastoma. (A-D) In the training cohort, enrichment of myeloid dendritic cells and M1 macrophages was observed in the low immune risk group, while enrichment of Th1 and Th2 subsets of CD4⁺ T cells was observed in the high immune risk group. (E-H) In the validation cohort, enrichment of myeloid dendritic cells and M1 macrophages was observed in the low immune risk group, while enrichment of Th1 and Th2 subsets of CD4⁺ T cells was observed in the high immune risk group.

Table 3. Univariate and Multivariate Cox Regression Analyses of Clinicopathological Factors in OS.

Datasets	Variables	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	P	HR (95% CI)	P
TARGET-NBL	Gender	1.05 (.67 ~ 1.67)	.822	1.43 (.89 ~ 2.32)	.141
	Age	4.04 (1.63 ~ 10.04)	.003	1.46 (.46 ~ 4.60)	.516
	Stage	6.46 (2.03 ~ 20.52)	.002	6.42 (1.56 ~ 26.40)	.01
	MYCN	1.39 (.82 ~ 2.36)	.226	1.34 (.78 ~ 2.32)	.289
	IPGPN	3.92 (2.35 ~ 6.53)	< .001	5.82 (3.33 ~ 10.16)	< .001
GSE62564	Gender	.82 (.55 ~ 1.20)	.299	.75 (.50 ~ 1.10)	.145
	Age	8.58 (5.26 ~ 14)	< .001	3.56 (2.05 ~ 6.17)	< .001
	Stage	8.54 (5.36 ~ 13.6)	< .001	3.36 (1.98 ~ 5.70)	< .001
	MYCN	7.78 (5.26 ~ 11.53)	< .001	3.33 (2.19 ~ 5.07)	< .001
	IPGPN	1.84 (1.24 ~ 2.71)	.002	1.13 (.76 ~ 1.70)	.538

Abbreviations: IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma.

validation cohort (Table 3). To further examine whether including clinicopathological parameters could improve the predictive performance of IPGPN, we then combined age, stage, MYCN, and IPGPN to establish an integrated model in the TARGET-NBL training cohort (integrated IPGPN = age*0.5578 + stage*1.6866 + IPGPN*1.6337), with the median score set as the optimal cut-off value, and then validate it in the GEO-NBL validation cohort. The integrated model achieved a satisfactory differentiation in terms of OS (training cohort: HR = 5.99, 95%CI = 3.53-10.16, $P < .0001$; validation cohort: HR = 12.26, 95%CI = 6.97-21.56, $P < .0001$; Figures 6A and 6C) and EFS (training cohort: HR = 4.92, 95%CI = 2.94-8.23, $P < .0001$; validation cohort: HR = 3.85, 95%CI = 2.80-5.28, $P < .0001$; Figures 7A and 7C) in both training and validation cohorts. Though integrated model failed to improve the predictive performance in training cohort considering 12 month-AUC value (OS: .741 vs .819, Figure 6B; EFS: .667 vs .688, Figure 7B) compared to IPGPN alone, it achieved significant improvements of prognosis prediction for both OS (12-month AUC, .703 vs .665; 36-month AUC, .775 vs .641; 60-month AUC, .791 vs .615; Figure 6D) and EFS (12-month AUC, .598 vs .562; 36-month AUC, .710 vs .567; 60-month AUC, .709 vs .559; Figure 7D) in the validation cohort benefited from a larger sample size. Furthermore, the nomogram including age, stage, and IPGPN was constructed to predict the OS and EFS patients in the training cohort (Figures 8A and 8C). Moreover, the calibration plot showed that the 1-year OS/EFS nomogram of the training cohort was very close to the ideal curve (Figures 8B and 8D), indicating good consistency between the prediction of the nomogram and the actual observed outcomes in the training cohort.

Functional Enrichment Analysis

A total of 10 009 DEGs were confirmed between the low and high immune-risk groups in the TARGET-training cohort. Functional enrichment analysis was then performed between

low and high immune risk groups in the TARGET-training cohort. GSEA demonstrated that gene sets of NK cells mediated cytotoxicity and Th1/Th2 CD4⁺ cells were significantly activated in the low immune risk group, indicating the critical roles of NK cells and CD4⁺ cells in neuroblastoma (Figure 8A and 8B). Enrichment of other cancer-immunity-related gene sets, such as antigen processing and lysosome, was also observed (Supplementary Table 1).

Discussions

There is emerging attention on tumor-associated immune cells and molecular events governing immune responses in NBL. Frequent spontaneous regression mediated by cellular immunity was observed in children less than 18 months.²³ Notably, neuroblastoma is the first pediatric tumor for which immunotherapy (dinutuximab, a chimeric anti-GD2 monoclonal antibody) was approved. Still, the cure rates remain less than 50%, and many patients failed to achieve a minimal residual disease state.²⁴ Therefore, systematic profiling of NBL immune signature is warranted to display the immune landscape in NBL and guide the following immunotherapy. This study established IPGPN, a prognostic immune signature for neuroblastoma, through the specific pairwise correlation between different immune-related genes' expression. With the TARGET-NBL cohort, we finally selected 7 IGP with 13 IGs to establish IPGPN, which successfully stratified NBL patients into high and low immune risk groups with distinctively different survival outcomes. Subsequently, the predictive value of IPGPN for both OS and EFS in neuroblastoma was verified in the GEO-NBL cohort. Moreover, immune cell infiltration analysis demonstrated that the low immune risk group might possess a more active immune response. Furthermore, an integrated model was established by including age, stage, and MYCN to IPGPN, which exhibited better predictive power than IPGPN alone (Figure 9).

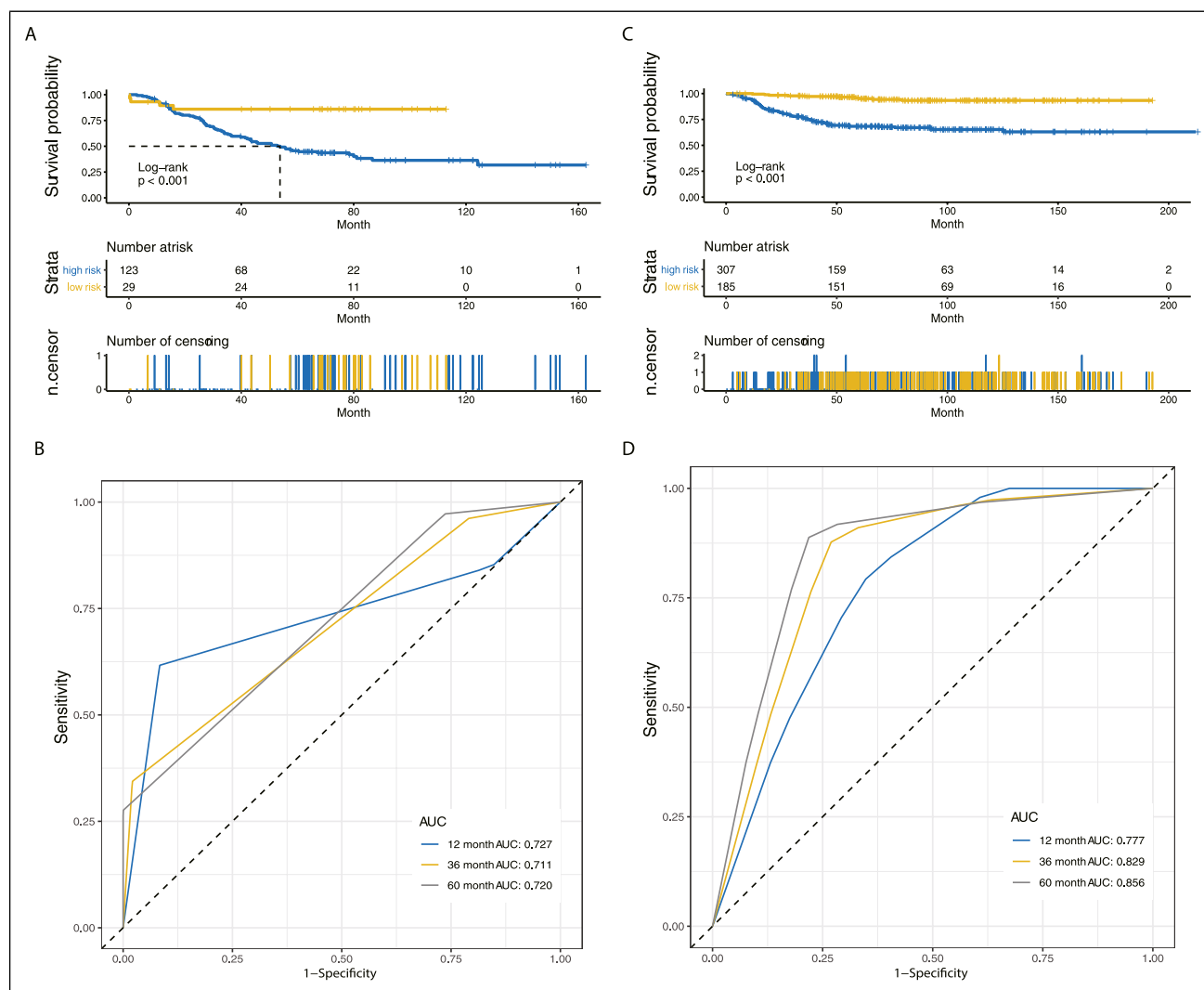


Figure 6. Integrated prognostic signature combining IPGPN and clinicopathological characteristics is prognostic for OS in NBL patients. (A-B) OS analysis of NBL patients in the high- and low-risk group considering integrated prognostic signature in the training and validation cohort. (C-D) ROC curve demonstrating the prognostic value of integrated prognostic signature in predicting OS in the training and validation cohort. Abbreviations: IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma; NBL, neuroblastoma; ROC, receiver operating characteristic.

Expression of immune-related genes could be a reliable predictor in neuroblastoma. It was found that 14% of neuroblastoma expressed programmed death-ligand 1 (PD-L1), and these patients were more prone to unfavorable prognosis than those PD-L1 negative ones.²⁵ Complementary to the current understanding of tumor immune in neuroblastoma, IPGPN was established with seven gene pairs involving 13 immune-related genes. Compared to a single-gene signature, a gene signature built on the pairwise correlation of gene expression is more robust and accountable across different datasets.²⁶ A handful of these genes are involved in the immune response under specific pathological conditions. For example, mutation of NFKBIZ was found to be associated with downregulation of pro-inflammatory factor and against colorectal carcinogenesis.²⁷ As a tripartite motif-containing

(TRIM) superfamily member, TRIM22 plays a vital role in immune response facing viral infection by modifying monocyte functions.^{28,29} Fewer of them have been discussed by activated NK cells, has already been reported to mediate the cytotoxicity of anti-GD2 antibody in neuroblastoma.³⁰ Other genes, including ABCC4,³¹ TNFRSF25,³² and NGF³³ are found to be dysregulated in neuroblastoma, yet future study into their immune response role is needed.

Interaction between immune-related genes and immune cells also attracted attention from researchers. On the one hand, several immune cells were found to be oncogenic in neuroblastoma. A subset of neuroblastoma cell lines was found to express membrane-bound TNF- α , which further induced TAMs to release CCL20.³⁴ Macrophages were prone

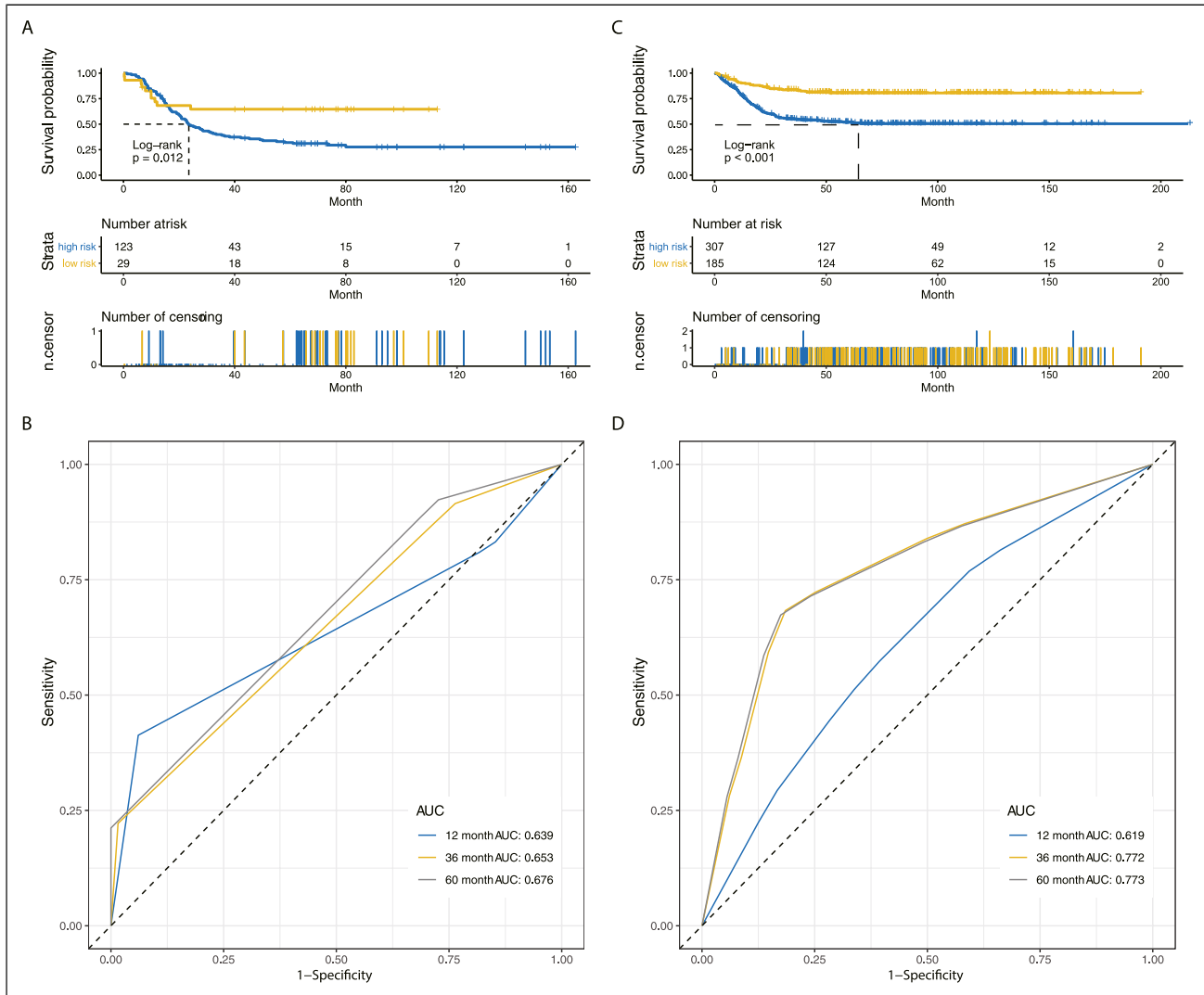


Figure 7. Integrated prognostic signature combining IPGPN and clinicopathological characteristics is prognostic for EFS in NBL patients. (A-B) EFS analysis of NBL patients in the high- and low-risk group considering integrated prognostic signature in the training and validation cohort. (C-D) ROC curve demonstrating the prognostic value of integrated prognostic signature in predicting EFS in the training and validation cohort. Abbreviations: EFS, event-free survival; IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma; NBL, neuroblastoma; ROC; receiver operating characteristic.

to be identified in PD-L1 positive neuroblastoma, associated with poor prognosis.²⁵ In particular, M2 macrophage infiltration was correlated with both the extent of disease and event-free survival in patients with MYCN-non-amplified neuroblastoma.³⁵ Depletion of TAMs by blockade of colony-stimulating factor 1 receptor (CSF-1R) could sensitize neuroblastoma cells to cyclophosphamide and topotecan regimen.³⁶ Likewise, the lack of regulatory T cells, which are immune suppressive, improved the efficacy of anti-GD2 antibody treatment.³⁷

On the other hand, activation of a specific type of immune cell could synergize immunotherapy and was associated with a favorable outcome. Increased signatures of activated NK cells and CD8⁺ T cells were associated with a favorable prognosis

in high-risk neuroblastoma.³⁸ Therefore, a reversal of the immune-suppressive environment through NK cells' modulation is promising. NKG2D, ζ -NK cell, a gene-modified type of NK cell that targeted myeloid-derived suppressor cells, enhances CAR-T cell activity in neuroblastoma.³⁹ Likewise, enhanced uptake of cancer-derived neoantigens by dendritic cells could stimulate CD8⁺ T cells' antitumor effect,⁴⁰ indicating the vital role of antigen processing in cancer immunity. Following this, we found prominent enrichment of NK cells, Th1/Th2 CD4⁺ cells, and antigen processing gene sets in the immune low-risk group, indicating an active immune response positively correlated with a favorable prognosis. Therefore, immune modulation focused on NK cells and CD4⁺ cells may synergize immunotherapy in the high immune risk group.

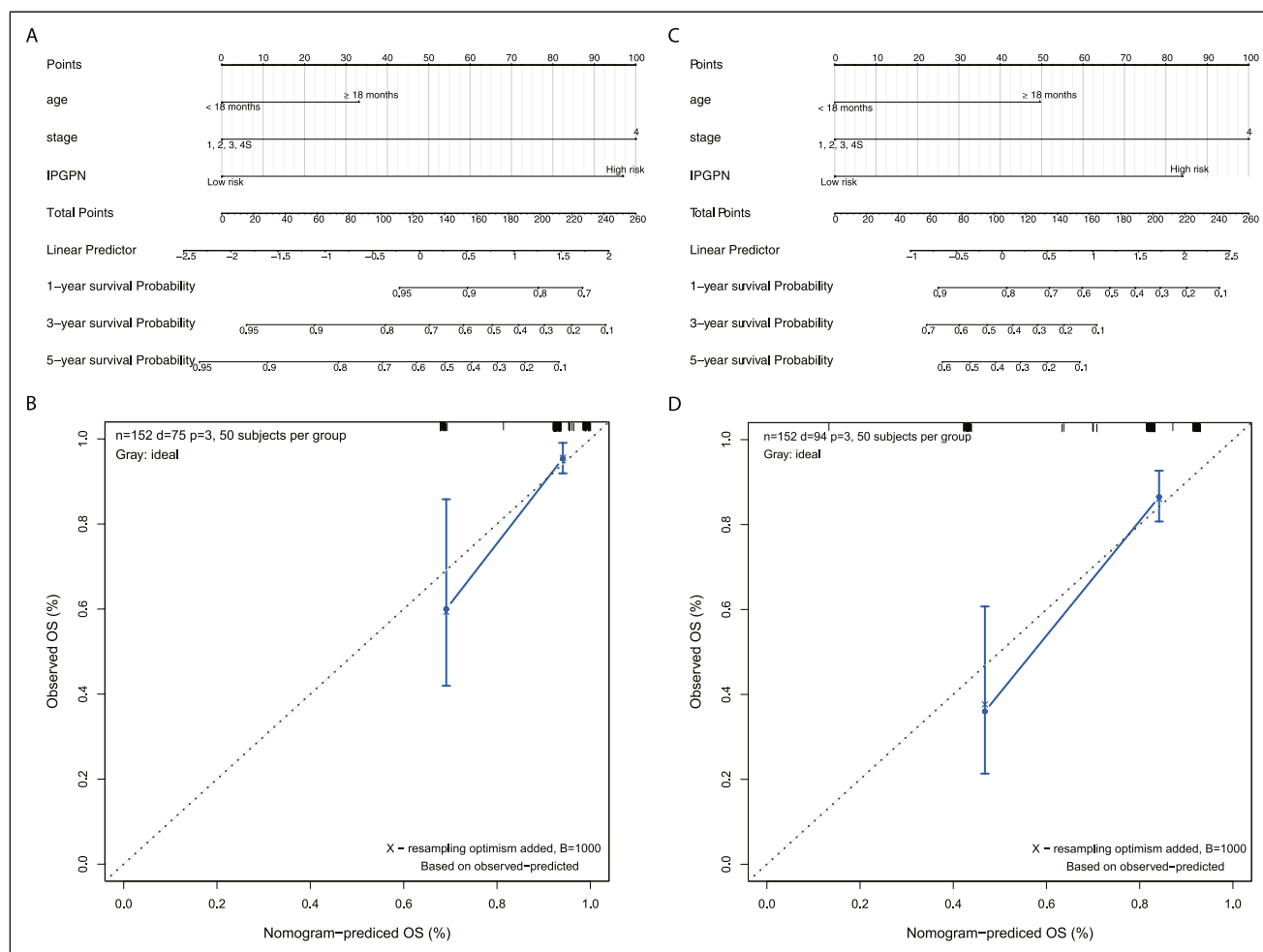


Figure 8. Nomogram for both OS and EFS prediction. (A) Nomogram combining IPGPN and clinicopathological characteristics for 1-year, 3-year, and 5-year OS prediction in the training cohort. (B) Calibration plot showing the ideal prediction (black dotted line) and the nomogram-prediction (blue line with the bars representing the 95% CI) in the training cohort. (C) Nomogram combining IPGPN and clinicopathological characteristics for 1-year, 3-year, and 5-year OS prediction in the validation cohort. (D) Calibration plot showing the ideal prediction (black dotted line) and the nomogram-prediction (blue line with the bars representing the 95% CI) in the validation cohort. Abbreviations: EFS, event-free survival; IPGPN, Immune-related Prognostic Gene Pairs for Neuroblastoma; OS, overall survival.

Though immune checkpoint inhibitors have achieved satisfying efficacy in several adult cancers, most of them failed in neuroblastoma due to their cold immunogenic nature.⁴¹ Several approaches have been suggested to improve the immune response of cold neuroblastoma by reprogramming innate immune systems. For those neuroblastomas with inadequate immune response, activation of the simulator of interferon genes (STING) pathway could convert them into a more T-cell enriched microenvironment and enhance their response to immune checkpoint blockade.⁴² While the addition of antibody against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), an immune checkpoint blockade depleting T regulatory cells (Tregs), to combined therapy of radiation and intratumor injection of immunocytokine could not lead to complete regression of N-MYC driven cold neuroblastoma, further addition of CpG-oligodeoxynucleotides and anti-

CD40 mAb to this combined regimen could result in complete response through activating innate immune system.⁴³ Furthermore, though inhibition of programmed cell death protein 1 (PD-1) alone could not lead to neuroblastoma regression, concurrent inhibition of CSF-1R could significantly promote T-cell infiltration through upregulating the expression of recruiting chemokines and downregulating suppressive myeloid cells.⁴⁴ Based on the comprehensive immune profiling of neuroblastoma in our research, neuroblastoma with low immune response and specific modification of innate immune to enhance their response to immune checkpoint blockade treatment could be further identified.

Besides of gene expression profiles, several clinicopathological characteristics could be predictive for prognosis and clinical efficacy in neuroblastoma. For example, patients with

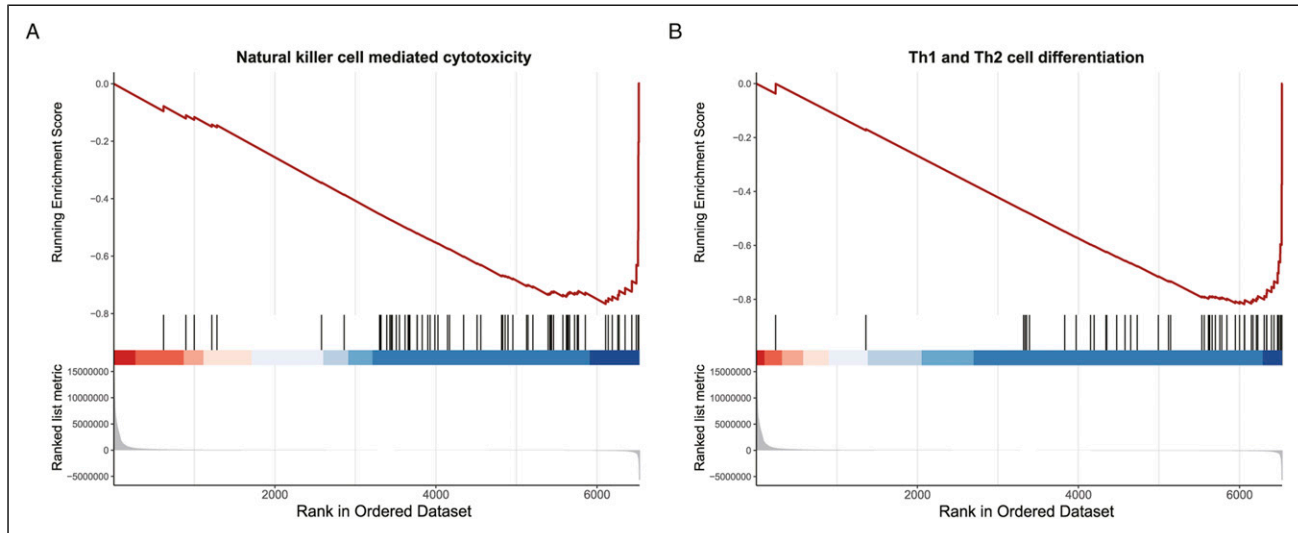


Figure 9. Functional enrichment analysis of DEGs between low- and high-risk groups. (A) Enrichment plots for the natural killer cell mediated cytotoxicity signature. (B) Enrichment plots for the Th1 and Th2 cell differentiation signature.

older age at diagnosis (>18 months), independent from the stage, usually had a worse prognosis.⁴⁵⁻⁴⁷ Moreover, MYCN status is associated with therapeutic response to immunotherapy. MYC and MYCN were found to regulate the expression of PD-L1 in neuroblastoma,⁴⁸ and MYCN could further inhibit NK cell activation in high-risk neuroblastoma. Consistently, we confirmed that age, stage, and MYCN are independent prognosis predictors. These three parameters could further complement IPGPN with better predictive power than IPGPN alone or clinicopathological factor alone.

Our study has several advantages compared with other existing models. First, compared with the previous NBL signature,¹⁸ IPGPN focused on immune-related genes and delineated the immune cell composition in NBL. Second, compared with the previous models established with immune-related genes in NBL,^{49,50} IPGPN was established with pairwise correlation, reflecting the landscape of immune-related tumor microenvironment more accurately across different populations and reducing specific cross-study batch effects. Third, while IPGPN was established with the TARGET neuroblastoma cohort in terms of OS, it presents with a satisfactory performance for both OS and EFS prediction in the GEO neuroblastoma cohort, indicating the robustness of this model among different neuroblastoma cohorts.

Limitations of our study should also be noted. Though gene-pair calculation reduced the batch effects to some extent, it led to the low AUC values around 0.6. Moreover, further studies to validate this model both *in vitro* and *in vivo* are needed.

In conclusion, our study provided a comprehensive understanding of immune signatures in neuroblastoma based on the pairwise correlation of immune-related genes and cellular compartment within different immune risk groups. IPGPN, a

prognostic immune signature proposed, is promising for prognosis prediction and distinguishing neuroblastoma with different immune responsiveness, which guides immunotherapy to overcome immune therapy resistance.

Acknowledgments

The study funders had no role in the study's design; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

Authors Contributions

YY and FC conceived, designed, or planned the study. YZ, YY, and JGZ analyzed the data. FC acquired data. JGZ and YZ helped interpret the results. JGZ and FC provided study materials or patients. YZ, YY, JGZ, XPX, and FC drafted the manuscript. All authors revised and reviewed this work, and all authors gave their final approval of the submitted manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was funded by the Natural Science Foundation of China (NSFC: 81660421, 81 860 450). Outstanding Youth Foundation of Zunyi medical university (2018-05).

ORCID iDs

Yunhu Yu  <https://orcid.org/0000-0002-5021-3739>
Fang Cao  <https://orcid.org/0000-0003-2328-8994>

Supplementary Material

Supplementary material for this article is available online.

References

1. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *Lancet*. 2007;369(9579):2106-2120. doi:10.1016/S0140-6736(07)60983-0.
2. Gatta G, Botta L, Rossi S, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCare-5-a population-based study. *Lancet Oncol*. Jan 2014;15(1):35-47. doi:10.1016/S1470-2045(13)70548-5
3. Smith MA, Seibel NL, Altekruse SF, et al. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. *J Clin Oncol*. 2010;28(15):2625-2634. doi:10.1200/JCO.2009.27.0421.
4. Brodeur GM, Bagatell R. Mechanisms of neuroblastoma regression. *Nat Rev Clin Oncol*. 2014;11(12):704-713. doi:10.1038/nrclinonc.2014.168.
5. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Canc*. 2003;3(3):203-216. doi:10.1038/nrc1014.
6. Matthay KK, George RE, Yu AL. Promising therapeutic targets in neuroblastoma. *Clin Canc Res*. 2012;18(10):2740-2753. doi:10.1158/1078-0432.CCR-11-1939.
7. Spill F, Reynolds DS, Kamm RD, Zaman MH. Impact of the physical microenvironment on tumor progression and metastasis. *Curr Opin Biotechnol*. 2016;40:41-48. doi:10.1016/j.copbio.2016.02.007.
8. Seebauer CT, Brunner S, Glockzin G, et al. Peritoneal carcinomatosis of colorectal cancer is characterized by structural and functional reorganization of the tumor microenvironment inducing senescence and proliferation arrest in cancer cells. *Oncol Immunology*. 2016;5(12):e1242543. doi:10.1080/2162402X.2016.1242543.
9. Tang T, Yang Z, Zhu Q, et al. Up-regulation of miR-210 induced by a hypoxic microenvironment promotes breast cancer stem cell metastasis, proliferation, and self-renewal by targeting E-cadherin. *Faseb J*. 2018;32:6965-6981. doi:10.1096/fj.201801013R.
10. Xia S, Lal B, Tung B, Wang S, Goodwin CR, Laterra J. Tumor microenvironment tenascin-C promotes glioblastoma invasion and negatively regulates tumor proliferation. *Neuro Oncol*. 2016;18(4):507-517. doi:10.1093/neuonc/nov171.
11. De Francesco EM, Sims AH, Maggiolini M, Sotgia F, Lisanti MP, Clarke RB. GPER mediates the angiocrine actions induced by IGF1 through the HIF-1 α /VEGF pathway in the breast tumor microenvironment. *Breast Canc Res*. 2017;19(1):129. doi:10.1186/s13058-017-0923-5.
12. Wang Y, Chen K, Cai Y, et al. Annexin A2 could enhance multidrug resistance by regulating NF- κ B signaling pathway in pediatric neuroblastoma. *J Exp Clin Canc Res*. 2017;36(1):111. doi:10.1186/s13046-017-0581-6.
13. Eschenburg G, Eggert A, Schramm A, Lode HN, Hundsdoerfer P. Smac mimetic LBW242 sensitizes XIAP-overexpressing neuroblastoma cells for TNF- α -independent apoptosis. *Canc Res*. 2012;72(10):2645-2656. doi:10.1158/0008-5472.CAN-11-4072.
14. Silverman AM, Nakata R, Shimada H, Sposto R, DeClerck YA. A galectin-3-dependent pathway upregulates interleukin-6 in the microenvironment of human neuroblastoma. *Canc Res*. 2012;72(9):2228-2238. doi:10.1158/0008-5472.CAN-11-2165.
15. Hashimoto O, Yoshida M, Koma Yi, et al. Collaboration of cancer-associated fibroblasts and tumour-associated macrophages for neuroblastoma development. *J Pathol*. 2016;240(2):211-223. doi:10.1002/path.4769.
16. Srinivasan P, Wu X, Basu M, Rossi C, Sandler AD. PD-L1 checkpoint inhibition and anti-CTLA-4 whole tumor cell vaccination counter adaptive immune resistance: a mouse neuroblastoma model that mimics human disease. *PLoS Med*. 2018;15(1):e1002497. doi:10.1371/journal.pmed.1002497.
17. McCarrick JW 3rd, Parnes JR, Seong RH, Solter D, Knowles BB. Tumor immunogenicity—the prime determinant of the nutritional influence on the host-tumor relationship. *Transgenic Res*. 1986;10(1):21-28.
18. Zhou J-G, Liang B, Jin S-H, et al. Development and validation of an RNA-seq-based prognostic signature in neuroblastoma. *Frontiers in oncology*. 2019;9:1361. doi:10.3389/fonc.2019.01361.
19. Consortium SM-I. A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the sequencing quality control consortium. *Nat Biotechnol*. 2014;32(9):903-914. doi:10.1038/nbt.2957.
20. Li B, Cui Y, Diehn M, Li R. Development and validation of an individualized immune prognostic signature in early-stage nonsquamous non-small cell lung cancer. *JAMA Oncology*. 2017;3(11):1529-1537. doi:10.1001/jamaoncol.2017.1609.
21. Bhattacharya S, Andorf S, Gomes L, et al. ImmPort: disseminating data to the public for the future of immunology. *Immunol Res*. 2014;58(2-3):234-239. doi:10.1007/s12026-014-8516-1.
22. Heagerty PJ, Lumley T, Pepe MS. Time-dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics*. 2000;56(2):337-344. doi:10.1111/j.0006-341x.2000.00337.x.
23. Auslander N, Zhang G, Lee JS, et al. Robust prediction of response to immune checkpoint blockade therapy in metastatic melanoma. *Nat Med*. 2018;24(10):1545-1549.
24. Ploessl C, Pan A, Maples KT, Lowe DK. Dinutuximab. *Ann Pharmacother*. 2016;50(5):416-422. doi:10.1177/1060028016632013.
25. Majzner RG, Simon JS, Grosso JF, et al. Assessment of programmed death-ligand 1 expression and tumor-associated immune cells in pediatric cancer tissues. *Cancer*. 2017;123(19):3807-3815.
26. Kim S, Lin C-W, Tseng GC. MetaKTSP: a meta-analytic top scoring pair method for robust cross-study validation of omics prediction analysis. *Bioinformatics*. 2016;32(13):1966-1973. doi:10.1093/bioinformatics/btw115.
27. Kakiuchi N, Yoshida K, Uchino M, et al. Frequent mutations that converge on the NFKBIZ pathway in ulcerative colitis. *Nature*. 2020;577(7789):260-265.

28. Mobasheri S, Irani N, Sepahi AA, et al. Evaluation of TRIM5 and TRIM22 polymorphisms on treatment responses in Iranian patients with chronic hepatitis C virus infection. *Gene*. 2018; 676:95-100. doi:10.1016/j.gene.2018.07.023.
29. Wang Y, Chen Y, Lin Y, Quan Y, Xiao X, Zhang R. TRIM22 inhibits respiratory syncytial virus replication by targeting JAK-STAT1/2 signaling. *J Med Virol*. 2020;93:3412-3419. doi:10.1002/jmv.26436.
30. Liu Y, Wu H-W, Sheard MA, et al. Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. *Clin Canc Res*. 2013;19(8):2132-2143. doi:10.1158/1078-0432.CCR-12-1243.
31. Yu DMT, Huynh T, Truong AM. ABC transporters and neuroblastoma. *Adv Cancer Res* 2015;125:139-170. doi:10.1016/bs.acr.2014.10.005.
32. Okawa ER, Gotoh T, Manne J, et al. Expression and sequence analysis of candidates for the 1p36.31 tumor suppressor gene deleted in neuroblastomas. *Oncogene*. 2008;27(6):803-810. doi:10.1038/sj.onc.1210675.
33. Dzieran J, Rodriguez Garcia A, Westermark UK, et al. MYCN-amplified neuroblastoma maintains an aggressive and undifferentiated phenotype by deregulation of estrogen and NGF signaling. *Proc Natl Acad Sci Unit States Am*. 2018;115(6):E1229-E1238. doi:10.1073/pnas.1710901115.
34. Liu D, Song L, Wei J, et al. IL-15 protects NKT cells from inhibition by tumor-associated macrophages and enhances antitumor activity. *J Clin Invest*. 2012;122(6):2221-2233. doi:10.1172/JCI59535.
35. Dondero A, Pastorino F, Della Chiesa M, et al. PD-L1 expression in metastatic neuroblastoma as an additional mechanism for limiting immune surveillance. *Oncol Immunology*. 2016; 5(1):e1064578. doi:10.1080/2162402X.2015.1064578.
36. Webb MW, Sun J, Sheard MA, et al. Colony stimulating factor 1 receptor blockade improves the efficacy of chemotherapy against human neuroblastoma in the absence of T lymphocytes. *Int J Canc*. 2018;143(6):1483-1493. doi:10.1002/ijc.31532.
37. Troschke-Meurer S, Siebert N, Marx M, et al. Low CD4⁺/CD25⁺/CD127⁻ regulatory T cell- and high INF- γ levels are associated with improved survival of neuroblastoma patients treated with long-term infusion of ch14.18/CHO combined with interleukin-2. *Oncol Immunology*. 2019;8(12):1661194.
38. Yang RK, Kalogriopoulos NA, Rakhmilevich AL, et al. Intratumoral treatment of smaller mouse neuroblastoma tumors with a recombinant protein consisting of IL-2 linked to the hu14.18 antibody increases intratumoral CD8⁺ T and NK cells and improves survival. *Canc Immunol Immunother*. 2013;62(8):1303-1313. doi:10.1007/s00262-013-1430-x.
39. Parihar R, Rivas C, Huynh M, et al. NK cells expressing a chimeric activating receptor eliminate mdscs and rescue impaired CAR-T cell activity against solid tumors. *Cancer Immunology Research*. 2019;7(3):363-375. doi:10.1158/2326-6066.CIR-18-0572.
40. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol*. 2020;20(1):7-24. doi:10.1038/s41577-019-0210-z.
41. Patel RB, Ye M, Carlson PM, et al. Development of an in situ cancer vaccine via combinational radiation and bacterial-membrane-coated nanoparticles. *Adv Mater*. 2019;31(43):1902626. doi:10.1002/adma.201902626.
42. Wang-Bishop L, Wehbe M, Shae D, et al. Potent STING activation stimulates immunogenic cell death to enhance antitumor immunity in neuroblastoma. *Journal for Immunotherapy of Cancer*. 2020;8(1):e000282. doi:10.1136/jitc-2019-000282.
43. Voeller J, Erbe AK, Slowinski J, et al. Combined innate and adaptive immunotherapy overcomes resistance of immunologically cold syngeneic murine neuroblastoma to checkpoint inhibition. *Journal for Immunotherapy of Cancer*. 2019;7(1):344. doi:10.1186/s40425-019-0823-6.
44. Eissler N, Mao Y, Brodin D, et al. Regulation of myeloid cells by activated T cells determines the efficacy of PD-1 blockade. *Oncol Immunology*. 2016;5(12):e1232222. doi:10.1080/2162402X.2016.1232222.
45. Rosswog C, Schmidt R, Oberthuer A, et al. Molecular classification substitutes for the prognostic variables stage, age, and MYCN status in neuroblastoma risk assessment. *Neoplasia*. 2017;19(12):982-990. doi:10.1016/j.neo.2017.09.006.
46. Chang H-H, Tseng Y-F, Lu M-Y, et al. MYCN RNA levels determined by quantitative in situ hybridization is better than MYCN gene dosages in predicting the prognosis of neuroblastoma patients. 2019:1-10.
47. Kreitz K, Ernst A, Schmidt R, et al. A new risk score for patients after first recurrence of stage 4 neuroblastoma aged ≥ 18 months at first diagnosis. *Cancer Medicine*. 2019;8(17):7236-7243.
48. Melaiu O, Mina M, Chierici M, et al. PD-L1 is a therapeutic target of the bromodomain inhibitor JQ1 and, combined with HLA class I, a promising prognostic biomarker in neuroblastoma. *Clin Canc Res*. 2017;23(15):4462-4472.
49. Jin W, Zhang Y, Liu Z, Che Z, Gao M, Peng H. Exploration of the molecular characteristics of the tumor-immune interaction and the development of an individualized immune prognostic signature for neuroblastoma. *J Cell Physiol*. 2020;236:294-308. doi:10.1002/jcp.29842.
50. Liu Z, Grant CN, Sun L, et al. Expression patterns of immune genes reveal heterogeneous subtypes of high-risk neuroblastoma. *Cancers*. 2020;12(7):1739. doi:10.3390/cancers12071739.