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Turning towards nonimmunoreactive tumors: Evaluation of cancer-associated fibroblasts enables prediction of the immune microenvironment and treatment sensitivity in pancreatic cancer

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ABSTRACT

Increasing evidence has confirmed that cancer-associated fibroblasts (CAFs) recruit and induce regulatory T cells (Tregs) and macrophages but inhibit cytotoxic T lymphocyte infiltration to a certain extent, indicating that CAFs have a significant influence on the immunosuppressive microenvironment. However, the effect of CAFs on the immune microenvironment and immunotherapy response in pancreatic cancer remains unclear. Our research identified remarkable variation in CAF-associated molecules in multiple cancer types at the genetic and transcriptome levels. Two phenotypes were identified for 476 pancreatic cancer samples, and the different phenotypes exhibited significant variation in immune and inflammatory characteristics. Phenotype 1 exhibited higher levels of immune infiltration and lower expression of tumor-associated gene signatures than phenotype 2. We used a multipart approach to assess the prognostic value of CAF-associated molecules and constructed a CAF score model that could accurately predict patient prognosis. The CAF score accurately predicted infiltrating immune cell abundance, chemosensitivity, and the response to immunotherapy. Additionally, we found that the CAF-associated molecule FGFR4 may promote the proliferation and migration and inhibit the apoptosis of pancreatic cancer cells and is correlated with immune infiltration, suggesting its potential role as an oncogene. CAFs may promote the malignant biological behavior of pancreatic cancer through FGFR4. In summary, our research highlights potential relationships of the dysregulation of CAF-associated molecules with genome alterations and carcinogenesis in multiple malignancies. Our CAF-associated phenotypes and scoring system may enhance the understanding of pancreatic cancer chemotherapy sensitivity and immunotherapy response, providing new insights for personalized chemotherapy and immunotherapy.

1. Introduction

Pancreatic cancer is a lethal and complex malignancy worldwide, with increasing incidence [1]. With the continuous improvement of chemotherapy drugs and combination regimens, the paclitaxel–gemcitabine and fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) regimens have partially improved the survival of pancreatic cancer patients, but late-stage drug resistance remains a barrier [2]. Immunotherapy, represented by immune checkpoint (such as PD-1, PD-L1, and CTLA4) inhibitors, has brought therapeutic benefits to many solid tumor patients [3]. However, patients with pancreatic cancer usually respond poorly to immunotherapy due to differences in the composition and proportions of immune cells in their immune microenvironment [4]. The tumor microenvironment contains tumor-suppressive components, such as tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs), and antitumor components, such as tumor-infiltrating lymphocytes and CD8+ T cells [5–7]. Analyzing the critical components and exploring new targets based on the immune

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microenvironment may bring new breakthroughs to pancreatic cancer immunotherapy.

Cancer-associated fibroblasts (CAFs) are one of the main components of the stroma of multiple solid tumors and lead to microenvironment heterogeneity [8]. CAFs have been reported to participate in multiple biological functions in malignant tumorigenesis, including remodeling of the stroma, acceleration of angiogenesis, and regulation of inflammatory responses and antitumor immunity [8,9]. In particular, CAFs can influence immune cell function and promote the formation of an immunosuppressive microenvironment through multiple pathways. Some researchers have proposed that CAFs affect cytotoxic T lymphocyte infiltration and *T*-cell migration by regulating CXCL12/CXCR4 and TGF-β signaling [10,11]. In addition, Cheng et al found that CAFs inhibit the differentiation and antigen presentation of dendritic cells by secreting vascular endothelial growth factor, subsequently inhibiting *T*-cell localization and cytotoxicity [12]. CAFs may secrete multiple cytokines to stimulate the biological functions of tumor-promoting immune cells; for example, CAFs secrete WNT16B to recruit Tregs [13]; SDF-1a and IL6 to induce MDSCs [13]; and IL33 and IL10 to stimulate macrophages [14]. These findings suggest the role of CAFs in the tumor immune microenvironment and their potential value in immunotherapy.

The aforementioned crosstalk between CAFs and the tumor microenvironment as well as the effects of CAFs on the immunotherapy response are often based on the regulation of one or two core molecules. Analysis of high-throughput data and analysis of the interactions of multiple molecules may be more valuable.

In the current study, we elucidated the transcriptomic characteristics of 28 CAF-associated molecules in 33 cancer types, identifying significant relationships of methylation and copy number variation (CNV) of CAF-associated molecules with CAF molecule expression and tumor survival. In addition, we assessed 476 samples from different databases and identified CAF-related phenotypes and scoring systems in pancreatic cancer, focusing on their relationships with chemotherapy, the immune microenvironment and immunotherapy.

2. Materials and methods

2.1. Data sources and acquisition

The data for the pancancer analysis were obtained from the TCGA and GSCA databases. Fig. S1 presents our experimental design and workflow in detail. Detailed data acquisition and analysis procedures are presented in the supplementary materials and methods.

2.2. Identification of CAF molecule-based phenotypes

We identified 28 CAF-associated molecules from previous studies [15–17] and the GeneCards database. A consensus clustering algorithm was performed to identify CAF molecule-based phenotypes based on these CAF-associated molecules. The cluster analysis details are elaborated in the supplementary materials and methods.

2.3. Construction of the CAF scoring model

We screened the aforementioned 28 molecules by univariate Cox, random forest, and LASSO regression and ultimately constructed a CAF-based scoring system with multivariate Cox regression analysis. The CAF score was calculated as follows: $expr_{gene1} * coff_{gene1} + expr_{gene2} * coff_{gene2} + expr_{gene3} * coff_{gene3} \ldots \ldots expr_{gene\ n} * coff_{gene\ n}$. We further evaluated the relationship of the CAF score with the immune microenvironment, chemotherapy and immunotherapy in pancreatic cancer.

2.4. Statistical analysis

Bioinformatics analysis and associated statistical calculations were performed with R 4.0.3, and statistical analysis of cytology experiments was completed with GraphPad Prism 8.1. The detailed computational methods and R packages used are elaborated in the supplementary materials and methods.

3. Results

3.1. Pancancer analysis of the transcriptome characteristics of CAF-associated molecules

We identified 28 CAF-associated molecules from previous research and comprehensively analyzed their transcriptome characteristics and genetic variation. Fig. 1A shows the crosstalk between CAFs and tumor cells in different tumor immune microenvironments. CAFs may be a significant element in the transformation of tumors from the immune-inflamed type to the immune-excluded or even the immune-desert type. To explore the genomic alterations of CAF-associated molecules in various cancers, 1203 samples containing at least one mutation of the above molecules were analyzed. A waterfall diagram was employed to visualize the mutation landscape of the 10 CAF-associated molecules with the highest mutation frequency, and mutations in these 10 molecules accounted for 76.23% of the total mutations (Fig. 1B). Additionally, SYNPO2 and PDGFRA exhibited the highest mutation frequency, and the most common type of mutation was missense mutation (Fig. 1B). We elucidated that the methylation of genes encoding CAF-associated molecules is a critical factor affecting their normal functions. Fig. 1C shows the effect of differences in methylation levels on the prognosis of different tumor types, and correlation analysis indicated that CAF-associated molecules with higher gene expression tended to show lower methylation levels (Fig. 1D). In addition, the methylation levels of more than half of the CAF-associated molecules were increased in multiple tumor types (Fig. S2A). We performed differential expression analysis of these CAF-associated molecules in paired cancer and paracancerous samples from TCGA and displayed the results in bubble plots (Fig. 1E). Several CAF-associated molecules, including FAP, CDK1, MET, PLAU, SPINT2, AGT, PDGFRB, MAN2B1, CA12, KRT19, GRB2, and FGFR4, exhibited elevated expression trends in multiple tumors (Fig. 1E). CNV is a significant type of tumor genome alteration. We found that the expression level of most CAF-associated molecules was positively correlated with the CNV level (Fig. 1F). Heterozygous amplification and deletion occurred in almost all CAF-associated molecules, with AGT and S100A4 having the highest frequencies, while homozygous mutation occurred infrequently (Figs. S2B, S2C, S2D).

In the current research, we mainly explored the effects and changes of CAF-associated molecules in pancreatic cancer. These CAF-associated molecules exhibited remarkable differences in expression between cancer and normal samples, and most molecules were upregulated in cancer (Fig. 1G). Furthermore, univariate regression analysis showed that most CAF-associated molecules had prognostic significance in pancreatic cancer (Fig. 1H).

3.2. Identification of two CAF-related phenotypes by unsupervised clustering

We further explored the clinical significance of the 28 CAF-associated molecules. Consistent with the trend shown in Fig. 1G,

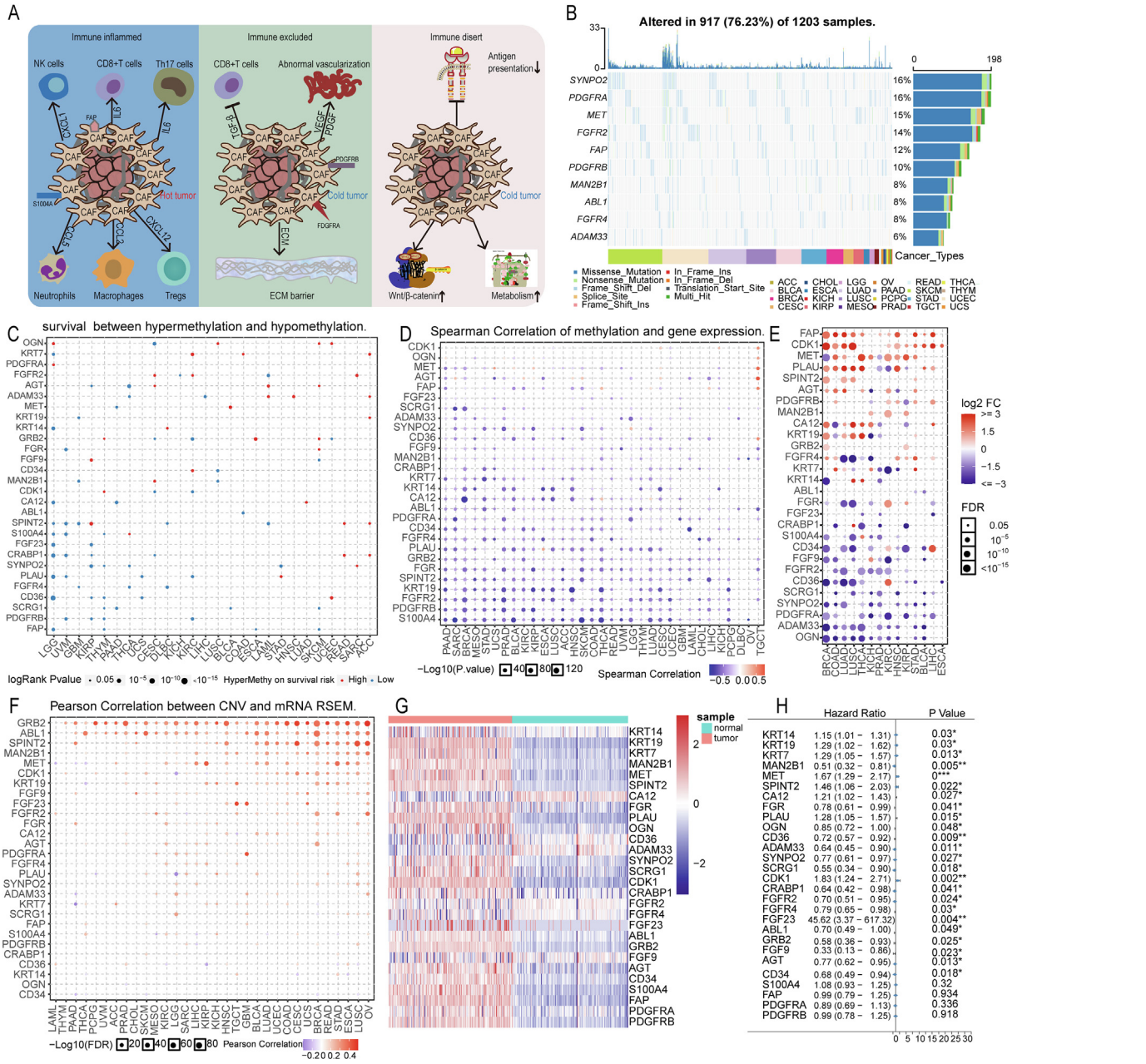

Fig. 1. Genetic and transcriptome alterations of CAF-associated molecules across cancers. (A) Crosstalk between CAFs and tumor cells in different tumor immune microenvironments. (B) Waterfall diagram exhibiting the mutation landscape of the 10 CAF-associated molecules with the highest mutation frequencies. (C) Bubble plot showing the effects of methylation level on survival. (D) Correlation analysis between methylation level and expression level for CAF-associated molecules. (E) Bubble plot showing the relative expression of CAF-associated molecules in cancer and normal tissues. (F) Bubble plot showing the correlation between CNV and expression level. (G) Heatmap showing the expression levels in cancer and normal tissues. (H) Univariate Cox analysis shows the prognostic value of CAF-associated molecules in pancreatic cancer (*P < 0.05, **P < 0.01, ***P < 0.001).

we found that only CA12, CD36, ADAM33, and FGFR2 were down-regulated in pancreatic cancer, while the other molecules were upregulated (Fig. S3A). In addition, we analyzed the expression differences of CAF-associated molecules between different T, N and M stages. FGFR4, S100A4, PDGFRA, and PDGFRB exhibited expression differences among samples with various metastatic statuses (Figs. S3B, S3C, S3D). To comprehensively analyze the underlying mechanisms of CAF regulators in pancreatic cancer and avoid the limitations of a single platform, we combined the expression data of 476 pancreatic cancer samples from five TCGA and GEO datasets and used the COMBAT package to exclude batch effects. Principal component analysis (PCA) graphs were used to visualize the gene expression data of the five datasets before and after removing the batch effects, and the results indicated the effective elimination of batch effects (Fig. 2A, B). Two distinct phenotypes were identified by the consensus clustering algorithm with the smallest area under the fitted curve and the best clustering effect (Fig. S4A, S4B). We further used PCA to assess the relative distribution of the two phenotypes, which could be perfectly differentiated based on the expression patterns of 28 CAF-associated molecules (Fig. 2C). A thermogram was generated to visualize the expression of these CAF-associated molecules: CDK1, SPINT2, MET, KRT19, KRT7, KRT14, PLAU and S100A4 exhibited elevated expression in phenotype 2, while the other CAF-associated molecules were

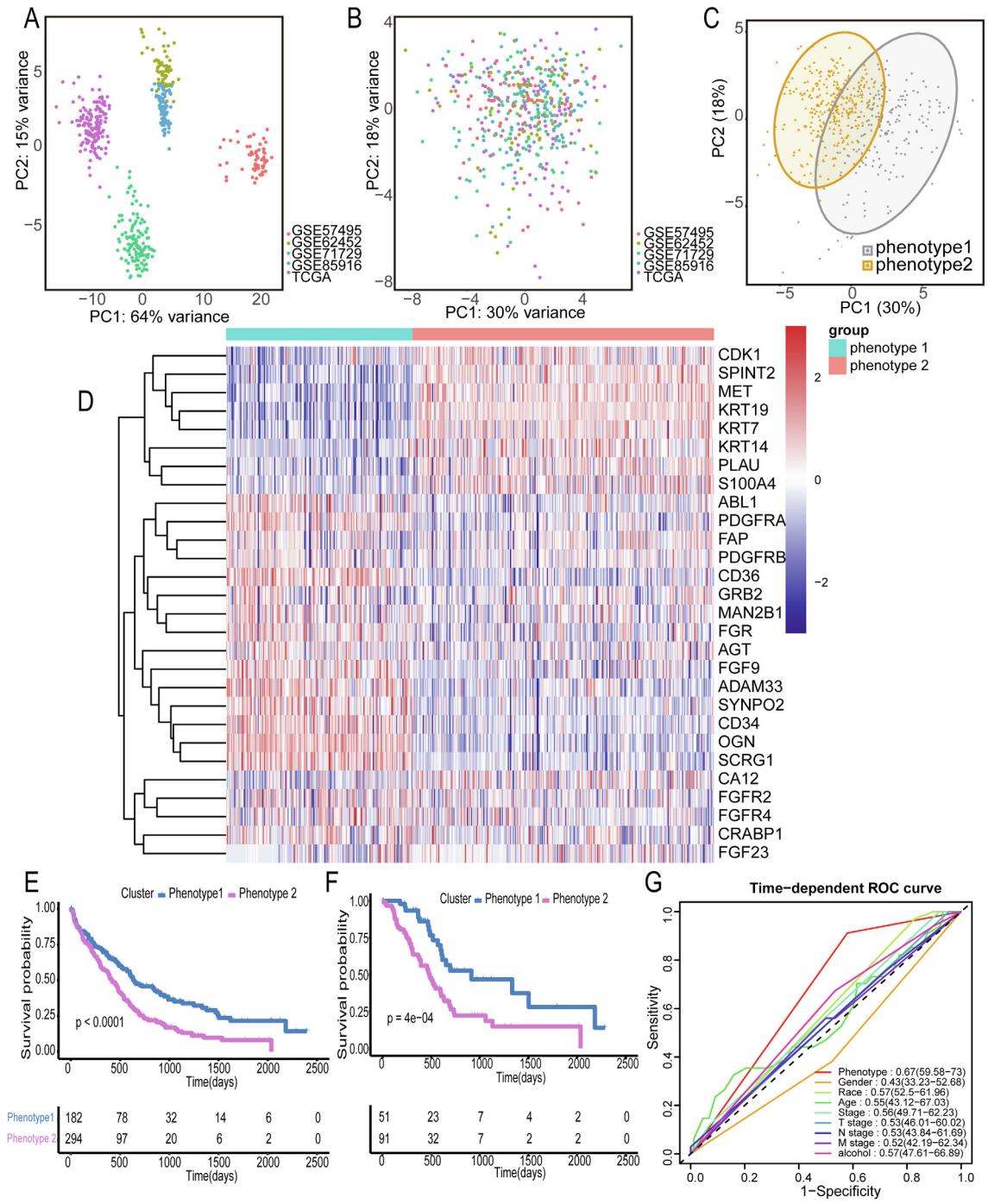

Fig. 2. Identification of two CAF phenotypes by unsupervised clustering. (A) The PCA graph shows the gene expression of the five datasets before removing the batch effect. (B) The PCA graph shows the gene expression of the five datasets after removing the batch effect. (C) PCA plot showing the relative distribution of the two phenotypes. (D) A thermogram showing the expression mode of these CAF-associated molecules in different phenotypes. (E) Survival differences between the two phenotypes in the combined dataset. (F) Survival differences between the two phenotypes in the TCGA cohort. (G) Multivariate Cox regression analysis of our phenotype with other clinical features.

downregulated (Fig. 2D). Three well-established subtypes of pancreatic cancer were compared to our phenotypes, which were identified as unique subtypes different from those of any classification method. Regarding Bailey subtypes, most of the squamous- and progenitor-subtype samples were concentrated in phenotype 2, and phenotype 1 had more immunogenic-subtype samples. Regarding Collison subtypes, phenotype 2 had more classical-subtype samples. Regarding Moffitt subtypes, phenotype 2 included more basic-subtype samples, and phenotype 1 had more classical-subtype samples (Fig.S4C). We compared survival between patients with the two phenotypes in the combined dataset and found that patients with phenotype 1 had a better prognosis (Fig. 2E). In the TCGA cohort, patients with phenotype 1 also exhibited a longer survival time (Fig. 2F). To further verify the reliability of the conclusion, we performed phenotype classification of 48 samples from our own cohort from Fudan University Shanghai Cancer Center (FUSCC) based on the expression levels of these 28 CAF-associated molecules. The results indicated that the cohort was divided into two phenotypes (with the best clustering effect) (Fig. S5A–S5D), and the phenotypes were significantly correlated

with the survival outcome (p = 0.0162) (Fig. S5E). Multivariate Cox regression analysis was used to compare our phenotypes with other clinical features and indicated that our phenotypes had better predictive performance and could be independent prognostic factors for pancreatic cancer (Fig. 2G).

3.3. Differences in inflammatory and immune microenvironment characteristics between the two CAF-related phenotypes

We explored the influence of CAFs on inflammatory factors and the immune microenvironment in pancreatic cancer. Chemokines and receptors were upregulated in phenotype 1; these included CXCL12, CCL5, CCL2, and CXCR4, which may recruit immunocompetent cells such as natural killer (NK) cells and CD8+ T cells. Interleukins, receptors, and other cytokines, such as PDGFRA and TGFB3, which may provide critical regulatory effects on inflammation and immune activity, also exhibited higher expression in phenotype 1 (Fig. 3A). In addition, some classical immune checkpoints, including CD274, CTLA4, and LAG3, were differentially expressed between the two phenotypes (Fig. S6A). Seven algorithms (ssGSEA, TIMER, quantiseq, XCELL, MCPcounter, EPIC, and CIBERSORT) were utilized to assess the immune microenvironment in pancreatic cancer. Our results indicated that tumor-killing immune cells, such as CD4+ T cells, CD8+ T cells, NK T cells, cytotoxic lymphocytes, and dendritic cells, were mainly distributed in phenotype 1. Interestingly, the levels of some immunosuppressive immune cells, including Tregs, M0 macrophages, and M2 macrophages, were increased in phenotype 2 (Fig. 3B). We used the GSVA algorithm to assess immune response processes in samples with different phenotypes and found that samples of phenotype 1 showed increased activity of immune response processes, including antigen presentation and tumor cell killing (Fig. 3B).

We further assessed the stromal and immune scores of the two phenotypes through the estimate algorithm. Phenotype 1 showed a higher stromal score, immune score and estimate score and lower tumor purity, in line with the abundant immune infiltration in this phenotype (Fig. S6B). The immunohistochemical images of samples from different phenotypes were selected to assess immune infiltration at the histological level, and it was shown that phenotype 1 had significantly more abundant immune cell infiltration (Fig. 3C). Some immune-related signatures were also assessed. Tumor progression-related signatures, such as the EMT1, DNA damage repair, and cell cycle signatures, were upregulated in samples of phenotype 2, while immune activity-related process signatures, such as the TME gene B, TME score, immune checkpoint, and CD8 T effector signatures, were enriched in samples of phenotype 1 (Fig. 3D).

3.4. Differences in carcinogenic signals and the metabolic microenvironment between the CAF-related phenotypes

We further focused on the differences in carcinogenic signaling pathways and biological processes between the two phenotypes. Pancancer analysis of signaling pathways suggested activation of the epithelial–mesenchymal transition (EMT), apoptosis, and receptor tyrosine kinase (RTK) pathways but inhibition of the DNA damage repair, estrogen receptor (ER) hormone and cell cycle pathways (Fig. 4A). In pancreatic cancer, EMT, the cell cycle and the ER hormone pathway were activated, and the PI3K/AKT and TSC/mTOR pathways were inhibited (Fig. 4B). We also assessed 10 classical tumor-related signaling pathways and analyzed their differences between the two phenotypes. The Wnt, PI3K, MYC, and cell cycle signaling pathways were clearly more activated in phenotype 2, and the TP53 and TGF-β signaling pathways were enriched in phenotype 1 (Fig. 4C).

We obtained 1757 differentially expressed genes between the two phenotypes and performed pathway enrichment analysis. GO annotation indicated that some metabolism-related terms, including “glucose metabolic process”, “retinoid metabolic process”, and “response to fatty acid”, were markedly enriched. In addition, several immune-related and cancer-related terms were also significantly enriched; these included “fibroblast proliferation”, “regulation of angiogenesis” and “leukocyte chemotaxis” (Fig. 4D). The KEGG analysis identified the enrichment of many typical carcinogenic pathways and metabolic regulatory pathways, including the “PI3K-Akt signaling pathway”, “MAPK signaling pathway”, “Wnt signaling pathway”, and “central carbon metabolism in cancer” (Fig. 4E).

We further assessed 724 metabolism-related pathways from the KEGG database with GSVA and compared their differences between the two phenotypes. >180 metabolism-related pathways were altered, and a heatmap was used to visualize the significant pathways (Fig. 4F). The altered metabolic processes included common metabolic processes such as glucose metabolism, lipid metabolism, protein metabolism, and nucleic acid metabolism, indicating that alterations in these metabolic processes may be important factors leading to poor prognosis in pancreatic cancer. The GSVA results were consistent with the aforementioned results that the lipid catabolic response and immune cell activation were significantly enriched in phenotype 1 (Fig. 4G).

3.5. Clinical application value of the CAF score

Previous experiments identified the immune microenvironment and metabolic and prognostic characteristics of our two phenotypes, and we further constructed a CAF scoring model to facilitate clinical application. Based on specific CAF-associated molecules, we performed univariate, random forest, LASSO regression, and multivariate cox analyses. Three CAF-associated molecules, FGFR4, SYNPO2, and MET, were incorporated into the final model, and their expression and correlation coefficients were used to determine the CAF score. We compared the CAF score between the two phenotypes and found that phenotype 2 showed a higher CAF score (Fig. 5A). Additionally, we found that the CAF score could accurately determine the prognosis of patients with pancreatic cancer. Groups with lower CAF scores tended to have longer survival and better prognosis in both the combined dataset and the TCGA cohort (Fig. 5B, 5C). Subtype analysis indicated that the high CAF score group was mainly concentrated in the phenotype 2 cluster and contained more basal-subtype samples, and the low CAF score group mainly included classic-subtype samples (Fig. 5D). Correlation analysis indicated that the CAF score was negatively correlated with the abundance of most immune cells, including antitumor CD8+ T cells and NK cells, while it was positively related to the abundance of Tregs, M0 macrophages and M2 macrophages (which are believed to be immunosuppressive) (Fig. 5E). Moreover, the CAF score was positively correlated with most tumorigenesis-related signatures, such as the EMT, cell cycle, and DNA damage repair signatures, but negatively correlated with immune-related signatures, such as the TME, CD8 effector, and immune checkpoint signatures (Fig. 5F). Analysis of TMB and microsatellite instability (MSI) suggested that TMB and MSI were higher in the high CAF score group (Fig. 5G, H). Additionally, the high CAF score group exhibited a higher mutation rate of typical genes than the low CAF score group (Fig.S7). In addition, the CAF score was generally negatively correlated with the IC50 value of most chemotherapy drugs (derived from the Cancer Genome Project (CGP)) and positively correlated with the IC50 value of a few drugs, including nilotinib and vorinostat (Fig. 5I).

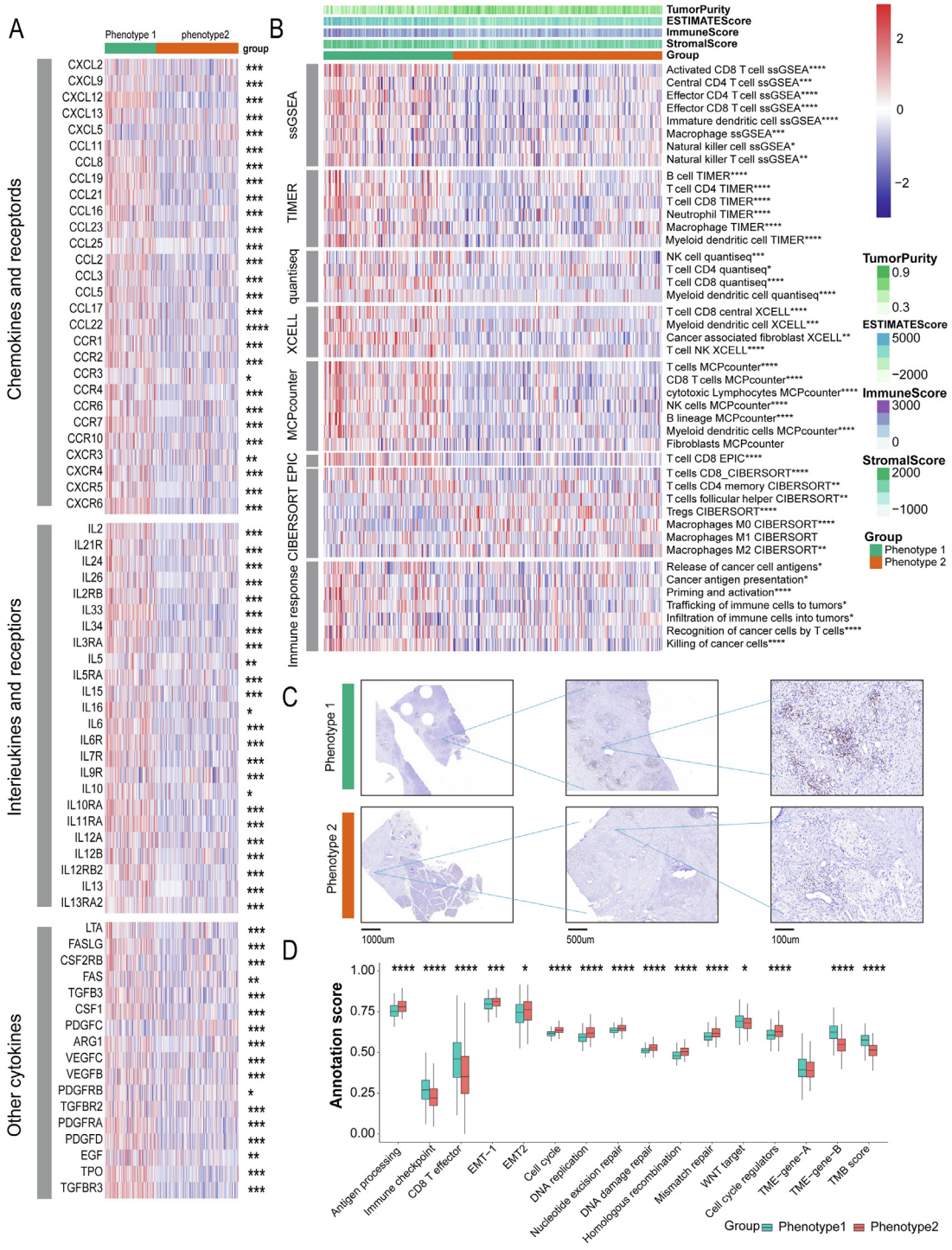

Fig. 3. Differences in inflammatory and immune microenvironment characteristics between the two CAF phenotypes. (A) The heatmap shows the alterations of chemokines and interleukins in different CAF phenotypes (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). (B) The heatmap shows differences in the abundance of infiltrating immune cells and the enrichment of immune response processes between the two CAF phenotypes (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). (C) The immunohistochemical images from FUSCC showing the immune infiltration landscapes of the two CAF-related phenotypes. (D) Variations in the TME-related signature between different CAF phenotypes (*P < 0.05, ***P < 0.001, ****P < 0.0001).

3.6. The CAF score predicts the sensitivity of pancreatic cancer patients to chemotherapy and targeted therapy

We further explored the interaction between CAF-associated molecules and chemotherapeutics in the Genomics of Drug Sensitivity in Cancer (GDSC) database. A Spearman correlation analysis was performed, with negative correlations representing synergy and positive correlations implying antagonism. The results indicated that most CAF-associated molecules had synergistic relationships with drugs, especially KRT14, PLAU, and SPINT2, which were identified as having possible synergistic relationships with gefitinib (EGFR inhibitor), afatinib (multikinase inhibitor), and erlotinib (EGFR inhibitor), respectively. Additionally, PLAU was identified as having a possible antagonistic relationship with

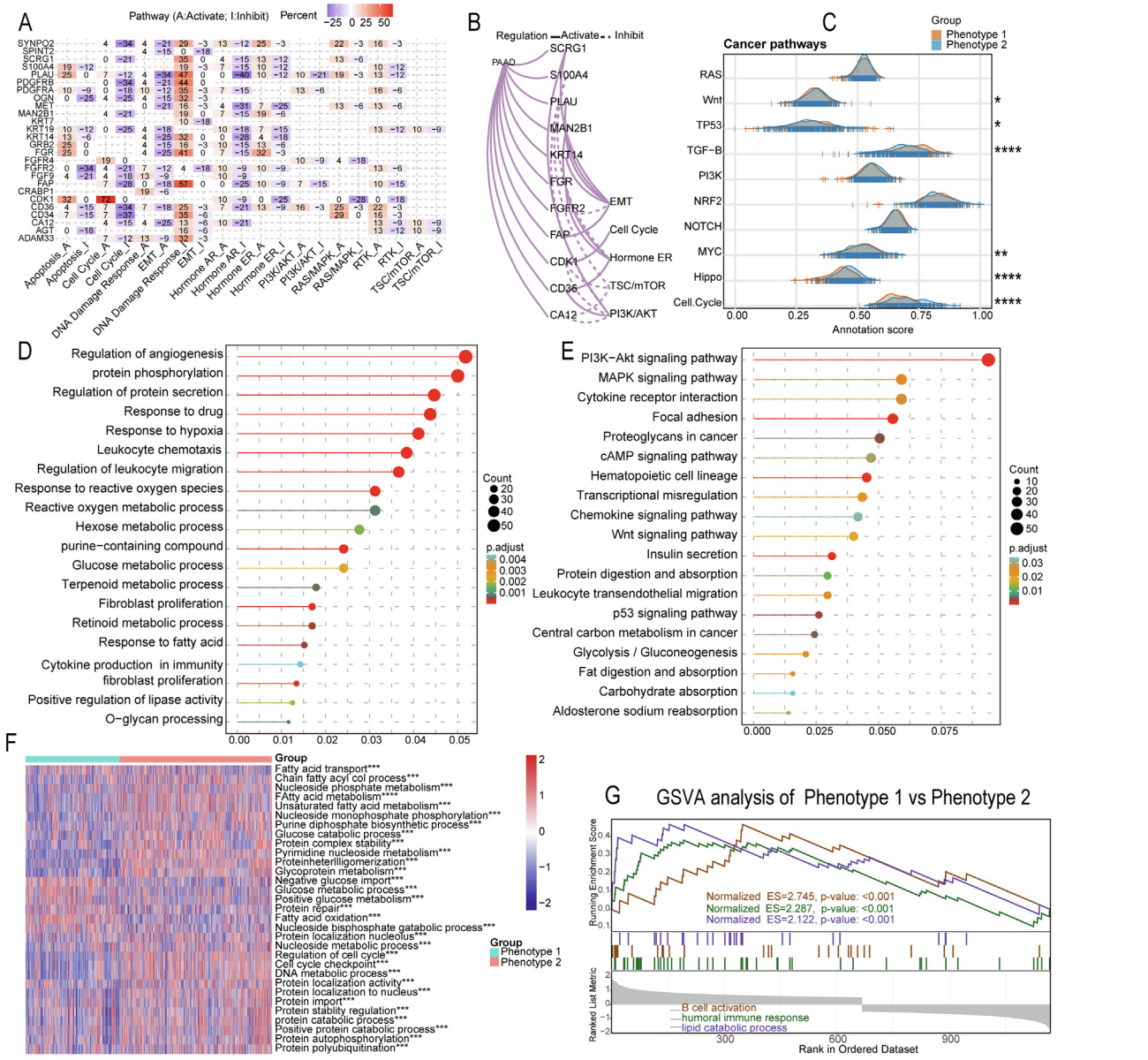

Fig. 4. Differences in carcinogenic signals and the metabolic microenvironment between CAF phenotypes. (A) Pancancer analysis revealed the effects of CAF-associated molecules on carcinogenic pathways. The percentage of cancer types in which a CAF molecule affects the pathway, and CAF-associated molecules affecting more than five cancer types are shown. (B) The correlation between CAF-associated molecules and significant cancer signaling pathways in pancreatic cancer. (C) The mountain plot shows the differences in 10 classical cancer pathway scores between the two CAF phenotypes (*P < 0.05, **P < 0.01, ****P < 0.0001). (D) Gene Ontology annotation of the differentially expressed genes between the two phenotypes. (E) KEGG analysis of the differentially expressed genes between the two phenotypes. (F) Metabolic reprogramming in the two CAF phenotypes (***P < 0.001, ****P < 0.0001). (G) GSEA of the biological pathways involved in the two CAF phenotypes.

YM201636 (PIKfyve inhibitor) and I-BET-762 (acetylated histone peptide inhibitor); CDK1 was identified to potentially inhibit RDEA119 (MEK inhibitor) and selumetinib (MEK inhibitor) (Fig. S8).

The aforementioned results indicated the influence of CAF-associated molecules on chemotherapeutic sensitivity, and we further explored the ability of the CAF score to distinguish sensitivity. We utilized the prrophetic and oncopredict algorithms to calculate the IC50 values for nearly a thousand chemotherapeutic drugs from the CGP, GDSC and Cancer Therapeutics Response Portal (CTRP) databases and compared the differences in IC50 values between the two CAF score groups. The results showed that both common chemotherapeutic drugs and targeted drugs showed substantial differences between the high and low CAF score groups. In the CGP database, the IC50 values of camptothecin, lenalidomide, vorinostat and nilotinib were lower in the low CAF score group, indicating that patients in this group may be more sensitive to these drugs (Fig. S9A, S9B). In the CTRP database, the IC50 values of azacitidine, tamoxifen, myricetin and etoposide in the high CAF score group were higher than those in the low CAF score group, suggesting that high CAF score samples may be insensitive to these drugs (Fig. S9C, S9D). In the GDSC database, the IC50 values of most drugs, including cisplatin, gemcitabine, oxaliplatin and nilotinib, were lower in the low CAF score group (Fig. S9E, S9F), further showing the correlation between the CAF score and chemotherapeutic drug sensitivity.

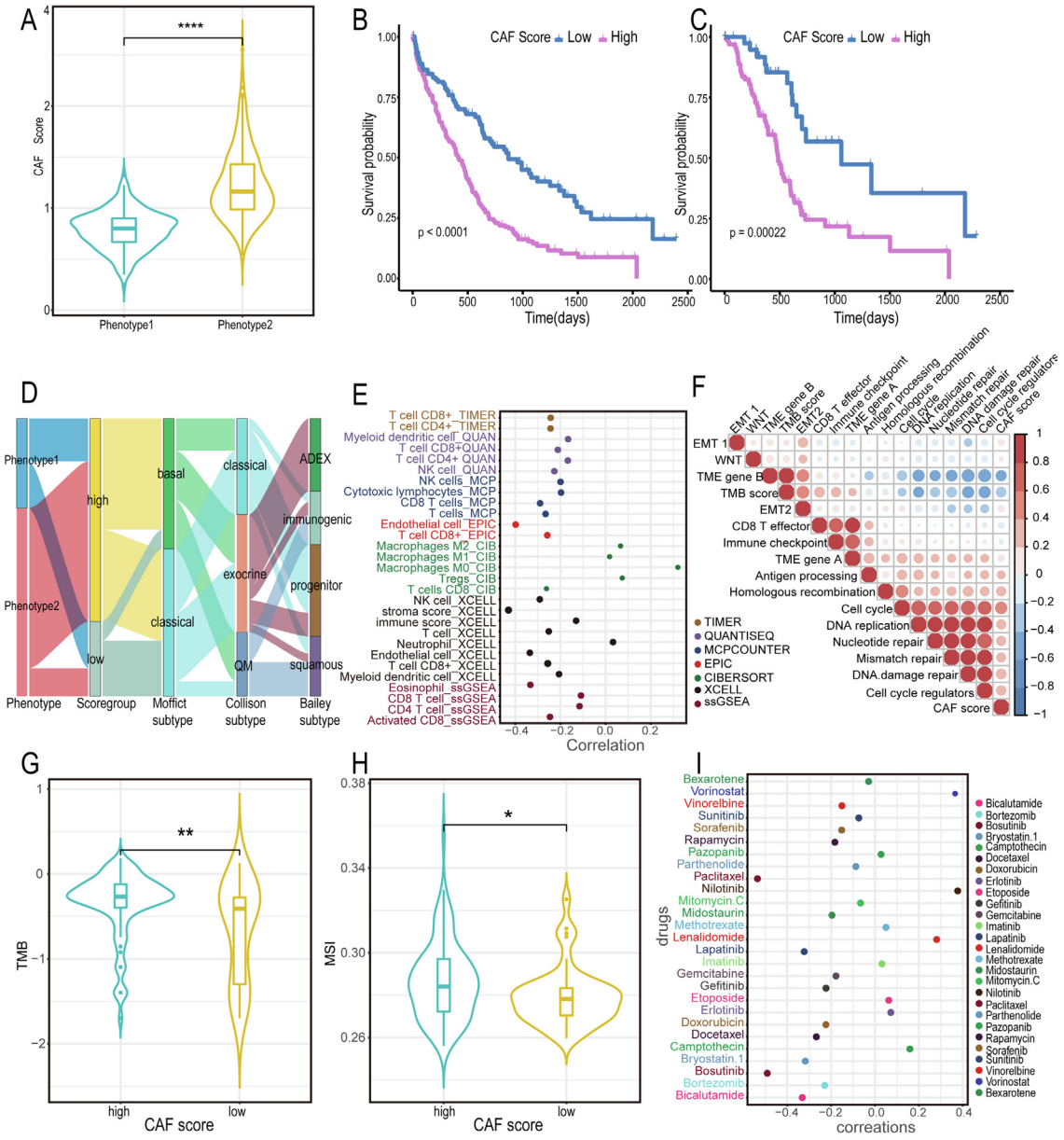

Fig. 5. Clinical application value of the CAF score. (A) The difference in CAF scores between the two phenotypes (****P < 0.0001). (B) The Kaplan–Meier curve shows a survival difference between patients with high and low CAF scores in the combined dataset. (C) The Kaplan–Meier curve shows a survival difference between patients with high and low CAF scores in the TCGA dataset. (D) The Sankey diagram shows the correlations among CAF score group, CAF phenotype and pancreatic cancer subtype. (E) The bubble plot shows the correlation between the CAF score and immune cell infiltration. (F) The correlation heatmap shows the correlation between the CAF score and immune-related signatures. (G) The difference in TMB between the high and low CAF score groups (**P < 0.01). (H) The difference in microsatellite instability between the high and low CAF score groups (*P < 0.05). (I) Correlation between CAF score and IC50 of chemotherapy drugs (from Cancer Genome Project).

3.7. The CAF score has potential for predicting the response to immunotherapy

Immune checkpoint inhibitor therapy targeting PD-1 and PD-L1 has become a hot topic in immunotherapy research. We first compared the differences in immune checkpoint expression between the high and low CAF score groups and found that most immune checkpoints were upregulated in the low CAF score group, including PDCD1, LAG3, and TMIGD2 (Fig. 6A, Fig. S10A, S10B). Furthermore, we used several immunotherapy cohorts, including the IMvigor210 cohort (anti-PD-L1 therapy), GSE78220 (anti-PD-1 therapy) and GSE35640 (anti-MAGE-A3), to validate the ability of the CAF score to predict immunotherapy response. Samples in these cohorts were divided into high and low groups according to CAF score. In the IMvigor210 cohort, patients with higher CAF scores exhibited poorer prognosis and appeared to benefit less from PDLI blockade therapy (Fig. 6B). In addition, patients' response to immunotherapy was related to CAF score; patients with progressive disease (PD) and partial response (PR) showed higher CAF scores than those with complete response (CR) (Fig. 6C). The high CAF score group exhibited higher PD and stable disease (SD) rates than the low CAF score group (Fig. 6D). Groups with low tumor cell levels also showed lower CAF scores (Fig. 6E). In addition, we assessed the CAF score across different immune phenotypes and groups with various overall responses (Fig. S10C, S10D). TMB has been found to be significantly related

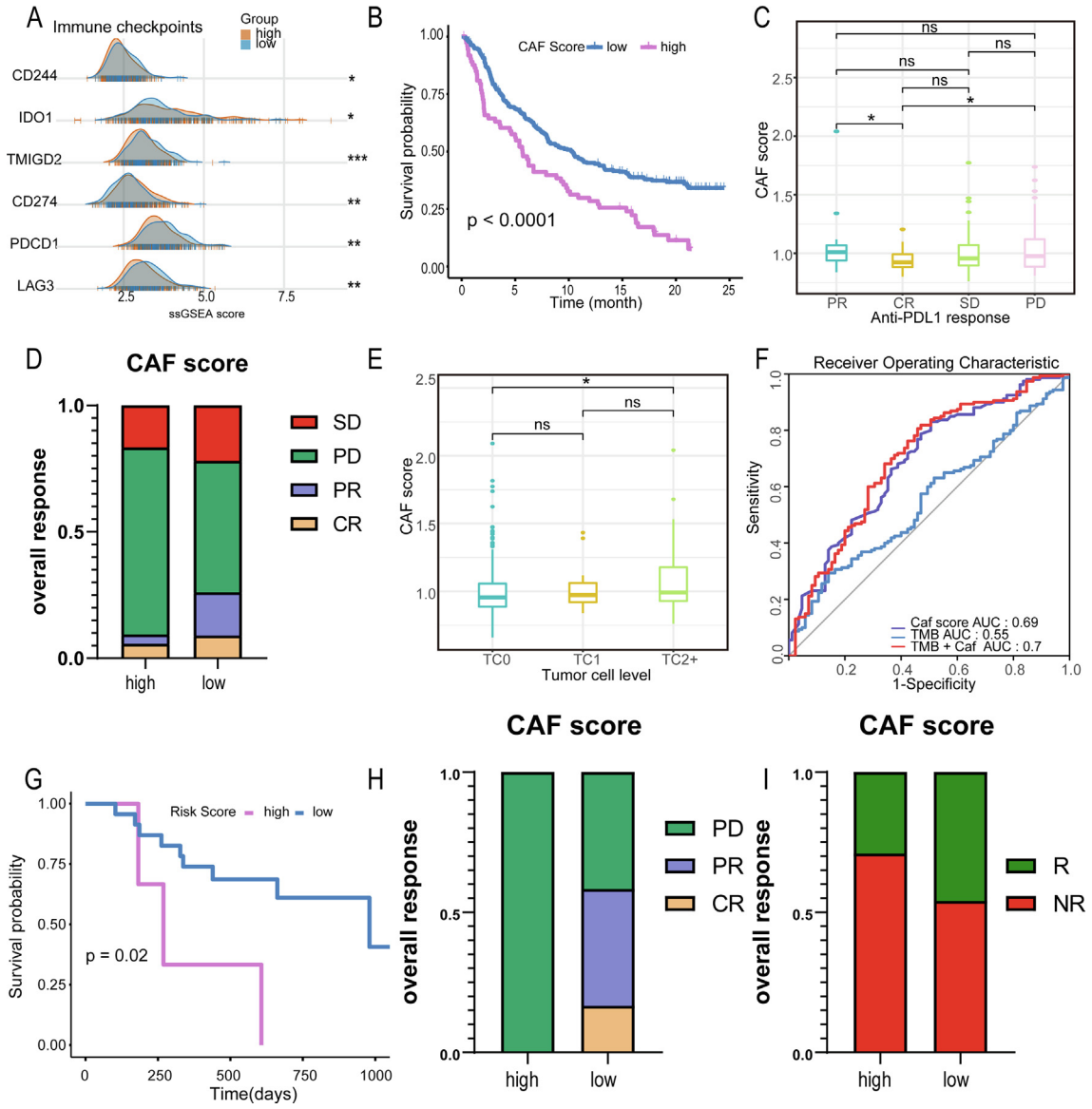

Fig. 6. The CAF score predicts the sensitivity of pancreatic cancer patients to chemotherapy and targeted therapy. (A) Comparison of differences in immune checkpoint expression between the high and low CAF score groups (*P < 0.05, **P < 0.01, ***P < 0.001). (B) The survival curve shows a significant difference in prognosis between the high and low CAF score groups after anti-PD-L1 therapy in the IMvigor210 cohort. (C) Differences in CAF scores in groups with various anti-PD-L1 responses (*P < 0.05, ns P > 0.05). (D) The cumulative histogram shows the difference in the anti-PD-L1 response between the high and low CAF score groups. (E) Differences in CAF scores in groups with various tumor cell levels (*P < 0.05, ns P > 0.05). (F) The ROC curve shows the predictive value of the CAF score in the IMvigor210 cohort. (G) The survival curve shows a significant difference in prognosis between the high and low CAF score groups after anti-PD-1 therapy in GSE78220. (H) The cumulative histogram shows the difference in the anti-PD-1 response between the high and low CAF score groups in GSE78220. (I) The cumulative histogram shows the difference in the MAGE-A3 response between the high and low CAF score groups in GSE35640.

to immunotherapy efficacy [18]. We compared the sensitivity of TMB and CAF score separately and in combination in predicting the response to immunotherapy. The results suggested that the CAF score (AUC = 0.69) was more sensitive than TMB (AUC = 0.55) in predicting immunotherapy outcome, and the CAF score combined with TMB had significantly improved accuracy compared with TMB alone (Fig. 6F). In the GSE78220 cohort, we explored the ability of the CAF score to predict the anti-PD-1 immunotherapy response. Patients with lower CAF scores exhibited better prognoses and appeared to benefit more from PD-1 blockade therapy (Figs. 6G, S10E). In addition, all patients in the high CAF score group had PD, while some patients in the low CAF score group exhibited CR and PR (Fig. 6H); the TMB AUC was 0.6 (Fig.S10F). In the GSE35640 cohort, the low CAF score group exhibited more responders (R) than the high CAF score group. Interestingly, no difference was found in the CAF score between the responder and nonresponder (NR) groups (Fig.S10G); the TMB AUC was 0.6 (Fig.S10H).

3.8. FGFR4 regulates crosstalk between pancreatic cancer cells and CAFs and is associated with immunity

In our article, FGFR4 was identified as a CAF-related molecule and was the key gene used to construct the CAF score. FGFR4 has been reported to play a role as an oncogene in the development of various tumors [19,20]. In addition, some scientists have reported that FGFR4 mediates the interaction between CAFs and colon cancer cells and promotes colon cancer progression [21].

Hence, in addition to verifying the oncogenic role of FGFR4 in pancreatic cancer, we wanted to explore the potential role of FGFR4 in the interaction between pancreatic cancer cells and CAFs. In pancreatic cancer, FGFR4 is mainly expressed in pancreatic duct cells and endocrine gland cells but rarely in CAFs according to the TISCH database [22]. Hence, we mainly applied different treatments to PANC-1 and MiaPaCa-2 cells to explore these potential regulatory mechanisms.

PANC-1 and MiaPaCa-2 cells were subjected to several treatment conditions: no treatment, coculture with CAF supernatant, FGFR4 gene silencing, and FGFR4 gene silencing combined with CAF supernatant coculture (the coculture method is shown in Fig. 7A). The results suggested that silencing FGFR4 promoted PANC-1 and MiaPaCa-2 cell apoptosis (Fig. 7B, C) and inhibited cell migration (Fig. 7D, E) and proliferation (Fig. 7F, G). Additionally, CAFs enhanced the malignant phenotype of PANC-1 and

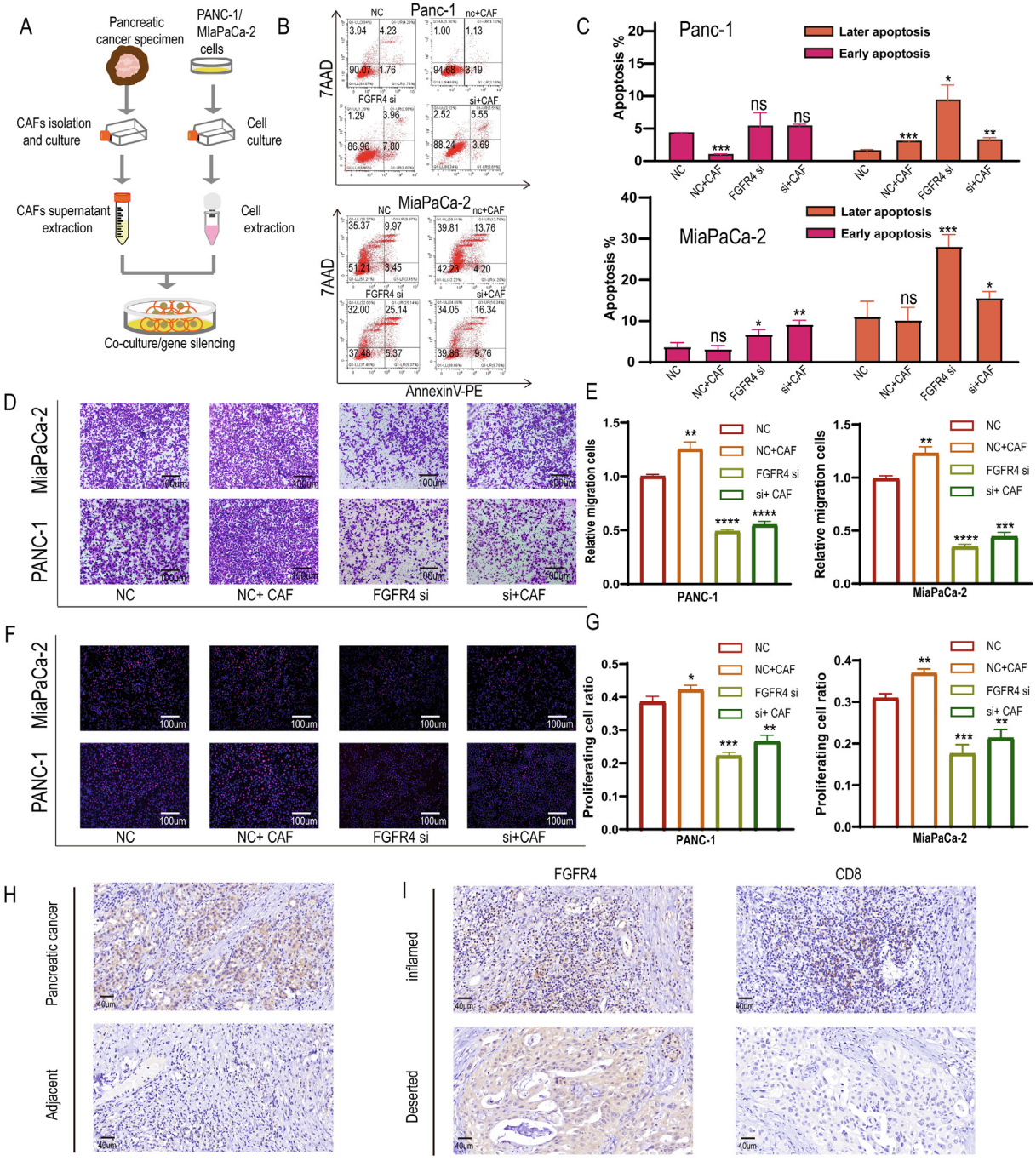

Fig. 7. FGFR4 is associated with immunity and regulates crosstalk between pancreatic cancer cells and CAFs. (A) Flowchart for the coculture protocol for PANC-1/MiaPaCa-2 cells and cancer-associated fibroblasts. (B) Flow cytometry analysis showing the apoptosis rate of PANC-1 and MiaPaCa-2 cells in different treatment groups. (C) Comparison of the apoptosis rates of PANC-1 and MiaPaCa-2 cells in different treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001, ns P > 0.05). (D) Transwell experiments demonstrated the migration ability of different PANC-1 and MiaPaCa-2 cell treatment groups. (E) Comparison of the migration ability of PANC-1 and MiaPaCa-2 cells in different treatment groups (**P < 0.01, ***P < 0.001, ****P < 0.0001). (F) EdU experiments demonstrated the proliferation ability of different PANC-1 and MiaPaCa-2 treatment groups. (G) Comparison of the proliferation ability of PANC-1 and MiaPaCa-2 cells in different treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001). (H) Immunohistochemical staining was used to detect the expression of FGFR4 in pancreatic cancer and adjacent tissues. (I) Immunohistochemical staining was used to detect the coexpression of FGFR4 and CD8 in pancreatic cancer.

MiaPaCa-2 cells to a certain extent (Fig. 7B–G). However, in FGFR4 knockdown PANC-1 and MiaPaCa-2 cells, the malignant phenotype-promoting effect of CAFs was not obvious (Fig. 7B–G). We further explored the potential role of FGFR4 in the immune microenvironment of pancreatic cancer. FGFR4 staining was significantly higher in pancreatic cancer tissues than in adjacent tissues (Fig. 7H). The tissue samples were divided into categories based on immune status (immune-inflamed and immune-desert types) according to CD8 histochemical staining; we further performed FGFR4 immunohistochemical staining on serial sections from the same patients to observe the colocalization of FGFR4 and CD8. The results showed that specimens with strong FGFR4 staining tended to be immune-desert samples, while those with weak staining tended to be immune-inflamed samples (Fig. 7I). Western blotting was used to validate our results. We examined the alterations in EMT markers in PANC-1 and MiaPaCa-2 cells after different treatments. ZO-1 expression was significantly downregulated in the FGFR4 silencing group and the silencing combined with coculture group. *N*-cadherin was also slightly decreased, suggesting that the changes in these two proteins may account for the inhibition of PANC-1 and MiaPaCa-2 cell migration (Fig. S11).

4. Discussion

Increasing evidence has illuminated the relationship between CAFs and multiple factors of malignant tumors, including interstitial heterogeneity and angiogenesis [8,9]. In addition, as an important component of the immune microenvironment, CAFs have been found to regulate the evolution and recruitment of various immune cells and may play an important role in immunosuppressive tumors [23]. In particular, Richards et al found that CAF exosomes significantly affect the proliferation of pancreatic cancer cells and the prognosis of pancreatic cancer [24]. A single-cell sequencing study showed that CAFs expressing CD74 and MHC II molecules regulated immune responses and antigen presentation in pancreatic cancer, indicating their indispensable role in carcinogenesis [25]. However, high-throughput analysis of CAFs is lacking, especially in pancreatic cancer, and high-throughput bioinformatics analysis of CAFs may identify new intrinsic mechanisms and provide insights. In this research, we first identified variations in the genomic characteristics of 28 CAF-associated molecules across cancers and further identified two phenotypes with completely different immune and metabolic features. We developed a prognostic scoring system and analyzed its relationship with various factors, including prognosis, the immune microenvironment, and chemotherapy and immunotherapy sensitivity.

CAFs can target different downstream effectors to exert tumorigenic or immunosuppressive effects, and different CAF-related phenotypes induce different effects to shape the tumor microenvironment [26]. Therefore, distinguishing different CAF-associated phenotypes in pancreatic cancer may have significance in predicting prognosis and therapy outcome. Based on the expression data of 28 CAF-associated molecules in 476 pancreatic cancer patient samples from different databases, we successfully identified two phenotypes with significantly different expression patterns of CAF-associated molecules. Patients with phenotype 1 had an obviously better prognosis than those with phenotype 2, and tumors of phenotype 1 exhibited enriched inflammatory factors and immune cell infiltration, especially infiltration of antitumor immune cells. EMT, DNA damage repair and other tumor-promoting signatures were higher in phenotype 2 tumors. In summary, phenotype 1 is closely related to the immune and inflammatory microenvironment, and patients with phenotype 1 tumors may be more sensitive to immunotherapy.

Focusing on variations in biological pathways and related molecules between different phenotypes may help identify downstream targets. Our results indicated that CAF-associated genes were correlated with EMT, cell cycle, and PI3K-AKT pathways [27–29], which are common pathways associated with tumorigenesis. We further analyzed the differences in 10 canonical pathways between the two phenotypes, and 6/10 pathways were significantly altered. In addition, we found that CAFs displayed close relationships with metabolic patterns. Metabolic processes, including glucose metabolism, fatty acid metabolism, protein metabolism, and nucleic acid metabolism, were significantly distinct between the two phenotypes. Furthermore, immune- and drug-resistance-related terms, such as drug response, fibroblast proliferation, lymphocyte migration, and B-cell activation, were also noticeably different between the two phenotypes. These findings suggest that the identification of CAF-related phenotypes may provide new insights into strategies targeting immune and metabolism-related factors in pancreatic cancer. Combining CAF-targeting strategies with inhibitors of metabolism or immune-related pathways may be a new treatment direction for pancreatic cancer.

The immune microenvironment has become a hot topic in tumorigenesis and treatment mechanism research in recent years, especially in pancreatic cancer [30]. As the main component of the pancreatic cancer stroma, CAFs play a pivotal role in the regulation of the microenvironment [31]. We constructed a refined scoring system based on CAF-related molecules to facilitate the evaluation of individual patient prognosis and various indicators. This CAF score accurately predicted patient prognosis, infiltrating immune cell abundance, TMB and MSI. We further applied the CAF score to predict the effect of immunotherapy. Patients with high CAF scores generally showed lower expression levels of immune checkpoints, and patients with low scores showed better prognosis and a better remission rate after immunotherapy. These results implied that the CAF score can accurately predict the response to antitumor immune therapy. Surprisingly, the CAF score optimally discriminated the IC50 values of multiple drugs according to analyses using three different chemotherapeutic drug databases, suggesting that this score may also be informative for chemotherapeutic drug use.

We focused on one CAF-associated regulator, FGFR4, which has been reported to be overexpressed in pancreatic cancer cells [32]. A recent study found that FGFR4 inhibitors promote the PD-1 immunotherapy response in hepatocellular carcinoma [33]. However, the underlying mechanisms of FGFR4 in the tumorigenesis of pancreatic cancer and its interaction with CAFs have not been determined. Our results suggest that knockdown of FGFR4 in pancreatic cancer inhibits the proliferation and migration ability of PANC-1 and MiaPaCa-2 cells and that CAFs are partly dependent on FGFR4 to exert their cancer-promoting effects. In addition, the results of immunohistochemical staining suggested that the expression of FGFR4 was correlated with immune infiltration, and patients with high expression of FGFR4 tended to have lower levels of immune infiltration, which may indicate poorer immunotherapy effects. In summary, we found that FGFR4 is a potential therapeutic target in pancreatic cancer and a potent marker predicting the efficacy of immunotherapy. Further experiments may be needed to verify our conclusions.

We recognize some shortcomings of our research. First, our results are based on sequencing data from previous samples, not from prospective studies. On the other hand, the data we analyzed were generated on multiple different platforms, and although we removed batch effects, this difference may still have influenced the results. In addition, the mechanism of FGFR4 in pancreatic cancer carcinogenesis is still relatively unclear, and we hope to explore the in-depth mechanism.

5. Conclusion

In conclusion, our research illuminates remarkable variation in CAF-associated molecules in multiple cancer types at the genetic level and indicates that CAFs exhibit close crosstalk with tumor metabolic pathways and the immune microenvironment. The CAF score may be able to accurately predict patient immune microenvironment status and response to immunotherapy in the clinic application, providing a theoretical basis for individualized immunotherapy for pancreatic cancer.

6. Data availability statement

The datasets used in the present study are publicly available.

7. Consent for publication

Not applicable.

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9. Authors' contributions

SYL performed the bioinformatic analysis. JH and JX cultured the cancer cells and performed the functional experiments. MYW, CL, QCM and JL performed the statistical analyses. BZ and WW designed the study. SS and XJY revised 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.

Ethical approval

The Institutional Research Ethics Committee of Fudan University Shanghai Cancer Center approved this study, and written informed consent was obtained from all patients prior to the investigation. The ethics approval number was 050432-4-1212B. We confirmed that the experiments on human tissue samples were performed in accordance with relevant guidelines and regulations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.07.029.

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