DNA Topoisomerase III Alpha Regulates p53-Mediated Tumor Suppression

Mei-Yi Hsieh1, Jia-Rong Fan1, Han-Wen Chang1, Hsiang-Chin Chen1, Tang-Long Shen2,3, Shu-Chun Teng1, Yen-Hsiu Yeh1, and Tsai-Kun Li1,3

Abstract

**Purpose:** Human DNA topoisomerase III alpha (hTOP3α) is involved in DNA repair surveillance and cell-cycle checkpoints possibly through formatting complex with tumor suppressors. However, its role in cancer development remained unsolved.

**Experimental Design:** Coimmunoprecipitation, sucrose gradient, chromatin immunoprecipitation (ChIP), real time PCR, and immunoblotting analyses were performed to determine interactions of hTOP3α with p53. Paired cell lines with different hTOP3α levels were generated via ectopic expression and short hairpin RNA (shRNA)-mediated knockdown approaches. Cellular tumorigenic properties were analyzed using cell counting, colony formation, senescence, soft agar assays, and mouse xenograft models.

**Results:** The hTOP3α isozyme binds to p53 and cofractionizes with p53 in gradients differing from fractions containing hTOP3β and BLM. Knockdown of hTOP3α expression (sh-hTOP3α) caused a higher anchorage-independent growth of nontumorigenic RHEK-1 cells. Similarly, sh-hTOP3α and ectopic expression of hTOP3α in cancer cell lines caused increased and reduced tumorigenic abilities, respectively. Genetic and mutation experiments revealed that functional hTOP3α, p53, and p21 are required for this tumor-suppressive activity. Mechanism-wise, ChIP data revealed that hTOP3α binds to the p53 and p21 promoters and positively regulates their expression. Two proteins affect promoter recruitments of each other and collaborate in p21 expression. Moreover, sh-hTOP3α and sh-p53 in AGS cells caused a similar reduction in senescence and hTOP3α mRNA levels were lower in gastric and renal tumor samples.

**Conclusion:** We concluded that hTOP3α interacts with p53, regulates p53 and p21 expression, and contributes to the p53-mediated tumor suppression. *Clin Cancer Res; 20*(6); 1–13. ©2014 AACR.

Introduction

It is generally believed that cancer results when cells acquire genetic alterations that cause uncontrolled proliferation. Consequently, genetic errors resulting in loss-of-function of tumor-suppressor genes and/or gain-of-function of oncogenes are critically involved in cancer development (1). Supportively, inherited diseases with genomic instability, such as Bloom syndrome, are prone to cancer development (2). In addition, genomic instability is recognized recently as an enabling characteristic of cancer. Two safeguard mechanisms, namely senescence and apoptosis, have evolved to prohibit neoplastic transformation (3), and thus the function and/or gene expression of the regulatory factors of senescence and apoptosis safeguards must be altered in a normal cell to become cancerous.

*TP53*, the most frequently mutated gene in cancers (~50%), encodes tumor suppressor p53, which functions as a transcription factor that drives the senescent (via p21), apoptotic (via BAX), and DNA repair (via GADD45) pathways (4). In addition to genetic alterations, other p53 inactivation mechanisms via BRD7 (bromodomain-containing 7) dysfunction or elevated microRNA expression have been reported in cancers (5–7). Chromatin modifying and remodeling complexes have also shown to regulate p53-dependent p21 expression (8, 9). Notably, recent reports on cancer genomic analyses revealed the potential contribution of chromatin factors to tumorigenesis (10). Together, these results suggest that cellular factors could contribute to tumorigenesis via directly modulating p53 expression and/or functionally cooperating with p53-regulated gene expression.

Two epigenetic mechanisms, chromatin modification and remodeling, are known to influence both chromatin structure and gene expression. Nevertheless, functional roles of the other epigenetic regulators of chromatin DNA topology...
in transcription programming remain to be elucidated. Through their enzymatic activity, DNA topoisomerases regulate various biological functions (11–13), and torsional stress accompanied with the separation of DNA strands during transcription can only be relieved by DNA topoisomerases. Recent studies revealed that the TOP2β isozyme is involved in regulated gene expression through specifically binding to transcription factors, for example, human TOP2β (hTOP2β) binds to the androgen receptor (AR) and subsequently contributing to AR-directed transcription programming and to DNA sequence rearrangements during the development of prostate cancer (14). Consistently, our recent study has demonstrated a specific involvement of hTOP3α in inflammation-associated DNA damage, mutagenesis, and skin melanoma formation (15). Interestingly, hTOP3α plays roles in maintaining genomic stability by forming DNA repair surveillance complexes with BLM, FANC2, and BRCA1 tumor-suppressor proteins (16–18) and/or acting in combination with BLM, RM1, and RM2 to promote the dissolution of double Holliday junctions (19, 20). Considering the importance of genomic instability in tumorigenesis and the specific interactions of hTOP3α with tumor suppressors, above reports suggest that hTOP3α might function as a tumor suppressor.

Six topoisomerases have been identified in human cells, namely nuclear hTOP1, 2α, 2β, 3α, 3β, and mitochondrial mTOP1 (12, 13). These enzymes change the topology of DNA using transesterification reaction involving the cleavage and religation of phosphodiester bonds. Although some functions of TOP1 and TOP2 are well studied (11–13), the biologic roles of TOP3 remain largely unresolved. Nevertheless, embryonic lethality occurs in mice lacking TOP3α (21), and TOP3β knockout mice have a reduced lifespan (22, 23). In addition, unexpected roles in checkpoints and chromosome segregation were suggested for yeast TOP3s (24–28). The expression of dominant-negative or truncated hTOP3α caused S-phase impairment and rescued some phenotypes of Ataxia telangiectasia cells (29). It is unclear how TOP3s carries out above specialized essential functions. Two explanations are offered: (i) TOP3 is present in multiprotein complexes that perform above functions; or (ii) both the enzymatic activities and properties of TOP3 are greatly modulated by its interacting proteins. Together, through physical interaction with specific DNA-binding factors, chromatin regulators such as TOP3α participate in the specialized biologic functions. Regarding the tumorigenic involvement, TOP3s have been shown to exist in different complexes whose component proteins function in genome stability, checkpoints, and possibly cancer development (16, 30, 31). Our group also reported that TOP3 functions in the alternative lengthening of telomeres via the recombination pathway and hTOP3α knockdown (sh-hTOP3α) using shRNA technology caused an elevated telomerase expression (32). Our result resembles telomerase reactivation during cancer progression thus consistent with a potential role for hTOP3α in tumorigenesis.

Here, we report a novel role for hTOP3α in tumorigenesis and describe its mechanism of action via physically and genetically interacting with p53 to regulate p53/p21-mediated transcription program. Our results demonstrated that hTOP3α binds to p53 through p53’s DNA-binding region. Similar to the phenotypes caused by p53 deficiency, sh-hTOP3α not only promoted anchorage-independent growth but also reduced β-galactosidase (β-gal) senescence staining of cells. Through modulating hTOP3α expression, we revealed that the cellular levels of hTOP3α are inversely correlated to tumorigenic growth in vitro and in vivo. Molecularly, hTOP3α regulates tumorigenic growth in a p53- and p21-dependent manner. Moreover, hTOP3α binds to both the p53 and p21 promoter regions and positively regulates their expression. Together, our results suggest that hTOP3α acts through and/or together with p53 as an anticancer block and subsequently provides the first example of how a general chromatin enzyme collaborates with a DNA-binding transcription factor to act on specific transcription programming and subsequent cellular functions.

Materials and Methods

Chemicals, plasmids, and antibodies

All chemicals and pFLAG-CMV2 plasmid were purchased from Sigma unless otherwise indicated. Plasmid pFLAG-hTOP3α was constructed by cloning the hTOP3α PCR fragment with pFLAG-CMV2 vector. Antibodies against p53, p5315, p21, BLM (cell signaling), GAPDH (Biodesign), PCNA, HP-1γ (Abnova), hTOP3α (Santa Cruz Biotechnology), and histone H3 K9 tri-methylation (Upstate) were obtained commercially.

Cell culture and cell proliferation assay

HCT116, RHEK-1, and H1299 cells were incubated in a 37°C incubator under 5% CO2. The culture medium consisted of Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mmol/L l-glutamine, 100
U/mL penicillin, and 100 μg/mL streptomycin, except for the AGS cells (RPMI media). Plasmid transfection was achieved using FuGENE reagent (Promega) as per the manufacturer’s instruction. After 2-week selection, resistant colonies were picked and transferred to 24-well plates. The hTOP3α levels of these clones were confirmed by immunoblotting analyses. For the cell proliferation assay, 10⁴ cells were seeded in 6 cm dishes for 4 days in triplicate at each time point. At each 24-hour interval, the cellular proliferation was determined using an MTT assay.

**Lentivirus-based RNA interference**

Helper plasmids and lentiviral vectors expressing RNA interference (RNAi) sequences specifically targeting sh-Luc, sh-hTOP3α, sh-p53, and sh-BLM were obtained from the National RNAi Core Facility. Nonreplicative viral particles were prepared as recommended (http://rnai.genmed.sicna.edu.tw). The targeted sequences were as follows:

5'-CGAGTTTATTTGTCGACATT-3' (sh1-hTOP3α)
5'-GCTCTCTCGAAGGTGAGAATA-3' (sh2-hTOP3α)
5'-CGGCCGCAGAGGAAGAGAAT-3' (sh-p53)
5'-GCTACATATCAGAGGTGAT-3' (sh-BLM)
5'-CTAAGGTTAAGTCGCCCTCG-3' (sh-luciferase; sh-Luc)

**Coimmunoprecipitation, immunoblotting, and real-time RT-PCR analyses**

Coimmunoprecipitation (co-IP) and immunoblotting experiments were performed as previously described (33). Cellular lysates for immunoblotting were prepared using the radioimmunoprecipitation assay buffer (0.5% NP40, 0.1% SDS, and 1× Roche protease inhibitor cocktail). For co-IP, lysates were prepared with lysis buffer (20 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 1% NP40, 100 μmol/L Na₃VO₄, 50 mmol/L NaF, and 30 mmol/L Na₃P₂O₇) and 1× EDTA-free protease inhibitor cocktail. The proteins in the extracts were immunoprecipitated with specific antibodies, separated by SDS-PAGE, transferred onto polyvinylidenedifluoride membranes, blocked with indicated antibodies, and then detected using the enhanced chemiluminescence procedure. In some experiments, cell extracts were treated with DNase I before being subjected to immunoprecipitation using p53 antibodies. Total cellular RNA was extracted using TRIzol reagent according to the manufacturer’s protocol (Invitrogen). and cDNA was synthesized using random primers (N6) and SuperScript III reverse transcriptase (Invitrogen). All PCR reactions were performed with a Bio-Rad real-time PCR detection system using DNA-binding SYBR Green dye for detection of the PCR products.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) analysis was performed in HCT116 cells as described in the manufacturer’s instructions (Millipore). For the p53 promoter, the primers for specific PCR products containing sequences −1206 to −1082, −320 to −208, and −171 to −90 relative to the transcriptional start site were used. For the p21 promoter, primers specific for the two 5′ p53-binding sites (−2313 to −2212 and −1452 to −1310) and an NS site (control, −4443 to −4199) were used.

**Tumorigenesis assays: anchorage-independent growth on soft agar and tumor formation in NOD/SCID mice**

For the soft agar assay, cells (5 × 10³) were mixed with 2 mL of 0.3% Bacto-agar (Difco)-containing DMEM and then overlaid onto a 2 mL layer of precoated 0.6% Bacto-agar DMEM in 6-well plates. After incubation for 3 weeks, the colonies were stained using 0.005% crystal violet and the number of colonies was scored.

The Animal Use Protocol was designed in accordance with the guidelines of and approved by the Institutional Animal Care and Use Committee (IACUC No. 20060072). For the tumorigenic xenograft model, 4- to 5-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were supplied by the Animal Center at the National Taiwan University College of Medicine and maintained in the SPF facility (5 mice/cage). A total of 5 × 10⁶ cells resuspended in serum-free DMEM were subcutaneously injected into the dorsal regions of the NOD/SCID mice. The injected mice were examined every 3 to 4 days for tumor appearance. The tumor volumes were estimated (V = 0.4ab²) from the length (a) and the width (b) measurements obtained using calipers. Some mouse tumors were excised and minced into 2- to 3-mm pieces at 37°C in DMEM medium, and the tumor cells were further cultivated.

**Statistical analyses**

Statistical analyses were performed using the simple Student t-test. The data were considered to be significant if the P value was less than 0.01.

**Results**

**The physical interaction between hTOP3α and p53**

Immunoprecipitation experiments were performed to analyze the putative interaction of hTOP3α with p53. Using an anti-p53 antibody, we have shown that hTOP3α could be coimmunoprecipitated with p53 and antibodies against hTOP3α also precipitated both proteins (Fig. 1A). Notably, p53 antibodies precipitated both proteins from the DNase I-treated cell lysates (Fig. 1B). Furthermore, we confirmed this interaction using an in vitro pulldown assay with recombinant hTOP3α and p53 proteins (Fig. 1C). Using a sucrose gradient assay, we found that hTOP3α and p53 proteins coexist in the same fractions that are different from gradients containing hTOP3α and BLM helicase (Fig. 1D). Immunoprecipitation experiments were further performed on cell lysates containing various HA-tagged deletion mutants of p53, allowing us to determine the hTOP3α-interacting region within the DNA-binding domain of p53 (residues 100-325; Fig. 1E). Therefore, our results demonstrated that hTOP3α interacts with p53 to potentially form a complex.
Figure 1. hTOP3α interacts physically with p53. A, co-IP experiments using p53 or hTOP3α antibodies revealed a physical interaction between hTOP3α and p53. Protein lysates of HCT116 cells expressing either the vector control or hTOP3α OE were immunoprecipitated using the control IgG or the indicated antibodies. B, the binding between hTOP3α and p53 is DNA independent. The co-IP assay with control IgG or anti-p53 antibodies was performed using lysates with (right) or without (left) DNase I pretreatment. C, the GST pull-down assay revealed that recombinant hTOP3α and p53 proteins bind to each other. Bacterial expressed GST-tagged p53 and His-tagged hTOP3α proteins were purified and a pull-down assay of GST-p53 with glutathione beads was performed as described. The immunoprecipitates and pull-down lysates were immunoblotted (IB) using the indicated antibodies. D, cosedimentation of hTOP3α and p53. Protein lysates from HCT116 hTOP3α OE cells were sedimented using a sucrose gradient. (Continued on the following page.)
Knockdown of hTOP3α expression promotes tumorigenic growth and reduces p53 and p21 expression

To investigate the impact(s) of hTOP3α deficiency on cellular growth and tumorigenic propensities, lentivirus-mediated knockdown (sh1- and sh2-hTOP3α) in various cell lines has been performed. Two targeting sequences could sufficiently, yet differentially, knockdown hTOP3α expression in RHEK-1 nontumorigenic, HCT116 colorectal, and AGS gastric cancer cells (Fig. 2A–C) with minimal effects on cell growth (Fig. 2D–F). We noticed that the expression levels of p53, p21, and p53\(^{15p}\) (phosphorylation at Ser15) were reduced in sh-hTOP3α cells (Fig. 2A–C), suggesting another potential interaction between hTOP3α and p53.

Interestingly, both sh1- and sh2-hTOP3α rendered RHEK-1, an immortalized human epidermal keratinocyte, to grow in soft agar (Fig. 2G), an indicative of neoplastic transformation. Moreover, sh-hTOP3α also promoted anchorage-independent growth of HCT116 and AGS cancer cells, in which tumor growth–promoting abilities of hTOP3α deficiency were proportional to sh-hTOP3α knockdown efficiencies (Fig. 2I and J). The above results suggest that hTOP3α might play roles in neoplastic transformation. Notably, when expression levels of hTOP3α and p53 were simultaneously knocked down (Fig. 2J), the double knockdown cells conferred the highest tumorigenic growth and the lowest p53 activity when compared with the single knockdown cells (Fig. 2K). Together, our data demonstrated a functional interaction between hTOP3α and p53. These two proteins function cooperatively to induce p21 expression and to act in tumor suppression.

In vitro and in vivo tumorigenic growth abilities of cells are inversely related to the cellular level of functional hTOP3α

To further support the above notion that hTOP3α functions as a tumor suppressor, we generated HCT116 hTOP3αOE clones that ectopically express hTOP3α (Fig. 3A). We showed that cells with higher hTOP3α expression always displayed reduced tumorigenic growth abilities in the soft agar assay and in a mouse xenograft model (Fig. 3B and C). Moreover, tumor samples were extracted from mice injected with different cell lines and established as paired vector control and hTOP3αOE (No. 2 and 3) cell lines. We showed that hTOP3αOE tumor cell lines exhibit higher hTOP3α levels than paired vector control cell lines (Fig. 3D) and sh-hTOP3α knockdown in the No. 2 tumor-derived cell line resulted in restoration of its anchorage-independent growth ability (Fig. 3E and F). Consistently, the complementation with the functional expression of a shRNA-resistant hTOP3α reduced the tumorigenic growth of HeLa sh-hTOP3α cells in soft agar (data not shown). Our results thus suggest a novel role for hTOP3α in tumor suppression possibly via the specific interactions with p53.

The collaborative action of and genetic interaction between hTOP3α and p53 on p21 expression and tumorigenesis

We investigated the potential mechanism(s) of action underlying hTOP3α-mediated tumor suppression. Two isozymes, TOP3α and TOP3β, were identified in mammalian cells and top3β\(^{−/−}\) mouse embryonic fibroblasts (MEF) have been reported to be defective in p53 engagement (34). Above results uncovered physical and functional interactions between hTOP3α and p53, prompting us to study the possible genetic link between hTOP3α and the p53-p21 pathway. First, we generated HCT116 p53\(^{+/+}\) and p53\(^{−/−}\) sh-hTOP3α clones with similar low hTOP3α levels (Fig. 4A) and found no enhanced tumorigenic growth in the sh-hTOP3α–expressing HCT116 p53\(^{−/−}\) cells (Fig. 4B–D).

Second, hTOP3α was ectopically expressed in HCT116 wild-type, p53\(^{−/−}\) or p21\(^{−/−}\) cells (Fig. 5A, denoted as hTOP3αOE) and our data revealed that hTOP3αOE reduced tumorigenic growth in HCT116 cells but not in HCT116 p53\(^{−/−}\) or p21\(^{−/−}\) cells (Fig. 5B), suggesting that hTOP3α requires functional p53 and p21 to reduce cellular tumorigenic growth. Nevertheless, ectopic hTOP3β expression did not affect tumorigenic propensity of cells (data not shown). Finally, we restored the protein levels of p53 by expressing p53 or the p53R248W DNA-binding mutant in HCT116 p53\(^{−/−}\) cells (Fig. 5C). Reintroduction of functional p53 not only restored both p53 and p21 expression but also revealed the cooperative action between hTOP3α and p53 to induce p21 expression (compare lane 3 to lane 4, Fig. 5C) and the suppression of tumorigenic growth (Fig. 5D), while p53\(^{R248W}\)OE could not restore p21 expression or tumor-suppressive activity.

Next, we performed cell-cycle analyses on sh-hTOP3α and hTOP3αOE cells. Although we found that sh-hTOP3α causes hypersensitivity to cell-cycle–disrupting agents (e.g., hydroxyurea), both knockdown and overexpression of hTOP3α only minimally affect the cell-cycle progression (data not shown). Moreover, our results also revealed that hTOP3α contributes to the DNA damage-induced activation of p53-mediated p21 expression, but only minimally affect ATM and γ-H2AX activation (Supplementary Figs. S1 and S2). As shown in the previous reports (35), ATM inhibitors caffeine and wortmannin effectively reduced phosphorylation of p53\(^{15p}\), ATM\(^{15p}\), and γ-H2AX and p21 expression (Supplementary Fig. S2B). Thus, our results
suggested that hTOP3α might play a role in p53-mediated tumor suppression and these two proteins might regulate p21 expression cooperatively. Consistently, we also found that hTOP3α could not suppress anchorage-independent growth of p53-deficient H1299 cells in soft agar (Fig. 5E).

The enzymatic activity of hTOP3α is required for its regulatory roles on tumorigenesis and p53 expression

DNA topoisomerases required a tyrosine residue in their active site for their enzymatic activity (12, 13) and...
we generated the catalytically dead hTOP3α Y337F mutant accordingly. Differing from HCT116 hTOP3α OE cells, hTOP3α Y337F OE expression in cells had no effect on the levels of p53, p21, and p53 15P, nor was there an effect on tumorigenic growth (Fig. 5F and G). These results suggested that the enzymatic activity of hTOP3α is not only required for tumorigenic propensity but also for the p53/p21 expression.

Next, we showed that the hTOP3α-mediated p53 and p21 upregulation occurs at the transcriptional level because sh-hTOP3α expression caused a reduction in both the p53 and p21 mRNA levels (Fig. 6A). Notably, the levels of p53 and p21 mRNA increased in hTOP3α OE cells but not in hTOP3α Y337F OE cells (Fig. 6B). As mRNA levels in cells could also be regulated by mRNA stability, RNA decay analyses were performed and transcription is inhibited by actinomycin D. As shown, TP53 mRNA amounts declined faster in the sh-hTOP3α cells than in the sh-Luc cells (Supplementary Fig. S3A; quantitative results in the right) and the half-life of p53 protein was...
notably shortened when the hTOP3α expression was knocked down (Supplementary Fig. S3B), revealing a potential role of hTOP3α in regulation of TP53 mRNA and protein stability. Our results thus suggested that hTOP3α regulates p53 expression not only at transcriptional level but also at the level of mRNA stability. We further proposed that hTOP3α regulates p53-mediated tumor suppression through the modulation of p53 expression and possibly by acting with p53 to upregulate p21 expression.

The hTOP3α isozyme binds to specific regions of p53 and p21 promoters

To support the mechanisms by which hTOP3α influences p53 and p21 transcription, ChIP experiments were performed. Figure 6C showed that hTOP3α was recruited specifically to the promoter region (−171 to −90) of the TP53 gene, which is a DNA region shown to be important for p53 promoter activity (36). In addition, hTOP3α also binds to the p53-binding sites of the p21 promoter (−1452 to −1310 and −2313 to −2212) but not to the nonspecific site (NS; −4443 to −4199). Importantly, the recruitment amounts of hTOP3α to the above promoter regions were reduced in HCT116 p53+/− and HCT116 sh-hTOP3α cells (Fig. 6C and D) and the p53 targeting to the p21 promoter regions was decreased in HCT116 sh-hTOP3α cells (Fig. 6E). Collectively, our results suggested that hTOP3α and p53 collaboratively modulate the expression of both p53 and p21 directly.

Both hTOP3α and p53 deficiency reduce β-gal senescent staining in AGS cells

Both senescence and apoptosis play important roles in the tumor-suppressor activity of p53 (5–7). The p53-targeting gene p21 encoded for a cell-cycle inhibitor that has been suggested to play a role in senescence (37). To better understand the tumor-suppressive mechanisms of hTOP3α, we determined the effect of sh-hTOP3α on cellular senescence. Unlike HCT116 cells (<5% of senescence cells), AGS cells contain approximately 25% of senescent cells during normal growing conditions (38). Therefore, we have chosen AGS cells for the following studies and we observed both sh-p53 and sh-hTOP3α reduced levels of β-gal senescent staining in AGS cells (Supplementary Fig. S4A). Further quantitative analyses of the levels of β-gal staining and tumorigenic growth exhibited by AGS sh-Luc control, sh-hTOP3α, and sh-p53 cells revealed an inverse relationship between senescence and tumorigenic propensity (compare Supplementary Fig. S4B with S4C). These results suggest that hTOP3α might, in part, regulate tumor growth in part through its ability to modulate cellular senescence in AGS cells. Nevertheless, two senescence marker proteins, histone H3 K9 tri-methylation and HP-1γ, remained unchanged in AGS sh-Luc control, sh-hTOP3α and sh-p53 cells (data not shown).

Cells from patients with cancer-prone Bloom syndrome undergo cellular senescence and genomic instability. The BLM helicase forms a complex specifically with hTOP3α to resolve recombination intermediates (31, 39) and also...
Figure 5. Genetic and functional interactions of hTOP3α and the p53-p21 pathway in the suppression of tumorigenic growth. Ectopic hTOP3α expression in HCT116 cell lines induces p53 and p21 expression (A), but reduces anchorage-independent growth in a p53- and p21-dependent manner (B). C, the p21 expression is collaboratively regulated by p53 and hTOP3α. D, the coordinative action of p53 and hTOP3α in tumor suppression. E, successful expression of hTOP3α in H1299 p53-null cells neither changed p53 level nor suppressed anchorage-independent growth. F, expression of hTOP3α and catalytically dead hTOP3αY377F mutant (Tyr to Phe mutation at the active site) in HCT116 cells. G, the suppression of anchorage-independent growth by hTOP3α requires hTOP3α enzymatic activity. Cells were transfected with control (Vec) and/or expressing plasmids (as indicated). Immunoblotting and soft agar assays were performed as mentioned above to analyze the protein expression levels (A, C, E, and F) and anchorage-independent growth abilities of cells (B, n = 8; D, E right and G, n = 4). The asterisk indicates a significant difference (***, P < 0.001).
interacts physically with p53 protein (40). We observed that sh-BLM caused an elevation in the level of β-gal senescent staining in AGS cells but with only a minimal effect on the tumorigenic growth (Supplementary Fig. S4D–S4F). As reported (31), we found that sh-hTOP3α cells have reduced BLM levels and exhibit chromosome instability phenotype (data not shown). These results suggest that hTOP3α acts with p53 but not with BLM to mediate senescence and tumor suppression.

**Reduced hTOP3α expression in human cancer samples**

The expression levels of hTOP1 and hTOP2α in tumor samples are generally higher than in matched normal tissues (41). Using the Cancer Profiling Array membrane containing 241 pairs of tumor specimens (T) and matched normal samples (N) from various anatomic origins, we examined the relative expression of hTOP3α (T/N ratio). In Supplementary Fig. S5A, the levels of the hTOP3α mRNA transcript from tumor samples extracted from stomach and kidney cancers were lower compared with the corresponding normal tissues. Quantitative results revealed that approximately 75%–80% tumor samples from patients express less hTOP3α mRNA (i.e., T/N ratio < 1; stomach, Supplementary Fig. S5B; kidney, Supplementary Fig. S5C). Furthermore, we have also obtained 10 pairs of cDNA originated from renal carcinoma (RCC) and matched normal tissues. We found that the hTOP3α mRNA levels were reduced in 80% of the RCC tumor samples (Supplementary...
TOP3α and p53 Cooperate in Tumor Suppression

Fig. S5D). In addition, based on gene expression profiling data from The Cancer Genome Atlas (TCGA), we found that the hTOP3α mRNA levels are downregulated 53.8% (59.2% altered, 316 cases) and 32.3% (41% altered, 195 cases) in ovarian serous cystadenocarcinoma and colon and rectum adenocarcinoma, respectively. These above results suggest a link of hTOP3α deficiency to tumorigenesis. Furthermore, hTOP3α deficiency may be a new mechanistic pathway to p53 dysfunction in certain carcinogenic processes.

Discussion

Tumor suppressor p53 plays a central role in blocking cancer development through its induction of senescence and apoptosis (1, 3, 5). Through the interactions with p53, chromatin factors modulate p53 expression and p53-mediated tumor suppression (5, 8, 9). Here, we have identified the topologizer-resolving hTOP3α protein as a candidate anticancer block in p53-mediated tumor suppression. The universal CDK inhibitor, p21, contributes to p53-mediated cell-cycle arrest and possibly senescence (37). Our results revealed physical, genetic, and functional interactions of hTOP3α with p53. Moreover, hTOP3α binds to p53 and p21 promoters, positively regulates p53 and p21 expression, and helps the recruitment of p53 to the p21 promoter. Knockdown of hTOP3α (sh-hTOP3α) in AGS cells, behaving similarly to the sh-p53 phenotypes, caused an elevated tumor growth but a reduction in senescence. Recently, it has been reported that p53 engagement of DNA damage response (DDR) is defective in the top3β−/− MEF cells (34). Our results also showed that p53-mediated DDR was defective in sh-hTOP3α cells and hTOP3αOE expression promoted p53 and p21 expression but reduced tumorigenic growth.

The importance of p53 in tumorigenesis is illustrated by the fact that p53 is not only highly mutated (~50%) in tumors (42) but it is also rendered inactive by a range of indirect mechanisms in other types of cancer (e.g., MDM2 amplification and loss of ARF). Recent studies have demonstrated microRNA-mediated translational repression as a novel mechanism for p53 inactivation (6, 7). Our results that p53 and p21 mRNA level are lower in sh-hTOP3α cells suggest that hTOP3α positively regulates p53 and p21 expression at the transcriptional level. We have observed that approximately 54.4% of cancer samples (more than 70% in renal and gastric carcinoma) have a reduction in hTOP3α expression. We therefore suggest that the reduction of hTOP3α expression might be an additional way to inactivate p53 during tumorigenesis.

Here, we suggest that hTOP3α acts functionally with p53 in tumor suppression by the following potential mechanisms: (i) promotion of p53 transcriptional expression; (ii) interaction with p53 to collaboratively regulate p21 expression; and/or (iii) p53-mediated senescence program. Nevertheless, our and other studies also suggest an existence of p53-independent mechanism(s) in tumor suppression. RNAi technology has allowed the search for novel tumor suppressors using a genetically defined tumorigenic system (43, 44), leading to the discovery of chromatin-modifying factors, including BRD7, TIP60, and HDAC4 (5, 9, 43). Similar to our findings on hTOP3α, these enzymes either serve as cofactors of p53 on tumor suppression or are required for its optimal transcriptional activity. In addition, topoisomerases have been shown to play important roles in gene expression regulation (12, 13). For example, hTOP2β plays important roles in regulated transcription (14, 45, 46) and TOP1 is involved in efficient chromatin disassembly at specific promoter regions (11). Collectively, our results suggested a novel regulatory mechanism via collaborative action of chromatin-modifying and/or topology-resolving enzymes with transcription factors for cellular processes, especially for those require regulated gene expression. Our observations that hTOP3α interacts with p53 and cooperates with p53 to regulate p21 expression and tumor suppression and the recent report on the transcription factor-dependent recruitment of Isw2 chromatin remodeling enzyme to specific genomic loci also support the above notion (47).

Our and other studies (48) have also revealed that hTOP3α is essential in certain cell lines. Consistently, the sh-hTOP3α–mediated lethality in HEK293T cells was confirmed by increases in the sub-G1 population of HEK293T sh-hTOP3α cells at 3 or 4 days after viral infection (data not shown). As for the p53-independent mechanism(s), we found that the level of active form Ras was reduced in HCT116 hTOP3αOE cells (data not shown), suggesting an involvement of Ras pathway in the hTOP3α-mediated tumor suppression. In agreement with previous studies in yeast (24, 25, 27), we observed that sh-hTOP3α results in hypersensitivity to cell-cycle–disrupting agents and an elevated genomic instability (data not shown). These results suggest that hTOP3α might suppress tumor growth through regulating the cell-cycle checkpoint and preventing genomic instability. Therefore, hTOP3α expression level in tumors may be a determinant for chemotheraphy, such as hydroxyl urea-based ones. In the results obtained from TCGA (http://cancergenome.nih.gov), we found that hTOP3α mRNA expression is commonly altered in cancer with different anatomic origins (15 types of cancer in total) and 86.67% (13/15) of cancer types are with the altered gene expression which is dominant by cancers with a lower hTOP3α level (downregulated). Coupled with our results, we suggested that altered expression of hTOP3α, especially downregulated one, is commonly associated with the tumorigenic process. It is thus important to study the underlying mechanisms for the reduction in hTOP3α expression during tumor development. It should be noted that hTOP3α could be a potential target for the newly identified oncogenic miRNA-372 (49). Further characterization of the potential role of miRNA-372 in the expression of hTOP3α will help to clarify whether hTOP3α deficiency contributes to the tumorigenic effect of miRNA-372 and its potential as a therapeutic target for the intervention of hTOP3α-deficient cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Authors' Contributions

Conception and design: T.-K. Li, M.-Y. Hsieh, J.-R. Fan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-C. Chen, Y.-H. Yeh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-Y. Hsieh, J.-R. Fan, H.-W. Chang, T.-L. Shen

Writing, review, and/or revision of the manuscript: T.-K. Li, J.-R. Fan

Study supervision: T.-K. Li

Acknowledgments

The authors thank Dr. M.-C. Huang for technical assistance and for the reagents for the Cancer Profiling Array assay, and Dr. P.-C. Yang for providing the expressing plasmids containing the HA-tagged p53 deletion mutants.

Grant Support

This work was supported by grants from the National Science Council, the National Health Research Institutes, and National Taiwan University.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 21, 2013; revised November 4, 2013; accepted December 17, 2013; published OnlineFirst February 13, 2014.