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Original Article

Transcriptional E2F1/2/3/6 as potential prognostic biomarkers in cutaneous melanoma

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Abstract: Although the abnormal expression of members of the E2F family has been reported to participate in carcinogenesis in many human types of cancer, the bioinformatics role of the E2F family in melanoma is unknown. This research was designed to detect the expression, methylation, prognostic value and potential effects of the E2F family in melanoma. We investigated E2F family mRNA expression from the Oncomine and GEPIA databases and their methylation status in the MethHC database. Meanwhile, we detected the relative E2F family expression levels by qPCR and immunohistochemistry. Kaplan-Meier Plotter was used to draw survival analysis charts, and gene functional enrichment analyses were applied through cBioPortal database analysis. E2F1/2/3/4/5/6 mRNA and proteins were clearly upregulated in cutaneous melanoma patients, and high expression levels of E2F1/2/3/6 were statistically related to high methylation levels. Increased mRNA expression of E2F1/2/3/6 was related to lower overall survival rates (OS) and disease-free survival (DFS) in cutaneous melanoma cases. Meanwhile, E2F1/2/3/6 carried out these effects through regulating multiple signaling pathways, including the MAPK, PI3K-Akt and p53 signaling pathways. Taking together, our findings suggest that E2F1/2/3/6 could act as potential targets for precision therapy in cutaneous melanoma patients.

Keywords: E2F1/2/3/6, cutaneous melanoma, database mining, prognostic value, bioinformatics analysis

Introduction

Melanoma is one of the most aggressive skin cancers, of which the pathogenic mechanisms remain unclear, as both environmental and genetic factors could contribute to its development. Cutaneous melanoma (CM) is one of the most prevalent skin malignancies, and its incidence and mortality are increasing around the world. According to the cancer statistics data from the National Center for Health Statistics (NCHS), an estimated 96,480 new CM cases and approximately 7,230 deaths due to CM occurred in the United States in 2019 [1].

CM is a heterogeneous and complex type of cancer whose main risk factors are: immunodeficiency, hereditary alterations and exposure to ultraviolet radiation (UVR) [2]. Although clinical diagnosis, surgical treatment, and drug therapy including immunotherapy and targeted therapy have made great progress, the overall survival rates for patients diagnosed with CM remain poor. Thus, it is urgent to investigate the initiation and developmental mechanisms in CM and to find novel combined molecular markers that may function as therapeutic or prognostic targets for CM.

E2Fs are a class of transcription factors that encode a family of transcription factors (TFs) in higher eukaryotes [3]. E2Fs are divided into two subgroups according to their structures and functions: 1) transcriptional activator protein-containing: E2F1, E2F2 and E2F3; and 2) transcriptional suppressor factors including E2F5, E2F6, E2F7 and E2F8 [3, 4]. In published research, E2Fs acted as an oncogene in some CM carcinogenesis [5, 6]. For instance, E2F1 mRNA expression was upregulated in melanoma, and the downregulation of E2F1 decreased the sensitivity of melanoma A375 cells to tar-

geted therapy, and it also induced the death of melanoma cells resistant to BRAF inhibitors [5]. E2F3 is highly expressed in melanoma, promoting tumor progression through regulating the cell cycle and acting as an important regulator of melanoma therapy [6]. However, the expression patterns, methylation status, relative survival analysis and potential mechanisms of the E2F family in CM remain unknown.

RNA and DNA exploration, a significant constituent of biomedical and biological research, has been undergoing milestone development with the rapid progression of RNA-sequencing technology and microarrays [7, 8]. In this research, using online gene expression analyses, qRT-PCR and immunohistochemistry, we detected the expression patterns, methylation status, relative survival analysis and potential mechanisms of E2F factors in CM patients to further clarify the effects of E2Fs in the etiopathogenesis and pathophysiological of CM.

Materials and methods

All clinical samples (40 CM and adjacent samples) were collected between 2006 and 2010 in the Department of Dermatology, Chongqing First People's Hospital and Chongqing Traditional Chinese Medicine Hospital. None of the patients received any radiotherapy or chemotherapy or used corticosteroid drugs before the operation and every patient signed the informed consent form. This research was permitted by the Hospital Bioethics Ethics Committee and it also conformed to the principles expressed in the Helsinki declaration.

Oncomine analysis

The Oncomine Platform (www.oncomine.org) provides solutions for individual researchers and multinational companies with robust, peer-reviewed analysis methods and a powerful set of analysis functions that compute gene expression signatures, clusters and gene-set modules, automatically extracting biological insights from the data. The database was applied to analyze relative E2Fs mRNA expression levels in cutaneous melanoma. The threshold settings were as below: *P*-value = 0.0001; fold-change = 2; THRESHOLD: top 10% and data type: mRNA. Analysis of different expression levels between the CM tissues and normal samples datasets was carried out for the E2Fs genes.

GEPIA dataset analysis

GEPIA2 is a web server for detecting the RNA sequencing expression data, which contain 9,736 tumors and 8,587 normal specimens from the TCGA (The Cancer Genome Atlas) and the GTEx (Genotype-tissue Expression dataset) projects. All the datasets on the server are computed by a standard pipeline and are compatible with each other. This platform allows users to perform all expression analyses, including expression analyses, cancer subtypes analyses, signature score analyses, signature score analyses, survival analysis, similar genes detection, correlation analysis and differential analysis [9].

Quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed as described previously [10]. Total RNA of clinical tissues was isolated by using TRIzol reagent (Thermo Fisher). Then, 2 μl separated RNA was reverse transcribed using the PrimeScript™ Reverse Transcription System with cDNA Eraser (Thermo Fisher) based on the manufacturer's instructions. qRT-PCR was carried out using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All PCR products were sequenced to confirm that the correct products were obtained. The experiments were repeated three times and the primers used are listed in Supplementary Table 1.

Immunohistochemistry (IHC)

Paraffin sections of preserved CM cancer samples and adjacent cancer tissue were subjected to immunohistochemistry to detect the expression of E2Fs proteins. E2Fs staining was based on the protocols of the VECTASTAIN® Elite ABC-Peroxidase KitElite ABC (Vector Laboratories, USA). Anti-E2Fs antibody (1:100, Abcam, UK) was selected as the primary antibody. Goat Anti-Rabbit IgG H&L (HRP) (1:1000, Biyuntuan, China) was selected as the second antibody. The primary antibody was incubated overnight at 4°C and the second antibody was incubated for 2 hours at 37°C. The slides were analyzed using a Leica TCS SP5 microscope (Leica microsystem) under five random fields

(400×) with the LAS AF Lite 4.0 image browser software [10].

MethHC dataset analysis

MethHC is a database containing a systematic integration of a series of DNA methylation data and mRNA/microRNA expression profiles of human cancer [11]. The database contains 18 human cancers in more than 6000 samples, 6548 microarrays and 12,567 RNA sequencing datasets, and could help authors to analyze epigenetic patterns that are essential for carcinogenesis.

cBioPortal analysis

The cBioPortal is a web platform of "gene-based" visualizations and analyses and it provides information related to cancer study and genomic profiles, such as mutations and copy number alterations, patients' case sets and gene sets of interest [12]. The genomic profiles of the E2F family gene alterations, including mutations, overall survival status, mRNA expression z-scores (RNA Seq V2 RSEM), protein expression Z-scores (RPPA) and co-expression network were analyzed based on the cBioPortal's online database [13].

Statistical analysis

We used GraphPad Prism 8 and SPASSI7.0 software to analyze the data in this study. The measurement data were expressed as the mean ± standard deviation. The comparison between the two groups was conducted by using t-tests and chi-square tests. The relationship between the E2Fs expression and prognosis was analyzed by Kaplan-Meier survival curves. $P < 0.05$ means that the difference is statistically significant.

Results

Expression patterns of E2Fs in patients with CM

We first analyzed all of the *E2F1-E2F8* mRNA expression levels in human CM by utilizing the Oncomine database. Through comparing relative mRNA levels of *E2F* between normal and cancer samples, we found that *E2F1 and E2F3* mRNA were upregulated in melanoma (**Figure 1**), while no obvious differences in expression were found in *E2F2, E2F4, E2F5, E2F6, E2F7, or E2F8* mRNA. However, we further analyzed the mRNA levels of the *E2Fs* in detail, and we found that in 6/8 genes, *E2Fs* mRNA expression was statistically upregulated in CM in five datasets with the following thresholds: *P* value = 0.0001; fold change = 2; gene rank = 10%. In the Talantov Melanoma database [14], *E2F1* mRNA was upregulated in CM compared to normal skin with a fold change of 1.797 and $P = 2.2\times10^{-4}$ (Reporter: 2028_s_at) and a fold change of 2.731 and $P = 7.67\times10^{-5}$ (Reporter: 204947_at). *E2F2* mRNA was up-regulated in CM compared to normal skin in the Talantov Melanoma database [14] with a fold change of 1.588 and P = 0.006 (Reporter: 2028_s_at) and a fold change of 2.731 and $P = 7.67\times10^{-5}$ (Reporter: 207042_at). *E2F3* mRNA was upregulated in the Riker Melanoma [15] and the Talantov Melanoma [14] databases with a *p*-value < 0.005. In Haqq Melanoma [16], *E2F4* and *E2F5* mRNA expression were obviously upregulated in CM with a *p*-value < 0.005 while *E2F4* mRNA was also overexpressed in the Talantov Melanoma [14] database. E2F7 overexpression was also found in CM with a fold change of 1.985 and $P = 7.67\times10^{-5}$ (Reporter: 228033_at) in Riker Melanoma [15]. However, there were no available results observed for *E2F6* and *E2F8* expression in CM. The data are shown in Supplementary Table 2.

E2Fs expression and clinicopathological characteristics of CM patients

Transcriptional mRNA expression of *E2F1-E2F8* between CM and normal skin samples was detected through analyzing the GEPIA dataset. The outcomes indicated that the relative mRNA levels of *E2F1*, *E2F3*, *E2F5* and *E2F7* in CM tissues were upregulated compared with normal samples with $P < 0.01$ (**Figure 2A**, **2B**), while the expression pattern levels of *E2F2*, *E2F4*, *E2F6* and *E2F8* between CM and normal tissues had no significant difference when the cutoff was $P < 0.01$ (**Figure 2A**, **2B**). We thus examined the relative E2F expression in CM and the results showed only *E2F7* and *E2F8* expression were related to the clinical stages in CM (**Figure 3A**). Furthermore, we detected E2F mRNA expression in CM and adjacent samples by qRT-PCR and found that the mRNA levels of the *E2Fs* factors were higher in CM samples than in the adjacent ones, with $P < 0.001$ (**Figure 3B**). There were selected difference of CM samples pathological stages and also cutoff values, thus the gene expression was difference among different databases. How-

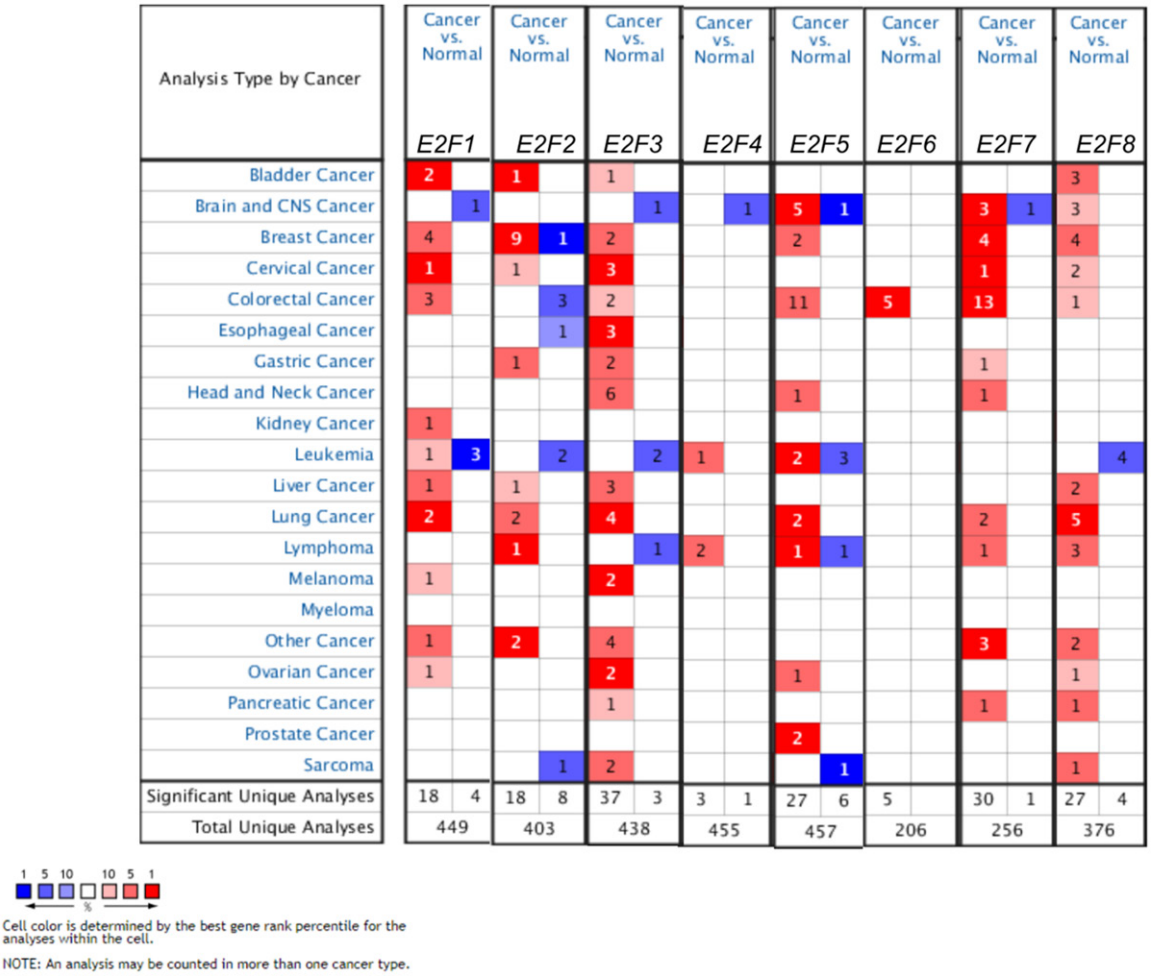


Figure 1. The transcription levels of E2F factors in different types of cancers (ONCOMINE).

ever, we examined E2Fs protein levels through IHC, and found that the protein expression of E2F1, E2F2, E2F3, E2F6 and E2F7 were statistically upregulated in CM samples (**Figure 4A**, **4B**). Taken together, abnormally high expression of E2Fs in CM may have a key role in CM progression.

E2Fs expression and the prognosis of CM patients

Survival plots were used to explore the correlation between the E2Fs mRNA levels and the survival of CM patients (**Figure 5A-C**). We first examined the survival of 40 CM patients and discovered that the E2F1, E2F2, E2F3, E2F4 and E2F6 expression levels were statistically associated with cumulative survival (**Figure 5A**). Furthermore, we verified the potential survival condition of these genes in the GEPIA dataset, and the results showed that *E2F1*, *E2F2*, *E2F3* and *E2F6* displayed statistical relationships with relatively worse overall survival (OS) rates, whereas only high expression of *E2F3* and *E2F6* mRNA were significantly correlated with lower disease-free survival (DFS) with *p*-values < 0.05 (**Figure 5B**, **5C**). These findings indicated that high *E2F1/2/3/4/6* mRNA expression levels were closely associated with a relatively poor prognosis of CM, which was consistent with the published research [5, 6].

Methylation of the E2F promoter in CM

Aberrant promoter methylation is a common mechanism involved in carcinogenesis [17, 18]. To determine whether the overexpression of E2F family transcription factors is connected with promoter methylation, we further investigated the methylation level of the E2F promoter in CM tissues by analyzing the online MethHC dataset and the results revealed *E2F1*, *E2F2*, *E2F3* and *E2F6* were hypomethylated in 374

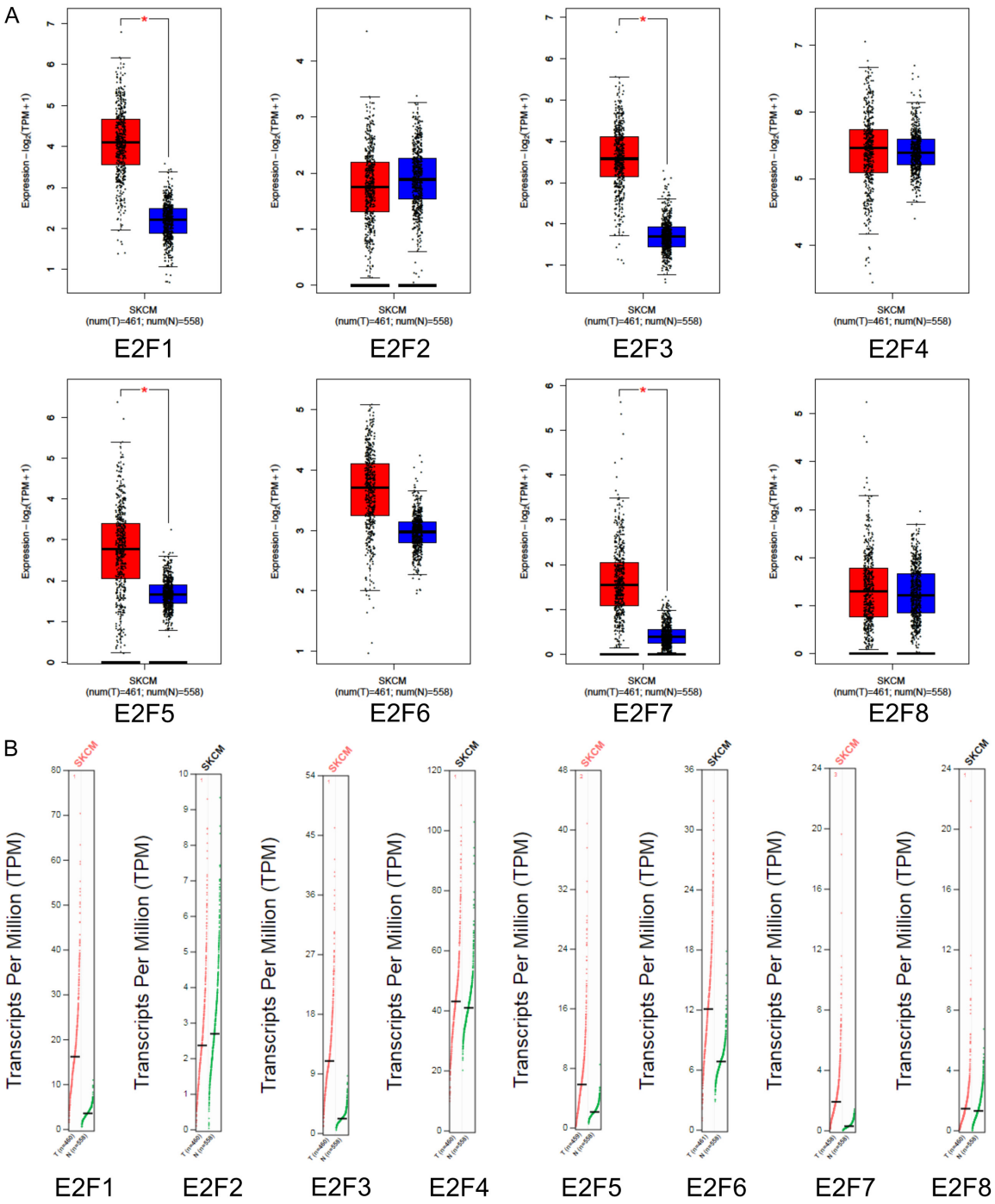


Figure 2. The expression of E2F family members in CM patients (GEPIA). A. Boxplot of E2Fs mRNA expression levels in CM; the *p*-value was set at 0.01. B. Transcripts per million (TPM) of E2Fs mRNA in CM; the *p*-value was set at 0.01.

cutaneous melanoma samples compared with normal tissues with P < 0.05 (**Figure 6A**). Additionally, data taken from the MethHC dataset showed that overexpression of *E2F1*, *E2F2*, *E2F3, E2F5* and *E2F6* were correlated with the promoter CpG methylation status with P < 0.05 (**Figure 6B**). Altogether, abnormal methylation of E2F family transcription factors in CM is involved in melanoma carcinogenesis.

Genetic alteration, co-expression and interaction analyses of E2Fs in CM

The cBioPortal database was applied to examine E2F genetic alterations, correlations, and

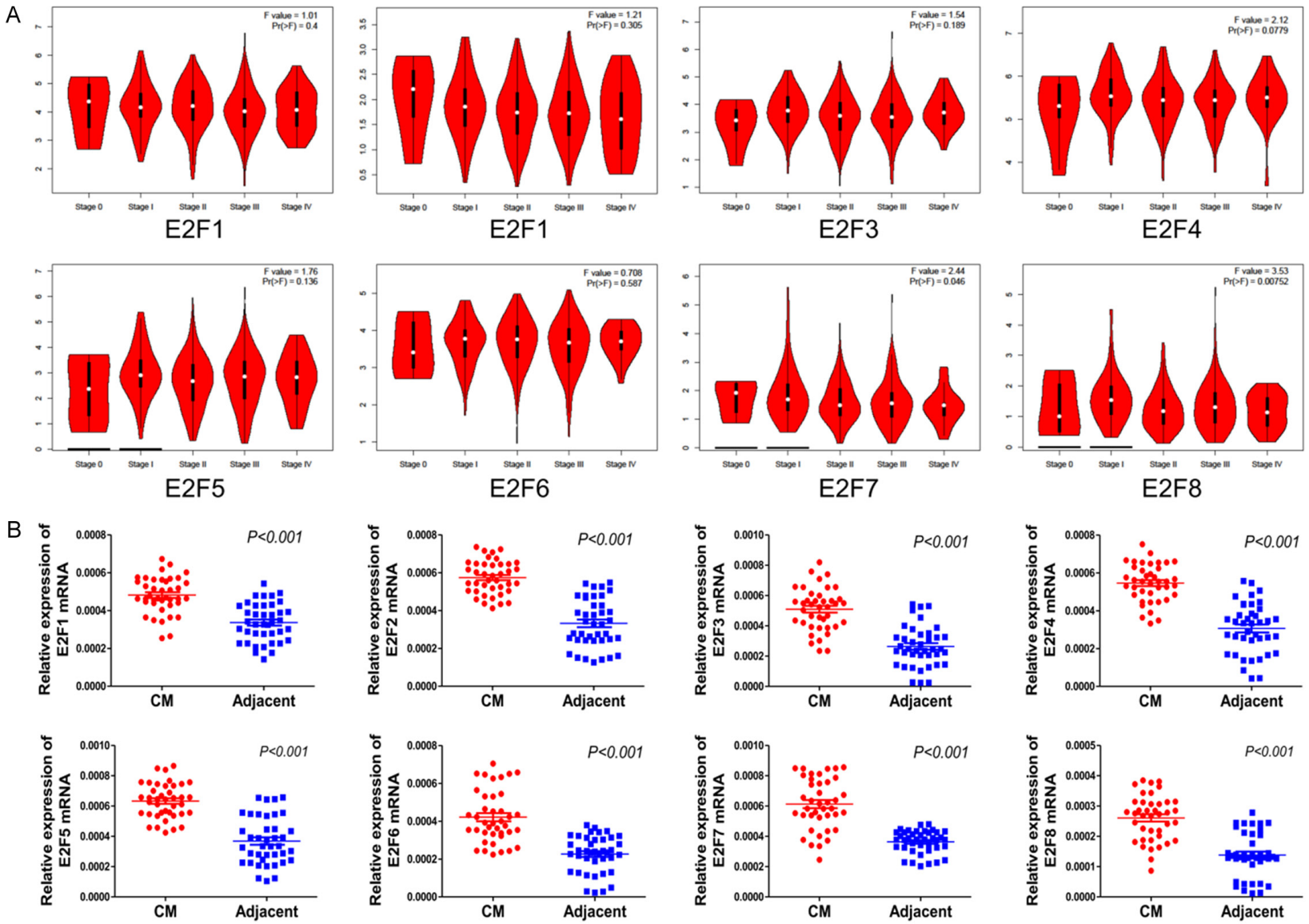


Figure 3. The expression of E2F family members in CM patients. A. Violin plot shows a correlation between E2F expression and tumor stage in CM patients (GEPIA); the *p*-value was set at 0.01. B. qRT-PCR showing E2Fs mRNA in CM and adjacent tissues.

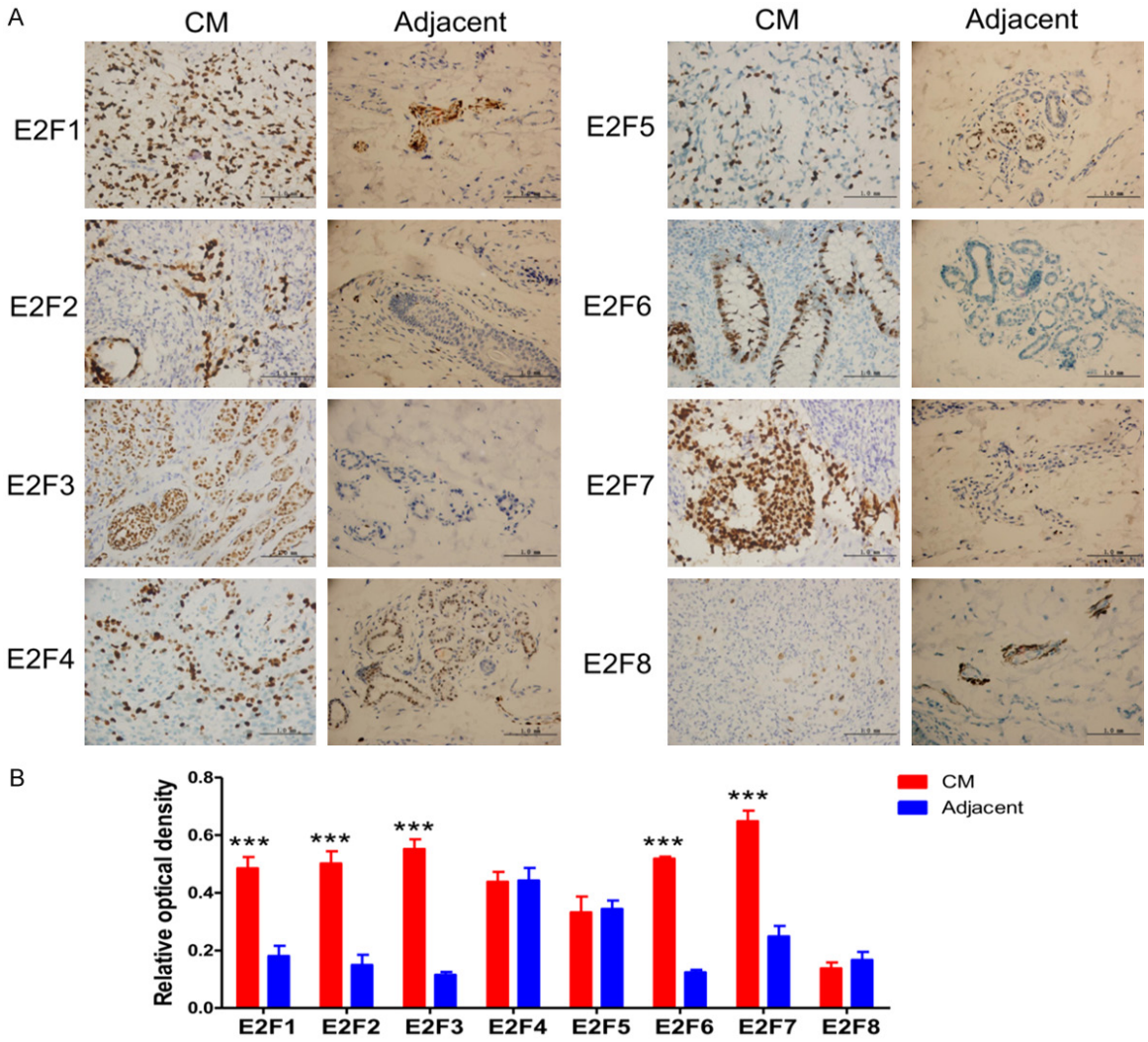


Figure 4. The expression of E2Fs in CM (IHC). A. The protein expression of E2Fs in CM andadjacent tissues. B. Histogram showing relative optical density, all of the results are shown as the mean ± SEM, ***P < 0.001.

networks simultaneously. The 448 samples obtained from the TCGA database (PanCancerAltas) were used in this study. As shown in **Figure 7A**, the rates of genetic alterations in E2Fs numbers for CM averaged from 1.6 to 26% individually (E2F1, 9%; E2F2, 7%; E2F3, 26%; E2F4, 8%; E2F5, 7%; E2F6, 8%; E2F7, 1.6%; E2F8, 2.9%; (**Figure 7A**). Meanwhile, we analyzed the mRNA expression relationship of *E2Fs* factors individually through using the cBioPortal online tool, and the results indicated statistical significance as follows: E2F1 with E2F4, E2F6; E2F2 with E2F4, E2F7 and E2F8; E2F5 with E2F7; E2F6 with E2F7; and E2F7 with E2F8 (**Figure 7B**). Moreover, STRING network analysis was applied to verify the protein interactions with every family member, and the outcomes also showed that E2Fs interacted with each other in CM progression (**Figure 7C**). Meanwhile, we thus found a connection between E2F family members and cutaneous melanoma-related downstream genes, including TP53, ESR1, MAP2K1, MAP3K3, MAP3K1, SMAD4, SMARCD1, STK11, CCND1, etc. Whereas the PPI network is composed of 40 nodes and 76 edges (average node degree of 1.15 and an average local clustering coefficient of 0.313) (**Figure 7D**). From this network, we found E2F1/2/3/6 directly interacted with SETD2, KRAS, MYC, PTEN, MAP3K13, CDKN2A, and AKT1. Among the related genes, PTEN, APC, CDKN2A and TP53 were identified as tumor suppressor genes [19-22]. KRAS and CASP8 were confirmed as oncogenes in

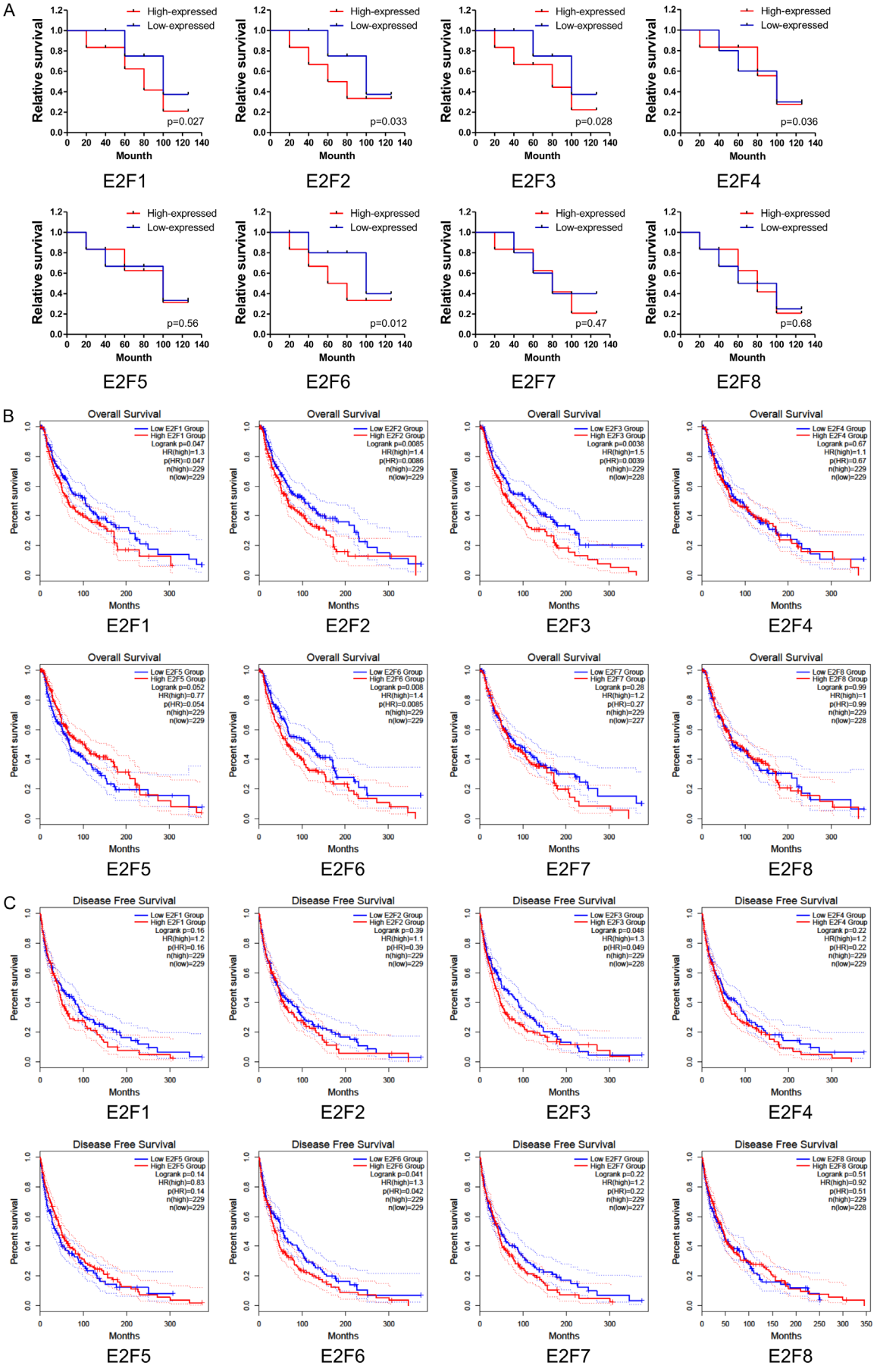

A
High-expressed
Low-expressed
Relative survival
Mounth
p=0.027
E2F1
p=0.033
E2F2
p=0.028
E2F3
p=0.036
E2F4
p=0.56
E2F5
p=0.012
E2F6
p=0.47
E2F7
p=0.68
E2F8
B
Overall Survival
Percent survival
Months
Low E2F1 Group
High E2F1 Group
Logrank p=0.047
HR(high)=1.3
p(HR)=0.047
n(high)=229
n(low)=229
E2F1
Low E2F2 Group
High E2F2 Group
Logrank p=0.0085
HR(high)=1.4
p(HR)=0.0086
n(high)=229
n(low)=229
E2F2
Low E2F3 Group
High E2F3 Group
Logrank p=0.0038
HR(high)=1.5
p(HR)=0.0039
n(high)=229
n(low)=228
E2F3
Low E2F4 Group
High E2F4 Group
Logrank p=0.67
HR(high)=1.1
p(HR)=0.67
n(high)=229
n(low)=229
E2F4
Low E2F5 Group
High E2F5 Group
Logrank p=0.052
HR(high)=0.77
p(HR)=0.054
n(high)=229
n(low)=229
E2F5
Low E2F6 Group
High E2F6 Group
Logrank p=0.008
HR(high)=1.4
p(HR)=0.0085
n(high)=229
n(low)=229
E2F6
Low E2F7 Group
High E2F7 Group
Logrank p=0.28
HR(high)=1.2
p(HR)=0.27
n(high)=229
n(low)=227
E2F7
Low E2F8 Group
High E2F8 Group
Logrank p=0.99
HR(high)=1
p(HR)=0.99
n(high)=229
n(low)=228
E2F8
C
Disease Free Survival
Percent survival
Months
Low E2F1 Group
High E2F1 Group
Logrank p=0.16
HR(high)=1.2
p(HR)=0.16
n(high)=229
n(low)=229
E2F1
Low E2F2 Group
High E2F2 Group
Logrank p=0.39
HR(high)=1.1
p(HR)=0.39
n(high)=229
n(low)=229
E2F2
Low E2F3 Group
High E2F3 Group
Logrank p=0.048
HR(high)=1.3
p(HR)=0.049
n(high)=229
n(low)=228
E2F3
Low E2F4 Group
High E2F4 Group
Logrank p=0.22
HR(high)=1.2
p(HR)=0.22
n(high)=229
n(low)=229
E2F4
Low E2F5 Group
High E2F5 Group
Logrank p=0.14
HR(high)=0.83
p(HR)=0.14
n(high)=229
n(low)=229
E2F5
Low E2F6 Group
High E2F6 Group
Logrank p=0.041
HR(high)=1.3
p(HR)=0.042
n(high)=229
n(low)=229
E2F6
Low E2F7 Group
High E2F7 Group
Logrank p=0.22
HR(high)=1.2
p(HR)=0.22
n(high)=229
n(low)=227
E2F7
Low E2F8 Group
High E2F8 Group
Logrank p=0.51
HR(high)=0.92
p(HR)=0.51
n(high)=229
n(low)=228
E2F8

Figure 5. The prognostic value of the mRNA level of E2F factors in CM (GEPIA). A. Kaplan-Meier analysis indicated that overall survival rates of E2F family member expression in CM, *p*-value of < 0.05. B. The overall survival rates of E2F family member expression in CM were plotted using the Kaplan-Meier plotter database at a threshold of a *p*-value of < 0.05. C. The disease-free survival of E2F family member expression in CM was plotted using the Kaplan-Meier plotter database at a threshold of a *p*-value of < 0.05.

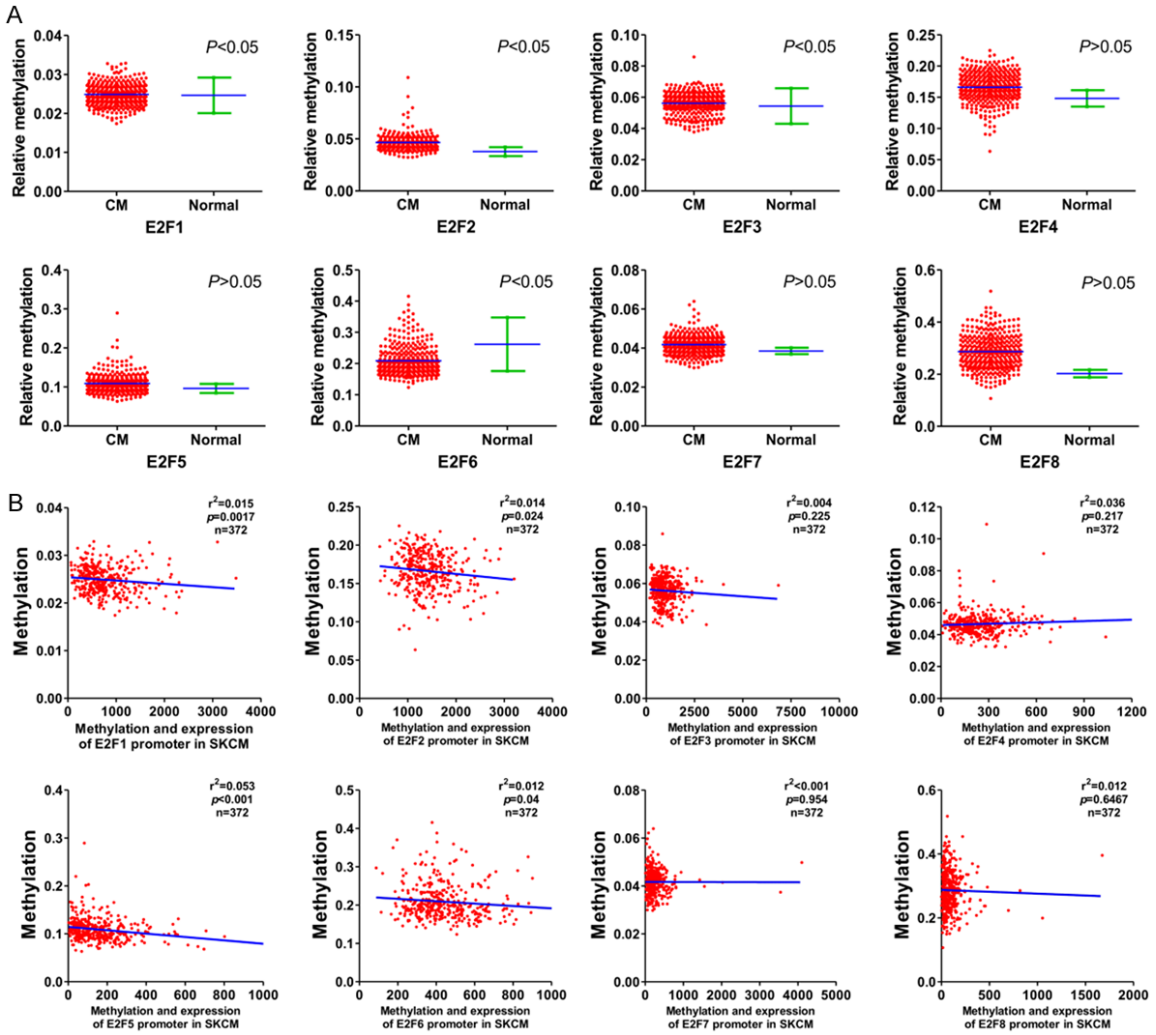
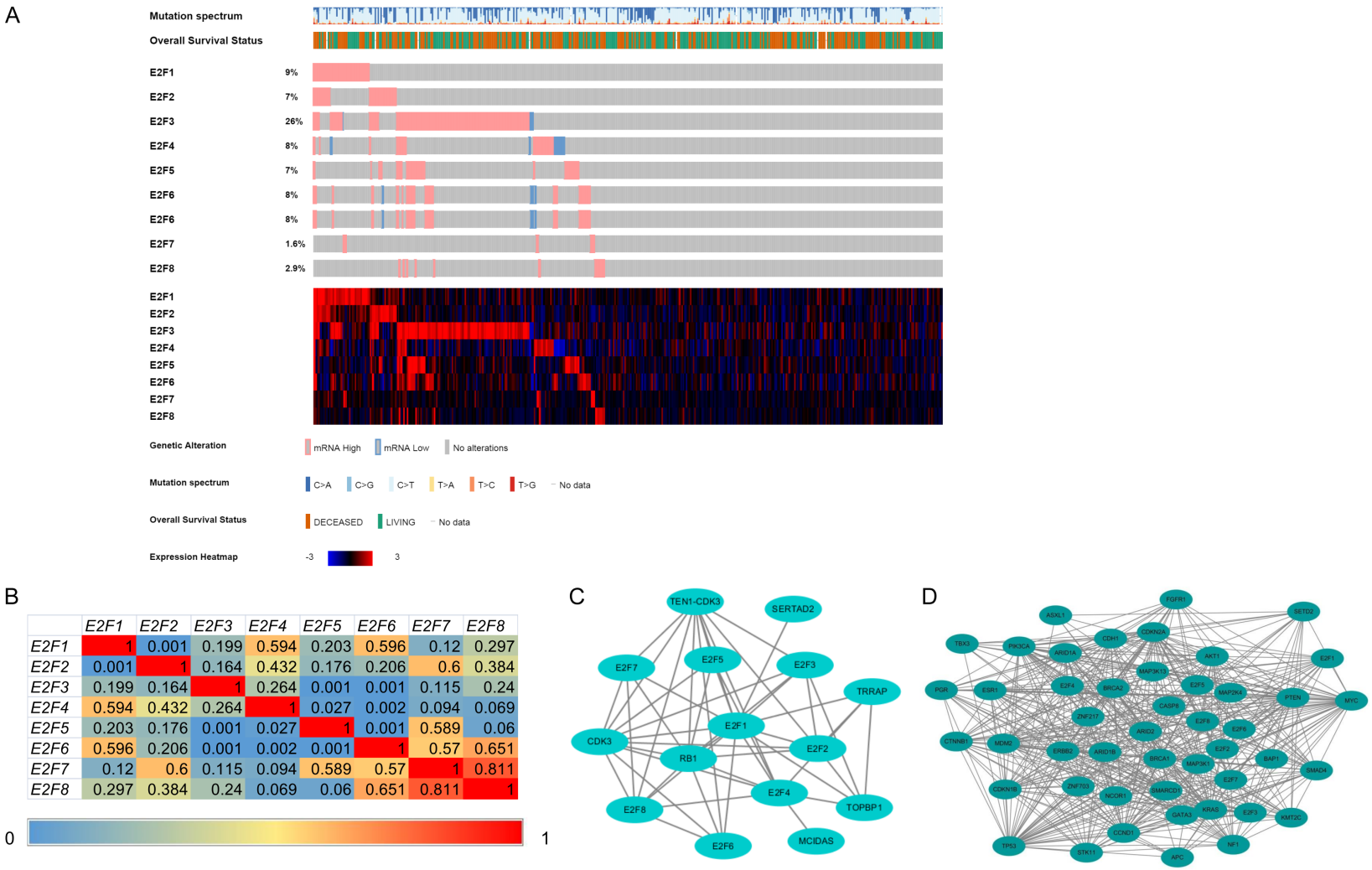


Figure 6. The methylation status of E2F genes in CM (MethHC). A. The methylation status of E2F factors in CM tissues and normal tissues, a *P*-value of < 0.05. B. Correlation analysis between E2Fs mRNA expression and methylation levels by Spearman's correlation analysis, respectively.

multiple cancers [23, 24]. Associated with the linkage verification of the PPI network, we thus speculated that E2F1/2/3/6 directly and indirectly reacted with SETD2, KRAS, MYC, PTEN, MAP3K13, CDKN2A, AKT1, etc., which are important components of the PI3K-Akt, MAPK and p53 signaling pathways (Supplementary Figure 1). Taken together, high-frequency mutations of E2F1/2/3/6 were confirmed in CM, indicating that the tumor-related functions of E2F1/2/3/6 depend on the synergistic effects of the oncogenes and tumor-suppressing gene.

Discussion

Abnormal dysregulation of E2Fs was demonstrated to participate in multiple cancers [5, 6]. E2Fs are either oncogenes or tumor suppressor genes in carcinogenesis and pathophysiological processes. However, their expression profiles and a bioinformatics analysis of CM



	E2F1	E2F2	E2F3	E2F4	E2F5	E2F6	E2F7	E2F8
E2F1	1	0.001	0.199	0.594	0.203	0.596	0.12	0.297
E2F2	0.001	1	0.164	0.432	0.176	0.206	0.6	0.384
E2F3	0.199	0.164	1	0.264	0.001	0.001	0.115	0.24
E2F4	0.594	0.432	0.264	1	0.027	0.002	0.094	0.069
E2F5	0.203	0.176	0.001	0.027	1	0.001	0.589	0.06
E2F6	0.596	0.206	0.001	0.002	0.001	1	0.57	0.651
E2F7	0.12	0.6	0.115	0.094	0.589	0.57	1	0.811
E2F8	0.297	0.384	0.24	0.069	0.06	0.651	0.811	1

0 1

Figure 7. Relative levels of E2F factors and mutation analysis in CM (cBioPortal). A. Summary of alterations in E2Fs genes. B. Correlation analysis of E2F by heatmap. C. Gene-gene interaction network among E2F factors in the cBioPortal database. D. The protein-protein network for E2F factors in CM was analyzed via a protein-protein interaction (PPI) network.

have not been reported until now. The purpose of our research was to detect the possible connections among E2Fs expression, methylation, prognostic values and possible mechanisms in CM. We hope that our findings will help to offer new cognitive perspectives and normative handling methods, and enhance the precision of molecular diagnostic levels in CM patients.

Among the E2Fs genes, E2F1 is the most investigated in cutaneous melanoma. There is some controversial research about E2F1 in CM carcinogenesis since E2F1 can act as either an activator or inhibitor in cancer progression [25, 26]. E2F1 induces cell cycle G1 to S phase transition via abnormal activating different genes transcription, including chromosomal DNA replication and its promoter [26]. E2F1 induced migration and invasion through regulating MYLK transcription and the associated changes in the stress fibers of the cytoskeleton [27]. Vijay Alla [28] found that high expression of E2F1 mRNA was common in high-grade cancers, which is related to poor patient survival. In our study, data from the Oncomine, TCGA datasets and qPCR showed E2F1 mRNA was highly expressed in human CM tissues compared to normal tissues. However, E2F1 expression is not related to tumor grade. Further, we found that highly expressed E2F1 RNA was associated with a worse OS, which is similar to the findings of a previous report [5].

E2F2 and E2F3 were also reported to act as tumor suppressors according to published studies [29, 30]. For instance, E2F2 mRNA and protein levels were upregulated in nonsmall cell lung cancer (NSCLC) and acted as an activator of the tumor progression of NSCLC [31]. In other literature, E2F2 inhibited embryo fibroblast cell proliferation and decreased expression of E2F2 suppressed cell viability in breast cancer [32]. E2F3 played a key role in regulating the transcriptional effects of multiple cancers [6, 29]. For example, E2F3 served to transactivate HIF-2α transcription and promoted clear cell renal cell carcinoma cell proliferation by influencing the E2F3-HIF-2α interaction [29]. Additionally, E2F3 was upregulated in human melanoma, and knocking down E2F3 gene expression could inhibit cell viability and cause cells to arrest in G0 phase [29]. In our research, E2F2 and E2F3 mRNA were highly expressed in CM samples. Notably, the upregulation of E2F2 mRNA was obviously related to lower OS rates, while a relatively high level of E2F3 mRNA showed a reduced OS and DFS rate.

As transcriptional suppressors of E2F members, E2F4 and E2F5 were reported to regulate cell viability, cell cycle or cellular transformation procession with a dimerization partner and inhibitory pocket proteins (Rbs) [33]. E2F4 interacted with chromatin regulators associated with gene activation and regulated transcriptional activation in embryonic stem cells independently of the RB family [34]. In another study, E2F4 could influence acute myeloid leukemia [35], breast cancer [36], bladder cancer [37], and some benign diseases [38] progression. E2F5 was confirmed to act as a transcriptional target in the pathogenesis of hepatocellular carcinoma [39], ovarian cancer [40], gastric cancer [41] and colon cancer [42]. However, the expression and prognostic role of E2F4 and E2F5 in CM have not been reported. Here, we found that E2F4 and E2F5 were highly expressed in CM samples, but their expression was not statistically associated with CM tumor stage, OS or DFS. The detailed function of E2F4 and E2F5 in CM needs to be further investigated.

E2F6, one of the unique E2F transcription members, participates in the control of a large number of genes by activating or repressing mechanisms regarding breast cancer [43], ovarian cancer [44], endometrial carcinoma [45] and lung cancer [46]. Until now, there was no research related to the effect of E2F6 in CM. We found E2F6 mRNA and protein expression were upregulated in CM samples compared to normal tissues. Meanwhile, high expression of E2F6 mRNA levels was related to CM patients' OS and DFS, which suggests it could be a new diagnostic and treatment target in CM.

E2F7 and E2F8 possessed analogous tumor-restraining effects while there was a difference in molecular mechanisms [47-49]. Although there was no research reporting its expression and roles in CM, our studies revealed that high expression of E2F7 and E2F8 were related to tumor stage, but apart from an association with OS and DFS. Thus more molecular assays are needed to reveal the possible functions of E2F7 and E2F8 in CM.

DNA methylation is an important epigenetic mechanism that plays an important role in reg-

ulating gene expression and other biological behaviors. Aberrant DNA promoter methylation participates in the alteration of multiple oncological pathways with relevant theranostic utility in almost all tumors [50, 51]. The detection of promoter methylation is an important indicator of tumor prognosis. In this study, we found that E2Fs were hypomethylated in CM samples and high expression of E2F1/2/3/6 was correlated with the promoter CpG methylation status, suggesting that E2F1/2/3/6 were acting as oncogenes in CM progression. Multiple signaling pathways are involved in CM progression, including the Wnt/β-catenin pathway [52], the MITF/IFNγ pathway [53] and the AKT pathway [54]. In this research, we noticed that E2F1/2/3/6 interacted with MAP3K1, MAP3K11, PTEN, MYC, and et al., which are involved in the PI3K-Akt, p53 and MAPK signaling pathways, respectively. The above results indicated that the MAPK, PI3K-Akt and p53 signaling pathways participated in E2Fs regulation of CM. Thus, the possible following bold assumption could be proposed: transcriptional E2F1/2/3/6 are potential prognostic biomarkers and may play a key role through influencing the MAPK, PI3K-Akt and p53 signaling pathways.

In our research, we found that the upregulation of E2F1/2/3/6 in CM samples regulates CM oncogenesis, and they may be promising diagnostic biomarkers for CM. Meanwhile, the abnormal high expression of E2F1/2/3/6 is statistically related to DNA promoter methylation, OS and DFS, hinting that they may also function as prospective diagnostic and therapeutic markers in CM.

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Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7-34.
[2] Rocca MS, Benna C, Mocellin S, Rossi CR, Msaki A, Nisio AD, Opocher G and Foresta C. E2F1 germline copy number variations and melanoma susceptibility. J Transl Med 2019; 17: 181.
[3] Tsantoulis PK and Gorgoulis VG. Involvement of E2F transcription factor family in cancer. Eur J Cancer 2005; 41: 2403-2414.
[4] Attwooll C, Denchi EL and Helin K. The E2F family: specific functions and overlapping interests. EMBO J 2004; 23: 4709-4716.
[5] Rouaud F, Hamoudatekaya N, Cerezo M, Abbe P, Zangari J, Hofman V, Ohanna M, Mograbi B, Elhachem N and Benfodda Z. E2F1 inhibition mediates cell death of metastatic melanoma. Cell Death Dis 2018; 9: 527-527.
[6] Feng Z, Peng C, Li D, Zhang D, Li X, Cui F, Chen Y and He Q. E2F3 promotes cancer growth and is overexpressed through copy number variation in human melanoma. Onco Targets Ther 2018; 11: 5303-5313.
[7] Raghavachari N, Barb J, Yang Y, Liu P, Woodhouse K, Levy D, O'Donnell CJ, Munson PJ and Kato GJ. A systematic comparison and evaluation of high density exon arrays and RNA-seq technology used to unravel the peripheral blood transcriptome of sickle cell disease. BMC Med Genomics 2012; 5: 28-28.
[8] Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, Barrette TR, Anstet MJ, Kincead-Beal C, Kulkarni P, Varambally S, Ghosh D and Chinnaiyan AM. Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia 2007; 9: 166-180.
[9] Tang Z, Li C, Kang B, Gao G, Li C and Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 2017; 45: W98-W102.
[10] Mu J, Hui T, Shao B, Li L, Du Z, Lu L, Ye L, Li S, Li Q and Xiao Q. Dickkopf-related protein 2 induces G0/G1 arrest and apoptosis through suppressing Wnt/β-catenin signaling and is frequently methylated in breast cancer. Oncotarget 2017; 8: 39443-39459.
[11] Huang W, Hsu S, Huang H, Sun Y, Chou C, Weng S and Huang H. MethHC: a database of DNA methylation and gene expression in hu-

man cancer. Nucleic Acids Res 2015; 43: 856-861.
[12] Hu XF, Yao J, Gao SG, Yang YT, Peng XQ and Feng XS. Midkine and syndecan 1 levels correlate with the progression of malignant gastric cardiac adenocarcinoma. Mol Med Rep 2014; 10: 1409-1415.
[13] Cerami E, Gao J, Dogrusoz U, Gross B, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer M and Larsson E. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-404.
[14] Talantov D, Mazumder A, Yu J, Briggs T, Jiang Y, Backus J, Atkins D and Wang Y. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res 2005; 11: 7234-7242.
[15] Riker AI, Enkemann SA, Fodstad O, Liu S, Ren S, Morris CG, Xi Y, Howell P, Metge BJ and Samant RS. The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. BMC Med Genomics 2008; 1: 13.
[16] Haqq CM, Nosrati M, Sudilovsky D, Crothers J, Khodabakhsh D, Pulliam BL, Federman S, Miller JR, Allen RE and Singer MI. The gene expression signatures of melanoma progression. Proc Natl Acad Sci U S A 2005; 102: 6092-6097.
[17] Herman JG and Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003; 349: 2042-2054.
[18] Jones PA and Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3: 415-428.
[19] Xu M, Liu X, Xu Y, Zhu S and Gao Y. Co expression of Axin and APC gene fragments inhibits colorectal cancer cell growth via regulation of the Wnt signaling pathway. Mol Med Rep 2017; 16: 3783-3790.
[20] Kim D, Suh J, Surh Y and Na H. Regulation of the tumor suppressor PTEN by natural anticancer compounds. Ann N Y Acad Sci 2017; 1401: 136-149.
[21] Duffy MJ, Synnott NC, Mcgowan PM, Crown J, Oconnor DP and Gallagher WM. p53 as a target for the treatment of cancer. Cancer Treat Rev 2014; 40: 1153-1160.
[22] Muscarella P, Bloomston M, Brewer AR, Mahajan A, Frankel WL, Ellison EC, Farrar WB, Weghorst CM and Li J. Expression of the p16INK4A/Cdkn2a gene is prevalently downregulated in human pheochromocytoma tumor specimens. Gene Expr 2008; 14: 207-216.
[23] Ogishima J, Taguchi A, Kawata A, Kawana K, Yoshida M, Yoshimatsu Y, Sato M, Nakamura H, Kawata Y and Nishijima A. The oncogene KRAS promotes cancer cell dissemination by stabilizing spheroid formation via the MEK pathway. BMC Cancer 2018; 18: 1-13.
[24] Liu D, Xu W, Ding X, Yang Y, Lu Y, Fei K and Su B. Caspase 8 polymorphisms contribute to the prognosis of advanced lung adenocarcinoma patients after platinum-based chemotherapy. Cancer Biol Ther 2017; 18: 948-957.
[25] Sheldon LA. Inhibition of E2F1 activity and cell cycle progression by arsenic via retinoblastoma protein. Cell Cycle 2017; 16: 2058-2072.
[26] Wang Y, Alla V, Goody D, Gupta SK, Spitschak A, Wolkenhauer O, Putzer BM and Engelmann D. Epigenetic factor EPC1 is a master regulator of DNA damage response by interacting with E2F1 to silence death and activate metastasis-related gene signatures. Nucleic Acids Res 2016; 44: 117-133.
[27] Lai Q, Giralt A, May CL, Zhang L, Cariou B, Denechaud P and Fajas L. E2F1 inhibits circulating cholesterol clearance by regulating Pcsk9 expression in the liver. JCI Insight 2017; 2: e89729.
[28] Alla V, Kowtharapu BS, Engelmann D, Emmrich S, Schmitz U, Steder M and Putzer BM. E2F1 confers anticancer drug resistance by targeting ABC transporter family members and Bcl-2 via the p73/DNp73-miR-205 circuitry. Cell Cycle 2012; 11: 3067-3078.
[29] Gao Y, Li H, Ma X, Fan Y, Ni D, Zhang Y, Huang Q, Liu K, Li X and Wang L. E2F3 upregulation promotes tumor malignancy through the transcriptional activation of HIF-2α in clear cell renal cell carcinoma. Oncotarget 2017; 8: 54021-54036.
[30] Yuwanita I, Barnes D, Monterey MD, Oreilly S and Andrechek ER. Increased metastasis with loss of E2F2 in Myc-driven tumors. Oncotarget 2015; 6: 38210-38224.
[31] Chen L, Yu JH, Lu ZH and Zhang W. E2F2 induction in related to cell proliferation and poor prognosis in non-small cell lung carcinoma. Int J Clin Exp Pathol 2015; 8: 10545-10554.
[32] Hollern DP, Honeysett J, Cardiff RD and Andrechek ER. The E2F transcription factors regulate tumor development and metastasis in a mouse model of metastatic breast cancer. Mol Cell Biol 2014; 34: 3229-3243.
[33] Liban TJ, Thwaites MJ, Dick FA and Rubin SM. Structural conservation and E2F binding specificity within the retinoblastoma pocket protein family. J Mol Biol 2016; 428: 3960-3971.
[34] Hsu J, Arand J, Chaikovsky AC, Mooney N, Demeter J, Brison CM, Oliverio R, Vogel H, Rubin SM and Jackson P. E2F4 regulates transcriptional activation in mouse embryonic stem cells independently of the RB family. Nat Commun 2019; 10: 2939.

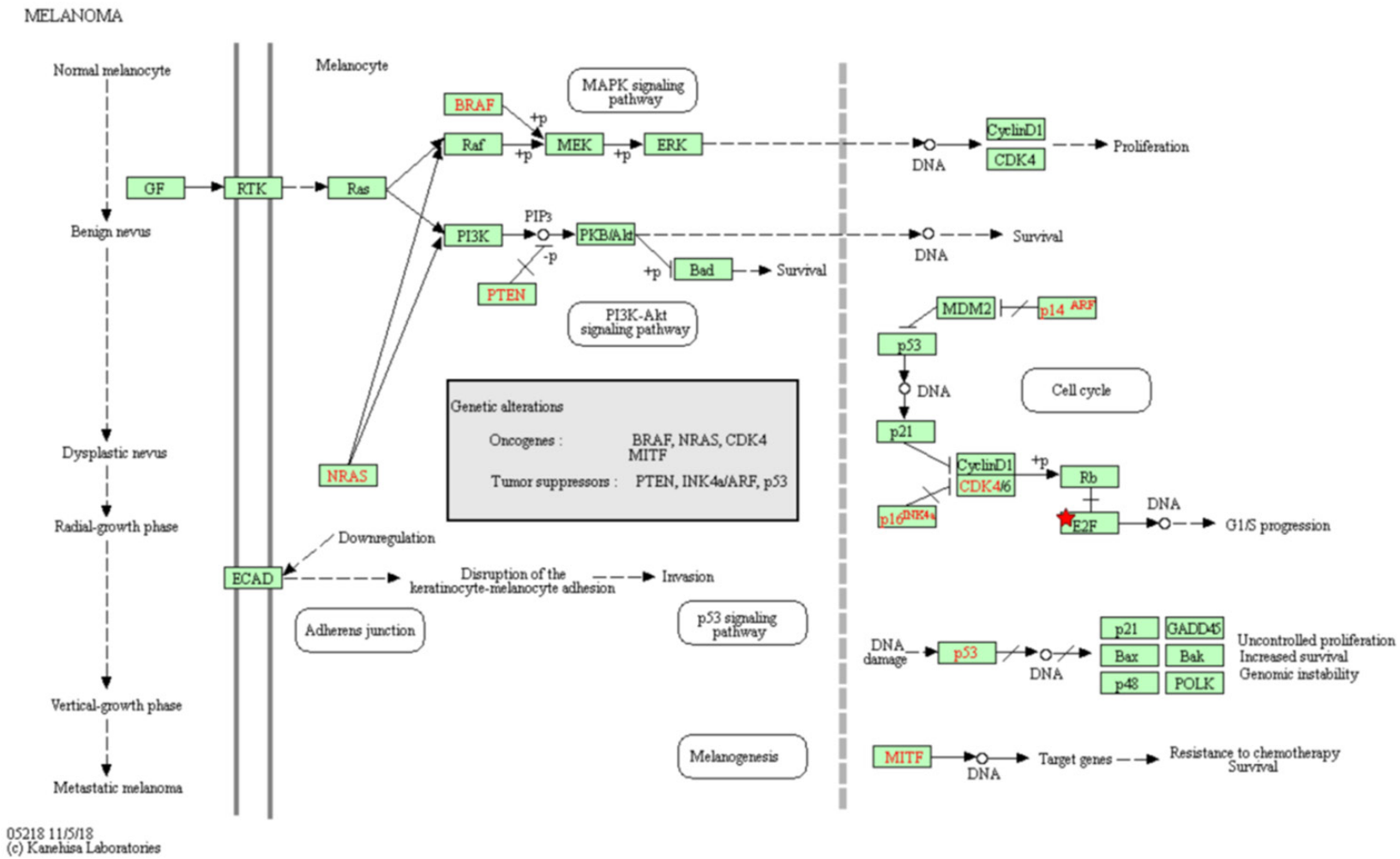
[35] Feng Y, Li L, Du Y, Peng X and Chen F. E2F4 functions as a tumour suppressor in acute myeloid leukaemia via inhibition of the MAPK signalling pathway by binding to EZH2. J Cell Mol Med 2020; 24: 2157-2168.
[36] Khaleel SS, Andrews E, Ung M, Direnzo J and Cheng C. E2F4 regulatory program predicts patient survival prognosis in breast cancer. Breast Cancer Res 2014; 16: 486-486.
[37] Cheng C, Varn FS and Marsit CJ. E2F4 program is predictive of progression and intravesical immunotherapy efficacy in bladder cancer. Mol Cancer Res 2015; 13: 1316-1324.
[38] Hsu J and Sage J. Novel functions for the transcription factor E2F4 in development and disease. Cell Cycle 2016; 15: 3183-3190.
[39] Jiang Y, Yim S, Xu H, Jung S, Yang SY, Hu H, Jung CK and Chung Y. A potential oncogenic role of the commonly observed E2F5 overexpression in hepatocellular carcinoma. World J Gastroenterol 2011; 17: 470-477.
[40] Kothandaraman N, Bajic VB, Brendan PN, Huak CY, Keow PB, Razvi K, Saltotellez M and Choolani M. E2F5 status significantly improves malignancy diagnosis of epithelial ovarian cancer. BMC Cancer 2010; 10: 64.
[41] Zhang X, Ni Z, Duan Z, Xin Z, Wang H, Tan J, Wang G and Li F. Overexpression of E2F mRNAs associated with gastric cancer progression identified by the transcription factor and miRNA co-regulatory network analysis. PLoS One 2015; 10: e0116979.
[42] Yao H, Lu F and Shao Y. The E2F family as potential biomarkers and therapeutic targets in colon cancer. PeerJ 2020; 8: e8562.
[43] Lafta IJ. E2F6 is essential for cell viability in breast cancer cells during replication stress. Turk J Biol 2019; 43: 293-304.
[44] Cheng FHC, Lin HY, Hwang TW, Chen YC, Huang RL, Chang CB, Yang W, Lin RI, Lin CW, Chen GCW, Mai SY, Lin JMJ, Chuang YM, Chou JL, Kuo LW, Li C, Cheng ASL, Lai HC, Wu SF, Tsai JC and Chan MWY. E2F6 functions as a competing endogenous RNA, and transcriptional repressor, to promote ovarian cancer stemness. Cancer Sci 2019; 110: 1085-1095.
[45] Lu Z, Nian Z, Jingjing Z, Tao L and Quan L. MicroRNA-424/E2F6 feedback loop modulates cell invasion, migration and EMT in endometrial carcinoma. Oncotarget 2017; 8: 114281-114291.
[46] Sun CC, Zhou Q, Hu W, Li SJ, Zhang F, Chen ZL. Li G, Bi ZY, Bi YY, Gong FY, Bo T, Yuan ZP, Hu WD, Zhan BT, Zhang Q, Tang QZ and Li DJ. Transcriptional E2F1/2/5/8 as potential targets and transcriptional E2F3/6/7 as new biomarkers for the prognosis of human lung carcinoma. Aging 2018; 10: 973-987.
[47] Lu Y, Zhang J, Li L, Li S and Yang Z. Carcinogenesis effects of E2F transcription factor 8 (E2F8) in hepatocellular carcinoma outcomes: an integrated bioinformatic report. Biosci Rep 2020; 40: bsr20193212.
[48] Mitxelena J, Apraiz A, Vallejorodriguez J, Malumbres M and Zubiaga AM. E2F7 regulates transcription and maturation of multiple microRNAs to restrain cell proliferation. Nucleic Acids Res 2016; 44: 5557-5570.
[49] Park S, Platt JT, Lee JW, Lopezgiraldez F, Herbst RS and Koo JS. E2F8 as a novel therapeutic target for lung cancer. J Natl Cancer Inst 2015; 107: djv151.
[50] Marzese DM and Hoon DS. Emerging technologies for studying DNA methylation for the molecular diagnosis of cancer. Expert Rev Mol Diagn 2015; 15: 647-664.
[51] Delpu Y, Cordelier P, Cho WC and Torrisani J. DNA methylation and cancer diagnosis. Int J Mol Sci 2013; 14: 15029-15058.
[52] Kovacs D, Migliano E, Muscardin L, Silipo V, Catricala C, Picardo M and Bellei B. The role of Wnt/β-catenin signaling pathway in melanoma epithelial-to-mesenchymal-like switching: evidences from patients-derived cell lines. Oncotarget 2016; 7: 43295-43314.
[53] Bai X, Fisher DE and Flaherty KT. Cell-state dynamics and therapeutic resistance in melanoma from the perspective of MITF and IFNγ pathways. Nat Rev Clin Oncol 2019; 16: 549-562.
[54] Wang L, Guo W, Ma J, Dai W, Liu L, Guo S, Chen J, Wang H, Yang Y and Yi X. Aberrant SIRT6 expression contributes to melanoma growth: role of the autophagy paradox and IGF-AKT signaling. Autophagy 2018; 14: 518-533.

Supplementary Table 1. List of qRT-PCR primers used in this research

Gene	Forward	Reverse
E2F1	AGCATGATCCGAGATGTGGAA	TGCTCGCACGATCGTAGCCCT
E2F2	ACGATGTCGATGCTAGCGTGG	CGTCGTACCCAACTGCTAGCT
E2F3	ACGTCGTAGCTGATGGGCAGT	CGGTGTACGTACCAAAACTG
E2F4	ACAAATGCATGGGTCCGTCGA	GACATGCCGCCTGGAGAAAC
E2F5	ACGTGGACTGGCCCAACTGCC	GACATGCCGCCTGGAGAAAC
E2F6	CGCGTAGCTACGCTACAGCTAC	ACGTGATCGTAGCTGATCGCC
E2F7	CACACACGTTAAACACCAACCT	CGTGTGGGGCACGTGGCAAC
E2F8	ACAAAGTGCGGTCACGTTTCAT	ACGATCGATGCTGATCGCGA

Supplementary Table 2. The expression of E2Fs expression in transcription level between cutaneous melanoma and normal tissues (ONCOMINE database)

Gene	Dataset	Reporter	Cutaneous melanoma vs. Normal (no. of cases)		Fold-change	t-Test	*P*-value
E2F1	TalantovMelanoma (15)	2028_s_at	Cutaneous Melanoma (45) vs. Skin (7)	Over-expressed	1.797	5.583	$2.2.\times10^{-4}$
	TalantovMelanoma (15)	204947_at	Cutaneous Melanoma (45) vs. Skin (7)	Over-expressed	2.731	5.615	7.67×10^{-5}
E2F2	TalantovMelanoma (15)	207042_at	Cutaneous Melanoma (45) vs. Skin (7)	Over-expressed	1.588	2.965	0.006
E2F3	Riker Melanoma (16)	203692_s_at	Cutaneous Melanoma (14) vs. Skin (4)	Over-expressed	1.938	3.612	0.005
	Riker Melanoma (16)	203693_s_at	Cutaneous Melanoma (14) vs. Skin (4)	Over-expressed	3.146	5.055	6.98×10^{-5}
	TalantovMelanoma (15)	203692_s_at	Cutaneous Melanoma (45) vs. Skin (7)	Over-expressed	2.648	10.756	1.03×10^{-6}
	TalantovMelanoma (15)	203693_s_at	Cutaneous Melanoma (45) vs. Skin (7)	Over-expressed	3.687	9.609	4.49×10^{-6}
E2F4	Haqq Melanoma (17)	AA448641 (2)	Cutaneous Melanoma (6) vs. Skin (3)	Over-expressed	3.987	1.510	0.003
	TalantovMelanoma (15)	38707_r_at	Cutaneous Melanoma (45) vs. Skin (7)	Over-expressed	1.290	3.349	0.006
E2F5	Haqq Melanoma (17)	AA455521	Cutaneous Melanoma (6) vs. Skin (3)	Over-expressed	4.479	4.077	0.003
E2F6	N/A						
E2F7	Riker Melanoma (16)	228033_at	Cutaneous Melanoma (14) vs. Skin (4)	Over-expressed	1.985	2.192	0.026
E2F8	N/A						



Supplementary Figure 1. Cutaneous melanoma downstream signal pathways regulated by the E2Fs alteration (cBioPortal).