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ORIGINAL RESEARCH

Low Expression of *CLEC2B* Indicates Poor Prognosis in Melanoma

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Background: Melanoma is a highly malignant skin tumor with a poor prognosis. Identification of novel biomarkers might potentially reveal the underlying mechanisms of melanoma progression.

Methods: We demonstrated the relationship between pan-cancer *CLEC2B* expression and melanoma samples in The Cancer Genome Atlas (TCGA) database. Next, the Kaplan-Meier plot and Cox regression analysis determined the prognostic value of *CLEC2B* in melanoma. Biological pathway enrichment was screened by Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA), enabling the correlation analysis between the immune infiltration level and *CLEC2B* expression in melanoma. Our final claim was validated using qPCR, immunohistochemistry, Western blot, cell colony formation assays, ethynyldeoxyuridine (Edu) analysis, and cell Invasion assays.

Results: Our study revealed that the high *CLEC2B* expression correlates with poor overall survival of melanoma patients. Moreover, a low expression of *CLEC2B* was found in the A375 cell line. In addition, *CLEC2B* has significant prognostic value in melanoma diagnosis, with an AUC of 0.896. Prognostic analysis showed the low expression of *CLEC2B* to be independently associated with melanoma patients. Moreover, the expression of *CLEC2B* was significantly correlated with B cells, eosinophils, macrophages, neutrophils, NK cells, T helper cells, Tregs, Th1 cells, Th17 cells, and Th2 cells. PCR and immunohistochemistry indicated *CLEC2B* to be significantly downregulated in melanoma. The cell colony formation assay showed *CLEC2B* knockout increased the proliferation of A375 cells.

Conclusion: Our study established low levels of *CLEC2B* to be poor prognostic markers, enabling immunosuppressive cell infiltration in melanoma.

Keywords: melanoma, *CLEC2B*, prognosis, immune infiltration, TCGA

Introduction

Melanoma is the deadliest type of skin cancer, with a rising yearly incidence.[1,2] Despite the rapid development of checkpoint immunotherapy and targeted therapies, the main melanoma treatment methods include surgical resection and chemotherapy.[3,4] Recent immunosuppressive agents increase the one-year survival rate of melanoma patients to more than 50%. However, many patients do not respond to these drugs, while many who have previously responded acquire secondary drug resistance.[4] Therefore, critically determining prognostic biomarkers will help doctors to timely and accurately predict the state of melanoma patients by using improved personalized treatment plans.

Increasing evidence suggests that *CLEC2B* is a crucial regulator in tumor evolution.[5,6] *CLEC2B* is a member of the C-type lectin domain family two member B and encodes a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. These family members share common protein folding techniques and have multiple functions, including intercellular signal transduction, glycoprotein conversion, and its role in inflammation.[7] *CLEC2B* is a well-known prognostic biomarker of endometrial cancer.[8] However, the expression and clinical significance of *CLEC2B* in melanoma have not been studied.

We studied *CLEC2B* expression in melanoma using patient data from TCGA database. We studied the transcriptional level and prognostic significance of *CLEC2B* by analyzing data obtained by TCGA-SKCM. We also explored its

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biological mechanism using GO, KEGG and GSEA to assess the association between *CLEC2B* and immune infiltration. qPCR, immunohistochemistry, Western blot, cell colony formation assays, ethynyldeoxyuridine (Edu) analysis, and cell invasion assays confirmed our conclusion. This study established the vital role of *CLEC2B* in melanoma and provided an in-depth understanding of the potential underlying mechanisms of *CLEC2B* and tumor immune interactions.

Materials and Methods

Data Preparation

RNA-sequencing and follow-up data for melanoma patients were acquired from TCGA-SKCM (n=471). We acquired data from UCSC Xena (https://xenabrowser.net/datapages/) to evaluate the pan-cancer expression level of *CLEC2B*.

Patients and Tissues

Twelve melanoma samples and matched non-tumor tissues were obtained from the First Affiliated Hospital of China Medical University. The ethics committee of the First Affiliated Hospital of China Medical University approved this study. And the study was conducted in accordance with the declaration of Helsinki. Melanoma tissues were obtained, frozen in liquid nitrogen, and stored at −80 °C after surgery for qPCR detection.

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA)

We used GO, KEGG, and GSEA to identify the enrichment patterns in the genome and pathways related to *CLEC2B* based on the transcriptional sequences of TCGA-SKCM. Functional enrichment in the DEGs was analyzed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) using the R package GOplot (version 1.0.2). Gene set enrichment analysis (GSEA) was carried out using the R package clusterProfiler, and an adjusted p-value <0.05 and false discovery rate (FDR) < 0.25 were regarded as statistically significantly enriched functions or pathway terms.

Immune Cell Infiltration

A total of 24 immune cells were used to investigate the level of immune infiltration. The relative enrichment score of these immune cells in breast cancer was assessed by single-sample GSEA (ssGSEA) using the R package GSVA. The correlation between *CLEC2B* expression and the immune cells was investigated using Spearman correlation analysis. The immune infiltration differences between the high and low *CLEC2B* expression groups were evaluated using the Wilcoxon rank-sum test.

Survival and Prognosis Analysis

The Kaplan-Meier method with the Log rank test was used for survival analysis, and the median expression level of *CLEC2B* was set as the cut-off value. Univariate and multivariate Cox regression analysis were used to assess the effect of clinical variables on patient outcomes. The prognostic variables $p < 0.1$ in the univariate Cox regression analysis were incorporated into the multivariate Cox regression analysis. The R package ggplot2 was used to visualize the forest map.

Construction and Validation of the Nomogram

A nomogram based on independent prognostic factors in multivariate Cox analysis was constructed to predict the overall survival probability. Calibration plots assessed the performance of the nomogram, while the concordance index (C-index) quantified the discrimination of the nomogram. This nomogram and calibration plots were created using the R package RMS (version 5.1–4)8. The time-dependent receiver-operating characteristic (ROC) curve evaluated the predictive accuracy using the timeROC package.

Cell Culture and Transfection

The A375 cell line was obtained from The Chinese Academy of Sciences. Small interfering RNA (siRNA) and Lipofectamine 2000 (Invitrogen, Shanghai, China) were used, as previously described,[9] for cell transfection.

RNA Isolation and qPCR Analysis

RNA extraction from tissues was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA, USA). qPCR analyses were performed using SYBR-Green (Takara, Otsu, Shiga, Japan), and the gene expression levels were normalized to GAPDH levels. Sequences of upstream and downstream primers were as follows: *CLEC2B*: 5′-GTTCCACTCAACATGCCGAC-3′ and 5′-TGCCATCTTCAGTCCAATCCA-3′.

Immunohistochemistry

Immunohistochemistry (IHC) staining was carried out as previously described.[10] Briefly, each group of melanoma samples was fixed in 10% formalin, embedded in paraffin, and processed as 5μm continuous sections. Samples were dewaxed with discontinuous ethanol concentration gradients and blocked to inhibit endogenous peroxidase. They were then heated in a microwave for antigen retrieval, cooled to room temperature, and blocked in goat serum for 30 minutes at 37°C. Samples were incubated in rabbit anti-*CLEC2B* (Abcam, Cambridge, UK; 1: 1, 200) overnight at 4°C, followed by horseradish peroxidase (HRP)-coupled goat anti-rabbit secondary antibody at 37°C for 30 minutes, and stained by 3,3′-diaminobenzidine. The cell nucleus was stained blue by hematoxylin. Sections were then dehydrated, cleared by xylene, and mounted. *CLEC2B* expression was detected by IHC using a streptavidin peroxidase method.

Cell Colony Formation Assay

The cells were seeded at a density of 1×10^3/mL per well in the 6-well plates per 2 mL of DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Single-cell-derived clones were grown for ten days. The culture was pre-cooled three times with PBS, fixed with methanol for 15 min, stained with crystal violet for 20 min, and the water was rinsed and air dried. The number of visible clones was visually counted, and the colony formation rate was calculated: clone formation rate(%)=(clone number/number of inoculated cells)×100%. This procedure was repeated three times.

Western Blotting Assay

Cells were lysed using RIPA Lysis containing Protease/Phosphatase Inhibitor Cocktail (Abcam). The extracted proteins were separated in 10% SDS-polyacrylamide gel and then transferred to immobilon-P membranes (Merck Millipore). The membrane was blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBST) for one h at room temperature and then incubated with primary antibodies overnight at 4°C. The next day, the membranes were washed three times with Tris-buffered saline Tween and then incubated with HRP-conjugated secondary antibody (1:1000, Santa Cruz Biotech) at room temperature for two hours. Finally, all the bands were detected using ECL Western blotting kit (Amersham Biosciences). GAPDH was used as an internal reference.[10]

Cell Invasion Assays

Cells were harvested, resuspended in serum-free media, and placed in the upper chamber of a Transwell membrane filter coated with Matrigel (Corning) for invasion assays. A culture medium with 10% FBS and 0/5/10 nM Tanespimycin was added to the lower compartment as a chemoattractant. After 24 h of incubation, cells were fixed with methanol, stained with 0.1% crystal violet, imaged, and counted using an Olympus microscope (Tokyo, Japan).

Ethynyldeoxyuridine (Edu) Analysis

The cells were cultured in 96-well plates at a density of 4×10^4 cells/well. Forty-eight hours after transfection, 20 μM Edu labeling media (Beyotime Biotechnology, Shanghai, China) was added to the wells and incubated for two h at 37 °C

and 5% CO2. The cells were then treated with 4% paraformaldehyde and 0.5% Triton X-100 and finally stained with an anti-Edu working solution. The percentage of Edu-positive cells was calculated after fluorescence microscopy analysis.

Statistical Analysis

The statistical tool SPSS 21.0 was used to statistically analyze the data (IBM Corporation, Armonk, NY, USA). GraphPad Prism 8.0 was used to generate the graphs (GraphPad Software, Inc., San Diego, CA). Student's t-tests were used for the statistical analysis of the data. A two-tailed $p < 0.05$ indicated a significant value, wherein "ns" represented $p > 0.05$, *represented $p <= 0.05$, **represented $p <= 0.01$ and ***represented $p <= 0.001$.

Result

Expression Analysis of *CLEC2B* in Pan-Cancer and Melanoma

The expression analysis of *CLEC2B* in 33 cancers showed that *CLEC2B* was less expressed in most cancers, including adrenocortical carcinoma (ACC), urothelial bladder carcinoma (BLCA), breast invasive carcinoma (BRCA), COAD (colon adenocarcinoma), kidney chromophobe (KICH), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), ovarian serous cystadenocarcinoma (OV), prostate adenocarcinoma (PRAD), rectal adenocarcinoma (READ), skin cutaneous melanoma (SKCM), thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC) and uterine carcinosarcoma (UCS). However, *CLEC2B* was highly expressed in cholangiocarcinoma (CHOL), glioblastoma (GBM), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), acute myeloid leukemia (LAML), pancreatic adenocarcinoma (PAAD) and stomach adenocarcinoma (STAD) and thymoma (THYM) (Figure 1A). We assessed the expression of *CLEC2B* in melanoma in the TCGA-SKCM, which confirmed its low expression in melanoma (Figure 1B). We further studied the association between *CLEC2B* expression and clinical outcomes. Survival analysis revealed significant differences between different cancer types. In the SKCM cohort, patients with high *CLEC2B* levels showed more prolonged overall survival (OS),

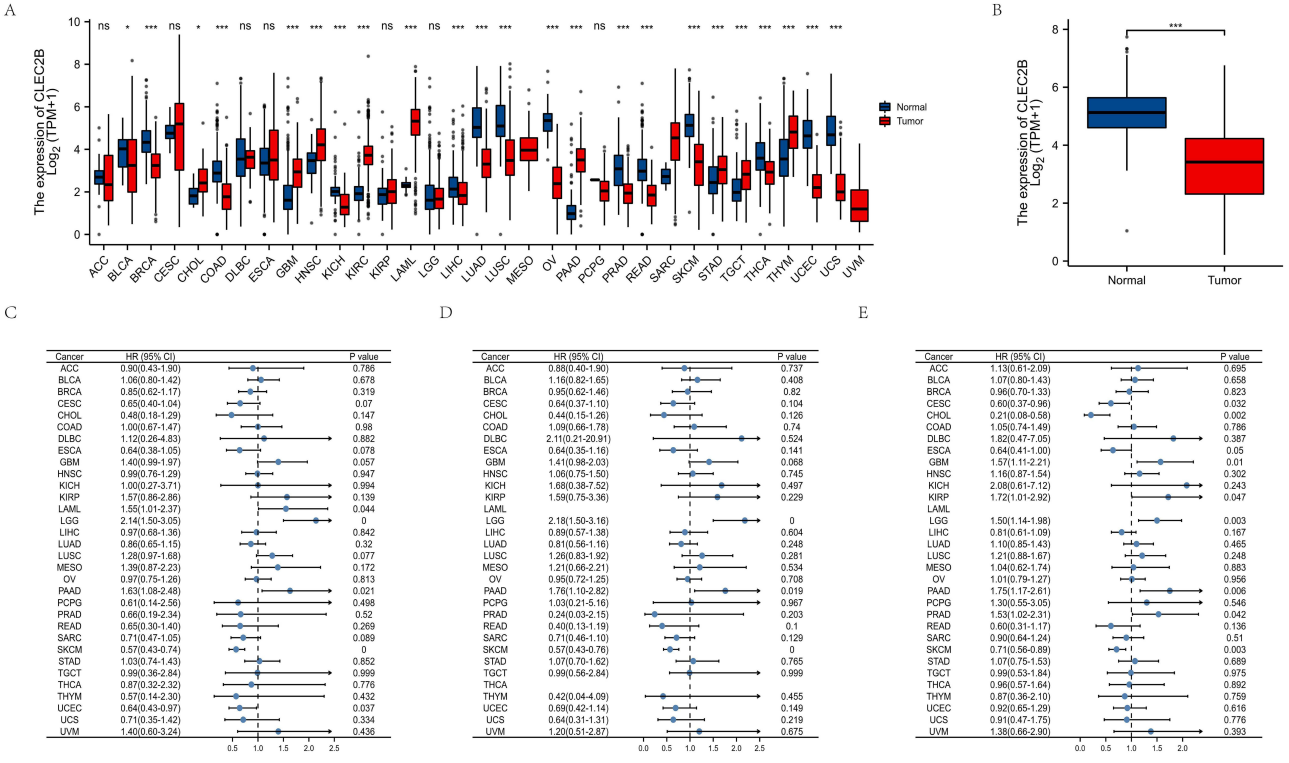

Figure 1 *CLEC2B* expression level in mRNA based on TCGA database. (**A**) Overview of *CLEC2B* mRNA expression in different tumor tissues and adjacent normal tissues. (**B**) Comparison of *CLEC2B* expression between SKCM and normal tissues. Risk plot of the correlation between *CLEC2B*: (**C**) OS. (**D**) PFI. (**E**) DSS. (right represents HR > 1 (risky); left represents HR < 1 (protective)) "ns" represented $p > 0.05$, "*" represented $p <= 0.05$ and "***" represented $p <= 0.001$.

progression-free interval (PFI), and disease-specific survival (DSS) than those with low *CLEC2B* levels (Figure 1C–E). In the following research, we focussed on exploring the role of *CLEC2B* in melanoma.

Clinical Correlation of CLEC2B Expression in Patients with Melanoma

The clinical characteristics and gene expression data of 471 patients with melanoma from TCGA-SKCM helped us divide the patients into *CLEC2B* high (n = 236) and low expression groups (n = 235) to investigate the correlation between *CLEC2B* expression and the clinicopathological features of patients. The expression of *CLEC2B* significantly correlated with T stage (P < 0.001), pathological stage (P < 0.001), radiation therapy (P = 0.018), age (P = 0.010), melanoma ulceration (P = 0.002), and Breslow depth (P < 0.001), and was also associated with age (P < 0.001) (Table 1).

Table 1 *CLEC2B* Expression in SKCM Patients with Different Clinical Parameters

Characteristic	Low Expression of *CLEC2B*	High Expression of *CLEC2B*	p
n	235	236	
T stage, n (%)			< 0.001
T1	14 (3.8%)	27 (7.4%)	
T2	37 (10.2%)	42 (11.5%)	
T3	43 (11.8%)	48 (13.2%)	
T4	105 (28.8%)	48 (13.2%)	
N stage, n (%)			0.435
N0	121 (29.2%)	114 (27.5%)	
N1	32 (7.7%)	42 (10.1%)	
N2	26 (6.3%)	23 (5.6%)	
N3	32 (7.7%)	24 (5.8%)	
M stage, n (%)			0.776
M0	213 (48.1%)	205 (46.3%)	
M1	14 (3.2%)	11 (2.5%)	
Pathologic stage, n (%)			< 0.001
Stage I	28 (6.8%)	49 (11.9%)	
Stage II	90 (21.8%)	50 (12.1%)	
Stage III	83 (20.1%)	88 (21.4%)	
Stage IV	13 (3.2%)	11 (2.7%)	
Radiation therapy, n (%)			0.018
No	200 (43.1%)	183 (39.4%)	
Yes	30 (6.5%)	51 (11%)	
Gender, n (%)			0.361
Female	84 (17.8%)	95 (20.2%)	
Male	151 (32.1%)	141 (29.9%)	
Race, n (%)			0.114
Asian	9 (2%)	3 (0.7%)	
Black or African American	0 (0%)	1 (0.2%)	
White	221 (47.9%)	227 (49.2%)	
Age, n (%)			0.010
<=60	112 (24.2%)	140 (30.2%)	
>60	120 (25.9%)	91 (19.7%)	
Weight, n (%)			0.194
<=70	46 (17.8%)	31 (12%)	
>70	91 (35.1%)	91 (35.1%)	
Height, n (%)			0.829
< 170	65 (25.6%)	53 (20.9%)	
≥170	72 (28.3%)	64 (25.2%)	

(*Continued*)

Table 1 (Continued).

Characteristic	Low Expression of *CLEC2B*	High Expression of *CLEC2B*	p
BMI, n (%)			0.181
<=25	51 (20.3%)	33 (13.1%)	
>25	85 (33.9%)	82 (32.7%)	
Tumor tissue site, n (%)			0.618
Extremities	106 (25.3%)	91 (21.7%)	
Trunk	87 (20.8%)	84 (20%)	
Head and Neck	22 (5.3%)	16 (3.8%)	
Other Specify	5 (1.2%)	8 (1.9%)	
Melanoma ulceration, n (%)			0.002
No	69 (22%)	78 (24.8%)	
Yes	108 (34.4%)	59 (18.8%)	
Melanoma Clark level, n (%)			0.085
I	2 (0.6%)	4 (1.2%)	
II	7 (2.2%)	11 (3.4%)	
III	37 (11.5%)	40 (12.4%)	
IV	84 (26.1%)	84 (26.1%)	
V	36 (11.2%)	17 (5.3%)	
Breslow depth, n (%)			< 0.001
<=3	80 (22.2%)	105 (29.2%)	
>3	114 (31.7%)	61 (16.9%)	
Age, median (IQR)	62 (52, 72)	56 (45, 68)	< 0.001

Correlation Between CLEC2B Expression and Survival Prognosis of Melanoma Patients

CLEC2B expression showed a significant correlation with overall survival (OS), disease-related survival (DSS), and progression-free survival (PFI) in melanoma patients. Kaplan-Meier plots showed that patients with lower *CLEC2B* had a worse prognosis for OS (HR = 0.57, 95% CI: 0.43–0.74, P < 0.001) (Figure 2A) and DSS (HR = 0.57, 95% CI: 0.43–0.76, P < 0.001) (Figure 2B). For PFI also, a similar trend was observed (HR = 0.71, 95% CI: 0.56–0.89, P = 0.003) (Figure 2C). In addition, we generated receiver operating characteristic (ROC) curves to assess the feasibility of distinguishing melanoma tissues from normal skin tissues based on *CLEC2B* expression levels, which revealed the area under the ROC curve (AUC) to be 0.896 (Figure 2D). Therefore, our results indicated that *CLEC2B* might be a potential prognostic biomarker in patients with melanoma.

Univariate and Multivariate Survival Analysis

We then conducted univariate and multivariate analyses, in which the former showed that low expression of T3 and T4 of T stage, N1 and N3 of N stage, M1 of M stage, age > 60, stage 2, stage 3 and 4 of the pathological stage, other of tumor tissue site and *CLEC2B* were associated with OS. The latter analysis revealed independent risk factors. We found that the main T4 stage and low expression of *CLEC2B* were independent prognostic factors of OS in SKCM inpatients (Table 2 and Figure 3).

CLEC2B Gene Enrichment Analysis

Three thousand four hundred twenty-eight genes were differentially expressed between groups with low and high levels of *CLEC2B* expression, including 879 downregulated and 2549 upregulated DEGs (adjusted p-value <0.05, |Log2-FC| > 1) (Figure 4A and Supplementary Table 1). GO, KEGG and GSEA analyses of the DEGs showed enrichment of GO terms, including molecular functions, cellular components, and biological processes, enriched in receptor ligand activity,

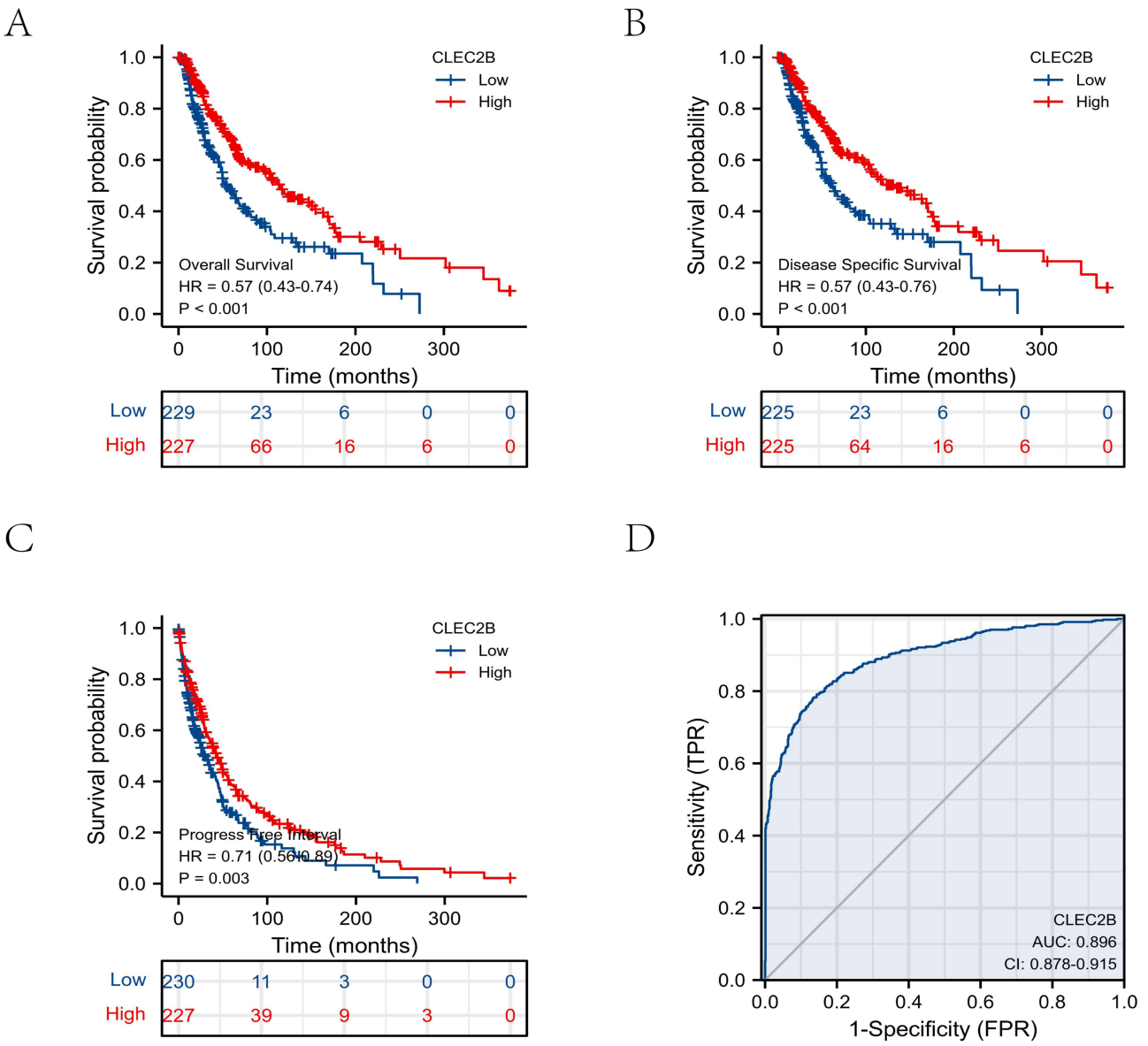

Figure 2 Survival in patients with high and low *CLEC2B*. (**A**) OS. (**B**) DSS. (**C**) PFI (**D**) ROC analysis illustrated that *CLEC2B* expression accurately discriminated SKCM tumor tissues from normal tissues.

antigen binding, skin development, and human immune response (Figure 4B). The KEGG pathway analysis demonstrated significant enrichment of the Cytokine cycloline receiver interaction, Neuroactive light receiver interaction, and Haematopoietic cell lineage (Figure 4C). In addition, GSEA of the DEGs identified some immune-related biological processes, such as GPCR ligand binding, interleukins signaling, and neuronal system (Figure 4D). Next, analyzing the relationship between the top 20 upregulated and downregulated DEGs and *CLEC2B* revealed most DEGs to be significantly associated with *CLEC2B* (Figure 4E and F).

Relationship Between *CLEC2B* Expression and Immune Cell Infiltration

We further evaluated the relationship between *CLEC2B* expression and immune cell infiltration using ssGSEA and Spearman's R to study the potential relationship between *CLEC2B* expression and 24 immune cell types. The analysis revealed *CLEC2B* expression to be significantly positively correlated with B cells, eosinophils, macrophages, neutrophils, NK cells, T helper cells, Tregs, Th1 cells, Th17 cells, and Th2 cells (Figure 5A). Varying

Table 2 Univariate Analysis and Multivariate Analysis of the Correlation Between Clinicopathological Characteristics and OS in SKCM

Characteristics	Total (N)	Univariate Analysis		Multivariate Analysis	
		Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
T stage	361				
T1	41	Reference			
T2	77	1.495 (0.811–2.756)	0.197	1.885 (0.444–8.011)	0.390
T3	90	2.097 (1.158–3.798)	0.015	4.367 (0.967–19.734)	0.055
T4	153	3.711 (2.070–6.653)	<0.001	6.045 (1.378–26.522)	0.017
N stage	402				
N0	224	Reference			
N1	73	1.497 (1.014–2.210)	0.043	4.228 (0.484–36.964)	0.192
N2	49	1.534 (0.972–2.419)	0.066	5.467 (0.599–49.872)	0.132
N3	56	2.731 (1.769–4.215)	<0.001	9.094 (0.893–92.613)	0.062
M stage	430				
M0	406	Reference			
M1	24	1.897 (1.029–3.496)	0.040	2.717 (0.147–50.365)	0.502
Pathologic stage	410				
Stage I	77	Reference			
Stage II	140	1.586 (1.054–2.385)	0.027	0.774 (0.184–3.258)	0.726
Stage III	170	1.983 (1.344–2.927)	<0.001	0.368 (0.031–4.349)	0.428
Stage IV	23	3.517 (1.781–6.944)	<0.001		
Radiation therapy	450				
No	374	Reference			
Yes	76	0.977 (0.694–1.377)	0.895		
Gender	456				
Female	172	Reference			
Male	284	1.172 (0.879–1.563)	0.281		
Age	456				
<=60	246	Reference			
>60	210	1.656 (1.251–2.192)	<0.001	0.957 (0.524–1.748)	0.886
Weight	249				
<=70	72	Reference			
>70	177	0.649 (0.389–1.083)	0.098	0.544 (0.251–1.178)	0.122
Height	244				
< 170	112	Reference			
≥170	132	0.855 (0.556–1.316)	0.477		
Tumor tissue site	405				
Extremities	190	Reference			
Trunk	166	0.940 (0.693–1.276)	0.694	0.956 (0.516–1.774)	0.888
Head and Neck	36	1.269 (0.756–2.131)	0.368	0.945 (0.311–2.874)	0.921
Other Specify	13	2.100 (1.091–4.044)	0.026	1.552 (0.455–5.289)	0.483
CLEC2B	456	0.746 (0.672–0.828)	<0.001	0.757 (0.620–0.925)	0.006

degrees of correlation between the ratios of 24 different types of tumor-infiltrating immune cell subsets were evaluated and visualized via heatmap (Figure 5B).

Nomogram Construction and Validation Based on the Independent Factors

A nomogram based on independent OS factors was generated to predict melanoma patients' prognosis. In this analysis, the higher the total score, the worse the prognosis (Figure 6A). In addition, the calibration curve was used to evaluate the prediction power of the nomogram (Figure 6B–D), indicating its suitability.

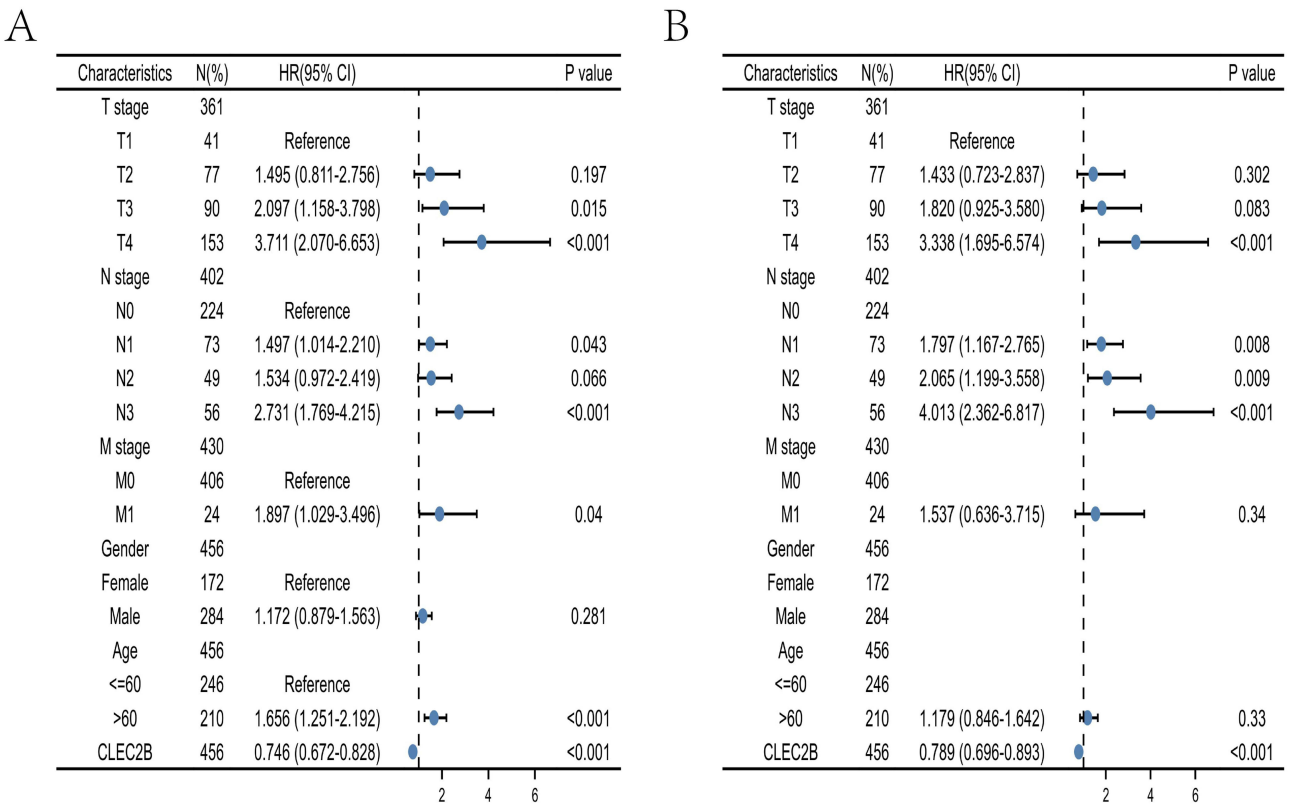

Figure 3 Univariate and multivariate survival analysis. (**A**) Univariate Analysis of the Overall Survival of Melanoma Patients (**B**) Multivariate Analysis of the Overall Survival of Melanoma Patients. (p-value is log rank test p).
Abbreviation: HR, hazard ratio.

CLEC2B is Expressed at Low Levels in Melanoma

Further verification of *CLEC2B* expression in melanoma tissues by qPCR, immunohistochemistry, Western blot, cell colony formation assays, ethynyldeoxyuridine (Edu) analysis, and cell Invasion assays validated the low levels of *CLEC2B* in melanoma (Figure 7A and B). Next, we transfected the *CLEC2B*-targeted pEZ-M03 vector into the A375 cell line. Western blot showed that *CLEC2B* was highly expressed in A375 cells after transfection (Figure 7C). The colony formation assay results showed decreased proliferation of A375 cells transfected with *CLEC2B* (Figure 7D). Moreover, the proliferation of A375 cells decreased upon *CLEC2B* overexpression, determined by Edu assays (Figure 7E). Cell invasion assays showed that *CLEC2B* overexpression inhibited the invasion ability of cells (Figure 7F). These findings collectively indicated that *CLEC2B* may act as tumor suppressor in melanoma.

Discussion

The mortality rate of melanoma remains high, and its occurrence results from multiple genetic changes.[11,12] Due to the high heterogeneity of melanoma, it is crucial to find different prognostic biomarkers.

In this study, we evaluated the role of *CLEC2B* as a prognostic melanoma biomarker. We evaluated the expression of *CLEC2B* in melanoma patients and across cancers by analyzing RNA-seq data from TCGA-SKCM, which revealed low *CLEC2B* expression in melanoma and worse prognosis in patients with low *CLEC2B* expression. The ROC curve also indicates that *CLEC2B* can be a potential biomarker for melanoma prognosis. Subsequently, univariate and multivariate survival analyses in melanoma patients showed that the T4 stage and low expression of *CLEC2B* were independent prognostic factors of OS. Moreover, the over expression of *CLEC2B* decreased the proliferation and invasion ability of

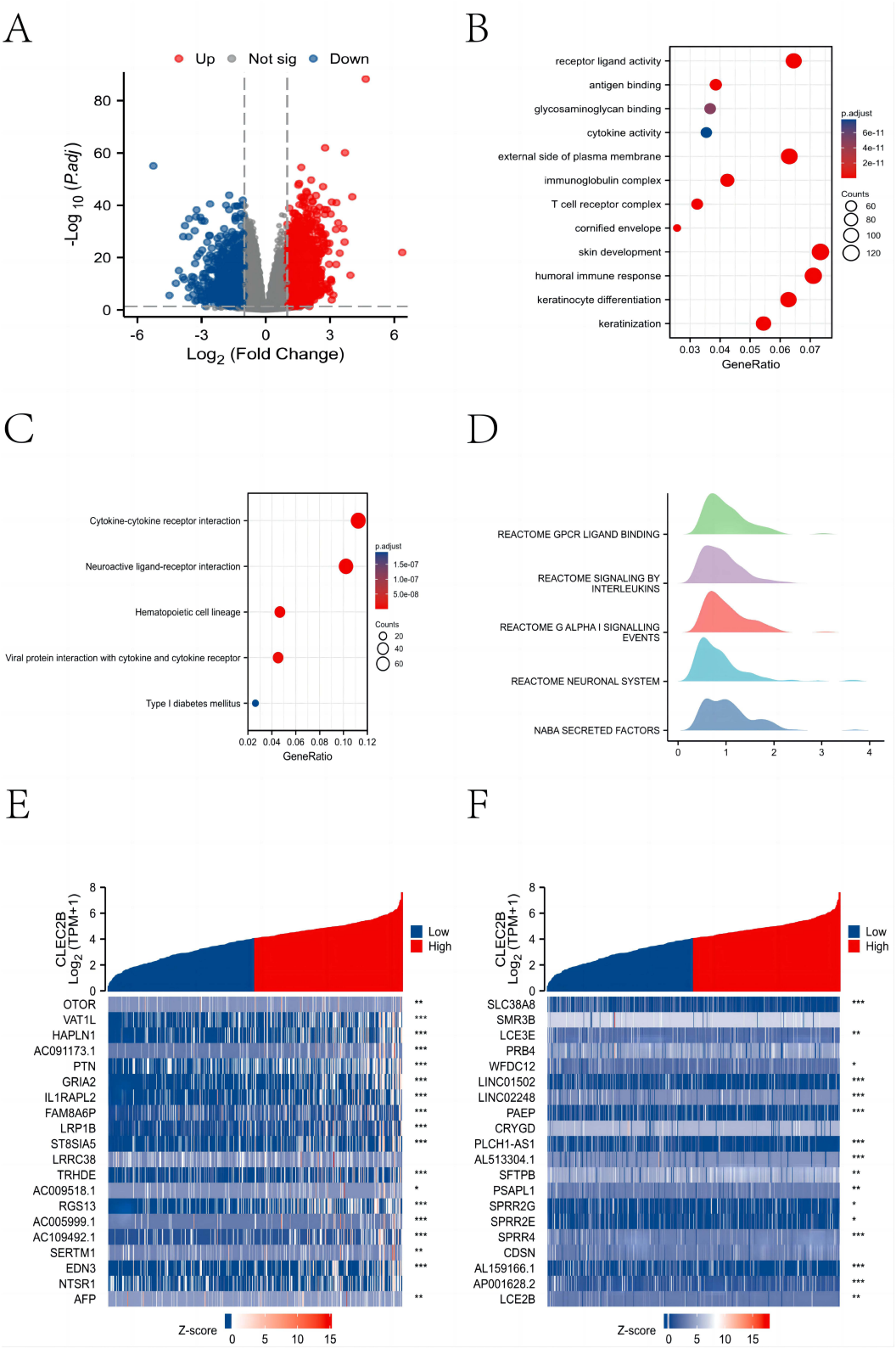

Figure 4 Differentially expressed genes (DEGs) related to *CLEC2B* and functional enrichment analysis of *CLEC2B* in melanoma. (**A**) Volcano plot. The blue dot and red dot indicate the DEGs that are significantly downregulated and upregulated, respectively. (**B**) GO analysis. (**C**) KEGG analysis. (**D**) GSEA. (**E**) Heatmap of the correlation between *CLEC2B* expression and the upregulated 20 DEGs. (**F**) Heatmap of the correlation between *CLEC2B* expression and the downregulated 20 DEGs. *p<0.05, **p< 0.01, ***p<0.001.

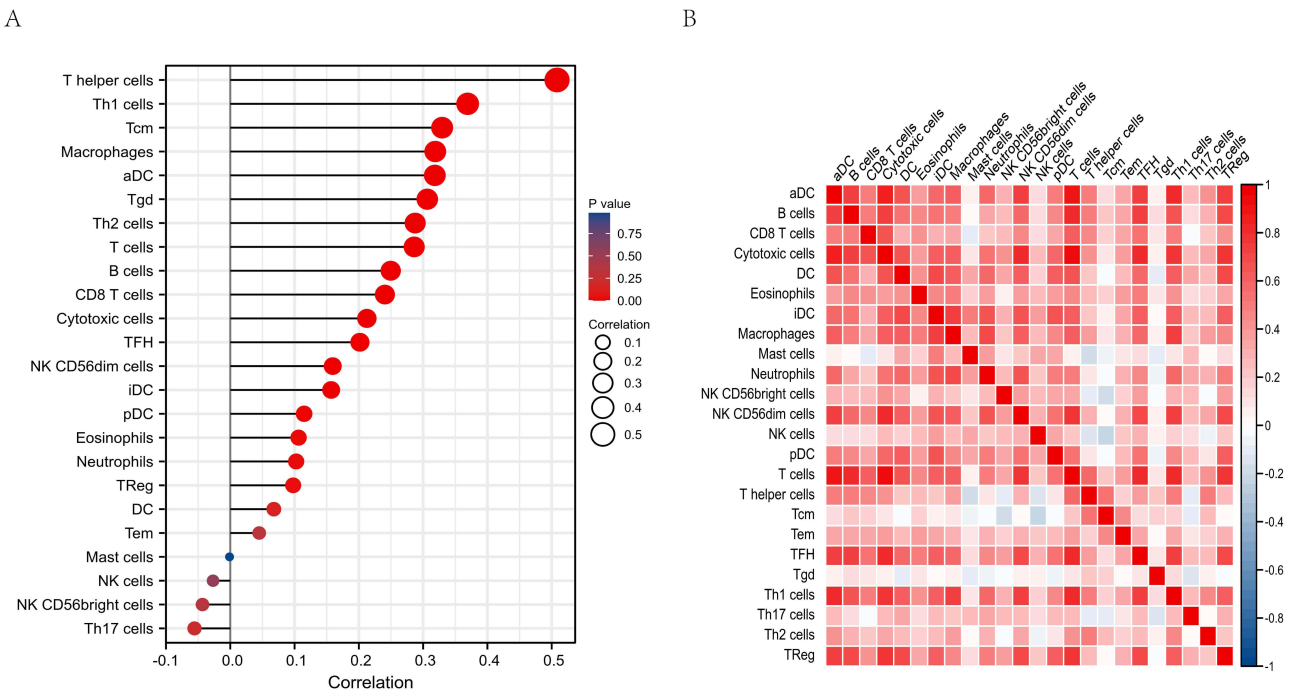

Figure 5 (**A**) Lollipop chart of *CLEC2B* expression levels in 24 immune cell types. (**B**) Heatmap of 24 infiltrating immune cells in SKCM and *CLEC2B* have the vital role in the immune infiltration in SKCM.

melanoma cells. Overall, these results suggest the possible effect of the expression of *CLEC2B* on the prognosis of melanoma.

GSEA on the *CLEC2B* coexpression data set revealed that *CLEC2B* was involved in the GPCR_LIGAND_BINDING and SIGNALING_BY_INTERLEUKINS pathways with positive correlation. These two pathways extensively affect cell proliferation, migration, differentiation, and metabolism. In melanoma, GPCR-mediated signal transduction is activated by combining paracrine factors and their receptors or by activating mutations to promote proliferation, invasion, and migration.[13,14] In addition, the interleukin signaling pathway is also implicated in the treatment of melanoma. Various interleukins inhibit melanoma development by regulating the tumor microenvironment's immunity and inflammation,[15–17] suggesting a possible role of *CLEC2B* in the immune response of melanoma.

ssGSEA and Spearman correlation to investigate the relationship between the expression of *CLEC2B* and the level of immune infiltration in melanoma revealed *CLEC2B* to be positively correlated with macrophages, B cells, neutrophils, T helper cells, Tregs, Th1 cells, and Th2 cells. B cells are activated in the germinal center of the tertiary lymphatic structure, increasing antigen presentation to T cells or killing tumor cells, thus producing beneficial clinical effects.[18] The macrophage infiltration of tumors is related to chemoresistance and poor prognosis in most cancers. Macrophages promote tumor malignancy and its occurrence by stimulating angiogenesis, increasing tumor cell invasion, migration, and intravascular infiltration, and inhibiting antitumor immunity.[19] The high ratio of circulating neutrophils to lymphocytes is a robust biomarker of poor clinical outcomes of various cancers.[20] Neutrophils may be reprogrammed to promote cancer in their microenvironment.[21] Treg cells adversely improve cancer progression by regulating immune monitoring and inhibiting the antitumor immune response. Increased Treg cell levels are related to disease progression and low survival in various cancers.[22] The cytokines and chemokines secreted by Th1 cells are the primary effector molecules downstream of immune cell signal transduction. Th2 cells conduct IL-4 signal transduction through STAT6 and upregulated transcription. IL-4 binds to the IL-4 receptor on immune cells, resulting in nuclear translocation, STAT6 phosphorylation, and GATA3 transcription factor expression, leading to 2-cell factor secretion and final tumor metastasis and growth.[23]

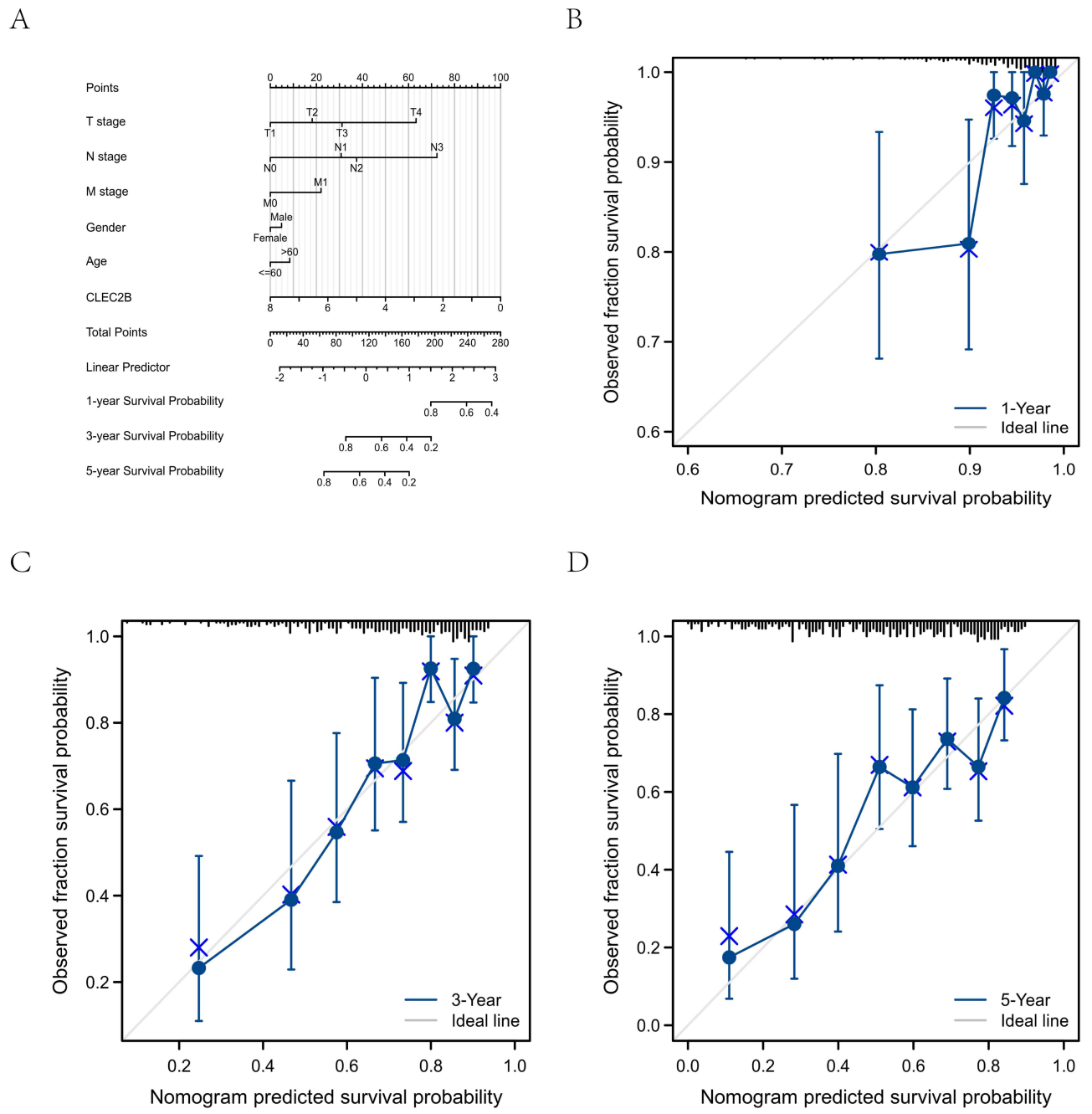

Figure 6 A nomogram and calibration curves for the prediction of one-, three-, and five-year overall survival rates of patients with melanoma. (**A**) A nomogram for the prediction of the one-, three-, and five-year overall survival rates of patients with melanoma. (**B**–**D**) Calibration curves of the nomogram prediction of one-, three-, and five-year overall survival rates of patients with melanoma.

Moreover, *CLEC2B* was negatively correlated with eosinophils, NK cells, and Th17 cells. Studies have shown that eosinophils have the regulatory function of other immune cell subsets in the tumor microenvironment or cytotoxic function to tumor cells, resulting in either antitumor or tumor-promoting effects.[24] Natural killer (NK) cells efficiently kill adjacent tumor cells, which increases in the context of targeted molecular therapy.[25] Th17 cells and related cytokines promote tumorigenesis or tumor inhibition, depending on the proportion of Th17 and Treg cells.[26] ssGSEA analysis showed how *CLEC2B* plays an essential role in regulating the immune infiltration of melanoma.

Finally, our results were validated using qPCR, immunohistochemistry, Western blot, cell colony formation assays, ethynyldeoxyuridine (Edu) analysis, and cell Invasion assays. We proved that the *CLEC2B* expression was significantly

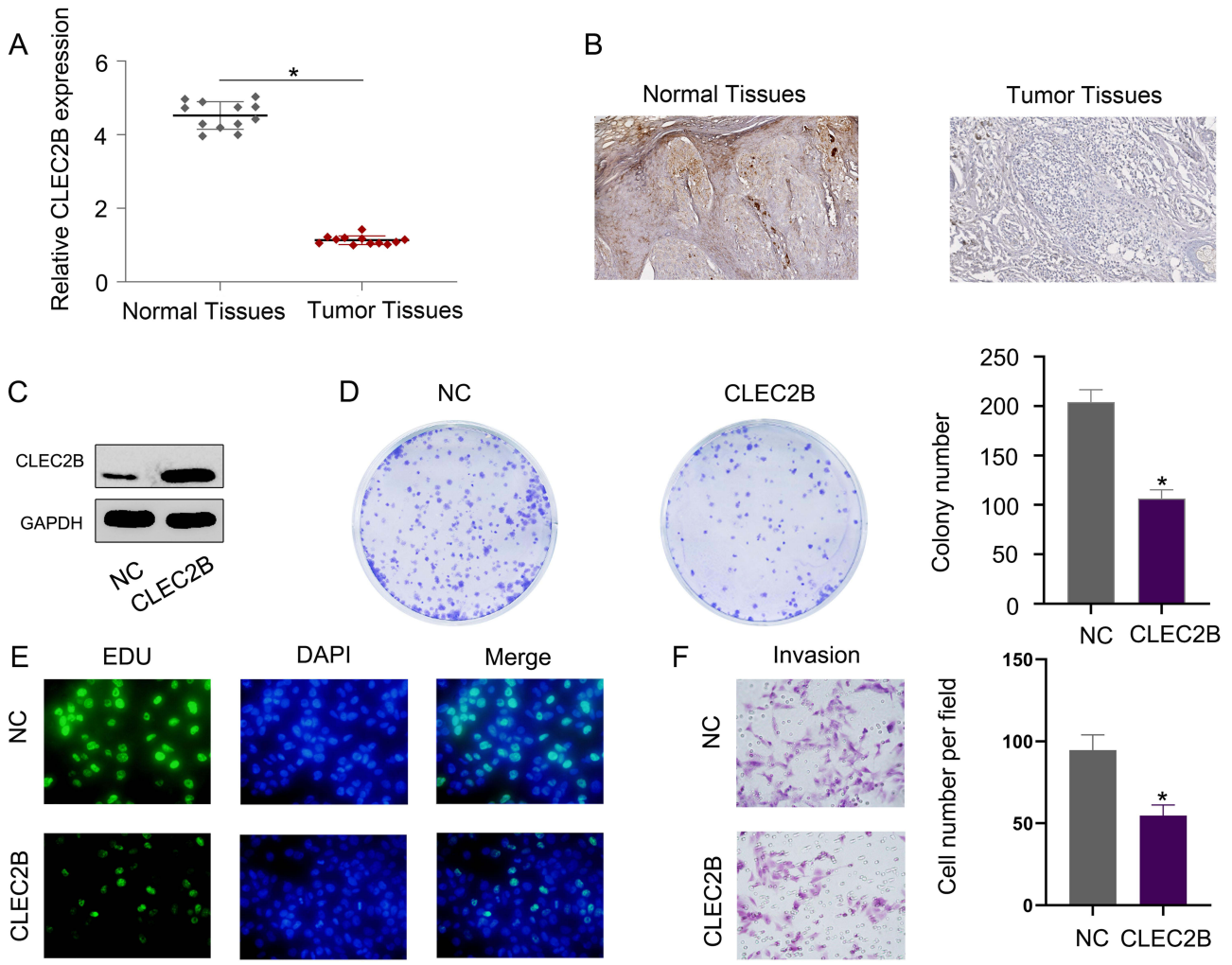

Figure 7 *CLEC2B* is expressed at low levels in melanoma. (**A**) The expression of *CLEC2B* mRNA was measured by qPCR. (**B**) The expression of *CLEC2B* proteins was measured by immunohistochemistry. (**C**) The expression of *CLEC2B* proteins was measured by Western blot. (**D**) Decreased proliferation of A375 cells transfected with *CLEC2B* vector. (**E**) The proliferation rate of A375 cells was measured by Edu assays. (**F**) Cell invasion assays were used to measure the effect of *CLEC2B* on cell invasion. *p<0.05.

lower than that in matched non-tumor tissues. In addition, the overexpression of *CLEC2B* decreased the proliferation and invasion ability of melanoma A375 cells. In conclusion, these results collectively suggested *CLEC2B* as a potential predictive tumor marker in patients with melanoma.

Our research has some shortcomings. First, the data has been used from public databases. Second, more volume of tissue samples from patients with melanoma is warranted to verify the potential of these biomarkers. Moreover, we need more experiments in cell lines and animal models to illustrate the mechanism to demonstrate the usefulness of the biomarkers.

Conclusion

Overall, our study highlights how the low expression of *CLEC2B* is associated with the prognosis of melanoma patients. In addition, *CLEC2B* was positively correlated with B cells, neutrophils, macrophages, T helper cells, Tregs, Th2 cells, and Th1 cells and negatively correlated with eosinophils, NK cells, and Th17 cells. Therefore, *CLEC2B* indicates a probable relation to immune infiltration, which can be used as a prognostic factor in melanoma patients.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

Before undergoing the treatment, all patients and their legal guardians provided written informed consent for their data to be published in the article. The study was conducted in accordance with the declaration of Helsinki and the study was also approved by the First Affiliated Hospital of China Medical University.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no conflicts of interest regarding the publication of this study.

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