Combination therapy of hTERTR and FAM96A for hepatocellular carcinoma through enhancing apoptosis sensitivity

WAN-PENG WANG and HAI-YING GAO

Department of Infectious Diseases, Weifang City People's Hospital, Weifang, Shandong 261041, P.R. China

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Abstract. Avoidance of apoptosis induced by anticancer drugs is an essential factor of carcinogenesis and a hallmark of resistance to cancer therapy. Human telomerase reverse transcriptase receptor (hTERTR) is a potential anti-cancer agent for inhibiting tumor growth. Family with sequence similarity 96 member A (FAM96A) is a ubiquitous, conserved protein and possesses apoptosome-activating and pro-apoptotic tumor suppressor potential in hepatocellular carcinoma (HCC). In the present study, hTERTR and FAM96A were identified as efficient anti-cancer agents for activating apoptosomes and reducing tumor growth. The potential tumor suppressor function of combination treatment with hTERTR and FAM96A in HCC was also investigated. hTERTR and FAM96A proteins were expressed by genetic engineering and their anti-cancer function was explored in vitro and in vivo. Effects of hTERTR and FAM96A on improvement of apoptotic sensitivity and inhibition of migration and invasion were examined in cancer cells and in a mouse model. The present results demonstrated that the therapeutic effects of hTERTR and FAM96A were effective for inhibiting tumor growth and inducing apoptosis of HCC cells in H22-bearing nude mice compared with single agent treatment. hTERTR and FAM96A were found to bind with apoptotic protease activating factor 1 and human telomerase reverse transcriptase, which enhanced the apoptosis of tumor cells and apoptosis sensitivity. In addition, hTERTR and FAM96A therapy enhanced cytotoxic effects by cytotoxic T lymphocyte responses, interferon-γ release, T lymphocytes infiltration and apoptosis on tumor cells. Furthermore, hTERTR and FAM96A protein inhibited tumor growth in HCC mice. In conclusion, the present findings suggested that combination therapy with hTERTR and FAM96A may serve as novel tumor suppressor agents.

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer, which exhibits a high recurrence and the second highest cancer-associated mortality rates following radiotherapy, chemotherapy and surgery in Chinese patients (1). HCC not only exhibits a high incidence among human cancers, but potential therapeutic strategies remain limited, especially for patients with advanced late-stage HCC (2). At present, the recommended clinical therapies of surgery, chemotherapy and radiotherapy present only modest efficacy due to tumor progression and side effects, including myelosuppression and digestive system toxicity during and following the treatment period (3-5). Therefore, novel clinical strategies are required in order to enhance curative effects, minimize adverse response and potentially prolong survival of patients with HCC (6,7).

Various oncolytic protocols for targeting different signal pathways have been characterized (8,9). These cellular signal pathways presented key signal transduction for various extracellular growth factors and receptors of HCC cells. However, the high occurrence (62-82%) of drug resistance is a major issue in clinical HCC therapy (10,11). Tumors have been demonstrated to acquire apoptotic-resistance during early stage apoptosis or incomplete apoptotic responses to oncolytic treatments and anticancer drugs, and the consequent requirement for long-term medical therapy presented a major clinical problem in HCC (12). Therefore, novel effective therapeutic agents for HCC therapy are required to inhibit metastasis for patients with HCC.

Human telomerase reverse transcriptase (hTERT) is a target of tumor therapy which is strongly expressed in the majority of tumor cells and is rarely expressed in normal cells (13). A previous study has reported that hTERT is overexpressed in 80% HCC cells, and suggest that telomere elongation is a potential target of gene therapy for HCC (14). In the present study, it was demonstrated that the anti-tumor efficacy of hTERT receptor (hTERTR) was mediated by altering endogenous telomerase activity; thereby significantly inhibiting HCC growth in vitro and strongly suppressing tumor cell proliferation and cancer cell metastasis in anti-telomerase therapy mice.

Family with sequence similarity 96 member A (FAM96A) is identified as a member of the cytosolic Fe/S protein in charge of regulator of cellular iron homeostasis and assembly machinery, which exhibits apoptosome-activating activities.
and pro-apoptotic tumor suppressor potential in HCC (15-17). The results of the present study demonstrated that FAM96A induced no adverse side effects for normal, non-cancerous human cells, which indicated that FAM96A protein may be able to enhance apoptosis sensitivity via the mitochondrial apoptosis pathway in vitro and promote immunologic cytotoxicity for apoptotic fragments in vivo (18).

In the present study, combination therapy of hTERTR and FAM96A for HCC through apoptosis was explored and their antitumor efficacies were confirmed in a murine model of HCC. Notably, compared with sole administration of FAM96A and hTERTR, combination therapy demonstrated markedly improved therapeutic effects for HCC in vitro and in vivo. These findings support the use of multi-target fusion protein drugs for treatment of HCC cells and suggest that hTERTR and FAM96A may be efficient anti-cancer agents for HCC treatment.

Materials and methods

Ethical approval. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (19) of Weifang City People's Hospital. All experimental protocols and animal maintenance were approved by the Committee on the Ethics of Weifang City People's Hospital (Shandong, China). All surgery and euthanasia were performed to minimize suffering.

Cells culture and reagents. HepG2 and H22 cells were obtained from Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository (Frederick, MD, USA) and normal mice liver cells (NCTC-1496 cells) were obtained from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 3 mM L-glutamine, 50 µg/ml gentamicin (all from Biowhittaker; Lonza Group, Ltd., Basel, Switzerland) and 1% penicillin/streptomycin under standard culture conditions (5% CO2, 37°C). Cells were treated with 0.10, 0.18, 0.25, 0.32 mg/ml hTERTR and/or FAM96A for 72 h at 37°C.

Construction of recombinant hTERTR and FAM96A. The hTERTR and FAM96A genes were obtained from lung tissue from the Microbiological Laboratory of Shandong University (Shandong, China). A PET-27b expression system (cat. no. addgene 0020; Shanghai North Connaught Biological Technology Co., Ltd, Shanghai, China) was used to construct the recombinant hTERTR and FAM96A proteins. DNA sequence encoding 130 bp of FAM96A (forward, 5'-AGA CCT GTG AGA TGA CCT CC-3') and reverse, 5'-AGA CCT GTG AGA TGA CCT CC-3') was amplified by polymerase chain reaction (PCR) from pMD-18-FAM96A (Weifang Medical University, Weifang, China) using 1 µl PCR clone product reaction (PCR) from pMD-18-FAM96A (Weifang Medical University, Weifang, China) was used to construct hTERTR and FAM96A proteins. DNA sequence encoding 130 bp of FAM96A (forward, 5'-AGA CCT GTG AGA TGA CCT CC-3') and reverse, 5'-AGA CCT GTG AGA TGA CCT CC-3') was amplified by polymerase chain reaction (PCR) from pMD-18-FAM96A (Weifang Medical University, Weifang, China) using 1 µl PCR clone product mixed with 10 µl of TaqMan Universal PCR MasterMix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). PCR was performed under the following conditions: 95°C for 30 sec, 30 cycles at 95°C for 15 sec and 55°C for 1 min and then 72°C for 1 min. FAM96A (50 ng) was subcloned into the rPET-27b plasmids and named rPET-27-FAM96A using electrottransformation (cat. no. 1359; Bio-Rad Laboratories, Inc., Hercules, CA, USA) following a previously described protocol (20). Gene edited cell-penetrating peptide (CPP; Weifang Medical University) was inserted into plasmids with proteins following 25-µl volume PCR to induce cells to take the plasmids up. The same method was used to clone recombinant hTERTR (forward, 5'-AGAAGATTTTGTAACAAAGGT-3' and reverse, 5'-AGACCTGTGAGATGACCTCC-3') with the CPP into PET-27b plasmids, which were then named rPET-27-hTERTR. PCR, as performed previously and gene sequencing (Invitrogen; Thermo Fisher Scientific, Inc.) were used to select the correct monoclonal sequence identified by Invitrogen (Invitrogen; Thermo Fisher Scientific, Inc.) (21). Recombinant rPET-27-hTERTR (4 µg) or rPET-27-FAM96A (4 µg) was expressed in E. coli in LB medium at 37°C for 12 h. hTERTR and FAM96A protein was extracted and purified as described previously (17).

Western blotting. The purified hTERTR or FAM96A was homogenized and separated by SDS-PAGE, and subsequently transferred to nitrocellulose membranes. For western blot analysis, hTERTR and apoptotic protease activating factor 1 ( APAF1) were prepared. The detection of protein was carried out by incubating the membranes with hTERTR and APAF1 with IL-1 as a negative control. The procedures were performed as previously described (17). All experiments were performed in triplicate and repeated at least three times.

Animal experiments. A total of 80 specific pathogen-free female BALB/c nude mice (age, 6-week old; weight, 30-35 g) were purchased from Harbin Veterinary Research Institute (Harbin, China). Mice were feed under pathogen-free conditions and maintained at a controlled environment (temperature, 23±1°C; humidity, 50-60%) with an artificial simulation of 12 h light/dark cycles. H22 cells (1x10⁶) in 200 µl PBS were injected into the right flank of female BALB/c nude mice to establish HCC. Therapy for tumor-bearing mice with hTERTR and/or FAM96A, or PBS was initiated when tumor diameters reached 6-8 mm at 7 days in the HCC mouse model following tumor inoculation. Mice with HCC were randomly divided into 4 groups (n=20 each) and injected intratumorally with 0.25 mg hTERTR and/or 0.32 mg FAM96A in PBS buffer (200 µl), or the same volume of PBS (200 µl), respectively. Each treatment was subsequently administered a further 7 times at two-day intervals, giving a total of 15 administrations. Tumor diameters were recorded once every two days and tumor volume was calculated using the following formula: 0.52x smallest diameter² x largest diameter.

Lactate dehydrogenase (LDH) and interferon (IFN)-γ release assays. HepG2 (1x10⁶), H22 (1x10⁶) and NCTC-1496 cells (1x10⁶) were incubated with hTERTR (0.20 µg/ml) and/or FAM96A (0.20 µg/ml) in DMEM for 96 h at 37°C. Cells were washed with PBS three times for 2 min at room temperature. Cells were subsequently incubated with 1% Triton-X-100 at 37°C for 30 min. LDH assay was determined using the CytoTox 96 assay kit (Promega Corporation, Madison, WI, USA) and recorded at 490 nm according to manufacturer’s protocol. For IFN-γ release assays, splenocytes were harvested from euthanized animals. Splenocytes (1x10⁶) were subsequently incubated with UV-inactivated H22 cells (1x10⁶) in DMEM at
Cells were analyzed by flow cytometry. Cells were collected at 37˚C for 72 h. Specific cytotoxic T lymphocyte (CTL) response to the target cells was determined using a Pierce™ LDH Cytotoxicity assay kit (cat. no. 88953; Thermo Fisher Scientific, Inc.) cytotoxicity assays as described previously (23).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted from H22 cells with an RNasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) and 1 µg RNA was subjected to a cDNA synthesis kit (Bio-Rad Laboratories, Inc.). A total of 10% of the cDNA sample was subjected to a 25-µl PCR (cDNA, 10 ng, 5 µl, PCR buffer, 2 µl, DNA polymerase, 0.5 µl, primers, 2 µl, water, 15.5 µl) carried out in an iCycler thermal cycler (Bio-Rad Laboratories, Inc.) using iQ SYBR-Green Supermix (Bio-Rad Laboratories, Inc.). The reaction conditions were performed as follows: 95°C for 10 min, 35 cycles of 95°C for 20 sec and 58°C for 1 min. The forward and reverse primers for B-cell lymphoma 2 (Bcl-2) and c-Myc were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). Primers were as follows: Bcl-2 forward, 5’-CTGGGGAGCAACATCAGTCTG-3’ and reverse, 5’-GGTCTGATCCTACCTGTG-3’; c-Myc forward, 5’-TTCT ATCCAGGTATCGAGACAG-3’ and reverse, 5’-GCCAGGCAAGT-3’; GAPDH forward, 5’-GGCCAAGAT CATCATTGACACT-3’ and reverse, 5’-GCCAGGACATGA GCTTGACAGAAT-3’. The amplified PCR products were quantified by measuring the calculated quantification cycles (Cq) of sample and GAPDH mRNA. Relative changes in mRNA expression were calculated by the 2^ΔΔCq method (24). The results are expressed as the n-fold difference relative to GAPDH (relative expression levels).

Tumor cell migration and invasion assays. HepG2 cells were treated with hTERTR and/or FAM96A and non-treated cells were used as control. For migration assay, HepG2 cells (1x10^5) were incubated with hTERTR (0.20 µg/ml) and/or FAM96A (0.2 µg/ml) in DMEM for 96 h at 37˚C using control culture inserts (BD Biosciences, Franklin Lakes, NJ, USA). For invasion assay, hTERTR and/or FAM96A-treated cells were suspended at a density of 1x10^6 cells in 500 µl serum-free DMEM. HepG2 cells were then placed in the upper chambers of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) for 96 h at 37˚C according to the manufacturer’s instructions. 0.1% crystal violet was used to stain the membrane for 30 min at 37˚C. After washing with PBS, tumor cell invasion and migration were counted in at least three random stained fields of view via microscopy (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Flow cytometry. HepG2 and H22 cells were cultured in DMEM medium supplied 10% fetal calf serum. HepG2 and H22 (1x10^5) cells were incubated with PBS, hTERTR and/or FAM96A at 37˚C for 72 h. Subsequently, apoptosis of suspended cells were analyzed by flow cytometry. Cells were collected and suspended with Annexin V-FITC and PI for 30 min at 4˚C according to the manufacturer protocol. Fluorescence was measured with a fluorescence-activated cell sorting flow cytometer (FCS Express™ 4 IVD; BD Biosciences) and analyzed using Quantity One software (version 3.0; Bio-Rad Laboratories, Inc.).

Immunohistochemical staining. Tumor tissues were fixed using 10% formalin solution for 12 h at 4˚C. Immunohistochemical staining was performed using an avidin-biotin-peroxidase technique on tumor tissues obtained from the BALB/c mice on day 30. Paraffin-embedded tissue sections 4 µm thick were prepared and epitope retrieval was performed for further analysis. The paraffin sections were incubated with hydrogen peroxide (3%) for 10-15 min at 37˚C and were subsequently blocked with a regular blocking solution (5% skim milk powder) for 10-15 minutes at 37˚C. Sections were incubated with anti-Annexin antibody (1:2,000, cat. no. ab14196; Abcam, Cambridge, UK) at 4˚C for 12 h following blocking. All sections were washed three times with PBS at 37˚C for 5 min and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG mAb (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 1 h at 37˚C and were counterstained with hematoxylin or DAPI for 1 h at 37˚C. Images were captured with a ZEISS LSM 510 confocal microscope (Zeiss AG, Oberkothen, Germany) at 488 nm.

Statistical analysis. All data are presented as the mean ± standard error of the mean. Comparisons of data between multiple groups were performed using one-way analysis of variance followed by a Tukey's multiple comparison post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of recombinant hTERTR and FAM96A, and in vitro activity. In order to construct the recombinant protein, two recombinant plasmids were structured, rPET-27b-hTERTR and rPET-27b-FAM96A. The structure of rPET-27b-hTERTR and rPET-27b-FAM96A are presented in Fig. 1A. The recombinant plasmids of rPET-27b-hTERTR and rPET-27b-FAM96A were respectively expressed by E. coli. The molecular weight of hTERTR was ~28 kDa and FAM96A was ~30 kDa (Fig. 1B). Western blotting revealed a band of ~27 kDa (hTERTR) and 30 kDa (FAM96A) and identified the purified protein of hTERTR or FAM96A specifically bound to hTERT and APAF1, respectively (Fig. 1C). ELISA also revealed that hTERTR-FAM96A was able to cross-bind both hTERT and APAF1 (Fig. 1D). These results indicated that the recombinant proteins hTERTR and FAM96A were purified successfully and possessed the expected binding potentials.

Cytolytic effects of hTERTR and/or FAM96A on normal cells and HCC cells. In the present study, the cytolytic effects of hTERTR and/or FAM96A on H22 and HepG2 tumor cells were investigated to determine the cytotoxic effects of hTERTR and/or FAM96A via LDH assay, using untreated cells as a control. Different dosages of hTERTR and/or FAM96A (0.10, 0.18, 0.25, 0.32 mg/ml) induced different cytotoxic effects on
tumor cell lines in a time-dependent manner, but were not toxic for normal hepatocytes (Fig. 2A-D). The different dosages of hTERTR or FAM96A (0.10-0.32 mg/ml) also demonstrated inhibiting effects for tumor cell growth in a dose-dependent manner at 72 h post-treatment. The present results indicated that hTERTR (0.18 mg/ml) and FAM96A (0.25 mg/ml) was enough to inhibit growth of hepatic tumor cells. Nevertheless, combination treatment with hTERTR and FAM96A resulted in great inhibition compared with either agent administered alone. It was observed that there was no significant difference in the growth-inhibiting rate at the maximum concentration levels between hTERTR and FAM96A at 72 h post-treatment. These data suggested that combination treatment of hTERTR and FAM96A significantly inhibited HCC cells.

**Improvement of apoptotic sensitivity in hTERTR and/or FAM96A-treated HCC cells.** The cytotoxic effects on human or mouse normal liver cell lines at the highest dose of hTERTR (0.18 mg/ml) or FAM96A (0.25 mg/ml) were also investigated. The present results indicated that hTERTR (0.18 mg/ml), FAM96A (0.25 mg/ml) or combination treatment [hTERTR (0.18 mg/ml) and FAM96A (0.25 mg/ml)] had no significant cytotoxic effect on NCTC-1496 cells (Fig. 3A). In addition, treatment with hTERTR and/or FAM96A induced apoptosis of tumor cells (Fig. 3B). Furthermore, the inhibitory effects of hTERTR and/or FAM96A on migration and invasion in HCC cells were investigated. The present results indicated that hTERTR and/or FAM96A administration significantly inhibited migration (Fig. 3C) and invasion (Fig. 3D) of HCC cells. Notably, these findings suggest that combination treatment with hTERTR and FAM96A markedly promoted apoptosis and inhibited migration and invasion of hepatocellular tumor cells. These results suggested that hTERTR and/or FAM96A significantly inhibited hepatocellular tumor cells growth, migration and invasion.

**Therapeutic effects of hTERTR-FAM96A in HCC tumor-bearing mice.** Therapeutic effects of hTERTR and/or FAM96A in HCC tumor-bearing mice were evaluated by measuring tumor volume. Also, tumor cellular immunity was analyzed at 10 days following treatment. The results in Fig. 4A showed that treatment with hTERTR and FAM96A exhibited significantly increased inhibitory effects for HCC tumor growth compared with hTERTR, FAM96A and PBS groups in a 30-day observation. The result in Fig. 4B showed that treatment with hTERTR and FAM96A markedly suppressed expressions of apoptosis-suppressing genes (Bcl-2 and C-myc) in tumors compared with hTERTR or FAM96A-treated tumors. In addition, the results in Fig. 4C showed that apoptosis rate was increased on tumor surface following combination treatment with hTERTR and FAM96A. In addition, CTL responses and IFN-γ release were also assessed in H22-bearing mice. Treatment with hTERTR and FAM96A resulted in significantly higher CTL activity and IFN-γ release when compared with control groups (Fig. 4D and E). Furthermore, the date in Fig. 4F indicated that hTERTR and FAM96A-treated tumors generated more apoptotic bodies analyzed by immunofluorescence compared with
single agent-treated tumors. These data suggest that hTERTR and FAM96A improved apoptosis of HCC cells and enhanced CTL responses, more apoptotic bodies and lymphocytes for HCC tumors.

Discussion

The majority of patients with HCC have exhibited drug resistance and reduced apoptosis-induced immunologic...
cytotoxicity in clinical trials (25). The ability to escape apoptosis induced by immunologic cytotoxicity is a distinguishing feature of tumorigenesis and an important factor in resistance to anti-cancer treatments (26,27). A recent study has indicated that HCC cells that developed resistance to the telomerase-activated prodrug acycloguanosyl 5'-thymidyltriphosphate may undergo spontaneous apoptosis (28). In addition, a previous report demonstrated that dominant negative p63-α induced drug resistance in HCC by interference with apoptosis signaling pathways (29). Furthermore, down-regulation of aquaporin expression has been demonstrated to induce an increasing resistance to apoptosis in HCC (30). These findings suggested that resistance to apoptosis was a major obstacle in clinical treatment of HCC. Therefore, it was hypothesized that inhibition of resistance to apoptosis may enhance the therapeutic efficacy for patients with HCC.

In previous studies, the therapeutic effects of targeting hTERT in patients with HCC were reported (31,32). The hTERT protein is identified as a component of the cytosolic Fe/S protein assembly machinery, and it is evolutionarily conserved and critical for cellular iron homeostasis (33). In the present study, hTERTR was identified as a novel target-regulating protein by binding with hTERT in human and mouse hepatic carcinoma cells. Apoptosis of tumor cells was also examined to confirm their association with hTERTR; however, administration of a single anticancer agent of hTERTR was not able to achieve the desired therapeutic effects in HCC tumor model.

FAM96A is a 21 kDa (including the cell-penetrating peptide) protein that is expressed at low levels in tumor cells (34). Stehling et al (15) previously demonstrated that FAM96A is associated with metabolism, DNA maintenance, protein translation and facilitating the effective induction of cell death via the mitochondrial apoptosis pathway by binding to pro-apoptotic APAF1 protein; however, it is poorly expressed in most tumor cells. FAM96A, as a pro-apoptotic tumor suppressor, presented potential therapeutic effects for tumor cells (15). Previous studies reported that reestablishment of FAM96A expression enhanced apoptosis sensitivity and inhibited tumor growth, leading to the hypothesis that the limited apoptosis sensitivity of cancer cells was associated with FAM96A loss (16,18). In the present study, it was observed that FAM96A with cell penetrating peptide efficiently inhibited tumor cells growth and induced apoptosis. However, in the HCC mouse model, tumor cell were not eliminated completely.

Several challenges remain in reducing HCC-associated resistance to apoptosis by targeted anti-cancer treatments (35-37). Combinations of anti-cancer agents have previously demonstrated a strong therapeutic effect (38-40). Therefore, combination treatment of hTERTR and FAM96A was investigated in HCC cells and a xenograft mouse model. The present study indicated that combination treatment with hTERTR and FAM96A was efficient for HCC inhibition, which elucidated a reference for protein drugs for HCC therapy (41,42). In addition, combination treatment with hTERTR and FAM96A presented a better outcome by inducing tumor cell apoptosis compared with single hTERTR or FAM96A treatments in murine HCC models. Furthermore, the efficacy of combination treatment hTERTR and FAM96A also generated tumor-specific CTL responses, which indicated that an increase in apoptotic bodies and debris was considered as potential cytotoxic toxicity mediated by cellular immunity.
In conclusion, combination treatment hTERT and FAM96A was identified as a hTERT-targeting and FAM96A-reestablishing molecular therapy, which has the potential to inhibit hepatic carcinoma tumor cells growth by prompting apoptosis, which suggest the beneficial effects of targeted therapy.

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