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The spliceosome pathway activity correlates with reduced anti-tumor immunity and immunotherapy response, and unfavorable clinical outcomes in pan-cancer

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ABSTRACT

Alterations in the spliceosome pathway (SP) have been associated with diverse human cancers. In this study, we explored associations of the SP activity with various clinical features, anti-tumor immune signatures, tumor immunity-related genomic and molecular features, and the response to immunotherapies and targeted therapies in 29 cancer types from The Cancer Genome Atlas (TCGA) database. We showed that the SP activity was an oncogenic signature, as evidenced by its hyperactivation in cancer and invasive cancer subtypes and correlations with unfavorable clinical outcomes and anti-tumor immunosuppression in various cancers. The SP activity showed positive correlations with tumor mutation burden (TMB) and aneuploidy in diverse cancers, suggesting its association with genomic instability. However, the negative association between the SP activity and anti-tumor immune response was independent of its associations with aneuploidy and TMB. Furthermore, we supported that the SP activity had a negative correlation with immunotherapy response in four cancer cohorts treated by immune checkpoint inhibitors. Moreover, elevated SP activity is correlated with increased drug sensitivity for a broad spectrum of anti-tumor targeted therapies. In conclusion, the SP activity is a negative biomarker for anti-tumor immune response, prognosis, and the response to immunotherapeutic and targeted drugs in pan-cancer.

1. Introduction

Recently, immunotherapy has achieved success in treating diverse cancers [1,2], particularly immune checkpoint inhibitors (ICIs) targeting PD-1 (programmed cell death protein 1) and its ligand PD-L1 [1]. However, only a subset of cancer patients respond to ICIs [3]. Certain molecular determinants of the response to ICIs have been identified, such as tumor mutation burden (TMB) [4], DNA mismatch repair deficiency (dMMR) [5], and PD-L1 expression [6]. In addition, the tumor immune microenvironment (TIME) is also crucial for the response to ICIs [7]. In general, "hot" tumors with dense tumor-infiltrating lymphocytes (TILs) in the TIME are more likely to respond to ICIs than "cold" tumors with sparse TILs [8]. Although some biomarkers for stratifying cancer patients responsive to ICIs are being utilized in clinical practice guidelines, e.g., PD-L1 expression, dMMR, and high TMB, the effectiveness of these biomarkers for pan-cancer immunotherapy remains controversial [9,10]. Thus, discovery of novel and effective biomarkers for cancer immunotherapy response remains an urgent need. With the recent rapid development of next-generation sequencing (NGS) technologies, large volumes of high-quality cancer genomics data have been produced through international cooperation, e.g., the Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov) and the International Cancer Genome Consortium (ICGC, https://daco.icgc.org/). Meanwhile, preclinical studies have generated a large number of data of immunotherapy response in cancer patients accompanying with their genomics data. Based on these data, many potential biomarkers for immunotherapy response have been explored, such as mutations of *TP53* [11], *ARID1A* [12], *KALRN* [13], *KRAS* [14], and

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HRAS [15], and elevated activities of glycolysis [16] and cell cycle pathways [17].

The spliceosome is a complex of ribonucleoproteins regulating RNA splicing, which is made up of five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4, U5, and U6, and several spliceosome-associated proteins (SAPs). Because RNA splicing plays an important role in gene regulation, alterations in the spliceosome pathway (SP) have been associated with diverse human cancers [18,19]. A comprehensive analysis of 32 TCGA cancer types revealed that alternative splicing events more frequently occur in cancer than in normal tissues [20]. Furthermore, tumor specific mRNA splicing events may derive neoepitopes to incite anti-tumor immune response and serve as immunotherapy target [21]. A recent study showed that spliceosome-targeted therapies may stimulate anti-tumor immune response in triple-negative breast cancer [22]. Despite these previous studies, a systematic investigation into the correlation between SP and anti-tumor immunity and immunotherapy response in pan-cancer remains lacking.

In this study, we first explored associations between SP and various clinical features, including survival prognosis, tumor progression phenotypes, and tumor subtypes in 29 TCGA cancer types. Next, we analyzed the association between SP and anti-tumor immune signatures in these cancer types. We also explored associations between SP and tumor immunity-related genomic and molecular features, including tumor mutation burden (TMB), aneuploidy, DNA damage repair pathways, and PD-L1 expression. Moreover, we explored associations of SP with the response to targeted therapies and immunotherapies. Finally, we revealed the splicing events that were significantly associated with SP and immune signatures in cancer. Our data demonstrated that SP was hyperactivated in various cancers, and elevated SP activity was correlated with unfavorable clinical outcomes, reduced anti-tumor immune response and immunotherapy response, and increased response to targeted therapies. Our findings provide new insights into the role of SP in pan-cancer.

2. Methods

2.1. Datasets

We downloaded transcriptome (RNA-Seq, RSEM normalized), somatic mutations, somatic CNAs, protein expression profiling, and clinical data for 29 TCGA cancer types from the genomic data commons data portal (https://portal.gdc.cancer.gov/). The 29 cancer types included adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous-cell carcinoma (CESC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian carcinoma (OV), and pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS). The transcriptome (RNA-Seq, RSEM normalized) data for 962 cancer cell lines and their drug sensitivities (IC50 values) to 265 compounds were obtained from the Genomics of Drug Sensitivity in Cancer website (https://www.cancerrxgene.org/downloads). RNAi sensitivity data (DEMETER dependency scores) for 662 cancer cell lines (DEMETER2 Data v6) was obtained from the Depmap website (https://depmap.org/portal/). Alternative splicing events (percentage of samples with Percent-Spliced-In (PSI) value = 0.75; minimum PSI standard deviation = 0.1) in TCGA cancers were obtained from the TCGA SpliceSeq (https://bioinformatics.mdanderson.org/TCGASpliceSeq/). The TCGA alternative splicing events were identified by SpliceSeq [23], a tool identifying genes with multiple spliceforms by aligning reads to gene splice graphs stored in a database involving known splicing patterns. For each splicing event in a tumor sample, PSI represents the proportion of reads falling on a certain exon as normalized by length. We obtained 75 key splicesome molecules (proteins) by the intersection of the spliceosome protein complex from the CORUM Protein Complexes dataset [24], the major spliceosome proteins from the HGNC (https://www.genenames.org/data/genegroup/#!/group/1518), and the KEGG SP [25]. Moreover, we obtained four datasets involving data of transcriptome and the response to ICIs in four cancer cohorts, including the Snyder cohort (melanoma) [26], the Nathanson cohort (melanoma) [27], the Braun cohort (renal cell carcinoma) [28], and the Snyder cohort (urothelial carcinoma) [29]. A summary of these datasets is provided in Supplementary Table S1.

2.2. Gene-set enrichment analysis

We used the single-sample gene-set enrichment analysis (ssGSEA) [30] to score activities of pathways, and enrichment levels of immune signatures and stemness signatures based on the expression levels of their marker genes. The marker genes of immune and stemness signatures were obtained from several publications, including CD8+ T cells [31], interferon (IFN) response [31], and tumor stemness [32]. The sets of marker genes for SP and other pathways were obtained from KEGG [33]. We present all sets of marker genes in Supplementary Table S2. To identify KEGG [33] pathways highly enriched in higher-SP-score (>median) tumors and lower-SP-score (<median) tumors, we first identified differentially expressed genes between both groups of tumors using a threshold of Student's *t* test adjusted *P* value < 0.05 and fold change of mean expression levels >1.5. The adjusted *P* values, namely false discovery rate (FDR), were evaluated by the Benjamini and Hochberg method [34]. The differentially expressed genes included the upregulated genes in higher-SP-score tumors and the upregulated genes in lower-SP-score tumors. By inputting the upregulated genes in higher-SP-score tumors into the GSEA web tool [35], we obtained the KEGG pathways highly enriched in higher-SP-score tumors with a threshold of adjusted *P* value < 0.05. We likewise obtained the KEGG pathways highly enriched in lower-SP-score tumors by inputting the upregulated genes in them into GSEA.

2.3. Calculation of immune score, stromal score, and tumor purity

We used the ESTIMATE algorithm [36] to calculate immune score, stromal score, and tumor purity for each tumor sample based on its gene expression profiles. The immune score and stromal score represent the enrichment levels of immune signatures and stromal signatures in the TIME, respectively. Tumor purity refers to the proportion of tumor cells in a bulk tumor. In evaluating the ratio of two immune signatures, we used the log2-transformed value of the geometric mean expression level of all marker genes in an immune signature over that in another immune signature.

2.4. Evaluation of TMB and homologous recombination deficiency (HRD) score

We defined the TMB of a tumor sample as the total number of somatic mutations detected in the tumor. From the publication by Knijnenburg et al [37], we obtained HRD scores (aneuploidy levels) of TCGA cancers.

2.5. Survival analysis

We used Kaplan–Meier curves to show the survival (overall survival (OS) or disease-free survival (DFS)) time differences between different groups and the Gehan-Breslow-Wilcoxon or log-rank test to assess the significance of survival time differences. To explore whether the association between SP scores and survival prognosis in cancer was affected by other confounding variables, we performed multivariate survival analysis using the multivariate Cox proportional hazards model. In the model, the response variable was OS or DFS time, and the predictor variables included SP score, immune score, stemness score, tumor purity, age, stage, grade, and metastasis status. The "SP score", "immune score", "stemness score", "tumor purity", and "age" were continuous variables, and the "stage" (early-stage (Stage I-II) versus late-stage (Stage III-IV)), "grade" (low-grade (G1-2) versus high-grade (G3-4)), and "metastasis status" (primary versus metastatic) were binary variables. We implemented the multivariate survival analysis with the function "coxph" in the R package "survival".

2.6. Logistic regression analysis

We predicted immune signature scores (high (>median) versus low (<median)) using univariate logistic regression models with three predictors (SP score, HRD score, and TMB). We also used logistic regression models to predict SP scores (high (>median) versus low (<median)) using two predictors (TMB and HRD score). In performing the logistic regression analyses, we first normalized all values by z-score and then used the R function "glm" to fit the binary model. We specified the parameter "family" as "binomial" and other parameters as default in "glm".

2.7. Statistical analysis

We calculated correlations between SP scores and other variables using the Spearman method with a threshold of $P < 0.05$ for statistical significance. In comparisons of two classes of samples, we used the Student's *t* test if they were normally distributed, otherwise we used the Mann-Whitney *U* test. We used the Benjamini and Hochberg method [35] to calculate adjusted *P* values for correcting for multiple tests. In all correlation analyses and class comparisons, we showed the cancer types in which the results were statistically significant. In addition, we present all raw data in the statistical analyses in Supplementary Table S3.

3. Results

3.1. Elevated SP scores correlate with unfavorable tumor phenotypes and clinical outcomes

In 9 cancer types (ACC, ESCA, KIRC, KIRP, LIHC, LUAD, PAAD, PRAD, and SARC), higher SP scores were correlated with better OS and/or DFS (Gehan-Breslow-Wilcoxon test, $P \leq 0.05$) (Fig. 1A). Furthermore, we analyzed associations between SP scores and tumor progression phenotypes, including tumor stage, grade, and metastasis. In seven cancer types (ACC, HNSC, KIRC, KIRP, LIHC, LUAD, and TGCT), SP scores were significantly higher in late-stage (Stage III-IV) than in early-stage (Stage I-II) tumors (one-tailed Mann-Whitney *U* test, $P < 0.05$) (Fig. 1B). In BLCA and COAD, SP scores were significantly lower in late-stage than in early-stage tumors. Among the 12 cancer types with tumor grade information available, SP scores were significantly higher in high-grade (G3-4) than in low-grade (G1-2) tumors in five cancer types (CESC, HNSC, KIRC, LIHC, and UCEC) (Fig. 1B). In addition, in three cancer types (KIRC, KIRP, and LUAD), SP scores were significantly higher in metastatic than in primary tumors (Fig. 1B).

Cancer stemness, known as tumor stem cell-like characteristics, has associations with tumor progression, relapse, and therapeutic resistance [38]. Strikingly, in 24 cancer types, SP scores had significant positive correlations with stemness scores (Spearman correlation, $P < 0.05$) (Fig. 1C). In 16 cancer types, SP scores had significant positive correlations with the expression levels of *MKI67*, a proliferation marker gene (Spearman correlation, $P < 0.05$) (Fig. 1C). ITH is common and is associated with tumor advancement, immune evasion, and drug resistance in various cancers [31]. We found that SP scores had significant positive correlations with ITH scores by DEPTH [31] in 23 cancer types (Fig. 1C). Notably, SP scores were significantly and positively correlated with the cell cycle pathway's scores in 27 cancer types, while they were negatively correlated with the apoptosis pathway's scores in 22 cancer types (Fig. 1C).

The multivariate survival analysis revealed that the SP score remained a risk factor for survival prognosis in multiple cancer types, including ACC, KIRC, KIRP, SARC, and SKCM, after adjusting for immune score, stemness score, tumor purity, age, stage, grade, and metastasis status (Supplementary Fig. S1).

Taken together, these results support that elevated SP activity is associated with unfavorable clinical outcomes in various cancers.

3.2. SP scores correlate negatively with immune signature scores in cancer

Among the 29 cancer types, SP scores showed a significant negative correlation with the enrichment levels of CD8+ T cells in 17 cancer types (Spearman correlation, $P < 0.05$) (Fig. 2A). In addition, in 15 cancer types, SP scores displayed a significant negative correlation with IFN response scores ($P < 0.05$) (Fig. 2A). Moreover, SP scores were significantly and negatively correlated with immune scores in 16 cancer types (Fig. 2A). Likewise, SP scores had a significant negative correlation with *PD-L1* expression levels in 21 cancer types ($P < 0.05$) (Fig. 2A). Furthermore, in 20 cancer types, SP scores were significantly and negatively correlated with the ratios of immune-stimulatory/immune-inhibitory signatures (CD8+/CD4 + regulatory T cells) ($P < 0.05$) (Fig. 2A). Because tumor-infiltrating CD8+ T cells are anti-tumor immune cells and CD4+ regulatory T cells are anti-tumor immunosuppressive cells, a higher ratio of them indicates a stronger anti-tumor immune response. Collectively, these results suggest that elevated SP activity is associated with reduced anti-tumor immune response in diverse cancers. Interestingly, in 25 cancer types, SP scores had a significant negative correlation with stromal scores ($P < 0.05$) (Fig. 2B). The negative correlation between SP scores and both immune and stromal scores indicates that SP scores are positively correlated with tumor purity. Indeed, SP scores were positively correlated with tumor purity in 22 cancer types ($P < 0.05$) (Fig. 2C). This also supports that tumor cells likely have higher SP scores than non-tumor cells.

3.3. SP scores correlate positively with genomic instability in cancer

We investigated associations between SP and several genomic features, including TMB and aneuploidy. We found that SP scores had a significant positive correlation with TMB in 13 cancer types

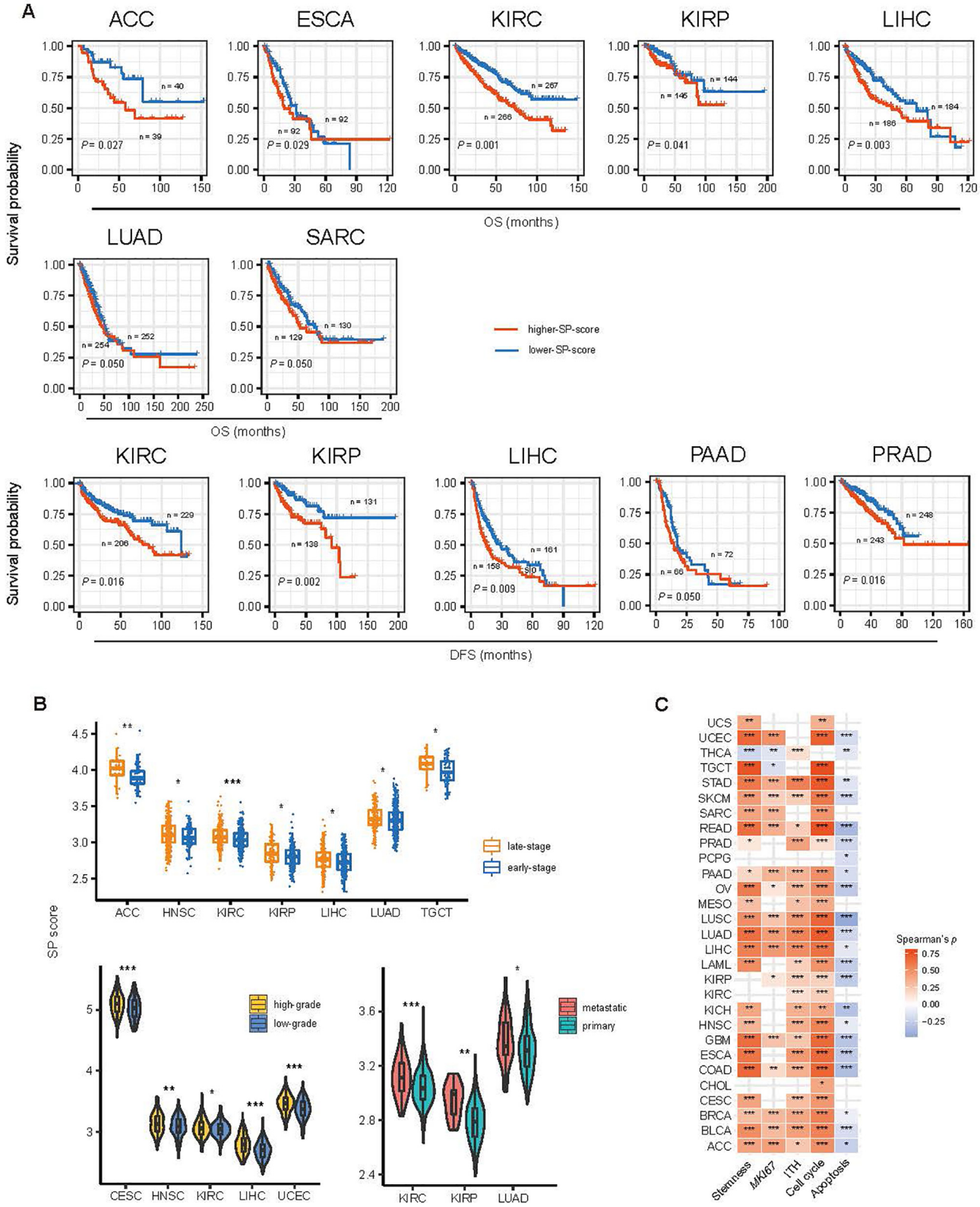

Fig. 1. Associations between SP and clinical outcomes in cancer. A. Kaplan-Meier curves showing that higher-SP-score (>median) tumors have worse survival than lower-NS-score (<median) tumors in diverse cancers. The Gehan-Breslow-Wilcoxon test *P* values are shown. B. SP scores are significantly higher in advanced (late-stage, high-grade, or metastatic) than in non-advanced (early-stage, low-grade, or primary) tumors in diverse cancers. The one-tailed Mann-Whitney *U* test *P* values are shown. C. Spearman correlations between SP scores and stemness scores, *MKI67* expression levels, ITH scores, cell cycle and apoptosis pathways' scores. The Spearman correlation coefficients (ρ) and *P* values are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. It also applies to the following figures. OS, overall survival; DFS, disease-free survival; ITH, intra-tumor heterogeneity.

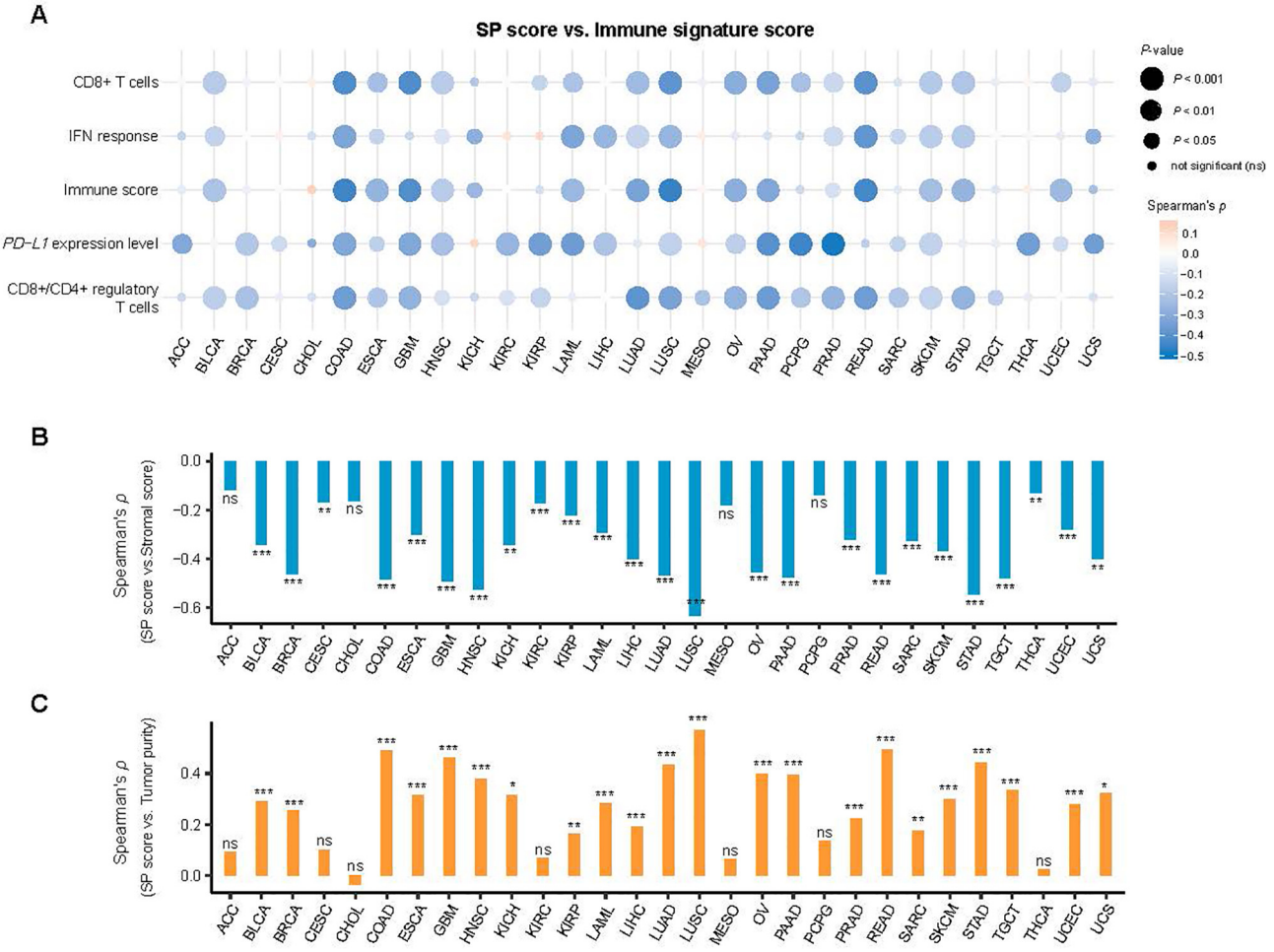

Fig. 2. Associations between SP and immune signatures in cancer. A. The significant negative correlations between SP scores and immune signature scores and the ratios of immune-stimulatory/immune-inhibitory signatures (CD8+/CD4+ regulatory T cells) in diverse cancers. B. The significant negative correlations between SP scores and stromal scores in diverse cancers. C. The significant positive correlations between SP scores and tumor purity in diverse cancers. The scores of CD8+ T cells and interferon response were calculated by the single-sample gene-set enrichment analysis (ssGSEA) [6] based on the expression levels of their marker genes. The immune scores, stromal scores, and tumor purity were calculated by ESTIMATE [25].

($P < 0.05$) (Fig. 3A). HRD may result in large-scale genomic instability, namely aneuploidy [37]. Knijnenburg et al. defined HRD scores (aneuploidy levels) in 9,125 TCGA cancer samples based on HRD loss of heterozygosity, large-scale state transitions, and the number of telomeric allelic imbalances. We found that SP scores had a significant positive correlation with HRD scores in 15 cancer types ($P < 0.05$) (Fig. 3A). Collectively, these results suggest a positive association between the SP activity and genomic instability in diverse cancers. The positive associations between the SP activity and TMB and aneuploidy implicate that increased TMB and aneuploidy may promote the SP activity in light of the impact of DNA damage on the RNA splicing response [39]. To compare the contributions of TMB and aneuploidy in altering SP, we used logistic regression models to predict SP scores (high (>median) versus low (<median)) using two predictors: TMB and HRD score. We found that HRD score was a significant positive predictor of SP scores in 15 cancer types, compared to TMB in 3 cancer types ($P < 0.05$) (Fig. 3B). It suggests that aneuploidy has a more significant impact on the SP activity than TMB.

Because both aneuploidy and TMB are correlated with anti-tumor immune response [40], and SP are significantly correlated with them in a variety of cancers, the significant correlation between SP and anti-tumor immune response could be attributed to its associations with aneuploidy and TMB. To explore this hypothesis, we used logistic regression models to predict immune signature (CD8 + T cells and IFN response) scores (high (>median) versus low (<median)) with three variables: SP score, HRD score, and TMB. Within the 29 individual cancer types, SP was a significant negative predictor of the CD8 + T cell score in 19 cancer types ($P < 0.05$, β ranging from −0.96 to −0.23) (Fig. 3C); HRD was a significant negative and positive predictor in 2 and 5 cancer types, respectively, and TMB was a significant positive predictor in 2 cancer types (COAD and SARC) ($P < 0.05$). In predicting the IFN response score, SP was a significant negative and positive predictor in 11 and 1 cancer types, respectively (Fig. 3C); HRD was a significant negative and positive predictor in 6 and 3 cancer types, respectively, and TMB was a significant positive and negative predictor in 1 and 1 cancer type, respectively. We also predicted immune scores (high (>median) versus low (<median)) using logistic regression models with the three variables in pan-cancer and in 29 individual cancer types. Consistently, SP was a significant negative predictor of the immune score in 17 cancer types ($P < 0.05$, β ranging from −1.12 to −0.24) (Fig. 3C); HRD was a significant negative and positive predictor in 4 and 4 cancer types, respectively, and TMB was a significant positive and negative predictor in 3 and 1 cancer type, respectively. In addition, we used logistic regression models to predict the ratios of immune-stimulatory/immune-inhibitory signatures (CD8+/CD4+ regulatory T cells) (high

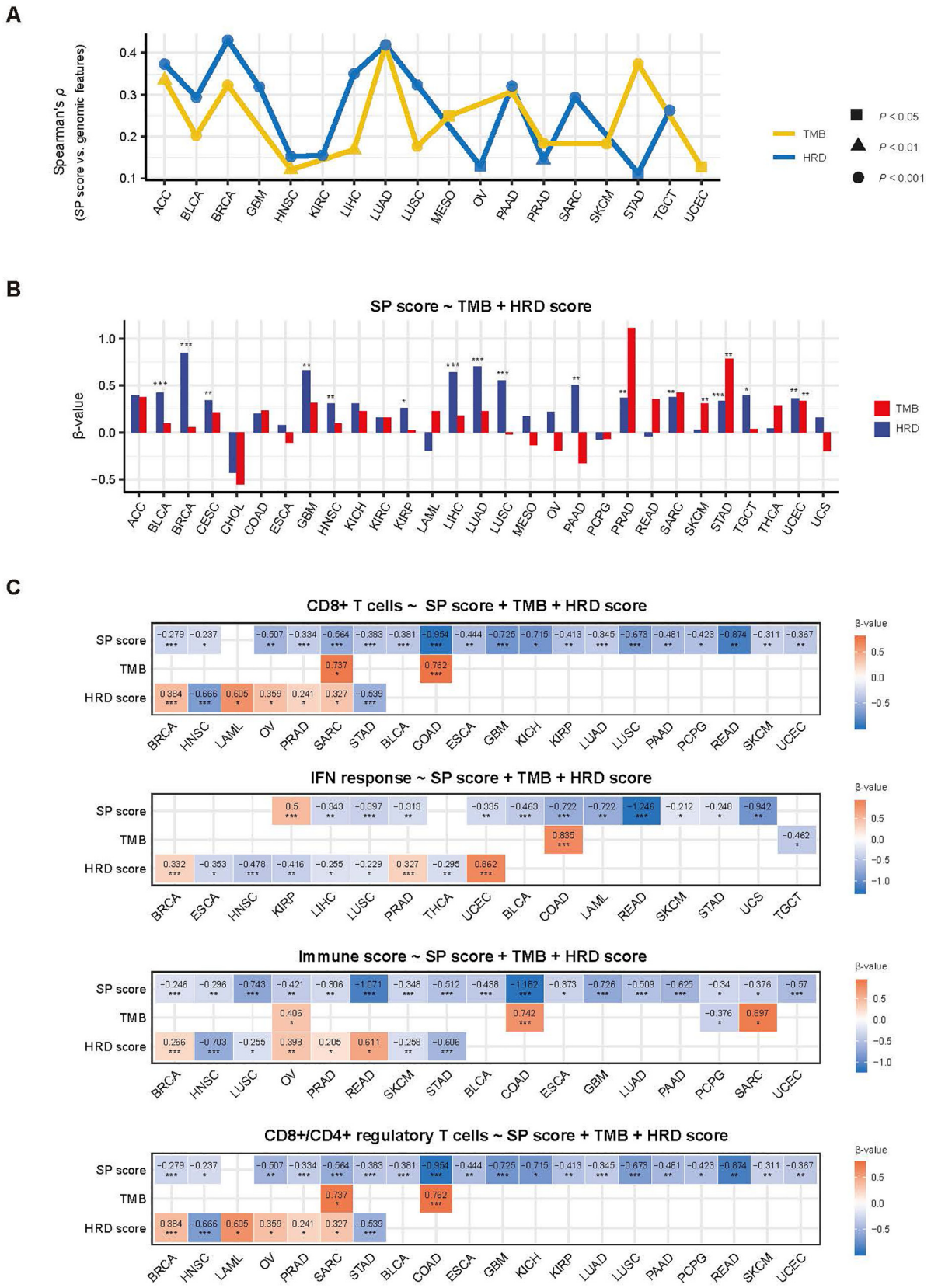

Fig. 3. Associations between SP and genomic features in cancer. A. Spearman correlations between SP scores and tumor mutation burden (TMB) and homologous recombination deficiency (HRD) scores. B. Logistic regression analysis to predict SP scores (high (>median) versus low (<median)) with TMB and HRD score. C. Logistic regression analyses to predict immune signature scores (high (>median) versus low (<median)) with SP score, TMB, and HRD score. The standardized regression coefficients (β values) are shown.

(>median) versus low (<median)) with the three variables in pan-cancer and in 29 individual cancer types. SP was likewise a significant negative predictor of the ratios in 19 cancer types (Fig. 3C); HRD was a significant negative and positive predictor in 2 and 5 cancer types, respectively, and TMB was a significant positive predictor in 2 cancer type (COAD and SARC).

Collectively, these results confirmed that SP was a negative predictor for anti-tumor immune response in diverse cancers, independent of its associations with aneuploidy and TMB.

3.4. Comparisons of SP scores between cancer subtypes

We found that SP scores were significantly higher in tumor than in normal samples in 17 cancer types (one-tailed Mann-Whitney *U* test, $P < 0.05$) (Fig. 4A). Only in KICH, PCPG, and THCA, SP scores were significantly lower in tumor than in normal samples. We further compared SP scores between cancer subtypes. We found that SP scores were significantly lower in *EGFR*-mutated than in *EGFR*-wildtype LUAD ($P = 0.009$) (Fig. 4B). Besides, *EGFR* mutations were associated with lower SP scores in LUSC and OV ($P < 0.05$) (Fig. 4B). It indicates that there is a negative correlation between SP scores and prognosis in lung cancer because *EGFR*-mutated lung cancer patients display better clinical outcomes than *EGFR*-wildtype patients due to their responses to EGFR inhibitors [41,42]. In contrast, *EGFR* mutations were associated with higher SP scores in several cancer types, including ACC, BRCA, SARC, and UCEC ($P < 0.05$) (Fig. 4B). *BRAF* mutations have been associated with worse prognosis in diverse cancers [43,44]. In CESC, ESCA, STAD, and THCA, *BRAF*-mutated tumors had significantly higher SP scores than *BRAF*-wildtype tumors ($P < 0.05$) (Fig. 4C). However, in SKCM, *BRAF*-mutated tumors displayed significantly lower SP scores than *BRAF*-wildtype tumors ($P = 0.047$) (Fig. 4C). In fact, *BRAF* mutations are correlated with a better prognosis in melanoma due to the use of BRAF inhibitors [45,46]. *KRAS* mutations are one of the most prevalent gene mutations driving cancer progression [47]. In BLCA and STAD, *KRAS*-mutated tumors had significantly higher SP scores than *KRAS*-wildtype tumors ($P < 0.05$) (Fig. 4D). In addition, in PAAD, PRAD, and SARC, SP scores were higher in *KRAS*-mutated than in *KRAS*-wildtype tumors ($P < 0.1$) (Fig. 4D).

In BRCA, SP scores were significantly higher in basal-like than in HER2-positive and luminal A&B (ER-positive) subtypes and significantly higher in HER2-positive than in luminal A&B subtypes (basal-like versus HER2-positive: $P = 7.04 \times 10^{-5}$; basal-like versus luminal A&B: $P = 4.36 \times 10^{-22}$; HER2-positive versus luminal A&B: $P = 3.11 \times 10^{-5}$) (Fig. 4E). Furthermore, SP scores were significantly higher in luminal B than in luminal A ($P = 6.29 \times 10^{-10}$). Again, SP scores were prognostic in breast cancer since basal-like is the most aggressive and luminal A is the most unaggressive among all breast cancer subtypes [48]. Collectively, these data suggest that SP is upregulated in various cancers and more highly enriched in the cancer subtypes with worse clinical outcomes.

GBM is a type of intrinsic brain tumor which lacks effective targeted therapies [49]. We analyzed the association between gene mutations and SP scores in GBM for 231 genes whose mutated tumor sample size exceeded 20. We found 29 genes whose mutations were associated with elevated SP scores, compared to 3 genes whose mutations were associated with reduced SP scores ($P < 0.05$) (Supplementary Fig. S2). These results suggest that gene mutations likely correlate with higher SP activity in GBM.

We further compared SP scores between spliceosome gene-mutated and spliceosome gene-wildtype tumors in the 29 cancer types. Spliceosome gene-mutated (SGM) tumors referred to the tumors with mutation in at least one of the 75 critical spliceosome genes, while spliceosome gene-wildtype (SGW) tumors referred to the tumors without such a mutation. We found 14 cancer types in which SGM tumors had markedly higher SP scores than SGW tumors, compared to 1 cancer type (OV) in which SGM tumors had lower SP scores than SGW tumors ($P < 0.05$) (Fig. 4F). Similarly, we compared SP scores between the tumors with mutation in at least one of 16 splicing factor genes and the tumors without such a mutation. The 16 splicing factor genes included *CRNKL1*, *SF3B6*, *PUF60*, *RP9*, *SF3A1*, *SF3B1*, *SF3B3*, *SF3B4*, *SRSF1*, *SRSF10*, *SRSF2*, *SRSF3*, *SRSF6*, *SRSF7*, *SRSF8*, and *SRSF9*. These splicing factor genes were identified based on their expression levels showing significantly negative correlations with immune scores in at least 10 cancer types (Spearman correlation, $P < 0.05$). We found 6 cancer types (LIHC, READ, SARC, SKCM, STAD, and THCA) in which SP scores were significantly higher in the splicing factor gene-mutated tumors ($P < 0.05$) (Fig. 4G). Only in CHOL, SP scores were significantly lower in the splicing factor gene-mutated tumors. These results imply that genetic alternations in key spliceosome molecules and splicing factors likely increase SP activity in cancer.

3.5. Identification of molecular features associated with SP in cancer

We found 11 proteins (Cyclin_B1, PCNA, MSH6, Chk2, MSH2, S6, TFRC, RBM15, FoxM1, ASNS, and FASN) showing significant higher expression levels in higher-SP-score (>median) than in lower-SP-score (<median) tumors in at least 10 cancer types (two-tailed Student's *t* test, FDR < 0.05) (Fig. 5A). Notably, many of these proteins function in cell cycle regulation (such as Cyclin_B1, Chk2, and FoxM1) and DNA damage repair (such as PCNA, MSH6, and MSH2). It is in line with previous results of the positive associations between SP scores and the cell cycle activity and genomic instability. RBM15 is a member of the SPEN (Split-end) family of proteins which interacts with spliceosome components [50,51]. This protein is also a component of m^6A methyltransferase complex, important in the regulation of RNA methylation [52]. RBM15 upregulation has been associated with tumor invasion and unfavorable prognosis [53]. This conforms to its positive association with the SP activity that is associated with unfavorable clinical outcomes in various cancers. S6 belongs to the S6E family of ribosomal proteins and plays a role in the regulation of cell growth and proliferation by translating certain classes of mRNA [54]. S6 hyperphosphorylation has been associated with tumor progression in diverse cancers [55–57]. Again, it conforms to the positive association between S6 expression and the SP activity which is an adverse factor in diverse cancers.

We identified KEGG pathways highly enriched in higher-SP-score tumors and lower-SP-score tumors in at least 10 cancer types (Fig. 5B). The pathways highly enriched in higher-SP-score tumors were mainly involved in cell cycle, DNA damage repair (such as DNA replication, homologous recombination, p53 signaling, mismatch repair, nucleotide excision repair, and base excision repair), and RNA regulation (such as ribosome, spliceosome, and RNA degradation). In contrast, the pathways highly enriched in lower-SP-score tumors were mainly involved in immune signatures (such as chemokine signaling, B/T cell receptor signaling, Toll-like receptor signaling, and Natural killer cell-mediated cytotoxicity), stromal signatures (focal adhesion, gap junction, cell adhesion molecules, and ECM-receptor interaction), neural regulation (neuroactive ligand-receptor interaction, axon guidance, and neurotrophin signaling), cell proliferation and differentiation regulation (such as MAPK, ErbB, Wnt, Hedgehog, TGF-β, and VEGF signaling), and apoptosis.

We further analyzed associations between SP and seven major DNA damage repair pathways [33] in cancer. The seven pathways included DNA replication, base excision repair, nucleotide excision repair, mismatch repair, homologous recombination, non-homologous end-joining, and fanconi anemia pathway. Strikingly, SP scores showed significant positive correlations with scores of five of the seven pathways in all the 29 cancer types (Spearman

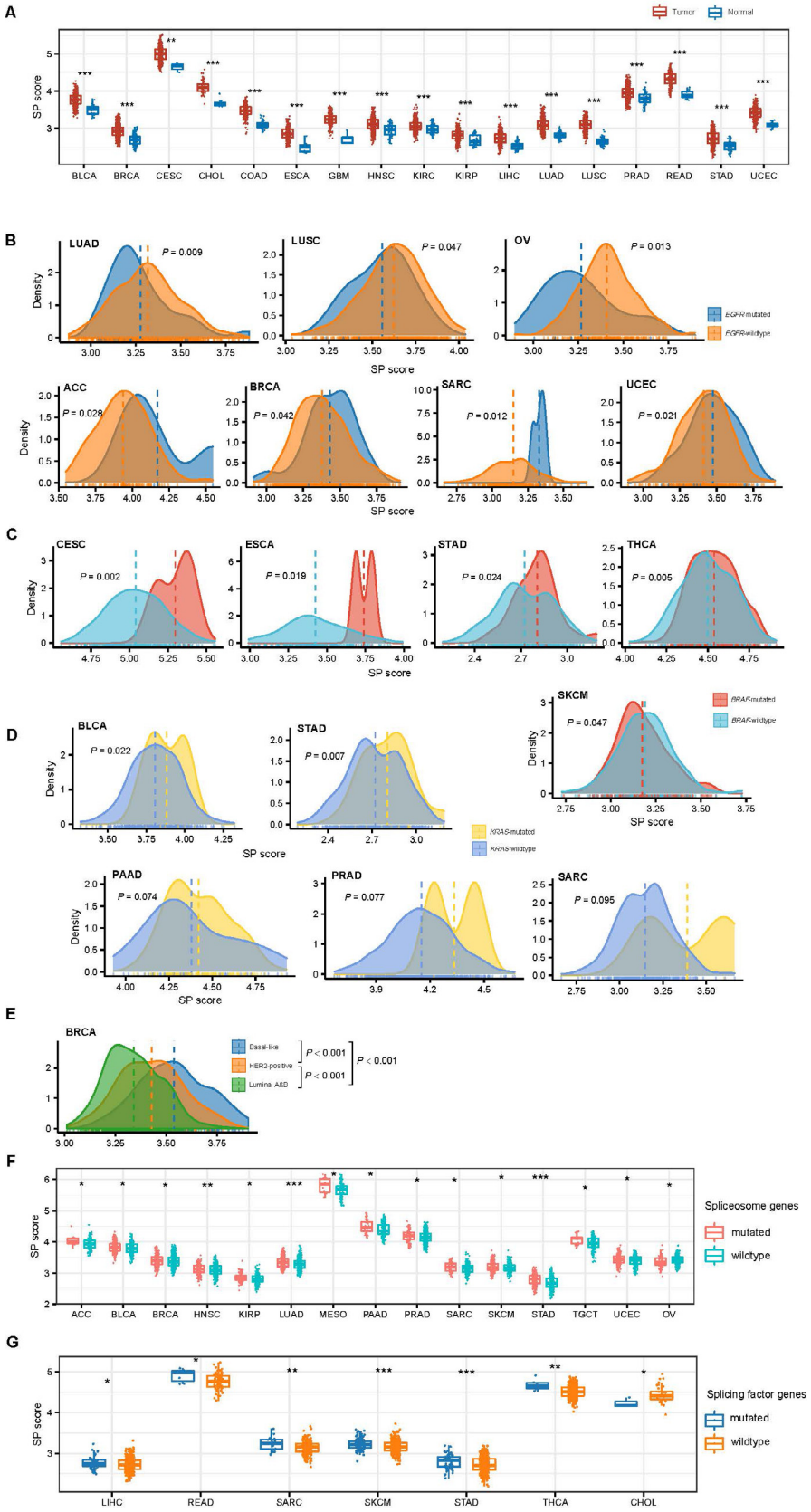

Fig. 4. Comparisons of SP scores between cancer subtypes. A. SP scores are significantly higher in tumor than in normal samples in 17 cancer types. Comparisons of SP scores between *EGFR*-mutated and *EGFR*-wildtype (B), between *BRAF*-mutated and *BRAF*-wildtype (C), between *KRAS*-mutated and *KRAS*-wildtype (D), between breast cancer (E), between spliceosome gene-mutated and spliceosome gene-wildtype (F), and between splicing factor gene-mutated and splicing factor gene-wildtype (G) subtypes of cancers. The one-tailed Mann-Whitney *U* test *P* values are shown.

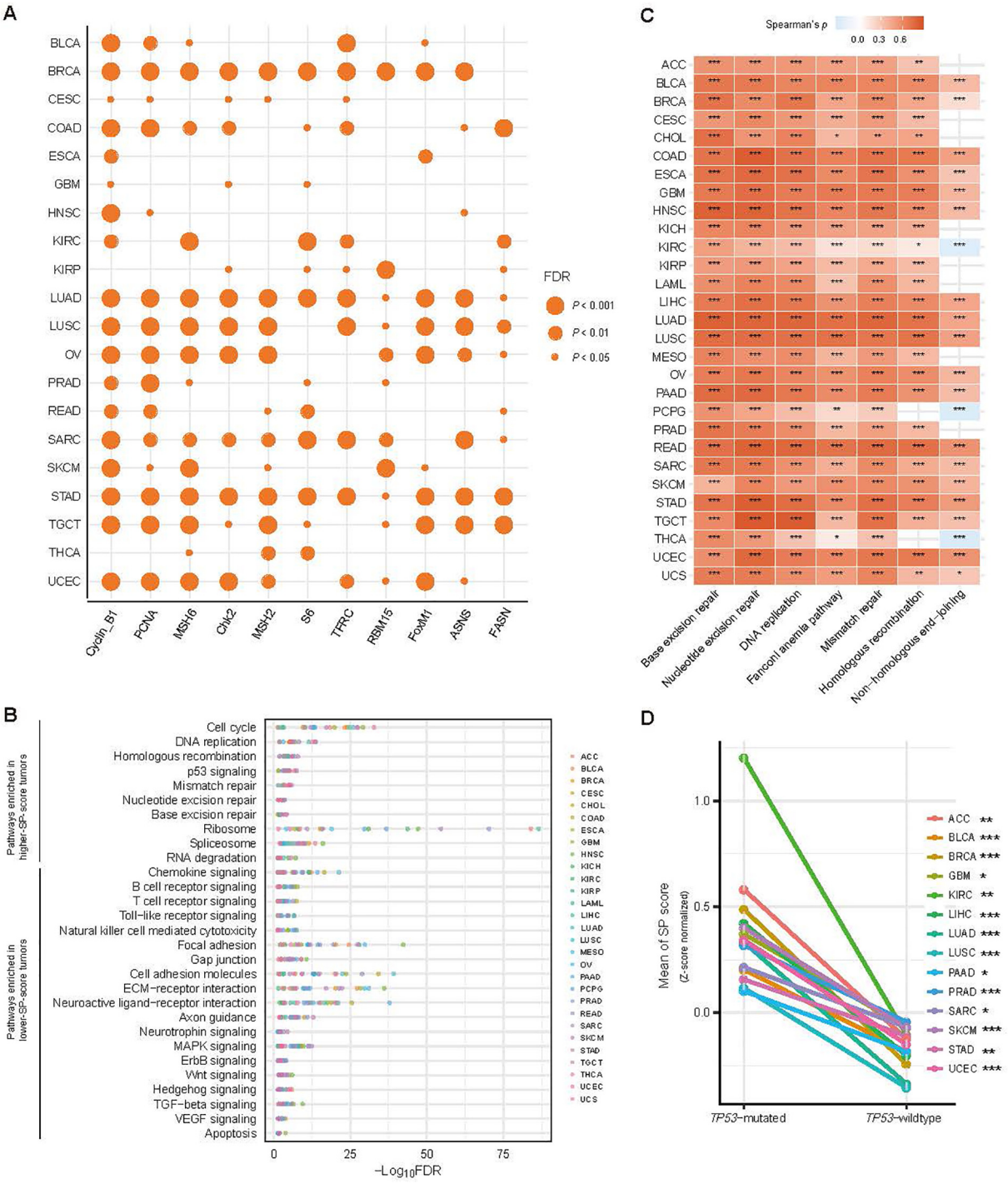

Fig. 5. Associations between SP and protein expression, pathways' activities, and DNA repair damage signatures. A. 11 proteins showing significant higher expression levels in higher-SP-score (>median) than in lower-SP-score (<median) tumors in at least 10 cancer types. The two-tailed Student's *t* test adjusted *P* values (FDRs) are shown. The FDRs were evaluated by the Benjamini and Hochberg method [34]. B. KEGG pathways highly enriched in higher-SP-score tumors and lower-SP-score tumors in at least 10 cancer types identified by [34]. C. Spearman correlations between SP scores and seven DNA damage repair pathways' scores in 29 cancer types. D. SP scores are significantly higher in *TP53*-mutated than in *TP53*-wildtype tumors in 14 cancer types. The one-tailed Mann-Whitney *U* test *P* values are shown.

correlation, $P < 0.05$) (Fig. 5C). Besides, SP scores had significant positive correlations with scores of non-homologous end-joining, and homologous recombination in 18 and 27 cancer types, respectively. The tumor suppressor p53 plays a key role in DNA damage repair [58]. We found that *TP53*-mutated tumors had significantly higher SP scores than *TP53*-wildtype tumors in 14 cancer types (Fig. 5D). Again, these results suggest a positive association between the SP activity and genomic instability.

3.6. SP scores likely correlate negatively with immunotherapy response and positively with targeted therapy response in cancer

Because both inflamed TIME and PD-L1 expression are determinants of the active response to ICIs, and SP scores were negatively correlated with them, we anticipated that there would be an association between elevated SP activity and reduced response to ICIs. This anticipation was supported in four cancer cohorts receiving ICI

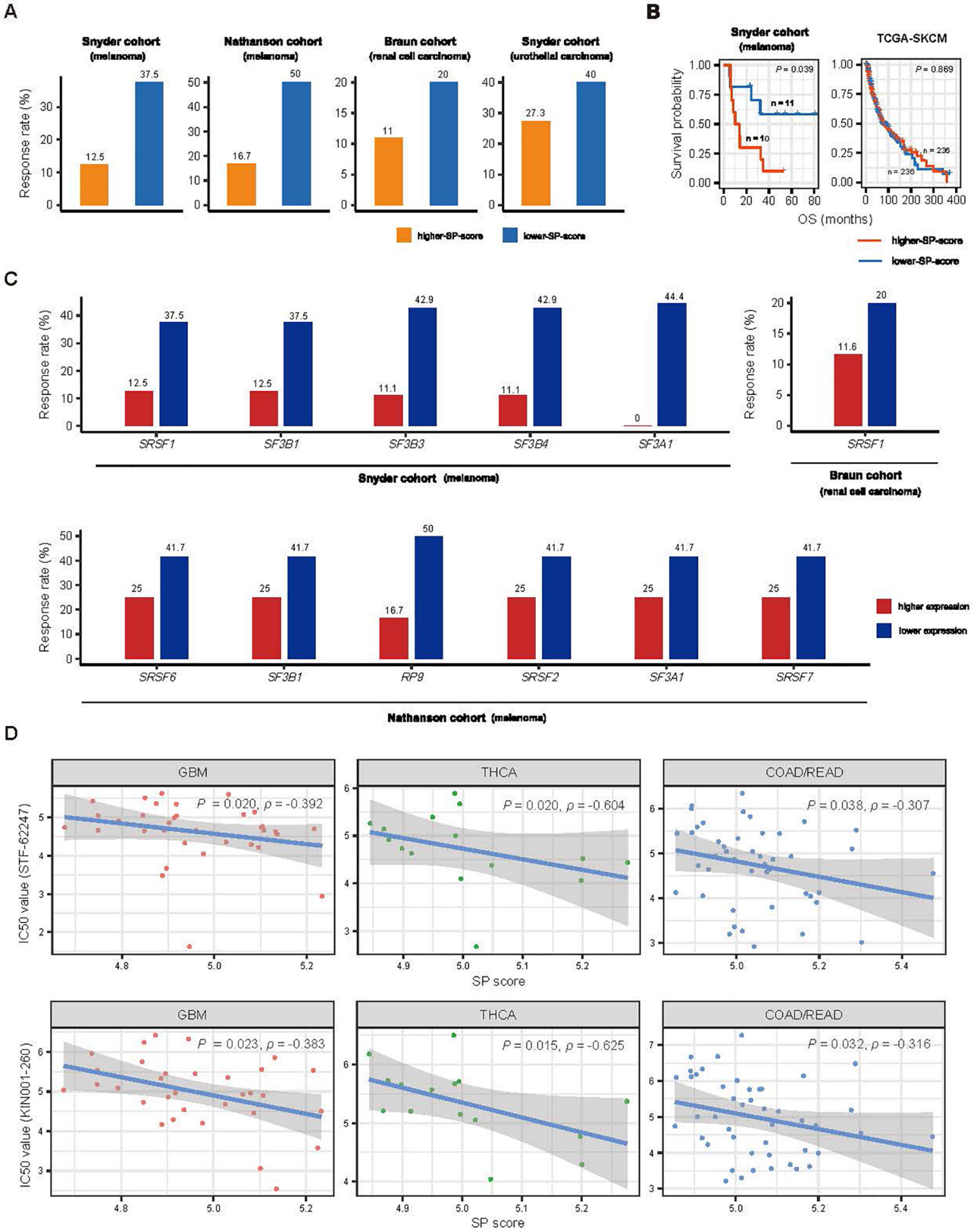

Fig. 6. Association between SP and immunotherapy in cancer. A. The tumors with lower SP scores (<median) show a higher response rate than the tumors with higher SP scores (>median) in four cohorts treated by immune checkpoint inhibitors (ICIs). B. In the Snyder (melanoma) cohort treated by ICIs, lower-SP-score tumors show better OS than higher-SP-score tumors, while in the TCGA melanoma cohort not treated by ICIs, higher-SP-score and lower-SP-score tumors show no significant difference of OS. The log-rank test *P* values are shown. C. The tumors with lower expression levels (<median) of several genes encoding splicing factors show higher response rates than the tumors with higher expression levels of them (>median) in three cohorts treated by ICIs. D. Two compounds (STF-62247 and KIN001-260) showing significant negative correlations of its IC50 values with SP scores in cell lines of GBM, THCA, and colorectal cancer. The Spearman correlation coefficient (ρ) and *P* value are shown.

treatments, in which the tumors with lower SP scores (<median) displayed a higher response rate than the tumors with higher SP scores (>median) (Fig. 6A). The four cohorts included the Snyder (melanoma) [26], Nathanson (melanoma) [27], Braun (renal cell carcinoma) [28], and Snyder (urothelial carcinoma) [29] cohorts. The response rates to ICIs in higher-SP-score versus lower-SP-score tumors were 12.5% versus 37.5%, 16.7% versus 50%, 11% versus 20%, and 27.3% versus 40% in these cohorts, respectively (Fig. 6A). Furthermore, in the Snyder (melanoma), lower-SP-score tumors showed a better OS prognosis than higher-SP-score tumors (log-rank test, $P = 0.039$) (Fig. 6B). The better outcome in the lower-SP-score tumors could be attributed to their more favorable response to ICIs since higher-SP-score and lower-SP-score tumors showed no significant difference of OS in the TCGA melanoma cohort without ICI treatments (log-rank test, $P = 0.869$) (Fig. 6B). Furthermore, we analyzed associations between the expression of the 16 splicing factor genes and the response to ICIs in these cohorts. In the Snyder (melanoma) cohort, the tumors with lower expression levels (<median) of *SRSF1*, *SF3A1*, *SF3B1*, *SF3B3*, and *SF3B4* showed higher response rates than the tumors with higher expression levels of them (>median) (Fig. 6C). Similar results were observed for *SF3A1*, *SF3B1*, *RP9*, *SRSF2*, *SRSF6*, and *SRSF7* in the Nathanson (melanoma) cohort and *SRSF1* in the Braun (renal cell carcinoma) cohort (Fig. 6C).

We also explored associations between SP scores and drug sensitivities (IC50 values, half maximal inhibitory concentration) of cancer cell lines to 265 anti-tumor targeted therapeutic compounds from the Genomics of Drug Sensitivity in Cancer (GDSC) project (https://www.cancerrxgene.org). Strikingly, the IC50 values of 187 (71%) of the 265 compounds were significantly and negatively correlated with SP scores in cancer cell lines (Spearman correlation, $P < 0.05$) (Supplementary Table S4). The PLK inhibitor NPK76-II-72-1 showed the strongest negative correlation of IC50 values with SP scores ($\rho = -0.46$, $P = 1.85 \times 10^{-47}$). In contrast, only 18 (7%) compounds had a significant positive correlation of IC50 values with SP scores (Supplementary Table S4). These results suggest that elevated SP activity is correlated with increased drug sensitivity for a broad spectrum of anti-tumor targeted therapies. We further analyzed correlations between drug sensitivities (IC50 values) and SP scores in cell lines from individual cancer types. We found that the IC50 values of many compounds had significant negative correlations with SP scores in diverse cancer types, such as GBM, THCA, and colorectal cancer (COAD/READ). Notably, STF-62247, an autophagy inducer, had significant negative correlations of its IC50 values with SP scores in GBM, THCA, and colorectal cancer (Fig. 6D). This result implicates a potential link between autophagy and spliceosome pathways. Indeed, previous studies have demonstrated the association between autophagy and spliceosome pathways

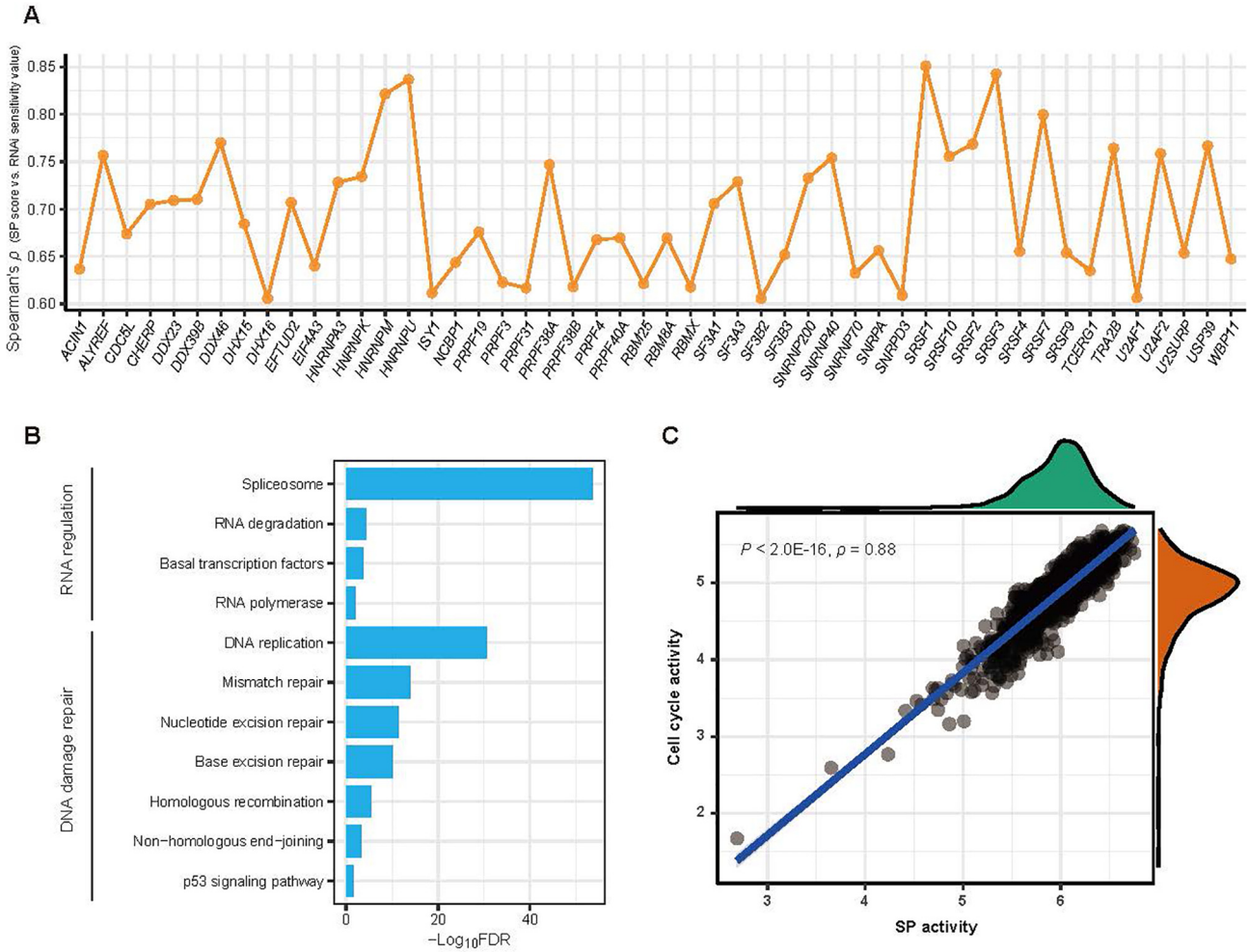

Fig. 7. Association between SP and RNAi sensitivity profile in cancer cell lines. A. 50 SP genes whose RNAi sensitivity values have a strong correlation with SP scores in cancer cell lines (Spearman correlation, $\rho > 0.6$). B. The genes having a strong correlation of their RNAi sensitivity values with SP scores in cancer cell lines are significantly involved in RNA regulation and DNA damage repair pathways. C. The strong positive correlation between SP scores and cell cycle activity in cancer cell lines. The Spearman correlation coefficient (ρ) and P value are shown.

[59,60]. KIN001-260, an inhibitor of IKK-β, known as an enzyme involved in immune regulation [61], also displayed significant negative correlations of its IC50 values with SP scores in GBM, THCA, and colorectal cancer (Fig. 6D). It supports the significant correlation between the SP activity and immune signatures in cancer.

3.7. Associations of SP with RNAi sensitivity profile in cancer cell lines

We analyzed associations between RNAi sensitivity values of 19,177 genes and SP scores in 662 cancer cell lines. RNAi sensitivity reflects the degree of cancer cell survival dependency on specific

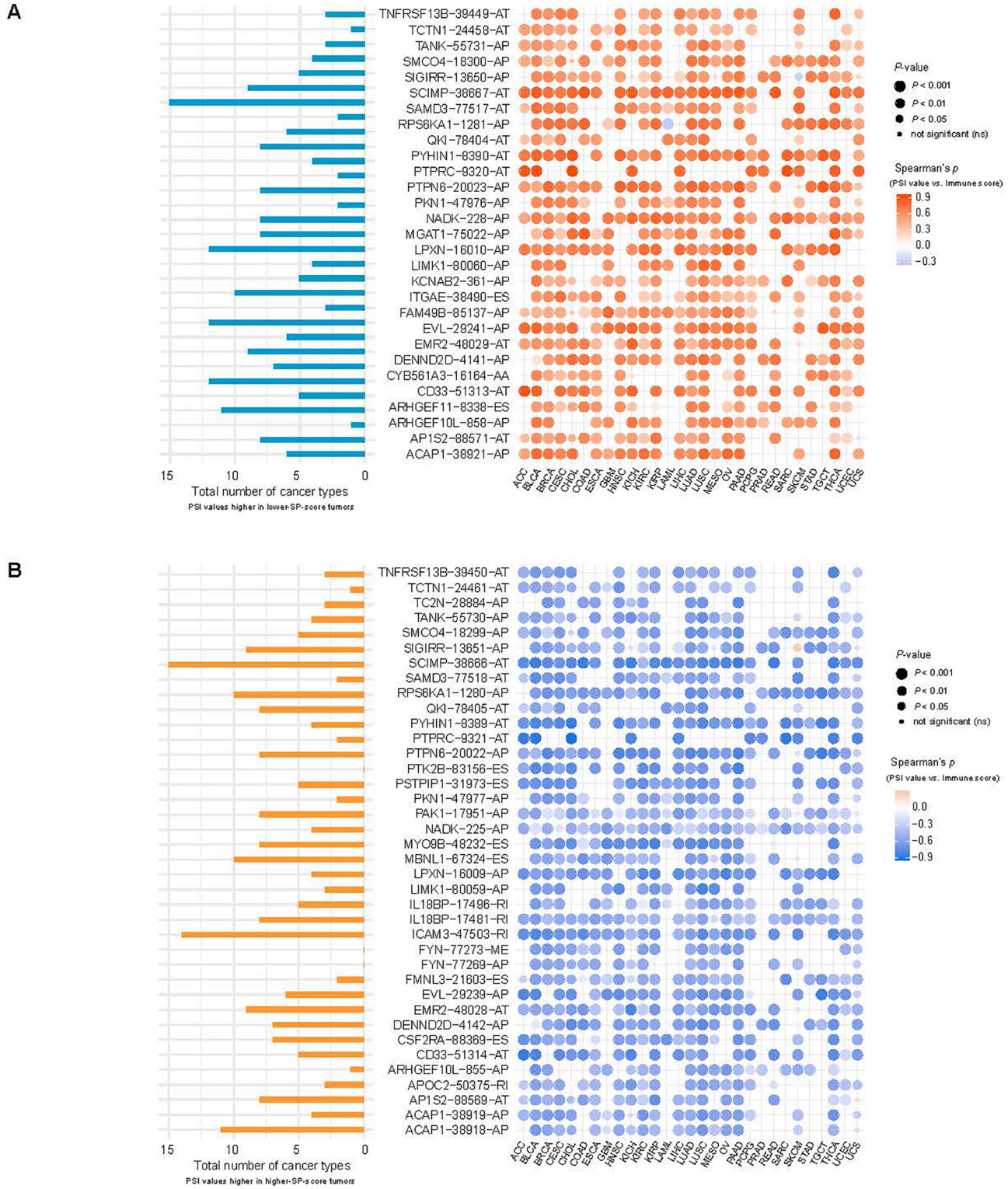

Fig. 8. Associations of splicing events with SP and immune signatures in cancer. A. 31 splicing events whose PSI values are positively correlated with immune scores in at least 10 cancer types (Spearman correlation, $P < 0.05$) and higher in lower-SP-score than in higher-SP-score tumors in diverse cancers (two-tailed Student's t test, FDR < 0.05). B. 38 splicing events whose PSI values are negatively correlated with immune scores in at least 10 cancer types and higher in higher-SP-score than in lower-SP-score tumors in diverse cancers. PSI (percent spliced in) defines the efficiency of splicing an exon into all the transcripts of a gene [25].

genes by genome-wide RNAi loss-of-function screens [62]. Higher RNAi sensitivity indicates a stronger dependency of cancer cell survival on specific genes. We found 628 genes whose RNAi sensitivity values had a strong positive correlation with SP scores (Spearman correlation, $\rho > 0.6$). Notably, there were 50 SP genes in the list of 628 genes, such as *SRSF3* and *SRSF1* (Fig. 7A). GSEA [63] identified 26 KEGG pathways significantly associated with the 628 genes (FDR < 0.05). The 26 pathways were mainly involved in RNA regulation (spliceosome, RNA degradation, RNA polymerase, and basal transcription factors) and DNA damage repair (mismatch repair, nucleotide excision repair, base excision repair, homologous recombination, non-homologous end-joining, DNA replication, and p53 signaling) (Fig. 7B). Again, the activity of cell cycle pathway had a strong positive correlation with SP scores in the cell lines ($\rho = 0.88$) (Fig. 7C). Collectively, these results implicate that the cancer cells with elevated SP activity have strong dependencies on the expression of the genes in the pathways of RNA regulation, DNA damage repair, and cell cycle.

3.8. Associations of splicing events with SP and immune signatures in cancer

The PSI defines the efficiency of splicing an exon into all the transcripts of a gene [64]. We first identified 100 splicing events whose PSI values showed the strongest correlations with immune scores in each of the 29 cancer types. Among these splicing events, 31 splicing events displayed significantly positive correlations of PSI values with immune scores in at least 10 cancer types (Spearman correlation, $P < 0.05$) (Fig. 8A). Notably, the PSI values of these splicing events were significantly lower in higher-SP-score than in lower-SP-score tumors in diverse cancers (Student's t test, FDR < 0.05) (Fig. 8A, Supplementary Table S5). In contrast, 38 splicing events had significantly negative correlations of their PSI values with immune scores in at least 10 cancer types, and their PSI values were significantly higher in higher-SP-score than in lower-SP-score tumors in diverse cancers (Student's t test, FDR < 0.05) (Fig. 8B, Supplementary Table S5). This conformed to previous results of the significant negative correlation between SP scores and immune scores in various cancer. Interestingly, around 80% of the genes involved in the 31 splicing events were involved in at least one of the 38 splicing events and vice verse (Fig. 7A&7B). It indicated that the genes involved in both types of splicing events were highly overlapped. For example, the PSI values of RPS6KA1-1280-AP had a significant negative correlation with immune scores in 24 cancer types and were significantly higher in higher-SP-score than in lower-SP-score tumors in 10 cancer types. However, the PSI values of RPS6KA1-1281-AP had a significant positive correlation with immune scores in 19 cancer types and were significantly lower in higher-SP-score than in lower-SP-score tumors in 6 cancer types. These data suggest that different splicing events for an identical gene may have opposite effects on anti-tumor immune response. A total of 39 genes were involved in the 38 and 31 splicing events, which were significantly associated with pathways of Fc gamma R-mediated phagocytosis, T cell receptor signaling, and primary immunodeficiency, identified by GSEA (adjusted P value < 0.05). It supports the strong correlation between their splicing events and immune signatures in cancer.

4. Discussion

For the first time, we systematically investigated associations of the SP activity with clinical features, anti-tumor immune signatures, tumor immunity-related genomic and molecular features, and targeted therapies and immunotherapies in pan-cancer. Our results showed that the SP activity was an oncogenic signature, as evidenced by its hyperactivation in cancer and invasive cancer subtypes and correlations with unfavorable clinical outcomes and anti-tumor immunosuppression in a wide variety of cancers. Our data suggest that the SP activity is correlated with genomic instability in diverse cancers, as evidenced by its positive correlations with TMB and aneuploidy. However, our data shows that aneuploidy has a more significant impact on the SP activity than TMB in cancer. Because TMB and aneuploidy are positively and negatively correlated with anti-tumor immune response, respectively, and the SP activity is positively correlated with both of them, the negative correlation between the SP activity and anti-tumor immune response could be a consequence of their joint influence on SP. However, our results indicate that the negative association between the SP activity and anti-tumor immune response is likely independent of its associations with aneuploidy and TMB, suggesting that the SP activity is an independent predictor of anti-tumor immune response. Furthermore, we supported that the SP activity had a negative correlation with immunotherapy response in four cancer cohorts receiving ICI treatments. Our data also revealed that the correlation of aneuploidy and TMB with anti-tumor immune response could be positive or negative, depending on cancer types. This is in accordance with findings from previous studies [9,65]. However, different from aneuploidy and TMB, the SP activity was a negative predictor of anti-tumor immune response consistently in various cancers. It suggests that the SP activity is a more reliable biomarker of anti-tumor immune response and immunotherapy response versus aneuploidy and TMB.

We found that the SP activity was strongly and positively associated with the activity of various DNA damage repair pathways in cancer. This could explain why SP is associated with anti-tumor immunosuppression since the deficiency of DNA damage repair pathways, e.g., dMMR [66], can stimulate anti-tumor immune response. In addition, the SP activity was positively associated with the cell cycle activity and negatively associated with the apoptosis activity. It may explain why the SP activity is an oncogenic signature.

Interestingly, although the SP activity is negatively correlated with immunotherapy response, it is positively correlated with the response to various anti-tumor targeted therapies. These targeted therapies mainly targeted pathways of cell cycle, EGFR, p53, Wnt, IGF1R, JNK/p38, RTK, ERK/MAPK, PI3K/MTOR, DNA replication, ABL, metabolism, apoptosis regulation, chromatin histone acetylation, chromatin histone methylation, protein stability and degradation, cytoskeleton, genome integrity, and hormone-related. It suggests that elevated SP activity could enhance drug sensitivities of a wide variety of targeted therapies.

The significant association between the SP activity and anti-tumor immunosuppression could explain why the tumors with high SP activity had more unfavorable tumor phenotypes and clinical outcomes than the tumors with low SP activity. Indeed, the higher-SP-score tumors had higher levels of stemness, proliferation potential, ITH, and cell cycle activity. The advanced tumors had significantly higher SP scores than the non-advanced tumors in multiple cancers. The higher-SP-score tumors had worse survival prognosis in multiple individual cancer types. Within single cancer types, the SP activity is higher in the subtypes with worse clinical outcomes.

It should be noted that the negative association between anti-tumor immune signatures and the SP activity does not conflict with their positive association with specific mRNA splicing events. Instead, it is consistent with a recent study showing that inhibition of spliceosome could promote anti-tumor immune response in triple-negative breast cancer [22]. Furthermore, our results suggest that inhibition of spliceosome can enhance anti-tumor immune response in various cancers. Thus, the combination of spliceosome

inhibitors and ICIs could be an effective strategy for improving immunotherapy response.

This study has several limitations. First, we used the SP score, which was obtained by the ssGSEA of all genes in the SP, to quantify the SP activity, namely spliceosome activation. We did not show the data on the correlations of the key spliceosome molecules with molecular and clinical features in pan-cancer. Nevertheless, we found 60 (80%) of the 75 critical spliceosome genes whose expression levels were significantly and positively correlated with SP scores in at least 20 cancer types and all 75 genes having significant positive expression correlations with SP scores in at least 12 cancer types ($P < 0.05$) (Supplementary Table S6). It indicates that high SP scores likely reflect high spliceosome activation. Second, the relationship between SP activity and molecular and clinical features in pan-cancer revealed by the bioinformatics analysis is the correlation relationship, but not the causal relationship. To prove the causal relationship, further experimental and clinical studies are necessary. Finally, we found that SP scores had a positive correlation with tumor purity in most cancer types. It suggests that the measure of SP scores in the tumors with relatively low tumor purity could be less accurate than that in the tumors with high tumor purity, whereas this is a common problem in the studies of bulk tumors. To address this issue, the use of single-cell transcriptomic data is a must.

In conclusion, our findings suggest that the SP activity is a negative biomarker of clinical outcomes, anti-tumor immunity, and immunotherapy response in various cancers. Spliceosome-targeted therapies may promote immunotherapy response.

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Authors' contributions

ZC performed data analyses and helped prepare for the manuscript. CC performed data analyses and helped prepare for the manuscript. LL performed data analyses and helped prepare for the manuscript. TZ performed data analyses. XW conceived of the research, designed the methods, and wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.09.029.

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