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BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

TRIM59 Is Up-regulated in Gastric Tumors, Promoting Ubiquitination and Degradation of p53

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BACKGROUND & AIMS: Little is known about factors that promote gastric carcinogenesis. We analyzed multiple microarray data sets for messenger RNAs (mRNAs) that were increased significantly in human gastric tumor samples, compared with the adjacent normal gastric tissue. We found expression of tripartite motif 59 (*TRIM59*), which encodes a putative ubiquitin ligase, to be increased, and investigated its effects in gastric cancer cell lines. **METHODS:** We analyzed microarray data sets from the Oncomine database. We used quantitative polymerase chain reaction and immunoblotting to measure levels of *TRIM59* mRNA and protein in 50 human gastric cancer and paired normal tissues, obtained from Renji Hospital and the First Affiliated Hospital of Nanchang University, in China. We also measured protein levels in the gastric epithelial cell line GES-1; the cancer cell lines MKN45, AGS, SGC7901, BGC823, Snu5, N87, and Snu1; and in tissue arrays of 108 human gastric tumors. TRIM59 was knocked down and overexpressed in gastric cancer cell lines, and the effects on proliferation, clone formation, migration, and growth of xenograft tumors in nude mice were assessed. TRIM59-related signaling pathways were examined by immunoblotting and quantitative polymerase chain reaction. We analyzed interactions among TRIM59, P53, and ubiquitin in immunoprecipitation studies. **RESULTS:** Levels of TRIM59 mRNA and protein were increased significantly in gastric tumors compared with nontumor tissues; increased levels were associated with advanced tumor stage and shorter patient survival times. TRIM59 knockdown reduced proliferation, clone formation, and migration of gastric cancer cell lines, as well as growth of xenograft tumors in nude mice; overexpression of TRIM59 had the opposite effects. TRIM59 interacted physically with P53, increasing its ubiquitination and degradation. Increased levels of TRIM59 in human gastric tumors correlated with reduced expression of P53 target genes. **CONCLUSIONS:** The putative ubiquitin ligase TRIM59 is up-regulated in human gastric tumors compared with nontumor tissues. Levels of TRIM59 correlate with tumor progression and patient survival times. TRIM59 interacts with P53, promoting its ubiquitination and degradation, and TRIM59 might promote gastric carcinogenesis via this mechanism.

Keywords: Oncogene; Stomach Cancer; Tumor Formation; Progression.

Gastric cancer is the fourth most frequent type of cancer and the second most common cause of death from cancer worldwide.[1] Strikingly, gastric cancer cases in China alone account for 42% of cases globally. This incidence increases steadily, likely owing to the dietary habits of, and the high prevalence of *Helicobacter pylori* infection in, the Chinese population.[2] Therefore, the discovery of new diagnostic and prognostic markers and a better understanding of molecular mechanisms for gastric tumorigenesis remain urgent problems.

P53, a key tumor suppressor, regulates multiple critical biological processes including apoptosis, cell-cycle arrest, DNA repair, and so forth.[3] Genetic and epigenetic alterations causing inactivation of P53 are implicated in approximately 50% of gastric cancer patients.[4–6] Notably, gastric cancer tissues from 50% of stomach cancer patients show positive immunostaining for the P53 protein. Nevertheless, it was later shown that the positive staining largely was attributable to mutant P53, whereas the expression of wild-type P53 protein became undetectable/lost in gastric tumors with the wild-type *p53* allele.[5,6] Re-activation of the P53 pathway is an attractive strategy for pharmaceutical interference in tumor initiation and progression. However, the underlying mechanisms for the P53 down-regulation in gastric cancer patients have not been understood fully to date.

The tripartite motif (TRIM) family proteins are evolutionarily conserved proteins that share a common N-terminal really interesting new gene (RING) finger domain followed by 1 or 2 B-boxes and coiled-coil sequences.[7] Because of the RING finger domain, many of the TRIM proteins act as E3 ubiquitin ligases.[7–9] The importance of TRIM proteins in cancers was first shown by the discovery of the translocation of several TRIM genes to other genes (eg, TRIM19 encoding the *pml* gene and *rarα* gene fusion, *trim24/braf* translocation, and *trim24/rets* fusion).[10–14] In addition, TRIM proteins including TRIM13, TRIM19, TRIM24, and TRIM25 were shown to be involved in leukemia, breast, and

Abbreviations used in this paper: MDM2, murine double minute 2; mRNA, messenger RNA; q-PCR, quantitative polymerase chain reaction; shRNA, short hairpin RNA; TRIM, tripartite motif.

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prostate cancers through the regulation of transcriptional factors or tumor suppressors, indicating crucial roles of the TRIM family in tumorigenesis.[15]

We performed searches and a meta-analysis on microarray data sets from the *Oncomine* database (www.oncomine.com) for possible targets in the TRIM family whose expressions are altered significantly in gastric cancer. Interestingly, TRIM59, with unidentified biological functions, was the top hit found in our searches. We report in this study that TRIM59 is up-regulated in gastric cancer and strongly associated with poor patient outcome. TRIM59 promotes gastric cancer cell proliferation, migration, and xenograft tumor growth. Mechanistically, TRIM59 interacts with the P53 tumor suppressor, and, as a result, facilitates the ubiquitination and degradation of P53. In addition, increased expression of TRIM59 in human gastric tumor samples correlates with down-regulation of P53 target genes.

Materials and Methods

Patient Samples

Fifty fresh samples of human gastric cancer and paired normal tissues were obtained during surgery at the Department of General Surgery from Renji Hospital and the First Affiliated Hospital of Nanchang University. All samples were collected with patients' informed consent. A total of 178-spot, paraffin-embedded, tissue array chips (HStm-Ade178Sur-01) including 67 paired gastric tumor and normal tissues, 39 tumor tissues, and 5 normal tissues with 7 to 9 years of follow-up information, were purchased from Shanghai Outdo Biotech, Ltd (Shanghai, China).

Immunoblotting, Immunofluorescence, and Immunohistochemistry

Detailed descriptions are available in the Supplementary Materials and Methods. Quantitative analysis of the immunostaining images was performed after color segmentation based on the fixed threshold value of hue, saturation, and intensity. Each image was assigned a score calculated by multiplying the staining intensity by the area of positive stained cells. "Up-regulation" denoted that the score of the cancer tissue was higher than that of the paired normal tissue, whereas "down-regulation" meant that the score of the cancer tissue was lower compared with the matched normal tissue.

Immunoprecipitation and Ubiquitination Assays

Cells were washed with ice-cold phosphate-buffered saline and lysed in a lysis buffer (50 mmol/L Tris-HCl, pH 8.0; 150 mmol/L NaCl; 1% NP-40) supplemented with protease and phosphatase inhibitors (Roche, Penzberg, Germany) at 36 hours after transfection. Cell lysates were incubated with primary antibodies overnight at 4°C. TrueBlot anti-Mouse IgG IP Beads (eBioscience, San Diego, CA) then were added and incubated for another 4 hours at 4°C. The immunoprecipitates were washed 4 times with the lysis buffer and boiled for 5 minutes at 98°C in protein loading buffer. Immunoprecipitated proteins were detected by following immunoblots. Antibodies used in the co-immunoprecipitation experiments were as follows: anti-Flag (Sigma, Saint Louis, MO), anti-P53 (Cell Signaling Technology, Boston, MA), mouse immunoglobulin G (Santa Cruz, Santa Cruz, CA), glyceraldehyde-3-phosphate dehydrogenase (Epitomics, Burlingame, CA), and anti-ubiquitin (Santa Cruz) antibodies.

Protein Half-Life Detection

HEK293T, MKN45, or MGC803 cells were co-transfected with the P53 expression plasmid and the TRIM59-Flag plasmid or the empty vector as described earlier. A total of 10 μg/mL of cycloheximide (purchased from Sigma) was added to the culture medium at 48 hours after the plasmid transfection to 293T cells or at 36 hours to MKN45 cells. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors as described earlier after cycloheximide treatment at indicated time points. For MG132 treatment, at indicated hours after transfection, cells were incubated with MG132 (10 μmol/L) for an additional 3, 6, or 9 hours. Cells then were collected for immunoblots to determine the P53 protein amount.

Cell Lines, Plasmids, Transfection, and Lentivirus Production

Detailed descriptions of the cell lines, plasmids, transfection, and lentivirus production are available in the Supplementary Materials and Methods.

Cell Proliferation, Clonogenic, Transwell Migration, and In Vivo Xenograft Assay

Detailed descriptions of the cell proliferation, clonogenic, Transwell (Corning, NY) migration, and in vivo xenograft assay are available in the Supplementary Materials and Methods.

Statistical Analysis

Statistical evaluation was conducted using the Student t test. Multiple comparisons were analyzed first by 1-way analysis of variance. The log-rank (Mantel–Cox) test was used for patient survival analysis. The Pearson correlation was used to analyze the strength of the association between expression levels of TRIM59 and its related genes in patient samples. A significant difference was defined as $P < .05$.

Results

TRIM59 Is Up-Regulated in Human Gastric Cancer and Correlates With Disease Progression as Well as Shortened Patient Survival

To determine the significance of TRIM59 in gastric cancer, we first analyzed multiple microarray data sets in the *Oncomine* database. As shown in Figure 1*A*, *TRIM59* messenger RNA (mRNA) levels were increased significantly in human tumor samples as compared with the adjacent normal gastric tissue. Importantly, up-regulated TRIM59 was associated strongly with shortened patient survival (Figure 1*B*). These data indicated a positive correlation of TRIM59 expression with gastric cancer.

To verify the microarray analysis results, we performed immunoblot and quantitative polymerase chain reaction (q-PCR) experiments on human gastric adenocarcinoma specimens and their matched normal tissues. Seven of 10