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Original Article

Comprehensive pan-cancer analysis identifies cellular senescence as a new therapeutic target for cancer: multi-omics analysis and single-cell sequencing validation

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Abstract: Although cellular senescence has long been recognized as an anti-tumor mechanism, mounting evidence suggests that in some circumstances, senescent cells promote tumor growth and malignancy spread. Therefore, research into the exact relationship between cellular senescence and tumor immunity is ongoing. We analyzed changes in the expression, copy number variation, single-nucleotide variation, methylation, and drug sensitivity of cellular senescence-related genes in 33 tumor types. The cellular senescence score was calculated using the single-sample gene-set enrichment analysis. The correlations between cellular senescence score and prognosis, tumor immune microenvironment (TIME), and expression of tumor immune-related genes were comprehensively analyzed. Single-cell transcriptome sequencing data were used to assess the activation state of cellular senescence in the tumor microenvironment (TME). The expression of cellular senescence-associated hub genes varied significantly across cancer types. In these genes, missense mutation was the major type of single nucleotide polymorphism, and heterozygous deletion and heterozygous amplification were the major types of copy number variation. Moreover, the cellular senescence pathway in tumors was sensitive to drugs such as XMD13-2, TPCA-1, methotrexate, and KIN001-102. Furthermore, the cellular senescence score was significantly higher in most cancer types, related to poor prognosis. The expression of immune checkpoint molecules such as NRP1, CD276, and CD44 was significantly correlated with the cellular senescence score. Monocyte cellular senescence was significantly higher in the TME of kidney renal clear cell carcinoma cells than in normal tissues. The findings of this study provide insights into the important role of cellular senescence in the TIME of human cancers and the effect of immunotherapy.

Keywords: Cellular senescence, tumor immunity, single-cell transcriptome sequencing, tumor microenvironment

Introduction

Cancer is a major global public health issue, with high morbidity and mortality rates in the population. The 2019 coronavirus disease (COVID-19) pandemic has impeded cancer diagnosis and treatment. The partial delay in diagnosis and treatment may result in a short-term decrease in cancer incidence, followed by an increase in the incidence of advanced disease and, ultimately, death [1]. According to estimates, cancer cases are expected to rise by 60% worldwide over the next 20 years [2]. Cancer is the second leading cause of death globally, with an annual increase in fatalities and occurrences. Therefore, it is crucial to study the mechanism of tumor development in greater detail.

Recent pan-cancer research has revealed the immune signature patterns of several tumor types. Global initiatives are currently underway

to perform a whole-cancer analysis of cancer's exome and genome, including non-coding regions [3, 4]. In 2018, the Cancer Genome Atlas (TCGA) research network used exome, transcriptome, and DNA methylation data to map commonalities among tumor types. The TCGA research network compiled data on thousands of tumor genomes' DNA, chromatin, and RNA aberrations relative to matched normal cell genomes and analyzed their protein interactions and epigenetics. The ICGC/TCGA Genome-Wide Pan-Cancer Analysis (PCAWG) project explored the expression of 1,542 RNA-binding proteins (RBPs), somatic cell copy number alterations (SCNAs), and mutational profiles in 7,000 clinical specimens from 15 cancer types in 2020 [5]. Pan-cancer research aims to identify transduced genes and recurrent genomic events or aberrations across tumor types. In addition to the clinical categorization of cancers, it provides new tools for genomic and bioinformatic investigations and the possibility of repurposing targeted medicines based on the molecular pathology of malignancies. Pan-cancer research may help with a molecular cancer diagnosis and therapeutic outcome-based cancer classification.

Cellular senescence is characterized by the induction of stable growth arrest, an irreversible growth-proliferation arrest that may evolve as a protective mechanism to maintain tissue homeostasis, ostensibly as a complement to programmed cell death [6]. Cellular senescence is often accompanied by marked phenotypic changes, such as chromatin remodeling, metabolic reprogramming, increased autophagy, the formation of a complex proinflammatory secretome [7], resistance to apoptosis, altered gene expression, and chromatin structure, expression of senescence-associated β-galactosidase, and acquisition of a senescence-associated secretory phenotype (SASP), all of which are cellular senescence programs evoked by morphological and metabolic changes. Cellular senescence has long been thought to be the underlying mechanism of therapy-induced senescence [8]. However, in some cases, senescent cells stimulate tumor development and malignant progression in a context-dependent manner [9, 10]. The main mechanism by which senescent cells promote malignant progression is the induction of SASP, in which signaling molecules (and proteases that activate and/or eliminate them) are delivered paracrinely to nearby cancer cells and other cells in the tumor microenvironment (TME). Thereby establishing tumors. However, the exact relationship between cellular senescence and tumor immunity remains elusive.

In this pan-cancer study involving 33 common tumor types, we provide a comprehensive analysis of differential expression, protein-protein interactions, enriched pathways, and prognostic significance of cellular senescence-associated genes. We identified 12 genes most closely associated with cellular senescence, established a cellular senescence score, and investigated cellular senescence-related functions at the single-cell level. Moreover, we investigated the relationship between cellular senescence and tumor immunity, including the tumor immune microenvironment (TIME), immune-related gene expression, and immune checkpoint blockade (ICB) therapy, to clarify the potential of cellular senescence as a new therapeutic target in cancer.

Methods

Data analysis and processing

Single-cell sequencing data were derived from GSE152938 [11]. The data sequencing and initial processing of the data were conducted as follows. The analyzed samples were initially sequenced using standard Hiseq X10 [12] (Illumina, San Diego, CA, USA) parameters. The sequencing file (BCL) was then converted to FASTQ format using Cell Ranger (Version 3.0.2) R (Version 3.5.2) [13]. Finally, the samples were quality control and secondary analyzed using the "Seurat" R package (Version 3.1.1).

Source and preprocessing of data

Clinical data and gene expression data used in this experiment were obtained from the TCGA database [14] (https://portal.gdc.cancer.gov/) and the Pan-Cancer Atlas Center at the University of California Santa Cruz database [15]. The analysis included samples from 33 solid cancers, namely adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), breast inva-

sive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), cholangiocarcinoma, colon adenocarcinoma (COAD), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), acute myeloid leukemia (LAML), brain low-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma, testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), uterine carcinosarcoma (UCS), and uveal melanoma (UVM).

Differential gene expression analysis

An in-depth analysis of gene expression levels was conducted to demonstrate differences in the expression of cellular senescence-associated genes between tumor and normal tissues. Tumor specimens were divided into two groups based on the optimal cutting point, and the survival time and survival status of the two groups were fitted using the "survival" R package.

Tumor immune dysfunction and elimination analysis

The tumor immune dysfunction and elimination (TIDE) algorithm predicts potential ICB responses. TIDE uses a suite of gene expression markers to assess two different tumor immune evasion mechanisms, including tumor-infiltrating cytotoxic T lymphocyte (CTL) dysfunction and CTL rejection by immunosuppressive factors. Patients with a high TIDE score had a higher probability of tumor immune escape than those with a low TIDE score. Therefore, patients with a high TIDE score had a lower ICB response rate.

Estimation of cellular senescence score

To quantify the expression level of cellular senescence-associated genes, single-cell exploration cellular senescence scores were calculated using single-sample gene set enrichment analysis (ssGSEA) from the cellular senescence-related gene sets (REACTOME_CELLULAR_SENESCENCE) downloaded from the GSEA database [16]. The ssGSEA analysis was performed in R using the "GSEABase [17]", "limma [18]", and "GSVA [19]" packages, with default parameters. The standardized enrichment score was used as an individual cell senescence score.

Construction of cellular senescence regulatory network and protein-protein interaction (PPI) analysis

For PPI analysis, the cellular senescence-related genes were imported into the STRING database (https://string-db.org/). The txt file was then downloaded and copied to excel for annotation before being imported into Cytoscape software to generate the core-gene PPI network diagram. Cytoscape's network function was used to analyze network topology, and genes with degrees greater than 30 were defined as hub genes.

Results

Differential gene expression analysis and prognostic analysis of hub genes associated with cellular senescence

First, we collected 196 cellular senescence-associated genes from the MSigDB database. We analyzed the PPI and its topology to identify the cellular senescence-associated hub genes. Based on a degree > 35, 12 cellular senescence-associated hub genes were identified (Supplementary Figure 1). We found nearly all of the 12 cellular senescence-associated hub genes in cancer and paracancerous tissues from the TCGA database were expressed differentially between tumor and normal tissues. *CBX2*, *CBX4*, and *TP53* genes were upregulated in most tumors, including BRCA, COAD, LUAD, LUSC, and LIHC. In contrast, *MAPK14* and *RING1* genes were downregulated in most tumors. The expression of cellular senescence-associated genes was significantly correlated with tumor progression ($P < 0.05$) (**Figure 1A**). The expression of cellular senescence-associated hub genes was linked to cancer patients' prognosis; upregulation of cellular senescence-associated hub genes was linked to a worse prognosis in most cancers. Some genes were identified as risk factors for overall survival

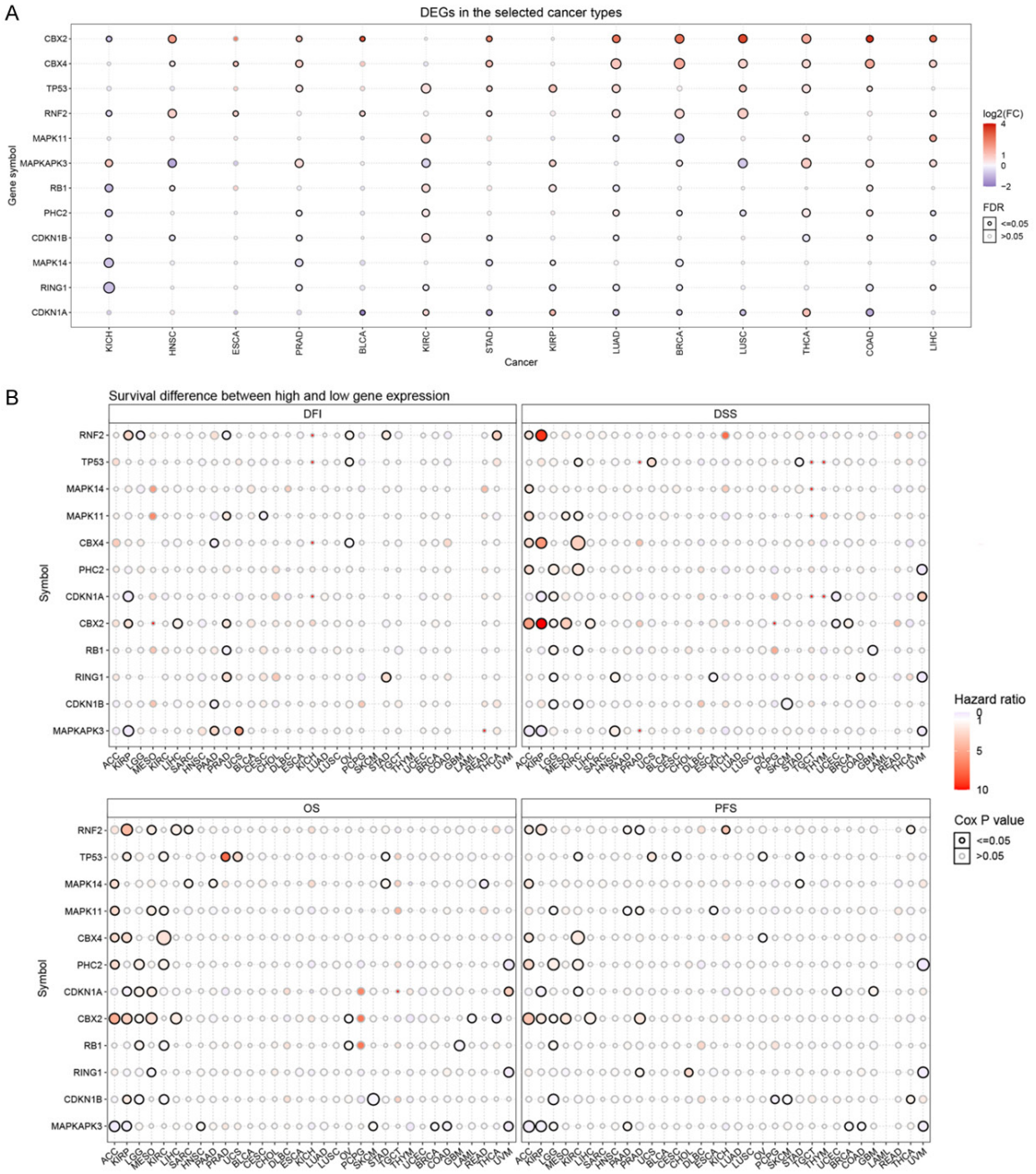


Figure 1. Expression and prognosis of cellular senescence-associated hub genes. A. Differential expression of cellular senescence-associated hub genes in cancer and normal tissues. FDR, false discovery rate. B. Relationship between cellular senescence-associated hub genes and disease-free interval (DFI), disease-specific survival (DSS), overall survival (OS), and progression-free survival (PFS).

(OS), including the *CBX2* gene in ACC, KIRP, MESO, and LIHC; the *CDKN1A* gene in LGG, MESO, and UVM; the *PHC2* gene in ACC, LGG, and KIRC; the *CBX4* gene in ACC, KIRP, and KIRC; the *MAPK11* gene in ACC and MESO; *MAPK14* gene in ACC; the *RNF2* gene in KIRP, MESO, and LIHC; the *TP53* gene in PRAD and UCS. Furthermore, *CBX2*, *CBX4*, and *RNF2* genes were risk factors for disease-specific survival (DSS) in some cancers, such as KIRP, ACC, and KIRC (**Figure 1B**).

Differences in the cell senescence score and its correlation with tumor stage

We determined the cellular senescence score to evaluate tumor tissue's degree of cellular senescence. The cellular senescence score

was significantly positively correlated with the expression of these cellular senescence-associated genes (P < 0.05), indicating that the cellular senescence score represents the degree of cellular senescence. Furthermore, most cellular senescence-associated hub genes were expressed positively. For example, *RB1 gene* expression was significantly positively correlated with *MAPK14*, *RNF2*, and *PHC2* gene expression (P < 0.05) (**Figure 2A**). We then investigated the TCGA database for the differential expression of cellular senescence-associated hub genes in cancer and normal tissues. The findings revealed that the cellular senescence score in cancer tissues was generally higher than in normal tissues. However, because the TCGA database contained fewer normal tumor samples, we selected normal samples from the GETx database as the control group for a combined analysis. The findings revealed that the cellular senescence score was substantially higher in 33 cancer types (P < 0.05) (**Figure 2B**). Therefore, the findings suggest that the cellular senescence score may be crucial in the emergence and progression of tumors.

Furthermore, we found that the cellular senescence score increased with increasing stages. The cellular senescence score was higher in stage III/IV cancer than in stage I/II cancer in ACC (P = 0.015), TGCT (P < 0.01), KIRP (P < 0.01), LIHC (P = 0.0049), and LUAD (P = 0.031) (Supplementary Figure 2).

Effects of cellular senescence on cancer prognosis

Comprehensive analysis showed that the degree of cellular senescence was a significant regulator in cancer patients. In terms of OS, a low degree of cellular senescence had a significant positive effect on BLCA (P = 0.007, hazard ratio (HR) = 1.51), BRCA (P = 0.04, HR = 1.5), LUAD (P < 0.001, HR = 1.8), and KIRC (P < 0.001, HR = 2.47) (Supplementary Figure 3). Furthermore, in terms of DSS, a low degree of cellular senescence was associated with a better prognosis for patients with BLCA (P = 0.011, HR = 1.59), BRCA (P = 0.009, HR = 2.41), LUAD (P = 0.001, HR = 1.92), CESC (P = 0.026, HR = 1.9), and OV (P = 0.046, HR = 1.35) (Supplementary Figure 4). Additionally, a low degree of cellular senescence was a positive factor for progression-free interval (PFI) in BLCA (P = 0.022, HR = 1.54), LIHC (P < 0.001, HR = 1.82), MESO (P = 0.033, HR = 1.89), and DLBC (P = 0.005, HR = 0.16) (Supplementary Figure 5). DSS prognostic analysis showed that the cellular senescence score was a risk factor for 20 cancer types, including ACC (P = 0, HR = 10.78), KICH (P = 0, HR = 83.96), KIRC (P = 0, HR = 3.26), KIRP (P = 0, HR = 4.48), LGG (P = 0, HR = 2.87), LIHC (P = 0, HR = 2.56), LUAD (P = 0, HR = 1.92), MESO (P = 0, HR = 3.36), PAAD (P = 0, HR = 2.58), SARC (P = 0, HR = 3.06) and UVM (P = 0, HR = 6.37) (P < 0.05) (**Figure 3A**). The results of OS were comparable to those of DSS, showing that the cellular senescence score was significantly negatively correlated with a worse prognosis in ACC (P = 0, HR = 11.27), KICH (P = 0, HR = 23.16), KIRC (P = 0, HR = 2.47), KIRP (P = 0, HR = 2.94), LGG (P = 0, HR = 2.67), LIHC (P = 0, HR = 1.95), LUAD (P = 0, HR = 1.8), MESO (P = 0, HR = 2.81), PAAD (P = 0, HR = 2.19), SARC (P = 0, HR = 2.62), THYM (P = 0, HR = 0.07), and UVM (P = 0, HR = 5.81) (**Figure 3B**). The results of DFI showed that the cellular senescence score could be significantly negatively correlated with a worse PFS in 23 out of 33 tumors (**Figure 3C**). These findings imply that cellular senescence is an independent prognostic marker for different tumors.

Single-cell analysis of the cellular senescence score

The correlation between the cellular senescence score and 14 functional states in different tumor types was analyzed using the TCGA database. In most tumor types, we found that processes such as invasion, DNA damage, DNA repair, epithelial-mesenchymal transition, apoptosis, stemness, angiogenesis, differentiation, inflammation, and quiescence were significantly positively correlated with the cellular senescence score, indicating that cellular senescence-associated hub genes promoted the above functions (P < 0.05). However, in most tumor types, the cellular senescence-associated hub genes inhibited processes such as cell cycle, proliferation, hypoxia, and metastasis (**Figure 4**). Based on the findings, we speculate that tumor malignancy is linked to abnormal cellular functions caused by cellular senescence.

Relationship between the cellular senescence score and TIME

Tumor purity is linked to immune and stromal scores in tumor tissue, which is linked to patient prognosis. We investigated the correlation

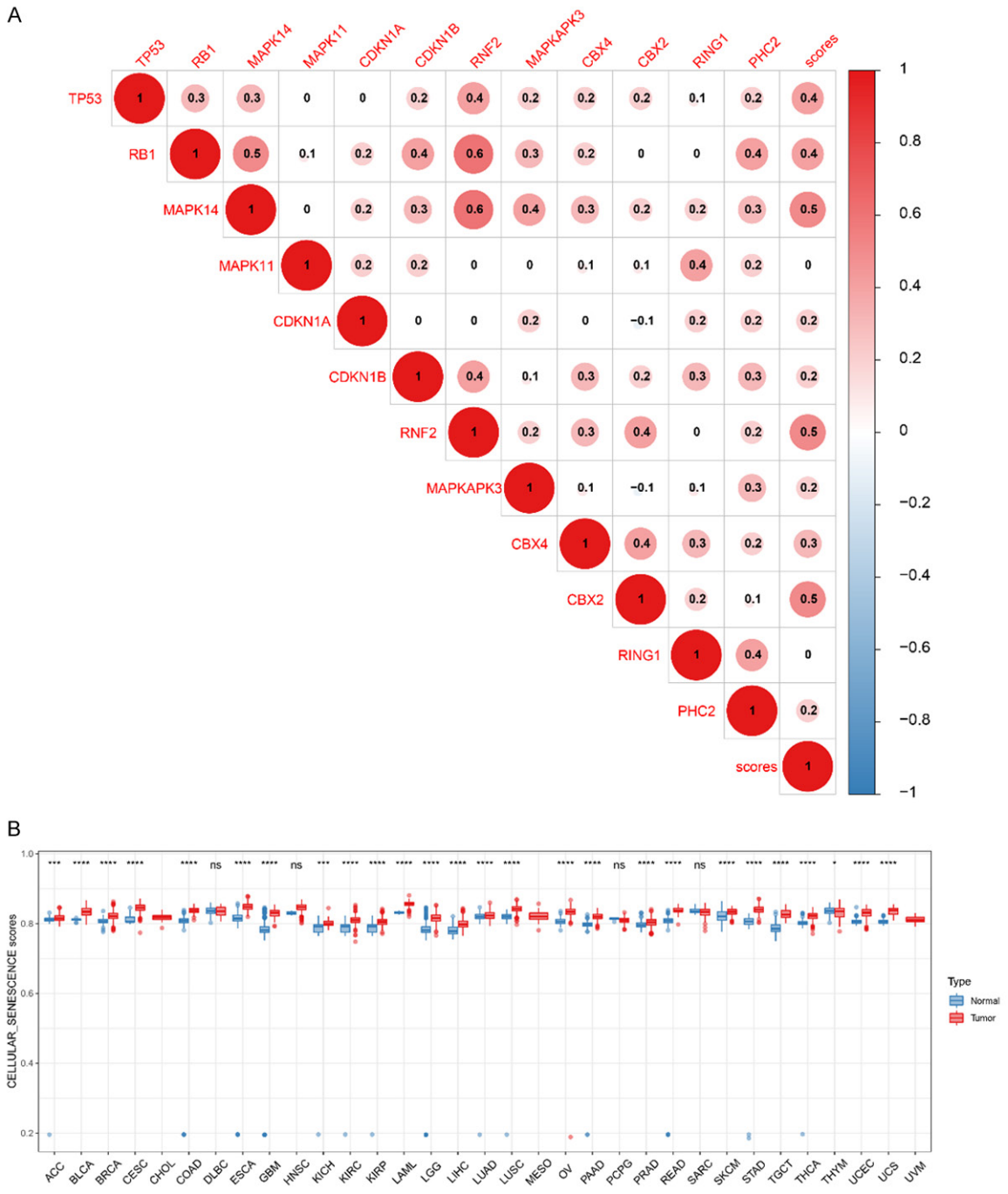


Figure 2. Cellular senescence score in different tissues and cancer stages. A. Heat map of correlations between the expression of cellular senescence genes and the cellular senescence score. The numbers in the circles represent correlation coefficients. B. Differences in the cellular senescence score between tumor and normal tissues. Red represents tumor tissue from The Cancer Genome Atlas, while blue represents normal tissue. *P < 0.05, **P < 0.01, ***P < 0.001.

between the immune scores of 22 types of immune cells and the cellular senescence score. The results revealed that the cellular senescence score was positively correlated with the immune score of several immune cells, such as M1 macrophages, M2 macrophages, activated memory CD4 T cells, neutrophils, resting cells, activated myeloid dendritic cells, M0 macrophages, and resting mast cells. However, the cellular senescence score was

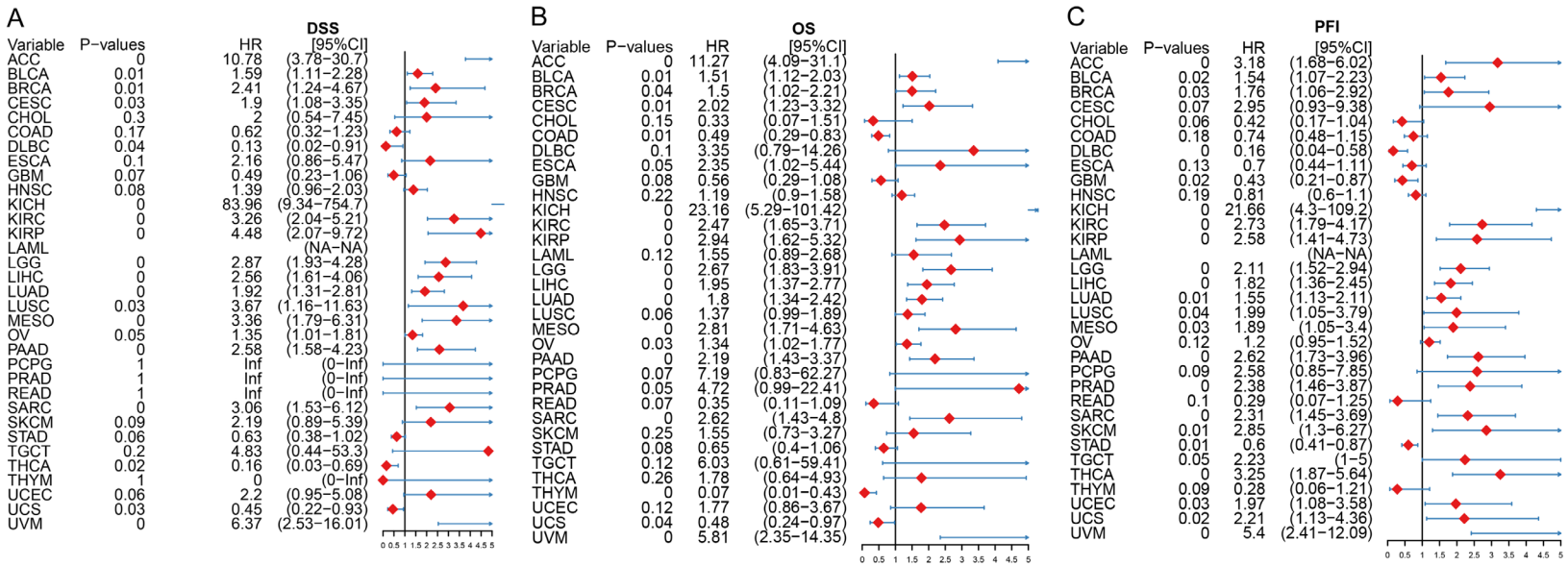


Figure 3. Prognostic value of the cellular senescence score. A. Effect of cellular senescence score on disease-specific survival (DSS). B. Effect of cellular senescence score on overall survival (OS). C. Effect of cellular senescence score on the progression-free interval (PFI).

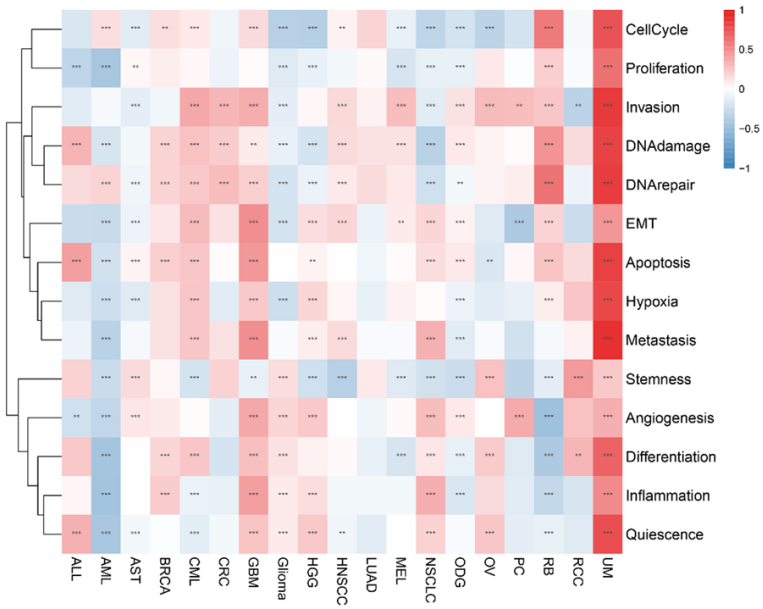


Figure 4. The function of the cellular senescence score at the single-cell level. Correlation of the cellular senescence score with 14 cellular functions in different tumors. *P < 0.05, **P < 0.01, ***P < 0.001.

negatively associated with naïve CD4 T cells, eosinophils, gamma delta T cells, plasma B cells, and activated mast cells (P < 0.05) (**Figure 5A**). In most tumor types, the cellular senescence score was significantly negatively correlated with the stromal score (**Figure 5B**). Their correlation was also investigated separately (Supplementary Figure 6). Contrastingly, the immune score was significantly positively correlated with the cellular senescence score in most cancer types, excluding TGCT, LUSC, UCEC, CESC, and SARC (P < 0.05) (**Figure 5C**). Their correlation was also investigated separately (Supplementary Figure 7). The cellular senescence score was significantly positively correlated with the TIME of LGG, KIRC, KICH, THYM, LAML, PAAD, PRAD, and BRCA. In contrast, it was significantly negatively correlated with the microenvironment score in the TIME of UCEC, TGCT, LUSC, SKCM, SARC, CESC, GBM, and HNSC (P < 0.05) (**Figure 5D**). Their correlation was also investigated separately (Supplementary Figure 8). These findings indicate that the cellular senescence score and the TIME are correlated.

Relationship between the cellular senescence score and the expression of immune-related genes

We showed that the cellular senescence score was significantly positively correlated with most immune checkpoint molecules, such as NRP1, CD276, CD44, and TNFSF9, and was significantly negatively correlated with TNFRSF14, CD27, TMIGD2, and HHLA2 (P < 0.05) (**Figure 6A**). Additionally, the cellular senescence score was significantly positively correlated with the expression of immunosuppressive genes such as *ULBP1*, *PVR*, *CD276*, *MICB*, *IL6*, *CD80*, *CD86*, *TNFRSF9*, and *IL2RA*. It was significantly negatively correlated with *TNFSF13*, *TNFRSF14*, *CD27*, *TMIGD2*, *TNFRSF17*, and *TNFRSF13B* genes (P < 0.05) (**Figure 6B**). The cellular senescence score was significantly positively correlated with the expression of *TGFBR1*, *KDR*, *TGFB1*, *PDCD1LG2*, *IL10*, *HAVCR2*, and *CD274* genes and was significantly negatively correlated with the expression of *CD160*, *CD244*, *KIR2DL3*, *KIR2DL1*, and *LAG3* genes (P < 0.05) (**Figure 6C**).

Furthermore, the cellular senescence score was significantly positively correlated with the expression of chemokine genes such as *CXCL8*, *CXCL2*, *CXCL5*, *CXCL3*, *CCL7*, *CXCL6*, *CXCL1*, *CCL20*, *CXCL9*, *CXCL11*, *CXCL10*, *CXCL16*, *CCL4*, *CCL3*, *CCL8*, and *CCL2* in most cancers (**Figure 6D**). Moreover, in most cancers, the cellular senescence score was positively correlated with the expression of chemokine receptor genes such as *CCR8*, *CXCR4*, *CCR4*, *CCR5*, and *CCR1* (P < 0.05) (**Figure 6E**).

Correlation between the cellular senescence score and markers of immunotherapy response

Tumor mutational burden (TMB) and microsatellite instability (MSI) refer to the tumor mutational load and microsatellite deletions, respectively. They are closely related to the therapeutic outcome in cancer patients. In this study, the cellular senescence score was significantly positively correlated with the MSI in LAML, suggesting that cancer immunotherapy is more effective in LAML (**Figure 7A**). Moreover, the cellular senescence score was significantly positively correlated with the TMB in ESCA,

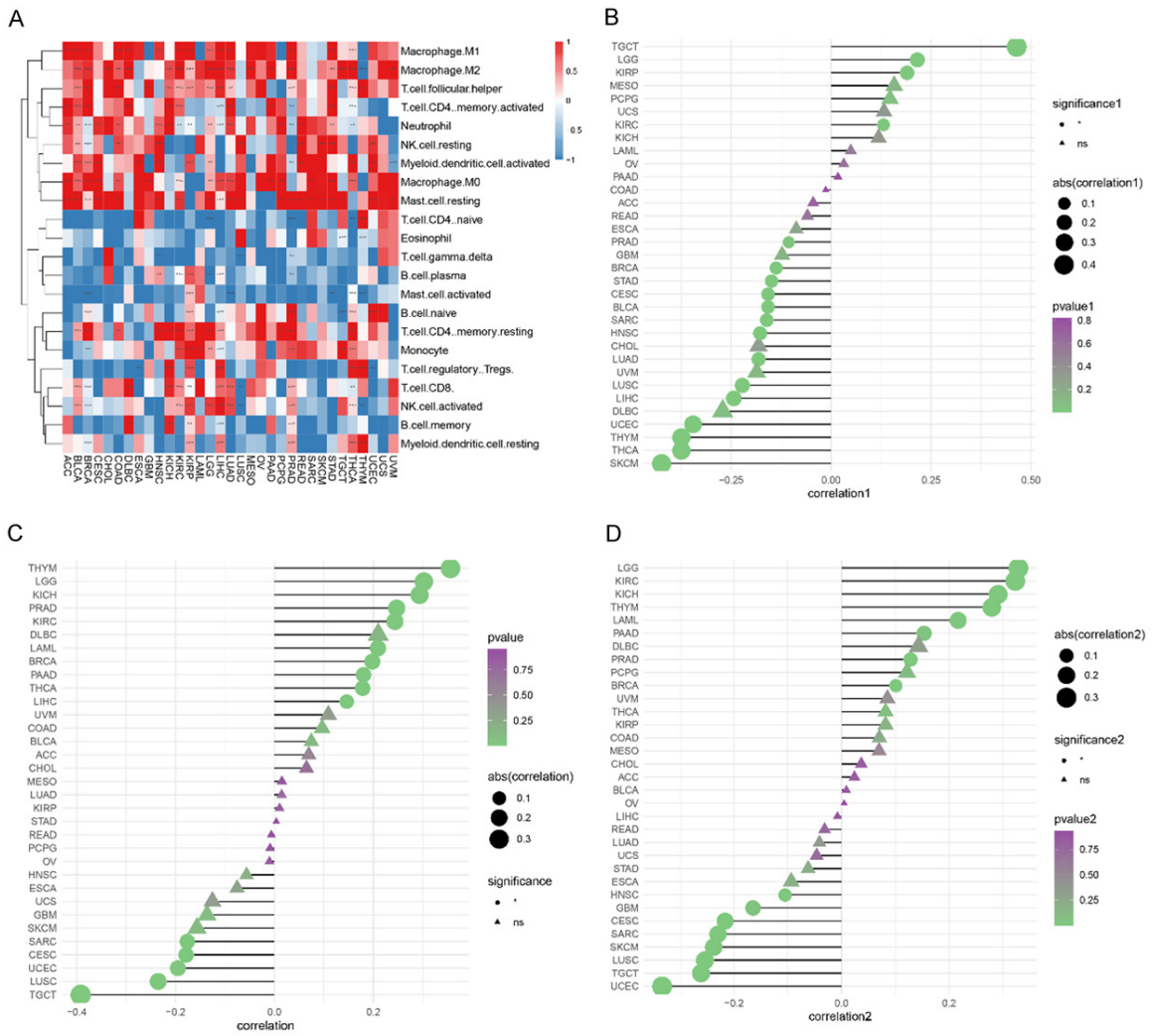


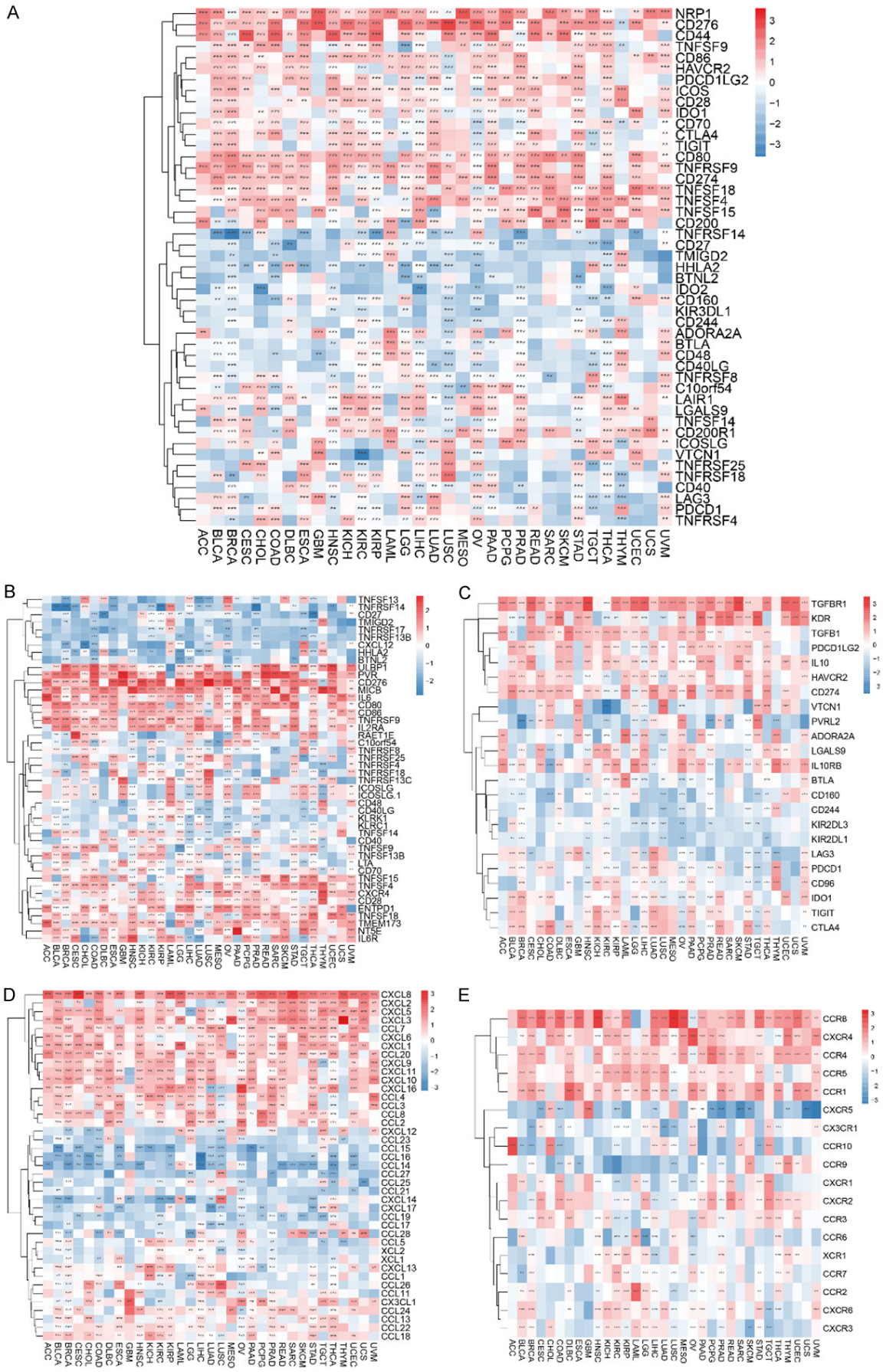
Figure 5. Relationship between the cellular senescence score and the tumor immune microenvironment. A. Correlation between the cellular senescence score and tumor immune infiltration. Red represents a positive correlation, and blue represents a negative correlation. B. Correlation between the cellular senescence score and tumor stroma. The larger the point, the larger the absolute value of the correlation coefficient. *P < 0.05. C. Correlation between the cellular senescence score and tumor immune score. The larger the point, the larger the absolute value of the correlation coefficient. *P < 0.05. D. Correlation between the cellular senescence score and tumor microenvironment. The larger the point, the larger the absolute value of the correlation coefficient. *P < 0.05, **P < 0.01, ***P < 0.001.

KIRP, SARC, READ, and PCPG. These findings indicate that cancer immunotherapy is more effective in the above-mentioned cancers (**Figure 7B**).

Oncologists can make decisions based on the TIDE score before administering immune checkpoint inhibition medication to cancer patients. We investigated the relationship between the cellular senescence score and the TIDE score to determine the scientific validity of the cellular senescence score. We found they were significantly positively correlated in most cancers, such as ESCA, LUSC, and LUAD (**Figure 7C**). Therefore, in immunotherapy the cellular senescence score is critical.

Analysis of single-cell transcriptional profile and cellular senescence score in the KIRC microenvironment

Next, single-cell RNA sequencing was performed on two interior KIRC samples. After performing quality control, high-quality single-cell transcriptome data from 13,124 cells were acquired. The t-SNE algorithm was used for cell


A
NRP1
CD276
CD44
TNFSF9
CD86
HAVCR2
PDCD1LG2
ICOS
CD28
IDO1
CD70
CTLA4
TIGIT
CD80
TNFRSF9
CD274
TNFSF18
TNFSF4
TNFSF15
CD200
TNFRSF14
CD27
TMIGD2
HHLA2
BTNL2
IDO2
CD160
KIR3DL1
CD244
ADORA2A
BTLA
CD48
CD40LG
TNFRSF8
C10orf54
LAIR1
LGALS9
TNFSF14
CD200R1
ICOSLG
VTCN1
TNFRSF25
TNFRSF18
CD40
LAG3
PDCD1
TNFRSF4
ACC BLCA BRCA CESC CHOL COAD DLBC ESCA GBM HNSC KICH KIRC KIRP LAML LGG LIHC LUAD LUSC MESO OV PAAD PCPG PRAD READ SARC SKCM STAD TGCT THCA THYM UCEC UCS UVM
B
TNFSF13
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TMIGD2
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TNFRSF13B
CXCL12
HHLA2
BTNL2
ULBP1
PVR
CD276
MICB
IL6
CD80
CD86
TNFRSF9
IL2RA
RAET1E
C10orf54
TNFRSF8
TNFRSF25
TNFRSF4
TNFRSF18
TNFRSF13C
ICOSLG
ICOSLG.1
CD48
CD40LG
KLRK1
KLRC1
TNFSF14
CD40
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TNFSF13B
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CD70
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TNFSF4
CXCR4
CD28
ENTPD1
TNFSF18
TMEM173
NT5E
IL6R
C
TGFBR1
KDR
TGFB1
PDCD1LG2
IL10
HAVCR2
CD274
VTCN1
PVRL2
ADORA2A
LGALS9
IL10RB
BTLA
CD160
CD244
KIR2DL3
KIR2DL1
LAG3
PDCD1
CD96
IDO1
TIGIT
CTLA4
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CXCL8
CXCL2
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CCR1
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CX3CR1
CCR10
CCR9
CXCR1
CXCR2
CCR3
CCR6
XCR1
CCR7
CCR2
CXCR6
CXCR3

Figure 6. Correlation between the cellular senescence score and the expression of immune-related genes. A. Heat map showing the correlation between the cellular senescence score and the expression of immune checkpoints. Red represents a positive correlation. *P < 0.05. B. Heat map showing the correlation between the cellular senescence score and expression of immune activation genes. Red represents a positive correlation. *P < 0.05. C. Heat map showing the correlation between the cellular senescence score and the expression of immunosuppressive genes. Red represents a positive correlation, *P < 0.05. D. Heat map showing the correlation between the cellular senescence score and the expression of chemokines. Red represents a positive correlation. *P < 0.05. E. Heat map showing the correlation between the cellular senescence score and the expression of chemokine receptors. Red represents a positive correlation. *P < 0.05, **P < 0.01, ***P < 0.001.

cluster analysis, and the results showed that the above cells could be classified into 11 clusters, including KIRC1, KIRC2, KIRC3, CD4+ T cells, CD8+ T cells, macrophages, monocyte 1, monocyte 2, mast cells, natural killer (NK) cells, and endothelial cells (**Figure 8A**). The expression of cell marker genes was displayed in Supplementary Figure 9. Furthermore, we analyzed tumor cells from two different KIRC samples and found that these two KIRC tumor cell samples contained two distinct clusters (KIRC1 and KIRC2) and one common cluster (KIRC3). The results above indicated heterogeneity in the KIRC cell types (**Figure 8B**). We estimated the cellular senescence score in different types of KIRC cells and found that it differed between them. We then determined the cellular senescence score in KIRC TME cells and looked at how it differed between cell types (**Figure 8C**). Remarkably, we discovered considerable variations in the cellular senescence score between different cell types (**Figure 8D**). The cellular senescence score in monocyte 1 was significantly higher than in other cells (P < 0.05). We investigated the expression of cellular senescence-associated hub genes in different cell populations and discovered that some of them had TIME-specific expression. For example, *CDKN1A* was primarily expressed in monocyte-macrophages, whereas *RB1* was primarily expressed in macrophages (**Figure 9**). These findings revealed that the cellular senescence score differed by TME in KIRC TME. Therefore, we hypothesized that the cellular senescence score might be a promising indicator in cancer treatment.

Protein expression of hub-genes

The hub-gene was identified as *RB1* with the highest 62-degree in the PPI network. The Human Protein Atlas immunohistochemistry results indicated that *RB1* expression was significantly low in lung, renal, and colorectal cancers (**Figure 10A-F**), consistent with our differential expression analysis.

Discussion

Cellular senescence occurs at all stages of development and growth, serving as an important mechanism for maintaining tissue homeostasis and preventing damaged cell expansion [20]. Previously, the active oncogene Ras was shown to cause premature senescence in primary rodent and human cells, correlating with the induction of p53 and p16 [21], suggesting that senescence acts as a tumor suppressor mechanism in cells, preventing oncogenic mutations from causing abnormal cell proliferation [22]. Despite this, severe extracellular or intracellular stress triggers cellular senescence [23], and evidence suggests that persistent senescence or escape causes secondary malignancies [24]. Tumors develop when the immune system cannot eliminate oncogene-induced senescent cells on time [26]. Because the relationship between cellular senescence and tumor immunity is poorly understood, we analyzed and investigated the relationship between tumor progression, prognostic values, and immune infiltration at the pan-cancer level and their correlation with the expression of immunotherapeutic markers of cellular senescence in this study. We discovered a strong correlation between the patient's responsiveness to immunotherapy and the expression of hub genes associated with cellular senescence.

We revealed that cellular senescence-associated hub genes are differentially expressed in tumors. Some of these genes are identified as risk factors in tumor prognosis, with lower cellular senescence-associated hub gene expression indicating a better prognosis. Subsequently, we estimated the cellular senescence score to express the degree of cellular senescence and discovered that a low level of cellular senescence was associated with a better prognosis for cancer patients. Cellular senescence-associated hub genes were found to be correlated with the expression of TME and immune-related genes. Therefore, central cel-

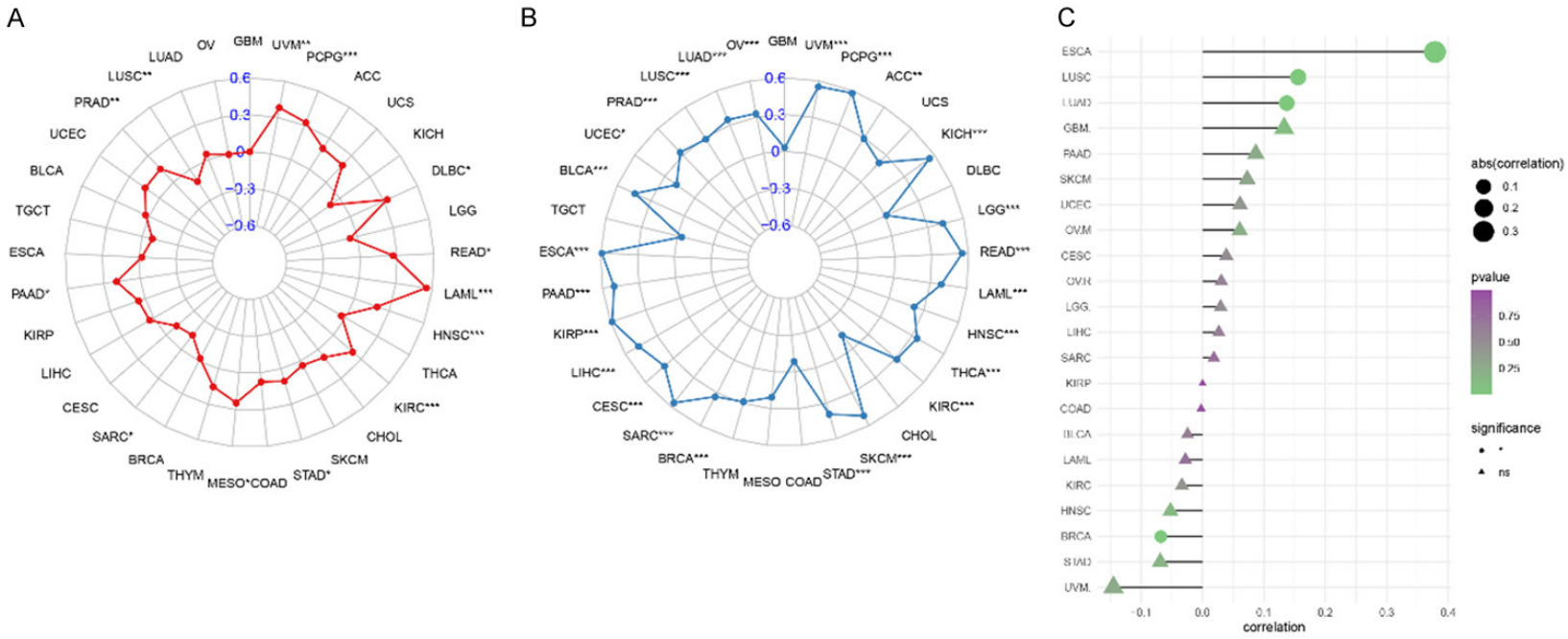

Figure 7. Correlation between the cellular senescence score and the expression of markers related to immunotherapy response. A. Correlation between the cellular senescence score and microsatellite instability; B. Correlation between the cellular senescence score and tumor mutational burden. C. Correlation between the cellular senescence score and tumor immune dysfunction and exclusion score.

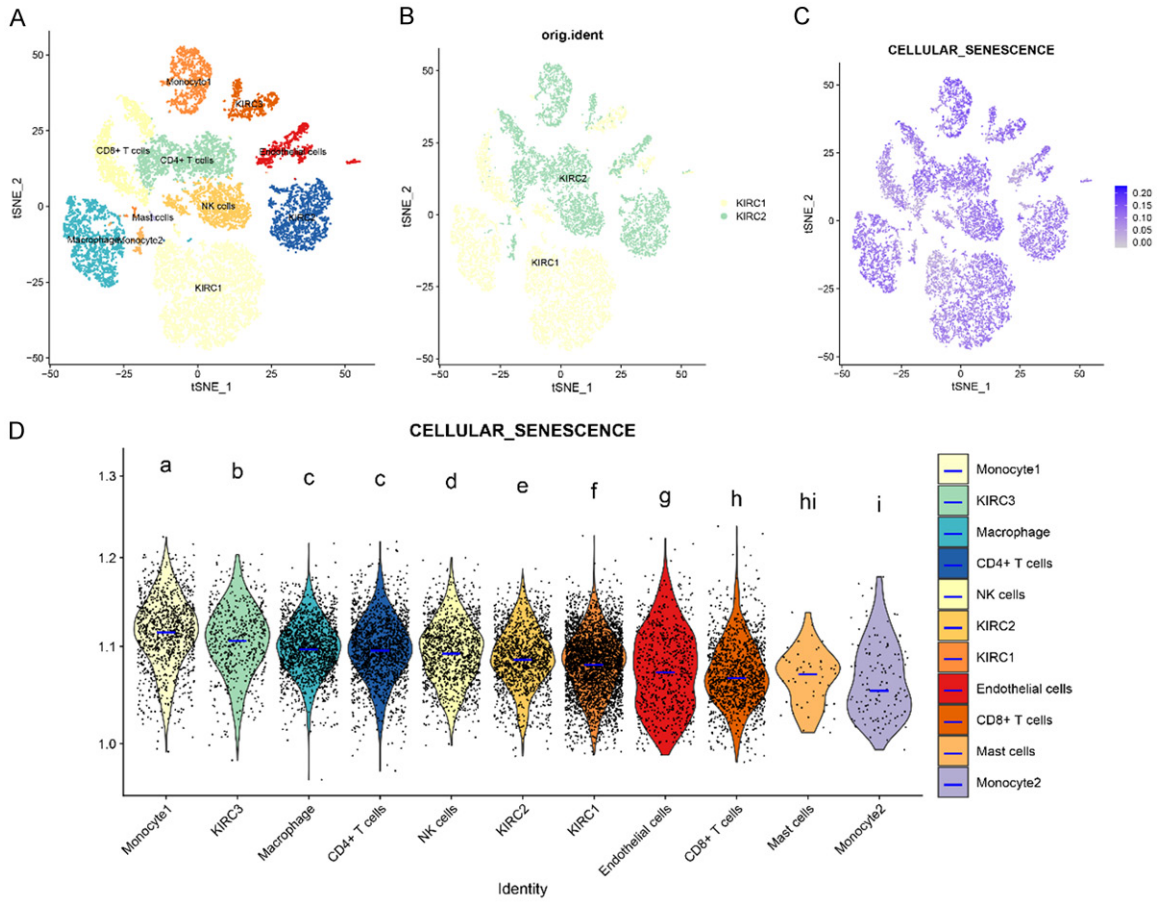

Figure 8. Cellular senescence score analysis based on kidney renal clear cell carcinoma (KIRC) single-cell transcriptome atlas. A. tSEN plot representation of KIRC samples with 11 distinct cell types. B. t-SNE analysis based on two different samples of KIRC. C. The different cellular senescence scores in different types of KIRC cells. D. Comparison of cell senescence scores in different cells.

lular senescence-associated genes could be used as prognostic and predictive markers for immunotherapy [25].

Using the TCGA database, we discovered that the central cellular senescence-associated genes, *CBX2* and *CBX4*, are highly expressed in most tumors. Recurrent upregulation of the *CBX2* gene in metastatic PRAD is associated with poor clinical outcomes in PRAD cohorts, which is consistent with our findings. The *CBX4* gene is a relatively specific Polycomb Group protein involved in tumorigenesis and cell cycle regulation [26]. In addition, cancer metastasis, clinically advanced stage, and a worse OS of patients are all highly correlated with increased *CBX4* gene expression in tumor tissues compared to non-tumor tissues in lung cancer, hepatocellular carcinoma, and breast cancer. Therefore, central genes associated with cellular senescence may be useful prognostic biomarkers and potential therapeutic targets in cancer.

We revealed a link between cellular senescence scores and TIME. Cellular senescence, associated with an altered TME, affects tumor proliferation and development. The TME includes immune cells such as macrophages, cytotoxic T lymphocytes, dendritic cells, NK cells, cancer-related fibroblasts, blood vessels, lymphatic endothelial cells, and the extracellular matrix, which are key factors in tumor development and progression. Because stromal cells are equally vulnerable to treatment-induced damage as cancer cells, developing a considerable number of senescent cells in the TME has a major impact on treatment outcomes [27]. Senescent cells can induce proliferation, angiogenesis, migration, and invasion

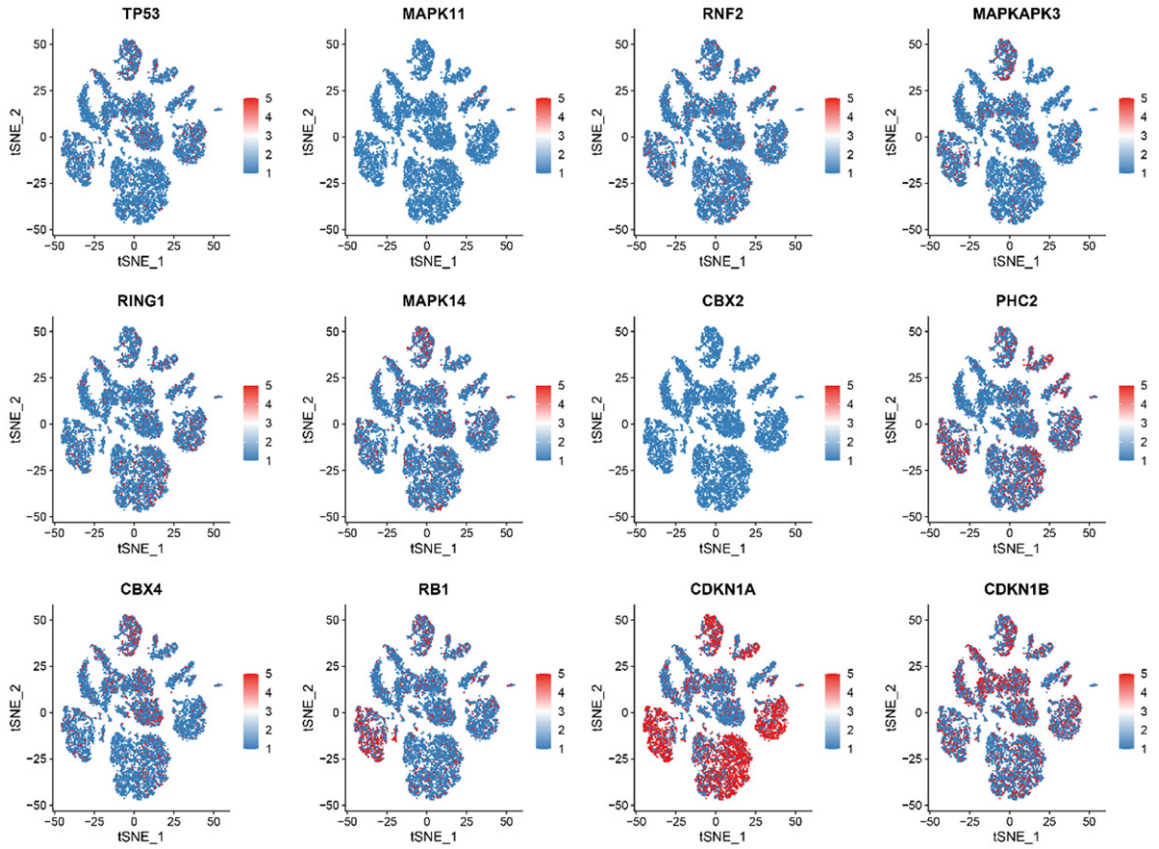

Figure 9. Feature plots of cellular senescence hub genes in different cell populations.

of neighboring cells through the secretion of inflammatory SASP [28-31]. SASP has protumorigenic properties. The vascular endothelial growth factor, a key component of SASP, promotes angiogenesis and provides access to growth factors, ultimately favoring malignant growth [32]. Consequently, senescence-inducing therapy could be an effective cancer treatment strategy.

Subsequently, immunological pathways such as immune checkpoints are linked to cellular senescence and can be used as immune indicators in patients with cancer. In this study, the cellular senescence score was positively correlated with the expression of immune checkpoints CD276 and CD44. Correlation studies have suggested that radiation-induced reprogramming of pre-senescent mammary epithelial cells may be crucial in developing cancer and non-oncogenic mammary epithelial populations. These cells are enriched for the putative CD44/CD24+/low stem cell phenotype [33]. CD276, also known as B7-H3 (B7 homolog 3), is overexpressed in various malignancies, such as colorectal, pancreatic, prostate, and ovarian cancers [34-37]. According to our findings [43], CD276 levels were significantly higher during tumor cell senescence because it prevented adriamycin-induced senescence in colon cancer cells *in vivo* [38]. Therefore, senescent cells can play an important role in tumor therapy.

Finally, we analyzed the potential role of cellular senescence as an immunotherapy marker. TMB, MSI, and TIDE scores are currently recognized ICB therapy markers. TMB is usually defined as the total number of non-anonymous mutations per megabase (Mut/Mb) in somatic cells, including frameshift mutations, insertions, point mutations, and deletions [39, 40]. These mutations produce abnormal proteins acting as neoantigens and activating anti-tumor responses [41]. Genetic material stability affects tumor cell expression; therefore, MSI can be a clinically important tumor marker. Currently, the TIDE score is the most promising marker of ICB response, and several studies

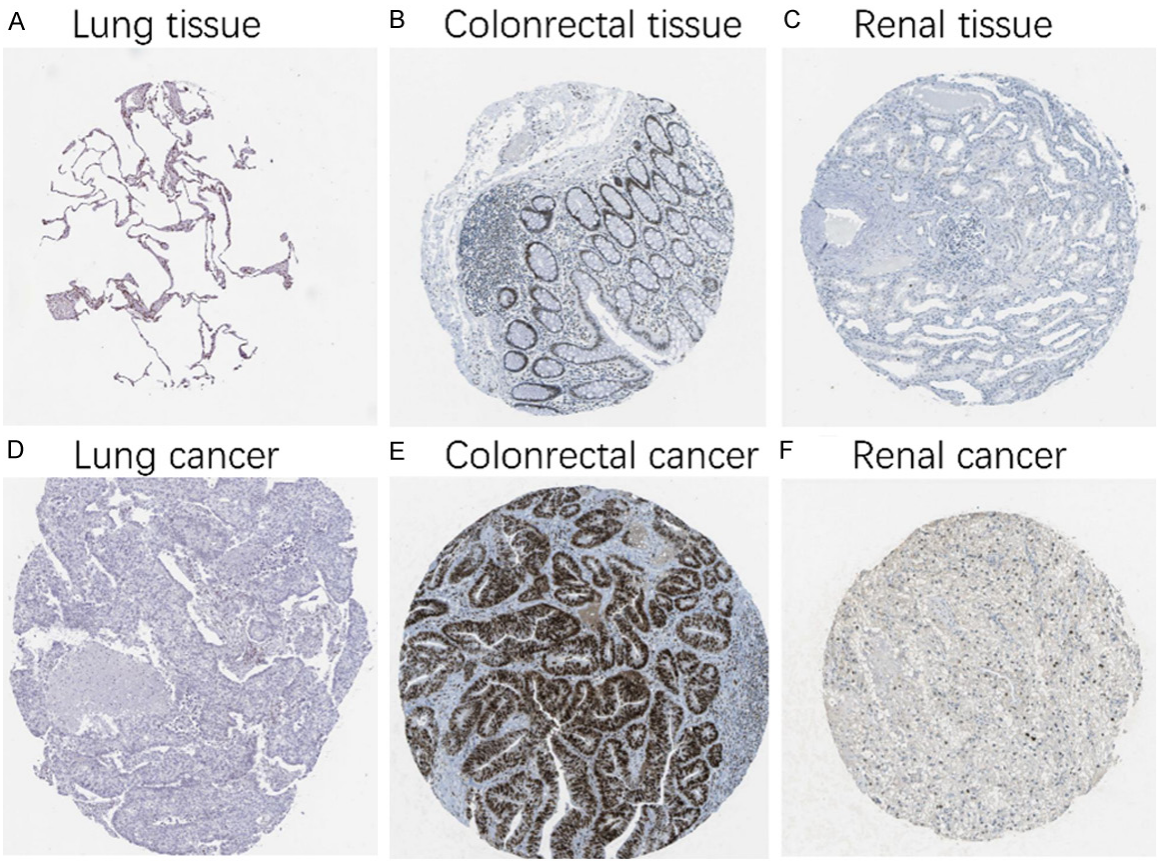

Figure 10. Protein levels of RB1 in human tumors and normal tissues. (A-C) normal lung, renal, and colorectal (D-F); lung cancer, renal cancer, and colorectal cancer.

have shown that it is more accurate than MSI and TMB in predicting survival outcomes in cancer patients treated with ICB drugs. Furthermore, we demonstrated that the cellular senescence score was positively related to the TIDE score. Therefore, we concluded that the expression of cellular senescence-associated genes was significantly and positively correlated with TMB and MSI scores. The cellular senescence score was significantly and positively correlated with the expression of immune activation genes *MICB* and *IL2RA*. However, in BLCA and BRCA, the cellular senescence score was significantly negatively related to the expression of the immune activation gene *TNFRSF14*. Therefore, the findings of this study suggest that cellular senescence-associated genes are an independent prognostic factor for ICB treatment.

However, there are certain limitations to this study. We found that the cellular senescence-associated genes could influence patient prognosis in various cancer types by changing the TME using a thorough bioinformatics approach. We also found that cellular senescence could be a prognostic biomarker in cancer. However, large-scale clinical trials are still required to confirm the immunotherapeutic role of cellular senescence at a pan-cancer level in the future.

Conclusion

To the best of our knowledge, this is the first study to comprehensively analyze the role of cellular senescence at the pan-cancer level. We discovered that the cellular senescence score was significantly higher in most cancer types and was closely related to TIME inhibition. The findings of this study suggest that cellular senescence is crucial in tumors and is intricately linked to tumor immunity. Therefore, cellular senescence targeting may represent a novel, ground-breaking approach to cancer therapy.

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Disclosure of conflict of interest

None.

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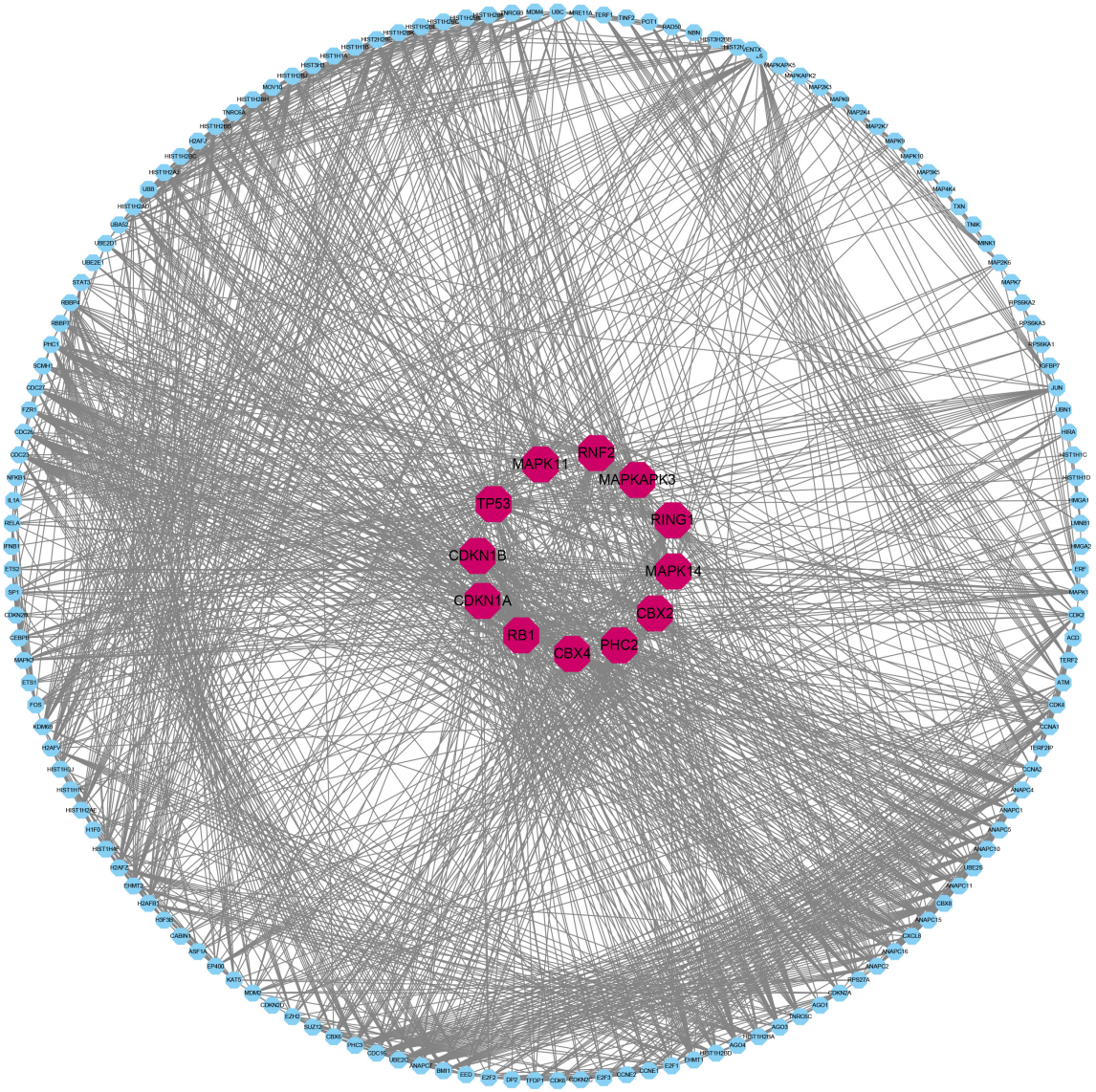
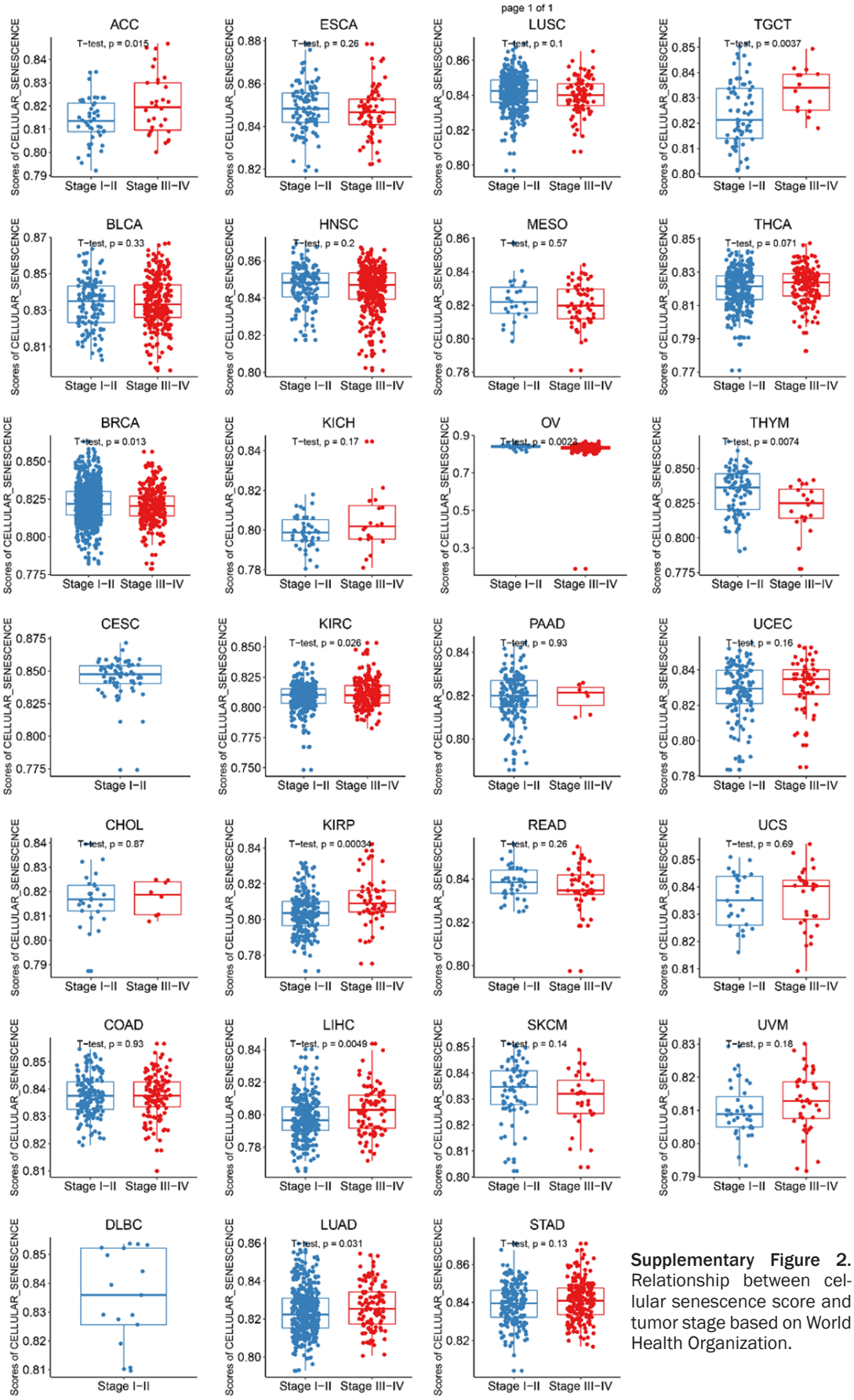
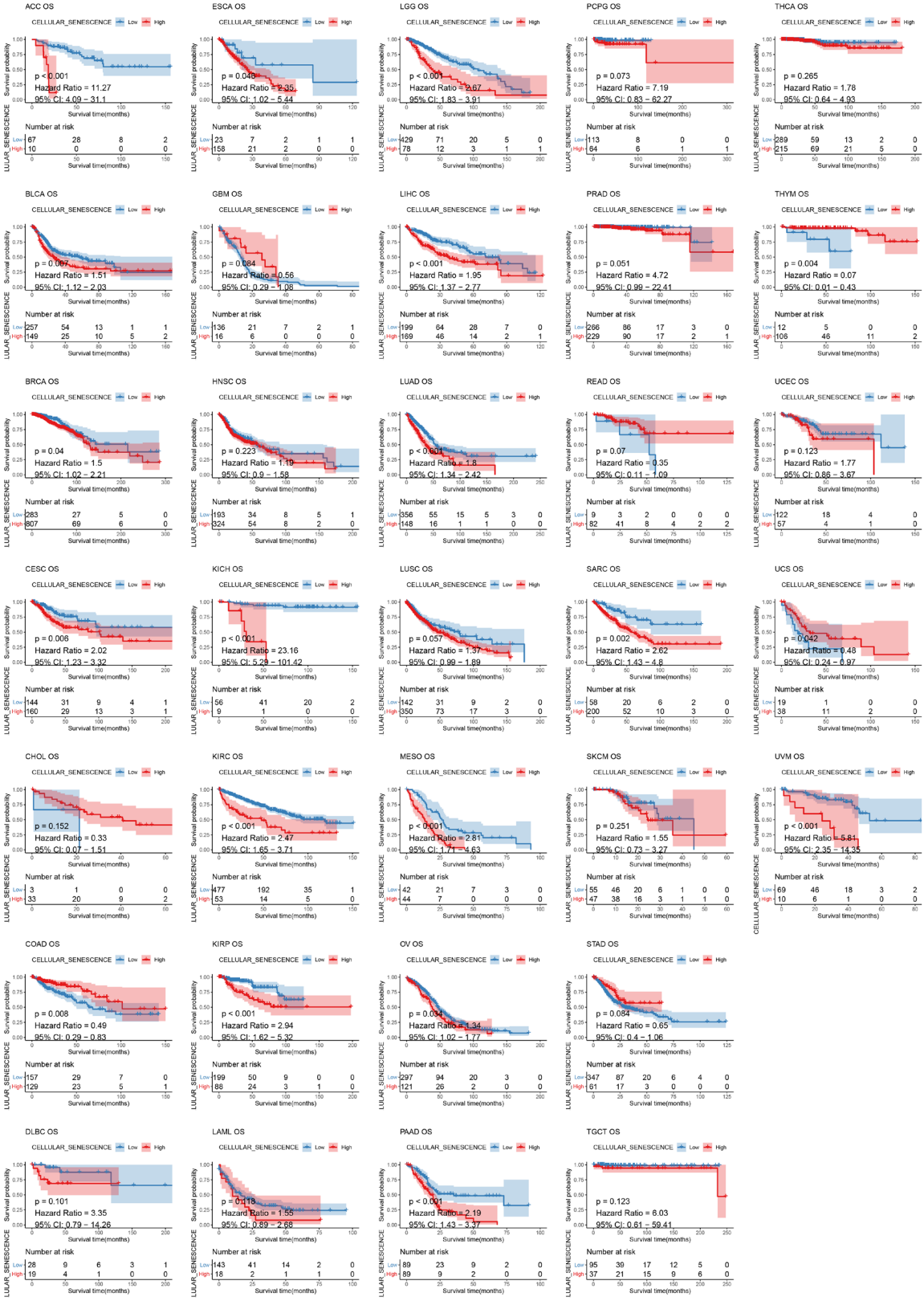


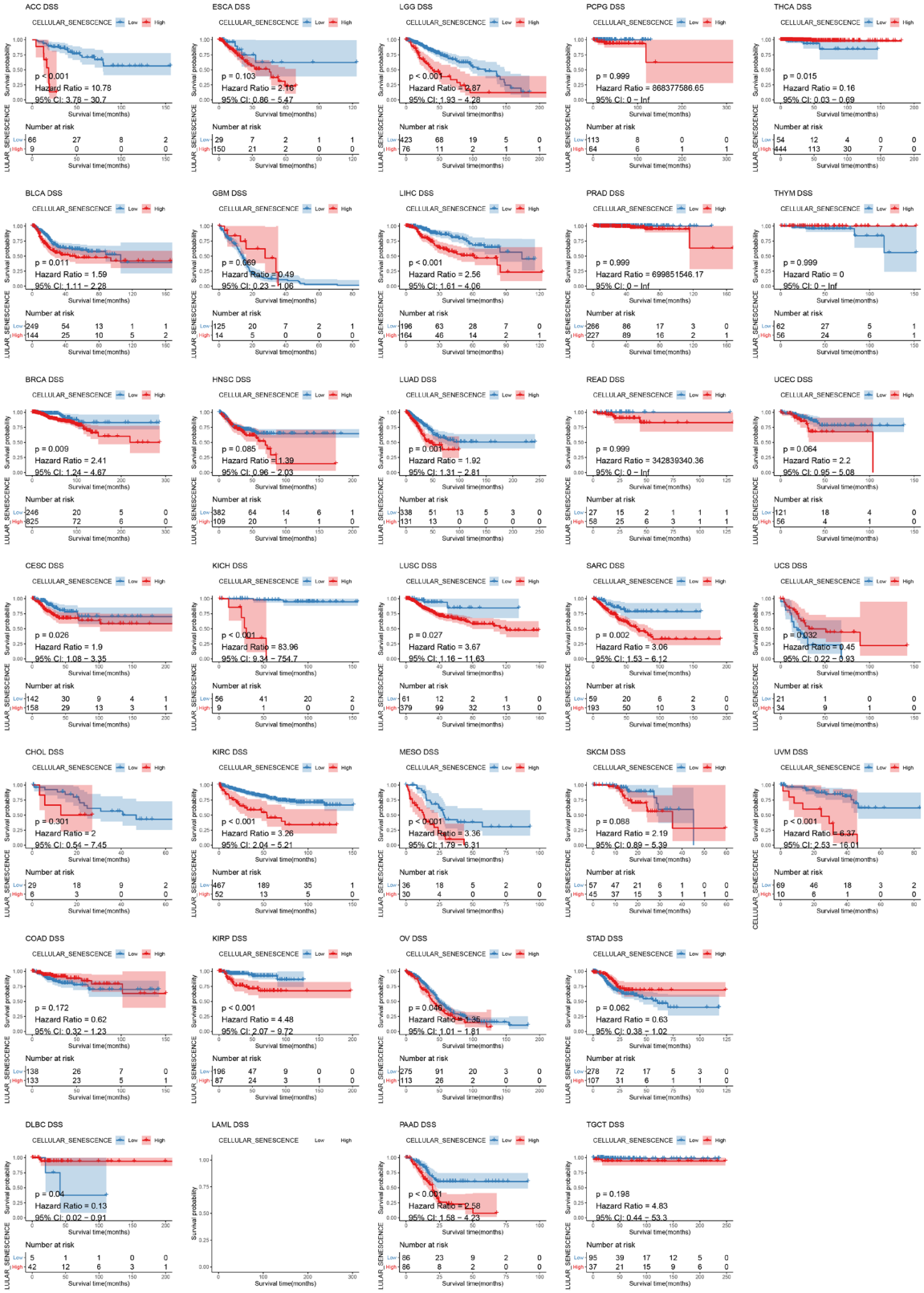
Supplementary Figure 1. Identification of cellular senescence-associated genes. Network map of cellular senescence-associated genes.



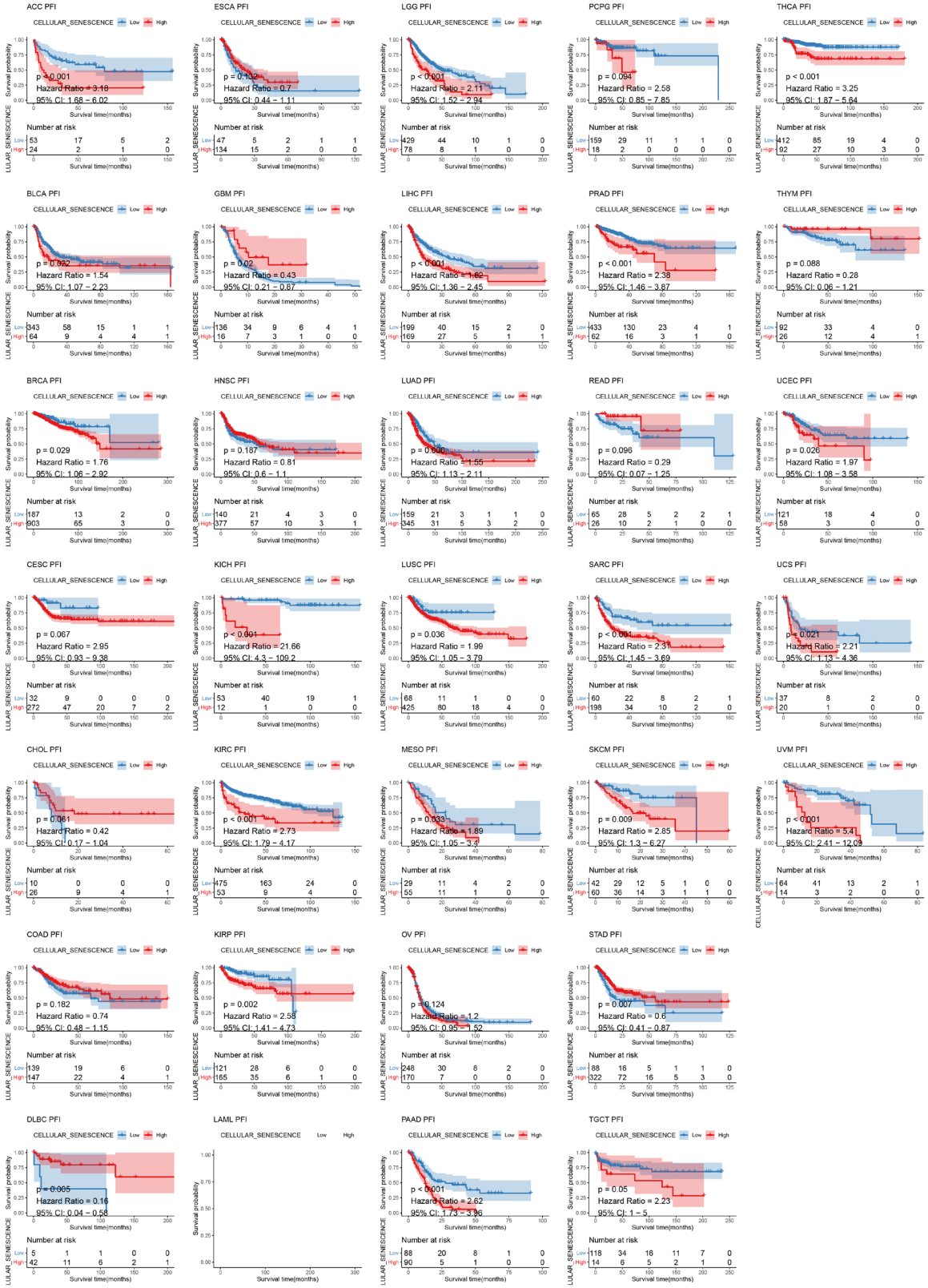
Supplementary Figure 2. Relationship between cellular senescence score and tumor stage based on World Health Organization.



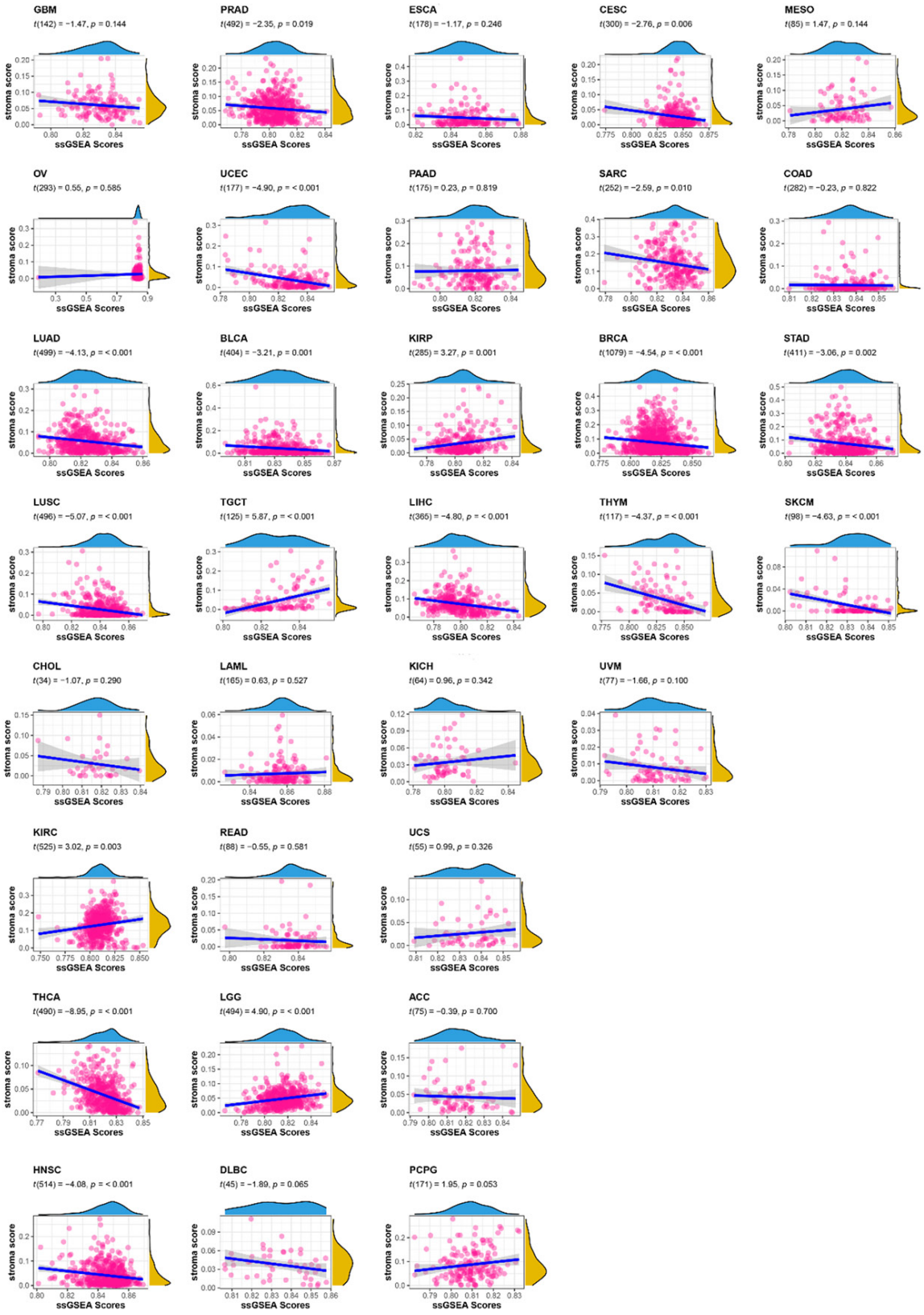
Supplementary Figure 3. Effect of cellular senescence on overall survival (OS).



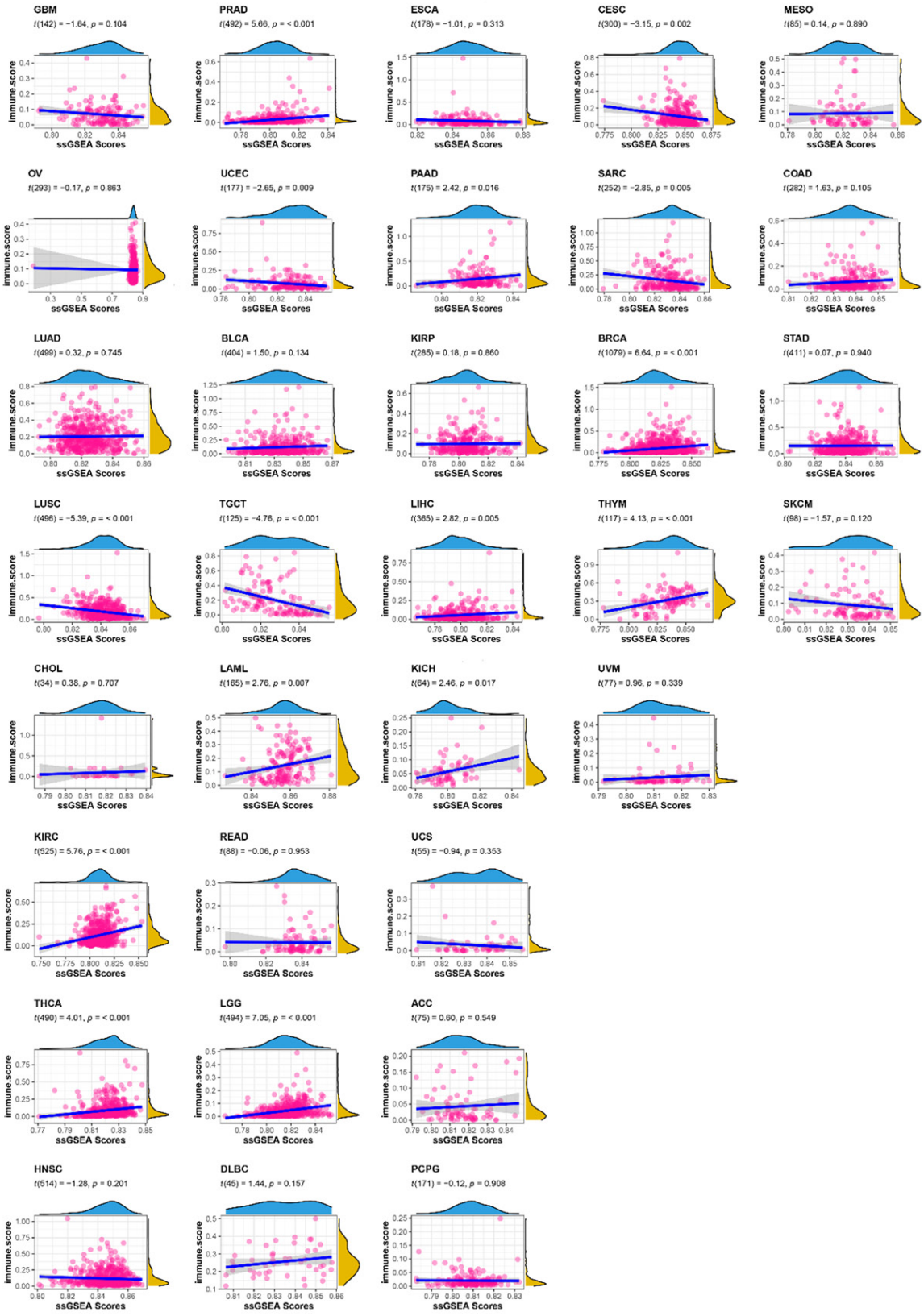
Supplementary Figure 4. Effect of cellular senescence on disease-specific survival (DSS).



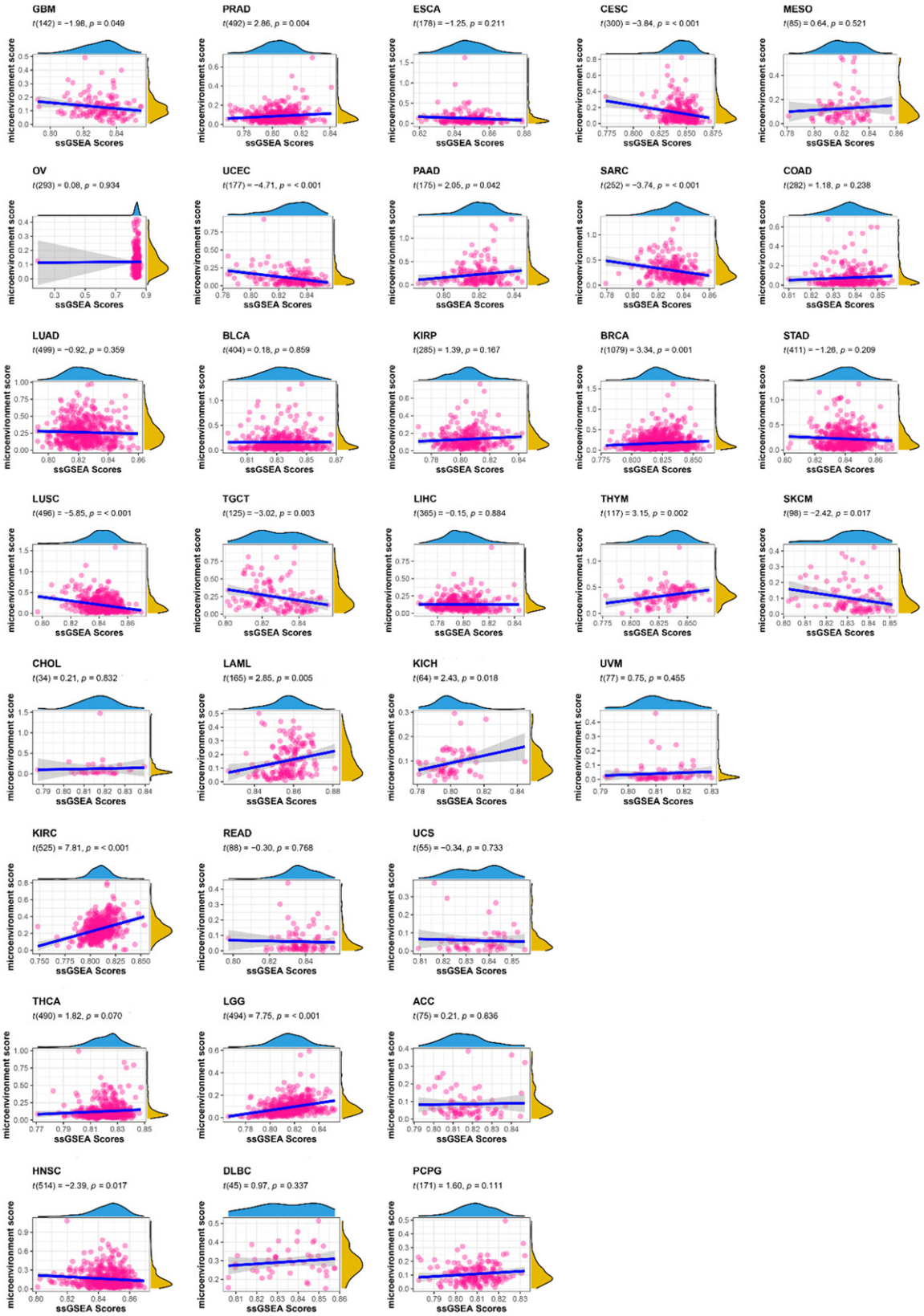
Supplementary Figure 5. Effect of cellular senescence on progression-free interval (PFI).



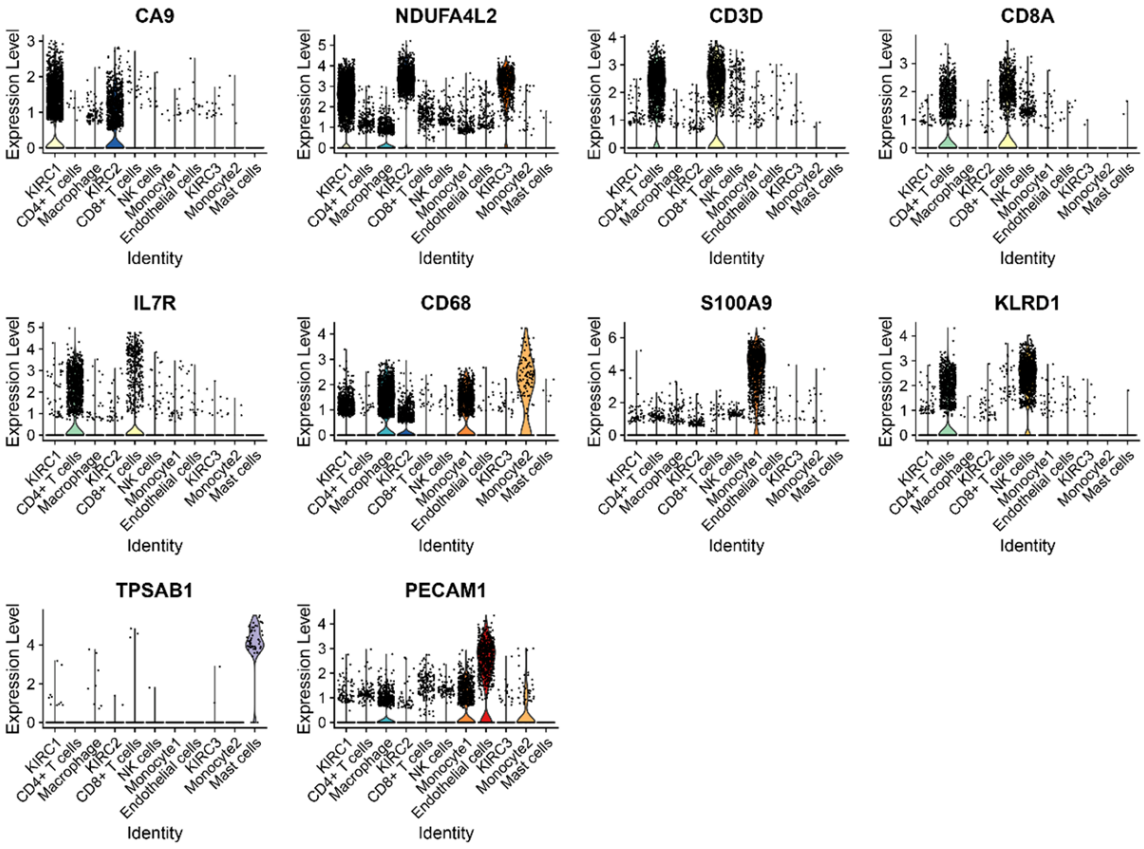
Supplementary Figure 6. The relationship between cellular senescence score and cancer stromal score.



Supplementary Figure 7. The relationship between cellular senescence score and cancer immune score.



Supplementary Figure 8. The relationship between cellular senescence score and cancer microenvironment score.



Supplementary Figure 9. Expression levels of different markers in different cells.