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ORIGINAL RESEARCH

Immune Score Indicator for the Survival of Melanoma Patients Based on Tumor Microenvironment

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Background: Tumor microenvironment (TME) refers to the cellular environment where tumors exist, including immune cells, fibroblasts, stromal cells, chemokines, etc. TME is closely related to the prognosis of various tumors; nevertheless, limited studies have established predictive prognosis models based on TME. This work aims to construct a survival prediction model for melanoma patients based on TME.

Methods: Data of 482 melanoma patients were extracted from The Cancer Genome Atlas (TCGA) database. Based on the infiltration of immune cells (Immune score), stromal cells (Stromal score), and tumor purity (Estimate score), the "Estimate" algorithm was used to construct 3 scores for each patient. To identify the differentially expressed genes (DEGs), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using DAVID database and visualized using the R software. The STRING database was used to construct the protein-protein interaction (PPI) network and functional modules. *FGD2* expression was confirmed via Western Blotting and quantitative reverse transcription PCR (RT-qPCR) analyses.

Results: Patients with higher immune scores estimate scores showed better OS than those with lower scores. All three scores were related to age and primary tumor stage. Further, DEGs between patients with high immune/stromal scores and low immune/stromal scores were screened. Eventually, 10 down-regulated DEGs and 201 up-regulated DEGs were identified as TME associated genes. Out of these, the *FGD2* gene demonstrated close association with survival and was confirmed in the included melanoma patients.

Conclusion: In summary, TME is closely associated with the prognosis of melanoma patients. Besides, genes including *FGD2* promote the TME-mediated regulation of melanoma.

Keywords: tumor microenvironment, the cancer genome atlas, melanoma, *FGD2*

Introduction

The tumor microenvironment (TME) is the initial internal environment where tumor cells proliferate. The main cell types in TME include stromal cells (fibroblasts, endothelial cells, and many more) and immune cells (T cells, B cells, etc.). Accumulating studies indicate that the tumor microenvironment regulates tumor immunosuppression, drug resistance, tumor invasion, metastasis, and growth.[1,2]

In the past decades, significant treatment efforts of cancers targeted tumor cells; nevertheless, with the growing research importance of TME, there has been a gradual shift in the concept of cancer treatment. Unlike the adaptive mutation and

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acquired drug resistance produced by tumor cell accumulation, the immunotherapy approach targeting TME is stable As the most promising therapy in various cancers, immune checkpoint inhibitors (ICIs) are based on the immune escape in TME. Immune checkpoints are molecules producing costimulatory or inhibitory signals in the immune response, thus regulating the host immune response. Recent studies focused on the immune checkpoint PD-1 and its ligand PD-L1 signal axis. PD-L1, highly expressed in tumors, binds to PD-1 on the surface of T cells, inducing their depletion, thereby causing immune escape of tumor cells. Thus, the treatment of PD-1 or PD-L1 monoclonal antibodies to rescue the suppression of TME on T cells restores the normal activation of T cells.[3,4] Although the stromal cells in TME are not as important as immune cells in tumor immunotherapy, they regulate anti-tumor therapy. Commonly used methods minimize matrix hardness and fibrosis, thereby promoting immune cell infiltration and drug delivery.[5,6] Besides the therapeutic effect, TME mediates the prediction of cancer progression and response to immunotherapy. Reportedly, a Tumor Inflammation Signature (TIS) based on the expression of 18-gene signatures demonstrate satisfactory performance in predicting adaptive immune response.[7] In digestive system cancers, a prognostic immune score based on 22 types of immune cells shows satisfactory performance in predicting the survival of patients.[8]

Melanoma is a tumor produced by melanocytes in the skin and other organs with high malignancy. Its early diagnosis and treatment are crucial for prevention. Melanoma incidence has increased at an annual rate of about 3% to 7%, hence one of the fastest-growing malignant tumors in recent years. The primary risk factors for melanoma include a history of long-term sun exposure, UV exposure history, local chronic injury, or irritation. Meanwhile, melanoma is cancer with highly activated TME.[9] As such, our research seeks to understand the prediction role of TME in melanoma and molecular mechanisms underlying TME regulation.

Materials and Methods

Data Acquisition and Score Construction

The data were obtained from the TCGA (The results here are in whole or part based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga). database. Transcriptome data of 482 melanoma patients were identified and downloaded from the TCGA database using the R package "TCGA-Assembler". Relevant clinical characteristics were also obtained and are shown in Table 1.

The TME score was analyzed using the R package "Estimate"; this algorithm was also used to obtain the three scores, including stromal score, immune score, and estimate score. A higher stromal score and immune score indicated higher infiltration of stromal and immune cells. The estimated score was the sum of the stromal and immune scores. A higher estimate score indicated lower purity of tumor cells.

Screening of Differentially Expressed Genes (DEGs)

The R software "Limma" package was used to normalize the expression of mRNAs based on transcript data derived from the TCGA database. Further, the "DEGseq" package was utilized to screen the DEGs between different groups. $P<0.05$ and Fold-change>1.5 or Fold-change<-1.5 were set as the screening filters of DEGs.

Gene Ontology, KEGG Pathway, and Gene Set Enrichment Analyses

For Gene Ontology (GO) and KEGG pathway analyses, all the screened DEGs were uploaded to the Database for Annotation Visualization and Integrated Discovery (DAVID, david.ncifcrf.gov/) online tool. Besides, concrete pathways and annotations were obtained using the above-mentioned tool and further visualized using the R software. GSEA database (http://software.broadinstitute.org/gsea/index.jsp) built-in standard datasets were used for gene set enrichment analysis (GSEA) analysis.

Protein Extraction and Western Blotting Analyses

Melanoma tissues samples were extracted from patients diagnosed with melanoma by (three independent) experienced physicians (based on Chinese guidelines for diagnosis and treatment of melanoma). The tissues of each group were digested and lysed using the 100ul RIPA lysate. After complete lysis, the lysate was centrifuged at 4 °C for 15 minutes. The supernatants were collected as the total protein extract. Then, the BCA assay was performed to quantify the proteins (Thermo Fisher Scientific, Waltham, MA, USA). Exactly 20μg proteins were then loaded and separated by 10% SDS-PAGE gels. The proteins were transferred to the PVDF membranes (0.45 mm,

Table 1 Relevant clinical characteristics of melanoma patients

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-DA-A95Z	396	0	87	MALE	Stage IV	TX	M1a	N0
TCGA-FS-A1ZF	470	1	78	FEMALE	Stage IIC	T4b	M0	N0
TCGA-D3-A2J8	1992	1	48	MALE	Stage IB	T2a	M0	N0
TCGA-ER-A2NC	1333	1	50	MALE	Stage IB	T2a	M0	N0
TCGA-RP-A693	10	0	77	MALE	Stage IV	TX	M1c	NX
TCGA-EB-A82C	17	0	70	FEMALE	Stage IIC	T4b	M0	N0
TCGA-W3-AA1R	3379	1	71	MALE	Stage II	T3	M0	N0
TCGA-EE-A2GU	2884	0	65	FEMALE	Stage IA	T1a	M0	N0
TCGA-BF-A1PZ	853	0	71	FEMALE	Stage IIB	T4a	M0	N0
TCGA-FS-A1ZQ	4062	1	31	MALE	I/II NOS	TX	M0	N0
TCGA-EE-A20I	412	1	79	MALE	Stage IV	TX	M1c	N0
TCGA-FR-A44A	5299	0	29	FEMALE	Stage II	T3a	M0	N0
TCGA-D3-A3C6	1766	1	54	FEMALE	Stage IB	T2a	M0	N0
TCGA-EE-A3AE	1658	0	52	FEMALE	Stage IA	T1a	M0	N0
TCGA-GN-A262	4255	0	47	FEMALE	unknow	unknow	unknow	unknow
TCGA-ER-A2NE	613	1	39	MALE	Stage 0	Tis	M0	N0
TCGA-D3-A51R	1941	0	60	MALE	Stage IIA	T3a	M0	N0
TCGA-EB-A97M	414	0	66	MALE	Stage IIC	T4b	M0	N0
TCGA-WE-A8ZQ	1923	0	48	MALE	Stage IIA	T3a	M0	N0
TCGA-ER-A42K	394	1	40	FEMALE	Stage IIIC	T4b	M0	N3
TCGA-EE-A2MT	2166	0	45	MALE	Stage IB	T2a	M0	N0
TCGA-DA-A960	804	0	73	MALE	Stage IIB	T3b	M0	N0
TCGA-XV-AAZY	405	0	76	FEMALE	Stage IIIC	T4	M0	N3
TCGA-EE-A2M6	3932	0	61	MALE	Stage I	T1	M0	N0
TCGA-GN-A264	3587	1	60	MALE	unknow	unknow	unknow	unknow
TCGA-ER-A19O		1	56	MALE	Stage IIIB	T3b	M0	N1b
TCGA-D9-A6E9	301	0	75	FEMALE	Stage IIIA	T3a	M0	N1
TCGA-EE-A2MN	1446	1	58	MALE	Stage I	T2	M0	N0
TCGA-DA-A1I4	1093	1	51	MALE	Stage IIIC	T3b	M0	N2b
TCGA-EE-A3AB	3733	0	30	MALE	Stage III	T0	M0	N2a
TCGA-DA-A3F8	1319	0	39	MALE	Stage IIIB	T2a	M0	N2b
TCGA-BF-AAP6	325	0	55	MALE	Stage III	T4b	M0	N2
TCGA-FS-A1ZD	1628	1	63	MALE	Stage IIA	T2b	M0	N0
TCGA-D9-A4Z3	505	0	73	FEMALE	Stage IIIC	T4b	M0	N1b
TCGA-D3-A8GB	938	1	48	MALE	Stage IIIB	T3a	M0	N1b
TCGA-DA-A95V	2193	0	83	FEMALE	Stage IIC	T4b	unknow	N0
TCGA-EE-A2A5	1195	1	43	MALE	Stage IB	T2a	M0	N0
TCGA-D9-A3Z4	519	1	54	MALE	Stage IIIC	T4b	M0	N3
TCGA-FR-A8YE	3176	0	41	MALE	Stage IA	T1a	M0	N0
TCGA-EE-A2GC	2051	0	82	MALE	Stage IIB	T3b	M0	N0
TCGA-EE-A29G	2192	1	53	MALE	Stage IIIA	T4a	M0	N2a
TCGA-EE-A29S	1864	1	79	MALE	Stage IIA	T3a	M0	N0
TCGA-D3-A3MO	284	1	47	MALE	Stage III	TX	M0	N2c
TCGA-WE-A8ZO	2145	0	73	FEMALE	Stage IIIB	T3a	M0	N1b
TCGA-BF-A9VF	440	0	77	MALE	Stage IIC	T4b	M0	N0
TCGA-YD-A89C	210	0	43	FEMALE	Stage IA	T1a	M0	NX
TCGA-EE-A2GT	1365	0	77	MALE	Stage IIA	T3a	M0	N0
TCGA-HR-A5NC	0	0	90	FEMALE	unknow	T4	M0	NX
TCGA-ER-A19G	9188	0	48	FEMALE	unknow	unknow	M0	N0
TCGA-D3-A3CB	5065	0	39	MALE	I/II NOS	T2	M0	N0
TCGA-EB-A44P	741	0	58	FEMALE	Stage IIC	T4b	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-EB-A6R0	608	1	58	FEMALE	Stage IIC	T4b	M0	N0
TCGA-D3-A8GD	718	0	63	FEMALE	Stage IIIC	T4b	M0	N3
TCGA-ER-A197	424	1	83	FEMALE	Stage IIIB	T4b	M0	N1a
TCGA-EE-A29X	545	1	58	FEMALE	Stage IB	T2a	M0	N0
TCGA-YD-A9TA	1496	0	75	MALE	unknow	unknow	unknow	unknow
TCGA-EE-A2GE	5286	0	44	MALE	Stage I	T2	M0	N0
TCGA-EB-A57M	472	1	56	MALE	Stage IIIB	T4b	M0	N1
TCGA-EB-A85J	360	0	66	FEMALE	Stage IIB	T4a	M0	N0
TCGA-D3-A2JB	5110	1	70	FEMALE	Stage 0	Tis	M0	N0
TCGA-D3-A1QB	2912	0	75	FEMALE	Stage III	T0	M0	N2c
TCGA-D3-A2JE	841	1	75	FEMALE	Stage IIIC	TX	M0	N3
TCGA-DA-A3F5	6873	1	45	MALE	Stage I	T1a	M0	N0
TCGA-EE-A2M5	659	1	49	MALE	Stage I	T2	M0	N0
TCGA-D3-A2JA	3514	0	68	MALE	Stage IIIA	T2a	M0	N1a
TCGA-ER-A19H	4634	1	40	MALE	unknow	unknow	M0	N0
TCGA-EE-A3JA	1618	1	44	MALE	Stage IB	T2a	M0	N0
TCGA-FS-A4F8	5318	1	52	MALE	Stage I	T1	M0	N0
TCGA-WE-A8ZR	274	1	49	MALE	Stage IIIC	T4b	M0	N1b
TCGA-EE-A3JD	832	1	70	MALE	Stage III	TX	M0	N2b
TCGA-Z2-AA3S	2950	0	58	MALE	Stage IA	T1a	M0	N0
TCGA-ER-A198	1544	1	45	MALE	unknow	unknow	M0	NX
TCGA-ER-A42L	4533	0	49	MALE	Stage II	T3	M0	N0
TCGA-FR-A7U8	847	0	50	MALE	Stage IIIC	TX	M0	N3
TCGA-EB-A41B	291	0	76	FEMALE	Stage IIC	T4b	M0	N0
TCGA-EB-A44O	81	0	69	MALE	Stage IIB	T4a	M0	N0
TCGA-GN-A267	1960	1	38	MALE	Stage IIIA	T4a	M0	N1a
TCGA-EE-A2MI	6225	1	43	MALE	Stage IIB	T4	M0	N0
TCGA-ER-A19N	1341	1	47	MALE	unknow	unknow	unknow	unknow
TCGA-EB-A3XC	650	0	74	MALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2M7	877	1	66	MALE	Stage II	T3a	M0	N0
TCGA-EB-A42Z	441	0	49	MALE	Stage IIIC	T4b	M0	N1b
TCGA-D3-A8GI	1780	1	68	MALE	Stage IA	T1a	M0	N0
TCGA-FR-A728	583	0	54	FEMALE	Stage IIIB	T4b	M0	N2a
TCGA-D3-A8GQ	884	1	66	MALE	Stage II	T3	M0	N0
TCGA-DA-A1I5	4107	0	27	FEMALE	Stage IV	T1a	M1c	N0
TCGA-EE-A2GK	1665	0	46	FEMALE	Stage I	T1	M0	N0
TCGA-BF-A5EO	703	0	65	MALE	Stage IIC	T4b	M0	N0
TCGA-ER-A3EV	1429	1	55	MALE	Stage III	T4	M0	N0
TCGA-EB-A4OY	977	0	65	FEMALE	Stage IIIB	T4b	M0	N1a
TCGA-D3-A3CF	746	1	61	FEMALE	Stage IIIC	T4b	M0	N3
TCGA-XV-AB01	403	0	54	FEMALE	Stage II	T3	M0	NX
TCGA-EE-A29E	1940	0	54	MALE	Stage IIIB	T3a	M0	N1b
TCGA-DA-A1IC	2071	1	81	MALE	Stage IIIB	T3a	M0	N2c
TCGA-EE-A180	2889	1	69	MALE	Stage III	T4a	M0	N0
TCGA-D3-A5GS	553	0	58	MALE	Stage IV	T1b	M1c	N1b
TCGA-EE-A3AC	1948	0	47	MALE	Stage III	T0	M0	N2b
TCGA-FS-A1ZZ	822	1	54	FEMALE	Stage IIB	T3b	M0	N0
TCGA-WE-A8K1	1492	0	74	MALE	Stage IIIC	T3b	M0	N3
TCGA-FR-A8YC	1059	1	78	MALE	Stage IIB	T3b	M0	N0
TCGA-FS-A1Z7	237	1	19	MALE	Stage IIIC	T4b	M0	N1b

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-FS-A1ZK	728	1	68	MALE	Stage II	T4	M0	N0
TCGA-D3-A3CC	2644	0	69	FEMALE	Stage IIC	T4b	M0	N0
TCGA-WE-A8JZ	731	0	70	MALE	Stage IIIB	T4b	M0	N1a
TCGA-ER-A19M	1857	1	36	MALE	Stage IB	T2a	M0	N0
TCGA-FS-A1ZN	730	1	43	MALE	Stage IIIA	T4b	M0	N1a
TCGA-D3-A8GL	2711	1	43	MALE	Stage IIIB	T2a	M0	N1b
TCGA-EB-A5UN	1792	0	49	MALE	Stage IIC	T4b	M0	NX
TCGA-EE-A17X	907	1	54	MALE	Stage IA	T1a	M0	N0
TCGA-EE-A2GD	10346	1	58	FEMALE	Stage IIB	T4	M0	N0
TCGA-EB-A3XE	180	0	77	FEMALE	Stage IIA	T3a	M0	N0
TCGA-FR-A726	0	0	90	MALE	Stage IIC	T4b	M0	N0
TCGA-D9-A4Z2	190	1	50	MALE	Stage IIIC	T4b	M0	N3
TCGA-FS-A4FC	1655	1	75	FEMALE	Stage IIA	T3a	M0	N0
TCGA-XV-AAZW	393	1	62	FEMALE	Stage II	T4	M0	N0
TCGA-EB-A1NK	1039	0	48	MALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2GJ	2270	0	83	MALE	Stage IA	T1a	M0	N0
TCGA-EE-A20B	4070	0	66	FEMALE	Stage II	T3	M0	N0
TCGA-EE-A2MK	5487	0	18	FEMALE	Stage III	T4a	M0	N0
TCGA-GF-A3OT	301	0	58	FEMALE	Stage IIIC	T3	M0	N3
TCGA-FS-A1ZG	295	1	60	FEMALE	Stage IIIC	T4b	M0	N2b
TCGA-EE-A182	447	1	84	FEMALE	Stage IIIC	T4b	M0	N1b
TCGA-FS-A1Z3	636	1	72	FEMALE	Stage IV	TX	M1	N0
TCGA-ER-A19W	4507	1	48	FEMALE	unknow	unknow	unknow	unknow
TCGA-ER-A19P	4930	1	47	FEMALE	unknow	unknow	M0	N0
TCGA-ER-A19C	1487	1	77	MALE	Stage I	T2a	M0	NX
TCGA-BF-A5ES	490	0	76	FEMALE	Stage IIC	T4b	M0	N0
TCGA-D9-A1X3	551	0	63	MALE	unknow	T4b	unknow	N2b
TCGA-D3-A1Q7	4053	0	42	FEMALE	Stage IB	T1b	M0	N0
TCGA-EB-A4P0	326	1	82	MALE	Stage IIC	T4b	M0	N0
TCGA-D3-A3MV	1378	0	38	FEMALE	Stage IIIB	T2b	M0	N2a
TCGA-EE-A2GM	2296	0	70	FEMALE	Stage IIC	T4b	M0	N0
TCGA-FR-A7U9	571	0	63	FEMALE	Stage IIIC	T3b	M0	N3
TCGA-FS-A1ZB	1486	1	57	MALE	Stage II	T3a	M0	N0
TCGA-QB-A6FS	220	0	49	MALE	Stage IIIC	T0	M0	N3
TCGA-WE-A8ZY	1506	1	62	MALE	Stage IIA	T3a	M0	NX
TCGA-EE-A2MF	8174	1	39	FEMALE	Stage I	T2	M0	N0
TCGA-EB-A4XL	777	0	56	FEMALE	Stage IIC	T4b	M0	NX
TCGA-EE-A185	151	1	55	FEMALE	Stage IIIC	T4b	M0	N3
TCGA-GN-A4U3	3708	0	30	MALE	Stage III	T3a	M0	N1a
TCGA-EB-A3XB	796	0	63	MALE	Stage II	T4	M0	NX
TCGA-EE-A2A0	1424	1	77	FEMALE	Stage IIA	T3a	M0	N0
TCGA-DA-A3F3	319	1	52	MALE	Stage IIIB	T0	M0	N2b
TCGA-EE-A3AF	420	1	48	FEMALE	Stage IIIC	T0	M0	N3
TCGA-D3-A3BZ	3976	0	63	MALE	Stage IIB	T4a	M0	N0
TCGA-ER-A3ET	2829	1	64	FEMALE	Stage IIIA	T3a	M0	N1a
TCGA-RP-A694	21	0	71	MALE	Stage IV	TX	M1c	NX
TCGA-EE-A29C	2402	1	20	MALE	Stage IB	T2a	M0	N0
TCGA-EE-A2MH	516	1	66	MALE	Stage III	T4a	M0	N0
TCGA-EB-A5UM	779	0	48	FEMALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2MC	1871	1	73	MALE	Stage I	T2	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-BF-A1PV	14	0	74	FEMALE	Stage IIC	T4b	M0	N0
TCGA-GF-A6C8	62	0	62	FEMALE	Stage IIB	T3b	M0	NX
TCGA-XV-A9VZ	0	0	90	FEMALE	Stage II	T4	M0	N0
TCGA-GN-A4U7	317	1	56	FEMALE	Stage IIIC	T2b	M0	N3
TCGA-DA-A95W	1136	0	52	MALE	Stage IIIB	TX	M0	N1b
TCGA-D3-A3C3		0	unknow	FEMALE	I/II NOS	TX	M0	N0
TCGA-D3-A51K	1002	0	51	MALE	Stage IIIB	Tis	M0	N2b
TCGA-D3-A2JD	361	1	58	MALE	Stage IIIC	T4b	M0	N1b
TCGA-EB-A5VU	321	1	56	MALE	Stage IIIB	T4b	M0	N1
TCGA-WE-A8ZN	1794	0	57	MALE	Stage IIB	T4a	M0	NX
TCGA-FS-A1ZH	996	1	71	FEMALE	Stage IV	T3b	M1c	N2c
TCGA-D3-A51F	1695	0	51	MALE	Stage IIIC	T4b	M0	N1b
TCGA-BF-AAP2	405	0	62	MALE	Stage IIB	T3b	M0	N0
TCGA-ER-A19L	4000	1	35	MALE	unknow	unknow	unknow	unknow
TCGA-W3-A825	1917	1	60	FEMALE	Stage II	T3	M0	N0
TCGA-GN-A265	2948	0	53	MALE	unknow	unknow	unknow	unknow
TCGA-D3-A2JG	3453	1	30	FEMALE	Stage IIIA	T3a	M0	N1a
TCGA-EB-A5SG	2076	0	57	FEMALE	unknow	unknow	unknow	unknow
TCGA-W3-A828	3683	1	66	MALE	Stage II	T3	M0	N0
TCGA-FS-A1ZA	843	1	45	FEMALE	Stage IIIB	T4b	M0	N2c
TCGA-OD-A75X	9061	1	49	MALE	unknow	TX	M0	NX
TCGA-EB-A5SE	401	1	73	MALE	Stage IIB	T3b	M0	NX
TCGA-D3-A8GK	5177	0	45	MALE	Stage IIA	T3a	M0	N0
TCGA-BF-AAP4	335	0	61	MALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2MP	7563	0	34	FEMALE	Stage I	T2	M0	N0
TCGA-FW-A5DY	587	0	48	FEMALE	Stage III	T3	unknow	N1
TCGA-EB-A5KH	619	1	55	MALE	Stage III	T0	M0	N1
TCGA-EE-A2MM	5107	1	63	FEMALE	Stage I	T2	M0	N0
TCGA-EB-A5FP	454	1	65	FEMALE	Stage IV	T4b	M1b	NX
TCGA-FS-A1ZR	347	1	36	MALE	Stage II	T2	M0	N0
TCGA-WE-A8ZT	359	0	25	FEMALE	Stage IV	T3b	M1b	N1b
TCGA-D3-A2J6	1321	1	65	MALE	Stage IIB	T3b	M0	N0
TCGA-EE-A29Q	2030	1	70	FEMALE	Stage IIB	T3b	M0	N0
TCGA-EE-A2GS	2470	1	28	FEMALE	Stage IB	T2a	M0	N0
TCGA-D3-A1QA	2765	0	55	MALE	Stage IB	T2a	M0	N0
TCGA-D9-A1JW	111	0	82	MALE	unknow	T1a	M0	N2a
TCGA-ER-A2ND	710	1	57	FEMALE	Stage IIIC	T1b	M0	N3
TCGA-GN-A26C	821	1	77	MALE	Stage IIIC	T4b	M0	N2b
TCGA-EE-A2ME	3141	1	51	MALE	Stage I	T2	M0	N0
TCGA-WE-AAA3	651	0	84	FEMALE	Stage IIIC	T4b	M0	N2b
TCGA-BF-A3DM	601	0	63	MALE	Stage IIA	T2b	M0	N0
TCGA-D3-A3MU	1209	0	53	MALE	Stage IIIA	T3a	M0	N2a
TCGA-FR-A3YN	2828	0	44	MALE	Stage IB	T2a	M0	N0
TCGA-D9-A3Z3	678	0	39	FEMALE	Stage IIIB	T3a	M0	N1b
TCGA-EE-A3J7	1949	0	43	MALE	Stage I	T2	M0	N0
TCGA-D3-A2JF	1888	0	74	MALE	Stage IA	T1a	M0	N0
TCGA-EE-A29L	79	1	78	MALE	Stage IIIC	T4b	M0	N3
TCGA-FS-A4FB	813	1	46	FEMALE	Stage III	T2	M0	N1a
TCGA-D3-A5GN	4129	0	15	FEMALE	Stage I	T1	M0	N0
TCGA-XV-AAZV	412	0	56	FEMALE	Stage II	T4	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-D9-A149	1663	0	65	FEMALE	unknow	TX	M0	N1b
TCGA-DA-A1HW	1096	1	37	FEMALE	Stage IIIB	T1a	M0	N1b
TCGA-ER-A2NB	857	1	57	MALE	Stage IIIB	T4b	M0	N2
TCGA-D3-A1Q3	507	1	64	MALE	Stage IIC	T4b	M0	N0
TCGA-D3-A2J7	3136	1	67	MALE	Stage IIIC	T3b	M0	N1b
TCGA-ER-A2NG	1490	1	43	FEMALE	Stage IIIC	T3b	M0	N3
TCGA-FS-A1ZE	1413	1	40	MALE	Stage IIC	T4b	M0	N0
TCGA-DA-A95X	2249	0	62	MALE	Stage IB	T2a	M0	N0
TCGA-FS-A4FD	2454	1	39	MALE	Stage IIIC	T2	M0	N3
TCGA-GN-A26D	1460	1	72	FEMALE	Stage IIC	T4b	unknow	N0
TCGA-3N-A9WC	2022	0	82	MALE	Stage IIA	T2b	M0	NX
TCGA-D3-A1Q6	2184	1	55	MALE	Stage III	T4	M0	N1b
TCGA-ER-A2NF	877	1	53	MALE	Stage IIIB	T3b	M0	N3
TCGA-FS-A1Z0	6164	1	32	FEMALE	Stage IA	T1a	M0	N0
TCGA-BF-A1Q0	831	0	80	MALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2GR	1301	1	78	MALE	Stage II	T4	M0	N0
TCGA-WE-AAA0	1229	0	47	MALE	Stage IA	T1a	M0	N0
TCGA-EE-A29P	1716	0	73	FEMALE	Stage IIC	T4b	M0	N0
TCGA-WE-A8K5	1860	1	65	MALE	Stage IV	T2a	M1c	N3
TCGA-YG-AA3N	306	0	67	MALE	Stage IIC	T4b	M0	N0
TCGA-DA-A1IB	1235	1	69	FEMALE	Stage IIIC	T2b	M0	N2b
TCGA-EB-A430		1	83	MALE	Stage IIC	T4b	M0	N0
TCGA-BF-A1PX	282	1	56	MALE	Stage IIIB	T4b	M0	N2a
TCGA-FS-A4F2	1525	1	46	FEMALE	Stage IIC	T4b	M0	N0
TCGA-GN-A8LN	772	0	68	MALE	Stage IIC	T4b	M0	NX
TCGA-EB-A299	378	0	63	MALE	Stage IIA	T2b	M0	N0
TCGA-EE-A2MU	1620	0	71	MALE	Stage IA	T1a	M0	N0
TCGA-ER-A199	279	1	86	FEMALE	Stage IIIC	T4b	M0	N3
TCGA-BF-AAP8	447	0	58	MALE	Stage IIC	T4b	M0	N0
TCGA-ER-A194	1354	1	77	MALE	unknow	unknow	M0	N0
TCGA-EB-A5UL	891	0	71	MALE	Stage III	TX	M0	N1
TCGA-EE-A29H	1966	0	59	FEMALE	Stage IA	T1a	M0	N0
TCGA-D3-A51N	688	0	56	FEMALE	Stage IV	T0	M1c	N3
TCGA-EB-A5SH	1643	0	60	FEMALE	Stage III	T4	M0	N0
TCGA-EE-A2MJ	2927	1	60	MALE	Stage III	T4b	M0	N0
TCGA-RP-A690	6	0	66	FEMALE	unknow	unknow	unknow	unknow
TCGA-EE-A29B	2588	1	67	MALE	Stage IIB	T3b	M0	N0
TCGA-QB-AA9O	549	1	73	MALE	Stage IIIC	TX	M0	N3
TCGA-EB-A550	264	1	75	FEMALE	Stage IIC	T4b	M0	NX
TCGA-FS-A1ZJ	1441	1	75	FEMALE	Stage I	T2	M0	N0
TCGA-EB-A3HV	39	0	37	MALE	Stage IIC	T4b	M0	N0
TCGA-3N-A9WB	518	1	71	MALE	Stage IA	T1a	M0	NX
TCGA-W3-AA21	3195	1	26	MALE	Stage I	T2	M0	N0
TCGA-D3-A8GC	2421	1	48	MALE	Stage IIIC	TX	M0	N3
TCGA-FS-A1ZT	1617	0	55	MALE	Stage III	T2	M0	N1b
TCGA-EE-A181	1026	1	82	FEMALE	Stage II	T3	M0	N0
TCGA-D3-A8GP	4638	0	77	MALE	Stage III	T2	M0	N2c
TCGA-BF-AAP0	454	0	40	FEMALE	Stage IV	T4	M1	NX
TCGA-DA-A1I8	1640	1	63	FEMALE	Stage IIC	T4b	M0	N0
TCGA-D3-A5GO	4195	0	61	MALE	Stage II	T4	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-D3-A51T	818	0	59	FEMALE	Stage IIIC	T4b	M0	N1b
TCGA-ER-A19F	802	1	82	MALE	unknow	unknow	M0	N0
TCGA-EB-A44R	315	1	52	MALE	Stage IIIB	TX	M0	N2b
TCGA-FS-A1Z4	854	1	62	MALE	Stage I	T1	M0	N0
TCGA-FR-A3YO		0	unknow	FEMALE	I/II NOS	T2	M0	N0
TCGA-BF-AAP1	409	0	86	MALE	Stage IIC	T4b	M0	N0
TCGA-D9-A3Z1	468	1	66	MALE	Stage IIIC	T2a	M0	N3
TCGA-EB-A6L9	1109	0	55	MALE	Stage IIIC	TX	M0	N3
TCGA-ER-A42H	426	1	76	MALE	unknow	unknow	unknow	unknow
TCGA-ER-A19S	1505	0	81	FEMALE	unknow	unknow	unknow	unknow
TCGA-ER-A1A1	3196	0	58	MALE	Stage IIIC	TX	M0	N3
TCGA-DA-A111	6768	0	55	MALE	Stage III	T0	M0	N2a
TCGA-D3-A3C1		0	unknow	MALE	I/II NOS	TX	M0	N0
TCGA-EB-A82B	390	0	58	FEMALE	Stage III	T4b	M0	N2
TCGA-EE-A29A	1927	1	68	MALE	Stage IIIA	T3a	M0	N1a
TCGA-EB-A431	568	0	34	MALE	Stage IIC	T4b	M0	N0
TCGA-FS-A4F5	874	1	77	FEMALE	Stage IB	T2a	M0	N0
TCGA-EB-A42Y	721	1	73	FEMALE	Stage IIC	T4b	M0	N0
TCGA-D3-A2JK	368	1	24	MALE	Stage IIIC	T4b	M0	N2b
TCGA-D3-A51J	4414	0	19	MALE	Stage III	T0	M0	N1b
TCGA-WE-A8ZX	1089	0	45	MALE	Stage IIIB	TX	M0	N1b
TCGA-EE-A29T	11252	0	51	FEMALE	unknow	TX	M0	NX
TCGA-ER-A19J	196	1	54	MALE	Stage IV	TX	M1	N3
TCGA-W3-AA1W	6666	0	64	MALE	Stage II	T3	M0	N0
TCGA-BF-A1PU	387	0	46	FEMALE	Stage IIC	T4b	M0	N0
TCGA-EB-A3XF	278	0	57	MALE	Stage IIC	T4b	M0	N0
TCGA-GN-A4U9	673	1	71	MALE	Stage IIIC	T2b	M0	N3
TCGA-EB-A4IS	774	0	77	MALE	Stage IIB	T3b	M0	NX
TCGA-FS-A4F0	2367	0	67	FEMALE	Stage IIB	T4a	M0	N0
TCGA-BF-A5EP	335	0	75	FEMALE	Stage IIIC	T4b	M0	N3
TCGA-EB-A41A	0	0	90	MALE	Stage IIC	T4b	M0	N0
TCGA-ER-A193	955	1	62	MALE	Stage IIB	T3b	M0	N0
TCGA-D3-A2JO	2010	0	50	FEMALE	Stage IIIC	TX	M0	N3
TCGA-LH-A9QB	11217	0	24	FEMALE	unknow	unknow	unknow	unknow
TCGA-D3-A3CE	1832	1	74	FEMALE	Stage III	T0	M0	N1b
TCGA-D3-A5GL	3826	0	74	MALE	Stage IB	T2a	M0	N0
TCGA-EE-A3J5	1124	1	71	MALE	Stage III	T4a	M0	N1
TCGA-EE-A29D	425	1	87	MALE	Stage IIIC	T3b	M0	N1b
TCGA-EE-A2A6	2620	0	43	MALE	Stage IA	T1a	M0	N0
TCGA-D3-A51E	5318	0	39	FEMALE	I/II NOS	T2	M0	N0
TCGA-EE-A2GH	6699	0	34	MALE	Stage I	T2	M0	N0
TCGA-EE-A2A2	1814	0	71	MALE	Stage IIIC	T4b	M0	N1b
TCGA-GN-A9SD	1807	1	59	FEMALE	Stage IA	T1a	M0	NX
TCGA-EE-A183	818	1	48	MALE	Stage 0	Tis	M0	N0
TCGA-EE-A17Z	263	1	57	MALE	Stage IIB	T4a	M0	N0
TCGA-GF-A6C9	480	0	78	MALE	Stage IIIB	unknow	unknow	unknow
TCGA-D9-A4Z5	218	0	68	MALE	Stage IIB	T4a	M0	N0
TCGA-D3-A1Q1	504	1	79	FEMALE	Stage IIIC	T1b	M0	N3
TCGA-EB-A3Y7	326	1	86	FEMALE	Stage IIIB	T3a	M0	N2c
TCGA-ER-A3PL	1010	0	30	MALE	Stage IV	T3b	M1a	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-D3-A5GU	3808	0	36	MALE	Stage IB	T1b	M0	N0
TCGA-EE-A2GP	423	1	80	MALE	Stage IIIB	T4b	M0	N1a
TCGA-FS-A1YW	6598	1	52	MALE	Stage IB	T1b	M0	N0
TCGA-D3-A2JN	2022	1	46	FEMALE	Stage III	T0	M0	N1b
TCGA-FS-A1ZC	10870	1	51	MALE	I/II NOS	TX	M0	N0
TCGA-EE-A2MS	4942	0	72	MALE	Stage II	T3a	M0	N0
TCGA-W3-A824	6940	0	63	MALE	Stage I	T2	M0	N0
TCGA-FS-A1ZW	1505	0	65	MALE	Stage IIIB	T2b	M0	N1a
TCGA-D9-A1JX	216	1	80	FEMALE	unknow	TX	M0	NX
TCGA-EE-A3JB	6138	0	60	FEMALE	Stage III	T3a	M0	N1
TCGA-EE-A2GI	1482	0	39	MALE	Stage IA	T1a	M0	N0
TCGA-EE-A3JH	4086	0	54	MALE	Stage IB	T2	M0	N0
TCGA-D3-A2JP	1812	0	37	MALE	Stage IIIC	T0	M0	N3
TCGA-ER-A19Q	1548	1	37	FEMALE	unknow	unknow	M0	N0
TCGA-FR-A8YD	1103	1	56	FEMALE	Stage IIC	T4b	M0	N0
TCGA-BF-A3DJ	464	0	36	FEMALE	Stage IIIB	T4b	M0	N1
TCGA-EE-A20F	2785	0	53	MALE	Stage I	T1	M0	N0
TCGA-EE-A3AG	1265	1	25	MALE	Stage III	T0	M0	N2c
TCGA-EE-A29V	787	1	85	MALE	Stage IIIC	T3b	M0	N1b
TCGA-EE-A20H	5118	1	56	MALE	Stage I	T2	M0	N0
TCGA-ER-A19E	396	1	36	FEMALE	Stage IB	T2a	M0	N0
TCGA-GN-A4U5	1156	0	61	FEMALE	Stage IB	T2a	M0	NX
TCGA-EE-A3J3	5237	1	42	MALE	Stage IB	T2	M0	N0
TCGA-FW-A3TU	1691	1	72	FEMALE	unknow	unknow	unknow	unknow
TCGA-EE-A2MD	1438	1	52	MALE	Stage II	T3a	M0	N0
TCGA-EE-A2GB	1803	0	51	MALE	Stage IIIB	T2b	M0	N1a
TCGA-XV-A9W5	392	0	51	MALE	I/II NOS	T2	M0	N0
TCGA-GN-A8LL	650	1	68	FEMALE	Stage IIC	T4b	M0	NX
TCGA-BF-A5ER	327	0	63	MALE	Stage IIC	T4b	M0	N0
TCGA-BF-AAOX	444	0	83	MALE	Stage IIC	T4b	M0	N0
TCGA-EB-A44Q	422	0	51	FEMALE	Stage IIIC	TX	M0	N3
TCGA-BF-AAP7	318	0	76	FEMALE	Stage IIC	T4b	M0	N0
TCGA-Z2-A8RT	839	0	42	FEMALE	Stage IIB	T3b	M0	N0
TCGA-D3-A1Q8	854	1	33	MALE	Stage IV	T0	M1b	N3
TCGA-EE-A2M8	601	1	54	FEMALE	Stage III	T3a	M0	N1
TCGA-EB-A553	226	0	62	MALE	Stage IIC	T4b	M0	N0
TCGA-BF-A3DN	717	0	81	FEMALE	Stage IIIC	T3b	M0	N3
TCGA-ER-A3ES	7514	1	25	MALE	unknow	unknow	unknow	unknow
TCGA-EB-A85I	362	0	66	MALE	Stage IIC	T4b	M0	N0
TCGA-FR-A69P	478	0	34	FEMALE	Stage IIIC	TX	unknow	N3
TCGA-EE-A3AD	875	1	50	MALE	Stage III	T0	M0	N1b
TCGA-EB-A24D	645	0	72	MALE	Stage IIIB	T4a	M0	N2b
TCGA-D9-A4Z6	561	1	54	MALE	Stage IIIC	T3b	M0	N1b
TCGA-FR-A3R1	685	0	69	MALE	Stage IIC	T4b	M0	N0
TCGA-FS-A1ZY	824	1	71	MALE	Stage IIB	T3b	M0	N0
TCGA-FW-A3I3	531	0	59	FEMALE	Stage IV	unknow	M1	N0
TCGA-EB-A4IQ	636	1	42	FEMALE	Stage IIIB	T4b	M0	N1
TCGA-ER-A19K	469	1	79	FEMALE	Stage IIC	T4b	M0	N0
TCGA-FW-A3TV	411	0	57	FEMALE	Stage IIIB	T1	M0	N2b
TCGA-EE-A2GN	3106	1	67	MALE	Stage IIA	T2b	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-FR-A7UA	1164	0	65	FEMALE	Stage IB	T2a	M0	N0
TCGA-DA-A3F2	1032	1	55	MALE	Stage IIIB	T4a	M0	N2b
TCGA-Z2-AA3V	486	0	57	FEMALE	Stage IA	T1a	M0	N0
TCGA-FR-A2OS	368	1	49	FEMALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2MQ	1315	1	70	FEMALE	Stage IIIA	T3a	M0	N2a
TCGA-FR-A729	6716	0	38	FEMALE	Stage I	T1	M0	N0
TCGA-FS-A1YY	6953	1	55	FEMALE	Stage IIA	T3a	M0	N0
TCGA-BF-A3DL	769	0	84	FEMALE	Stage IIIB	T3b	M0	N2
TCGA-YG-AA3P	439	0	63	FEMALE	Stage IIB	T4a	M0	N0
TCGA-DA-A1I7	2703	0	62	MALE	Stage IIIB	T0	M0	N2b
TCGA-WE-A8K4	614	0	85	MALE	Stage IIB	T4a	M0	NX
TCGA-EE-A2MR	4088	0	61	MALE	Stage I	T2	M0	N0
TCGA-EB-A3Y6	126	0	56	FEMALE	Stage IIC	T4b	M0	N0
TCGA-BF-AAOU	476	0	73	FEMALE	Stage IIC	T4b	M0	N0
TCGA-ER-A19D	383	1	46	FEMALE	Stage IB	T2a	M0	N0
TCGA-D3-A1Q9	961	1	72	MALE	Stage IIIB	T4b	M0	N2a
TCGA-D3-A2JC	2639	0	53	FEMALE	Stage III	T0	M0	N2b
TCGA-DA-A1HV	2329	0	75	FEMALE	Stage IIIB	T0	M0	N2b
TCGA-EE-A2GL	2423	0	40	FEMALE	Stage IIA	T3a	M0	N0
TCGA-ER-A19T	270	1	51	MALE	Stage IV	T4a	M1a	N3
TCGA-D3-A2JH	1280	0	68	MALE	Stage IB	T1b	M0	N0
TCGA-GN-A268	1910	1	83	FEMALE	Stage IIB	T4a	M0	N0
TCGA-WE-A8K6	546	0	79	MALE	Stage IIIB	TX	M0	N1b
TCGA-GF-A2C7	21	0	48	MALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2ML	6590	1	35	MALE	Stage II	T3a	M0	N0
TCGA-D3-A1Q4	3408	0	53	FEMALE	Stage IIIC	T2b	M0	N1b
TCGA-D3-A51G		0	unknow	MALE	Stage 0	Tis	M0	N0
TCGA-EE-A2A1	3527	0	46	MALE	Stage IB	T2a	M0	N0
TCGA-GN-A269	170	1	70	MALE	Stage IIIC	T4b	M0	N3
TCGA-D3-A8GN	4897	0	27	FEMALE	I/II NOS	TX	M0	N0
TCGA-D3-A8GJ	7342	0	18	MALE	Stage II	T3	M0	N0
TCGA-D3-A3ML	422	1	70	MALE	Stage IIIA	T3a	M0	N2a
TCGA-W3-AA1Q	2101	1	57	MALE	Stage III	TX	M0	N1
TCGA-HR-A2OG	7	0	50	FEMALE	unknow	unknow	unknow	unknow
TCGA-EE-A3AA	3781	0	47	MALE	Stage III	T0	M0	N2a
TCGA-FS-A4F4	2028	1	64	MALE	Stage II	T3a	M0	N0
TCGA-EE-A29M	1729	0	33	FEMALE	Stage IB	T2a	M0	N0
TCGA-WE-AAA4	760	0	56	FEMALE	Stage IIIC	TX	M0	N3
TCGA-DA-A1I2	5370	1	45	MALE	Stage III	T4b	M0	N2b
TCGA-WE-A8ZM	3082	0	70	MALE	Stage IIIB	TX	M0	N1b
TCGA-FS-A1ZU	808	1	70	FEMALE	Stage IIC	T4b	M0	N0
TCGA-D3-A2JL	5219	0	43	FEMALE	I/II NOS	TX	M0	N0
TCGA-EB-A4OZ	620	0	41	FEMALE	Stage IIIC	T4a	M0	N3
TCGA-ER-A196	1785	0	64	FEMALE	Stage IIC	T4b	M0	N0
TCGA-FW-A5DX	640	0	71	MALE	Stage IIIC	T4a	unknow	N3
TCGA-EB-A6QZ	352	1	76	FEMALE	Stage IIA	T3a	M0	N0
TCGA-D3-A8GS	3564	1	52	MALE	Stage I	T1	M0	N0
TCGA-DA-A95Y	430	1	68	MALE	Stage IIC	T4b	M0	N0
TCGA-EE-A2GO	3857	0	66	FEMALE	Stage II	T3b	M0	N0
TCGA-EE-A29W	5932	0	42	MALE	Stage 0	Tis	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-EE-A29N	566	1	78	MALE	I/II NOS	TX	M0	N0
TCGA-EB-A551	590	0	78	FEMALE	Stage IIIC	T4b	M0	N2b
TCGA-D3-A2J9	723	1	75	MALE	Stage IIIC	T4b	M0	N3
TCGA-EE-A3JE	1562	0	75	MALE	Stage IIIB	T3b	M0	N1a
TCGA-EE-A17Y	828	1	69	MALE	Stage IIIB	T3b	M0	N1a
TCGA-D3-A3C8	1409	0	58	FEMALE	Stage IIIC	TX	M0	N3
TCGA-D3-A3C7	1429	0	57	FEMALE	Stage III	T0	M0	N1b
TCGA-EE-A2MG	3139	1	23	MALE	Stage I	T2	M0	N0
TCGA-D3-A1Q5	3424	1	60	MALE	I/II NOS	TX	M0	N0
TCGA-EB-A24C	632	0	56	MALE	unknow	T4b	M0	NX
TCGA-XV-A9W2	417	0	81	MALE	Stage I	T1	M0	N0
TCGA-D9-A6EC	2359	0	56	MALE	Stage IIIA	T3a	M0	N1
TCGA-BF-A5EQ	323	0	63	MALE	Stage IIC	T4b	M0	N0
TCGA-W3-AA1V	1280	1	63	MALE	Stage II	T3	M0	N0
TCGA-FS-A1ZP	2273	1	52	MALE	Stage II	T3	M0	N0
TCGA-GN-A4U4	1197	0	73	MALE	Stage IIA	T2b	M0	NX
TCGA-D3-A8GE	804	0	26	MALE	Stage IV	TX	M1b	N0
TCGA-EE-A3J8	1044	1	59	MALE	Stage IIIA	T4a	M0	N1a
TCGA-EB-A5SF	369	1	78	FEMALE	Stage IIC	T4b	M0	NX
TCGA-GF-A769	1070	1	39	MALE	Stage IIC	T4b	M0	NX
TCGA-D3-A8GM	3259	1	73	MALE	Stage IIB	T3b	M0	N0
TCGA-FS-A1ZM	3080	0	74	MALE	Stage III	T2	M0	N2c
TCGA-YD-A9TB		0	unknow	FEMALE	unknow	unknow	unknow	unknow
TCGA-EE-A3AH	4222	1	30	MALE	Stage II	T3b	M0	N0
TCGA-GN-A266	308	1	45	MALE	unknow	unknow	unknow	unknow
TCGA-EB-A5VV	214	0	74	FEMALE	Stage IIIB	T3b	M0	N1
TCGA-EB-A3XD	1160	0	53	FEMALE	Stage IIC	T4b	M0	NX
TCGA-EE-A29R	440	0	48	FEMALE	Stage IIIC	T3b	M0	N1b
TCGA-3N-A9WD	395	1	82	MALE	Stage IIIA	T2a	M0	N1a
TCGA-EE-A20C	4601	1	59	MALE	Stage 0	Tis	M0	N0
TCGA-D3-A8GV	5101	1	25	MALE	I/II NOS	TX	M0	N0
TCGA-ER-A19A	2365	0	79	MALE	Stage IV	TX	M1	N0
TCGA-ER-A2NH	1264	0	49	MALE	Stage IIIC	T3a	M0	N3
TCGA-EE-A3J4	3869	1	72	MALE	Stage II	T3a	M0	N0
TCGA-D9-A148	4609	0	40	MALE	unknow	TX	M1b	N3
TCGA-FS-A1ZS	4526	0	54	MALE	Stage I	T2	M0	N0
TCGA-ER-A19B	2993	1	42	MALE	unknow	TX	M0	N0
TCGA-GN-A8LK	1524	1	70	MALE	Stage IB	T1b	unknow	NX
TCGA-W3-AA1O	122	1	85	MALE	Stage III	TX	M0	N2
TCGA-RP-A6K9		0	unknow	FEMALE	unknow	unknow	unknow	unknow
TCGA-WE-AA9Y	370	0	37	MALE	Stage IIIC	T2a	M0	N3
TCGA-EE-A3JI	4648	1	48	MALE	Stage I	T2	M0	N0
TCGA-EB-A6QY	382	0	71	MALE	Stage IIC	T4b	M0	N0
TCGA-GF-A4EO	591	0	74	FEMALE	Stage IIIC	T0	M0	N3
TCGA-D3-A5GR	5424	0	23	FEMALE	Stage III	T1b	M0	N1
TCGA-D9-A6EG	698	1	56	MALE	Stage IIIA	T4a	M0	N1
TCGA-DA-A1I0	620	1	63	MALE	Stage IV	T4b	M1a	N3
TCGA-FW-A3R5	1124	0	68	MALE	Stage III	TX	M0	N2
TCGA-D3-A5GT	487	0	43	MALE	Stage IIIC	T2b	M0	N3
TCGA-EE-A184	2073	1	72	MALE	Stage IB	T2a	M0	N0

(*Continued*)

Table 1 (Continued).

Id	Futime	Fustat	Age	Gender	Stage	T	M	N
TCGA-YG-AA3O	1154	1	62	MALE	unknow	unknow	unknow	unknow
TCGA-GN-A4U8	1487	0	51	MALE	unknow	unknow	unknow	unknow
TCGA-RP-A695		0	unknow	MALE	Stage IV	TX	M1c	NX
TCGA-FS-A4F9	1035	0	80	MALE	Stage IIIC	T4b	M0	N3
TCGA-EB-A44N	205	1	59	MALE	Stage IIC	T4b	M0	N0
TCGA-D9-A6EA	766	0	70	MALE	Stage IIIC	T4a	M0	N3
TCGA-GN-A263	467	1	24	MALE	Stage IV	T4b	M1c	N3
TCGA-EB-A51B	931	0	53	MALE	Stage IIC	T4b	M0	NX
TCGA-D3-A3MR	3151	0	42	MALE	Stage III	T0	M0	N1b
TCGA-GN-A26A	988	1	63	FEMALE	Stage IIIA	T3a	M0	N1a
TCGA-DA-A1HY	4407	0	42	MALE	Stage III	T2b	M0	N1
TCGA-D3-A8GO		1	unknow	FEMALE	I/II NOS	T2	M0	N0
TCGA-FS-A1YX	1478	1	39	FEMALE	Stage I	T2	M0	N0
TCGA-HR-A2OH	2004	1	46	FEMALE	Stage IIIB	T3b	M0	N2a
TCGA-D3-A51H	1714	0	60	MALE	Stage IIIC	T1b	M0	N3
TCGA-ER-A195	1078	1	46	MALE	unknow	TX	M0	N0
TCGA-IH-A3EA	524	0	61	MALE	Stage IIC	T4b	M0	N0
TCGA-D3-A8GR	3943	1	54	FEMALE	Stage 0	Tis	M0	N0
TCGA-DA-A1IA	2005	1	32	FEMALE	Stage IIIB	T2a	M0	N1b

Merck Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% bovine albumin (BSA) at room temperature for 1 h, then overnight incubated with *FGD2* and *GAPDH* rabbit polyclonal antibodies (1:4000, Abcam, UK) at 4°C. The secondary antibodies were used at a dilution of 1:4000 and incubated at room temperature for 1 h. Eventually, the bands were visualized using the ECL reagents (Merck Millipore).

RNA Extraction, Reverse Transcription, and Quantitative PCR (RT-qPCR)

Melanoma tissues samples were extracted from patients diagnosed with melanoma by (three independent) experienced physicians (based on Chinese guidelines for diagnosis and treatment of melanoma). The total RNA was extracted using the Trizol Reagent (Invitrogen) from tissues based on the manufacturer's instructions (Trizol, chloroform, and isopropanol were added in turn; the supernatant was centrifuged and quantified by absorbance value of 260nm and stored at - 80 °C). Subsequently, a reverse transcription kit (Takara Bio, Inc., Otsu, Japan) was used to reverse-transcribe RNA into cDNA in a 20ul system. Subsequently, the cDNA was used as a template, detected by the SYBR Green (Takara Bio) and ABI 7900HT Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers used are shown in Table S1. The comparative cycle threshold values (2-ΔΔCt) were used to analyze the final results.

Statistical Analysis

The IBM SPSS 19.0 software was used for statistical analyses of all experimental data. Data were expressed as mean ± sd. Graphpad Prism version 7.0 software was used to visualize the statistical results. *T*-test was used to compare data between two groups, whereas One-way ANOVA was used to compare data between multiple groups; LSDt-test was used for pairwise comparison within the group. Overall Survival (OS) curves were drawn through the Kaplan–Meier analysis. The difference with $P < 0.05$ was considered statistically significant.

Results

Construction of Tumor Microenvironment Score

In total, transcript data of 482 melanoma patients were extracted from the TCGA-SKCM database; the R software "Limma" package was used for data standardization. The "Estimate" package was utilized to obtain three TME scores for each patient, respectively. Notably, higher stromal and immune scores indicated higher infiltration of stromal cells and immune cells. Estimate scores were the sum of the stromal score and immune score. A higher estimate score indicated lower purity of tumor cells. Patients with higher immune and

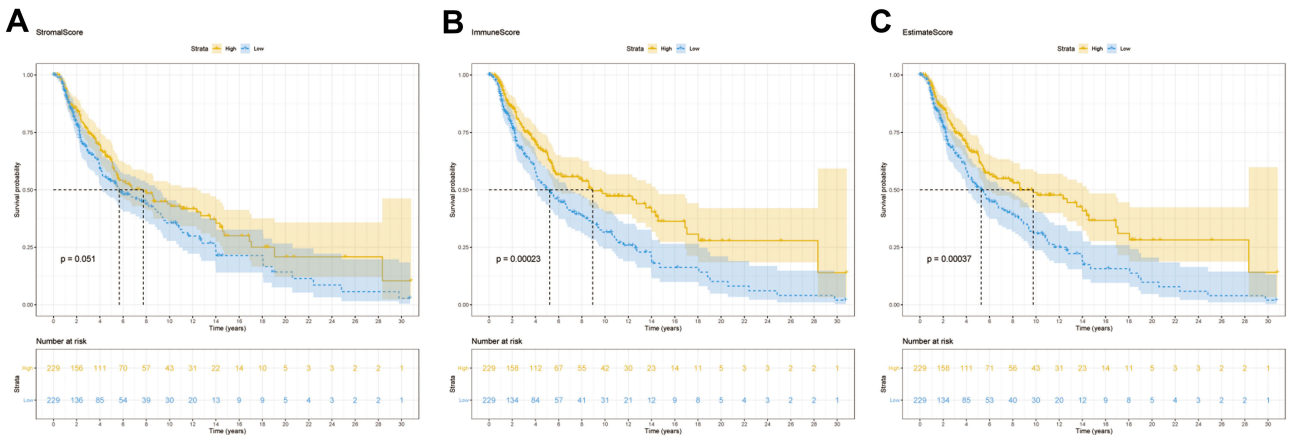

Figure 1 Construction of tumor microenvironment score. (**A**) Kaplan-Meier analysis for the survival of patients based on the stromal score; (**B**) Kaplan-Meier analysis for the survival of patients based on the immune score; (**C**) Kaplan-Meier analysis for the survival of patients based on the estimated score. Patients were divided by the median of all these three score systems.

estimate scores displayed better OS than those with lower scores (Figure 1).

Tumor Microenvironment Score is Associated with Age and Tumor Size

The relationship between TME scores (stromal score: Figure 2A, immune score: Figure 2B, estimate score: Figure 2C) and clinical features of patients (age, gender, pathological stages, etc.) was analyzed. Interestingly, higher TME scores were closely related to younger age (Figure 2 left panel) and earlier primary tumor stage (Figure 2 right panel).

Screening for Tumor Microenvironment Associated Genes

To evaluate the molecular mechanisms underlying the relationship between TME and survival, the patients were divided into two groups based on the median of the stromal and immune scores, respectively (Figure 3A, B, C and D). DEGs were screened between the two groups and further intersected based on stromal score and immune score. Consequently, 10 down-regulated DEGs and 201 up-regulated DEGs were identified. These DEGs were closely related to the TME, hence defined as TME associated genes (Figure 3E and F).

Gene Ontology, KEGG Pathway, and Protein-Protein Interaction Analyses of Tumor Microenvironment Associated Genes

Furthermore, GO and KEGG analyses were performed based on the TME associated genes. As a result, TME associated genes were closely related to T cell activation, cytokine-cytokine receptor interaction, etc. (Figure 4A and B). Moreover, a PPI network for TME associated genes was constructed, and Top20 hub-genes were calculated using the Cytoscape software (Figure 4C and D). The association of all DEGs and survival was analyzed through the Cox and Kaplan-Meier analyses. Consequently, 138 genes were confirmed to be associated with the survival of melanoma patients (Table S2). Further, 12 intersected genes were finally obtained between the Top 20 hub-genes and 138 survival-associated genes (Figure 4E). Among them, *FGD2* showed the smallest q-value, hence was selected for subsequent analyses (Figure 4F).

FGD2 is Associated with the Progression of Melanoma

FGD2 was found to be associated with the progression of pan-cancer, including adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), and so on (Figure 5A). Further, were analyzed the association of *FGD2* and clinical features. Consequently, higher expression of *FGD2* indicated better survival (Figure 5B) and earlier primary tumor stage (Figure 5C). Nonetheless, *FGD2* expression was not associated with lymph nodes metastasis, distant metastasis, pathological stage, and age (Figure 5D–G).

Validation of FGD2 in Clinical Specimens

To further verify the *FGD2* expression, melanoma specimens and paired non-tumor skin tissues were used to perform Western blotting and RT-qPCR analyses (Figure 6A and B). As expected, *FGD2* expression was significantly downregulated in melanoma

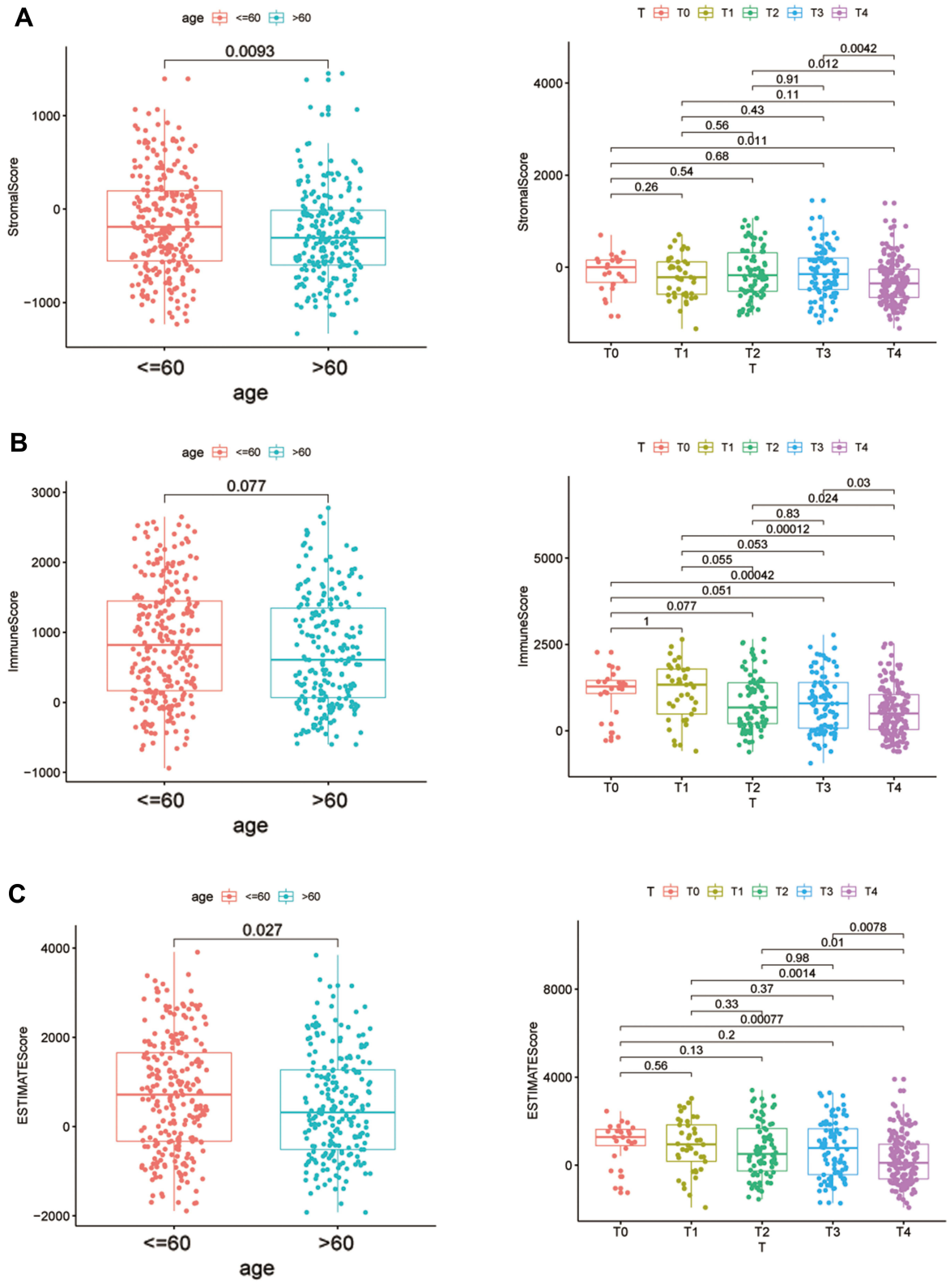

Figure 2 Tumor microenvironment score associated with age and tumor size (**A–C**) The relationship between TME score and clinical features analyzed using One-way ANOVA.

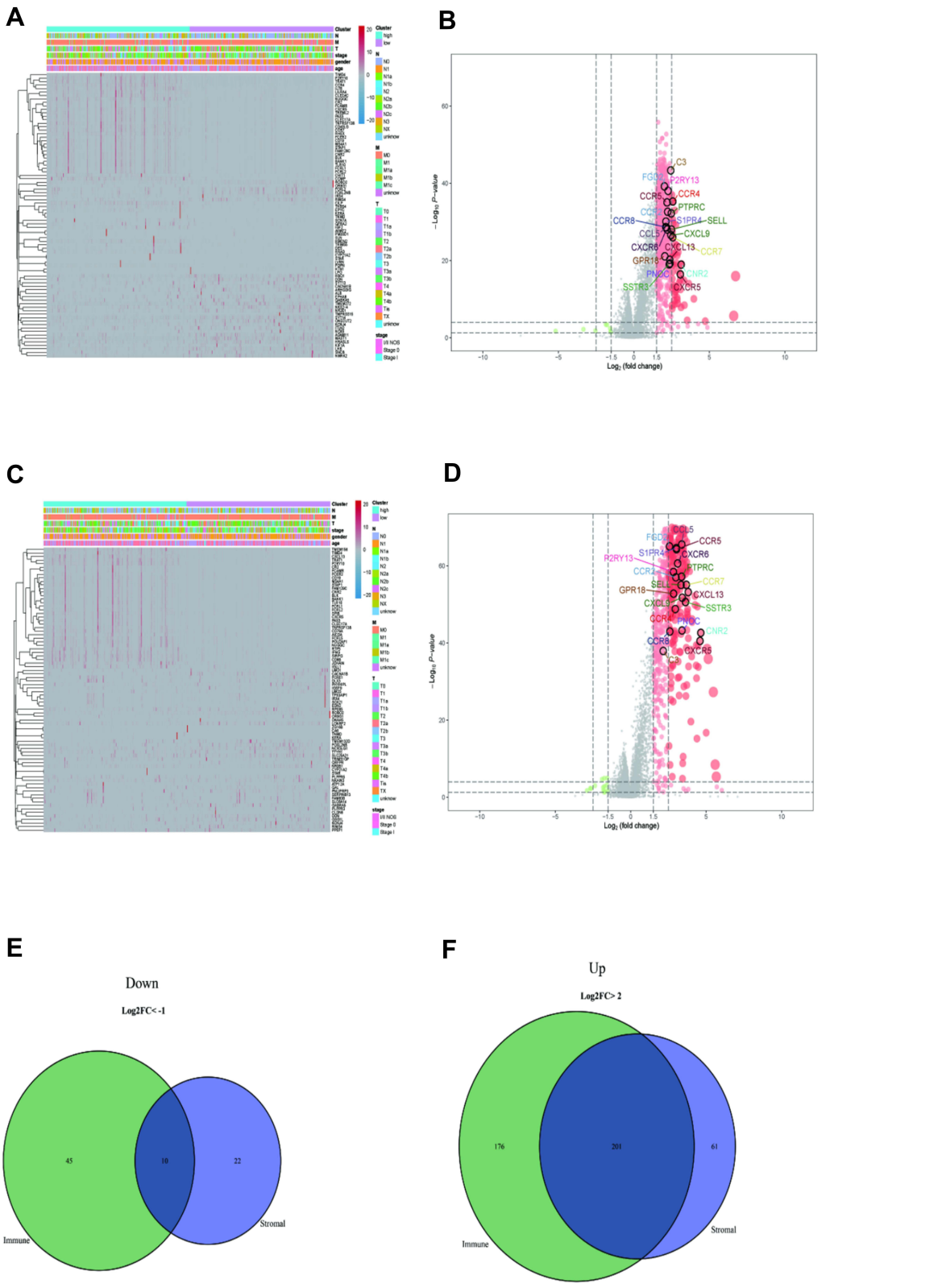

Figure 3 Screening for tumor microenvironment associated genes. (**A**) Heatmap of DEGs between patients with high and low stromal scores; (**B**) Volcano map of DEGs between patients with high and low stromal scores; (**C**) Heatmap and of DEGs between patients with high and low immune scores; (**D**) Volcano map of DEGs between patients with high and low immune scores; (**E**) Downregulated genes of the intersection of DEGs derived from immune and stromal scores; (**F**) Upregulated genes of the intersection of DEGs derived from immune and stromal scores.

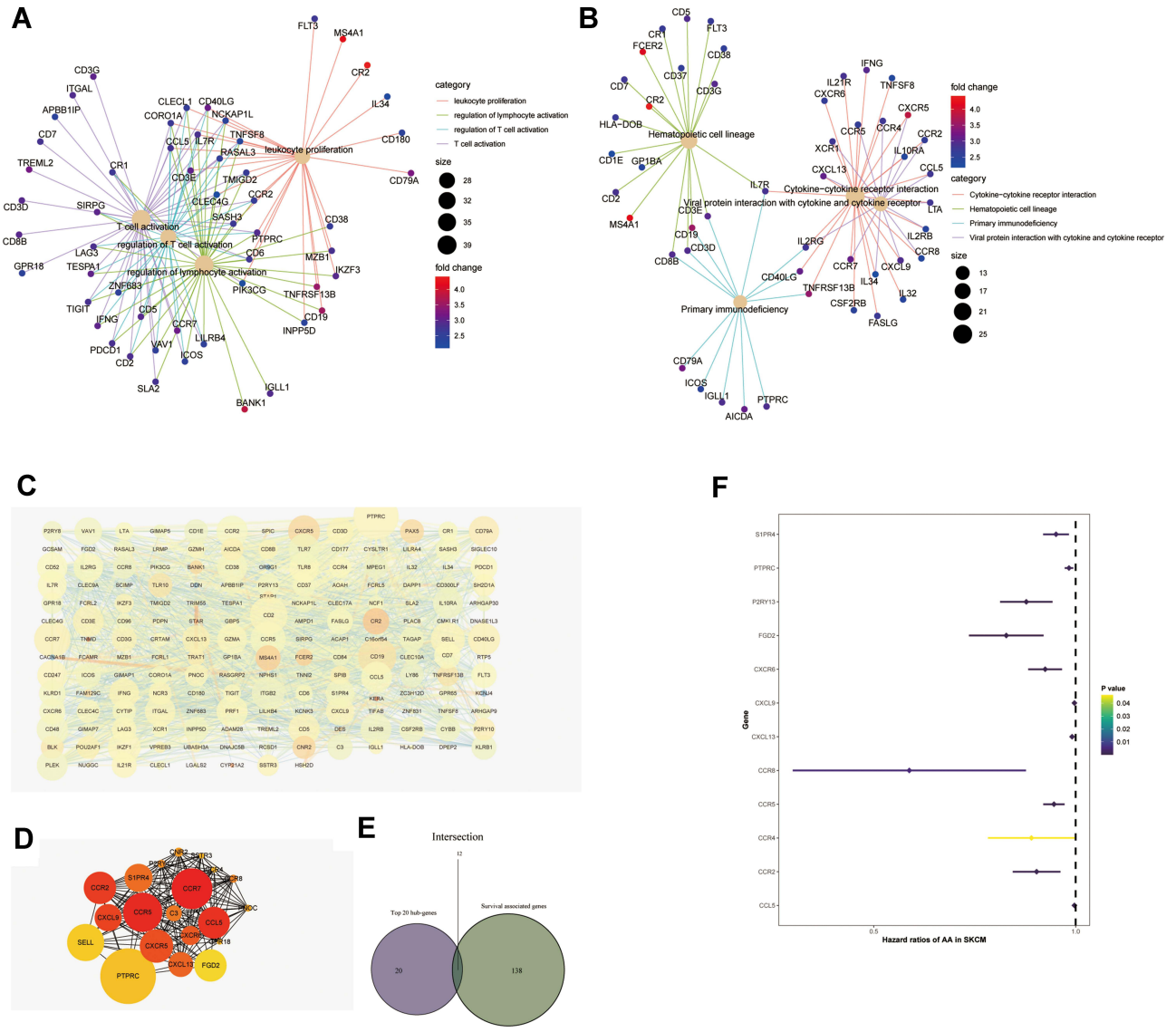

Figure 4 Gene ontology, KEGG pathway, and protein-protein interaction analysis of tumor microenvironment associated genes. (**A**) Gene ontology (GO) analysis of TME associated genes; (**B**) KEGG pathway analysis of TME associated genes; (**C**) protein-protein interaction (PPI) analysis of TME associated genes; (**D**) Identified of Top 20 hub-genes via the Cytoscape software; (**E**) Intersection of Top 20 hub-genes and 138 survival associated genes; (**F**) Survival analysis of 12 intersected genes based on Cox method and visualized by forest map.

compared to that in paired normal tissues (P<0.001). Also, *FGD2* associated pathways were examined through GSEA analysis. As a result, *FGD2* was associated with *IL6-JAK, KRAS, TNF-α* pathways, etc. All these pathways were closely related to the progression of melanoma and TME (Figure 6C). The relationship between *FGD2* and various types of immune cells was assessed through ssGSEA analysis. As a consequence, *FGD2* expression was closely related to the infiltration of T cells, B cells, and so on (Figure 6D and E). Generally, we confirmed the *FGD2* downregulation in melanoma. Besides, downregulated *FGD2* may modulate the TME by regulating the infiltration of immune cells. (WB original pictures are shown in the Supplementary Material)

Discussion

The tumor microenvironment is vital in the development of various tumors. Several studies have reported the role of part cells or factors in the TME of melanoma, including immune cells, immune checkpoints, etc.[10,11] Nevertheless, limited information is available on the regulatory mechanisms of TME as a whole.

CIBERSORT is a gene expression-based deconvolution algorithm developed to examine the proportion of stromal and cells in tumor samples.[12] Because of its excellent

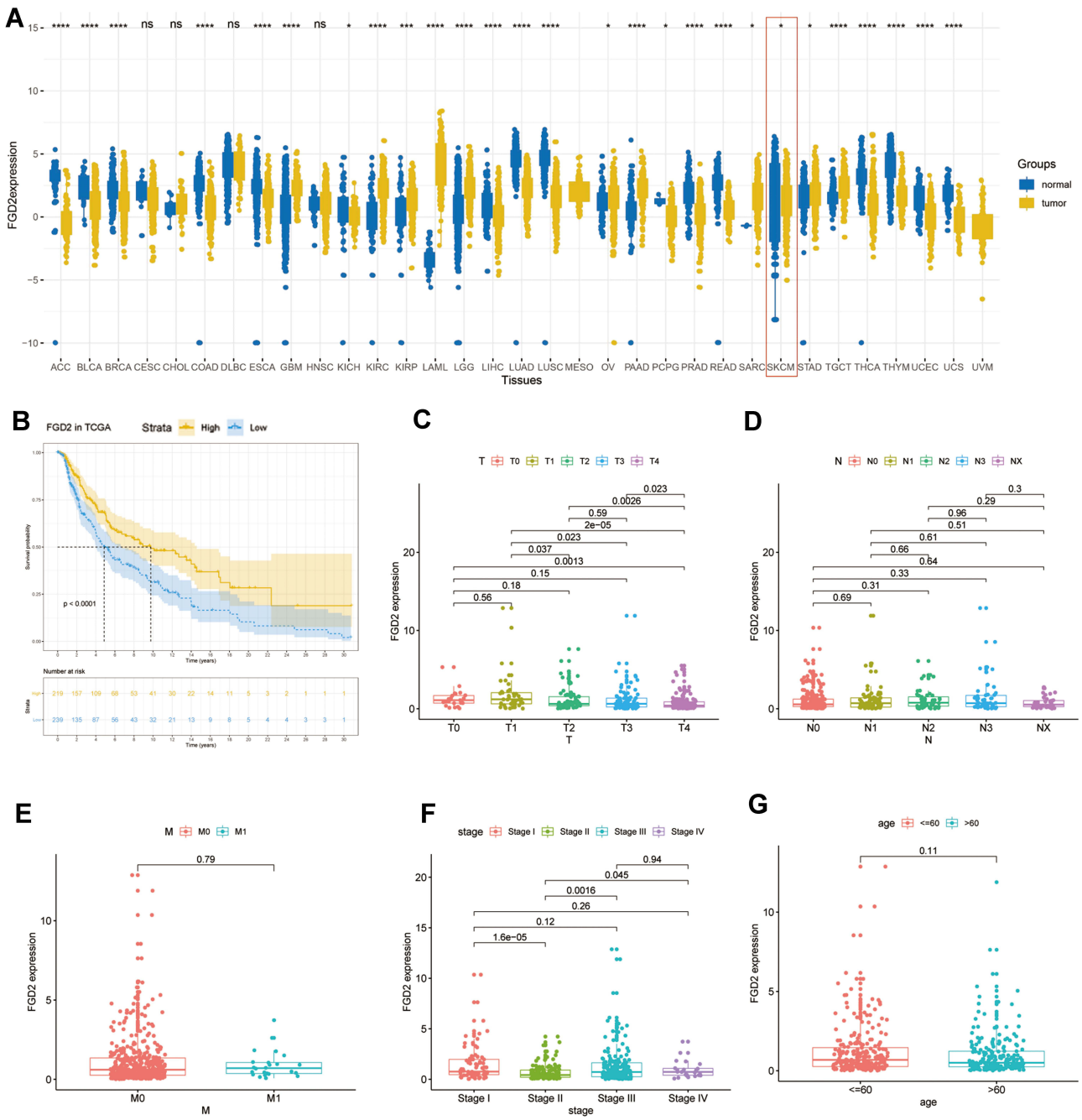

Figure 5 *FGD2* associated with the progression of melanoma. (**A**) The association of *FGD2* with survival in pan-cancer; (**B**) The association of *FGD2* with survival in melanoma performed by Kaplan-Meier analysis; (**C–G**) The association of *FGD2* with clinical features of melanoma patients analyzed by One-way ANOVA.

performance, CIBERSORT has been utilized in TME research.[13] Based on this algorithm, we calculated three TME scores for each patient, respectively. Stromal and immune scores indicated the infiltration of stromal and immune cells. The estimated score is the sum of stromal and immune scores indicating lower purity of tumor cells. In this scoring system, patients with higher immune and estimate scores demonstrated better survival. This also meant that patients with high infiltration of immune cells displayed better survival. Similar to other solid tumors, melanoma comprises a large number of immune cells, which potentially reflects tumor response. TME with high immune infiltration revealed strong antigenicity and can easily be detected by the immune system. Nonspecific innate immune mechanisms (including phagocytes, natural killer cells, etc.) and specific acquired immune

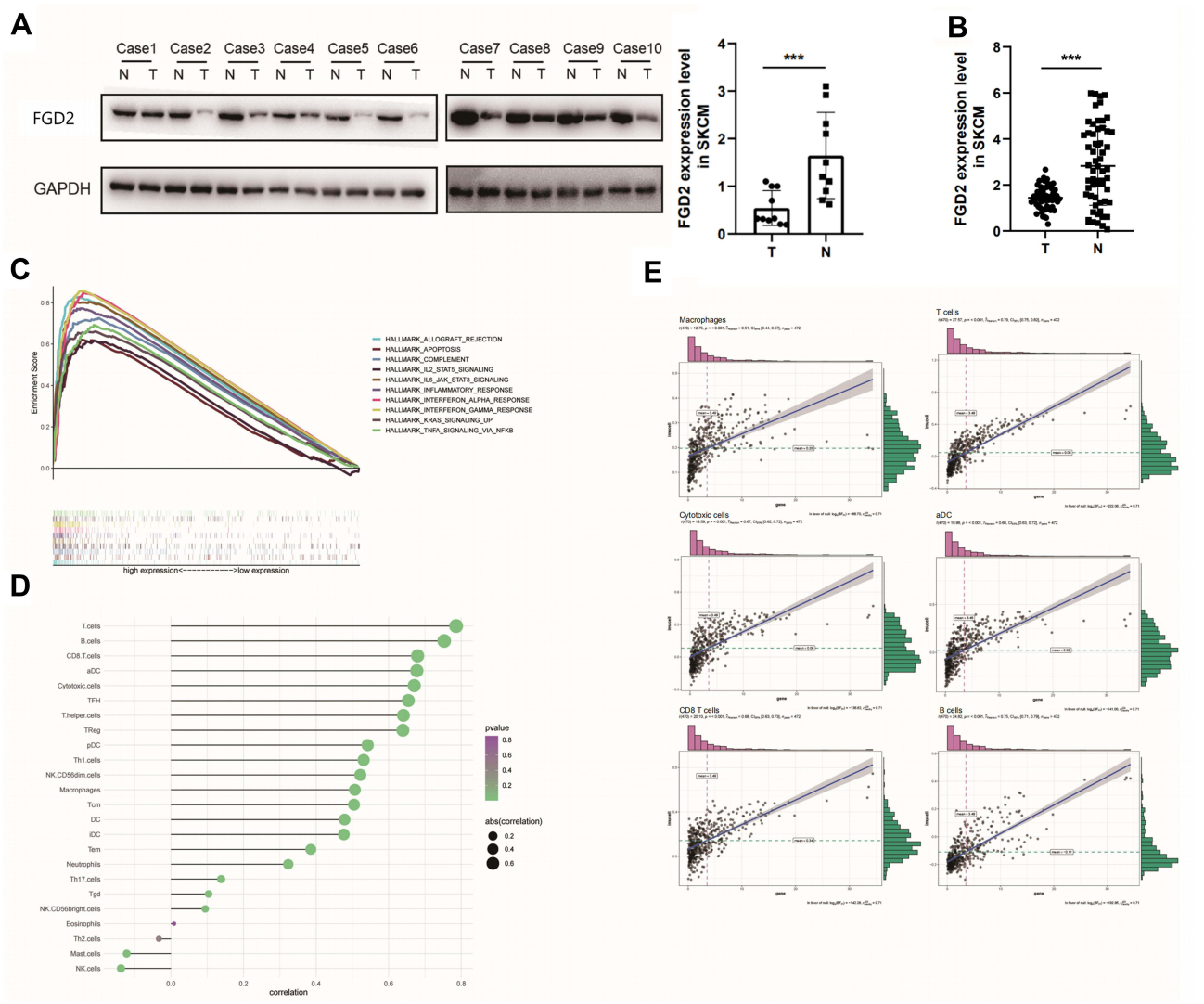

Figure 6 Validation of *FGD2* in clinical specimens. (**A**) *FGD2* expression in melanoma and paired non-tumor skin tissues validated by Western Blotting analysis (n=10). N: Normal tissues; T: Tumor tissues; (**B**) *FGD2* expression of in melanoma and paired non-tumor skin tissues validated by RT-qPCR analysis (analyzed by Student's *t*-test); (**C**) *FGD2* associated pathways assessed by GSEA analysis; (**D**) The correlation of *FGD2* and various types of immune cells assessed by ssGSEA analysis; (**E**) Top 6 *FGD2* associated immune cells derived from the ssGSEA analysis. All experiments were conducted in triplicate.

mechanisms (including CD4 + T cells, CD8 + T cells) are involved in the process of tumor cell clearance.[14] Further, we analyzed the relationship between estimate score and clinical features. As a result, a higher estimate score was related to younger age and earlier primary tumor stage. That is, highly immune infiltration in the early stage inhibits tumor progression. With the secretion of cytokines in the TME, immune cells are inhibited, immune escape occurs, causing tumor progression.[15]

We screened DEGs between patients with different TME scores to establish the related mechanisms underlying the regulation of TME. These DEGs were enriched in the T cell activation, cytokine-cytokine receptor interaction, and so on. T cells participate in killing tumors and the effective recognition of tumor cells is the premise of this role. In the TME, tumor cells exhibit selective inhibitory ligands and receptors, which regulate the function of T cells. In recent years, pharmacological modulators of these pathways (known as immune checkpoint therapy, specifically monoclonal antibody forms against PD-1 and CTLA-4) have been widely studied and utilized as novel immunotherapeutic agents against melanoma.[16] Considering the early success of immune checkpoint therapy, the development of immunotherapy targeting other costimulatory receptors activating the anti-tumor immune response is seemingly a convincing treatment approach.[17]

In subsequent analyses, we verified that *FGD2* may be the hub-gene of TME regulation in melanoma. Additionally, *FGD2* was closely related to the progression of melanoma.

Patients with high *FGD2* expression demonstrated better survival. The protein encoded by this gene is a member of the guanine nucleotide exchange factors (GEFs) family which regulate cytoskeleton-dependent membrane rearrangements by activating the cell division cycle 42 (CDC42) protein. This gene is expressed in B lymphocytes, macrophages, and dendritic cells. In the B lymphocyte lineage, *FGD2* levels change with the developmental stage. In both mature splenic and immature bone marrow B cells, *FGD2* expression is suppressed upon activation through the B cell antigen receptor.[18] Also, previous research approved *FGD2* as a biomarker for head and neck squamous cell carcinoma.[19] However, the roles of *FGD2* in the response of tumors remain unclear. Through GSEA analysis, we established that *FGD2* may regulate immune infiltration of various types of immune cells, including T cells, B cells, etc. Future studies should explore the role of *FGD2* in the immune response of tumors.

In conclusion, we established a relationship between TME and the survival of melanoma patients. Consequently, we discovered a novel *FGD2* gene that potentially regulates the TME in melanoma.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

All patients provided and signed informed consents. The study was approved by the Ethical Committee of the Affiliated Hospital of Qingdao University and experiments were performed as per the Ethical Committee's guidelines and regulations. All procedures involving human participants were performed based on the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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Author Contributions

All authors made a significant contribution to the work reported, including in the conception, study design, execution, data acquisition, analysis, and interpretation. Also, the authors participated in drafting, revising, or critically reviewing the article; provided final approval of the version to be published; agreed on the journal to which the article has been submitted, and remain accountable for all aspects of the work. Xuchao Ning and Renzhi Li equally contributed to this paper.

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Disclosure

The authors declare no conflicts of interest concerning the publication of this article.

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