Effects of TIN2 on telomeres and chromosomes in the human gastric epithelial cell line GES-1

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Abstract. TERF1-interacting nuclear factor 2 (TIN2) is a key member of the protein complexes that protect telomeres. TIN2 contributes an important role in biological processes. In a previous study by the present authors, an association was reported between high TIN2 protein expression and gastric cancer. Therefore, it was hypothesized that abnormal TIN2 expression may cause the development of malignancies, including, gastric carcinomas. To investigate this hypothesis, the present study employed peptide nucleic acid fluorescence *in situ* hybridization technology to analyze the human gastric epithelial GES-1 cells with high TIN2 expression or inhibited TIN2 expression. The results indicated that GES-1 cell lines with high TIN2 expression exhibited greater telomere dysfunction-induced damage compared with GES-1 cell lines with inhibited TIN2 expression. Chromosome analysis indicated that GES-1 cells with high TIN2 expression exhibited 2.48±1.30 aberrant chromosomal changes per 100 cells, that may contribute to telomere DNA damage. Therefore, aberrant chromosomal alterations may provide a novel perspective for the pathogenesis of gastric cancer.

Introduction

Telomeres are special structures on the linear chromosome ends of eukaryotic cells and are masked with shelterin (1). Shelterin, a protective protein complex, consists of telomeric repeat-binding factor (TRF)1, TRF2, tripeptidyl peptidase 1 (TPP1), TERF1-interacting nuclear factor 2 (TIN2), protection of telomeres 1 (POT1) and ras-related protein 1 (Rap1) (2). These proteins maintain telomere integrity and protect chromosomes (3). TIN2 is a core part of this system of protective proteins.

TIN2 is a crucial telomere-associated protein that interacts with telomeric double-stranded DNA and TRF1 and 2 binding proteins (4). TPP1 performs its role in the shelterin complex by interacting with TIN2, which directly combines and interacts with TPP1 (5). In human cells, the interactions between TIN2 and TPP1 have a major role in the recruitment of telomerase to the telomere. These interactions between TIN2 and TPP1 are independent, and are independent of the interactions between POT1 and shelterin on a single strand region of the telomere (6,7). In dyskeratosis congenita, more serious symptoms are associated with mutant TIN2 genes compared with normal TIN2 genes (8). Therefore, TIN2 has become the focus of research.

Bhanot and Smith (9) and Frescas and de Lange (10) reported that the depletion of TIN2 alters other shelterin-associated proteins, including, TRF1, TRF2 and POT 1, thereby resulting in chromosomal instability. Previous studies on shelterin have been reported, where high expression levels of TRF1 and TRF2 mRNA have been observed in lung (11), liver (12,13) and gastric cancer (14) as well as lymphoma (15). However, low levels of TRF1 and TRF2 mRNA expression have also been observed in breast (16) and gastric cancer (17). Therefore, it was hypothesized that high or low protein expression may induce instability of shelterin, which may lead to the occurrence of malignant tumors. In a previous study by the present authors, it was detected that TIN2 protein expression was higher in the precancerous lesion, gastric cancer and metastasis groups compared with the normal group (18). Furthermore, the expression of TIN2 protein was higher in the gastric cancer and metastasis groups compared with the precancerous group (18). Therefore, it was hypothesized that abnormal TIN2 expression may induce shelterin instability, thereby leading to the occurrence of malignant tumors.

In the present study, peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH) and chromosome analysis were utilized to analyze the human gastric epithelial cell

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line, GES-1, with overexpressed or inhibited TIN2 expression. The results from the present study demonstrated that TIN2 overexpression by GES-1 cells resulted in increased telomere dysfunction-induced damage and increased the number of aberrant chromosomal alterations. To the best of our knowledge, the present study is the first to report on the association between gastric cancer and TIN2. This association may provide a novel perspective for the pathogenesis of gastric cancer.

Materials and methods

Cell culture. Human gastric epithelial GES-1 cells (Shanghai Innovation Biotechnology Co., Ltd., Shanghai, China) and transfected cells were cultured in Dulbecco's modified Eagle's medium (HyClone; GE Healthcare, Chicago, IL, USA) complete medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

Establishment of human gastric epithelial GES-1 cell lines with overexpressed or inhibited TIN2 expression. A total of $2x10^5$ cells/well GES-1 cells were transfected with 8 μl GES-1-GTP-hTIN2 (with 400 μl 10% FBS), GES-1-hTIN2-short hairpin RNA (SH)1, GES-1-hTIN2-SH2, GES-1-hTIN2-SH3 (target gene was TIN2; sequence, 5'-GCAGGAACTTGAACAAGAGTA-3' synthesized by Shanghai Innovation Biotechnology) in a lentivirus vector constructed by Shanghai Innovation Biotechnology, (Shanghai, China) and 6 μl control lentivirus in 24-well plates. The medium was replaced at 8 h post-transfection. The cells were observed by inverted fluorescence microscopy 96 h after transfection. The GES-1 cells with stable TIN2 overexpression or inhibited TIN2 expression and their corresponding control cell lines were screened by the addition of puromycin (0-15 μg/ml).

Detection of TIN2 with reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TIN2 gene expression was examined by RT-qPCR. Total RNA was extracted from GES-1 cells using a RNA extraction kit (cat no. K08MG; Omega Bio-Tek, Inc., Norcross, GA, USA), according to the manufacturer's protocol (Takara Biotechnology Co., Ltd., Dalian, China). Total cell RNA was reverse transcribed to cDNA (cat no. K1622; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cDNA of normal GES-1 cells, GES-1 cells overexpressing TIN2, and GES-1 cells with inhibited TIN2 expression was subjected to qPCR (QuantiFast™ SYBR® Green PCR kit; Qiagen GmbH, Hilden, Germany). The Real-time PCR cycling machine was purchased from Applied Biosystems, Thermo Fisher Scientific, Inc. The thermocycling conditions were as follows: Predenaturation for 2 min at 93˚C, then at 93˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min and extension for 7 min at 72˚C for 40 cycles. The reference gene used was GAPDH, and the experiment was repeated three times. The primer sequences used were as follows: GAPDH forward, 5'-GGAGTCCAC TGGCGTCTTC-3'; and reverse, 5'-GCTGATGATCTTGAG GCTGTTG-3'. The TIN2 primer sequences were as follows: Forward, 5'-AAGTCCTGAAAGCCCTGAATCAC-3'; and reverse 5'-GGTTCCCCATACTCTTGTTCAAGTT-3'. The formula $2^{-\Delta\Delta Cq}$ was used to calculate the relative expression of the target gene (19).

Analysis of TIN2 protein expression by western blotting. TIN2 protein expression was examined by western blotting. The cells in the flasks were washed with refrigerated phosphate-buffered saline (PBS) three times. Then, 100 μl cell lysate was added to the cells. Following lysis for 30 min on ice, the cells were scraped off the flasks. The cells were centrifuged at 14,000 x g for 15 min at 4˚C. Protein concentration in the supernatant was detected by bicinchoninic acid assay. Each well contained 30 μg of protein. To denature protein, 4X loading buffer was added to each well. The samples were then heated to 100˚C for 5 min. SDS-PAGE was performed on 10% gel, and the proteins bands were transferred to a polyvinylidene difluoride membrane. Blocking was performed in TBST containing 5% skimmed milk for 2 h at 37˚C. The membrane was incubated a TIN2 primary antibody (dilution, 1:1,000; cat no. ab13791; Abcam, Cambridge, UK.) overnight at 4˚C. The membrane was then washed with TBST 3 times for 15 min. The membrane was incubated with the corresponding horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (dilution, 1:3,000; cat no. CW0156S; Bejing Comwin Biotechnology, Co., Ltd., Beijing, China) for 1 h at room temperature and then washed three times with TBST for 15 min each time. Protein detection was performed using an enhanced chemiluminescence system, and images of the blots were analyzed by BioRad system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated three times.

PNA-FISH analysis. The cells were fixed onto microscope slides. The cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 15 min at 37˚C. The cells were permeabilized with 0.5% Triton X-100 (Solarbio Science and Technology, Co., Ltd, Beijing, China) for 20 min and washed with PBS three times. The slides were placed in 4% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.), blocking buffer (70% formamide; 0.5% blocking reagent; Tris-HCl, pH 7.2) for 2 h at 37˚C. The slides were incubated at 37˚C with rabbit antibody against H2AX (dilution, 1:1,000; cat no. 9718; Cell Signaling Technology, Inc., Danvers, MA, USA) in a humidified chamber overnight. The film was incubated at 37˚C with goat anti-mouse IgG secondary antibodies (dilution, 1:5,000; cat no. 5257; Cell Signaling Technology, Inc.) for 1 h and then washed three times with PBS. PNA probes (cat no. F1002; Panagene Inc., Daejeon, Republic of Korea) were joined on each climbing flake and then denatured at 80˚C for 10 min. The cells were incubated for 2 h at 37˚C, and then washed with Tris-HCl (pH 7.2) and 70% formamide. The fixed cells were sequentially dehydrated in 70, 95 and 100% ethanol at 5 min for each step. The cells were observed under a fluorescence microscope following DAPI staining at 37˚C for 30 sec. A total of 500 cells were analyzed for each group.

Chromosome preparation. A total of 1 μl colchicine (0.2-0.4 mg/ml) was added to the cells in culture bottles. The cells were incubated for 2 h and then digested with trypsin

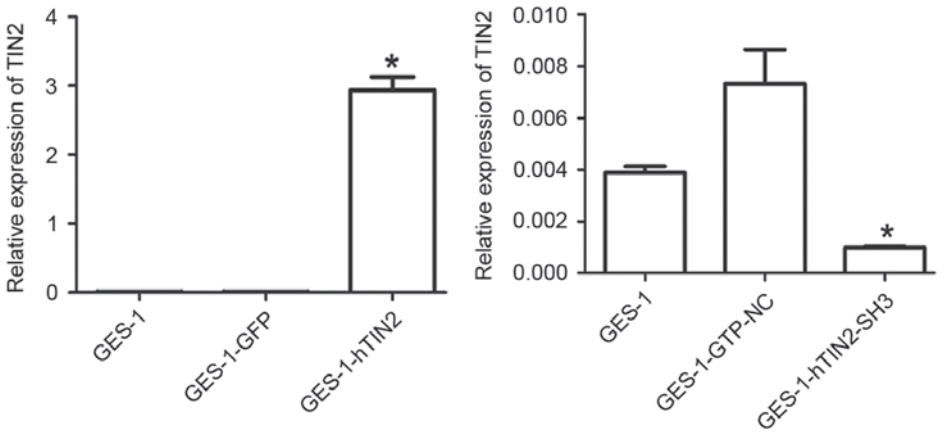

Figure 1. Relative expression levels of TIN2 in GES-1 and corresponding transfected cell lines. *P<0.05 vs. GES-1 cells. GES-1, normal human gastric epithelial cell line; GES-1-GFP, TIN2 overexpression vector group; GES-1-hTIN2, TIN2 overexpression group; GES-1-GTP-NC, low TIN2 expression vector group; GES-1-hTIN2-SH3, inhibited TIN2 expression group; TIN2, TERF1-interacting nuclear factor 2.

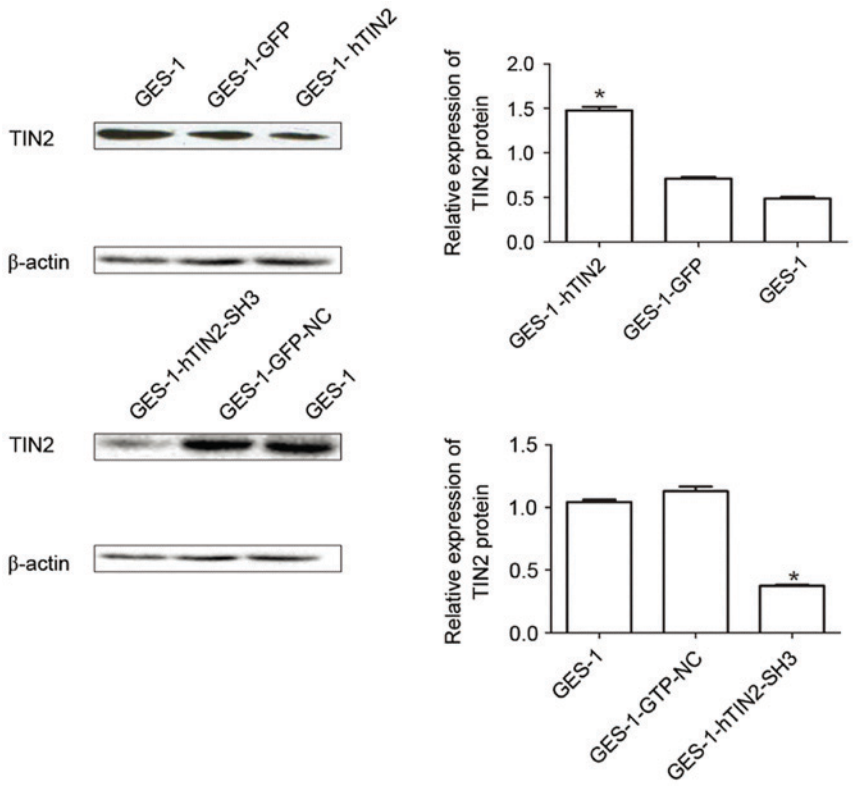

Figure 2. Detection of TIN2 protein expression in GES-1 and corresponding transfected cells lines by western blotting. *P<0.05 vs. GES-1 cells. GES-1-hTIN2-SH3, inhibited TIN2 expression group; GES-1-GTP-NC, low TIN2 expression vector group; NC, negative control; TIN2, TERF1-interacting nuclear factor 2.

(cat no. A600322; Sangon Biotech, Co., Ltd., Shanghai, China). The supernatant was discarded after the cells were centrifuged at 200 x g for 5 min at 37˚C. KCl (0.075 mol/l) was added to the cells, and the cells were incubated at 37˚C for 20 min. Then, 1 ml chromosome fixative (methanol: Acetic acid, 3:1) was added to the cells. The cells were centrifuged at 400 x g for 10 min 5257; Cell Signaling Technology at 37˚C. The addition of chromosome fixative and the centrifugation step was repeated. A chromosome fixative was added drop-wise to prepared slides. Finally, the chromosomes were observed by 10% Wright-Giemsa staining for 10 min at 37˚C. The chromosomal aberrations of 100 cells per group were recorded.

Statistical analysis. The data are presented as the mean ± standard deviation. Experimental data were analyzed using SPSS statistical software (version 18.0; SPSS, Inc., Chicago, IL, USA). The significance of the group difference was evaluated by single-factor analysis of variance (ANOVA). Multiple comparisons between the groups were performed using Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

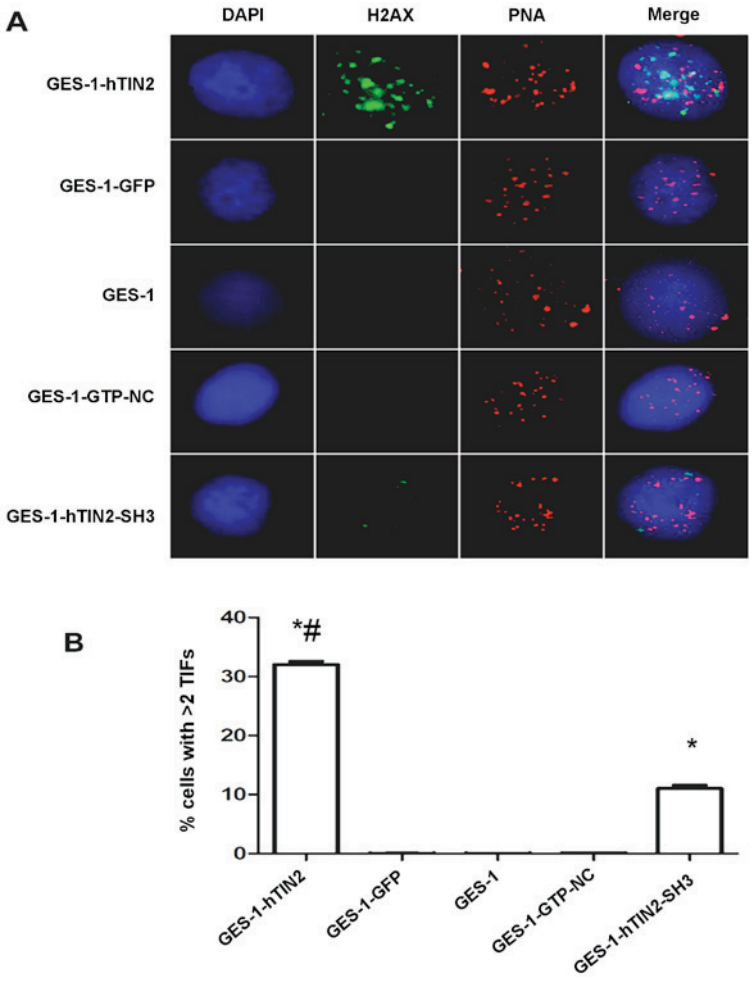

Figure 3. PNA-fluorescence *in situ* hybridization analysis of TIF formation. (A) Fluorescent staining of nuclei (blue), H2AX (green) and PNA (red). An increased number of TIFs were detected in the TIN2 high expression group compared with the group with inhibited TIN2 expression. TIFs were not detected in the other groups. (B) Percentage of cells with >2 TIFs in GES-1 and corresponding transfected cells lines. *P<0.05 vs. GES-1, GES-1-GFP and GES-1-GTP-NC. #P<0.05 vs. GES-1-hTIN2-SH3. Magnification, x1,000. GES-1, normal human gastric epithelial cell line; GES-1-GFP, TIN2 overexpression vector group; GES-1-hTIN2, TIN2 overexpression group; GES-1-GTP-NC, low TIN2 expression vector group; GES-1-hTIN2-SH3, inhibited TIN2 expression group; PNA, peptide nucleic acid; TIF, telomere dysfunction induced-foci; TIN2, TERF1-interacting nuclear factor 2.

Results

Levels of TIN2 expression as detected by RT-qPCR. GES-1 cell lines with overexpressed or inhibited TIN2 expression and their corresponding control cell lines were established. TIN2 gene expression was detected with GAPDH as the internal control. The level of TIN2 expression in normal GES-1 cells was set as 1 and acted as a control. As indicated in Fig. 1, the expression of TIN2 was higher in the GES-1-hTIN2 group and lower in the GES-1-hTIN2-SH3 group, compared with the level of TIN2 expression in normal GES-1 cells.

TIN2 protein expression in GES-1 cells with overexpressed or inhibited TIN2. Western blot analysis was performed with β-actin as the internal reference to evaluate TIN2 protein expression in GES-1 cells. The results from image analysis indicated high expression of TIN2 protein in the GES-1-hTIN2 group, compared with GES-1 cells (P<0.05; Fig. 2). Additionally, low expression of TIN2 protein was detected in the GES-1-hTIN2-SH3 group compared with the GES-1 cells (P<0.05; Fig. 2).

Detection of telomere dysfunction-induced damage by PNA-FISH analysis. The cells were observed under oil immersion for telomere dysfunction-induced damage. Green fluorescence signal indicated a positive H2AX immunofluorescence signal, and red fluorescence indicated *in situ* hybridization signals. Yellow fluorescence indicated telomere signals, and the nuclei were counterstained with DAPI (blue). Overlapping green and red fluorescence indicated telomere dysfunction induced-foci (TIFs). As indicated in Fig. 3A, overlapping green and red fluorescent signals were detected in the high TIN2 expression group, whereas clear signal overlaps were not detected in other groups. A total of 500 cells were analyzed for each group. It was detected that one cell was positive for two TIFs. The positive (signal overlaps) rate for TIFs was 31% for the TIN2 overexpression group, 11% for the TIN2-knocked down group and <1% for the other groups (Fig. 3B).

Chromosome analysis. The chromosome morphology of each cell line group was observed under oil immersion. The chromosomal aberrations of 100 cells per group

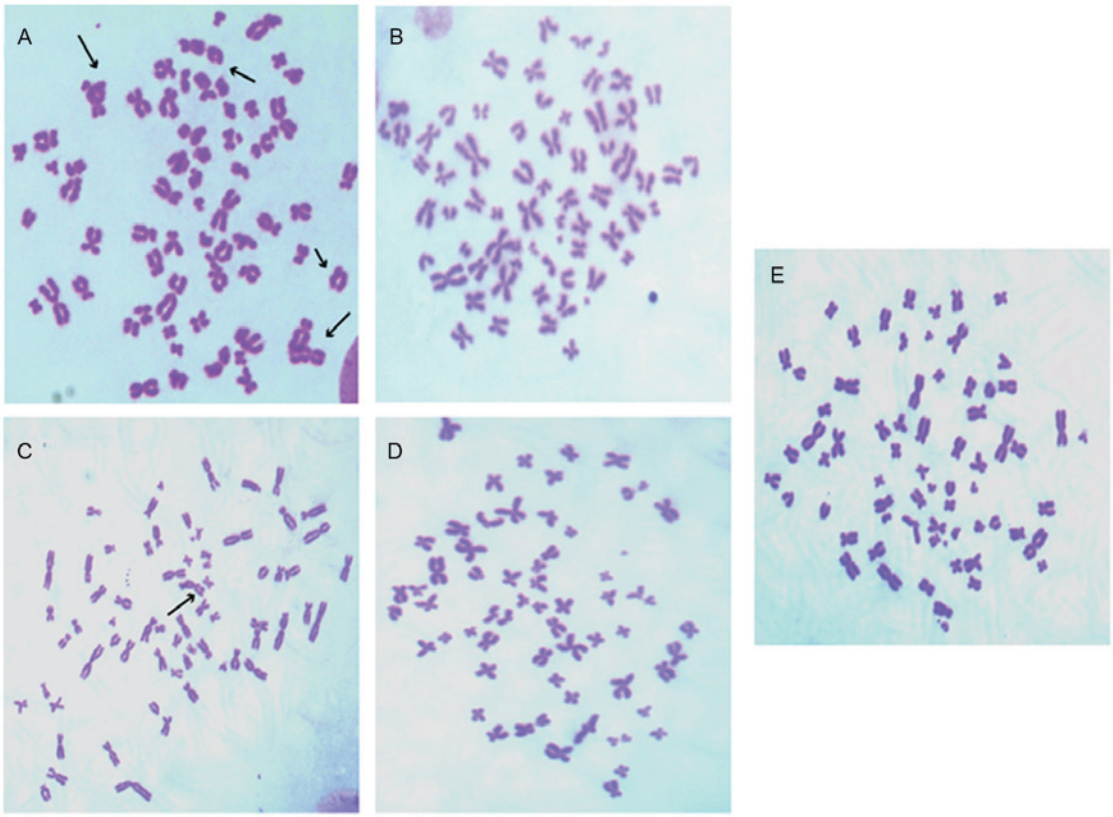

Figure 4. Chromosome morphology in (A) GES-1-hTIN2, (B) GES-1-GTP, (C) GES-1-hTIN2-SH3, (D) GES-1-GTP-NC and (E) GES-1 cells. The arrows indicate chromosomal aberrations. Chromosomal aberrations are detected in the GES-1-hTIN2 and GES-1-hTIN2-SH3 groups. Furthermore, the GES-1-hTIN2 group demonstrated more circular chromosome structures and chromosome end fusions, compared with the GES-1-hTIN2-SH3 group. Magnification, x1,000. GES-1-hTIN2, TIN2 overexpression group; GES-1-hTIN2-SH3, inhibited TIN2 expression group; GES-1-GTP-NC, low TIN2 expression vector group; GES-1-GFP, TIN2 overexpression vector group.

were recorded. As indicated in Fig. 4, circular chromosome structures and chromosome end fusions were detected in GES-1-hTIN2 chromosomes. Only 0.65±0.46 per 100 cells chromosomal aberrations were detected in GES-1-hTIN2-SH3 chromosomes, whilst all other cell lines exhibited no chromosomal abnormalities. Statistical analysis was performed to compare the means of the treatment groups, and the data are presented in Table I. The comparisons were made between the GES-1-hTIN2 and GES-1-hTIN2-SH3 groups, and it was detected that the number of chromosomal aberrations significantly differed between these groups.

Table I. Chromosomal aberration in each group.

Groups	Number of chromosomal aberration
GES-1-hTIN2	2.48±1.30[a,b]
GES-1-hTIN2-SH3	0.65±0.46[a]
GES-1	0
GES-1-GFP	0
GES-1-GTP-NC	0

[a]P<0.05 vs. GES-1, GES-1-GFP and GES-1-GTP-NC. [b]P<0.05 vs. GES-1-hTIN2-SH3. GES-1-GTP-NC, low TIN2 expression vector group; GES-1-hTIN2-SH3, inhibited TIN2 expression group. NC, negative control.

Discussion

The interaction between telomere DNA and shelterin is necessary to protect the ends of chromosomes because shelterin safeguards chromosomes (20). Chromosome end fusion occurs during chromosome breakage, thereby resulting in genomic instability, chromosome recombination, telomerase activation or upregulation and ultimately the development of malignant tumor (21). Telomere loss, breakage fusion bridge cycle and genome instability have been observed in the early stages of carcinogenesis in a model of melanoma cell malignancy (22). One previous study demonstrated that the knockdown of TRF1 and TIN2 genes resulted in alterations in telomere structure, thereby leading to chromosomal aberrations (23). This series of processes is linked to the occurrence of malignant tumors in the blood system. In the present study, it was revealed that chromosomal aberrations were associated, not only with inhibited TIN2 expression but also with TIN2 overexpression. Notably, chromosomal aberrations associated with high TIN2 expression in GES-1 cells were more serious.

H2AX can be hallmarks of DNA damage sites. Telomere DNA damage can lead to the recruitment of H2AX, which results in the formation of TIFs (24). TIFs occur during the early stages of disease and carcinogenesis (25). In the present

study, PNA-FISH was performed to investigate the causes of chromosome abnormalities including circular chromosome structures and chromosome end fusions. Compared with the GES-1 cells with inhibited TIN2 expression, there was an increased number of TIFs detected in the GES-1 cells overexpressing TIN2. The difference in the number of TIFs detected between the two groups was statistically significant.

In conclusion, abnormal TIN2 expression leads to damage in telomere DNA, which results in chromosomal aberrations. Compared with GES-1 cells with inhibited TIN2 expression, TIN2-overexpressing GES-1 cells exhibited greater marked telomere DNA damage and chromosomal aberrations. Shelterin negatively regulates telomeres (26), and consequently increased levels of shelterin correspond with increased telomere damage. TIN2 is a core part of the shelterin complex (27), which may account for why an increased number of TIFs was detected in GES-1 cells that overexpress TIN2 compared with GES-1 cells with inhibited TIN2 expression. TIN2 overexpression contributes a major role in telomere dysfunction. In the present study, it is concluded that telomere DNA damage is able to cause chromosomal aberrations. This process may be a contributory factor for the pathogenesis of gastric cancer.

In future studies, the aim is to further investigate the effect of TIN2 expression on TRF1, TRF2 and POT1 proteins in telomere-protective protein complexes. We further aim to investigate whether telomere dysfunction and chromosomal aberrations that are associated with high TIN2 expression are able to induce gastric cancer.

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Competing interests

The authors declare that they have no competing interests.

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