p53 suppresses 14-3-3γ by stimulating proteasome-mediated 14-3-3γ protein degradation

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Abstract. 14-3-3 proteins are a family of highly conserved polypeptides that interact with a large number of proteins and play a role in a wide variety of cellular processes. 14-3-3 proteins have been demonstrated overexpressed in several cancers and serving as potential oncogenes. In a previous study we showed one isoform of the 14-3-3 family, 14-3-3γ was negatively regulated by p53 through binding to its promoter and inhibiting its transcription. In the present study we investigated both p53 and 14-3-3γ protein levels in human lung cancerous tissues and normal lung tissues. We found 14-3-3γ expression correlated to p53 overexpression in lung cancer tissues. Ecotopic expression of wild-type p53, but not mutant p53 (R175H) suppressed both endogenous and exogenous 14-3-3γ in colon and lung cancer cell lines. Further examination demonstrated that p53 interacted with C-terminal domain of 14-3-3γ and induced 14-3-3γ ubiquitination. MG132, a specific inhibitor of the 26S proteasome, could block the effect of p53 on 14-3-3γ protein levels, suggesting that p53 suppressed 14-3-3γ by stimulating the process of proteasome-mediated degradation of 14-3-3γ. These results indicate that the inhibitory effect of p53 on 14-3-3γ is mediated also by a post-transcriptional mechanism. Loss of p53 function may result in upregulation of 14-3-3γ in lung cancers.

Introduction

The 14-3-3 proteins are a family of highly conserved acidic polypeptides that are expressed in all eukaryotic cells (1-3). Seven isoforms, encoded by seven distinct genes have been identified in mammals (named β, γ, ε, η, σ, τ and ζ). The 14-3-3 family proteins are thought to act as scaffolding proteins and interact with a wide variety of protein partners which participate in many biological processes, such as cell cycle progression (4-7), apoptosis (8-10), cell adhesion and migration (11,12) and signal transduction regulation (13-15). Importantly, high expression of 14-3-3 family proteins have been observed in a number of human cancers, including lung (16,17), prostate (18,19), pancreatic (20,21), gastric (22) and colon cancer (23) which suggests that they may have a critical role as tumor oncogenes (24). However, few studies concerning regulation of 14-3-3 protein expression on transcriptional or post-transcriptional levels have been published.

The p53 tumor suppression protein has been defined as a critical component of the DNA damage checkpoint machinery (25). p53 can transcriptionally activate or silence a number of target genes (26,27). One of the seven human 14-3-3 isoforms 14-3-3σ is directly transactivated by p53 after DNA damage (28,29). 14-3-3σ induces cell cycle arrest in G2 by sequestering proteins in the cytoplasm which are required for entry into mitosis (29,30). Noyably, 14-3-3σ can bind to p53 and leads to p53 stabilization and enhances transcriptional activity of p53 (31). Therefore, as a target gene of p53, 14-3-3σ appears to have a positive feedback effect on p53 activity. In addition to 14-3-3σ, several 14-3-3 isoforms, including 14-3-3γ have been demonstrated to interact with p53 both *in vitro* and *in vivo* and in turn, this interaction increases the DNA binding activity of p53 (32,33).

In a previous study, we found that the 14-3-3γ protein is elevated in human lung cancerous tissues (16) and overexpression of this protein in lung cancer cells affects cell cycle progression and results in DNA polyploidization (34). In addition, wild-type p53 can bind the 14-3-3γ promoter and reduce its mRNA (35). In the present study we investigated the expression of p53 and 14-3-3γ in human lung cancer tissues and discovered that 14-3-3γ expression is significantly correlated with p53 mutation. Further investigation using cultured colon and lung cancer cells showed wild-type of p53 but not mutant p53 (R175H) could suppress 14-3-3γ expression. Importantly, inhibition of 26S proteasome by MG132 blocked the reduction of 14-3-3γ by p53. These results indicate that p53 negatively regulates 14-3-3γ by stimulating proteasome-mediated 14-3-3γ protein degradation.

Materials and methods

Antibodies and reagents. Anti-14-3-3γ (specific) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz,

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CA, USA). Antibodies to flag the β-actin were purchased from Sigma (St. Louis, MO, USA). Anti-p53 (DO-1) and GFP antibodies were obtained from Oncogene (La Jolla, CA, USA) and BD Pharmingen (San Diego, CA, USA), respectively. Cycloheximide and MG132 were purchased from Calbiochem (La Jolla, CA, USA).

Plasmid construction and cell transfection. cDNA encoding human 14-3-3γ was cloned behind the CMV promoter in pCMV-Tag2 (with flag tag at N-terminal) expression vector (Stratagene, La Jolla, CA, USA). Human non-small cell lung carcinoma cell line H358 (p53 null) from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum. Transfection was conducted using LipoTAXI mammalian transfection kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. In brief, $3x10^5$ H358 cells were seeded in 60-mm dish and the cells cultured until they reached a confluence of 50-70%. The cells were washed with serum/antibiotic-free DMEM medium prior to transfection. Plasmid pCMV-flag-14-3-3γ (5 μg) purified with Perfectprep Plasmid midi (Eppendorf, Hamburg, Germany) and 50 μl of LipoTAXI reagent diluted in 900 μl of serum/antibiotic-free DMEM medium were mixed gently and incubated at room temperature for 30 min. After adding 1.5 ml of serum/antibiotic-free DMEM medium the mixture was placed onto the cells. Transfection was performed at 37˚C in an incubator under 5% CO_2 for 5 h and then further 2.5 ml DMEM containing 20% serum was added. After incubation overnight the DNA mixture was replaced by fresh, complete medium and the cells were incubated for 48 h and split into three 10-cm plates. When the cells grew to 95% confluence, 0.5 μg/ml of G418 (Invitrogen, Carlsbad, CA, USA) was added to medium to select the clones. After three weeks, 24 clones were collected and expression of 14-3-3γ protein was assessed by immunoblotting using anti-Flag antibody (Sigma).

Adenovirus construction and infections. Ad-GFP, Ad-p53 (wild-type) and Ad-p53 (mutant, R175H) expressed GFP, wild-type and mutant p53 were kindly provided by Dr Cyrus Vaziri (Boston University School of Medicine) and Dr Bernard Futscher (Arizona Cancer Center, The University of Arizona), respectively. HCT116 cells with p53 knocked out were plated in 60-mm culture dishes (in DMEM containing 10% fetal bovine serum) and infected by adding virus directly to the medium when the cultures had attained a confluence of ~60%. After 24-, 32- and 48-h infection, the cells were harvested and endogenous 14-3-3γ protein level was analyzed by immunoblotting. H358 cells stably expressing flag-14-3-3γ were also infected by adenovirus-GFP, wild-type and mutant p53 for 24 h and then treated with cycloheximide alone or together with proteasome inhibitor MG132. Eight and 16 h later, cells were lysed and exogenous flag-14-3-3γ was analyzed by immunoblotting.

The 14-3-3γ truncated constructs and protein expression. Human 14-3-3γ fragments (1:1-78 amino acid, N-terminal; 2:79-131 amino acid, central part; 3:132-184 amino acid, central part; and 4:185-247 amino acid, C-terminal) were amplified by PCR and inserted in-frame into pGEX2T vector (Amersham Pharmacia Biotech) for expression of GST fusion proteins. All constructs were sequenced to make sure the sequences were correct. The recombinant glutathione-S-transferase (GST) tagged proteins were expressed in *E. coli* strain DH5α with 0.5 mM isopropyl-β-L-thiogalactopyranoside (IPTG) at 37˚C for 3 h. Cells were collected and lysed in B-PER bacterial protein extraction reagent (Pierce). After incubation on ice 30 min, the samples were centrifuged and the supernatants were purified using glutathione sepharose 4B beads (Amersham Pharmacia Biotech).

Glutathione-S-transferase pull-down assay and western blotting. Confluent cells (70%) were harvested and lysed in 0.5% Nonidet P-40 lysis buffer (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl, 0.5% NP-40, 50 mM NaF and ptotease inhibitors). The pull-down assay was conducted by incubating cell extracts in lysis buffer with GST-14-3-3s or GST bound to 40 μl glutathione sepharose beads for 2 h at 4˚C. After five washes with lysis buffer, the bound proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with primary antibodies.

Protein analysis. Frozen human lung cancerous (squamous cell carcinoma and adenocarcinoma) and normal tissues were obtained from the Department of Pathogenic Biology, Affiliated Hospital of Jiangsu University (Zhenjiang, China). Frozen tissues and cultured cells were lysed in NP-40 lysis buffer containing 50 mM Tris-Cl (pH 7.4), 0.15 M NaCl, 0.5% NP-40, 1 mM DTT, 50 mM sodium fluoride, and 2 μl/ml protease inhibitor cocktail (Sigma). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and 50 μg protein was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and non-specific binds were blocked by incubating with 5% non-fat milk in TBST buffer (0.01 M Tris-Cl, pH 8.0, 0.15 M NaCl, 0.5% Tween-20) at room temperature for 1 h. The membrane was subjected to the indicated antibodies and the proteins were detected by the SuperSignal West Pico detection system (Pierce, Rockford, IL, USA).

Ubiquitination assay. H358 cells were co-transfected with Flag-tagged 14-3-3γ expression vector and His-tagged ubiquitin expression vector. At 16 h after transfection, cells were infected with Ad-GFP, Ad-p53 (wt) and Ad-p53 (mt) for 32 h and then harvested. Cells were lysed in NP-40 lysis buffer (50 mM Tris-Cl, pH 7.4, 0.25 M NaCl, 0.5% NP-40, 50 mM NaF and ptotease inhibitors). Co-immunoprecipitation was conducted using Anti-Flag M2 affinity gel (Sigma). Briefly, 200 μg of total protein in 500 μl lysis buffer was incubated with 20 μl Anti-Flag M2 affinity gel at 4˚C overnight with rotation and the beads were then washed four times with lysis buffer. The proteins were eluted by boiling in SDS-PAGE sample buffer and applied to SDS-PAGE gels. 14-3-3γ ubiquitin was detected by immunoblotting using the anti-ubiquitin antibody (Upstate).

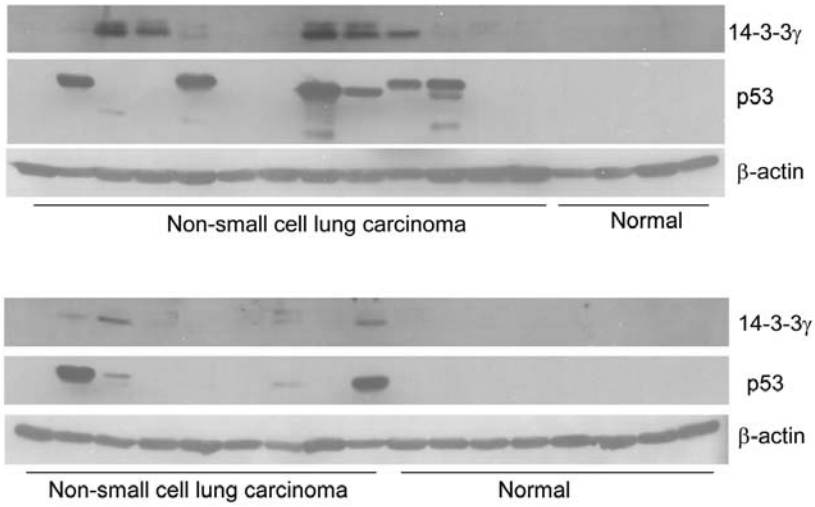

Figure 1. Representative expression profile of 14-3-3γ and p53 proteins in human non-small cell lung carcinoma and normal lung tissues. Total protein (50 μg) extracted from human lung cancerous and normal tissues was resolved by electrophoresis on a 10% SDS-PAGE gel and immunoblotting was performed using specific anti-14-3-3γ antibody (C16) and anti-p53 antibody (DO-1). β-actin protein was used as a loading control.

Table I. Expression of 14-3-3γ correlates with overexpression of p53 in human lung cancer tissues.

	No. of cancer tissues	% of 14-3-3γ expression in p53 overexpressed tissues	P-value
p53 positive	35		
14-3-3γ positive	33	94.3%	0.0001
14-3-3γ negative	2		

Statistical analysis. The Chi-squared test was performed to determine the correlation between mutant p53 and 14-3-3γ protein expression and $P<0.05$ is considered significant.

Results

14-3-3γ protein expression correlates with overexpression of p53 in human lung cancerous tissues. Our previous study indicates that high levels of 14-3-3γ protein exist in human lung cancer tissues as compared with the normal lung (16). Importantly, overexpression of 14-3-3γ protein can cause genomic instability by inducing polyploidization in human lung cancer cells (34). p53, a tumor suppressor protein, is reported to interact with several 14-3-3 isoforms including 14-3-3γ and this interaction appears to be functionally significant (32,33). In an attempt to understand the functional relationship between p53 and 14-3-3γ in lung cancers we determined the expression of p53 and 14-3-3γ by western blotting. In total 80 lung cancer tissues and 35 lung normal tissues were used in this experiment. A typical representative expression profile was shown in Fig. 1. As expected, we could not detect any signals of p53 in the 35 normal tissues. Consistent with our previous study, only one of 35 normal tissues had a band of 14-3-3γ. This normal tissue was probably contaminated with cancer cells. However, out of 80 cancer tissues there were 35 (43.8%) with p53 overexpression of (Fig. 1). In these 35 specimens, 33 expressed 14-3-3γ protein. Statistical analysis between 14-3-3γ and p53 proteins expression showed that 14-3-3γ expression significantly correlated with overexpression of p53 protein ($P=0.0001$; Table I). This result indicated that 14-3-3γ expression in lung cancers was dependent on overexpression of p53.

Wild-type p53 protein suppresses the 4-3-3γ protein level. p53 acts as a transcription factor to activate or silence a number of downstream target genes (27,28). To investigate the effect of p53 on 14-3-3γ protein level we introduced both wild-type p53 and mutant p53 (R175H) into human colon cancer cell line HCT116 in which endogenous wild-type p53 gene had been knocked out. We infected the cells with adenovirus containing GFP, wild-type p53 (wt) and mutant p53 (mt) cDNA for 24, 32 and 48 h, and then the endogenous 14-3-3γ protein level was examined by immunoblot analysis using a specific antibody against 14-3-3γ. The same blot was also probed with antibodies against GFP and p53 to detect the efficiency of adenovirus infection. Anti-human β-actin antibody was also used on the same blot to control for variations in loading. The results showed that after 32 h the wild-type p53 caused a considerable decrease (~50%) in 14-3-3γ protein level (Fig. 2). However, neither GFP nor mutant p53 (R175H) had any effect on levels of 14-3-3γ in HCT116 cells ($p53^{-/-}$). Hence, wild-type p53, but not mutant p53 (R175H) suppressed endogenous 14-3-3γ protein levels in colon cancer cells.

p53 suppresses 14-3-3γ through protein degradation by proteasome. Like other genes, 14-3-3γ regulation may occur at multiple levels, including transcription, post-transcriptional regulation of mRNA, translation and post-translational modification (36). Our previous study demonstrated that one of mechanism of p53 suppression of 14-3-3γ is through binding its promoter and inhibiting 14-3-3γ transcription (35). In order to clarify whether p53 suppresses 14-3-3γ post-transcription we stably transfected 14-3-3γ with Flag tag into human lung

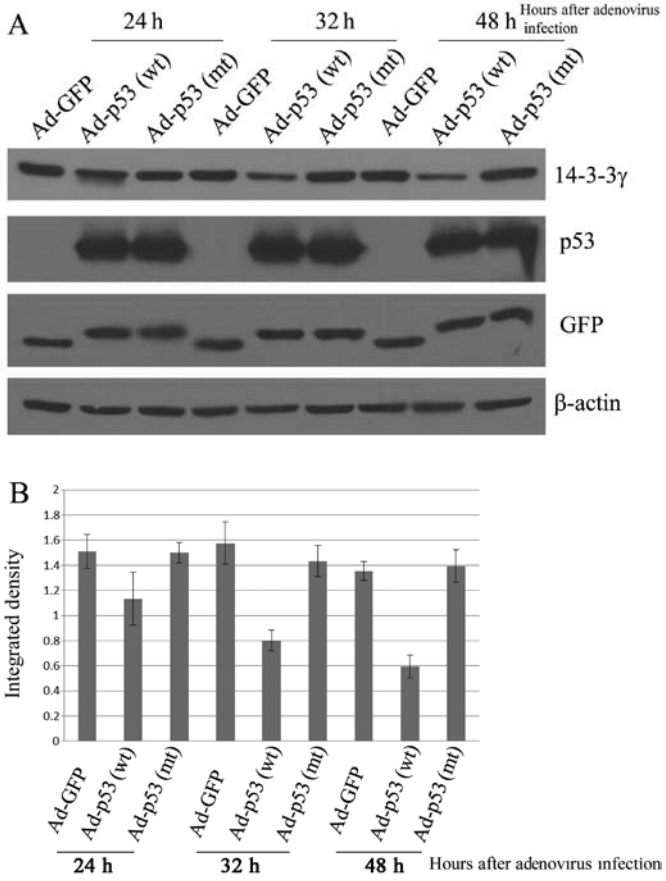

Figure 2. Wild-type p53 but not mutant p53 (R175H) suppressed endogenous 14-3-3γ protein. HCT116 ($p53^{-/-}$) cells were infected by adenovirus expressed GFP, wild-type p53 and mutant p53 (R175H). After 24, 32 and 48 h, cells were lysed and endogenous 14-3-3γ protein level was analyzed by immunoblotting. Ecotopic expressions of p53 and GFP proteins were also detected using the same membrane. β-actin was used as a loading control. (A) The experiment was repeated three times and representative data are shown. The expression of 14-3-3γ protein seen in the immunoblotting analysis was quantitated on a Strantagene Eagle Eye II, normalized against β-actin and graphed. (B) The graph represents the mean number of the integrated density ± SD, (n=3).

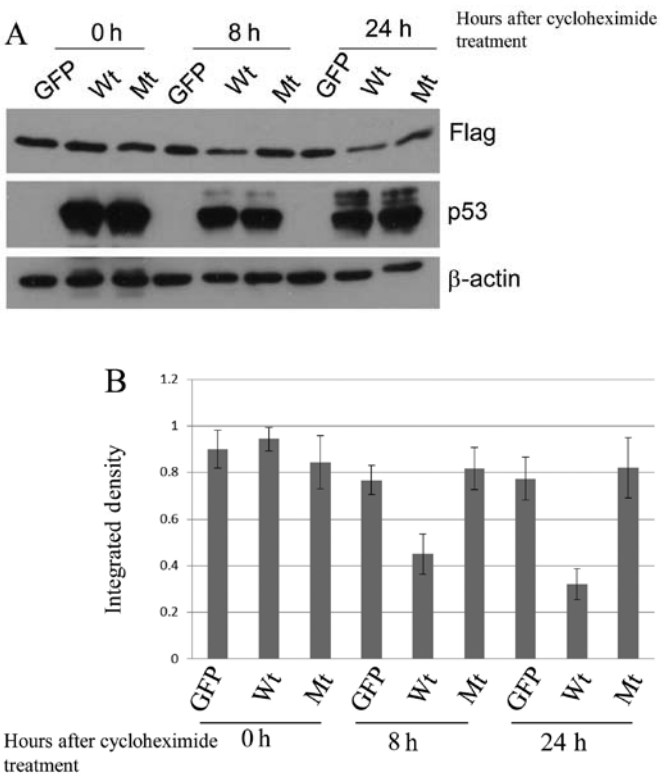

Figure 3. Wild-type p53, but not mutant p53 (R175H) suppresses exogenous 14-3-3γ protein. (A) H358 cells that stably expressed Flag-14-3-3γ protein were infected by Ad-GFP, Ad-p53 (wt) and Ad-p53 (mt). Twenty-four hours after infection, the cells were treated with 20 μg/ml cycloheximide for 0, 8 and 24 h and then harvested for detecting Flag-14-3-3γ protein level by immunoblotting. Both wild-type and mutant p53 protein were determined. β-actin was used as a loading control. The experiment was repeated three times and representative data are shown. (B) The expression of Flag-14-3-3γ protein seen in panel A was quantitated on a Strantagene Eagle Eye II, normalized against β-actin and graphed. The graph represents the mean number of the integrated density ± SD, (n=3).

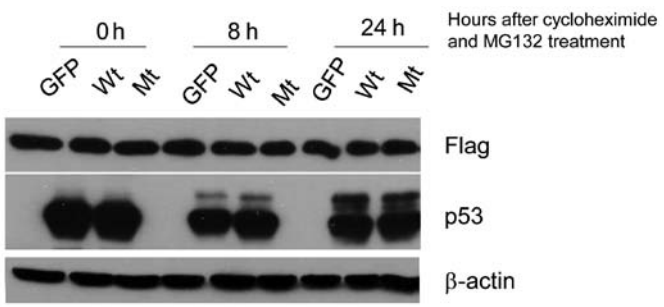

Figure 4. p53 suppresses 14-3-3γ by stimulating the process of proteasome-mediated degradation of 14-3-3γ. H358 cells that stably expressed Flag-14-3-3γ protein were infected by Ad-GFP, Ad-p53 (wt) and Ad-p53 (mt). Twenty-four hours after infection, the cells were treated with 20 μg/ml cycloheximide + 10 μm MG132 for 0, 8 and 24 h and then harvested for detecting Flag-14-3-3γ protein level by immunoblotting. Both wild-type and mutant p53 protein were determined. β-actin was used as a loading control.

cancer cell line H358 which is p53 null and then examine whether p53 suppresses this exogenous 14-3-3γ protein. H358 cells which constitutively express Flag-14-3-3γ protein were infected by adenovirus-GFP, wild-type p53 and mutant p53 (R175H) for 24 h and cycloheximide was added to inhibit protein synthesis. At 0, 8 and 24 h after cycloheximide treatment, the Flag-14-3-3γ protein levels were determined by immunoblot analysis using an antibody against Flag. Similarly to the effect of p53 on the endogenous 14-3-3γ in colon cancer cell line HCT116, wild-type p53 decreased 50% of Flag-14-3-3γ protein (Fig. 3). This result clearly indicates that p53 can negatively regulate 14-3-3γ by post-translational modification.

p53 suppression of 14-3-3γ could be through stimulating proeasome-mediated protein degradation. To determine whether p53 mediated 14-3-3γ reduction by stimulating proteasomal degradation of 14-3-3γ a specific inhibitor of proteasomal activity, MG132, was used and its effect on p53-mediated 14-3-3γ reduction was examined. The results showed that MG132 treatment completely abolished the p53-mediated reduction of 14-3-3γ protein (Fig. 4) which suggests that p53-mediated suppression of 14-3-3γ is accomplished by stimulating the process of proteasomal degradation of the protein.

C-terminal of 14-3-3γ binds to p53. It has been reported that wild-type of p53 can bind to 14-3-3γ (32,33). This interac-

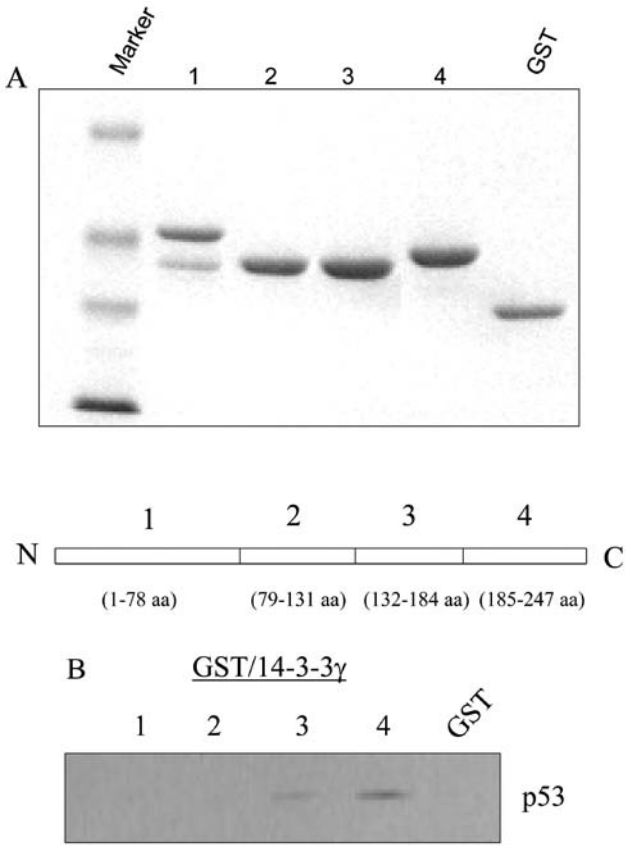

Figure 5. p53 interacts with C-terminal domain of 14-3-3γ. Human 14-3-3γ fragments (1:1-78 amino acid, N-terminal; 2:79-131 amino acid, central part; 3:132-184 amino acid, central part; and 4:185-247 amino acid, C-terminal) were amplified by PCR and inserted in-frame into pGEX2T vector to make truncated constructs. The GST-fusion proteins were expressed in bacteria and purified with glutathione sepharose 4B beads. Equal amount of fusion proteins were loaded on SDS-PAGE and then stained with Coomassie blue (A). The purified GST and GST-14-3-3γ fragments were incubated with 200 μg of HCT116 cell lysate for 4 h at 4˚C. The complex was pulled down with 40 μl of glutathione sepharose beads for 2 h at 4˚C. After five washes with lysis buffer, the bound proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-p53 antibody (B).

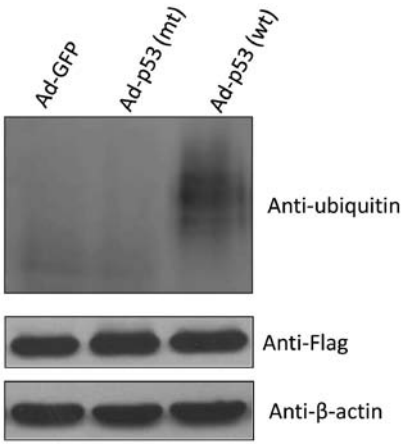

Figure 6. p53 enhances 14-3-3γ ubiquitination. H358 cells were co-transfected with Flag-tagged 14-3-3γ expression vector and His-tagged ubiquitin expression vector. After 16 h, cells were infected with Ad-GFP, Ad-p53 (wt) and Ad-p53 (mt) virus, respectively, for 32 h and then harvested. Immunoprecipitation was performed using Anti-Flag M2 affinity gel. The beads were washed successively and ubiquitin binding to 14-3-3γ was detected by SDS-PAGE and immunoblotting using the anti-ubiquitin antibody. 14-3-3γ expression and loading control were also evaluated by anti-Flag and β-actin antibodies, respectively.

tion may lead to proteasomal degradation of 14-3-3 protein. However, the detailed binding domain of 14-3-3γ is still unclear. To further explore the possible region of 14-3-3γ that is involved in binding to p53, we made GST-14-3-3γ truncated fusion proteins including N-terminal, GST-14-3-3γ1 (1-78 amino acids); central regions, GST-14-3-3γ2 and 3 (79-131 and 132-184 amino acids); and C-terminal GST-14-3-3γ4 (185-247 amino acids) (Fig. 5A). HCT116 cells were lysed and pull-down assay experiment was performed to determine the interaction. Fig. 5B showed that the C-terminal region of 14-3-3γ bound to p53 very efficiently compared with other domains, demonstrating that p53 could interact with C-terminal of 14-3-3γ.

p53 enhances 14-3-3γ ubiquitination. Since ubiquitination is required in the process of protein degradation by 26S proteasome which can be blocked by MG132, we next determined whether p53 increases 14-3-3γ protein ubiquitin. H358 cells were co-transfected with Flag-tagged 14-3-3γ expression vector and His-tagged ubiquitin expression vector and then infected with Ad-GFP, Ad-p53 (wt) and Ad-p53 (mt) viruses. After immunoprecipitation by anti-Flag antibody, 14-3-3γ ubiquitination was evaluated, and Fig. 6 clearly shows that wild-type p53 stimulated 14-3-3γ ubiquitination *in vivo*. Taken together, these data indicated that p53 could bind to 14-3-3γ, and enhanced its ubiquitin and proteasome-mediated degradation.

Discussion

14-3-3γ protein is overexpressed in human lung cancer tissues (16) and plays a role in the development of cancer (37). Notably, 14-3-3γ also has been demonstrated to interact with tumor suppression protein p53 and enhance transcriptional activity of p53 (33). Therefore, it is important to characterize the expression regulation of 14-3-3γ. In the present study, we investigated the expression profile of both 14-3-3γ and p53 in human lung cancer tissues and found that 14-3-3γ protein expression correlated with overexpression of p53. Since wild-type p53 protein level is usually too low to detect we supposed the p53 see in the bands on the blot to be mutated. Therefore, it is possible that 14-3-3γ expression correlated with p53 mutation in lung cancer patients. Ecotopic expression experiments showed wild-type p53, but not mutant p53 (R175H) could reduce both endogenous and exogenous 14-3-3γ protein levels in colon and lung cancer cells. In a previous study we showed that wild-type p53 inhibited 14-3-3γ on mRNA level (35). In the present study, we further demonstrated that p53 also can suppress 14-3-3γ by stimulating proteasome-mediated 14-3-3γ protein degradation. Together, these data indicate that p53 negatively regulate 14-3-3γ on both of transcriptional and post-translational level.

p53 has been studied intensely for its function as a genome stability guardian by regulating cell cycle arrest, apoptosis and DNA repair after DNA damage (26,38). It is also well known that p53 is the most commonly mutated gene with up to

50% mutations in different types of cancers. Consistent with previous studies, we found here p53 was overexpressed in 35 out of 80 human lung cancerous tissues. Importantly, high expression of 14-3-3γ was observed in 94% of these tissues with overexpression of p53 (33 of 35). Because overexpression of 14-3-3γ protein can cause DNA polyploidization in human lung cancer (34) and induced cell oncogenic transformation (37), it is possible that p53 guards genome stability and prevents tumorigenesis through inhibiting 14-3-3γ protein in the lung. Therefore, 14-3-3γ could be a novel target of p53 for maintaining genomic stability.

As a tumor suppressor gene, 14-3-3σ expression levels are significantly reduced or totally lost in a number of cancers, such as oral squamous cell carcinomas (39), primary bladder tumors (40), most types of breast cancer (41), gastric cancer (42) and hepatocellular carcinoma (43). One of the mechanisms resulting in 14-3-3σ reduction in cancers is due to p53 mutation because 14-3-3σ is transactivated directly by p53 (29). On the contrary, p53 negatively regulates 14-3-3γ by promoting its protein degradation. Taken together, these data suggest that p53 has diverse effects on 14-3-3 proteins in an isoform specific manner. p53 has been demonstrated to be mutated in almost half of all human cancers. In response to this, tumor suppression protein 14-3-3σ will be lost and the potential oncoprotein 14-3-3γ will progress. Therefore, p53 may act as the center of a complex network in tumorigenesis by regulating differently the 14-3-3 family proteins.

Recently, 14-3-3 proteins were identified to bind to an E3 ubiquitin-protein ligase, tripartite motif-containing protein 32 (TRIM32) and to prevent TRIM32 auto-ubiquitylation (44). Notably, Sato *et al* (45) also discovered the ubiquitin ligase ATL31 associated with and ubiquitinated 14-3-3 proteins for degradation via the ubiquitin-proteasome system during the response to cellular carbon (C)/nitrogen (N) status in *Arabidopsis*. Therefore, it is possible that p53 can regulate the interaction between 14-3-3γ and ubiquitin ligase. However, the mechanism of 14-3-3γ ubiquitination, especially enhanced by p53 is still unclear and further studies are needed.

Acknowledgements

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