MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 4573-4582
DOI: 10.12659/MSM.910903

Received: 2018.04.30
Accepted: 2018.06.18
Published: 2018.07.03

Programmed Death Receptor 1 (PD1) Knockout and Human Telomerase Reverse Transcriptase (hTERT) Transduction Can Enhance Persistence and Antitumor Efficacy of Cytokine-Induced Killer Cells Against Hepatocellular Carcinoma

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Source of support: The project was supported by grants from the National Natural Science Foundation of China (81372604, 91742203)

Background: The weak antitumor efficacy and limited lifespan are the main obstacles that hinder the therapeutic effect of cytokine-induced killer (CIK) cell immunotherapy. In the study, we enhanced the persistence and the antitumor efficacy of CIK cell through PD-1 knockout and hTERT transduction.

Material/Methods: CIK cells were cultured from patients with hepatocellular carcinoma and PD-1 gene was knocked out through the Cas9 ribonucleoproteins (Cas9 RNPs) electroporation. TIDE assay, T7E1 mismatch cleavage assay, and clone Sanger sequencing were used to detect PD-1 knockout efficiency. The immunophenotype was analyzed by flow cytometry. After PD-1 knockout, the hTERT gene was transduced into PD-1 KO/CIK cells with lentiviral transduction. The hTERT expression and persistence of hTERT/PD-1 KO/CIK cells were evaluated by Western blotting and proliferation curve. The antitumor efficacy was detected by ELISPOT and cytotoxicity assay. The telomere length was measured by the Q-FISH and qPCR method. The karyotype assay was used to analyze the chromosome structural stability.

Results: The optimal knockout efficiency of PD-1 gene in CIK cells could reach 41.23±0.52%. PD-1 knockout did not affect the immunophenotype of CIK cells. The hTERT transduction enhanced persistence and increased the telomere length. ELISPOT and cytotoxicity assay showed hTERT/PD-1 KO/CIK cells had an enhanced antitumor efficacy. Meanwhile, PD-1 KO/CIK cells transduced with hTERT showed a normal karyotype.

Conclusions: PD-1 knockout combined with hTERT transduction could prolong the lifespan and enhance antitumor efficacy of CIK cells against hepatocellular carcinoma cell line.

MeSH Keywords: Carcinoma, Hepatocellular • Cytokine-Induced Killer Cells • Programmed Cell Death 1 Receptor • Telomerase

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/910903

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Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, ranking fifth in prevalence and second in mortality [1]. The 5-year survival rate of HCC patients remains about 10–20% [2]. To improve the survival rate, scientists have developed a variety of novel treatments, of which, adoptive CIK cell therapy is one of the promising [3]. CIK cells as adjuvant therapy for patients with HCC could significantly improve their median survival time and reduce the tumor recurrence rate [4]. However, the killing function of CIK cells is not very powerful, and the CIK cells cannot persist *in vivo* very long. These are the main obstacles that limit the antitumor efficacy of CIK cells and so their clinical application.

PD-1, a T cell surface inhibitory receptor, is mainly expressed on activated T cells [5], and it is also one of the molecular markers of T cell exhaustion [6]. PD-1 exerts negative effects on the effector function of $CD8^+$T cells and blockade of PD-1 with antibodies could improve the function of intratumoral effector T cells [7]. Some researchers have proved that PD-1 knockout using the gene editing technology such as the CRISPR/Cas9 system could enhance antitumor efficacy of primary T cells and Chimeric Antigen Receptor (CAR) T cell [8,9]. However, the study on the function of PD-1 knockout CIK cells has not been reported. Here we hypothesize that PD-1 knockout can enhance the antitumor efficacy of CIK cells.

Another factor that affects the therapeutic effects of CIK cells is the limited replicative lifespan, which can lead to the replicative senescence in CIK cells. Senescent CIK cells have lost the proliferative capacity and antitumor efficacy. The lifespan of the cells has been found to be related to telomere length, which can be increased by the hTERT gene. Longer telomeres of the infused cells have been found to be associated with objective response of cell transfer therapy in patients with metastatic melanoma [10].

The aim of our study was to develop an efficient and feasible strategy to knock out the PD-1 gene and transduce the hTERT gene into CIK cells. On this basis, we also investigated whether the Cas9 RNP-mediated PD-1 knockout in CIK cells could enhance their antitumor ability and hTERT transduction could prolong the lifespan of PD-1 KO/CIK cells. Through our study, we hope to develop a new adoptive immunotherapeutic strategy for HCC patients with CIK cells modified by CRISPR technology and hTERT transduction.

Material and Methods

Reagents and cell culture

Human peripheral blood was obtained from HCC patients of Beijing Shijitan Hospital, Capital Medical University. Written informed consent was obtained from these patients, and the study was approved by the hospital ethics committee. The human hepatocellular carcinoma cell line SMMC-7721 was purchased from American Type Culture Collection (ATCC) and cultured in DMEM high-glucose medium (GIBCO, US) supplemented with 10% FBS (GIBCO, US), 100 U/ml penicillin, and 100 μg/ml streptomycin; all cells were cultured in a humidified cell incubator at 37°C and 5% CO_2.

Expansion of CIK cells

CIK cells were prepared as previously described [11]. In short, PBMCs separated from peripheral blood by Ficoll-Hypaque gradient centrifugation were suspended in GT-T551 serum-free medium supplemented with 10% FBS and 1000 U/mL IFN-γ (PeproTech, US). The next day, 50 ng/mL anti-CD3 antibody (eBioscience, US) and 100 U/mL recombinant human IL-2 (eBioscience, US) were added to the cell culture medium. Half of the volume of the cell culture medium was exchanged with the fresh GT-T551 serum-free medium (Takara, Japan) containing 100 U/mL recombinant human IL-2 every 2 days to maintain the cell concentration at 2×10^6 cells/ml. CIK cells were collected on the 14^{th} day to analyze the phenotype and cytotoxicity of CIK cells.

In vitro transcription of sgRNAs

Three gRNAs (Supplementary Table 1) were designed with 2 CRISPR design tools (*http://crispr.mit.edu* and *https://portals.broadinstitute.org/gpp/public/*). PX330 plasmid (Addgene plasmid #4223) was used as a template and the T7 promoter+20-bp target sequence oligonucleotide +20-bp sgRNA scaffold was used as forward primer (Supplementary Table 1). After the *in vitro* transcription template of T7-sgRNAs was amplified by PCR, the sgRNAs were transcribed *in vitro* using a HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB, US). The *in vitro* transcription single-guide RNAs (IVT sgRNAs) were purified by using RNA clean & concentratorTM-25 (Zymo Research, US), eluted in RNase-free water, and used immediately after elution or stored at –80°C.

Preparation of PD-1 knockout CIK cells

PD-1 knockout CIK cells were obtained by the electroporation of Cas9 RNPs as described in a previous report [12]. 5×10^6 CIK cells were transfected with 10 μg Cas9 protein (NEB, US) and 12 μg IVT sgRNA using 4D-Nucleofector System X (Lonza,

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Germany). After electroporation, the cells were resuspended in 2.5 ml pre-warmed cell culture medium in 6-well cell plates. 48 h later, the cells were harvested for the next experiments.

TIDE analysis, T7E1 mismatch cleavage assay, and Sanger sequencing

The mutations were checked by TIDE analysis, T7E1 mismatch cleavage assay, and clone sequence analysis. Cells were harvested 48 h after electroporation and genomic DNA was extracted. The genome region, including the PD-1 knockout site, was amplified and Sanger sequencing was performed on the purified PCR products. The sequencing results were used to perform TIDE analysis with online TIDE software (*http://tide.nki.nl*) to assess the knockout efficiency. A T7E1 assay of the purified PCR product was also performed using an EnGen Mutation Detection Kit (NEB, US) to validate the knockout efficiency. To analyze the indels of the PD-1 target site, the purified PCR fragment was cloned into the pGEM-T EASY vector (Promega, US) to assess the mutant alleles. Using blue-white screen experiments, a total of 40 positive colonies were obtained and sequenced by using universal primer M13F. The primer sequences used in these assays were listed in Supplementary Table 1.

Flow cytometry

The immunophenotype of wild-type CIK cells and PD-1 knockout CIK cells were analyzed using flow cytometry. Briefly, 5×10^6 wild-type CIK cells and PD-1 KO/CIK cells were resuspended in 100 µL PBS buffer. The monoclonal antibodies CD3-PerCP-Cy5.5, CD4-FITC, CD8-APC, CD56-PE, CD45RO-PE, CD45RA-FITC, CD27-Brilliant Violet 421, and CD279(PD-1)-PE were added. All antibodies were purchased from BD Bioscience (San Diego, CA, US). Samples were incubated for 30 min at 4°C in the dark and then tested with a FACSAriar flow cytometer. Flow cytometry data were analyzed with FlowJo v.10.2 (TreeStar, USA).

Lentiviral transduction of hTERT gene into the PD-1 KO/CIK cells

To transduce hTERT gene into PD-1 KO/CIK cells, the lentiviral transduction was used as described in a previous study [13]. The hTERT lentiviral particles were produced by a lipofectamine transfection system with hTERT lentiviral vector (Hanbio Biotechnology, China). Lentiviral particles were concentrated and virus titration was examined with HIV-1 p24 core profile ELISA (PerkinElmer, Waltham, MA, US). After electroporation of Cas9 RNPs, PD-1 KO/CIK cells were transduced with hTERT lentiviral particles at multiplicity of infection of 50. The cells are harvested for the next step in our experiments 96 h later.

Western blot analysis

The hTERT expressions were detected by Western blotting and compared to β-actin, as previously described [14]. The proteins were obtained from the cell and quantified. After separation using SDS-PAGE, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked using 5% non-fat milk for 1 h at room temperature and then incubated with rabbit anti-hTERT antibody (Abgent, US) overnight at 4°C followed by incubation with an infrared secondary antibody (LI-COR, US) for 1 h at room temperature prior to detection with the Odyssey imager (LI-COR, US). ImageJ software was used to assay the intensity of each band.

Cell proliferation assay

Based on the cell number count, the proliferation curves were obtained by the population doubling (PD) level. The PD levels were determined using the formula: $PD(n)=\log2(Nn/N_0)$ (n, passage; Nn and N_0 are the number of cells at the passage n and passage 0) [15].

ELISPOT assay

We performed an ELISPOT assay to assess IFN-γ production of CIK cells after activation with SMMC-7721 cells. A total of 5×10^3 SMMC-7721 cells (target cells) and 1×10^5 effector cells (wild-type CIK cells, PD-1 KO/CIK cells or hTERT/PD-1 KO/CIK cells) were added to precoated wells at a ratio of E: T=20: 1. After incubation for 16–18 h, the frequency of CIK cells stimulated was measured using an IFN-γ ELISPOT Kit (Dakewei, China). Plates were analyzed using an ELISPOT analyzer (Cellular Technology Ltd., US).

Luciferase-mediated cytotoxicity assay

The cytotoxicity of CIK cells was examined using luciferase-mediated cytotoxicity assay as previously described [23]. In short, the hepatocellular carcinoma cell line SMMC-7721 with luciferase and GFP proteins were established by lentivirus infection. Based on different effect target ratios, SMMC-7721 cells and effector cells (wild-type CIK cells, PD-1 KO/CIK cells or hTERT/PD-1 KO/CIK cells) were mixed and cultured in white 96-well fluorescence-detected plates at 37°C for 16–18 h. After the 100 µL substrate mixture was added, fluorescence values were tested immediately. The killing rate was calculated using the following formula: {%special lysis= [1-(fluorescence value of target cell co-culture well)/(fluorescence value of target cell well)] ×100}.

Measurement of telomere length

Telomere lengths were quantified using Quantitative Fluorescent *in situ* Hybridization (Q-FISH) as previously described. In brief,

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hTERT/PD-1/CIK cells were harvested at 7 days after hTERT transfection and treated with colcemid for 3 h before added into hypotonic KCl solution and fixed in methanol-acetic acid. Cy-3 labeled (CCCTAA)3 PNA probe (Panagene, Korea) was used to bind the telomere in the end of the chromosome. The fluorescence images of telomeres and chromosomes were obtained using a laser confocal microscope using a 100×oil objective. The relative telomere length was determined by fluorescence intensity using TFL-Telo software. Absolute telomere length was measured using the qPCR method described previously [16]. Two oligomer standards were introduced to generate the standard curve of telomere and single copy gene. Based on both standard curves, the absolute telomere length was measured using the PCR data.

Karyotype analysis

Karyotype analysis of hTERT/PD-1 KO/CIK cells was performed using Giemsa staining at 60 days after hTERT transfection. Briefly, preparation of metaphase spreads was performed as the Q-FISH procedure above. After digestion with 0.25% trypsin, G-banding of metaphase slides was obtained by Giemsa staining and analyzed by a karyotyping system from Applied Imaging Corporation.

Statistical analysis

All experiments were performed independently 3 times. The results are presented as mean ± standard error. Two-tailed Student's *t*-test was applied for analyzing the data, which was performed using GraphPad Prism6 software. *P* values of 0.05 or less were defined as statistically significant.

Results

Screen for the most effective sgRNA and generation of PD-1 knockout CIK cells

To achieve effective gene knockout, 3 sgRNAs targeting the first exon of PD-1 (Figure 1A) were designed. Both T7E1 mismatch cleavage assay and TIDE analysis showed that knockout efficiency of sgRNA1 to be highest, reaching 41.23±0.52% (TIDE method) (Figure 1B, 1C). The sgRNA1 target region was amplified and sub-cloned to identify mutant alleles. Of the 40 sequenced alleles, 16 were mutants, confirming the mutation of the PD-1 knockout site. As shown in Figure 1D, all the mutations occurred precisely in the sgRNA1 targeting region.

Phenotype of CIK cells after PD-1 knockout

As shown in Figure 2A, the percentage of $PD\text{-}1^{+}CD3^{+}$ cells in wild-type CIK cells was 4.54±0.28%, but the percentage was 1.81±0.31%in PD-1 KO/CIK cells which was significantly different from that of wild-type CIK cells. The immunophenotype of CIK cells was assessed according to the expression of CD4, CD8, and CD56 and the frequencies of naive T cells ($CD45RA^{+}/CD27^{+}$, TN), central memory T cells ($CD45RA^{-}/CD27^{+}$, TCM), effector memory T cells ($CD45RA^{-}/CD27^{-}$, TEM), and effector T cells ($CD45RA^{+}/CD27^{-}$, Teff). As shown in Figure 2B, 2C, the immunophenotype of PD-1 KO/CIK cells was approximately similar to that of wild-type CIK cells, which was roughly similar to that the CIK cell immunophenotype reported in previous literature [17].

Delivery of hTERT increased hTERT protein expression and enhanced proliferation of PD-1 KO/CIK cells

To verify the feasibility of the lentiviral transduction of hTERT gene to PD-1 KO/CIK cells, we performed Western blotting to assess the expression of hTERT protein. As shown in Figure 3B, 3C, a higher level of hTERT protein can be confirmed significantly in hTERT/PD-1 KO/CIK cells. To assay cell proliferation, PDs were calculated by cell count. As shown in Figure 3A, the CIK cells and PD-1 KO/CIK cells could expand 19–25 population doublings (PDs), whereas hTERT/PD-1 KO/CIK cells continue expand 30–40 PDs, which was a significant difference.

PD-1 knockout enhanced antitumor function of CIK cells whereas hTERT transduction did not affect antitumor function of PD-1 KO/CIK cells.

To assess the functional characteristics of CIK cells, we performed IFN-γ ELISPOT and cytotoxic assays. In the IFN-γ ELISPOT assay, IFN-γ secreting capacity of cells was detected by counting the number of spots. As shown in Figure 3D, there were significantly more spots in PD-1 KO/CIK cells than wild-type CIK cells (90.00±1.52 *vs.* 24.00±2.52), which indicated that knockout of PD-1 enhanced the cellular immune response of CIK cells to HCC cell line. However, the number of spots in hTERT/PD-1 KO/CIK cells was similar to that of PD-1 KO/CIK cells, which showed that hTERT transduction did not alter the IFN-γ secreting capacity of PD-1/CIK cells.

Figure 3E showed luciferase-based cytotoxicity assay against the hepatocellular carcinoma SMMC-7721 cell line. As shown in Figure 3E, the killing efficacy of PD-1 KO/CIK cells derived from patients with HCC were significantly stronger than wild-type CIK cells ($P<0.05$), whereas among PD-1 KO/CIK cells and hTERT/PD-1 KO/CIK cells, there was no difference in killing efficacy, which indicated that hTERT transduction did not alter the cytotoxicity of PD-1/CIK cells.

hTERT transduction could prolong the telomere of PD-1 KO/CIK cells

Figure 4A, 4D showed Q-FISH imagine and relative telomere length of wild-type CIK cells, PD-1 KO/CIK cells, and hTERT/PD-1

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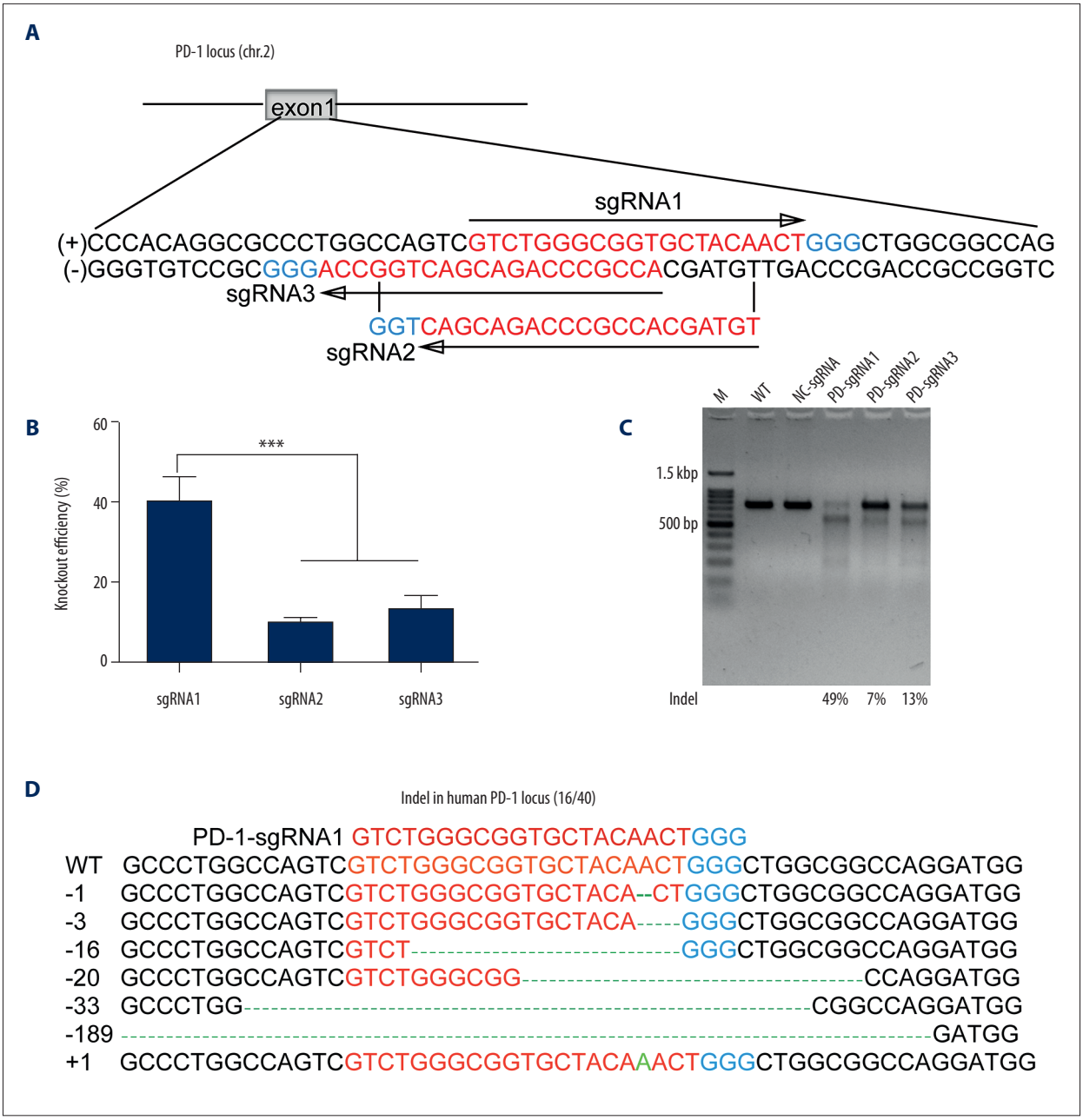

Figure 1. The CRISPR-Cas9 gene editing technique was used to knock out the PD-1 gene in CIK cells. (**A**) Schematic representation of 3 sgRNAs targeting sites on the PD-1 gene. Red indicates the sgRNAs targeting sequence and blue indicates the PAM sequence. (**B**) Analysis of PD-1 knockout efficiency of different sgRNAs by using TIDE method (mean ±SEM, n=3). (**C**) Detection of PD-1 knockout efficiency of different sgRNAs by using T7E1 mismatch cleavage assay. M – marker; WT – wild-type. (**D**) The indel situation of the PD-1 knockout site compared to the wild-type sequence (WT). The sgRNA target site is colored in red, the PAM sequence is colored in blue, and the indel mutations are colored in green; the PCR product of each sample was sub-cloned, and the alleles of each clone were sequenced. 16/40 indicated the number of clones containing the mutant allele in the total clones sequenced. *** $P<0.001$.

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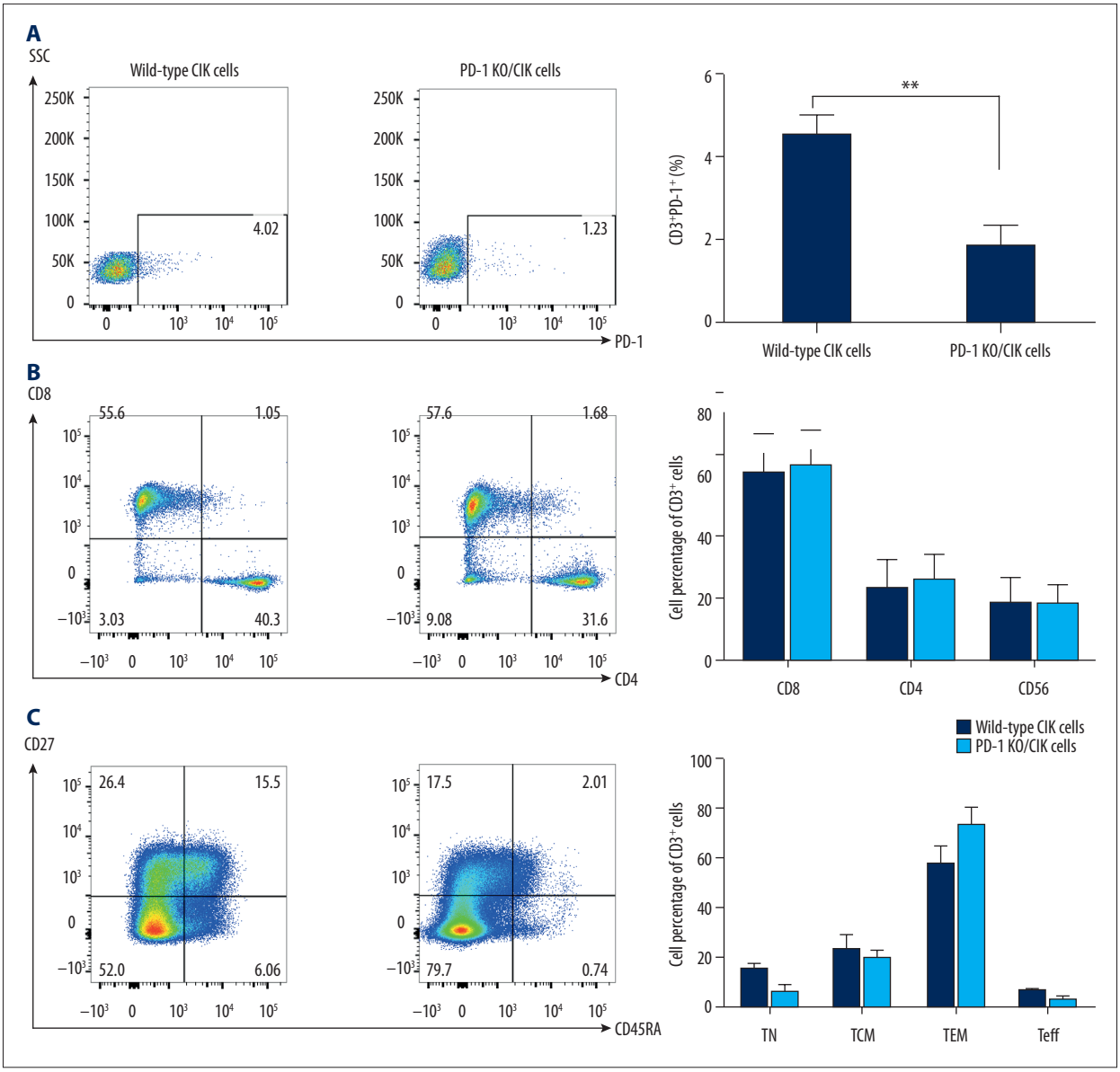

Figure 2. Changes in the immunophenotype of PD-1 KO/CIK cells versus wild-type CIK cells. (**A**) Analysis of PD-1$^+$ cells by gated CD3$^+$ cells. (**B**) Analysis of CD4$^+$, CD8$^+$, CD56+cells by gated CD3$^+$ cells. C) Analysis of CD45RA$^+$/CD27$^+$, CD45RA$^-$/CD27$^+$, CD45RA$^-$/CD27$^-$ and CD45RA$^+$/CD27$^-$ cells by gated CD3$^+$ cells. naive T cells (CD45RA$^+$/CD27$^+$, TN), central memory T cells (CD45RA$^-$/CD27$^+$, TCM), effector memory T cells (CD45RA$^-$/CD27$^-$, TEM) and effector T cells (CD45RA$^+$/CD27$^-$, Teff). The data shown are mean ±SEM of 3 independent experiments. ** P<0.01.

KO/CIK cells. According to the histogram of the telomere fluorescence, the relative telomere length of hTERT/PD-1 KO/CIK cells was significantly longer than that of wild-type CIK cells and PD-1 KO/CIK cells, whereas the relative telomere length of wild-type CIK cells and PD-1KO/CIK cells showed no difference. The result was validated by the findings obtained using the qPCR method (Figure 4B).

hTERT transduction did not affect the karyotype of PD-1 KO/CIK cells

To determine whether hTERT transduction could lead to the malignancy of PD-1 KO/CIK cells, the integrity of the chromosome structure was assessed using a karyotype assay. As shown in Figure 4C, the karyotype assay of hTERT/PD-1 KO/CIK cells demonstrated a normal karyotype without structural chromosome changes.

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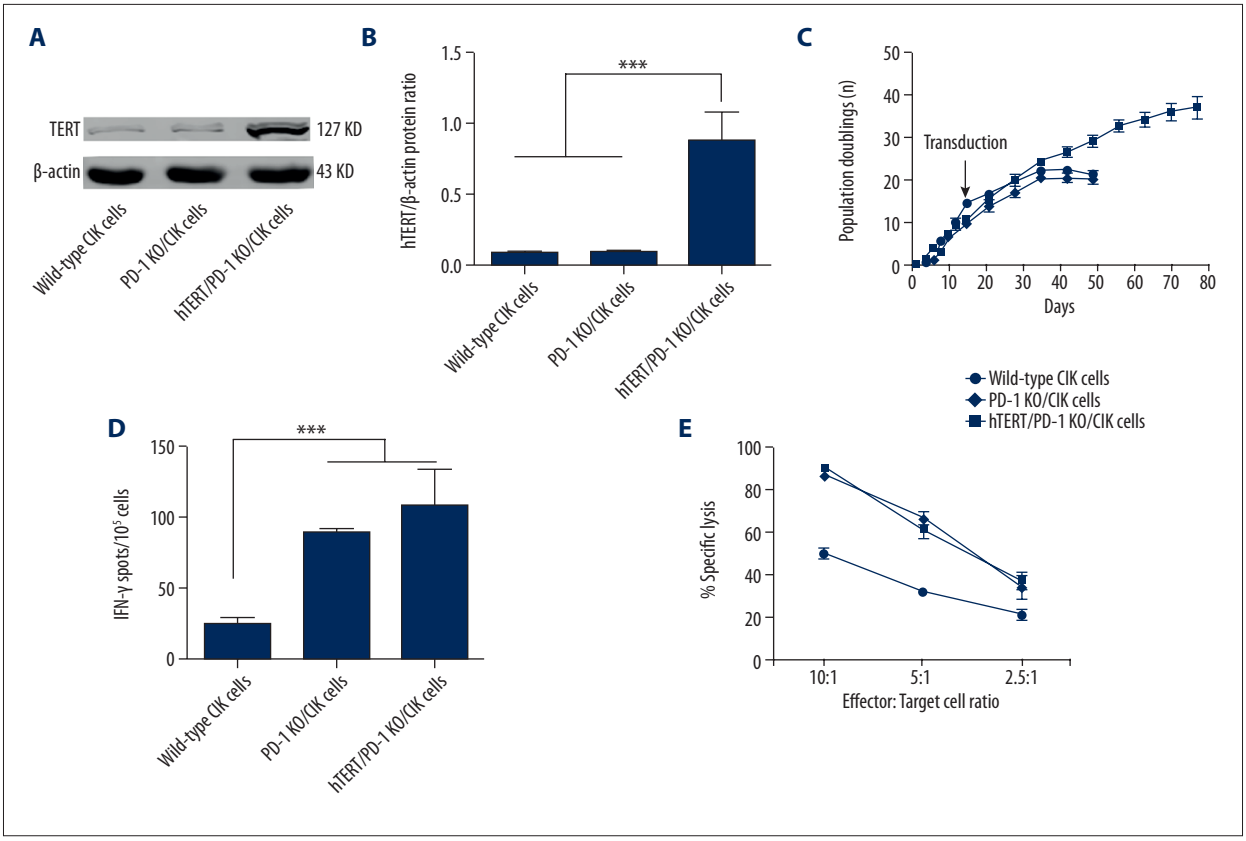

Figure 3. Proliferation and antitumor function of hTERT/PD-1 KO/CIK cells after hTERT transduction. (**A, B**) The expression of hTERT protein was measured using Western blotting. (**C**) Proliferation curves of wild-type CIK cells, PD-1 KO/CIK cells and hTERT/PD-1 KO/CIK cells were obtain based on the PDs. (**D**) IFN-γ secretion of PD-1 KO/CIK cells and hTERT/PD-1 KO/CIK cells was detected by ELISOPT assay after co-culture with SMMC-7721 cell line at E/T ratio (20: 1). (**E**) Cytotoxicity of wild-type CIK cells, PD-1 KO/CIK cells and hTERT/PD-1 KO/CIK cells was evaluated at different E/T ratio after co-culture with HCC cell line SMMC-7721 for 16–18 h. The data shown are mean ±SEM of 3 independent experiments. *** $P<0.001$.

Discussion

In our study, we explore a promising strategy to enhance the antitumor efficacy and the lifespan of CIK cells through CRISPR/Cas9 system and hTERT transduction. First, we screened the optimal sgRNA to knock out PD-1 effectively. Using the sgRNA, we knocked out the PD-1 gene of CIK cells was knocked out at an acceptable rate of efficacy. PD-1 knockout was found to significantly enhance the antitumor efficacy of CIK cells and to have no effect on the phenotype. After PD-1 knockout, PD-1 KO/CIK cells with hTERT overexpression were obtained by lentiviral transduction of hTERT. hTERT/PD-1 KO/CIK cells had a longer lifespan and more pronounced antitumor ability against hepatocellular carcinoma cell line than CIK cells.

The expression of PD-1 is low in CIK cells (Figure 2), but it can be upregulated when CIK cells are activated by antigen or stimulant. PD-1 knockout can inhibit the redistribution of PD-1, which can mitigate the negative effect of PD-1 and enhance the function of CIK cells. In our study, the PD-1 expression of CIK cells was very low (4.54±0.28%), but after PD-1 knockout, the antitumor capacity was improved significantly. Furthermore, PD-1 is not involved in the process of T cell differentiation, so PD-1 knockout had no effect on the phenotype of CIK cells (Figure 2). These results were consistent with those of previous studies [8,9].

There are many factors that affect the *in vivo* efficiency of adoptive cell therapy, and the persistence in the body is one of the most significant [18]. Long-term persistence of immune cells *in vivo* has been found to be related to a durable response effect to tumor [19]. However, when immune cells of *in vitro* culture reach 19-25 PDs, the immune cells began to enter the senescent state and both the proliferative capacity and antitumor efficacy are decreased [20]. In the present study, after transfection of hTERT gene, the telomere length of PD-1 KO/CIK cells was

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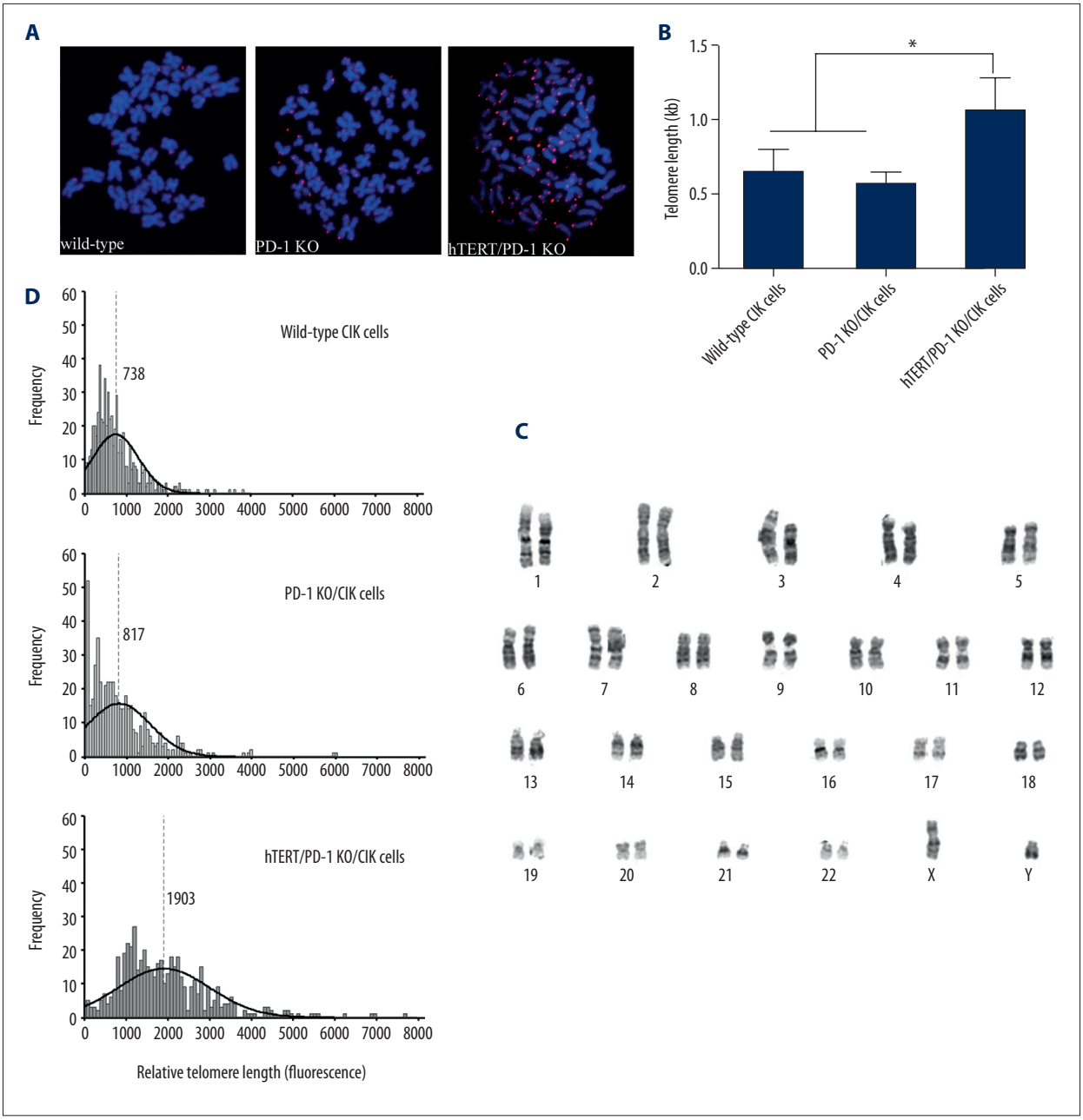

Figure 4. Telomere and karyotype assay of hTERT/PD-1 KO/CIK cells. (**A, D**) Q-FISH analysis and relative length (fluorescence) measurement of telomere were performed in wild-type CIK cells, PD-1 KO/CIK cells, and hTERT/PD-1 KO/CIK cells, respectively. In the Q-FISH image, red fluorescence indicates the telomere and blue fluorescence the chromosome. The mean relative fluorescence unit of telomere is shown in the histograms of relative telomere length. (**B**) The absolute telomere lengths of wild-type CIK cells, PD-1 KO/CIK cells, and hTERT/PD-1 KO/CIK cells were measured using the qPCR method. * $P<0.05$. (**C**) The karyotype of hTERT/PD-1 KO/CIK cells was analyzed with G-banding at 60 days after hTERT transduction. A normal karyotype was shown in hTERT/PD-1 KO/CIK cells.

doubled. When PD-1 KO/CIK cells reached 40–50 PDs, the cells remained in the cell proliferation phase, which showed that the lifespan of PD-1 KO/CIK cells had been prolonged. These were consistent with the results of previous studies [14]. More importantly, hTERT transduction does not alter the chromosomal structure. However, this was a mid-term (60-day) observation of the chromosomal structure. Long-term chromosome observations need to be further developed.

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CIK cells are heterogeneous cell populations composed of a variety of types of cells. $CD3^+CD56^+$ T cells of CIK cells are the main cell component that plays an important role in antitumor effects [21,22]. However, for a variety of cellular components, it was still unclear which component of CIK cells plays the most significant role after PD-1 knockout and hTERT transduction. This should be established in further studies performed *in vitro* and *in vivo*.

Conclusions

Our study showed that PD-1 knockout combined with hTERT transduction could prolong the lifespan and enhance antitumor efficacy of CIK cells against hepatocellular carcinoma cell line, which is an efficient and safe method to prepare robust engineered immune cells for adoptive cell immunotherapy against hepatocellular carcinoma.

Acknowledgments

We would like to thank Pengfei Yuan for his help with gene editing techniques. We would like to thank LetPub (*www.letpub.com*) for providing linguistic assistance during the preparation of this manuscript.

Cnflict of interest.

None.

Supplementary Table

Supplementary Table 1. Target sites and sequence.

Name	Sequence (5'→3')
Section 1: sgRNAs targeting PD-1	
PD-sgRNA1	GTCTGGGCGGTGCTACAACT
PD-sgRNA2	TGTAGCACCGCCCAGACGAC
PD-sgRNA3	ACCGCCCAGACGACTGGCCA
NC-sgRNA	CTAAATGGGGATTTCCGCAA
Section 2: Primers for generating *in vitro* transcription template	
Forward	TAATACGACTCACTATAGNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGC (N, 20bp target sequence)
Reverse	AGCACCGACTCGGTGCCACT
Section 3: Genotyping primers for T7E1, TIDE analysis and single clone sequencing primer	
PD-F	CCAGCACTGCCTCTGTCACTCTCG
PD-R	ACGTCGTAAAGCCAAGGTTAGTCCC
M13F	GGAGAGGCTTCACTAGGTGAG

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