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Research article

THE INTRABODY TARGETING OF hTERT ATTENUATES THE IMMORTALITY OF CANCER CELLS

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Abstract: hTERT (human telomerase reverse transcriptase) plays a key role in the process of cell immortalization. Overexpression of hTERT has been implicated in 85% of malignant tumors and offers a specific target for cancer therapy. In this paper, we describe an effective approach using a single-chain variable fragment (scFv) intrabody derived from monoclonal hybridoma directed against hTERT to attenuate the immortalization of human uterine cervix and hepatoma cells. The scFv we constructed had a high affinity to hTERT, and specifically neutralized over 70% of telomere synthesis activity, thereby inhibiting the viability and proliferation of the cancer cells. Our results indicate that this anti-hTERT intrabody is a promising tool to target hTERT and intervene in the immortalization process of cancer cells.

Key words: Cancer, Intrabody, Anti-hTERT ScFv, Immortality

INTRODUCTION

Telomerase is a ribonucleoprotein that maintains the length of the telomeres by adding G-rich repeats to the ends of eukaryotic chromosomes, using its own RNA component as a template [1]. This telomere maintenance mechanism is indispensable for genome stability in cell immortalization [2], which is a hallmark of cancer. Indeed, 85-90% of human tumors [3], including prostatic carcinoma [4], pancreatic carcinoma [5], breast carcinoma [6], pulmonary

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Abbreviations used: DAPI – diamidino-phenyl-indole; ECL – enhanced chemi-luminescence; ELISA – enzyme linked immunosorbent assay; FITC – fluorescein isothiocyanate; HRP – horseradish peroxidase; mAb – monoclonal antibody; MIFs – mouse intraperitoneal fluid; MTT – monotetrazolium; PI – propidium iodide

carcinoma [7] and hepatocellular carcinoma [8], show telomerase activity which bypasses replicative senescence yielding an unlimited proliferative capacity. There is evidence that the protein subunit human telomerase reverse transcriptase (hTERT) could be a specific target for cancer therapy because of its essential role in enzymatic activity [9-11] and the maintenance of cell proliferation activity [10, 12].

Since the beginnings of cancer therapy, delivering curative agents specifically to tumor cells has been a major concern. As antibodies are specific and have a high affinity for the antigen, they are an attractive means of therapy. Microinjection of antibodies [13, 14] or hybridoma mRNA into the cytoplasm of various cell types [15, 16] has been successfully used to block the activity of intracellular proteins. ScFv, which is composed of linked V region fragments of integrated antibody, overcomes the limitations of traditional monoclonal antibodies and provides an attractive approach for cancer therapy [17-19]. Equipped with suitable targeting signals, intracellular antibodies have been reported to successfully neutralize the targeted proteins in the cytoplasm [20, 21], the nucleus [22, 23] and the secretory pathway [24] of animal cells. Based on the above, in this study, we creatively attempted to target hTERT and block its function intracellularly using the scFv intrabody. We also estimated its affinity for endogenous hTERT and its effect on the proliferative capacity of cancer cells using the models of human uterine cervix and hepatoma cells.

MATERIALS AND METHODS

Plasmids, bacteria, cell lines and animals

The plasmids pET28a, pCDNA3.1/*myc*-HisA, pEGFP-C1 and pGEX-5X-1, *E. coli* DH5α and *E. coli* BL21 (DE3), and the HeLa, HepG2 and SP2/O myeloma cells were all from our laboratory stores. IMR-90, as a hTERT-negative cell line [25], was provided by the Peking University Health Science Center. 4- to 6-week old male Balb/c mice were purchased from the Shanghai SLAC Laboratory Animal Co. LTD.

Main reagents

RPMI-1640, DMEM, MEM, Fetal Calf Serum, HAT and HT medium supplements, and complete and incomplete Freund's adjuvant were purchased from GIBCO-BRL Company. AMV Reverse transcriptase and TMB were purchased from Promega. PEG4000, MTT, PI, DAPI and the antibodies were purchased from Sigma. An annexinV/FITC and PI apoptosis detection kit was purchased from BD Biosciences Pharmingen. The TRAP-Hyb kit was purchased from the Sino-American Biotechnology Company. TRIzol agent and Lipofectamine^{TM} 2000 were purchased from Invitrogen.

Construction and expression of the antigen

The cDNA-encoding T motif and RT domain (aa 540-946) of hTERT [26] were amplified from the plasmid pLPC-hTERT (Clontech) and subcloned into the

pGEX-5X-1 plasmid. Then the vector hosted by DH5α was introduced via 0.5 mM IPTG at 25ºC. The fusion proteins were purified by GST affinity chromatography and dialysed against PBS.

Generation and screening of the monoclonal antibody

Six-week old male BALB/c mice were immunized subcutaneously and intraperitoneally with a mixture containing 50 μg purified recombinant hTERT protein in 100 μL PBS and an equal volume of Freund's complete adjuvant. A booster injection with the same amount of antigen in Freund's incomplete adjuvant was administered at 2-week intervals. 3 days after the last booster, the immunized splenocytes were mixed with the SP2/0 myeloma cells at a ratio of 10:1, and fusion was carried out with 50% PEG-4000. The fusion products were seeded in 96-well plates with feeder cells in complete RPMI 1640 medium supplemented with HAT. The cultured supernatants from each well were screened for hTERT binding via indirect ELISA, and for hTERT neutralization via TRAP ELISA. After 4 to 6 limiting dilutions, the clones that possessed a high affinity and neutralizing effect against hTERT were expanded, and then injected into the intraperitoneal cavity of BALB/c mice for antibody production.

TRAP-ELISA

The telomerase activity was measured using the telomeric repeat amplification protocol-enzyme linked immunosorbent assay (TRAP-ELISA) method. Briefly, 2×10^6 of HeLa cells were harvested and suspended with lysis buffer on ice for 30 min, and the lysis supernatant was used as a TRAP template. Then the telomeres were synthesized and amplified as described in the manufacturer's instructions. After chromogenization for another 30 min, the level of OD_{570} was assessed.

Plasmids

Total RNA was isolated from the AhtA12 hybridoma cells and reverse transcripted. The V_H and V_L regions were amplified and joined by a $(Gly_4Ser)_4$ linker. In order to increase the stability in the eukaryotic cytoplasm, the scFv gene AHTSCFV was assembled with the nuclear localization signal (NLS) of the simian virus 40 large T-antigen on its 5' terminal [27] and the human IgG Vκ on its 3' terminal [23], and then cloned into the pEGFP-C1 or pCDNA3.1/*myc*-HisA vector, respectively. The control plasmids were constructed with an unrelated scFv gene (against the hepatitis C virus conserved epitope), URSCFV instead of AHTSCFV.

Cell culture and transfection

The telomerase-positive cell strains HeLa and HepG2 were maintained in DMEM. The telomerase-negative cell stain IMR-90 [28] was maintained in MEM. The cells were cultured in a medium supplemented with 10% FBS at 37ºC in a 5% CO_2-humidified atmosphere. Transfection was performed at 90%

cell confluence in serum-free medium using Lipofectamine^{TM} 2000. The medium was replaced with fresh complete medium after 5 h of incubation.

Western blot

Proteins were electrophoresed on 10% SDS-PAGE and then electroblotted onto PVDF membranes with a Transblot apparatus (Bio-Rad) following the manufacturer's instructions. After blockage with 5% skim milk in TBST, the membrane was probed with MIFs, followed by incubation with HRP goat anti-mouse antibody. Finally, ECL-based detection was performed as previously described [29].

Immunocytochemistry

Cells grown on glass coverslips were fixed in ice cold 4% paraformaldehyde for 10 min and then permeabilized in 0.2% Triton X-100 for 5 min at room temperature. The cells were then blocked in PBS containing 10% horse serum and 5% BSA for 1 h at room temperature. Labeling was performed with the diluted AhtA12 MIF for 2 h at 37ºC in a moist chamber. The cells were then washed and incubated with Cy3-conjugated anti-mouse antibody for 1 h in the dark. Then the fluorescence signal was captured under a confocal microscope (Olympus).

MTT assay

1×10^4 cells/well were seeded in a 96-well plate, and the standard MTT assay was performed as previously described [30]. The absorbance of the samples was measured at a wavelength of 570 nm.

Apoptosis and cell cycle analysis via flow cytometry

Surface exposure of phosphatidylserine in apoptotic cells was measured using an annexinV/FITC and PI apoptosis detection kit. The cells were collected and resuspended in the binding buffer provided in the kit, then mixed with FITC-conjugated annexin V and PI. After incubation for 15 min, the cells were assessed via flow cytometric analysis.

The cell cycle was analyzed via PI staining. Briefly, floating and adherent cells were collected and fixed by pre-chilled 70% ethanol at 4ºC overnight. The cells were treated with 10 μg/ml RNase A and 50 μg/ml PI at 37ºC for 15 min, and then assessed via flow cytometric analysis.

Statistical analysis

All of the experiments were repeated three times, and the results presented are the averages. Student's *t*-test was used to calculate the significance of the statistical comparisons. Values of $P < 0.05$ were considered to be statistically significant differences. The data is presented as means ± SD.

RESULTS

Construction of the scFv vector

In positive hybridoma clones identified via indirect ELISA and Western blot analysis, AhtA12 showed the most effective neutralization of hTERT, and was chosen for scFv construction (Fig. 1). The V_H and V_L genes were successfully amplified from the cDNA of the AhtA12 clone and assembled by $(Gly_4Ser)_4$ linker (GenBank accession No. EU219997).

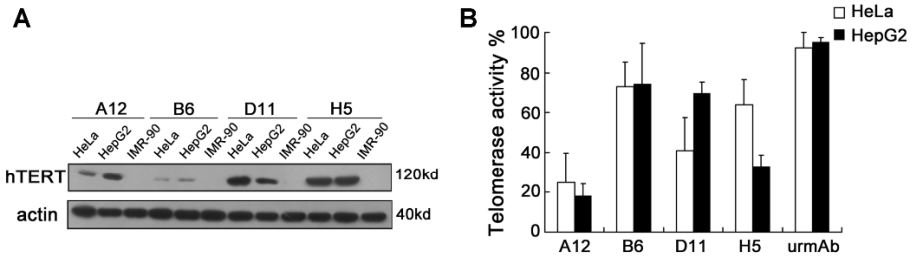

Fig. 1. hTERT in cancer cells is recognized and neutralized by mAbs. A – Equal amounts of HeLa, HepG2 and IMR-90 cell proteins were electrophoresed on 10% SDS-PAGE and then electroblotted onto PVDF membranes. The membranes were respectively probed with equal amounts of MAFs produced from different clones of hybridoma or anti-actin antibody as standardization, and then traced by goat anti-mouse HRP. B – The telomerase activity in the total cell lysis supernatant was assessed via TRAP-ELISA. The telomerase activities of untreated HeLa and HepG2 cells were considered as 100%. The cell lysis supernatant was incubated respectively with 10 μg/ml MAF or an unrelated mAb (urmAb), and then the telomerase activity was assessed.

Intracellular expression and localization of the *AhtscFv* intrabody in human cancer cells

AhtscFv and *Urscfv* with the NLS signal were successfully expressed and localized in the nucleus of HeLa cells (Fig. 2A). Colocalization of the *AhtscFv* intrabody with endogenous hTERT was observed inside the nucleus (Fig. 2B).

The *AhtscFv* intrabody neutralized endogenous telomerase activity

Telomerase activity was examined by TRAP-ELISA. As shown in Fig. 3, the telomerase activity had significantly decreased in *AhtscFv*-treated HeLa and HepG2 cells, while no obvious alteration was observed in *UrscFv*-treated cells.

The decreased viability of *AhtscFv*-treated cells

The MTT assay was carried out in order to investigate cell viability influenced by the *AhtscFv* intrabody. Untreated HeLa, HepG2 and IMR-90 cells were considered to be 100% viable. As shown in Fig. 4, a significant decrease was observed in *AhtscFv*-treated cancer cells but not in *Urscfv*-treated counterparts or IMR-90 cells. 60 h after transfection, the cell viability of AHTSCFV_EGFP/HeLa had decreased to 41.2% and the cell viability of AHTSCFV_EGFP/HepG2 had decreased to 56.4%.

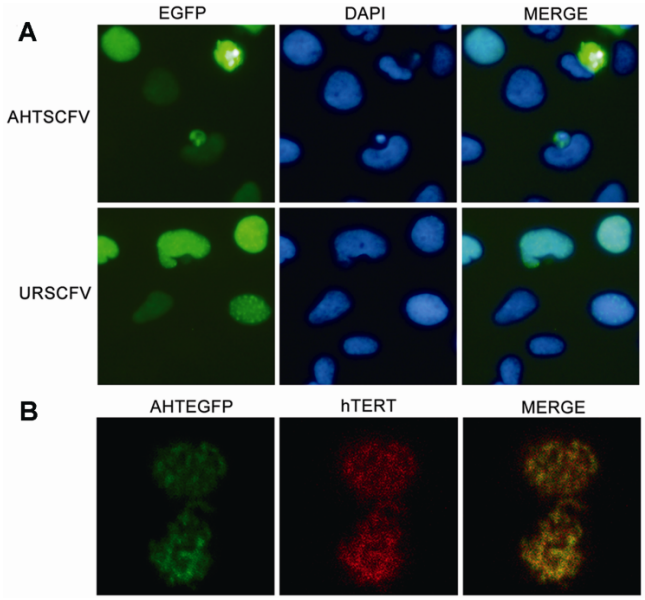

Fig. 2. The *AhtscFv* intrabody localized in the cell nucleus and interacted with hTERT. A – The localization of the *AhtscFv* intrabody in HeLa cells. URSCFV_EGFP/HeLa cells and AHTSCFV_EGFP/HeLa cells seeded on a coverslip were permeabilized and stained with DAPI; the EGFP signal showed that *AhtscFv* and *UrscFv* localized exclusively in the nucleus. B – Co-localization of hTERT and the *AhtscFv* intrabody in HeLa cells. AHTSCFV_EGFP/HeLa cells were permeabilized and blocked, and then probed with AhtA12 MAF followed by Cy3 conjugated goat anti-mouse IgG. The overlapped signal suggests the existence of the hTERT-Aht (Ag-Ab) complex.

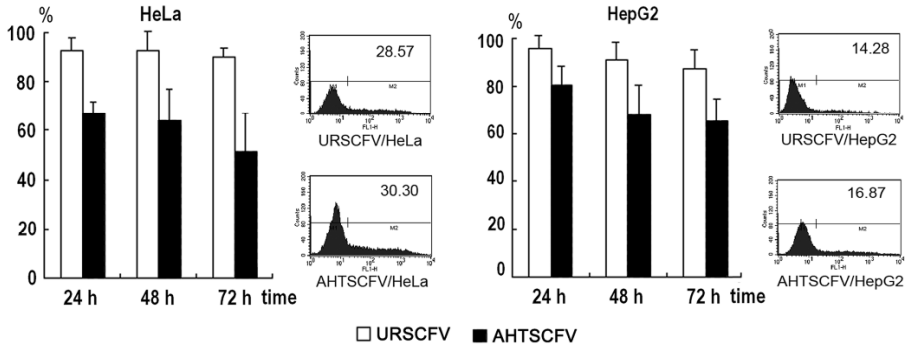

Fig. 3. Telomerase activity neutralization mediated by the *AhtscFv* intrabody. Hela and HepG2 cells were transiently transfected with AHTSCFV_EGFP or URSCFV_EGFP. 24, 48 and 72 h after transfection, the cells were collected and subjected to TRAP-ELISA analysis. The transfection efficiency was indicated by the GFP-positive cell analysis. The telomerase activity of the untreated cancer cells were taken as 100%, and the telomerase activity of the treated cells was assessed by means of three independent experiments. *AhtscFv* but not *UrscFv* obviously reduced endogenous telomerase activity.

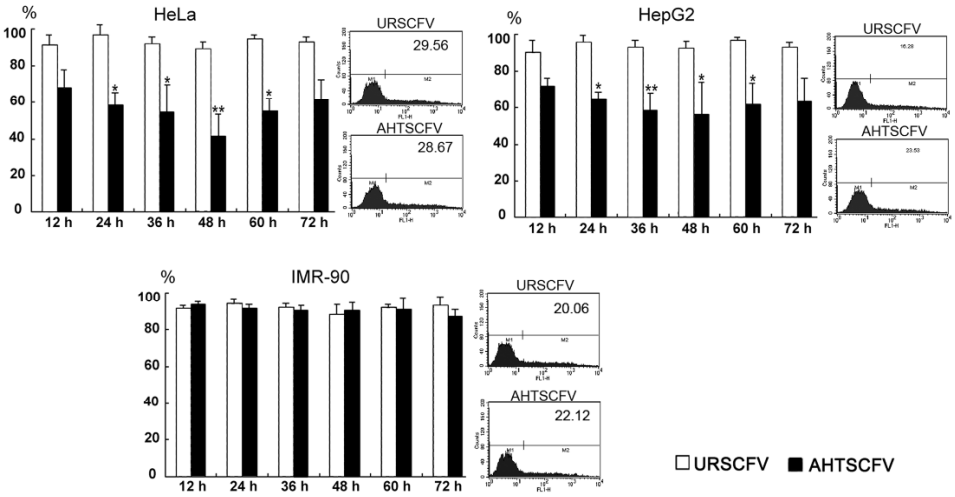

Fig. 4. The reduction in cell viability mediated by the *AhtscFv* intrabody. Hela, HepG2 and IMR-90 cells were transiently transfected with AHTSCFV_EGFP or URSCFV_EGFP. 24, 36, 48, 60, 72 and 84 h after transfection, the cells were seeded in 96-well plates and subjected to a standard MTT assay. Untreated cancer cells were taken as 100% viable. The viability of the *AhtscFv*- or *UrscFv*-treated cells was shown in three independent experiments. The transfection efficiency was indicated by the GFP-positive cell analysis. As shown, after treatment with *AhtscFv* but not after treatment with *UrscFv*, the cell viability significantly decreased. Neither *AhtscFv*- nor *UrscFv*-treated IMR-90 cells showed a significant change in cell viability. The asterisk indicates a significant difference. $*p < 0.05$, $**p < 0.01$.

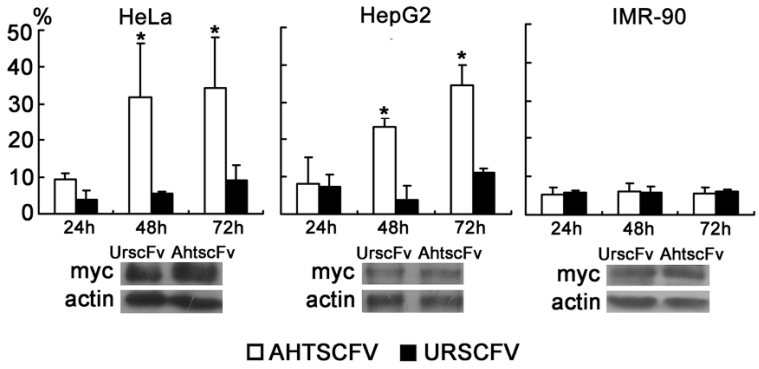

Fig. 5. Apoptosis evoked by the *AhtscFv* intrabody. Hela, HepG2 and IMR-90 cells were transiently transfected with AHTSCFV_pCDNA3.1or URSCFV_pcDNA3.1. 24, 48 and 72 h after transfection, the cells were collected and stained with AnnexinV/FITC and PI, and then analyzed via flow cytometry. The transfection efficiency was assessed via Western blot analysis**.** Significant apoptosis was observed in the *AhtscFv* intrabody-treated cells but not in the *UrscFv*-treated cells. Neither *AhtscFv*- nor *UrscFv*-treated IMR-90 cells showed significant levels of apoptosis. $*p < 0.05$.

Apoptosis induced by the *AhtscFv* intrabody

The assessment of cell proliferation was performed after treatment with *AhtscFv* or *Urscfv* (Fig. 5). Apoptotic morphological characteristics were observed in *AhtscFv*-treated cells but not in the *Urscfv*-treated counterparts or in the IMR-90 cells. In the AHTSCFV_pCDNA3.1/HeLa group, there were 31.7% apoptotic cells 48 h after transfection and 34.3% apoptotic cells 72 h after transfection, while in the AHTSCFV_pCDNA3.1/HepG2 group, there were 24.3% apoptotic cells 48 h after transfection and 34.6% apoptotic cells 72 h after transfection.

Cell cycle alteration caused by the *AhtscFv* intrabody

Transfected cells were stained with PI and subjected to flow cytometry analysis at 12-h intervals (only EGFP-positive cells were assessed). As shown in Fig. 6A-C, 47.2% sub-G1 cells were observed in the AHTSCFV_EGFP/HeLa group and

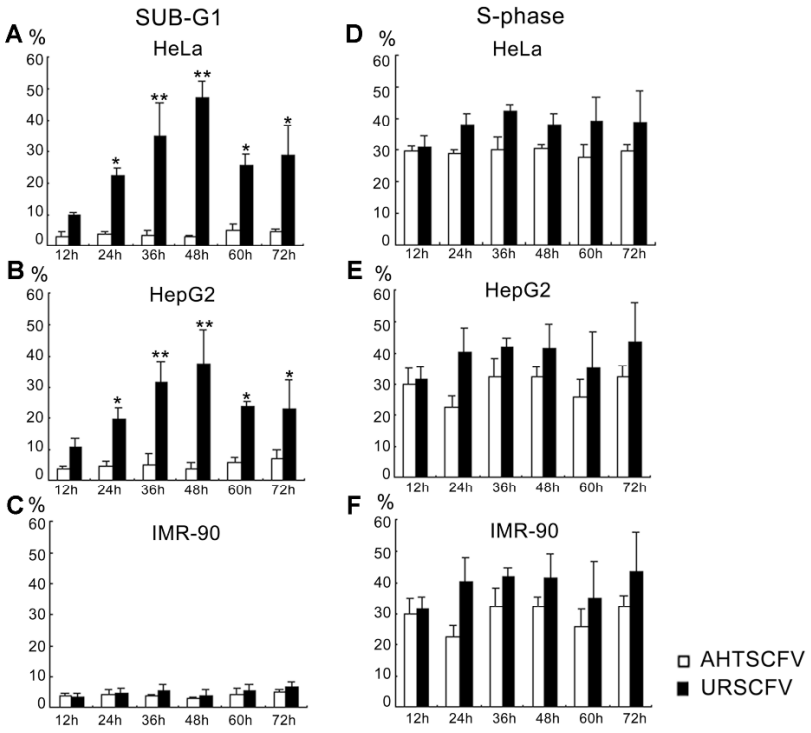

Fig. 6. Cell cycle alteration induced by the *AhtscFv* intrabody. Hela, HepG2 and IMR-90 cells were transfected with AHTSCFV_EGFP or URSCFV_EGFP. 24, 36, 48, 60, 72 and 84 h after transfection, the cells were stained with PI and subjected to flow cytometry analysis. Only EGFP-positive cells were analyzed to exclude the effects of transfection efficiency. The percentages of cells in the different phases including sub-G1 phase cells are shown. A significant increase in sub-G1 (A, B) and S-phase (D, E) cells was observed in *AhtscFv* intrabody-treated cancer cells but not in *UrscFv*-treated cancer cells. Neither the *AhtscFv*- nor *UrscFv*-treated IMR-90 cells showed cell cycle arrest (C, F). $*p < 0.05$, $**p < 0.01$.

37.2% of sub-G1 cells in the AHTSCFV_EGFP/HepG2 group. By contrast, neither of the treated IMR-90 groups showed obvious cell cycle alteration or apoptosis.

Furthermore, as shown in Fig. 6D-F, cell cycle redistribution was observed in both AHTSCFV_EGFP/HeLa cells and AHTSCFV_EGFP/HepG2 cells. 48 h after transfection, there were 12.5% more S-phase AHTSCFV_EGFP/HeLa cells than S-phase URSCFV_EGFP/HeLa cells, while 36 h after transfection, there had been 17.95% more S-phase AHTSCFV_EGFP/HepG2 cells than S-phase URSCFV_EGFP/HepG2 cells.

DISCUSSION

There has been a variety of research projects focused on hTERT, including studies with reverse transcriptase inhibitors, chemically diverse molecules, and siRNAs targeting hTERT mRNA [31-33]. However, they have not been particularly effective in inhibiting cell proliferation. Low specificity, poor uptake and low stability in the biological environment were the main problems. Furthermore, hTERT has a long half-life and is a highly stable protein with high activity [34], which protects it from being effectively silenced by interference at the mRNA level [35]. Our study looked at a novel approach using the anti-hTERT scFv intrabody derived from hybridoma mAb, to phenotypically neutralize the enzymatic activity of hTERT and hinder the unlimited proliferation of human cancer cells. However, although the eukaryotic expression vector was constructed, the expression in mammalian cells remained to be addressed. As the cytosol is a reducing environment, the intrachain disulfide bond in the functional antibody will be difficult to form, and the antibody fragments may be highly aggregated, leading to instability or dysfunction of the antibody [36]. A chimeric protein comprising scFv fused to a targeting sequence such as NLS was reported to increase the stability of scFv in the cytosol [35]. In our study, although the NLS sequences increase the intrabody localizing inside the nucleus, they did not enhance the neutralization of the recombinant antibody against hTERT. The neutralization function of the *AhtscFv* intrabody was significantly increased by adding the human Cκ light chain at the C-terminus. Colocalization of the Aht intrabody and hTERT inside the nucleus of the cancer cells, along with neutralization of the hTERT enzymatic activity, as detected by TRAP-ELISA, indicates that the scFv with NLS sequences and human Cκ light chain that we constructed successfully expressed in the nuclei of human cancer cells and specifically interacted with hTERT inhibiting its activity of telomere synthesis.

HeLa and HepG2 cells, which have been reported to exhibit high telomerase activity and express a high level of hTERT, were used as cell models in this study. The interaction of hTERT and the *AhtscFv* intrabody, coupled with the significant inhibition of telomere synthesis, suggests that *AhtscFv* could effectively block the enzymatic activity of hTERT. After treatment with

AhtscFv, both the HeLa and HepG2 cell groups exhibited cytoplasmic blebbing and chromatin condensation: this appearance indicates programmed cell death. The apoptotic cells were then evaluated by flow cytometry analysis. Since hTERT has been reported to interact with several cell cyclins [37], we performed a cell cycle analysis via PI staining. A significant increase in the content of S-phase cells was observed in *AhtscFv*-treated cancer cell groups but not in *UrscFv*-treated cancer cell groups. Moreover, we continuously cultivated the transfected cells in the presence of 400 μg/ml G418. From the 28th day after transfection, AHTSCFV_EGFP/HeLa and AHTSCFV_EGFP/HepG2 cells as a whole population died, while URSCFV_EGFP/HeLa cells, URSCFV_EGFP/HepG2 cells and AHTSCFV_EGFP/IMR-90 cells underwent a slightly slower but normal proliferation (data not shown). There are several possible reasons for the observed anti-hTERT intrabody-mediated cell cycle arrest. The first is genomic instability caused by shortened telomeric repeats. Once the telomere reaches a critically short length, the cells encounter a proliferative barrier that may be overcome by the induction of telomerase activity [38]. The second is the uncapping state of the telomeres. The capping state of telomeres is functionally defined as preserving the physical integrity of the genome, allowing cell division to proceed [39]. Once the uncapped telomeres accumulate to a critical level, they may signal cells to exit the cell cycle and enter programmed cell death. The third is DNA damage caused by interference with several check-point sensor and DNA repair proteins such as Rad1, Tel1 and Ku70 [40]. Dysfunctional telomerase may also cause DNA damage by sensitizing the cells to p53-dependent signals for growth arrest. Moreover, taking stereo-specific blockade effects into consideration, interaction between the intrabody and hTERT could probably prevent the holoenzyme from correct folding, modification or activation [41, 42], and other telomere protective mechanisms such as recombinant repair may also be effected by hTERT neutralization.

This is for the first time that we utilized the scFv intrabody to neutralize native hTERT in cancer cells. Since the anti-hTERT intrabody can disrupt telomerase activity and attenuate the immortality of hepatoma and cervix cancer cells, this approach may also be applied to other significant cancers with abnormally increased telomerase activity. There are two main concerns about the use of telomerase inhibitors in cancer therapy: one is the time lag caused by length of the telomeres in cancer cells, and the other is the adverse effect on normal proliferative cells like germ cells and gastrointestinal mucosal stem cells [43]. The *AhtscFv* intrabodies show significant cytotoxic effects to cancer cells within 48 h, so we can expect a much shorter time lag in the *in vivo* trials. The adverse effect will be reduced to a large extent by the specificity of the *AhtscFv* intrabody. Moreover, the telomeres in germ and stem cells are considerably longer than those of cancer cells, so they are more likely to be maintained during the therapy and more likely to be regained at the end of the therapy. Therefore, the *AhtscFv* intrabodies are likely to be very effective and safe in limiting the growth of many types of human cancer cells.

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