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A recombinant cell-permeable p53 fusion protein is selectively stabilized under hypoxia and inhibits tumor cell growth

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ABSTRACT

More than 50% of human tumors contain a mutation in p53. Over 90% of tumors are solid tumors. Solid tumors have low oxygenated regions, called hypoxic regions where the tumor cells are more resistant to radio- and chemo-therapy than their well-oxygenated counterparts. In this study, we constructed a cell-permeable p53 fusion protein with selective stability in the hypoxic region. The fusion protein contained the TAT peptide for transduction across membranes, the oxygen-dependent degradation domain of hypoxia-inducible factor-1alpha and wild-type p53. This protein was effectively delivered into tumor cells where it exerted anticancer activity leading to the inhibition of cancer cell growth *in vitro* and the reduction of tumor weight *in vivo*. Hence, the fusion protein can be a novel protein drug for antitumor therapies, especially for hypoxic tumor cells.

1. Introduction

The p53 tumor suppressor protein serves as an efficient guardian against cancers. Over 50% of human tumors harbor a dysfunctional p53 caused by genetic mutation or deletion [1]. Wild-type p53 is capable of preventing tumor formation and progression via multiple mechanisms [2,3]. The major pathway relies on its transcriptional activity to up-regulate numerous downstream genes associated with cell-cycle arrest, senescence, autophagy, and apoptosis [4–8]. Dysfunctional p53 not only fails to act as a tumor suppressor, but also plays a key role in accelerating tumorigenesis. Mutant p53 proteins possess novel oncogenic activities both in tissue culture and in experimental animal models [9]. Thus, p53 has been a target for anticancer treatment and reagents have been developed for the restoration of p53 function in tumor cells [10]. However, their utility is limited by poor permeability and low targeting-delivery.

More than 90% of human tumors are solid tumors. Low-oxygenated regions, called hypoxic regions, are present in solid tumors [11]. In these regions, the oxygen tension is low and the cancer cells adaptive to hypoxia are resistant to conventional radiotherapy and chemotherapy [12]. Furthermore, hypoxic tumor cells are predisposed to a more malignant phenotype. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that activates gene expression in a hypoxic environment [13,14]. In oxygenated cells, the HIF-1α subunits are rapidly destroyed via ubiquitylation by the von Hippel-Lindau tumor suppressor E3 ligase complex [15]. However, in hypoxic cells degradation of HIF-1α is suppressed, leading to transcriptional activation of target genes. Moreover, the full length oxygen-dependent degradation domain of HIF-1α (ODD) as well as its minimum domain (557–574) are capable of inducing expression of a fusion partner in a hypoxic environment [16,17].

For efficient delivery of therapeutic proteins, various protein transduction domains have been used [18,19].

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The positively charged transduction domain of the HIV-1 TAT protein is used for transmembrane delivery of proteins [20,21]. In this study, we constructed a novel fusion protein consisting of wild-type p53 fused with TAT_{47-57} and the minimum oxygen-dependent degradation domain of HIF-1α for antitumor therapy.

2. Materials and methods

2.1. DNA construction

pET28a/p53 construction: p53 cDNA was prepared from human embryonic total RNA by following the cDNA synthesis protocol (Promega, USA) and amplified by PCR with primers P5 and P4. The 1.2 kb product was purified using a Clontech Chromospin column (Promega, USA), and digested with EcoR I and Sal I. The fragment was cloned into pET28a (Novagen), sequenced, and matched with the p53 cDNA in GenBank (Accession No. BC003596).

pET28a/TAT-p53 construction: The TAT-p53 fragment was made with primers P6 and P4 by PCR using pET28a/p53 as a template. The PCR product was purified and extracted using Gel recycling Kit (TianGen CO., LTD. Beijing). The fragment was digested with EcoR I and Sal I and inserted into pET28a to yield pET28a/TAT-p53.

pET28a/TAT-ODD-p53 construction: pET28a/TAT-ODD-p53 was constructed by nested-PCR with a series of primers. Briefly, a portion of the oxygen-dependent degradation domain (ODD) was amplified with primers P1 and P2. Another portion of ODD as well as p53 was amplified with primers P3 and P4. The 54 bp ODD sequence was encoded in primers P2 and P3. After the two PCR products were purified, they were mixed and used as a template for amplification with primers P1 and P4 to yield TAT-ODD-p53. The TAT-ODD-p53 fragment was digested with EcoR I and Sal I and inserted into the pET28a vector to construct pET28a/TAT-ODD-p53 Fig. 1. The primers used in PCR are listed in Table 1. The inserted sequence contained 1278 base pairs (Sfig. 1).

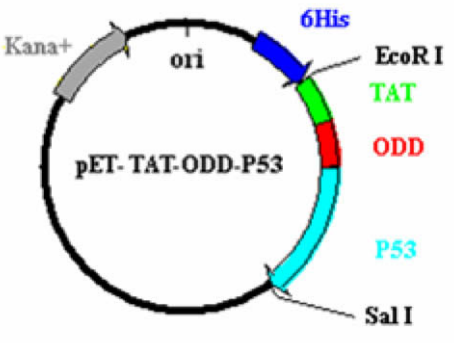

Fig. 1. TAT-ODD-p53 expression vector. Full length p53 cDNA was fused with TAT and ODD encoding sequences and cloned into the pET28a vector.

2.2. Generation of p53 fusion proteins

The p53 control and p53 fusion proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS (TianGen BIOTHEC CO., LTD. Beijing) and purified as previously described [21]. Briefly, transformed bacteria were cultured in LB medium containing 30 μg/ml kanamycin for 8 h, and then seeded into 2YT medium containing kanamycin at 1:100 (v/v) dilution. The bacteria were grown for 3–5 h at 37 °C, and then 1 mM IPTG was added for the final 5–6 h for induction of protein expression. After centrifugation at 3500*g* for 10 min, pellets were resuspended with PBS, and supersonicated on ice for 25 cycles (10 s on, 30 s off). The sonicates were centrifuged at 14,000*g* for 10 min at 4 °C. The pellets were washed with buffer A (2 M urea, 100 mM NaCl, 50 mM PBS, pH 8.0) overnight at 4 °C. The washed pellets were dissolved in buffer B (8 M urea, 50 mM PBS pH 7.5) containing 10 mM imidazole, and loaded onto a pre-equilibrated Ni-NTA column at 4 °C. Proteins were eluted with 300 mM imidazole. The purified fractions were pooled and dialyzed in buffer C (0.1 mM GSH, 0.01 mM GSSG, 0.1 mM EDTA and 50 mM PBS, pH 7.5) to remove urea and allow refolding of protein, and finally dialyzed against PBS. Protein concentration was measured according to Bradford [22] using bovine serum albumin as standard. Approximately 8 mg protein was recovered per liter of bacterial culture. Protein was stored in 50 mM PBS at −80 °C, and used within 6 months.

2.3. Western blotting

The purified fusion proteins were detected by 12% SDS-PAGE and blotted onto Nitrocellulose membrane (Amersham) with transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH, pH 8.3) through semi-dry transfer cell. The membrane was blocked with 5% skim milk and incubated with 1/1000 dilution of primary antibody against human p53 (DO-1, MBL, Japan) at 4 °C overnight. The membrane was washed three times with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Tween-20), incubated with 1/1000 dilution of horseradish peroxidase-labeled secondary antibody (Jackson, America) at 37 °C for 45 min. After being washed with TBST for 5 min ×6, the membrane was detected by ECL (Applygen Technologies Inc., Beijing, China).

2.4. Cell culture

Human colorectal adenocarcinoma cell line SW480 (p53 mutation) was obtained from ATCC and cultured in IMDM containing 10% fetal bovine serum. Mouse melanoma B16 cell line was kindly provided by the Beijing Center of Disease Prevention and Control, and maintained in 1640 medium containing 10% fetal bovine serum, in humidified 5% CO_2, 37 °C incubator (NAPCO or Thermo Forma).

2.5. Hypoxic condition

Hypoxic condition was achieved by incubating cells at 37 °C in the humidified CO_2 incubator maintaining 1% O_2, 5% CO_2 balanced with 99.5% N_2 (Thermo Forma).