Observational Study

Medicine

OPEN

APOL6 as a potential biomarker of immuno-correlation and therapeutic prediction in cancer immunotherapy

Jialin Yao, BMed[a], Wenyuan Gan, BMed[a], Jiukang Sun, BMed[a], Zhihang Han, BMed[a], Dongqing Li, BMed[a], Lei Cao, MMed[a,b], Lingjun Zhu, MD[a,*]

Abstract

The emergence of immune checkpoint inhibitors (ICIs) has significantly revolutionized the approach to treating advanced cancers. Despite their remarkable efficacy, not all patients exhibit favorable responses to ICI therapy. Hence, more biomarkers for therapeutic prediction need to be discovered. In this study, we utilized public cohorts to investigate the predictive significance and immunological associations of apolipoprotein L6 (APOL6) in cancers. The expression of APOL6 was found to be enhanced in tumors of patients who exhibited strong immunotherapeutic responses across various types of cancer. Furthermore, APOL6 showed immune correlations in pan-cancer and was confirmed by the tissue microarray cohort and in vitro experiments. Overall, this study highlights that APOL6 serves as a beneficial biomarker for immune checkpoint inhibitors in patients with cancer. Additional research involving larger numbers of patients and the underlying mechanism is necessary to determine its effectiveness as a biomarker for predicting the benefits of ICIs.

Abbreviations: APOL6 = apolipoprotein L6, BLCA = bladder urothelial carcinoma, CRT = calreticulin, HMGB1 = high mobility group box 1, IC = immune cells, ICIs = immune checkpoint inhibitors, IFITM3 = interferon induced transmembrane protein 3, IFN-g = interferon-gamma, OS = overall survival, PD-1 = programmed death protein 1, PD-L1 = programmed death-ligand 1, PFS = progression-free survival, TC = tumor cells, TCGA = The Cancer Genome Atlas, TILs = tumor-infiltrating lymphocytes, TIME = tumor immune microenvironment, TMA = tissue microarray, TMB = tumor mutation burden.

Keywords: APOL6, biomarker, cancer, immunotherapy, prognosis

1. Introduction

In the last several decades, the field of oncology has witnessed the pioneering approach of immunotherapy which has steered the therapeutic landscape towards a promising horizon, predominantly for the advanced stages of human cancer.[1] Tumor immunotherapy operates on the compelling premise of stimulating the patient's immune system to eliminate tumor cells (TC).[2] Immune checkpoints (ICIs) represent these predominant strategies, interrupting the interaction of ICIs and their receptors to exert efficacy.[3] The immunosuppressive ICI programmed death-ligand 1 (PD-L1) binding with the programmed cell death protein 1 (PD-1) receptor is the most important type, among which the former is expressed on TC, while the latter is located on immune cells (IC), notably contributing to initiating immune escape in TC.[4] Despite the consistent improvement in cancer patients' prognosis at advanced stages treated with immunotherapy, not every individual can obtain advantages from the treatment alternatives accessible.[5,6] It's broadly acknowledged that PD-L1 expression serves a pivotal role when evaluating patient response to anti-PD-1 or anti-PD-L1 immunotherapy. Conversely, many patients lacking PD-L1 expression seem to derive considerable benefits from immunotherapy.[7,8] This certainly sheds a new outlook of perspective and prompts that an urgent need in clinical practice for complementary biomarkers to predict immunotherapeutic responses.

Apolipoprotein L6 (APOL6) is a member of the lipid-binding L family proteins whose functions haven't been explored clearly.[9] As far as current research is concerned, APOL6 is a

JY, WG, and JS contributed equally to this work.

This study is supported in part by the National Natural Science Foundation of China (grant numbers 82273407), the Natural Science Foundation of Jiangsu Province (grant numbers BK20201495), and Jiangsu Province Capability Improvement Project through Science, Technology and Education (grant numbers CXZX202204).

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are publicly available.

This study involving tissue microarray slides obtained ethical approval (YB M-05-02) from the Clinical Research Ethics Committee, Outdo Biotech (Shanghai, China).

Supplemental Digital Content is available for this article.

[a] Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China, [b] Department of Oncology, The Affiliated Suqian First People's Hospital of Nanjing Medical University, Suqian, China.

** Correspondence: Lingjun Zhu, Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, China (e-mail: zhulingjun@njmu.edu.cn).*

How to cite this article: Yao J, Gan W, Sun J, Han Z, Li D, Cao L, Zhu L. APOL6 as a potential biomarker of immuno-correlation and therapeutic prediction in cancer immunotherapy. Medicine 2025;104:19(e42406).

Received: 23 April 2024 / Received in final form: 26 December 2024 / Accepted: 22 April 2025

http://dx.doi.org/10.1097/MD.0000000000042406

lipid droplet-associated protein primarily located in adipocytes. It directly interacts with the N-terminal domain of perilipin 1, disrupting the interaction between perilipin 1 and hormone-sensitive lipase, consequently impeding lipolysis in adipocytes. Elevated expression of APOL6 in adipose tissue may result in obesity and insulin resistance.[9] APOL6, located in the cytoplasm, is also recognized as a BH3-only protein of the BCL-2 family who serves significantly in the apoptosis process.[10] It has been reported that Apol6 is involved in the secretion of cytochrome c into the cytosol, thereby promoting apoptosis and restoring functional apoptosis in TC.[11] Based on basic experiments, APOL6 can enhance immunotherapy by inducing immunogenic cell death through apoptosis and necrosis in pancreatic cancer.[12] Therefore, further exploration is needed to investigate the specific function of APOL6 and its correlation with immune responses to tumor therapy.

Our research focused on investigating the immune correlations of APOL6 in multiple cancers. To achieve this, we analyzed some related public cohorts to study the predictive significance of APOL6 in immunotherapic responses and its associations with immunity. Moreover, we conducted a systematic examination of immunological connections of APOL6 in various cancers utilizing The Cancer Genome Atlas (TCGA) dataset, validated by a tissue microarray (TMA) cohort and in vitro experiments. Through this analysis, we highlight the meaningful role of APOL6 in facilitating antineoplastic immunity. Also, our identification of APOL6 as an innovative and potential biomarker holds profound implications for predicting immunotherapeutic responses in multiple cancers.

2. Materials and methods

2.1. Acquisition of public data and bioinformatics analysis

The Gene Expression Omnibus and the Tumor Immune Dysfunction and Exclusion databases are mainly used to explore the cohorts. The datasets chosen are GSE176307,[13] GSE194040,[14] GSE173839,[15] GSE100797,[16] and IMvigor210[17] datasets containing RNA-sequencing data with treatment of immunotherapy. Only patients who derived no kind of therapies were involved in these datasets. We explored the UCSC database to get the TCGA dataset with standardized pan-cancer data. Moreover, the data containing the influence of interferon-gamma (IFN-g) stimulation on APOL6 was obtained from the GSE199107,[18] GSE163067,[19] and GSE85898[20] datasets. The acronyms denoting different types of cancer are provided in Table S1, Supplemental Digital Content, https://links.lww.com/MD/O860. The selected publicly available datasets chosen in this study and their sources have been summarized in Table S2, Supplemental Digital Content, https://links.lww.com/MD/O861.

2.2. Exploring the features of tumor immune microenvironment (TIME)

The interactive tool Sangerbox was utilized for pan-cancer analysis. We identified the expression levels of immunomodulators, including chemokine, major histocompatibility complex, receptor, immunoinhibitors, and immunostimulators as assessment in TIME characteristics. We used both TIMER[21] and MCP-counter[22] algorithms to estimate the levels of tumor-infiltrating lymphocytes (TILs). Moreover, we searched the Genomic Data Commons and used the "MuTect2" software to get the genomic data on single nucleotide variations from TCGA samples. The calculation of the tumor mutation burden (TMB) for every specimen was performed using a related R package named "maftools."[23] We assessed the correlations between APOL6 and immunomodulators, tumor purity, TILs, neoantigen mutation, and TMB.

2.3. The bladder urothelial carcinoma (BLCA) TMA analysis

The HBlaU079Su01 TMA comprised 63 cancer samples and 16 adjacent tissue samples. The microarray was acquired from Outdo Biotech (Shanghai, China). This study involving TMA slides obtained ethical approval (YB M-05-02) from the Clinical Research Ethics Committee, Outdo Biotech (Shanghai, China).

2.4. Immunohistochemistry

We utilized standard procedures to conduct immunohistochemistry staining with the HBlaU079Su01 microarray. The antibodies employed were as listed: 1. Anti-APOL6 (1:1000dilution, Goat. Ab92273, Abcam, Cambridge, UK), 2. Anti-PD-L1 (Ready-to-use, Cat. GT2280, GeneTech, Shanghai, China), 3. Anti-CD8 (Ready-to-use, Cat. PA067, Abcarta, Suzhou, China), 4. Anti-IFITM3 (1:3000 dilution, Cat. ab109429, Abcam, Cambridge, UK). The process of staining was accomplished through DAB, complemented with hematoxylin counterstain. Following staining, sections were scanned with Aperio Digital Pathology Slide Scanners.

2.5. Semiquantitative scoring

Two pathologists evaluated the stained TMA independently. For a semiquantitative assessment of APOL6 and PD-L1 expression specifically on TC, the positively stained cells were classified into the following scores based on percentage: 0 (<1%), 1 (1–5%), 2 (6–25%), 3 (26–50%), and 4 (>50%). On a scale of 0 to 3, staining intensity was evaluated as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The calculation of the Immunoreactivity Score involved multiplying the percentage of positively stained cells by the staining intensity. Tumors were classified into 3 phenotypes – inflamed, excluded, and deserted dependent on the spatial distribution of CD8 + T cells.

2.6. Cell lines and culture

T24 cells and SCaBER cells were provided by Jiangsu Kaiji Biotechnology Co., Ltd. The culture conditions for T24 cells were 90% RPMI1640 + 10% CS + P/S, and for SCaBER cells were 90% MEM + 10% FBS + 1% non essential amino acids + 1 mM Sodium Pyruvate (NaP) + P/S. The cells were cultured in a 37 ℃, 5% CO_2, and saturated humidity incubator.

5-FU, IFN-g, and siRNAs are commercially provided by Jiangsu Kaiji Biotechnology Co., Ltd. in Fengtai, China. The primer sequences of APOL6 and siRNAs are shown in Table S2, Supplemental Digital Content, https://links.lww.com/MD/O861.

2.7. siRNA transfection studies

On the day before transfection, T24/SCaBER cells were seeded into cell culture plates, and antibiotic free medium was added to each well to achieve a cell density of 70% to 80% during transfection. Then 2.5 μL of 20 μM siRNA were diluted with 250 μL serum-free Opti MEM medium incubated at room temperature for 5 minutes. Next, 5 μL of KeygenMAX 3000(KGA9705-1.5) were diluted with 250 μL of serum-free Opti MEM medium at room temperature and incubated for 5 minutes, and then incubated for 20 minutes. The mixture of siRNA KeygenMAX 3000 were added to a culture containing 500 μL of T24/SCaBER cells for 4 to 6 h. Then the culture medium containing the mixture of siRNA KeygenMAX 3000 were removed from the well and replaced with fresh medium. The culture plate were placed in a CO2 incubator at 37 ℃ and cultured for 48 hours. Finally, cells were obtained and performed for apoptosis analysis or immunoblotting analysis.

2.8. Quantitative real-time q-PCR

TRIzol (KGF5101-100, Jiangsu Kaiji Biotechnology Co., Ltd., Fengtai, China) was used to extract total RNA from cells. The cDNA first strand synthesis kit (TaKaRa RR036B from Japan) and PCR kit TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa RR820A, Japan) were used for RT-qPCR assay. SYBR Green method was used to perform RT-qPCR reaction. The 2-ΔΔCt value were calculated for quantitative analysis. The primer sequences for RT-qPCR are shown in Table S2, Supplemental Digital Content, https://links.lww.com/MD/O861.

2.9. Cell counting kit-8 (CCK-8) assay

The survival rate of transfected cells was measured by the Cell Counting Kit-8. The cell suspension was seeded onto a 96 well flat plate and incubated overnight with 5% CO_2 at 37 °C. Then, 100 μL of 10% CCK-8 solution was added to each plate and the cells were incubated again for 2 hours. Then the enzyme-linked immunosorbent assay reader was used to measure the absorbance at 450 nm and counted the inhibition rates of each group.

2.10. Flow cytometry

The transfected T24/SCaBER cells were digested, counted, and prepared into suspension with a concentration of 7 × 104 cells/mL. Then, 2 mL of them was inoculated into a 6 well plate. The next day, they were added to the corresponding drug containing culture medium according to the group settings, and a negative control was set up. After 48 hours of drug action, the culture was washed, treated with trypsin, centrifuged, and processed. For apoptosis assay, cells were resuspended in binding buffer and stained sequentially with Annexin-V-FITC and DAPI. The samples were analyzed on a flow cytometer (BECKMAN COUPTER CytoFLEX). Each experiment was repeated at least 3 times.

2.11. Western blot

The total protein was extracted using RIPA buffer containing phosphatase inhibitors and protease inhibitors. After SDS-PAGE separation, the protein was transferred onto a polyvinylidene fluoride membrane at 250 mA for 100 minutes and the membrane was blocked with 5% skim milk. Next, the membrane was incubated with the primary antibodies at 4 °C for 12 hours. Subsequently, the membrane was incubated with the secondary antibodies at 37 °C for 60 minutes. After washing with TBST 3 times, imaging was performed using ChemiDoc MP Imaging System, and the results were subjected to grayscale analysis using Gel-Pro32 software.

The primary antibodies used are as follows: CRT (#10292-1-AP, Proteintech, 1:1000 diluted), HMGB1 (#10829-1-AP, Proteintech, 1:5000 diluted), APOL6 (#abcam ab92273, Proteintech, 1:1000 diluted), and VINCULIN (#26520-1-AP, Proteintech, 1:5000 diluted).

2.12. Statistical analysis

The software used to conduct statistical analyses are SPSS 26.0, Graphpad Prism 6.0, and R 4.0.2. All data are expressed as means ± standard deviations. To compare the 2 groups, either the parametric Student *t* test or the nonparametric Mann–Whitney U test was employed. Differences among multiple groups were analyzed using either parametric one-way ANOVA or nonparametric Kruskal–Wallis test. The log-rank test was employed for conducting survival analysis. The Pearson test was utilized to analyze the correlation between 2 variables. All statistical tests were two-sided, with a significance level of $P < .05$ indicating statistical significance, and labeled with $*P < .05$; $**P < .01$; and $***P < .001$.

3. Results

3.1. APOL6 stands as the beneficial biomarker to predict the effectiveness of immunotherapy in BLCA

We initially identified the predictive significance of APOL6 in immunotherapy using the GSE176307 dataset, with samples taken before the treatment (n = 88). The expression of APOL6 was found to be notably higher in cancer samples showing CR/PR responses in comparison to that showing standard deviation/PD responses (Fig. 1A). Depending on the median APOL6 expression, patients in this cohort were categorized into 2 groups, and the result revealed that patients who had a greater objective response rate (31.82% vs 4.55%, Fig. 1B) showed higher APOL6 expression. Regarding the overall survival (OS) time and progression-free survival (PFS) time, the expression of APOL6 was found to be significantly correlated with OS. Additionally, it showed a more significant correlation with PFS than OS (Fig. 1C). Notably, individuals showing elevated APOL6 expression levels displayed improved OS and PFS outcomes (Fig. 1E and F). The results also indicated that APOL6 exhibited positive effectiveness in patients of BLCA receiving immunotherapy using the Cox regression analysis (Fig. 1D). Furthermore, APOL6 expression was correlated with TMB positively (Fig. 1G) and the expression was enhanced in the high TMB type (Fig. 1H). Moreover, we examined the relationship between APOL6 and the expression of immunomodulators, which could reflect the characteristics of TIME, thus predicting the immunotherapic effectiveness. Our findings revealed that APOL6 expression was affirmatively linked to the majority of immunomodulators (Fig. 1I). In general, we speculate APOL6 could be a promising classifier for predicting the effectiveness of immunotherapy at least in bladder urothelial cancer.

3.2. APOL6 can predict immunotherapic responses across other tumor types

Supplementary public datasets were obtained to confirm the predictive significance of APOL6 in cancer immunotherapy. There were 4 validated datasets used in this study, namely the GSE194040 dataset (breast cancer, n = 69), the GSE173839 dataset (breast cancer, n = 71), the GSE100797 dataset (melanoma, n = 21, only specimens gathered prior to the presence of TILs were incorporated), the IMvigor210 dataset (urothelial cancer, n = 132, only samples gathered preceding the administration of platinum were used). Encouragingly, it was found that patients with positive treatment responses had significantly higher levels of APOL6 expression in these cohorts (Fig. 2A–D). Furthermore, we investigated the relationship between APOL6 and features of TIME in the IMvigor210 cohort. There was a notable association between the expression of APOL6 and PD-L1 of different scores of IC and TC, within which tumors of the immuno-inflamed type showed elevated APOL6 expression (Fig. 2E–G). In the IMvigor210 cohort, APOL6 expression exhibited a positive correlation with the load of neoantigens (Fig. 2H). Moreover, APOL6 displayed a favorable association with the bulk of immunomodulatory factors within these cohorts (Fig. 2I). These findings collectively indicate that APOL6 has the potential to predict immunotherapic responses in other types of cancer.

3.3. APOL6 is correlated with TIME in multiple types of cancer

Considering APOL6 demonstrated potential significance in the preeminent portion of immunomodulators within the cohorts

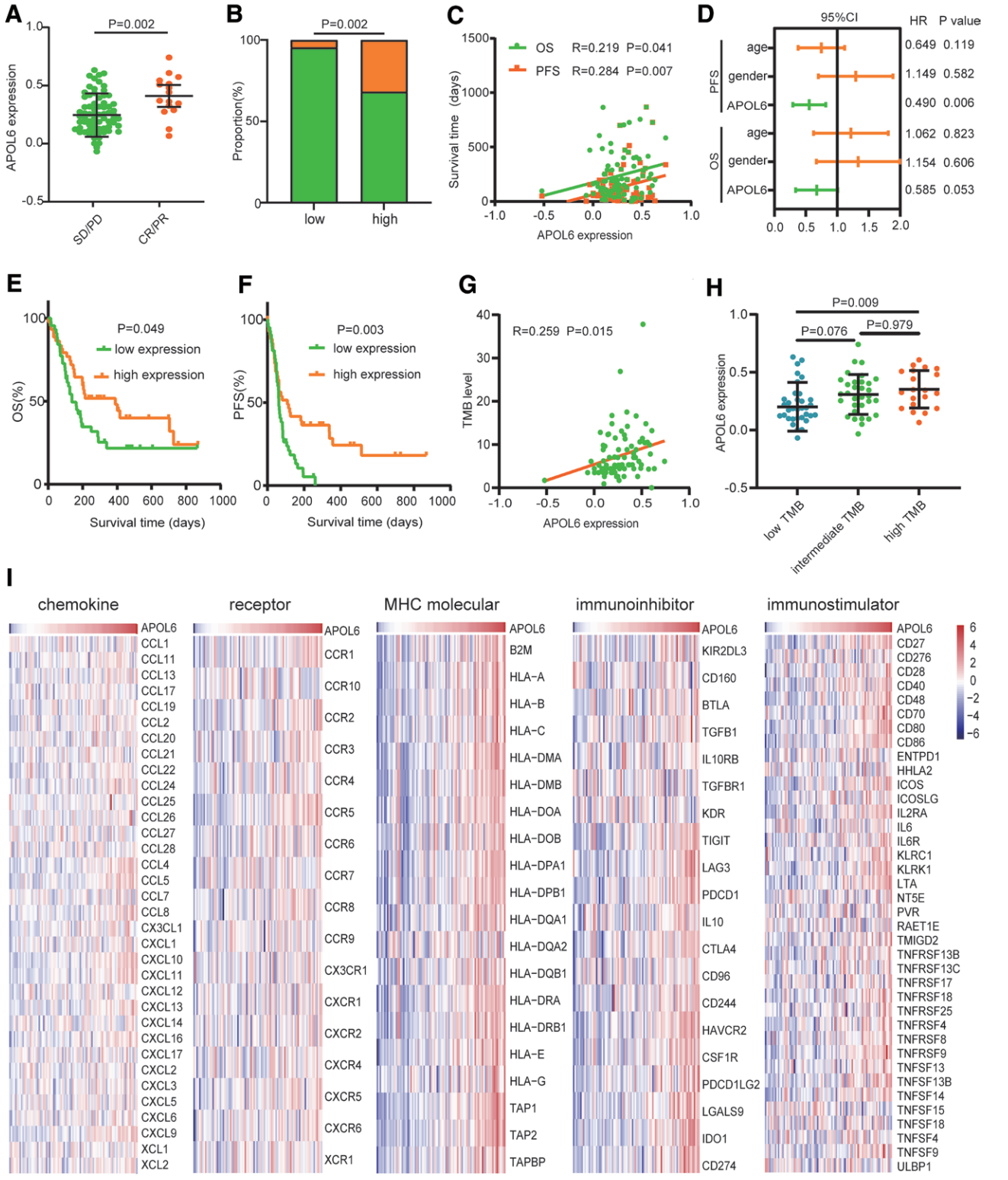

Figure 1. Predictive value and immunological associations of APOL6 within GSE176307. (A) APOL6 expression in BLCA among patients with distinct responses. (B) ORR among patients with different APOL6 expression. (C) Associations between APOL6 expression and OS and PFS time. (D) Cox regression analysis of prognostic factors in BLCA patients. (E and F) The prognostic significance of APOL6 in terms of OS and PFS. (G) Correlation between APOL6 expression and TMB levels.(H) APOL6 expression with different types of TMB levels. (I) A heatmap displaying correlations between APOL6 and TIME features. APOL6 = apolipoprotein L6, BLCA = bladder urothelial carcinoma, ORR = objective response rate, OS = overall survival, PFS = progression-free survival, TMB = tumor mutation burden, TIME = tumor immune microenvironment.

mentioned above, our next step analyzed the TCGA dataset to investigate whether APOL6 was linked to features of TIME in pan-cancer. The connections between APOL6 and chemokine, receptor, major histocompatibility complex, immunoinhibitors, and immunostimulators have been examined. APOL6 exhibited a favorable correlation with the levels of expression of these

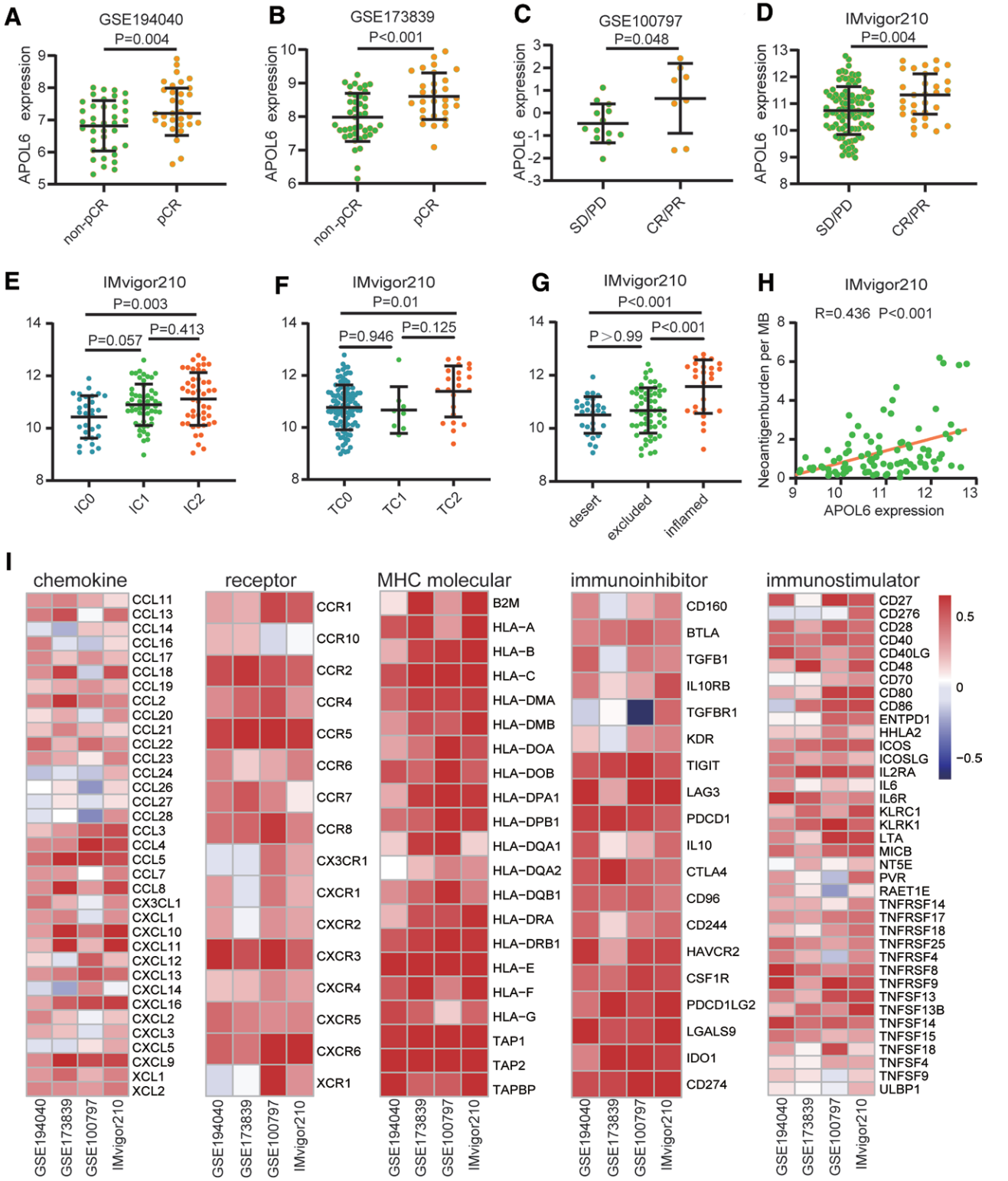

Figure 2. The predictive significance and immunological associations of APOL6 in other datasets. (A–D) APOL6 expression levels in cancer patients exhibiting various responses, (A) the GSE194040 dataset, (B) the GSE173839 dataset, (C) the GSE100797 dataset, (D) the Imvigor210 dataset. (E) APOL6 expression with different PD-L1 IC score. (F) APOL6 expression with different PD-L1 TC score. (G) APOL6 expression with different immuno-subtypes. (H) The relationship between APOL6 expression and neoantigen burden. (I) A heatmap displaying correlations between APOL6 and TIME features in related datasets. APOL6 = apolipoprotein L6, PD-L1 = programmed death-ligand 1, IC = immune cells, TC = tumor cells.

immunomodulators across multiple types of cancer, except for cholangio carcinoma and kidney chromophobe (Fig. 3A). Moreover, APOL6 was positively correlated with levels of TILs and exhibited a negative correlation with tumor purity across a majority of tumor categories (Fig. 3B and C). Furthermore, the associations between APOL6 and TMB, along with the load of neoantigens, exhibited distinct characteristics across various cancer types, where APOL6 demonstrated significant

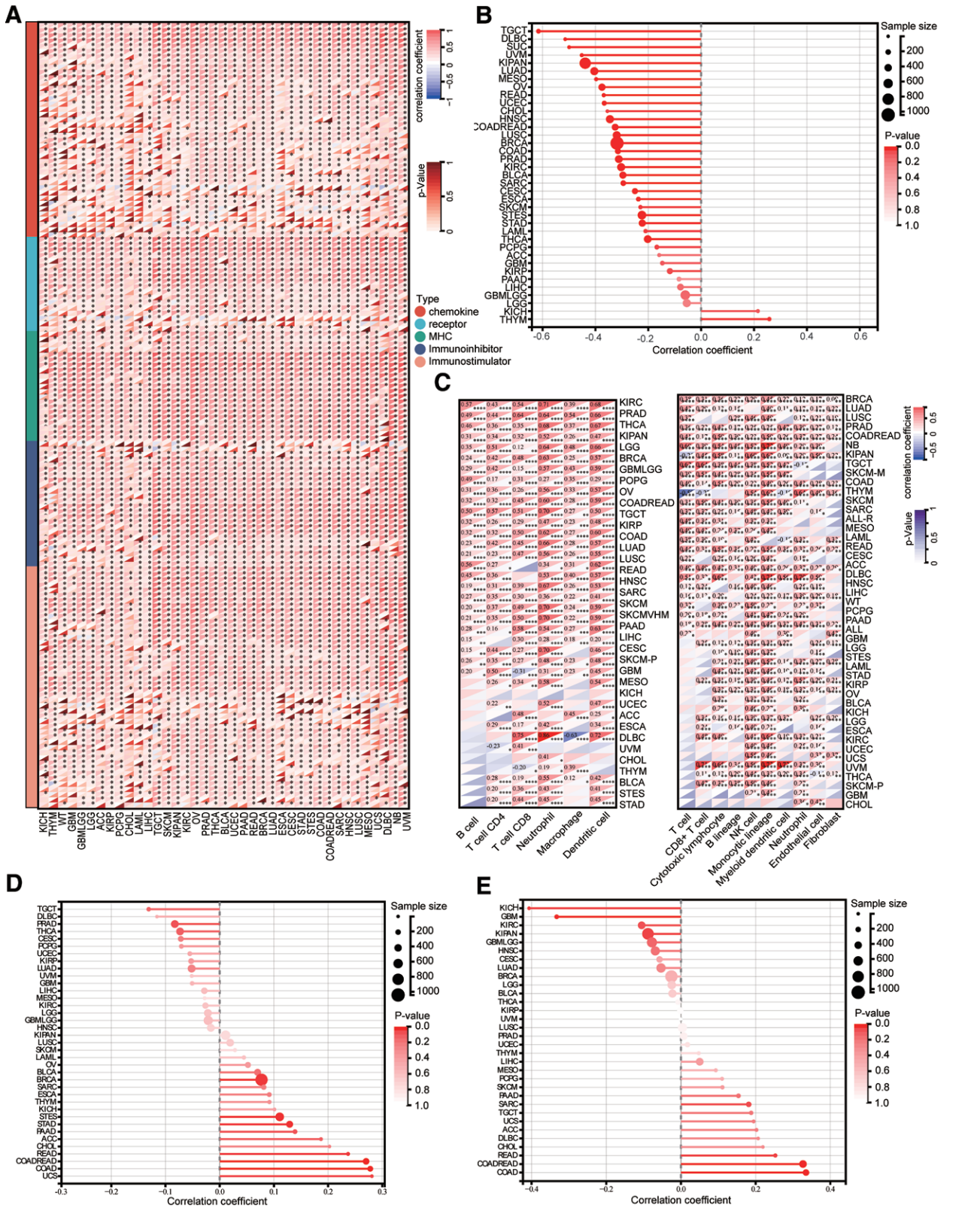

Figure 3. A pan-cancer investigation analyzing the immunological function of APOL6. (A) Association between APOL6 and immunomodulators expression reflecting TIME. (B) Association between APOL6 and tumor purity. (C) Association between APOL6 and TILs. (D) Association between APOL6 and TMB levels. (E) Association between APOL6 and neoantigen burden. APOL6 = apolipoprotein L6, TILs = tumor-infiltrating lymphocytes, TIME = tumor immune microenvironment, TMB = tumor mutation burden.

association with TMB in colorectal cancer and uterine carcinosarcoma (Fig. 3D) and was related with the neoantigen burden in colorectal cancer (Fig. 3E). In conclusion, these findings reveal that APOL6 acts as an immunological related classifier in major types of cancer, with the exclusion of certain specific cancer types.

3.4. Comparison and correlation of APOL6 and IFN-g in immunotherapy

Three cohorts of patients showed that APOL6 was more discriminating than PD-L1 when it came to detecting therapeutic responses, and APOL6 combined with PD-L1 improved predictive power in 4 cohorts (Fig. 4A–E). The PD-L1 expression is stimulated by IFN-g originating from lymphocytes within the tumor microenvironment.[24] IFN-g is one of the cytokines released by CD8 + T cells, exhibits effects in inhibiting the TC proliferation and promoting apoptosis, and its levels are elevated in immuno-hot tumors.[25] In our research, IFN-g displayed satisfactory discrimination in 2 datasets (Fig. 4B and C), and APOL6 expression displayed more satisfying discrimination than IFN-g in 3 cohorts (Fig. 4A, D and E), while the stability of IFN-g did not appear to be good. Considering PD-L1 expression can be upregulated by IFN-g and the similar predicting ability of APOL6 and PD-L1, to further explore the association between APOL6 and IFN-g, we chose several public datasets and in vitro assays conducted on NCI-H1299 and MDA-MB-231 cells, which have consistently demonstrated a significant upregulation of APOL6 in response to IFN-g (Fig. 4F–I). In summary, APOL6 expression shows similar predicting performance compared with IFN-g in immunotherapy and has an increase in response to IFN-g.

3.5. Predictive values of APOL6 in the BLCA TMA cohort

Following this, we confirmed the expression of APOL6 with various immuno-subtypes of cancer utilizing the HBlaU079Su01 cohort (Fig. 5A). The antibodies used were APOL6, PD-L1, CD-8, and interferon induced transmembrane protein 3 (IFITM3) antibodies. The expression of IFITM3 is associated with TC proliferation, invasion, and metastasis, and it participates in the activation of the TGF β-MAPK-Smads pathway.[26] IFITM3 is also a predicting biomarker in cancer immunotherapy studied previously.[27] Firstly, We compared the expression of APOL6 and PD-L1 in tumor and tumor-adjacent tissues, observing that both the expression of them showed higher levels in tumor tissues (Fig. 5B and C). Depending upon the CD8 + T cells distribution, immuno-subtypes of the samples were classified as 3 phenotypes: the inflamed, excluded, and deserted, with representative images provided for illustration (Fig. 5D). These findings indicated a promising correlation of APOL6 and PD-L1 across 3 immuno-subtypes of BLCA, and in immuno-inflamed tumors, their expression reached its peak, while in immuno-deserted or immuno-excluded tumors, it was at its lowest (Fig. 5E). Furthermore, APOL6 expression exhibited a positive association between PD-L1 and IFITM3 expression, which is also a pan-cancer biomarker in immunotherapy response studied before, but showed no significance in association with CD8 expression (Fig. 5F–H). Overall, The BLCA TMA cohort confirms the ability of APOL6 as an innovative and promising biomarker to predict response in cancer immunotherapy.

3.6. APOL6 can promote apoptosis of BLCA cells in vitro

Firstly, we screened for siRNAs targeting APOL6 using q-PCR to achieve effective knockdown of APOL6 (Fig. 6A and B). Subsequently, through the CCK-8 assay, we found that the cell viability of the APOL6 knockdown group after handled with 5-FU was significantly higher than that of the non knockdown group, indicating that knockdown of APOL6 may enhance cell tolerance to 5-FU (Fig. 6C and D). To further validate the effect of APOL6 knockdown on cell survival, we used flow cytometry to detect the degree of cell apoptosis (Fig. 6E). The results were consistent with the CCK-8 experiment, showing that the apoptosis rate of APOL6 knockdown group after handled with 5-FU was significantly lower than that of the non knockdown group (Fig. 6F and G). According to reports before, APOL6 induced cell apoptosis is associated with immunogenic cell death.[12] Therefore, we selected the immunogenic cell death marker proteins CRT (Calreticulin) and HMGB1 (High Mobility Group Box 1) for Western blot analysis. In the APOL6 knockdown group, the expression level of CRT was significantly upregulated, while the expression of HMGB1 was significantly downregulated (Fig. 6H and I). Finally, we observed a significant increase in APOL6 expression induced by IFN-g (Fig. 6J and K), suggesting that IFN-g may affect cell apoptosis and immune response by upregulating APOL6 expression. Taken together, APOL6 promotes apoptosis in bladder cancer cells and may play an important role in TC tolerance to chemotherapy and immune responses.

4. Discussion

Tumor immunotherapy focuses on enhancing the natural immune system and utilizes the inherent and adaptive immune system to target and eradicate TC and tissue.[2] Immunotherapy has revolutionized cancer treatment through the effective use of monoclonal antibodies, cytokines, cellular immunotherapy, and vaccines.[28] The levels of expression of genes related to immunity are regarded as biomarkers in diverse cancer immunotherapies for predicting efficacy.[29] TIME exerts a significant influence in cancer occurrence, progression, and immune evasion. The elements of TIME, which include IC, inflammatory cells, endothelial cells and so on, are able to change the immune status of cancer and the immunotherapic response.[30] Indicators that reflect the characteristics of TIME are suggested as potential biomarkers for cancer immunotherapy.[3]

PD-L1 is one of the most significant indicators of TIME, which engages with PD-1 on T cell surfaces, consequently leading to T cell inactivation, and TC have the ability to elevate PD-L1 expression to trigger immune escape.[28] ICIs function by blocking the interplay between PD-1 and PD-L1 to reactive immune responses against tumors.[31] In specific solid tumors like NSCLC and melanoma, PD-L1 expression has been considered the favorable biomarker in immunotherapic responses.[8] While high expression of PD-L1 typically correlates with a more favorable prognosis in individuals with cancer, it's noteworthy that some patients with low PD-L1 expression still demonstrate improved efficacy with immunotherapy.[5] This phenomenon may arise from significant variations in the expression of PD-L1 across distinct tumor types and exhibiting significant heterogeneity within individual patients.

Therefore, there is currently no ideal definitive predictive biomarker for cancer immunotherapy, as each biomarker comes with its own limitations. Enhanced precision in identifying biomarkers for predicting cancer immunotherapy outcomes will aid in patient stratification, also enhancing prognosis and OS rate.

Our present investigation noted that APOL6 is elevated in cancer patients who show positive responses to immunotherapy and is linked to immune modulators in the TIME. Furthermore, we conducted a pan-cancer analysis, revealing a positive association between APOL6 and inflamed TIME, and used the BLCA cohort to validate the favorable association between APOL6 and PD-L1. APOL6, a BH3-only protein belonging to the BCL-2 family, which is categorized into 3 subgroups (survival-promoting proteins, apoptotic effectors, and BH3-only proteins) based on its function and sequence homology at the amino acid level, is crucial for modulating cell apoptosis.[32] Apoptosis evasion represents a hallmark of cancer, and abnormal expression of members within the BCL-2 family is often detected across various cancer types,[33] which can markedly contribute to the development, expansion, and resistance to treatment within tumors.

It was found that BH3-only protein stands an important role in initiating apoptosis induced by various anticancer agents.[34]

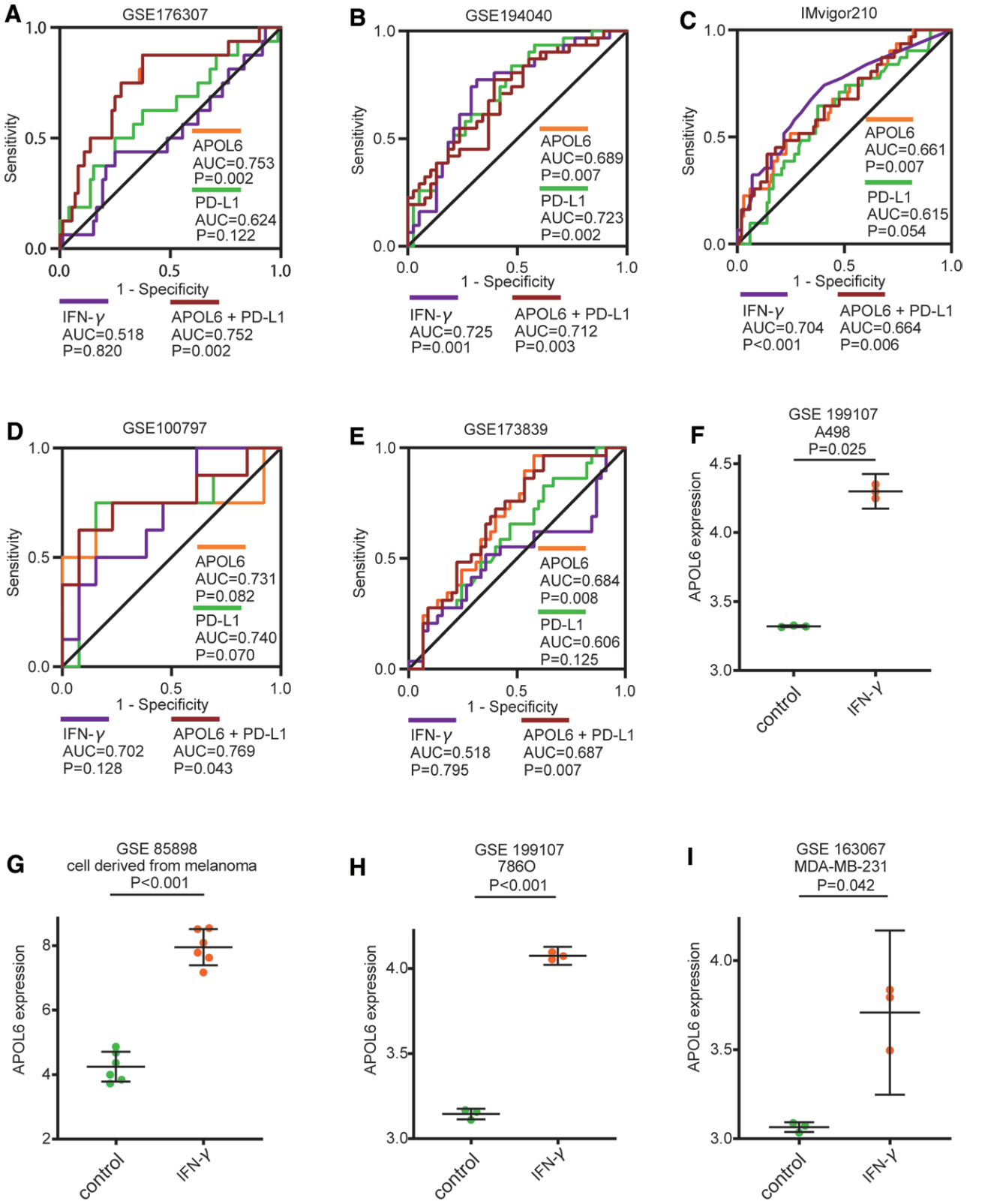

Figure 4. Contrasts of predicting immunotherapy efficacy of APOL6, PD-L1, IFN-γ and the APOL6/PD-L1 combination and transcriptional change of APOL6 expression under the stimulation of IFN-γ. (A-E) ROC curve analysis of potential value in immunotherapy with the APOL6, PD-L1, IFN-γ and the APOL6/PD-L1 combination. (A) The GSE176307 cohort, (B) The GSE194040 cohort, (C) The IMvigor210 cohort, (D) The GSE100797 cohort, (E) The GSE173839 cohort. (F) APOL6 expression stimulated by IFN-g in A498 cells from the GSE199107 dataset. (G) APOL6 expression stimulated by IFN-g in melanoma cells from the GSE85898 dataset. (H) APOL6 expression stimulated by IFN-g in 786O cells from the GSE199107 dataset. (I) APOL6 expression stimulated by IFN-g in MDA-MB-231 cells from the GSE163067 dataset. APOL6 = apolipoprotein L6, IFN-g = interferon-gamma, PD-L1 = programmed death-ligand 1.

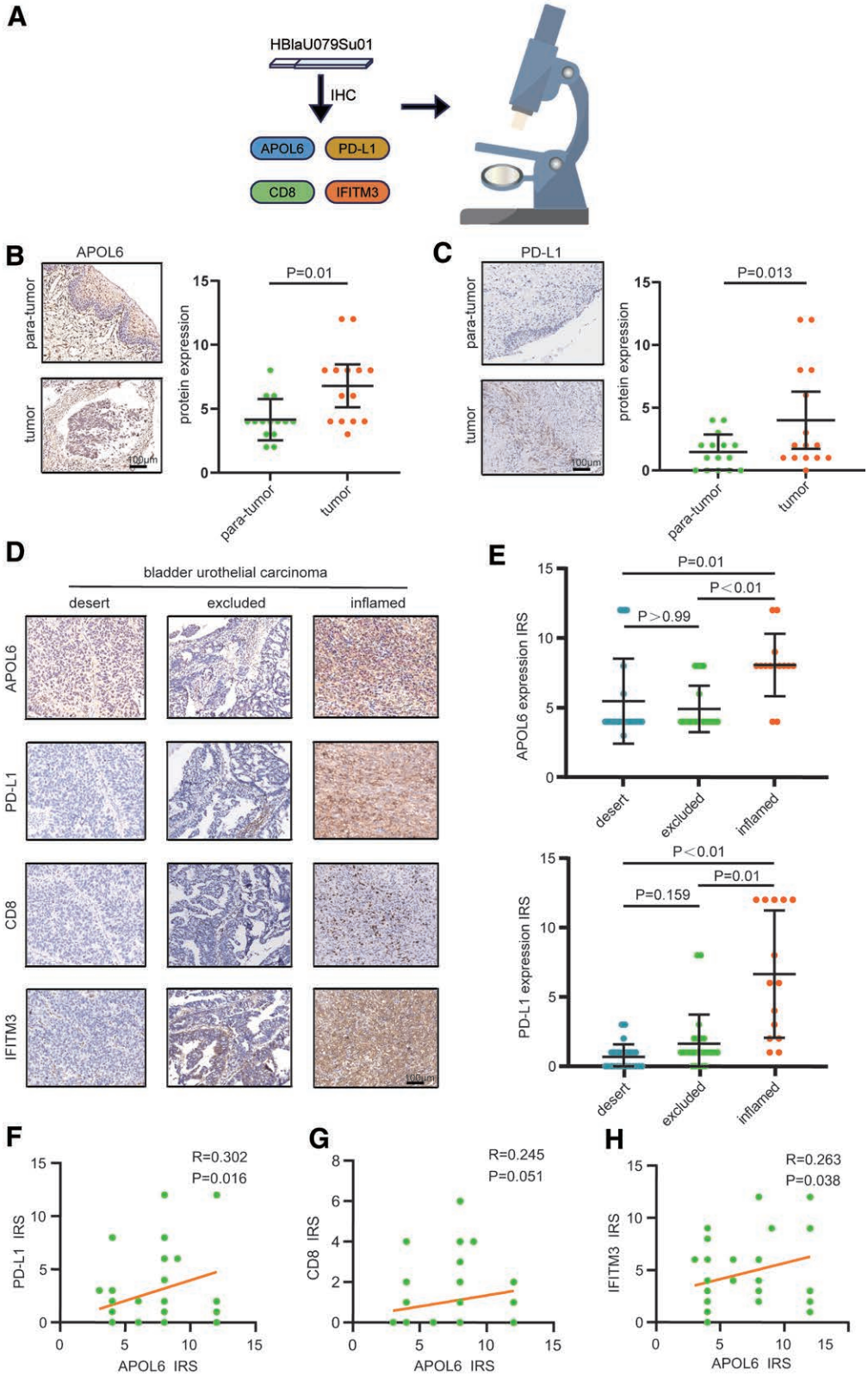

Figure 5. Research of APOL6 expression with immunologic correlations in BLCA TMA. (A) Schematic representation of the TMA validation cohort. (B) APOL6 expression levels in tumor and para-tumor tissues, with representative images. (C) PD-L1 expression levels in tumor and para-tumor tissues, with representative images. (D) Representative pictures illustrating the protein expression in BLCA, antibodies used are APOL6, PD-L1, CD8, IFITM3. (E) Semiquantitative analysis of APOL6 and PD-L1 expression in different immuno-subtypes of bladder urothelial carcinoma. (F) Association between APOL6 and PD-L1 expression in the HBlaU079Su01 TMA. (G) Association between APOL6 and CD8 expression in the HBlaU079Su01 TMA. (H) Association between APOL6 and IFITM3 expression in the HBlaU079Su01 TMA. APOL6 = apolipoprotein L6, PD-L1 = programmed death-ligand 1, TMA = tissue microarray, IFITM3 = interferon induced transmembrane protein 3.

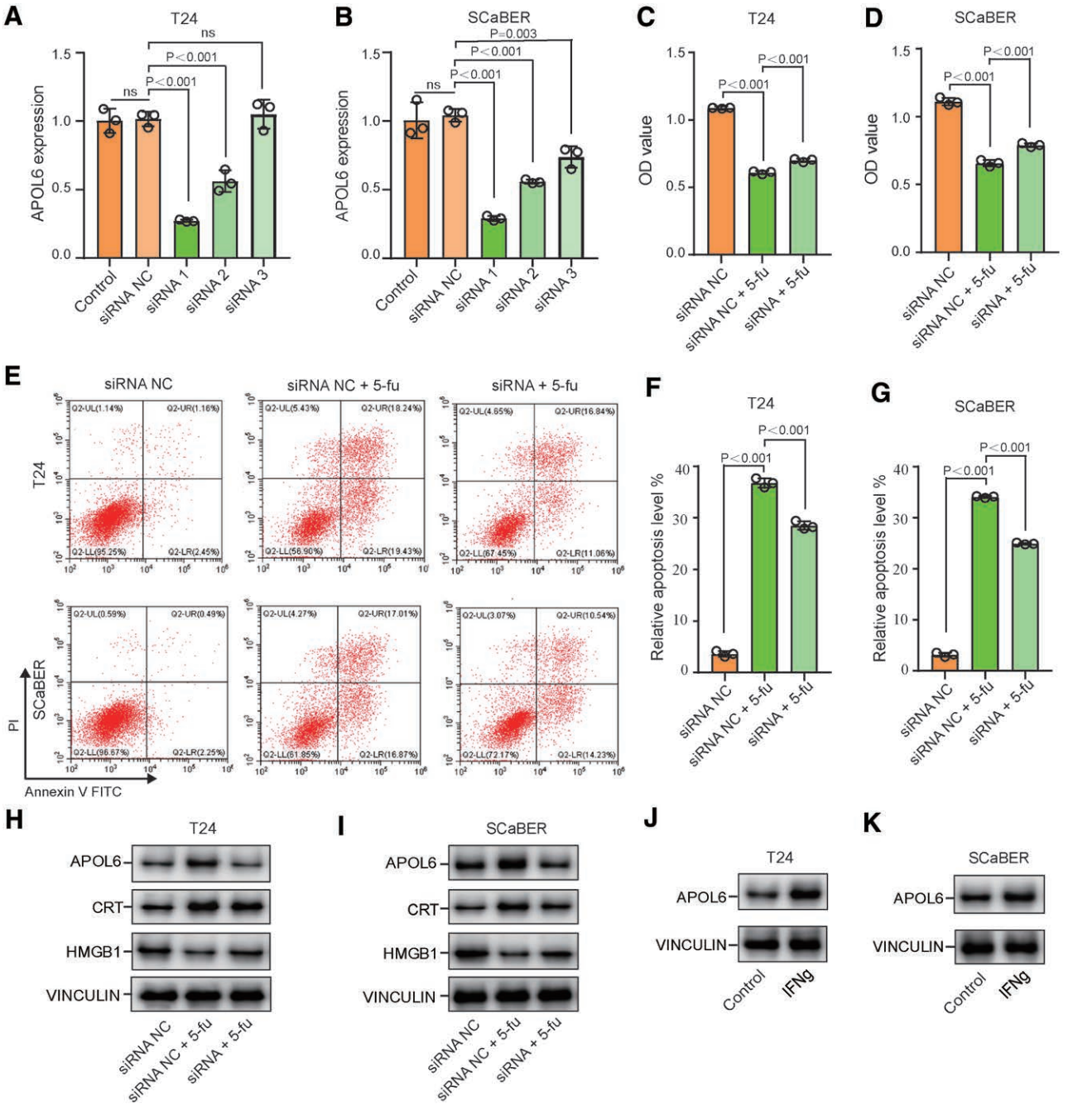

Figure 6. In vitro experiment verification of the role of APOL6 in BLCA. (A) Screening of siRNAs for APOL6 in the T24 cell line. (B) Screening of siRNAs for APOL6 in the SCaBER cell line. (C) In the T24 cell line, the viability of cells treated with 5-fu after APOL6 knockdown was significantly increased compared to the non knockdown group. (D) In the SCaBER cell line, the viability of cells treated with 5-fu after APOL6 knockdown was significantly increased compared to the non knockdown group. (E) Flow cytometry was used to detect cell apoptosis levels. (F) In the T24 cell line, the apoptosis rate of cells treated with 5-fu after APOL6 knockdown was significantly reduced compared to the non knockdown group. (G) In the SCaBER cell line, the apoptosis rate of cells treated with 5-fu after APOL6 knockdown was significantly reduced compared to the non knockdown group. (H, I) APOL6 knockdown inhibited CRT expression but promoted HMGB1 expression. (J, K) APOL6 expression was increased under the induction of IFN-g. APOL6 = apolipoprotein L6, CRT = Calreticulin, HMGB1 = High Mobility Group Box 1, WB = Western Blot.

APOL6 expression shows a positive correlation with pancreatic necrosis score, pyroptosis score, and ferroptosis score, which shows potential to reverse "cold" tumors to "hot" tumors, thus improving ICI treatment outcomes.[12] APOL6 exerts its influence through fas-induced and mitochondria-mediated apoptosis, in addition to generating reactive oxygen species and activating caspases, thereby predicting immune response in hepatocellular carcinoma.[35] A signature including TOP2A, MATR3, APOL6, JOSD1, and HOXC6 is associated with radiosensitivity, which has been identified as a potential indicator for prognostic outcomes in rectal cancer.[36] As a target in the downstream of interferon-g, APOL6 is found to be elevated by this cytokine, consistent with our research findings mentioned above.[37] Furthermore, the screening process identified APOL6 as a drug-sensitive target linked to cancer cell ferroptosis, which has been validated by scRNA-seq in colon cancer cell lines.[38] All of these results support the correlation between APOL6 and antitumor immunity, contributing to the screening

and evaluation for cancer patients who could derive advantages from cancer immunotherapy.

The present study nonetheless possesses certain constraints. Firstly, the biological role of APOL6 remains unclear and its potential ability on immunotherapy and TIME requires additional research. Moreover, the predictive efficacy of APOL6 in immunotherapy has not been validated within in-house cohorts, necessitating validation with cohorts including a substantial number of patients across multiple tumors. In addition, it is a well-established fact that tumors identified the "immuno-hot" subtype do not consistently lead to better prognosis or positive responses to immunotherapy.[39] Our findings suggest that APOL6 holds potential for predicting responses to immunotherapy, and its predictive ability depends upon immuno-inflamed characteristics. Therefore it is worth noting that APOL6 may not consistently work in all cases.

To conclude, our present research systematically examines the predicting utility of APOL6 in response to immunotherapy and its relevance in immunology. APOL6 has been identified as a promising indicator to predict effectiveness in immunotherapy of various tumors across many public datasets. Despite this, there remains a lack of further research on the precise role of APOL6 in tumors; therefore, the functional significance of APOL6 is still unclear. Additional research, involving a larger number of patients and in vivo assays, is still required to determine its potential to be a predicting biomarker for the benefit of ICIs.

Author contributions

Conceptualization: Jialin Yao.
Data curation: Jialin Yao, Wenyuan Gan.
Methodology: Wenyuan Gan.
Software: Dongqing Li, Lingjun Zhu.
Supervision: Lingjun Zhu.
Validation: Lei Cao.
Visualization: Zhihang Han.
Writing – original draft: Jialin Yao, Jiukang Sun.
Writing – review & editing: Lei Cao, Lingjun Zhu.

References

[1] Zhang Y, Zhang Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. Cell Mol Immunol. 2020;17:807–21.
[2] Kraehenbuehl L, Weng CH, Eghbali S, Wolchok JD, Merghoub T. Enhancing immunotherapy in cancer by targeting emerging immunomodulatory pathways. Nat Rev Clin Oncol. 2022;19:37–50.
[3] Rui R, Zhou L, He S. Cancer immunotherapies: advances and bottlenecks. Front Immunol. 2023;14:1212476.
[4] Marei HE, Hasan A, Pozzoli G, Cenciarelli C. Cancer immunotherapy with immune checkpoint inhibitors (ICIs): potential, mechanisms of resistance, and strategies for reinvigorating T cell responsiveness when resistance is acquired. Cancer Cell Int. 2023;23:64.
[5] Zhu Y, Zhu X, Tang C, Guan X, Zhang W. Progress and challenges of immunotherapy in triple-negative breast cancer. Biochim Biophys Acta Rev Cancer. 2021;1876:188593.
[6] Lahiri A, Maji A, Potdar PD, et al. Lung cancer immunotherapy: progress, pitfalls, and promises. Mol Cancer. 2023;22:40.
[7] Passiglia F, Galvano A, Rizzo S, et al. Looking for the best immune-checkpoint inhibitor in pre-treated NSCLC patients: an indirect comparison between nivolumab, pembrolizumab and atezolizumab. Int J Cancer. 2018;142:1277–84.
[8] Prasad V, Kaestner V. Nivolumab and pembrolizumab: monoclonal antibodies against programmed cell death-1 (PD-1) that are interchangeable. Semin Oncol. 2017;44:132–5.
[9] Wang Y, Nguyen HP, Xue P, et al. ApoL6 associates with lipid droplets and disrupts Perilipin1-HSL interaction to inhibit lipolysis. Nat Commun. 2024;15:186.
[10] Zhaorigetu S, Yang Z, Toma I, McCaffrey TA, Hu CA. Apolipoprotein L6, induced in atherosclerotic lesions, promotes apoptosis and blocks Beclin 1-dependent autophagy in atherosclerotic cells. J Biol Chem. 2011;286:27389–98.
[11] Qubi W, Zheng J, Wang Y, et al. Goat miR-92a-3p targets APOL6 gene to regulate the differentiation of intramuscular precursor adipocytes. Genes (Basel). 2023;15:57.
[12] Liu K, Chen Y, Li B, et al. Upregulation of apolipoprotein L6 improves tumor immunotherapy by inducing immunogenic cell death. Biomolecules. 2023;13:415.
[13] Rose TL, Weir WH, Mayhew GM, et al. Fibroblast growth factor receptor 3 alterations and response to immune checkpoint inhibition in metastatic urothelial cancer: a real world experience. Br J Cancer. 2021;125:1251–60.
[14] Wolf DM, Yau C, Wulfkuhle J, et al. Redefining breast cancer subtypes to guide treatment prioritization and maximize response: predictive biomarkers across 10 cancer therapies. Cancer Cell. 2022;40:609–23.e6.
[15] Pusztai L, Yau C, Wolf DM, et al. Durvalumab with olaparib and paclitaxel for high-risk HER2-negative stage II/III breast cancer: results from the adaptively randomized I-SPY2 trial. Cancer Cell. 2021;39:989–98.e5.
[16] Lauss M, Donia M, Harbst K, et al. Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. Nat Commun. 2017;8:1738.
[17] Necchi A, Joseph RW, Loriot Y, et al. Atezolizumab in platinum-treated locally advanced or metastatic urothelial carcinoma: post-progression outcomes from the phase II IMvigor210 study. Ann Oncol. 2017;28:3044–50.
[18] Garige M, Ghosh S, Norris A, et al. PD-L1 mediates IFNγ-regulation of glucose but not of tryptophan metabolism in clear cell renal cell carcinoma. Front Oncol. 2022;12:858379.
[19] Goncalves G, Mullan KA, Duscharla D, et al. IFNγ modulates the immunopeptidome of triple negative breast cancer cells by enhancing and diversifying antigen processing and presentation. Front Immunol. 2021;12:645770.
[20] Gao J, Shi LZ, Zhao H, et al. Loss of IFN-γ pathway genes in tumor cells as a mechanism of resistance to anti-CTLA-4 therapy. Cell. 2016;167:397–404.e9.
[21] Li T, Fan J, Wang B, et al. TIMER: a web server for comprehensive analysis of tumor-infiltrating immune cells. Cancer Res. 2017;77:e108–10.
[22] Becht E, Giraldo NA, Lacroix L, et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. Genome Biol. 2016;17:218.
[23] Beroukhim R, Mermel CH, Porter D, et al. The landscape of somatic copy-number alteration across human cancers. Nature. 2010;463:899–905.
[24] Razaghi A, Durand-Dubief M, Brusselaers N, Björnstedt M. Combining PD-1/PD-L1 blockade with type I interferon in cancer therapy. Front Immunol. 2023;14:1249330.
[25] Farhood B, Najafi M, Mortezaee K. CD8(+) cytotoxic T lymphocytes in cancer immunotherapy: a review. J Cell Physiol. 2019;234:8509–21.
[26] Gómez-Herranz M, Taylor J, Sloan RD. IFITM proteins: understanding their diverse roles in viral infection, cancer, and immunity. J Biol Chem. 2023;299:102741.
[27] Cai Y, Ji W, Sun C, et al. Interferon-induced transmembrane protein 3 shapes an inflamed tumor microenvironment and identifies immune-hot tumors. Front Immunol. 2021;12:704965.
[28] Bagchi S, Yuan R, Engleman EG. Immune checkpoint inhibitors for the treatment of cancer: clinical impact and mechanisms of response and resistance. Annu Rev Pathol. 2021;16:223–49.
[29] Yan Y, Huang L, Liu Y, et al. Metabolic profiles of regulatory T cells and their adaptations to the tumor microenvironment: implications for antitumor immunity. J Hematol Oncol. 2022;15:104.
[30] Kirchhammer N, Trefny MP, Auf der Maur P, Läubli H, Zippelius A. Combination cancer immunotherapies: emerging treatment strategies adapted to the tumor microenvironment. Sci Transl Med. 2022;14:eabo3605.
[31] Pang K, Shi ZD, Wei LY, et al. Research progress of therapeutic effects and drug resistance of immunotherapy based on PD-1/PD-L1 blockade. Drug Resist Updat. 2023;66:100907.
[32] Liu Z, Lu H, Jiang Z, Pastuszyn A, Hu CA. Apolipoprotein l6, a novel proapoptotic Bcl-2 homology 3-only protein, induces mitochondria-mediated apoptosis in cancer cells. Mol Cancer Res. 2005;3:21–31.
[33] Kaloni D, Diepstraten ST, Strasser A, Kelly GL. BCL-2 protein family: attractive targets for cancer therapy. Apoptosis. 2023;28:20–38.

[34] Hafezi S, Rahmani M. Targeting BCL-2 in cancer: advances, challenges, and perspectives. Cancers (Basel). 2021;13:1292.
[35] Wang XK, Guo YX, Wang M, et al. Identification and validation of candidate clinical signatures of apolipoprotein L isoforms in hepatocellular carcinoma. Sci Rep. 2023;13:20969.
[36] Liu Y, Yang Y, Ni F, et al. Research on radiotherapy related genes and prognostic target identification of rectal cancer based on multi-omics. J Transl Med. 2023;21:856.
[37] Liu X, Chan VSF, Smith KGC, et al. Recapitulating primary immunodeficiencies with expanded potential stem cells: proof of concept with STAT1 gain of function. J Allergy Clin Immunol. 2023;153:1125–39.
[38] Liu Y, Zhao J, Huang B, et al. Identification and validation of an immunotherapeutic signature for colon cancer based on the regulatory patterns of ferroptosis and their association with the tumor microenvironment. Biochim Biophys Acta Mol Cell Res. 2024;1871:119698.
[39] Chen G, Luo D, Zhong N, et al. GPC2 is a potential diagnostic, immunological, and prognostic biomarker in pan-cancer. Front Immunol. 2022;13:857308.