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Sustained release of therapeutic gene by injectable hydrogel for hepatocellular carcinoma

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ABSTRACT

Gene therapy has shown remarkable effectiveness in the management of disease like cancer and inflammation as a revolutionary therapeutic. Nonetheless, therapeutic drug target discovery, efficient gene delivery, and gene delivery vehicles continue to be significant obstacles. Due to their effective gene transport capabilities and low immunogenicity, supramolecular polymers have garnered significant interest. Herein, ABHD5 is identified as a potential therapeutic target since it is dysregulated in hepatocellular carcinoma (HCC). Interestingly, the downregulation of ABHD5 could induce programmed death-ligand 1 (PD-L1) expression in liver cancer, which may contribute to the immunosuppression. To overcome the immunosuppression caused by PD-L1, an injectable hydrogel is designed to achieve efficient abhydrolase domain containing 5 (ABHD5) gene delivery via the host-guest interaction with branched polyethyleneimine-g-poly (ethylene glycol), poly (ethylene oxide) and poly (propylene oxide) block copolymers and α-CD (PPA/CD), demonstrating the capability for sustained gene release. The co-assembly hydrogel demonstrates good biocompatibility and enhanced gene transfection efficiency, efficiently triggering tumor cell apoptosis. Overall, the results of this study suggest that ABHD5 is a potential therapeutic target, and that a host-guest-based supramolecular hydrogel could serve as a promising platform for the inhibition of HCC.

1. Introduction

The primary objective of gene therapy is to treat or avert diseases through manipulation of genes within cells, which mainly achieved by correction of genetic flaws or induction of a new ones (High and Roncarolo, 2019; Wang et al., 2019). Clustered regularly interspaced short palindromic repeats (CRISPR) genome editing technique is a well-established technology that has the ability to correct genetic flaws, which is famous for translational medicine, demonstrating the promising perspective in clinical implementation (Chen et al., 2019; Manghwar et al., 2019). And the induction of a new gene is also a prevalent technique employed in gene therapy, chimeric antigen receptor (CAR) T-cell therapy is achieved by induction of the chimeric immuno receptors in T cells, which could restore the antitumor capability and suppress the immunosuppression (Schubert et al., 2021; Zhang et al., 2018). In recent years, a number of novel cancer therapeutic approaches that are based on CAR-T technology have also emerged, and they have shown encouraging curative potential (Elahi et al., 2021; Gong et al., 2021).

Compared to conventional therapeutic approaches such as chemical small molecules and antibody macromolecules, gene therapy provides a higher degree of precision (Jain, 2020; Su et al., 2019). However, the direct insertion of genes into cells is not feasible, gene vectors are necessary for effective gene delivery. Viral vectors, a frequently

Abbreviations: HCC, Hepatocellular carcinoma; PD-L1, Programmed death-ligand 1; CRISPR, Clustered regularly interspaced short palindromic repeats; CAR-T, Chimeric antigen receptor T-cell; AAV, Adeno-associated virus; EPR, Enhanced permeability and retention; α-CD, Alpha cyclodextrin; VLDL, Very low-density lipoprotein; ABHD5, Abhydrolase domain containing 5; PBMC, Peripheral blood mononuclear cells; IHC, immunohistochemistry; OS, Overall survival; PFS, Progression-free survival; PEO-PPO-PEO, Poly (ethylene oxide) and poly (propylene oxide); PP, PEI-g-PEG; PBS, Phosphate-buffered saline; FBS, Fetal bovine serum; DMEM, Dulbecco's Modified Eagle Medium; RLU, Relative light unit; CI, Confidence interval; HR, Hazard ratio; FL, Fluorescence intensity.

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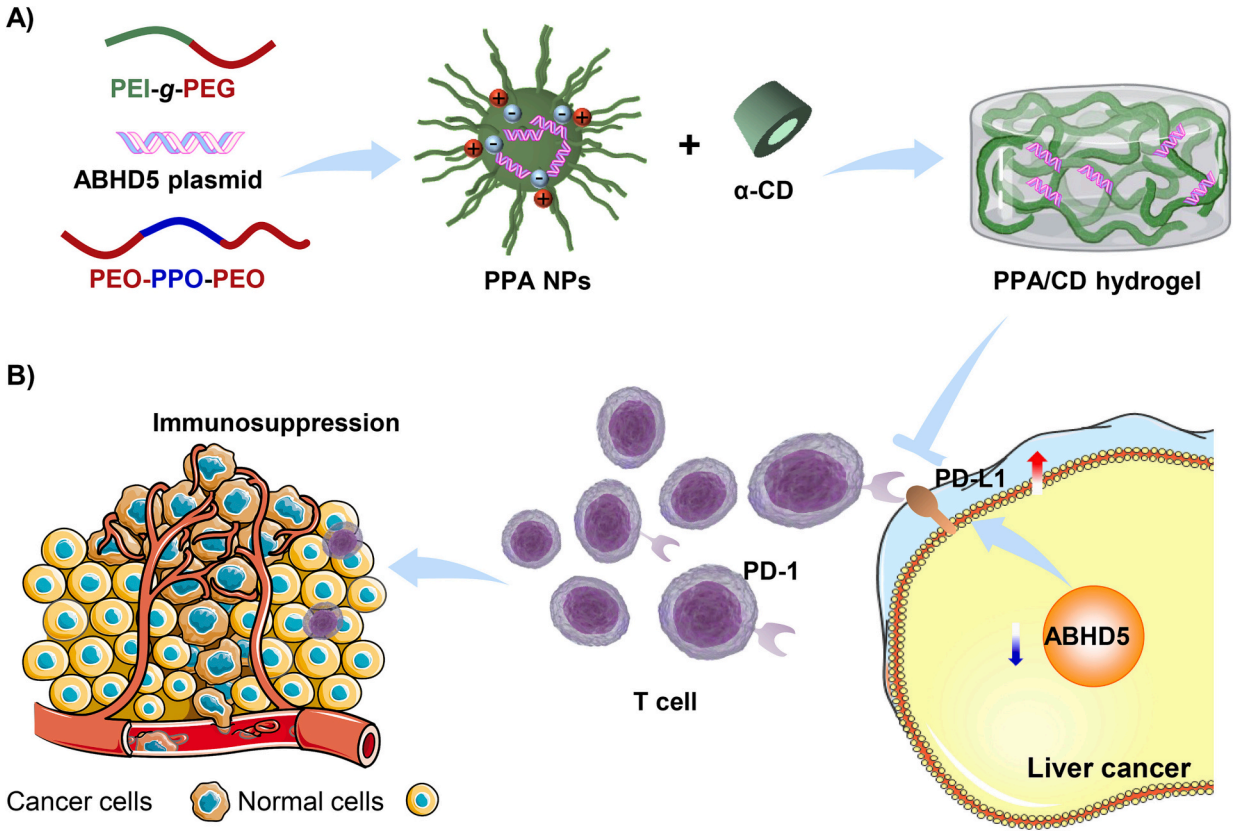
employed tool in gene therapy, exhibit a high degree of immunogenicity and thus pose potential hazards, including cytokine storms (Anguela and High, 2019; Riyad and Weber, 2021). Another deadly flaw with viral vectors is off-target effect, which have potential to induce adverse health outcomes (Goertsen et al., 2022; Rapti and Grimm, 2021). It is indisputable that viral vectors offer significant clinical transformative value as a result of their high transfection efficiency as well as the simplicity of their manufacturing on a large scale (Zolotukhin and Vandenberghe, 2022). In fact, a number of clinical investigations have already made use of adeno-associated virus (AAV) for many treatments (Mendell et al., 2021).

Considering the good biocompatibility, enhanced specific capability and the improved delivery efficacy of non-viral nanomaterials in the gene delivery, the recent progress demonstrates the outstanding performance of non-viral vectors in gene therapy (Cao et al., 2021; Zhou et al., 2017). Cationic supramolecular polymers as the typical non-viral nanomaterials have shown promising perspectives in gene deliver. The unique nano-size of supramolecular polymers could favor passive targeting through the enhanced permeability and retention (EPR) effect, which viral vectors do not possess this capacity (Cheng et al., 2022; Cheng et al., 2018; Hatakeyama et al., 2011). In addition, supramolecular polymers can be readily adapted to function as gene carriers and to facilitate the design of multifunctional joints, such as the simultaneous delivery of genes and drugs for synergistic therapeutic effects (Wang et al., 2017).

Supramolecular hydrogels are a typical kind of polymer used in biological applications. As an in situ treatment method, supramolecular hydrogels can achieve the slow drug release and multiple drug combination therapy (Bernhard and Tibbitt, 2021; Hu et al., 2018). Particularly, prolonged drug release can be accomplished using supramolecular hydrogel materials. On the one hand, it can increase the therapeutic effectiveness and bioavailability of medications (Zou et al., 2021). On the other hand, it may lessen the adverse effects of systemic drug distribution and enhance biological safety, which might improve the prognosis of the patients (Bercea, 2022; Chan et al., 2018). In recent years, there has been considerable interest in the application of cyclodextrin-based supramolecular hydrogel for cancer treatment due to their high drug loading and responsive drug release. Wu et al., reported alpha cyclodextrin (α-CD) could be assembled into a supramolecular hydrogel with PEG chain through host-guest interaction (Liu et al., 2017; Liu et al., 2019), and this specific polypseudorotaxane hydrogel has been numerously reported (Domiński et al., 2019; Hwang et al., 2021; Lan et al., 2021). In this study, we conducted an injectable cyclodextrin-based supramolecular hydrogel (Scheme 1), which comprised with branched polyethyleneimine-g-poly (ethylene glycol), poly (ethylene oxide) and poly (propylene oxide) block copolymers and α-CD, the interactions between the host (α-CD) and the guest molecules (PEG chain polymers) are necessary for the assembly of the supramolecular hydrogel (PPA/CD).

Abhydrolase domain containing 5 (ABHD5) was identified as a cofactor regulating hepatic lipolysis and very low-density lipoprotein (VLDL) secretion, which was associated with non-alcoholic steatohepatitis (Carlsson et al., 2020; Yang and Mottillo, 2020). And the mutation of ABHD5 could contribute to the dyslipidemia and non-alcoholic fatty liver disease (Youssefian et al., 2019). The mechanism studies about ABHD5 demonstrated ABHD5 could facilitate the lipolysis of triacylglycerols (Brown et al., 2007; Caviglia et al., 2009; Yamaguchi et al., 2007). All of these publications suggest that ABHD5 plays an important role in the liver, but there is no evidence demonstrating the role of ABHD5 in hepatocellular carcinoma (HCC). This study firstly reported ABHD5 was an antitumor gene, which was downregulated in liver tumor tissues, and the HCC patients with lower ABHD5 expression also



Scheme 1. Scheme of the injectable hydrogel for sustained gene release to inhibit PD-L1 mediated immunosuppression in liver cancer. (**A**) The scheme of the PPA/CD supramolecular hydrogel construction. (**B**) PPA/CD supramolecular hydrogel for ABHD5 gene delivery to liver tumor to reverse the negative regulation between ABHD5 and PD-L1, and reversed the PD-L1-mediated immunosuppression through enhance tumor killing ability of T cells.

displayed a poor prognosis. In addition, this study also verified ABHD5 as a promising therapeutic target, which could induce the apoptosis of liver tumor cells. Therefore, the delivery of ABHD5 to liver tumor cells might be a feasible strategy for HCC treatment. In this work, the supramolecular hydrogel (PPA/CD) with injectable capability was used for the delivery of ABHD5 gene to treat HCC (Scheme 1). Interestingly, this study also proposed for the first time that ABHD5 could negative regulate programmed death-ligand 1 (PD-L1), and verified that the effective delivery of ABHD5 gene can increase the tumor-killing efficacy of peripheral blood mononuclear cells (PBMC), which are mainly composed with T lymphocytes. In summary, the cyclodextrin-based supramolecular hydrogel with injectable capability could achieve an outstanding gene delivery performance with good gene loading and sustained gene release, and the efficient ABHD5 delivery is a promising strategy for HCC treatment.

2. Material and methods

2.1. Clinical study

The liver tumor tissues from the surgical resection were collected from the Second Affiliated Hospital of Fujian Medical University. A total of 72 cases liver tumor tissues and corresponding adjacent tissues were subjected to the immunohistochemistry (IHC) staining of ABHD5, and the relative staining intensity was scored by three different pathology technologists, and the median level of the score was set as the cut-off to divide the patient group. In detailed, 25 cases of liver cancer patients were identified as the low ABHD5 group, and 47 cases of liver cancer patients as high ABHD5 group. The patient's information was collected to follow-up the statue of overall survival (OS) and progression-free survival (PFS). And the log-rank method was subjected to evaluate the prognostic value of ABHD5 for hepatocellular carcinoma. The human study protocol was reviewed and approved by the Second Affiliated Hospital of Fujian Medical University (Approval number: 20220474), and the patients offered the signed informed.

2.2. Western blotting assay

The liver tumor tissues and cell lines were pre-treated with homogenizer and lysed with RIPA lysis buffer on ice for 30 min, and the protein quantification was achieved by BCA method. The samples with equal protein concentration were subjected to SDS-PAGE electrophoresis. The primary ABHD5 antibody (Cat. ab183739, Abcam, Cambridge, MA, USA) was incubated at 4 °C for overnight, and subjected to secondary antibody for 1 h at room temperature, and ECL detection system (Cat. 36208ES60, Yeasen Biotechnology, Shanghai, China) was conducted to visualize the protein band.

2.3. Flow cytometry analysis

For PD-L1 expression analysis, SMMC7721 and SK-Hep-1 cells were planted in 6-well plate, and the knockdown plasmid of ABHD5 with pLKO.1-GFP-shRNA (Addgene, Cambridge, MA, USA) system (primer for ABHD shRNA, forward: 5'-CCG GGC ACC AAC AGA CCT GTC TAT GCT CGA GCA TAG ACA GGT CTG TTG GTG CTT TTT G-3′, reverse: 5′-AAT TCA AAA AGC ACC AAC AGA CCT GTC TAT GCT CGA GCA TAG ACA GGT CTG TTG GTG C-3′) was transfected with Lipofectamine 3000 transfection reagent (Cat. L3000001, ThermoFisher, USA) and further incubation for 24 h, the cells were collected, and washed with PBS for twice time, the collected cells were suspended with staining buffer and APC-conjugated PD-L1 antibody (Cat. 17–5982-82, ThermoFisher, USA) for 15 min, and the subjected to flow cytometry testing (CytExpert, BD, USA). For gene transfection efficiency evaluation, GFP-ABHD5 plasmid was constructed with pEGFP-C1 vector (Addgene, Cambridge, MA, USA), and transfected with PPA/CD hydrogel, and polyethylenimine (PEI-25 K, Cat. 408,727, Sigma-Aldrich, St Louis, MO, USA) as positive control were also subjected to fluorescence intensity testing by flow cytometry after 48 h incubation. For apoptosis analysis, the SMMC7721 and SK-Hep-1 cells were stained with Annexin-V and PI reagent (Cat. 40304ES60, Yeasen Biotechnology, Shanghai, China) for 30 min, and APC and PE channels were collected by flow cytometry analysis (CytExpert, BD, USA).

2.4. PPA/CD hydrogel preparation and rheological testing

According to the previous reports (Domiński et al., 2019; Hwang et al., 2021; Lan et al., 2021), branched polyethyleneimine-*g*-poly (ethylene glycol) (PEI-*g*-PEG, PEG molecular weight: 5000, PEI molecular weight: 25000) was purchased from Sigma-Aldrich (Cat. 900,743, St Louis, MO, USA). Firstly, PEI-*g*-PEG polymer was prepared at the concentration of 1 mg/mL, and a total 10 μg amount of plasmid was mixed with 100 μL PEI-*g*-PEG polymer solution at room temperature for 30 min. Secondly, poly (ethylene oxide) and poly (propylene oxide) (PEO-PPO-PEO) block copolymers (Cat. P2443, Sigma-Aldrich, St Louis, MO, USA) were mixed with the above solution at the final concentration at 20 μg/mL. Finally, α-CD solution was added at the final concentration of 100 μg/mL at 4 °C overnight, and the complex nanomaterials could assembly to hydrogel. The strain value of the hydrogel was analyzed from 1 to 100% with angular frequency at 10 rad/s. The injectable capability was conducted by shear thinning with increasing shear stress, and the complex viscosity was achieved. Also, the modulus value of hydrogel at 0.1% strain was conducted from different frequency (1–100 Hz) and 500 s time period.

2.5. Evaluation of gene loading efficiency and gene release rate

The gene loading efficiency was achieved by nuclei dye competition binding assay. Simply, the plasmid-containing hydrogels were prepared as above mentioned, and 50 μg and 100 μg amount of PEI-*g*-PEG (PP) polymer were mixed with PEO-PPO-PEO copolymer and α-CD to construct the complex hydrogel. The gene loading was measured through nuclei dye competition binding assay as reported methods (Fan et al., 2018), and the emission fluorescence intensity was measured after 15 min incubation of the above complex materials. And the complex hydrogel was incubated in 2 mL phosphate-buffered saline (PBS) solution at 37 °C, and the supernatant was collected at indicated time, and the DNA concentration was examined by microplate reader at OD260/280. The relative gene release rate was calculated as the ratio of the amount of total released plasmid between the total plasmid amount.

2.6. Cell culture and viability testing

HEK293T (accession number: CRL-11268), SMMC7721 (accession number: CVCL-0534) and SK-Hep-1 (accession number: HTB-52) cells were obtained from American Type Culture Collection (ATCC). These cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) with containing 10% fetal bovine serum (FBS) and 100 U penicillin-streptomycin solution. The cell viability was measured by CCK-8 assay (Cat. 40203ES92, Yeasen Biotechnology, Shanghai, China) as following steps. The cells were plated in 96-wells plate at the density of 5000 cells per well, and the cells were treated with PEI-25 K and PPA/CD hydrogel after 12 h culture. And after 48 h incubation, the cells were incubated with CCK-8 reagent for 3 h, and the OD value at 450 nm was measured to calculate the cell viability rate. The determination of drug concentration and treatment times are based on previous reports (Cheng et al., 2018; Li et al., 2017; Liu et al., 2017).

2.7. Gene delivery efficiency evaluation

The Rellina luciferase reporter assay was conducted to evaluate the gene transfection efficacy as our previous reports (Cheng et al., 2018; Liu et al., 2019). Simply, the cells were plated in 24-wells plate, and after

12 h culture, the cells were treated with containing-Rellina reporter gene plasmid (dose: 2 μg, Addgene, Cambridge, MA, USA) PEI-25 K and PPA/CD hydrogel as the above mentioned in the preparation of PPA/CD hydrogel. After 48 h incubation, the cells were washed with cold PBS solution twice, and the cells were lysed with lysis buffer for 30 min on ice, and the luciferase substrates were incubated with the cell lysis for 30 min, the relative light unit (RLU) was measured by bioluminescence microplate reader. And the protein concentration was measured by BCA method, the ratio of RLU and total protein (RLU/mg) was applied to evaluate the relative transfection efficacy. Furthermore, the GFP-expressing plasmid (pEGFP-C1 vector, 2 μg) using PEI-25 K and PPA/CD hydrogel were also conducted as the cell processing process of above mentioned in Rellina reporter gene assay, and the cells were collected for flow cytometry testing.

2.8. Apoptosis analysis of tumor cells by co-culture with PBMC

SMMC7721 and SK-Hep-1 cells were plated in 6-wells plate, after 12 h culture, the cells were treated with PPA/CD@ABHD5 (ABHD5 plasmid: 2 μg) and PPA/CD hydrogel alone for 48 h incubation. Then, PBMC cells were co-cultured with the pre-treated cells for 24 h, and the cells were collected by cell scraper. The collected cells were washed with cold PBS solution for twice times, and stained with Annexin-V-PI staining reagent for 30 min, and then subjected to flow cytometry testing. The PBMC cells were extracted from the blood of HCC patients according to the protocol of PBMC extraction kit.

2.9. Bioinformatic analysis of ABHD5

The ABHD5 expression in various liver cancer cell lines, which was obtained from the CCLE dataset (https://portals.broadinstitute.org/ccle), and a total of 25 liver tumor cell lines were subjected to evaluate the ABHD5 mRNA expression distribution. The analysis was conducted by the R v4.0.3 software package ggplot2 (v3.3.3).

2.10. Statistical analysis

The survival analysis was conducted with log-rank method and hazard ratio with 95% confidence interval (CI), and the median level of ABHD5 score was set as the cut-off for grouping. The difference between two groups were calculated by Mann-Whitney test with unpaired two-tailed p value. And $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ was considered the significant statistical difference. GraphPad Prism (Version 8.0) software (GraphPad Software, San Diego, CA, USA) was subjected to perform the statistical analysis.

3. Results and Discussions

3.1. ABHD5 as an antitumor gene in HCC

To validate the clinical value of ABHD5 in HCC, this study firstly evaluated the ABHD5 expression in 12 cases liver tumor tissues (T) and corresponding adjacent tissues (N) by western blotting, and the results showed ABHD5 protein was highly expressed in adjacent normal tissues

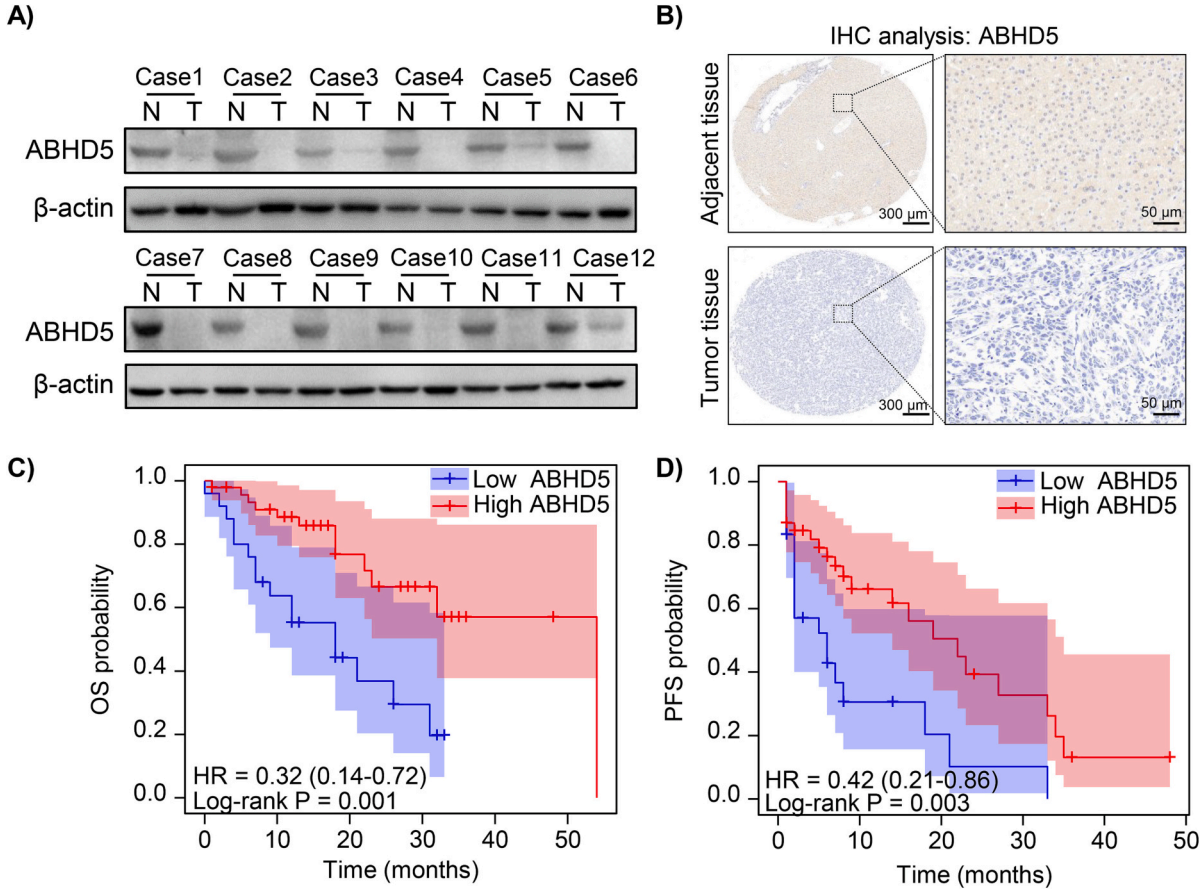


Fig. 1. Dysregulation of ABHD5 in liver cancer. (**A**) A total of 12 cases HCC samples with tumor tissues (T) and corresponding adjacent normal tissues (N) were subjected to western blotting for the analysis of ABHD5 protein. (**B**) Immunohistochemistry (IHC) staining analysis of the liver tumor tissues and adjacent normal tissues. (**C**) A total of 72 cases tumor tissues from HCC patients were included to the overall survival (OS) and (**D**) progression-free survival (PFS) analysis based on the ABHD5 expression statue. And the median level of ABHD5 was set as the cut-off for high ($n = 47$) and low ($n = 25$) ABHD5 group, the log-rank method was conducted for the statistical analysis between the two groups.

(Fig. 1A). To comprehensively define the expression statue of ABHD5 in HCC, including the protein location, IHC staining of ABHD5 was conducted in 72 cases HCC samples, the immunohistochemistry staining intensity was strong in adjacent normal tissues compared with liver tumor tissues (Fig. 1B). Furthermore, ABHD5 was both expressed in cytoplasm and nucleus. Considering the significant different expression of ABHD5 in adjacent normal and tumor tissues, this study present hypothesis that ABHD5 might be an antitumor gene in HCC. To confirm the hypothesis, the effect of ABHD5 expression on the prognostic performance of HCC was performed, and the overall survival (OS) analysis demonstrated the patients with low ABHD5 expression exhibiting the poor OS rate. Compared with the low ABHD5 group, the hazard ratio (HR) of OS was 0.32 ($p = 0.001$), which was significantly lower than 1.0, suggesting that ABHD5 was a favor prognostic biomarker for HCC (Fig. 1C). Similarly, the progression-free survival (PFS) analysis also verified the finding of ABHD5 as a significant favor prognostic factor for HCC (Fig. 1D). From all above findings, ABHD5 was downregulated in HCC, and the lower ABHD5 showed the poor survival rate, demonstrating ABHD5 was a potential therapeutical target for HCC.

3.2. Decreased ABHD5 induces PD-L1 expression in liver cancer

ABHD5 was lowly expressed in liver tumor tissues identified as a novel therapeutical target for HCC. To further study the underlying mechanism about the downregulation of ABHD5 in HCC and the potential association between the tumor immune checkpoint molecules. This study further analyzed the effect of ABHD5 on the programmed death-ligand 1 (PD-L1), which is a typical immune checkpoint and the known target of immune checkpoint blockade (ICB) therapy. Firstly, the CCLE dataset was used to collect information regarding the expression distribution of ABHD5 mRNA in several liver tumor cells, and the results showed ABHD5 was also expressed in various liver tumor cells, and it was expressed to a moderate degree in SK-Hep-1 cells (Fig. 2A). Consequently, SK-Hep-1 and SMMC7721 cells with similar ABHD5 expression were selected for the follow-up research. As shown in Fig. 2B-C, the ABHD5 protein level of SK-Hep-1 and SMMC7721 cells was significantly decreased in second ABHD5 knocking down group, and the second ABHD5 knockdown sequence was included to the subsequent study. Interestingly, the significant decreased ABHD5 expression could induce PD-L1 expression in both SK-Hep-1 and SMMC7721 cells. Furthermore, the inducible PD-L1 expression after ABHD5 knockdown

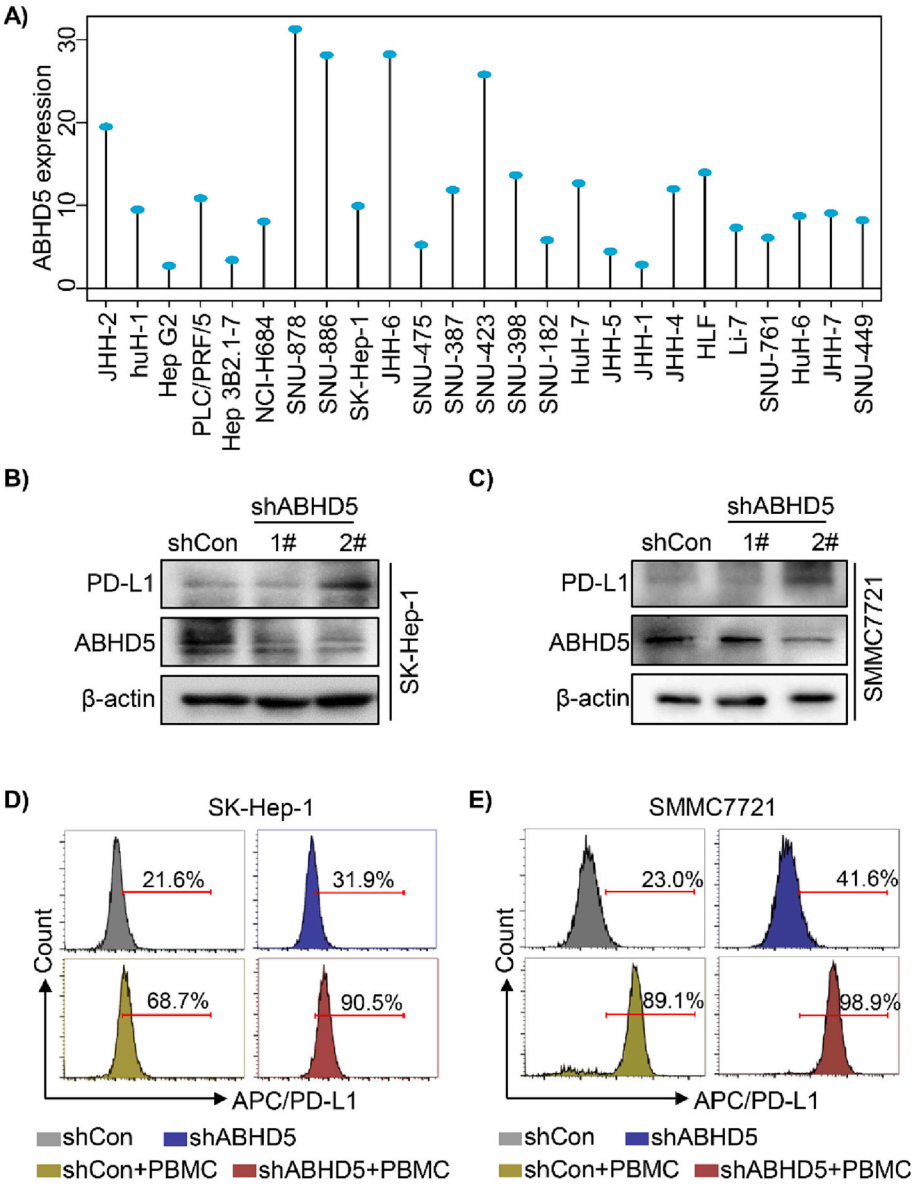


Fig. 2. Downregulation of ABHD5 induces PD-L1 expression in liver cancer. (**A**) The CCLE dataset was used to determine the ABHD5 mRNA expression pattern in different liver tumor cells lines. (**B**) SK-Hep-1 cells and (**C**) SMMC7721 cells were subjected to knockdown analysis of ABHD5, after transfected knockdown ABHD5 (shABHD5) for 24 h, the cells were collected for PD-L1 analysis by western blotting. (**D**) Flow cytometry analysis of PD-L1 expression in SK-Hep-1 and (**E**) SMMC7721 cells, after knockdown of ABHD5 for 24 h, the cells were co-cultured with peripheral blood mononuclear cells (PBMC) or not. With further 24 h culture, the cells were stained with APC conjugated PD-L1 antibody and the subsequent flow cytometry analysis.

was examined by flow cytometry in SK-Hep-1 and SMMC7721 (Fig. 2D-E). The study further verified the inducible effect after co-culture with peripheral blood mononuclear cells (PBMC). When interaction with PBMC, the PD-L1 expression in tumor cells was increased, and an enhanced effect on PD-L1 expression was found in ABHD5 knocking down group. From above evidence, the knockdown of ABHD5 could induce PD-L1 expression. Considering the decreased ABHD5 in liver tumor tissues (Fig. 1) and negative regulation on PD-L1 expression, ABHD5 might be a therapeutic target for HCC immunotherapy based on PD-L1.

3.3. *Efficient gene loading and sustained gene release by the injectable hydrogel*

Considering the significant value of ABHD5 in HCC, ABHD5 gene delivery could be a potential therapeutical platform to inhibit liver tumors. Based on the outstanding performance of hydrogel in the drug delivery, such as the sustained drug release and the enhanced bioavailability. This study constructed an assembly hydrogel with complex nanomaterials of branched polyethyleneimine-g-poly (ethylene glycol) (PEI-*g*-PEG) and poly (ethylene oxide) and poly (propylene oxide) (PEO-PPO-PEO) block copolymers and α-CD through host-guest interaction, the specific assembly was achieved by threading effect between α-CD and linear polyethylene glycol chain (Li and Loh, 2008; Li et al., 2012; Liu et al., 2021; Liu et al., 2017; Liu et al., 2019). The scheme of the prepared hydrogel was shown in Fig. 3A, the assembly complex materials displayed as the gel-like characteristics. Next, the rheological analysis was conducted to evaluate the characteristics of hydrogel. Firstly, the results of strain modulus scanning showed that the strain intensity was over 10%, displaying a good mechanical strength (Fig. 3B). Secondly, the modulus intensity was also measured with an increasing frequency, and the storage modulus (G') and loss modulus (G") exhibited a good stability (Fig. 3C). And the stability test over 500 s was also conducted, and the results also confirmed the good characteristics of the complex hydrogel (Fig. 3D). Moreover, the hydrogel displayed the shear thinning capability with increasing shear stress,

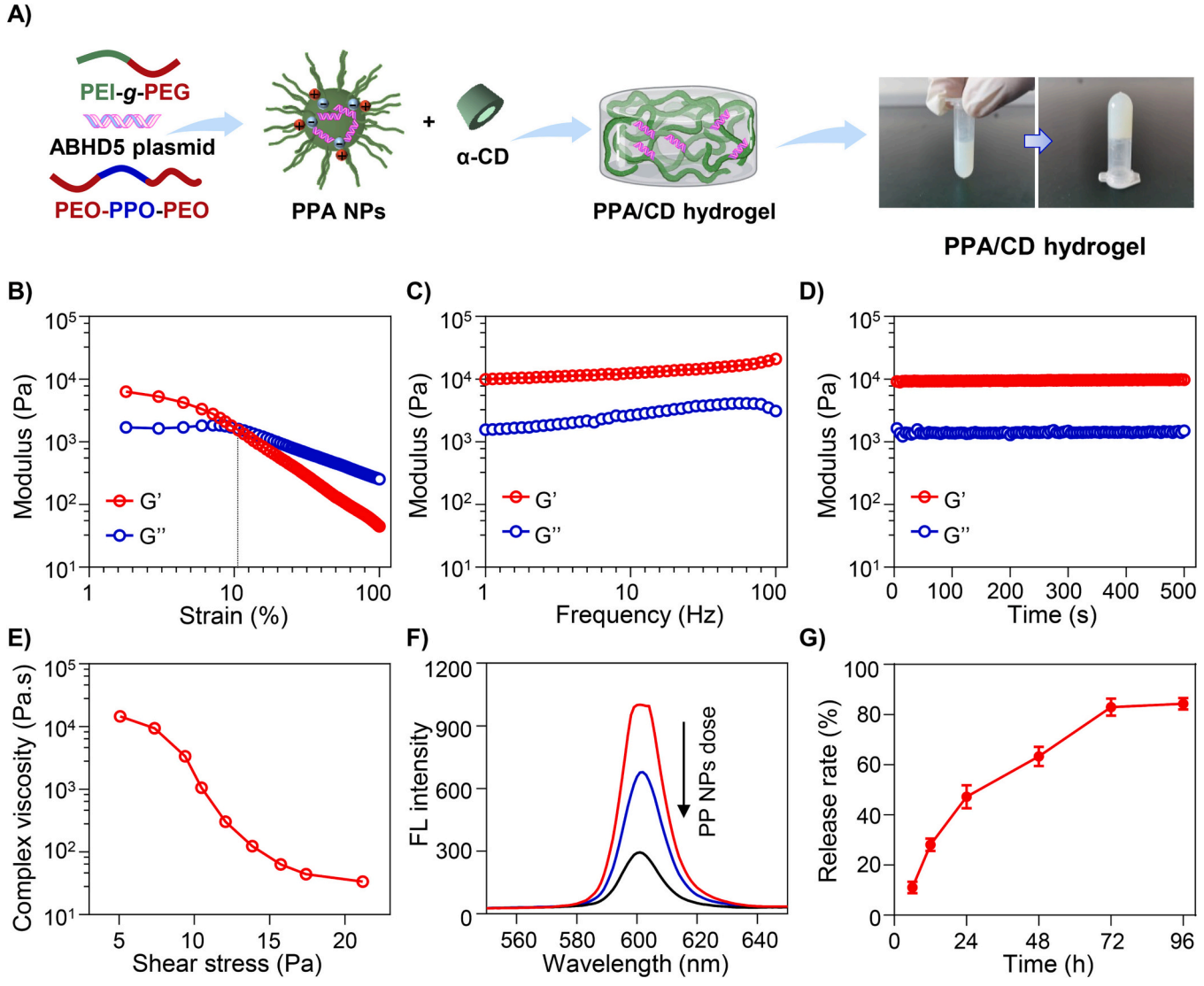


Fig. 3. Physical characterization of the injectable hydrogel for sustained gene release. (**A**) Scheme of the PPA/CD hydrogel preparation, and the photograph of the prepared complex hydrogel. The two nanomaterials and plasmid gene were mixed at indicated concentration for overnight at 4 °C. (**B**) The strain modulus scanning was conducted by rheometer from 1 to 100% with angular frequency at 10 rad/s, the modulus value was measured at 25 °C with CP25 cone plate. (**C**) The frequency and (**D**) time modulus scanning of hydrogel was conducted at 0.1% strain. (**E**) The complex viscosity of the complex hydrogel was measured with increasing shear stress. (**F**) The increasing concentration of PP nanoparticles were subjected to containing-plasmid PPA/CD hydrogel construction, and then nuclei dye competition binding was conducted for 15 min, and then the emission fluorescence intensity (FL) of nuclei dye was measured. (**G**) The plasmid-containing complex hydrogel was incubated in PBS solution at 37 °C, and the supernatant was collected at the indicated time, and the DNA concentration was examined by microplate reader.

suggesting the good injectable performance (Fig. 3E). Overall, the complex hydrogel by host-guest interaction displayed a good gelation property, which could endow the subsequent sustained performance and achieve the enhanced gene delivery efficacy.

Next, the gene loading effciency of complex hydrogel materials was measured by competition binding assay. With the increasing concentration of PEI-b-PEG nanoparticles (PP NPs), the emission fluorescence intensity of nuclei dye showed a decreased fluorescence intensity, suggesting the efficient gene loading with complex hydrogel materials (Fig. 3F). Considering the sustained release capability of hydrogel in drug delivery, the plasmid gene release was examined at indicated time, and the results exhibited a sustained gene release rate. Within 72 h, as much as 80% of plasmid gene is continually released (Fig. 3G). After all, the supramolecular hydrogel was composed of complicated nanomaterials by host-guest interaction, exhibited favorable gelation characteristics, gene loading, and sustained gene release capacity. Since ABHD5 was not a liver-specific gene, and active targeting was not included in the injectable hydrogel. However, the unique nanoscale dimensions of supramolecular polymer could also enhance efficiency of gene by EPR-mediated passive targeting (Cheng et al., 2022). Therefore, we believe it could still work in vivo application of HCC. Local treatment is the most common method in the clinical treatment of liver cancer, such as hepatic artery embolization, ablation therapy, etc. (Chen et al., 2020). Due to the good injectability and embolic properties of hydrogel, it can be used in the treatment of liver cancer through local drug delivery to realize the efficient delivery of ABHD5 gene.

3.4. The injectable hydrogel shows the good biocompatibility and gene delivery efficiency

Firstly, this study examined the biocompatibility and gene transfection efficacy of PPA/CD hydrogel in HEK293T normal cells, and SMMC7721 tumor cells, and SK-Hep-1 tumor cells. As the golden standard, PEI-25 K was conducted as a positive control. As shown in Fig. 4A-C, the cell viability of PPA/CD hydrogel treatment was significantly higher than the treatment with PEI-25 K, which was consistent with

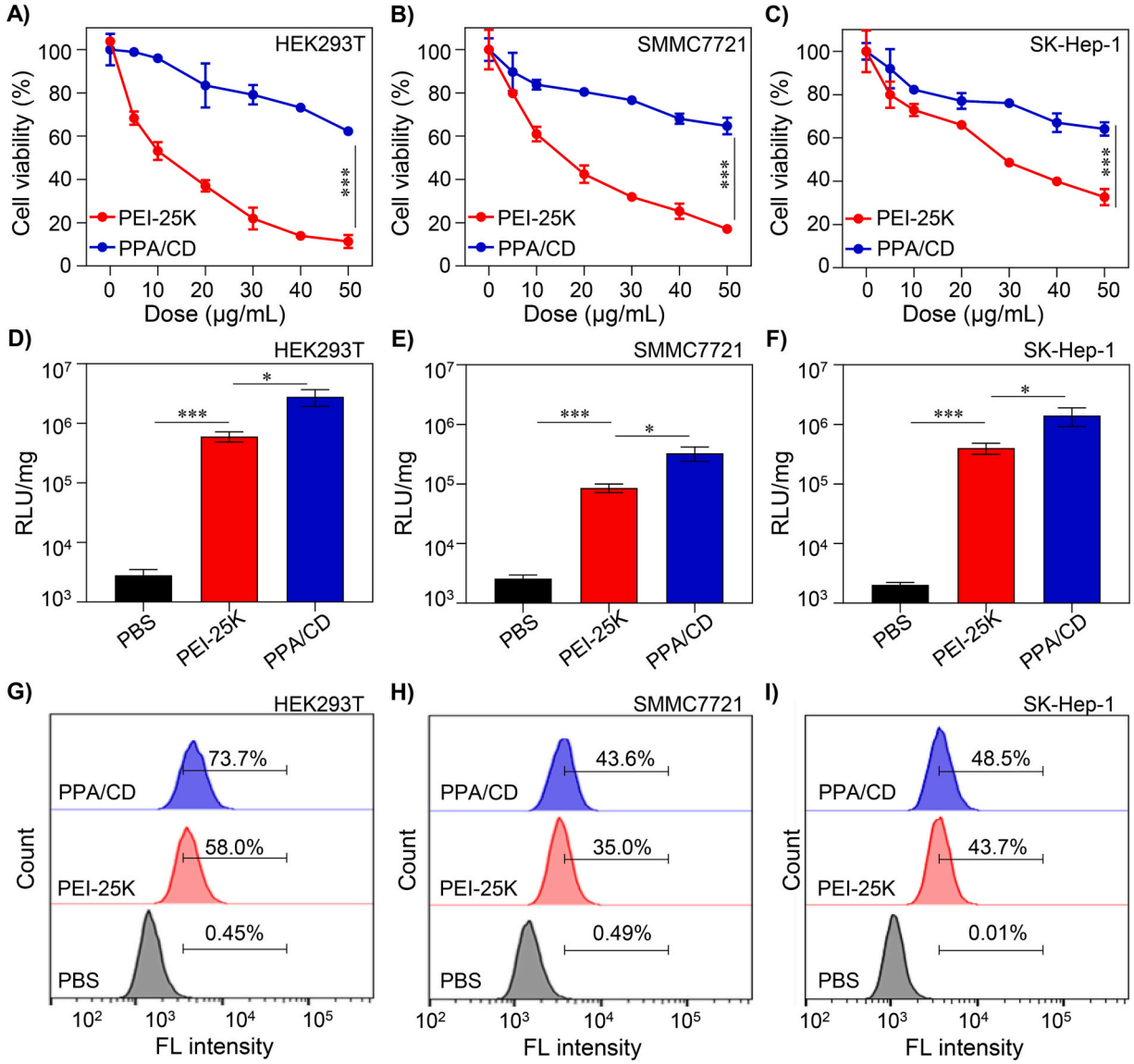


Fig. 4. The cellular biocompatibility and gene delivery efficiency evaluation of the injectable hydrogel in vitro. (**A**) HEK293T and (**B**) SMMC7721 and (**C**) SK-Hep-1 cells were subjected to examine the cell viability after the treatment with PEI-25 K and PPA/CD hydrogel for 48 h. (**D**) The Rellina luciferase reporter assay was subjected to test the efficacy of transfection in HEK293T and (**E**) SMMC7721 and (**F**) SK-Hep-1 cells. The Rellina luciferase reporter plasmid was transfected with PEI-25 K and PPA/CD hydrogel for 48 h. (**G-I**) HEK293T, SMMC7721, and SK-Hep-1 cells were transfected for 48 h with a GFP-expressing plasmid using PEI-25 K and PPA/CD hydrogel, and then the cells were collected for flow cytometry analysis.

previous reports, PEI-25 K had a poor biocompatibility (Cheng et al., 2018; Hashemzadeh et al., 2021; Ke et al., 2021). In addition, the transfection efficacy was evaluated using a luciferase reporter system, and the findings demonstrated that the luciferase intensity of the PPA/CD hydrogel treatment group was able to exhibit a higher intensity than PEI-25 K (Fig. 4D-F). It is possible that the good biocompatibility of the PPA/CD hydrogel contributed to the improved gene transfection efficacy. And the similar finding was also verified in a GFP-expressing system, the finding demonstrated the fluorescence intensity of PPA/CD hydrogel treatment was higher than PEI-25 K in HEK293T, SMMC7721, and SK-Hep-1 cells (Fig. 4G-I). Overall, the injectable PPA/CD hydrogel with sustained gene release had a good biocompatibility and gene transfection efficacy, it is a promising gene delivery platform for gene therapy.

3.5. *The injectable hydrogel exhibits a favor antitumor response*

ABHD5 was a potential therapeutic target for HCC immunotherapy based on PD-L1, and the PPA/CD supramolecular hydrogel displayed a good gene delivery capability. The SMMC7721 and SK-Hep-1 tumor cells were incubated with ABHD5 plasmid-containing PPA/CD hydrogel (PPA/CD@ABHD5), and the further co-culture with PBMC to examine the tumor apoptosis by PBMC-mediated immunotherapy (Fig. 5A). As shown in Fig. 5B-C, the efficient ABHD5 gene alone by PPA/CD hydrogel could remarkably induce apoptosis in both SMMC7721 and SK-Hep-1 cells, which further confirmed that ABHD5 was a potential antitumor gene. As indicated design, the PBMC treatment was able to significant induce the apoptosis of tumor cells, and the combination with ABHD5 gene therapy demonstrated a synergistic effect on cell viability inhibition. The major cells of PBMC are lymphocytes, which including T cells, B cells and NK cells, the PD-1 expressing T cells contribute to the immunosuppression of PD-L1 positive tumors (Chinnadurai et al., 2021; Liang et al., 2022; Pisani et al., 2022). ABHD5 was downregulated in liver tumor and showed a negative regulation on PD-L1 expression. Therefore, the efficient ABHD5 gene delivery was able to decrease the PD-L1 expression of tumor cells, and further enhance the antitumor response of T cells of PBMC, which could impair the immunosuppression by PD1/PD-L1interaction.

4. Conclusions

In summary, this study firstly reports a cyclodextrin-based supramolecular hydrogel for ABHD5 gene delivery in HCC therapy. The host-guest interaction of PPA/CD hydrogel endows a good gene loading, sustained gene release, and stable mechanical properties. The efficient gene delivery by the injectable supramolecular hydrogel could induce the apoptosis of liver tumor cells, and facilitate the tumor-killing efficacy of PBMC-based immunotherapy.

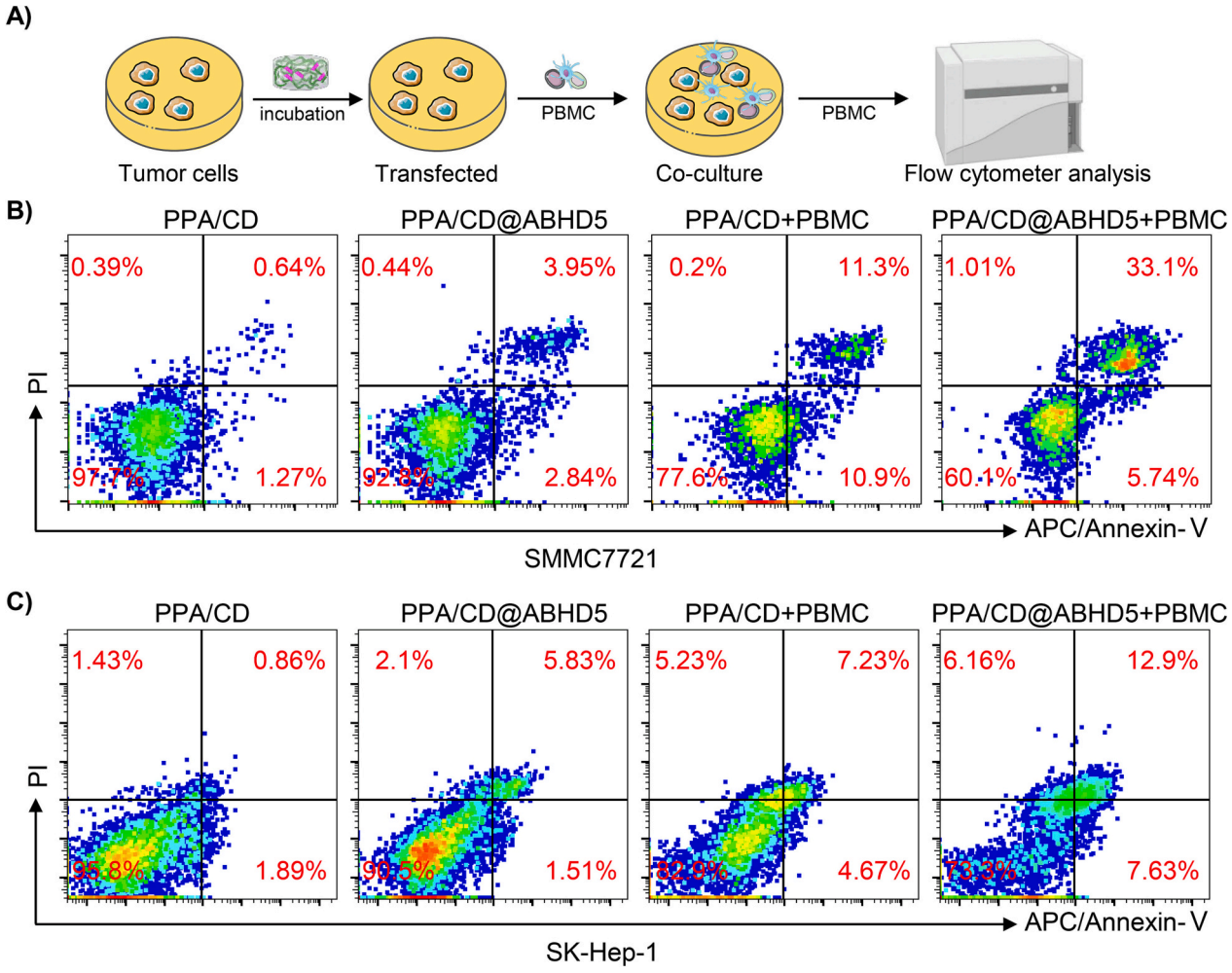


Fig. 5. Efficient ABHD5 plasmid delivery by the injectable hydrogel could enhance antitumor response to PBMC by analyzing tumor cell apoptosis. (**A**) The scheme of the co-culture with PBMC to examine the antitumor response with Annexin V-PI staining analysis by flow cytometry. (**B**—**C**) The SMMC7721 and SK-Hep-1 cell lines were subjected to a 48 h incubation period with PPA/CD@ABHD5 and PPA/CD hydrogel, respectively. Following this, the tumor cells were collected and subjected to Annexin V-PI staining analysis after being co-cultured with PBMC for 24 h.

Author contributions

Conceptualization, H. Cheng, S. Xu. and W. Wang; methodology, S. Xu and J. Cai; software, J. Cai; validation, S. Xu and J. Cai; formal analysis, S. Xu; resources, S. Xu; data curation, S. Xu and J. Cai; writing-original draft preparation, S. Xu and J. Cai; writing-review and editing, H. Cheng and W. Wang; visualization, J. Cai and W. Wang; supervision, H. Cheng and W. Wang; project administration, S. Xu and W. Wang; funding acquisition, S. Xu and W. Wang All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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