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TRIM65 negatively regulates p53 through ubiquitination

Yang Li [a], Chengyuan Ma [b], Tong Zhou [c], Ying Liu [a], Luyao Sun [d], Zhenxiang Yu [a, *]

[a] Department of Respiration, The First Hospital of Jilin University, Changchun 130021, China
[b] Department of Neurosurgery, The First Hospital of Jilin University, Changchun 130021, China
[c] Department of Endocrinology, The First Hospital of Jilin University, Changchun 130021, China
[d] Department of Infectious Diseases, The First Hospital of Jilin University, Changchun 130021, China

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ABSTRACT

Tripartite-motif protein family member 65 (TRIM65) is an important protein involved in white matter lesion. However, the role of TRIM65 in human cancer remains less understood. Through the Cancer Genome Atlas (TCGA) gene alteration database, we found that TRIM65 is upregulated in a significant portion of non-small cell lung carcinoma (NSCLC) patients. Our cell growth assay revealed that TRIM65 overexpression promotes cell proliferation, while knockdown of TRIM65 displays opposite effect. Mechanistically, TRIM65 binds to p53, one of the most critical tumor suppressors, and serves as an E3 ligase toward p53. Consequently, TRIM65 inactivates p53 through facilitating p53 poly-ubiquitination and proteasome-mediated degradation. Notably, chemotherapeutic reagent cisplatin induction of p53 is markedly attenuated in response to ectopic expression of TRIM65. Cell growth inhibition by TRIM65 knockdown is more significant in p53 positive H460 than p53 negative H1299 cells, and knockdown of p53 in H460 cells also shows compromised cell growth inhibition by TRIM65 knockdown, indicating that p53 is required, at least in part, for TRIM65 function. Our findings demonstrate TRIM65 as a potential oncogenic protein, highly likely through p53 inactivation, and provide insight into development of novel approaches targeting TRIM65 for NSCLC treatment, and also overcoming chemotherapy resistance.
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1. Introduction

Tripartite-motif protein family members (TRIM) have been proposed to involve in various human diseases, such as developmental disorders, viral infections and cancer [1–4]. Interestingly, most of TRIM proteins contain a RING-finger domain, which is a critical functional domain for ubiquitin E3 ligase, and therefore they are defined as a class of E3 ligases [5]. As ubiquitination is an important post-translational modification of proteins, it has drawn increasing attention to characterize the function of TRIM family members and their role in human diseases. For example, as a p53 binding protein identified by mass spectrometry of tandem-affinity-purification (TAP)-purified p53 complex, TRIM24 was reported to negatively regulate p53 through its E3 ligase activity, and therefore is a potential therapeutic target to restore the tumor suppressor role of p53 [6]. However, more information about the TRIM family members still remains largely unknown, especially in human cancers.

In the present study, by analyzing the Cancer Genome Atlas (TCGA) gene alteration database available in cBioportal [7,8], we found that TRIM65 expression is elevated in a significant portion of non-small cell lung carcinoma (NSCLC) patients. Although TRIM65 has been reported as a gene associated with white matter lesions [9–11], and one of its E3 ligase substrates is trinucleotide repeat containing six (TNRC6) involved in microRNA pathway, little is known about the role of TRIM65 in cancer, particularly in NSCLC. We for the first time showed that TRIM65 could negatively regulate p53 through mediating p53 ubiquitination and proteasomal degradation in NSCLC cell lines, and therefore is a potential oncogenic protein.

2. Materials and methods

2.1. Cell lines and reagents

H460, A549 and H1299 cell lines were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with

* Corresponding author. Department of Respiration, The First Hospital of Jilin University, Xin Ming Road, 71, Changchun, Jilin 130021, China.
E-mail address: zhenxiangyu2015@gmail.com (Z. Yu).

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10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), at 37 °C in the presence of 5% CO_2. Cisplatin was purchased from Sigma (St. Louis, MO) and treatment was performed as previously reported [12]. The antibodies used for Western Blotting were from Santa Cruz (anti-p53, DO-1; anti-p21, CP-19; anti-PUMA, N-19; anti-TRIM65, S-15), Sigma–Aldrich (anti-Flag M2, anti-Myc) and Millipore (anti-glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Control small interference RNA (siRNA) and siRNA against TRIM65 or p53 were purchased from Ambion and transfected into cells at 40 nM using Lipofectamine 2000 (Invitrogen) per manufacture's protocol.

2.2. Plasmids and in vivo ubiquitination assay

pcDNA3-Flag-p53 plasmid was a gift from Thomas Roberts (Addgene plasmid #10838). pCl-His-hUbi was a gift from Astar Winoto (Addgene plasmid #31815). TRIM65 cDNA was obtained through reverse transcription-PCR from H460 mRNA using the following primers, forward: 5′-CGAATTCGGatggccgcgcagctgc, 3′-GCTCGAGtcagctgagcacctcttcct. The PCR product was digested with EcoRI and XhoI and inserted into pCMV-Myc-N (Clontech). Sequence of the insertion was confirmed by sequencing. In vivo ubiquitination assay was carried out as previously described [13].

2.3. Cell counting assay

Cells were transfected with indicated plasmids or siRNAs using Lipofectamine 2000 (Invitrogen) and 5000 cells/well were seeded in 96-well plates in triplicate 3 h post-transfection. Cell Counting Kit-8 (Dojindo) was added to each well for 2 h at 37 °C before the absorbance was measured at 450 nm using a microplate reader.

2.4. Statistical analysis

The student's two-tailed *t* test was employed to determine the difference between control and treatment groups (at least three independent experiments performed for analysis) and the p value less than 0.05 was considered statistically significant. Data are presented as Mean ± SD.

3. Results

3.1. TRIM65 is upregulated in non-small cell lung carcinoma (NSCLC)

TRIM65 has been described as a gene associated with white matter lesion [9–11]. Functional studies have shown that TRIM65 possesses ubiquitin E3 ligase activity with trinucleotide repeat containing six (TNRC6) as its substrate and therefore acts as a cofactor for regulating microRNA pathway [14,15]. However, little is known about the role of TRIM65 in cancer. We analyzed the Cancer Genome Atlas (TCGA) gene alteration database, particularly in NSCLC, available in cBioportal [7,8], and found TRIM65 is either copy number amplified or mRNA up-regulated in 12% of 178 lung squamous cell carcinoma and 14% of 230 lung adenocarcinoma (Supplementary. Fig. 1A & 1B). To confirm the biological impact of TRIM65, we constructed Myc-TRIM65 overexpression plasmid and transfected H460 and A549 cells with this construct for cell growth analysis. As shown in Fig. 1A, B, the growth rate of both cell lines was significantly increased when TRIM65 was overexpressed as compared to vector transfectants. In contrast, when TRIM65 was knocked down using siRNA, these two cell lines showed suppressed cell proliferation (Fig. 1C, D). These observations suggest that TRIM65 may be a potential oncogene.

3.2. TRIM65 binds to p53 and inactivates p53

One of the tripartite-motif protein family members, TRIM24, has been reported as a p53 binding protein that acts as an E3 ligase for p53 [6]. As p53 is one of the most important tumor suppressors, we wanted to examine the association of TRIM65 and p53 in lung cancer. To this end, we transfected H1299 cells with Myc-TRIM65 alone or Myc-TRIM65 and Flag-p53, and the protein samples were collected and subjected to immunoprecipitation (IP) and Western blot analysis. As shown in Fig. 2A, TRIM65 was pulled down by anti-Flag antibody only in the presence, but not in the absence of Flag-p53. To further validate this observation, we also used anti-p53 antibody to pull down endogenous p53 in A549 cells, and the results in Fig. 2B show that TRIM65 co-precipitated only in the samples incubated with anti-p53 antibody, but not IgG. These findings indicate that TRIM65 is a p53-associated protein.

We next tested the consequence of TRIM65 binding to p53 by ectopically expressing TRIM65 in A549 cells. As shown in Fig. 2C, Myc-TRIM65 decreased the expression of p53 in a dose-dependent manner. Consistently, the p53 target genes, including p21 and PUMA, were also downregulated in response to Myc-TRIM65 overexpression. In contrast, when TRIM65 was knocked down using siRNA in A549 cells, the expression of p53, p21 and PUMA was all increased (Fig. 2D). To further investigate the effects of TRIM65 on p53 signaling pathway, we transfected A549 cells either with vector plasmid or Myc-TRIM65, and 48 h later treated the cells with 2.5 μM cisplatin for 16 h followed by Western blot analysis. The results showed that cisplatin induction of p53 and its target genes p21 and PUMA were significantly compromised in response to ectopic TRIM65 (Fig. 2E). Collectively, these findings suggest that TRIM65 negatively regulates p53 expression and activity, and impairs cisplatin-induced p53 activation.

3.3. TRIM65 ubiquitinates p53 and promotes its degradation

TRIM65 has been reported as an E3 ligase against TNRC6 [14,15]. Therefore we speculated that TRIM65 might also have the same function against p53. To test this hypothesis, we performed ubiquitination assay by co-transfecting H1299 cells with His-Ub and FLAG-p53 in the absence or the presence of Myc-TRIM65. As shown in Fig. 3A, ectopic expression of Myc-TRIM65 dramatically increased the amount of poly-ubiquitinated FLAG-p53. We next sought to explore whether Myc-TRIM65-mediated p53 ubiquitination promotes p53 degradation through proteasome by transfecting A549 cells with vector plasmid or Myc-TRIM65, followed by treatment with MG132, a proteasome inhibitor. As presented in Fig. 3B, while p53 was down-regulated by Myc-TRIM65 consistently with our previous observation (Fig. 2C), this effect was markedly rescued by MG132, indicating a proteasome-dependent degradation of p53 by TRIM65. In line with this finding, the protein half-life of p53 was significantly shortened in the presence of Myc-TRIM65 as compared to vector control (Fig. 3C). Collectively, these data suggest that TRIM65 mediates the process of p53 ubiquitination, leading to p53 proteasomal degradation.

3.4. TRIM65 affects cancer cell viability dependently of p53

We have demonstrated that overexpression of TRIM65 significantly promoted cell proliferation of NSCLC cancer cells, highly likely through negative regulation of p53. To further confirm the oncogenic property of TRIM65 is p53 dependent, we compared cell growth rate of H460 (p53 wild-type) and H1299 (p53 null) cells in response to TRIM65 knockdown. We found that although TRIM65 knockdown slightly suppressed the growth of H1299 cells, this suppression is much less than that observed in H460 cells (Fig. 4A).

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