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Systemic Analysis of the Prognosis-Related RNA Alternative Splicing Signals in Melanoma

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Background: Alternative splicing (AS), the mechanism underlying the occurrence of protein diversity, may result in cancer genesis and development when it becomes out of control, as suggested by a growing number of studies. However, systemically analyze of AS events at the genome-wide level for skin cutaneous melanoma (SKCM) is still in a preliminary phase. This study aimed to systemically analyze the bioinformatics of the AS events at a genome-wide level using The Cancer Genome Atlas (TCGA) SKCM data.

Material/Methods: The SpliceSeq tool was used to analyze the AS profiles for SKCM clinical specimens from the TCGA database. The association between AS events and overall survival was analyzed by Cox regression analysis. AS event intersections and a gene interaction network were established by UpSet plot. A multivariate survival model was used to establish a feature genes prognosis model.

Results: A total of 103 SKCM patients with full clinical parameters available were included in this study. We established an AS network that investigated the relationship between AS events and clinical prognosis information. Furthermore, 4 underlying feature genes of SKCM (MCF2L, HARS, TFR2, and RALGPS1) were found in the AS network. We performed function analysis as well as correlation analysis of AS events with gene expression. Using the multivariate survival model, we further confirmed the 4 genes that impacted the classifying SKCM prognosis at the level of AS events as well as gene expression, especially in wild-type SKCM.

Conclusion: AS events could be ideal indicators for SKCM prognosis. The key feature gene MCF2L played an important role in wild-type SKCM.

MeSH Keywords: Alternative Splicing • Melanoma • Prognosis • Survival Analysis

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Background

Protein diversity contributes to the regulatory and functional complexity of eukaryotes [1]. Alternative splicing (AS) of pre-mRNA represents the mechanism in which a small number of genes are used for producing mRNA isomers [2]. The AS process deletes the introns of multi-exon genes in humans, and at the same time, specific exons are added or ruled out alternatively [3]. In addition to protein diversity, the translation level of mRNA isomer may be reduced with the introduction of AS, which may thus degrade the early termination codon [4]. Consequently, AS is a vital process, and any changes to the splicing pattern is strongly related to protein functions, which in turn impacts numerous physiological functions of the human body, like hematopoiesis, brain development, and muscular activity [5,6].

Numerous studies in recent years have confirmed the correlation of AS with the genesis and development of cancer [7,8]. Uncontrolled AS has been shown to be involved in numerous carcinogenic processes such as proliferation, apoptosis inhibition, angiogenesis, immune escape, and metastasis [9,10]. Thus, it is important to explore cancer pathogenesis at the AS level to help establish a new approach to identifying biomarkers related to tumor prognosis as well as identify new therapeutic targets.

Skin cutaneous melanoma (SKCM) is one of the most frequently occurring skin malignancies [11,12]. However, melanoma studies have been rarely reported as this area of study is in a preliminary phase. According to genomic classification, SKCM could be divided into 4 subtypes: BRAF hotspot mutants subtype (52%, mainly BRAF V600E, V600K, and V600R mutations), RAS hotspot mutants subtype (28%, mainly RAS Q61R, Q61K, Q61L, Q61H, G12R/D/A, and G13R/D mutations), NF1 any mutants subtype (14%, NF1 any mutations) and triple wild-type subtype (6%, lack of hot-spot BRAF, N/H/K-RAS, or NF1 mutations) [13,14]. SKCM patient prognosis is poor, with high mortality for malignant melanoma when it enters a rapid growth period [15,16]. Approximately 50% of patients are diagnosed at the advanced disease stage even though great effort has been made to develop medical technology and techniques for early melanoma diagnosis [17]. Tremendous achievements have been attained in terms of surgical method, radiotherapy, and chemotherapy within the last 2 decades; however, the 5-year survival for melanoma has not markedly improved, which is particularly true for patients at the advanced stage [18]. The current study aimed to systemically analyze the bioinformatics of melanoma specimens using data extracted from the TCGA database, and to identify a new way to explore the related pathogenesis, prognostic biomarkers, and therapeutic targets of melanoma in terms of AS, thereby informing more accurate clinical treatment as well as prognosis judgement.

Material and Methods

AS events curation from TCGA RNA-seq data

RNA-seq AS events in melanoma specimens were retrieved from TCGASpliceSeq [19], and 103 specimens of primary SKCM were included in this study. RNA-seq data and clinical prognosis information of TCGA-SKCM cohorts were downloaded from TCGA data portal (*https://portal.gdc.cancer.gov/*). The SpliceSeq tool was used to evaluate the mRNA splicing patterns for the selected melanoma patients [20]. The PSI (percent spliced in) value, a common intuitive ratio (0-1) was used for quantifying a splicing event, and was calculated for 7 types of AS events [exon skip (ES), mutually exclusive exons (ME), retained intron (RI), alternate promoter (AP), alternate terminator (AT), alternate donor site (AD), and alternate acceptor site (AA)] [21]. Each coding gene in the 7 data types was analyzed among the melanoma specimens in terms of its distribution.

Screening of the AS events related to prognosis (overall survival and stage)

The study included 103 melanoma samples which had complete clinical parameters and at least 30 days of overall survival (OS) prognostic information. Various gene-splicing events were collected for the specimens, and univariate survival analysis was performed using the survival of R package. Genes conforming to the significance level at $P<0.05$ were screened as differential AS events related to OS. In addition, patients were divided into 2 groups according to the following cutoff value: patients in stage I and stage II were defined as early stage, patients in stage III and stage IV were deemed to be in an advanced stage. Correlation analysis were performed to evaluate the interaction of AS events and SKCM stages.

Survival analysis

All patients were divided into 2 groups for each parameter. Univariate Cox regression was performed to analysis the association between AS events and OS. Multivariate Cox regression was applied to remove any gene which might not be an independent prognostic predictor. To evaluate the prognostic predictors, survivalROC package in R was used to generate the area under curve (AUC) of receiver operating characteristic (ROC) for each model [22]. We compared the predictable validity of prognostic models at 5 years by Kaplan-Meier analysis. All reported *P* values were 2-sided.

UpSet plot and gene interaction network construction

We used UpSet plot [23] instead of Venn diagram to present relationships between interactive sets. UpSet is a new visualization technique for quantitative analysis of interaction

Table 1. Characteristics and baselines for TCGASpliceSeq-SKCM cohort.

Characteristics and baselines	
No. of Cases	103
Age (Year)	57.4±18.9
Gender M/F	64/39
Stage I/II/III/IV/NA	2/62/27/3/9
Median follow-up time (months)	69.1±1.1
OS median time (to event/months)	61.7±4.6

sets. We used it to analyze the intersection between 7 types of AS events.

The genes were mapped to the String database to detect gene interactions for AS events that displayed marked correlation with prognosis. Subsequently, gene interactions were acquired at the threshold of score >0.4 (top 25%), while Cytoscape was used for visualization.

Function analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed on genes from various AS types that showed a marked correlation with prognosis through cluster-Profile in R package. The enrichment pathways involved in the aforementioned genes were observed to allow us to detect related gene functions among different AS event types displaying marked correlation with prognosis.

Feature genes selection and prognosis model of melanoma establishment

Genes that had the Pearson correlation coefficient of gene expression with AS events of >0.4 or <−0.4 (top 25%) were screened to be feature genes for prognosis. To establish the appropriate factors for predicting melanoma prognosis and to facilitate clinical best practice, the feature genes for prognosis were screened for establishing a multivariate survival model, which was then used in detecting the accuracy of prognosis classification by the aforementioned feature genes for prognosis at the levels of AS events as well as expression profiles.
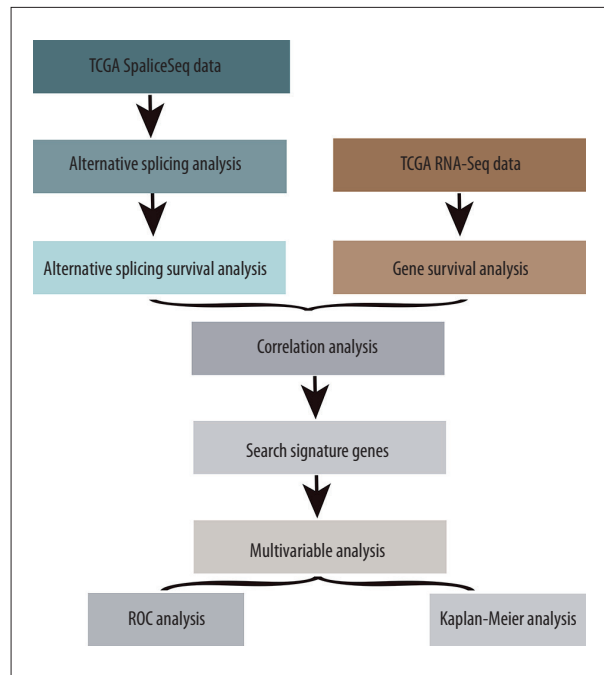

Figure 1. The schematic diagram of research methodology.

Results

AS event analysis among melanoma cases at mRNA level

Data were pre-processed according to the following methods: the FPKM (fragments per kilobase of exon model per million reads mapped) dataset regarding the RNA-Seq expression profiles was collected, which was then transformed as the TPM (Trusted Platform Module) data; meanwhile, ID was converted by the GENCODE (GRCh38.p2) genome file, and protein-coding genes were also collected. Afterwards, 103 ordinary specimens were collected from TCGASpliceSeq as well as RNA-Seq for inclusion in this study (Table 1). Specifically, 2 cases were at stage I, 62 cases at stage II, 27 cases at stage III, and 3 cases at stage IV, while the remaining 9 cases had no available information regarding staging. Meanwhile, 19 754 genes that had available expression profiles were acquired, which served as the total gene set in the current study (Figure 1).

The AS events among the 103 cancer specimens are shown in Supplementary Table 1, and the 7 AS patterns are displayed in Figure 2A. Altogether, 41 446 mRNA AS events were included, involving 9781 genes. The distribution of AS events in the 7 types is presented in Figure 2B. One gene was related to multiple AS event types at the mRNA level. AS events that had a standard deviation of >0.1 were selected, resulting in 10 348 mRNA AS events, and 4564 genes which were subsequently analyzed. The distribution of AS events in the 7 types are displayed in Figure 2C. The ES type was dominant, accounting for about 1/3 of the total AS events.
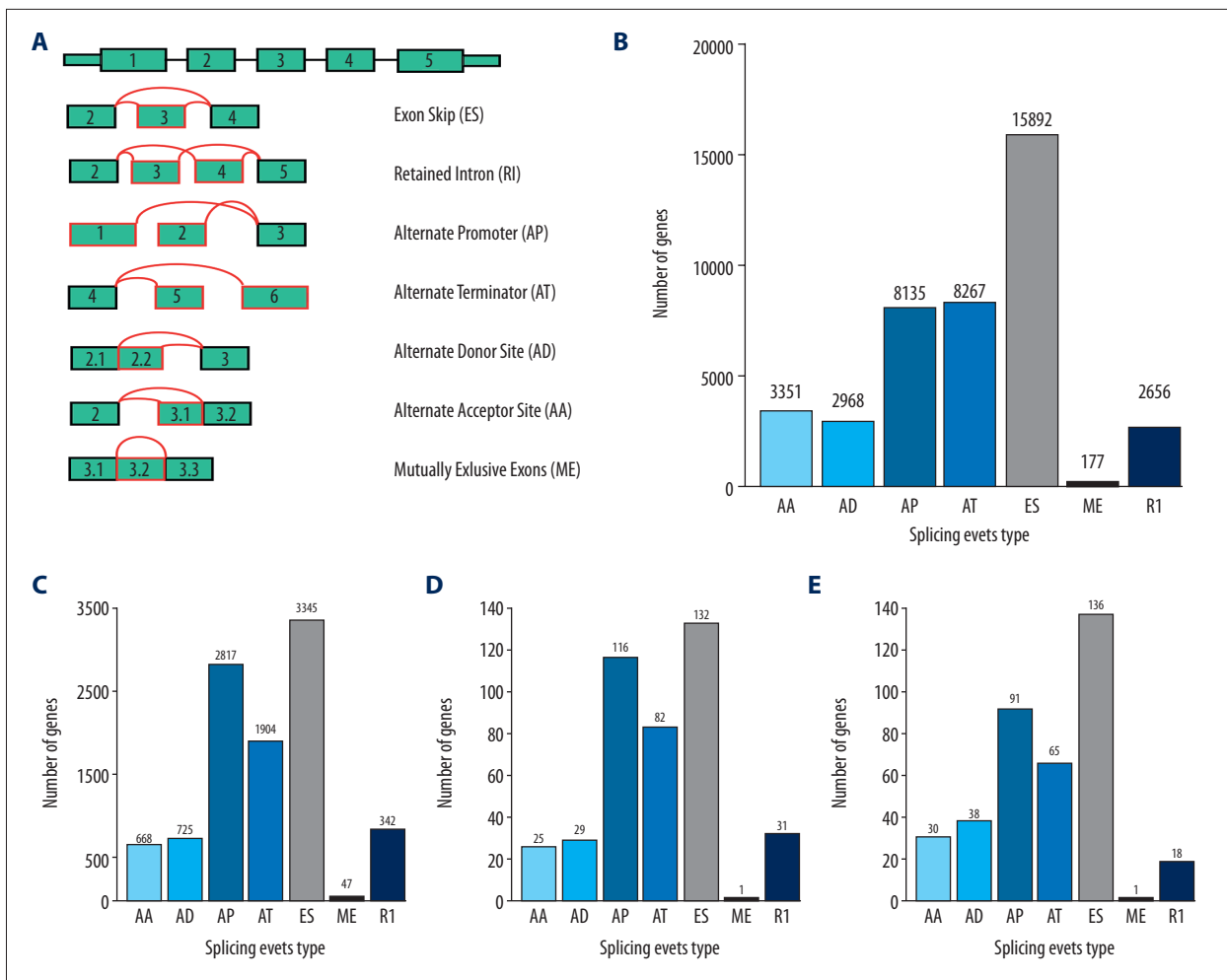
Figure 2. Comparison of all 7 alternative splicing (AS) events types. (**A**) Schematic representation of all 7 AS events types. (**B**) Number of all AS events types in melanoma. (**C**) The distribution of AS events (with standard deviation >0.1) in all 7 types. (**D**) The distribution of overall survival (OS) associated AS events in all 7 types. (**E**) The distribution of stage associated AS events in all 7 types. AA – alternate acceptor; AD – alternate donor; AP – alternate promoter; AT – alternate terminator; ES – exon skip; RI – retained intron; ME – mutually exclusive exons.

Prognosis (OS and stage) associated AS events in the TCGA melanoma cohort

To examine the correlation of AS events with cancer prognosis, the follow-up information was combined (Supplementary Table 2). The univariate survival analysis was carried out on 10 348 AS events to detect correlation of the aforementioned AS events with melanoma prognosis. Altogether, there were 416 AS events displaying remarkable correlation with overall survival (OS) at a threshold of $P<0.05$, and 294 genes were involved. Furthermore, patients at stage I and stage II were defined as early stage, while those at stage III and stage IV were deemed in advanced stage. According to our results, 379 AS events were markedly correlated with stage, and 282 genes were involved (Supplementary Table 3). The distribution analysis regarding AS events displaying marked correlation with OS

are presented in Figure 2D, which shows that the ES, AT, and AP type of events were dominant. The statistics regarding AS events displaying marked correlation with stage are shown in Figure 2E, which shows a consistent trend with that of OS. In addition, we noticed that 1 gene might have 2 or more events which were significantly associated with patient prognosis. Thus, the UpSet plot was generated and is shown in Figure 3, which suggests that 1 gene could be related to different types of AS events, while the latter could be related to prognosis.

AS event-related prognostic predictors for melanoma patients

We selected the top significant prognoses associated in 7 types of AS events as candidates to carry out a multivariate regression model analysis to detect the potential for using these
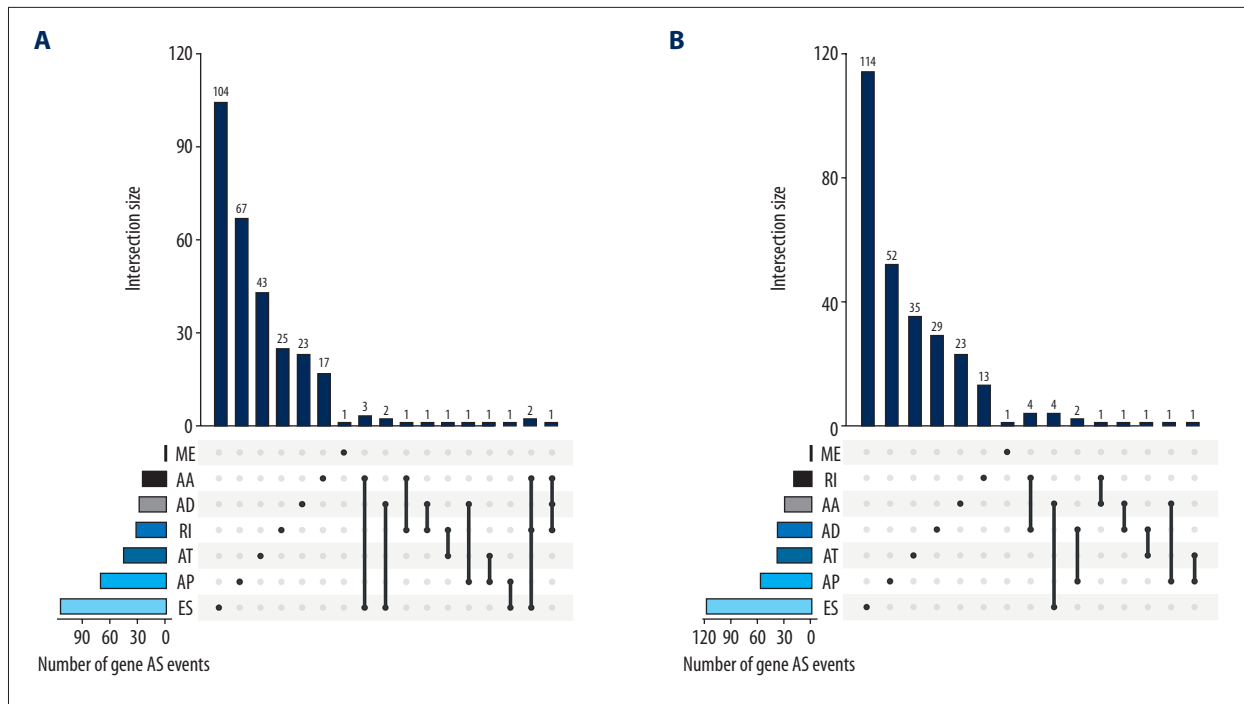
Figure 3. UpSet plots in melanoma. The UpSet intersection diagram shows all 7 types of overall survival (OS) (**A**) and stage (**B**) associated alternative splicing (AS) events in melanoma.

selective AS events as the prognostic factors. Then, their accuracy in prognosis classification was observed. We found large AUCs regarding prognosis classification among these 7 AS event types; specifically, AS events of the AP, AT, and RI types had optimal OS, whereas those of the AP and ES types were associated with the optimal performance of the selected AS events displaying marked correlation with stage (Figure 4). However, the AS events of the ME type showed insignificant correlation with prognosis. By contrast, the remaining 6 AS event types displayed favorable effect on classifying prognosis. In addition, the forest maps for the top 5 most significant survival associated AS events in the 7 types are shown in Figure 5. We found 1 respective AS events of AA and RI splicing types that had a hazard ratio (HR) of <1, while 4 had HR of >1 for OS and stage. These results suggested that AS could potentially be used as the novel method for classifying melanoma patients.

Network and function analysis of genes with prognosis associated AS events in different types

Top genes with prognosis associated AS events were mapped to the String database with the threshold of >0.4 (top 25%) to detect the gene interactions in various AS events displaying marked correlation with prognosis. Typically, gene interactions were acquired in establishing the gene interaction network for different AS events showing evident correlation with prognosis, while Cytoscape was utilized in visualization. The ES type

showed the highest interactions. In addition, a large proportion of genes related to the prognosis-associated AS events displayed correlation with protein-protein interaction (PPI), which revealed the involvement of a majority of these genes among various biological functions (Figure 6). Besides, each type of AS event exhibiting marked correlation with prognosis had a KEGG enrichment analysis performed to detect the gene functions among different AS event types. From these results (Figure 7), we found that the aforementioned genes were enriched in several disease-associated pathways, indicating their involvement in multiple biological functions.

Selecting the feature genes of melanoma and constructing the prognosis model

Genes that had the Pearson correlation coefficient regarding gene expression with AS events of >0.4 or <–0.4 (top 25%) were screened, which covered 4 genes that were markedly correlated with stage (including MCF2L [(MCF.2 cell line derived transforming sequence like], HARS [histidyl-tRNA synthetase], TFR2 [transferrin receptor-2], and RALGPS1 [ral gef with ph domain and sh3 binding motif-1]). Their associations at the transcriptome level are presented in Figure 8A, which shows 2 genes with negative correlation, among which TFR2 mutation might result in abnormality in the cell cycle [24], while RALGPS1 was found to be associated with lung cancer as well as acute lymphoblastic leukemia [25]. By contrast, the remaining 2 genes showed positive correlation, among which, MCF2L
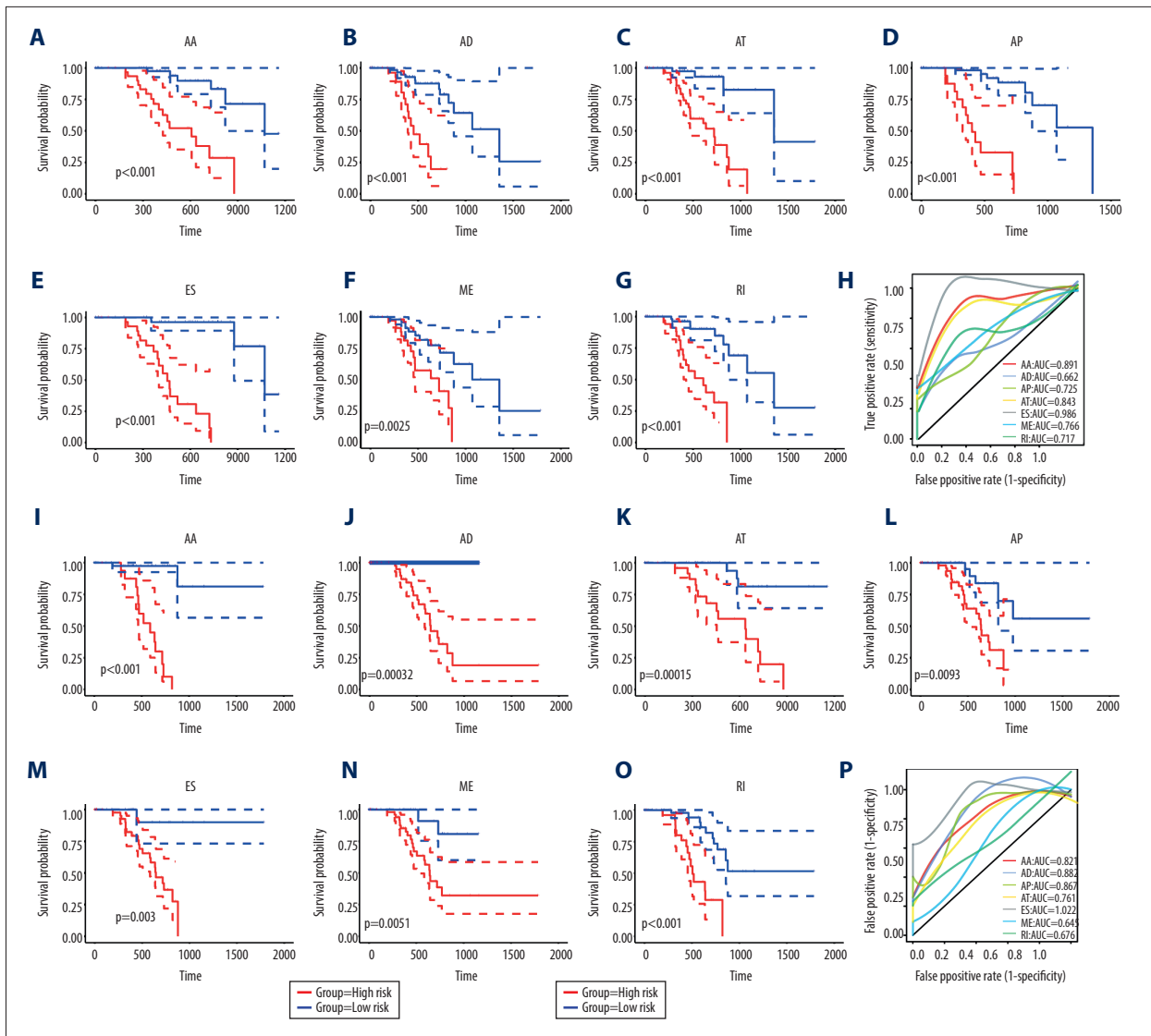
Figure 4. Kaplan-Meier and receiver operating characteristic (ROC) curves of overall survival (OS) and stage associated alternative splicing (AS) events in melanoma. Kaplan-Meier curves of OS (**A–G**) and stage (**I–O**) associated AS events. (Red: high risk group; blue: low risk group.) (**H**) ROC curves built by OS associated AS events in all 7 types in melanoma. (**P**) ROC curves built by stage associated AS events in all 7 types in melanoma.

could serve as the diagnostic biomarker for inflammatory response [26,27], whereas HARS could serve as the biomarker for monitoring disease progression [28]. Taken together, these results indicated that many of these genes play a vital role in cancer. Moreover, the 4 selected feature genes were used in constructing the multivariate survival model. Their accuracy in prognosis classification was observed at the level of AS events as well as expression profiles to establish suitable factors for predicting the prognosis for melanoma patients. These 4 genes exhibited good effects on classifying prognosis in the 2 datasets, with huge AUCs, which revealed that the 4 selected genes could be used as the prognosis biomarkers for melanoma (Figure 8B–8E).

Validating 4 feature genes prognosis model in different subtypes of melanoma

To validate the efficacy of 4 feature genes model under different subtypes of melanoma (BRAF_Hotspot_Mutants, NF1_Any_Mutants, RAS_Hotspot_Mutants, and Triple_WT), subgroup analysis was performed. The analysis showed the feature genes prognosis model works well under Triple_WT subtype rather than BRAF_Hotspot_Mutants, NF1_Any_Mutants, and RAS_Hotspot_Mutants subtype indicating that the model is more suitable in wild-type melanomas (Figure 9A–9D.) Further analysis demonstrated MCF2L had a significant higher expression under Triple_WT subtype (Figure 9E, Table 2). HARS, TFR2,
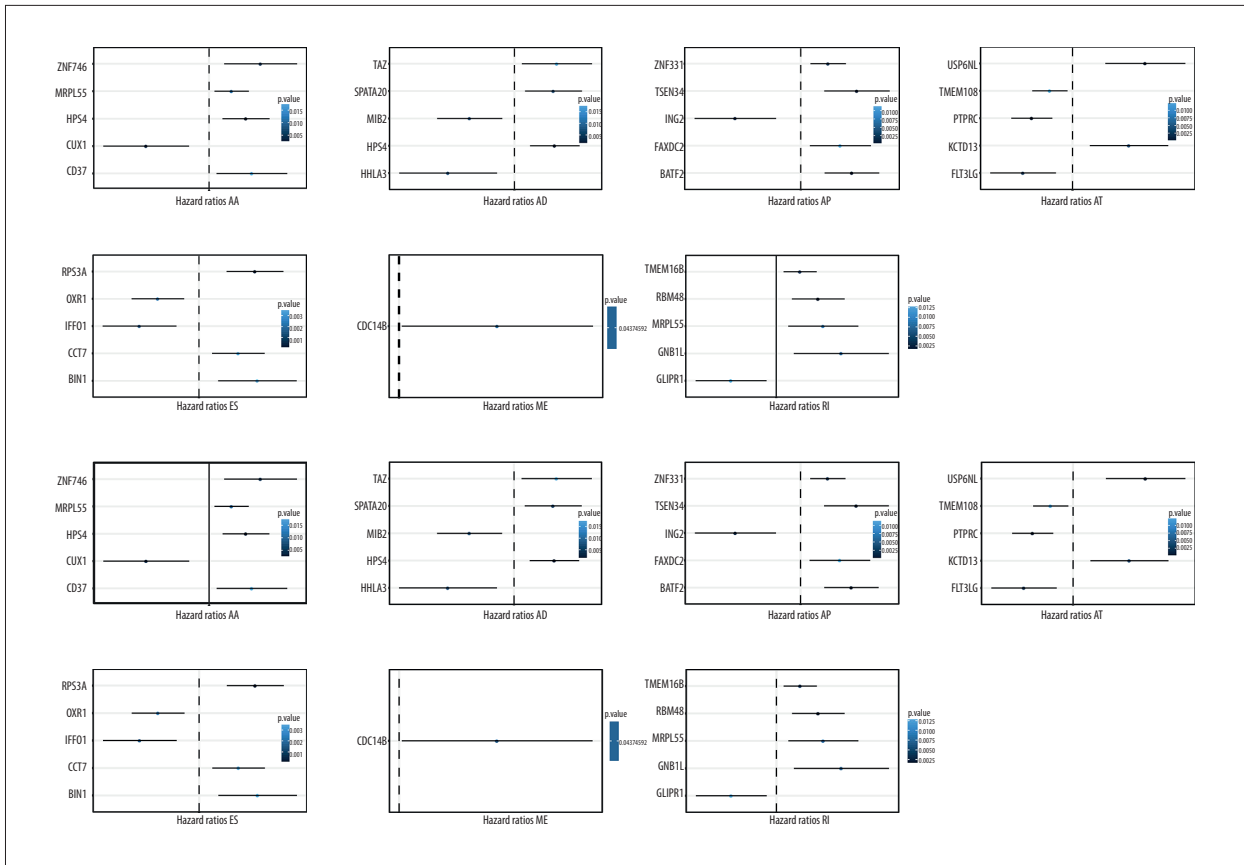
Figure 5. Forest plots for subgroup analyses of overall survival (OS) and stage associated alternative splicing (AS) events in melanoma. Forest plots of hazard ratios (HRs) for top OS (**A**) and stage (**B**) associated AS events in melanoma. (The color scale of circles: *P*-values; horizontal bars: 95% confidence intervals.)

Figure 6. Gene network of prognosis associated AS events in melanoma. Gene network of overall survival (OS) (**A**) and stage (**B**) associated alternative splicing (AS events interaction network created by Cytoscape.

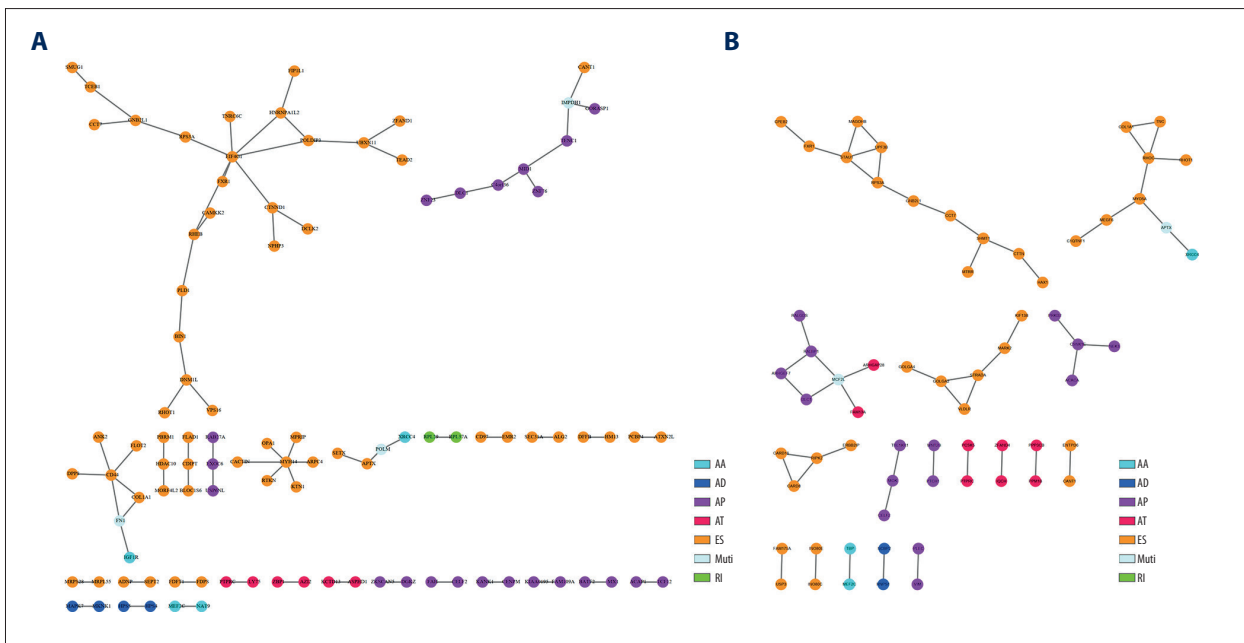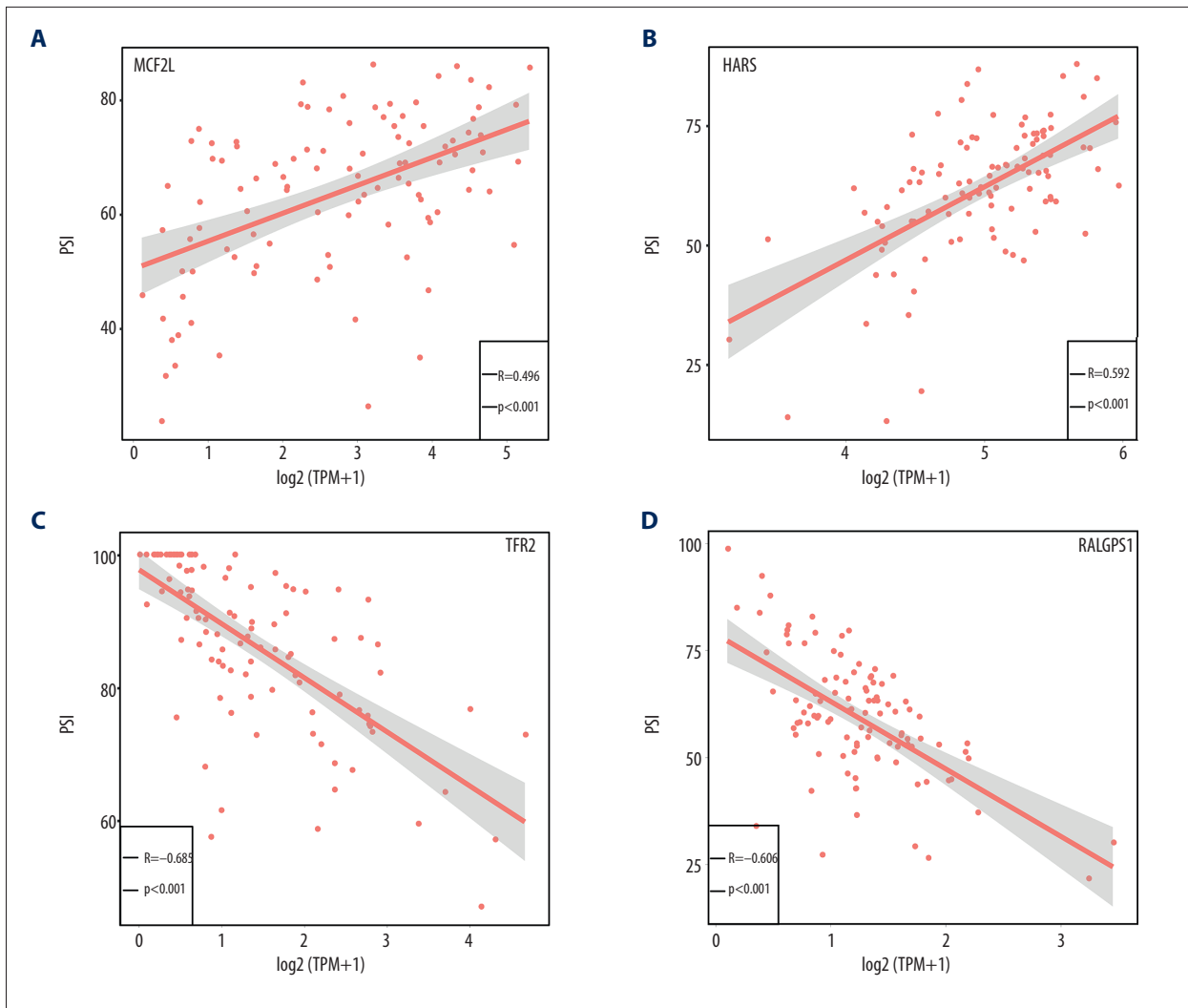**Figure 7.** (**A–D**) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of genes with prognosis associated alternative splicing (AS) events in each type.

and RALGPS1 did not have any expression difference under different melanoma subtypes. MCF2L might be a potential key biomarker and therapeutic target for wild-type melanomas.

Discussion

Melanoma, one of the skin cancers with the highest malignant grade, affects about 200 000 new cases annually. Melanoma only accounts for 4% of all skin malignancies, but it causes about 80% of skin cancer-related deaths. The survival rate for melanoma depends on the clinical stage at the time of diagnosis, and the 5-year survival rate ranges from 15% to 60% in cases developing distant as well as local metastases [29,30].

Melanoma is a kind of disease with high complexity and heterogeneity. Tumor heterogeneity mediates numerous clinical

subtypes which results in distinct sensitivities to chemotherapeutics as well as targeted preparations, along with different prognoses [31,32]. It is critical to investigate and apply clinical biomarkers when assessing prognosis, classifying molecular type, determining grade, judging recurrence, and selecting suitable drugs and treatment schemes [33].

AS represents a process in which various exon combinations can be integrated and produce numerous mRNA isoforms based on a single transcript. As a result, proteins with different structures, functions, and locations can be produced. In humans, AS can be observed in over 95% of multi-exonic genes encoding proteins. AS has a key great part to play in regulating gene expression; as a result, any abnormality in splicing may be related to various human diseases [34]. Specifically, abnormalities in AS have been frequently detected in different cancers, exemplified by p53 and PTEN, BRCA1 and PRMT2
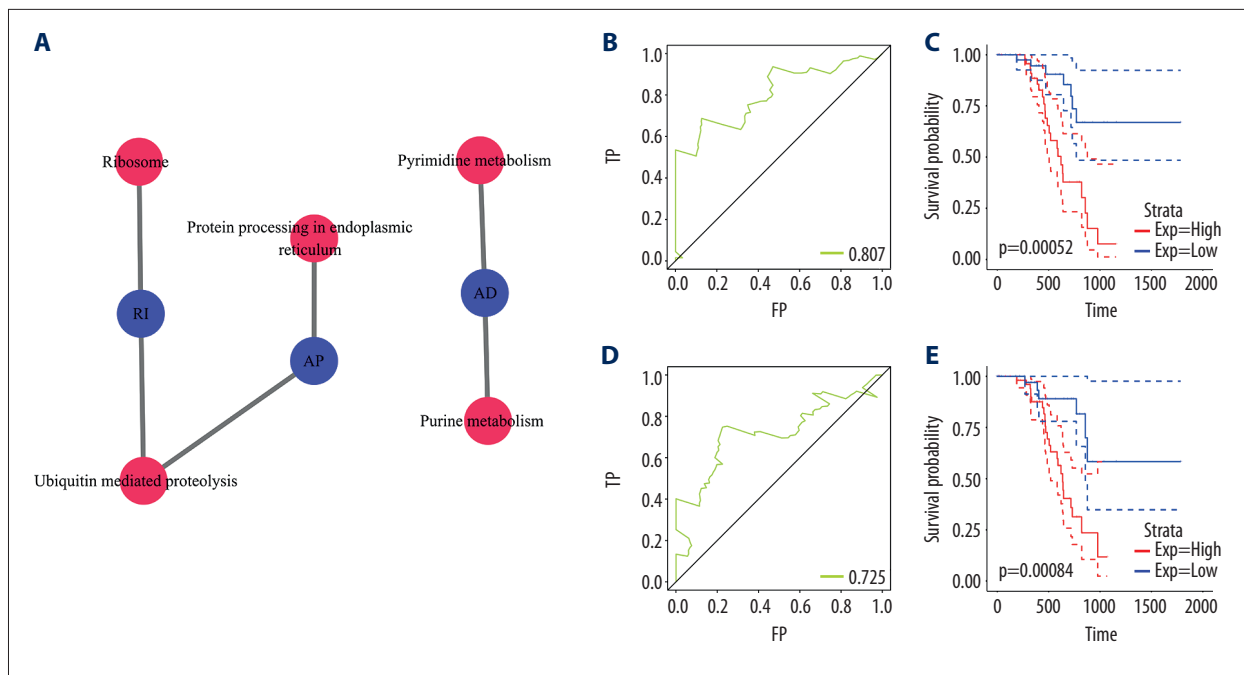
Figure 8. Correlation between 4 feature gene expression and PSI (percent spliced in) values of alternative splicing (AS) events and multivariate survival analysis of 4 characteristic genes. (**A**) Dot plots of correlation between expression of 4 feature genes and PSI values of AS events. (**B**) Receiver operating characteristic (ROC) curves of multivariate survival analysis of transcriptome level and stage. (**C**) Kaplan-Meier curves with multivariate survival analysis of transcriptome level and stage. (**D**) ROC curves of multivariate survival analysis of PSI values and stage. (**E**) Kaplan-Meier curves with multivariate survival analysis of PSI values and stage.

in breast cancer [35], TIMP1 and CD44 in colon cancer [36], together with Bcl-xL and CD44 in lung cancer [37].

Several preliminary studies discovered abnormal AS in melanoma. It has been reported in the literature that aberrant JMJD6 splicing level boosts the carcinogenesis of melanoma by regulating the PAK1 AS [38]. At the same time, MDA-7/IL-24 was found to change the AS level of Bcl-x pre-mRNA through the SRC/PKCδ signaling axis, thus causing cytotoxicity [39]. In addition, CD44 (CD44v8-10) AS has been reported to result in the metastasis of melanoma cells by suppressing U2AF2 ubiquitination as well as degradation [40]. In conclusion, these findings have confirmed the vital role of AS in melanoma genesis as well as development. Therefore, it is necessary to carry out systemic analysis of AS among melanoma specimens and to screen for relevant prognosis biomarkers as well as therapeutic targets.

In this study, 103 melanoma specimens extracted from TCGA were systemically analyzed, and the relationships of AS events with prognosis were examined. In the meantime, key genes that affected melanoma prognosis were also analyzed and identified using the AS events at a genome-wide level. In addition, the melanoma specimens were divided into high- or low-risk groups through the prognosis model established on the basis

of gene expression profiles and AS events. According to our findings, AS events and their regulated genes might be used as factors to predict prognosis and potential therapeutic targets, with a large sample size, and they could facilitate more accurate instruction for clinical treatment as well as prognosis judgement. Consequently, 4 possible feature genes were identified: MCF2L, HARS, TFR2, and RALGPS1. According to multivariate survival model analysis, the 4 selected feature genes were effective for classifying melanoma prognosis at the level of AS events as well as gene expression. In subgroup analysis, the feature genes prognosis model was more suitable for wild-type melanomas, and MCF2L had a higher expression in wild-type rather than the other subtypes. MCF2L is widely studied in the field of inflammatory diseases, and variants of MCF2L could aggravate osteoarthritis immune response [41]. HRS could induce mouse myositis model through MyD88-dependent Toll-like receptor (TLR) pathways, confirming the importance of HRS in the innate immune system [42]. TFR2 plays an important role in iron homeostasis regulation, which is localized on membrane lipid rafts and is highly expressed during the cell cycle S-M phase [43]. TFR2 can induce ERK1/2 phosphorylation to promote the growth of colorectal cancer cells [44]. But its relationship with SKCM has not been reported. RALGPS1 activates RalA and RalB by stimulating the exchange of Ral bound GDP to GTP, thus regulating
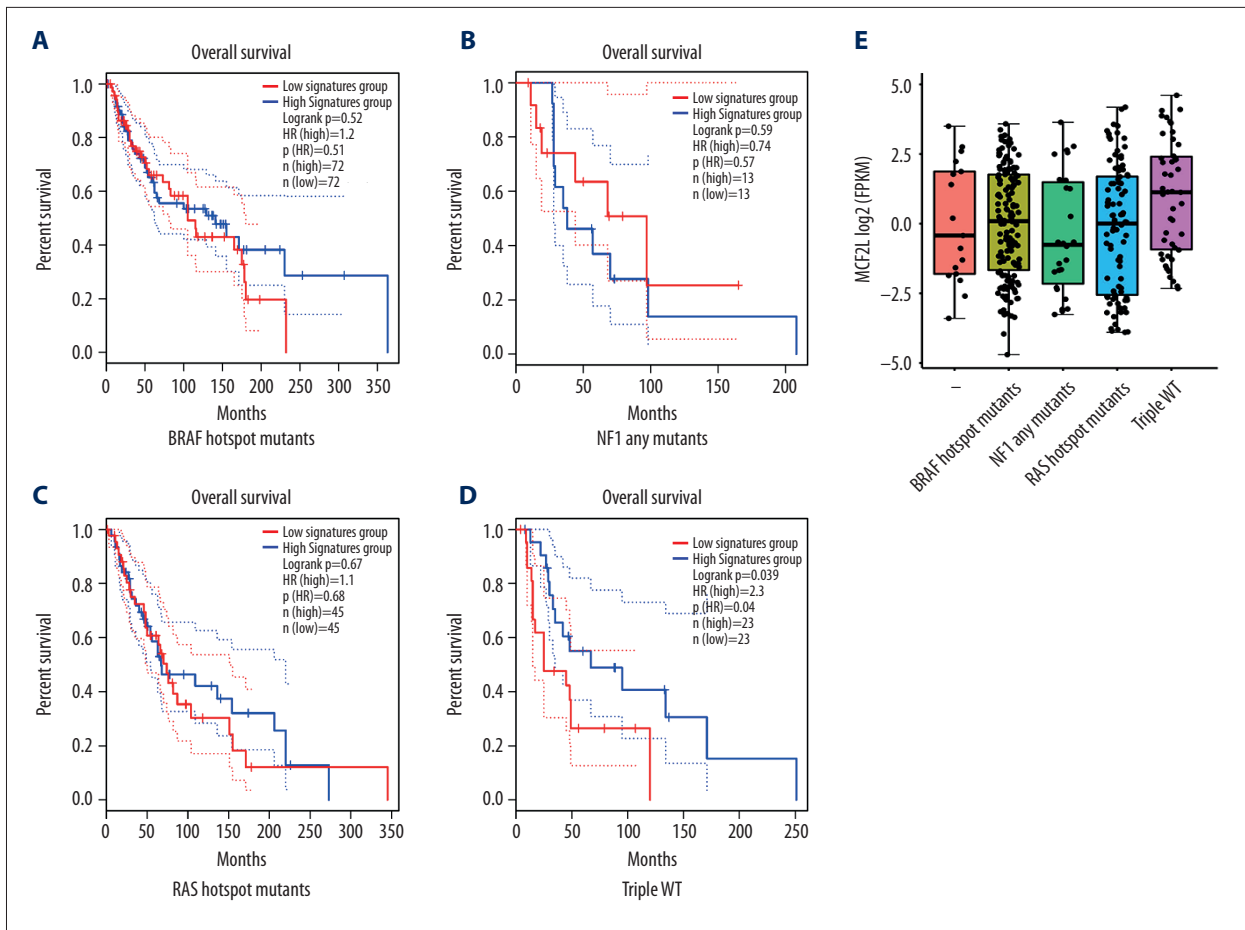
Figure 9. Efficacy of 4 feature genes model under different melanoma subtypes. (**A**) BRAF_Hotspot_Mutants. (**B**) NF1_Any_Mutants. (**C**) RAS_Hotspot_Mutants. (**D**) Triple_WT. (**E**) Gene expression of MCF2L in different melanoma subtypes.

Table 2. Differences in MCF2L gene expression among 4 melanoma subtypes.

P-value	Triple_WT	BRAF_Hotspot_Mutants	RAS_Hotspot_Mutants	NF1_Any_Mutants
Triple_WT	1	0.009	0.005	0.012
BRAF_Hotspot_Mutants	0.009	1	0.355	0.272
RAS_Hotspot_Mutants	0.005	0.355	1	0.709
NF1_Any_Mutants	0.012	0.272	0.709	1

various downstream cellular processes [45]. RalA has been reported to be widely activated in human SKCM cell lines and shRNA-mediated knockdown of RalA could inhibit SKCM cell line growth [46]. MCF2L, HARS, TFR2, and RALGPS1 may serve as potential key biomarkers and therapeutic targets for SKCM and play roles in oncogenesis and tumor immune microenvironment formation.

Conclusions

Our results suggest a prognostic value of different types of AS events in SKCM. Based on our analysis of AS and feature genes, 4 prognosis-related feature genes (MCF2L, HARS, TFR2, and RALGPS1) were revealed and a multivariate survival model was constructed. Further SKCM-subtype analysis indicated that the 4 feature genes prognosis model and key feature gene MCF2L played an important role in wild-type SKCM. These findings could provide new opportunities for SKCM survival prediction and targeted therapy.

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Supplementary Data

Supplementary Table 1. AS events among included 103 cancer specimens.

Supplementary Table 2. SKCM clinical prognosis information of included specimens

Supplementary Table 3. AS events correlated with SKCM stage

Supplementary/raw data available from the corresponding author on request.

Conflicts of interest

None.

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