Overexpression of Tetraspanin31 contributes to malignant potential and poor outcomes in gastric cancer

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Abstract
Tetraspanin has important functions in many cancers by aggregating with various proteins that interact with intracellular signaling proteins. The molecular function of Tetraspanin31 (TSPAN31), located in the 12q14 amplified region in various cancers, remains unclear in gastric cancer (GC). We tested whether TSPAN31 acts as a cancer-promoting gene through its activation or overexpression in GC. We analyzed seven GC cell lines and 189 primary tumors, which were curatively resected in our hospital between 2011 and 2013. Overexpression of the TSPAN31 protein was frequently detected in three GC cell lines (42.9%) and 62 primary GC specimens (32.8%). Overexpression of TSPAN31 was significantly correlated with lymphatic invasion, venous invasion, more advanced pT and pN stages, and a higher recurrence rate. Moreover, TSPAN31 positivity was an independent factor predicting worse patient outcomes (p = 0.0283, hazard ratio 3.97). Ectopic overexpression of TSPAN31 facilitated cell proliferation of GC cells, and knockdown of TSPAN31 inhibited cell proliferation, migration, invasion, and epithelial–mesenchymal transition of GC cells through the PI3K-Akt pathway and increased cell apoptosis in a TP53 mutation-independent manner. In vivo analysis also revealed knockdown of TSPAN31 suppressed tumor progression. In addition, knockdown of TSPAN31 improved chemosensitivity to cisplatin through the suppression of ABCC2. These findings suggest that TSPAN31 plays a crucial role in tumor-malignant potential through overexpression, highlighting its utility as a prognostic factor and a potential therapeutic target in GC.

Keywords
chemosensitivity, gastric cancer, overexpression, prognosis, TSPAN31
1 | INTRODUCTION

Gastric cancer is the fifth most frequently diagnosed cancer and the third leading cause of cancer deaths worldwide.1 Despite progress in surgical techniques, perioperative chemotherapy regimens, and perioperative management, GC has long been a global health problem.2,3 Although evidence is accumulating that alterations in several genes cause tumorigenesis and progression in GC,4 few therapeutic targets have been identified.5 These include gene amplification of MET and ERBB2, mutation in E-cadherin, APC, and TP53,6-8 hypermethylation of p16,9,10 oncogenic activation of K-ras and β-catenin,11 and inactivation of the mismatch repair gene hMLH1, which is associated with microsatellite instability.12 However, few genes have been identified as biomarkers or therapeutic targets for GC in clinical practice.13-15 Thus, we aimed to identify clinical biomarkers and molecular targets for GC.

Tetraspanin is a four-transmembrane protein that can bind to various other transmembrane receptors (or each other) to form TEMs. The TEMs are essential for fundamental biological activities such as cell adhesion, proliferation, and motility.16,17 An abundance of evidence indicates that TSPAN has a pivotal role in cancer progression.18-20 Within the TSPAN family, TSPAN31 was first reported as a gene amplified in malignant fibrous histiocytoma and liposarcoma, and it was thought to be involved in growth-related cellular processes.21 Additionally, TSPAN31 is located in the critical region of chromosome 12q14, which is a genomic high-copy amplification region in several cancers, such as in sarcoma,22 chronic lymphocytic leukemia,23 parosteal osteosarcomas,24 malignant tumors of the salivary glands,25 glioblastoma,26 nasopharyngeal carcinoma,27 malignant melanoma,28 and bladder cancer.29 Moreover, TSPAN31 plays an important role in the progression of hepatocellular carcinoma.30 However, there have been no reports on the molecular function of TSPAN31, its contribution to gastric carcinogenesis, or its clinical or prognostic significance in patients with GC.

In this study, we investigated the clinical effects of TSPAN31 overexpression and activation in GC. We observed that TSPAN31 was frequently overexpressed in GC cell lines and primary GC specimens; the overexpression of TSPAN31 was an independent risk factor for poor prognosis. Next, we clarified that knockdown of TSPAN31 expression in GC cells overexpressing TSPAN31 suppressed cell proliferation, migration, and invasion, as well as chemotherapy resistance to CDDP. These results provide evidence that TSPAN31 could be an important molecular marker of tumor malignancy and a promising therapeutic target for GC patients.

2 | MATERIALS AND METHODS

2.1 | Cell lines and primary tissue samples

Seven GC cell lines (KATO-III, NUGC4, HGC27, MKN7, MKN28, MKN45, and MKN74) and a fibroblast cell strain (WI-38) were used in this study. All cell lines and cell strain were purchased from RIKEN BioResource Center Cell Bank and were authenticated by short tandem repeat profiling before distribution. The HGC27 cells were cultured in DMEM; all other cells were cultured in RPMI-1640 medium. All media were purchased from Nacalai Tesque and added to 100 ml/L FBS (Corning). All cell lines were cultured in a humidified incubator at 37°C with 5% CO₂.

Paraffin-embedded primary GC tissue samples were collected from 189 consecutive patients with GC who had undergone curative gastrectomy at the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine between January 2011 and December 2013. Paraffin blocks were stored at room temperature in the dark, and the sliced specimens were stained within 2 weeks. Relevant clinical and survival data were obtained for all patients. All experimental methods were carried out in accordance with relevant guidelines and regulations. Written informed consent was obtained from all patients for the use of their tissue specimens for research purposes. The study was designed in accordance with the Declaration of Helsinki and was approved by the institutional review board of the Kyoto Prefectural University of Medicine. None of the patients underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy, or radiotherapy, and none had synchronous or metachronous cancers in other organs. Clinical and pathological stages were defined according to the UICC TNM classification.31

2.2 | Quantitative RT–PCR

Total RNA was extracted from the cell lines using an RNeasy Mini Kit (Qiagen). The reverse transcription reaction was carried out using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The abundance of mRNA was measured by quantitative PCR using a StepOnePlus PCR System (Applied Biosystems), and cycle threshold (Ct) values were calculated with StepOne Software version 2.0 (Applied Biosystems) using TaqMan Gene Expression Assays (Hs0019595_g1 for TSPAN31, Hs00170423_m1 for E-cadherin, Hs00958111_m1 for vimentin, Hs00960489_m1 for ABCC2; Applied Biosystems) according to the manufacturer’s instructions. The gene expression results were calculated as the ratio between TSPAN31 and an internal reference gene (Hs01060665_g1 for β-actin; Applied Biosystems) that provided a normalization factor for the amount of RNA isolated from a specimen. This assay was carried out in triplicate for each sample.

2.3 | Western blot analysis

Anti-TSPAN31 rabbit mAbs (ab180502) were purchased from Abcam, and anti-cyclinD1 (2978S), p21 (2947S), phospho-Rb (9308S), AKT (9272S), phospho-AKT (#4060S), caspase-3 (14220S), cleaved caspase-3 (9664S), PARP (9542S), cleaved PARP (5625S), E-cadherin
(5296S), Vimentin (3390S), and Snail (#3879S) Abs were purchased from Cell Signaling Technology. Cells were lysed, and their proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific).

2.4 | Immunofluorescence staining

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde at room temperature for 20 min, permeabilized in 0.25% Triton X-100 in PBS, and incubated in blocking buffer containing 1% BSA. Cells were then incubated with the anti-TSPAN31 and anti-ABCC2 Ab (ab3373) overnight at 4°C. After three washes in PBS, cells were incubated for 1 h at room temperature with Alexa Fluor 488-labeled goat anti-mouse and Alexa Fluor 594-labeled goat anti-rabbit secondary Abs. After three washes in PBS, cells were incubated with rhodamine phalloidin and DAPI for 30 min. Then DAPI staining was carried out, and the slides were mounted with Vectashield Mounting Medium (Vector Laboratories). The distribution of TSPAN31 and ABCC2 proteins was examined using BZ-X700 (Keyence).

2.5 | Knockdown by siRNA and cell growth analysis

For analyzing loss-of-function due to the knockdown of endogenous gene expression, each of the siRNAs targeting TSPAN31 (siRNA, 5′-GAUUGUUGUGGCUUAUUCAACCUC-3′ for siRNA-TSPAN31 1 and 5′-GACUCGGGAUGAACUGGAAAGU-3′ for siRNA-TSPAN31 2; Invitrogen) or Luc (5′-CGUACCGGAAUACUCGA-3′; Sigma) were transfected into cells (10 nmol/L) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Knockdown of the target gene was confirmed by western blot analysis.

2.6 | Proliferation assays and cell cycle analysis

For measurements of cell growth, the number of viable cells at various posttransfection time points was assessed by a colorimetric water-soluble tetrazolium salt assay (Cell Count Reagent SF; Nacalai Tesque). Cell cycle position was evaluated 72 h posttransfection by FACS, as described elsewhere. 34,3233

2.7 | Apoptotic cell analysis

At 72 h posttransfection, siRNA-transfected cells were harvested and stained with FITC-conjugated annexin V and phosphatidylinositol using an Annexin V Kit (Beckman Coulter). The proportion of apoptotic cells was determined with a Becton Dickinson Accuri C6 flow cytometer. To assess the chemoresistance of GC cell lines to CDDP, NUGC4 (WT TP53) and MKN74 (mutant TP53), transfected with siRNA-TSPAN31 and its control, were plated onto 6-well plates (5 × 10^5 cells per well) and incubated overnight under normal culture conditions. The cells were then incubated with CDDP (4 μM). At 48 h after adding the anticancer drug, apoptotic cell analysis was undertaken as described above.

2.8 | Transwell migration and invasion assays

Transwell migration and invasion assays were carried out in a 24-well Transwell chamber using a cell culture insert with 8.0-μm pores. The upper surface of the 6.4-mm-diameter filters with 8-μm pores was precoated with (Corning BioCoat Matrigel Invasion Chamber; Corning) or without (Falcon Cell Culture Inserts; Corning) Matrigel. The siRNA transfectants (2.5 × 10^5 cells per well) were seeded into the upper chamber with serum-free medium. Complete growth medium was added to the lower well of each chamber. The transfectants were incubated for 22 h, then migrated or invasive cells on the lower surface of the filters were fixed and stained with Diff-Quik stain (Sysmex). The stained cell nuclei were counted directly, in triplicate, as described elsewhere. 35,36,37

2.9 | Plasmid construction and overexpression

To undertake transient expression assays, Halo-tagged TSPAN31 clone (FHC08492/pFN21AE2829) was purchased from Promega. Overexpression analysis was carried out with MKN45, HGC27, and MKN7 cells. Cells were transfected with either the empty vector (pCI-neo Mammalian Expression Vector; E1841; Promega) or the HaloTag-TSPAN31 expression vector using Lipofectamine 3000 reagent (Invitrogen). The expression of TSPAN31 protein in transfected cells was confirmed by western blotting using anti-HaloTag mouse mAb (G9211; Promega). Then proliferation assay was undertaken using these transfected cells.

2.10 | Immunohistochemistry

Anti-TSPAN31 rabbit polyclonal Abs (21987-1-AP) were purchased from Proteintech. Primary tumor samples were fixed with 10% formaldehyde in PBS and embedded in paraffin. Paraffin blocks were stored at room temperature in the dark, and the sliced specimens were stained within 2 weeks using an HRP method. Following deparaffinization, antigen retrieval was carried out by heating the samples in 10 mmol/L citrate buffer (pH 9.0) at 95°C for 60 min. Endogenous peroxidases were quenched by incubating the sections in 3% H₂O₂ for 20 min. Following treatment with Block Ace (Dainippon Sumitomo Pharmaceutical) for 30 min at room temperature, the sections were further incubated at room temperature for 1 h with anti-TSPAN31 Abs (1:2000). All dilutions and washings were undertaken with PBS. Bound primary Abs were detected using the EnVision+ HRP System.
Survival analysis, Kaplan–Meier survival curves were constructed for each group based on univariate predictors; differences between the groups were assessed with the log-rank test. Univariate western blotting as previously described.

Extraction Reagent (Thermo Fisher Scientific). A Dynabeads protein and their proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). A Dynabeads protein G IP kit and Magnet Starter Pack (Thermo Fisher Scientific) were used to immunoprecipitate the Dynabeads-Ab-Ag complex following the manufacturer's protocol, and samples were analyzed using western blotting as previously described.

2.11 Coimmunoprecipitation assay

NUGC4 cells were grown to 80%–90% confluence in 10-cm plates, and three plates were used for each IP reaction. Cells were lysed, and their proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). A Dynabeads protein G IP kit and Magnet Starter Pack (Thermo Fisher Scientific) were used to immunoprecipitate the Dynabeads-Ab-Ag complex following the manufacturer's protocol, and samples were analyzed using western blotting as previously described.

2.12 Animal experimental protocol

The animal protocol was approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine (M2021-571) and all experiments were carried out strictly in accordance with the NIH Guide for Care and Use of Laboratory Animals. For the xenograft model, siRNA-Luc or siRNA-TSPAN31 transfected NUGC4 cells (5 × 10⁶ cells) were subcutaneously inoculated on one side of the ventral surface in the lower flank region of 5-week-old female BALB/cSlc nu/nu mice (SLS Inc.). As the efficacy of pretransfected siRNA was transient, we administered siRNA around the tumor nodules using AteloGene Local Use Quick gelation (Koken) on days 7 and 14 after injection. On day 21 after tumor cell implantation, the mice were killed. Tumor sizes were measured on days 7, 14, and 21 after injection.

2.13 Statistical analysis

Categorical clinicopathologic variables were compared between the high and low TSPAN31 expression groups using the χ²-test or Fisher's exact test. Subgroup differences in noncategorical variables were tested using the nonparametric Mann–Whitney U-test. For survival analysis, Kaplan–Meier survival curves were constructed for each group based on univariate predictors; differences between the groups were assessed with the log-rank test. Univariate and multivariate survival analyses were undertaken using the likelihood ratio test of the stratified Cox proportional hazards model. Differences were assessed with two-sided tests and were considered significant at the p < 0.05 level.

3 RESULTS

3.1 Overexpression of TSPAN31 in GC cell lines

Quantitative RT-PCR and western blot analyses using TSPAN31-specific Abs determined TSPAN31 mRNA levels and protein levels in GC cell lines and the fibroblast cell strain WI-38. The TSPAN31 protein expression was shown with mRNA expression in GC cell lines (Figure 1A). Three out of the seven GC cell lines (42.9%) showed overexpression of TSPAN31 (NUGC4, MKN7, and MKN74), suggesting that this gene is a target for activation in these cell lines.

3.2 Immunohistochemical analysis of TSPAN31 expression in primary tumors of GC

We examined the clinicopathologic significance of TSPAN31 expression in primary tumor samples of GC based on its immunohistochemical staining pattern. We classified 189 GC tumors into positive (n = 62, 32.8%) and negative (n = 127, 67.2%) groups according to the intensity and the proportion of TSPAN31 staining among tumor cells. In primary cases, TSPAN31 protein expression was negative in most of the nontumorous gastric mucosal cell population (Figure 1B). The distribution of patients based on the intensity and proportion scores of TSPAN31 immunoreactivity in tumor samples is shown in Table S1. Kaplan–Meier survival estimates revealed that TSPAN31 immunoreactivity in tumor cells was significantly associated with worse cancer-specific survival according to the extent of the intensity (Figure 1C) and proportion scores (Figure 1D). The high TSPAN31 expression group had significantly poorer prognoses than the low expression group for cancer-specific survival (p = 0.0018, log-rank test; Figure 1F), overall survival (p = 0.0041; Figure S1A), and disease-free survival (p = 0.0270; Figure S1B).

3.3 Association between TSPAN31 protein abundance and clinicopathologic characteristics in primary cases of GC

To test the hypothesis that TSPAN31 protein abundance was associated with malignant features in GC, we assessed the expression of TSPAN31 in primary GC tissues by immunohistochemistry. The relationships between the expression of TSPAN31 and clinicopathologic characteristics are summarized in Table 1. High expression levels of TSPAN31 were significantly associated with larger tumor size,
lymphatic invasion, more advanced pT and pN stages, and higher recurrence rates. A Cox proportional hazards regression analysis (Table 2) identified TSPAN31 immunoreactivity in tumor cells as an independent factor predicting worse cancer-specific survival rates (hazard ratio 3.97; 95% confidence interval, 1.2–13.6), as well as advanced pT and pN stages. Following gastrectomy, TSPAN31 protein expression tended to be associated with recurrence ($p = 0.0413$), hematogenous recurrence in particular ($p = 0.1041$; Table 3).
3.4 | Suppression of cell proliferation by TSPAN31 knockdown and its effect according to TP53 mutation status and proliferation promotion by ectopic TSPAN31 overexpression in GC cell lines

To gain insights into the potential role of TSPAN31 as an oncogene whose overexpression might be associated with gastric carcinogenesis, we first undertook a cell proliferation assay. Using siRNAs specific to TSPAN31, we investigated whether TSPAN31 knockdown would suppress the proliferation of GC cells that overexpress TSPAN31. In the TP53 WT cell lines, NUGC4 and MKN45, and the TP53 mutant cell lines, MKN74 and MKN7, expression of the TSPAN31 protein was efficiently knocked down by introducing a TSPAN31-specific siRNA (siRNA-TSPAN31), compared

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**TABLE 1** Associations between clinicopathologic features and expression of Tetraspanin31 (TSPAN31) in gastric cancer patients

|                                | n | TSPAN31 expression | Univariate $^a$ |
|--------------------------------|---|--------------------|-----------------|
|                                |   | High | Low | p-value |
| Sample size                    | 189 | 62  | 127 |
| Sex                            |    |      |     |         |
| Male                           | 124 | 43  | 81  | 0.5155  |
| Female                         | 65  | 19  | 46  |         |
| Age (years)                    |    |      |     |         |
| <65                            | 75  | 26  | 49  | 0.7517  |
| ≥65                            | 114 | 36  | 78  |
| Tumor location                 |    |      |     |         |
| U                              | 40  | 16  | 24  | 0.4611  |
| M                              | 96  | 28  | 68  |
| L                              | 53  | 18  | 35  |
| Tumor major axis               |    |      |     |         |
| <60 mm                         | 130 | 36  | 94  | 0.0305  |
| ≥60 mm                         | 59  | 26  | 33  |
| T stage                        |    |      |     |         |
| T1                             | 106 | 31  | 75  | 0.0142  |
| T2                             | 26  | 4   | 22  |
| T3                             | 31  | 13  | 18  |
| T4                             | 26  | 14  | 12  |
| N stage                        |    |      |     |         |
| N0                             | 135 | 37  | 98  | 0.0304  |
| N1                             | 25  | 9   | 16  |
| N2                             | 13  | 8   | 5   |
| N3                             | 16  | 8   | 8   |
| Histopathological grading      |    |      |     |         |
| Differentiated                 | 94  | 30  | 64  | 0.8771  |
| Undifferentiated               | 95  | 32  | 63  |
| Venous invasion                |    |      |     |         |
| Present                        | 62  | 20  | 42  | 1.0000  |
| Absent                         | 127 | 42  | 85  |
| Lymphatic invasion             |    |      |     |         |
| Present                        | 81  | 35  | 46  | 0.0120  |
| Absent                         | 108 | 27  | 81  |
| Recurrence                     |    |      |     |         |
| Present                        | 20  | 11  | 9   | 0.0413  |
| Absent                         | 169 | 51  | 118 |

Note: Significant values are bold.
Abbreviations: L, lower region; M, middle region; U, upper region.

$^a$p values are from the $\chi^2$-test or Fisher’s exact test.
with a luciferase-specific siRNA (siRNA-Luc) as a negative control. The proliferation of these cell lines was particularly suppressed following the knockdown of endogenous TSPAN31 expression (Figures 2A and S2A). To examine the proliferation-promoting effect of the ectopic overexpression of TSPAN31 in GC cells, we carried out transient expression assays by transfecting with expression constructs of TSPAN31 into MKN45 (TP53 WT), MKN7 (TP53 mutant), and HGC27 (TP53 mutant) cells. Ectopic expression of HaloTag-TSPAN31 in these cell lines was verified by western blotting using a HaloTag-specific Ab. We observed increased cell proliferation through TSPAN31 overexpression compared to the empty vector in these GC cell lines. Moreover, western blot analyses showed that overexpression of TSPAN31 induced phosphorylation activation of AKT (Figure 2D). The results suggest that TSPAN31 promotes cell proliferation activity in GC cells.

### 3.5 Investigation of the tumor proliferation function of TSPAN31 in vivo

To confirm whether TSPAN31 has tumor proliferation function, we undertook in vivo analyses using SCID mouse and siRNA-Luc or siRNA-TSPAN31 transfected NUGC4 cells. siRNA-TSPAN31/atelecollagen complexes or siRNA-Luc/atelecollagen complexes were subcutaneously injected around the tumors to maintain the effect of TSPAN31 knockdown. As a result, the volume of tumors with TSPAN31 knockdown was statistically smaller than the volume of control tumors (Figure 2E). The results suggest that TSPAN31 promotes cell proliferation activity in GC cells.

### 3.6 Cell cycle analyses and apoptosis assays by TSPAN31 knockdown using FACS

To investigate the molecular mechanisms by which TSPAN31 knockdown suppressed cell proliferation, we undertook

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**Table 2** Multivariate analysis using Cox proportional hazards model for cancer-specific survival among gastric cancer patients who received gastrectomy

| Variable                              | Univariate | Multivariate |
|---------------------------------------|------------|--------------|
|                                       | p-value    | HR | 95% CI | p-value |
| Sex                                   | 0.5399     | 0.88 | 0.2–3.1 | 0.8373 |
| pT category                           | <0.0001    | 11.88 | 3.0–47.3 | 0.0004 |
| pN category                           | <0.0001    | 10.95 | 1.2–99.8 | 0.0337 |
| Tumor size (mm)                       | <0.0001    | 3.28 | 0.7–16.0 | 0.1403 |
| Histopathological type                | 0.9984     | 2.13 | 0.6–7.8 | 0.2563 |
| Venous invasion                       | 0.3806     | 3.40 | 0.7–8.3 | 0.1936 |
| Lymphatic invasion                    | 0.0001     | 3.74 | 0.4–34.8 | 0.2460 |
| TSPAN31 expression                    | 0.0018     | 3.97 | 1.2–13.6 | 0.0283 |

Note: Significant values are bold.

Abbreviations: CI, confidence interval; HR, hazard ratio; TSPAN31, Tetraspanin31.

*Kaplan–Meier method; significance determined by log–rank test.

Multivariate survival analysis undertaken using Cox proportion hazards model.

**Table 3** Associations between expression of Tetraspanin31 (TSPAN31) and disease recurrence in gastric cancer patients with gastrectomy

|          | TSPAN31 expression | p-value |
|----------|--------------------|---------|
|          | High | Low |        |
| Number of patients | 189  | 62  | 127    | 0.0413 |
| Total of recurrence | 20   | 11 (18%) | 9 (7%) |
| Hematogenous recurrence | 4   | 3 (5%) | 1 (1%) | 0.1041 |
| Liver    | 2    | 1 (2%) | 1 (1%) | 0.5496 |
| Lung     | 2    | 2 (3%) | 0 (0%) | 0.1064 |
| Lymphatic recurrence | 4   | 2 (3%) | 2 (2%) | 0.5986 |
| Peritoneal recurrence | 12  | 6 (10%) | 6 (5%) | 0.2124 |

Note: Significant values are bold.

Fischer’s exact test.
a cell cycle analysis and an apoptosis assay. The FACS analysis indicated that transfection of TP53 WT NUGC4 cells and TP53 mutant MKN74 cells with siRNA-TSPAN31 induced sub-G1 phase arrest and G1 phase arrest of cell cycle progression compared with their transfection with control siRNA (Figure 2B). Apoptotic cell analysis showed that transfection of TP53 WT NUGC4 cells...
and TP53 mutant MKN74 cells increased early apoptosis (annexin V-positive/PI-negative) and late apoptosis (annexin V/PI-double positive), respectively, at 72 h posttransfection compared with transfection with control siRNA (Figure 2C). Western blot analysis showed that transfection of TP53 WT NUGC4 and TP53 mutant MKN74 with siRNA-TSPAN31 induced the cleavage of caspase 3 and PARP (Figure 3A). These findings suggest that the knockdown of TSPAN31 overexpression in TP53 WT cells induces cell apoptosis through caspase activation. Similar results were observed in the TP53 WT cell line, MKN45, and the TP53 mutant cell line, MKN7 (Figure S2B and S2C).

### 3.7 Suppression of cell migration, invasion, and EMT by downregulation of TSPAN31 expression

As shown in Table 1, protein expression of TSPAN31 was significantly associated with the presence of lymphatic invasion in clinical
samples. Transwell migration and invasion assays were carried out to confirm the molecular function of TSPAN31 in both cell migration and invasion in vitro. We examined the ability of TP53 WT NUGC4 and TP53 mutant MKN74 cells transfected with siRNA-TSPAN31 to move through pores under various conditions. An uncoated membrane was used for the migration assays, whereas a Matrigel-coated membrane was used for the invasion assays. As shown in Figure 4A, the number of siRNA-TSPAN31-transfected NUGC4 and MKN74 cells that migrated into the lower chamber was significantly lower compared with siRNA control-transfected cells under both conditions. These results suggest that TSPAN31 overexpression could enhance the ability of GC cells to migrate and invade in both TP53 WT and TP53 mutant cell lines.

Next, we investigated the effect of TSPAN31 on EMT. We identified MKN7 cells as the appropriate GC cell lines as Vimentin mRNA expression was higher and E-cadherin mRNA expression was lower than in the other TSPAN31-overexpressing GC cell lines (Figure 4B). The knockdown of TSPAN31 induced significant morphological change, increased the expression of E-cadherin protein, and reduced the expression of Vimentin and Snail proteins in MKN7 cells (Figure 4C).

3.8 | Molecular mechanisms by which overexpression of TSPAN31 contributes to malignant potential in GC cells

The PI3K/AKT pathway is frequently activated in cancers and is important for tumor cell growth and survival. Knockdown of TSPAN31 expression by transfection with siRNA-TSPAN31 suppressed the phosphorylation activation of AKT, induced the production of p21, suppressed the production of cyclin D1, and induced phosphorylation inactivation of Rb in NUGC4 cells (WT TP53) and MKN74 (mutant TP53) (Figure 3A). Figure 3B shows a hypothetical model of the overexpression/activation of TSPAN31 in GC cells. These results were also verified in the TP53 WT cell line, MKN45, and the TP53 mutant cell line, MKN7 (Figure 3C).

3.9 | Effect of TSPAN31 overexpression on chemoresistance in GC

Next, we examined whether TSPAN31 was also associated with chemoresistance in GC. When treated with CDDP, the transfection of TP53 WT NUGC4 and TP53 mutant MKN74 with siRNA-TSPAN31 increased both early apoptosis and late apoptosis (48 h posttransfection) compared to transfection with control siRNA (Figure 5A). To elucidate the molecular mechanism, we analyzed the interaction between TSPAN31 and ABCC2, which is a member of the multidrug resistance-associated protein family. Immunofluorescence staining was used to evaluate the distribution of TSPAN31 and ABCC2 in NUGC4 and MKN74 cells treated with siRNA-Luc and siRNA-TSPAN31. As a result, these two proteins were found to be colocalized (Figure 5B). Also, the knockdown of TSPAN31 induced a weak deterioration in ABCC expression (Figure 5C). The co-IP assay showed that TSPAN31 and ABCC2 interacted with each other in NUGC4 cells (Figure 5D). These results strongly suggest that overexpression of TSPAN31 induces chemoresistance through the suppression of ABCC2 in GC cells.

4 | DISCUSSION

In the present study, we showed that TSPAN31 is frequently overexpressed in primary GC specimens and cell lines. The overexpression of TSPAN31 significantly contributed to aggressive tumor features and poor outcomes. Overexpression of TSPAN31 promoted cell proliferation, and knockdown of TSPAN31 inhibited cell proliferation, migration, and invasion in a manner independent of TP53. Knockdown of TSPAN31 also inhibited tumor growth in vivo. Furthermore, the knockdown of TSPAN31 enhanced chemotherapy sensitivity to CDDP through the suppression of ABCC2. These results suggest that TSPAN31 plays an important role in tumor malignant features through overexpression, highlighting its usefulness as a prognostic factor and therapeutic target for GC.

New insights into the function of TSPANs in various cancers are accumulating. In contrast to other membrane proteins, TSPANs do not function as obvious receptors. Instead, TSPANs could affect the activation of various signaling pathways through interactions with other TSPANs, integrins, receptors, or cytoplasmic proteins. A previous study identified that TSPANs interact with PI3Kγ and activate cell survival in hepatocellular carcinoma. In contrast, TSPAN31 reportedly acts as a sense transcript and suppresses cancer proliferation in cervical cancer. However, these findings were only revealed in cell experiments and animal models. The present study is the first to report the significance of TSPAN31 expression in clinical specimens. Immunoreactivity to TSPAN31 in clinical specimens of GC was shown to be a poor prognostic factor, even after adjusting for other confounding factors in multivariate analysis.
A recent study suggested that TSPAN31 interacts with PI3K, resulting in the activation of Akt in hepatocellular carcinoma. Indeed, our in vitro analyses revealed the molecular mechanism affecting malignant features in GC cells with overexpression of TSPAN31 was through AKT pathway activation. Moreover, AKT/GSK3β pathway activation is required for the induction and maintenance of EMT. In this study, the knockdown of TSPAN31 suppressed cell migration and invasion through inactivation of the AKT/Snail pathway, which resulted in the inhibition of EMT and metastasis. These findings concur with the results of lymphatic invasion and recurrence rates in patients with high expression of TSPAN31 and strongly suggest that TSPAN31 plays a pivotal role in the malignant potential of GC.

Moreover, a fascinating chemotherapy study identified that the downregulation of several other TSPAN family proteins contributes to CDDP-induced cell apoptosis in GC cells and head and neck squamous cells. In this study, we found that knockdown of TSPAN31 also improved sensitivity to CDDP, a key chemotherapy drug for patients with locally advanced or metastatic GC. Interestingly, we clarified that TSPAN31 distribution was colocalized with that of ABCC2, which is a family of the ATP-dependent drug efflux pumps known as ABC transporters. Also, knockdown of TSPAN31 induced ABCC2 downregulation. Previously, strong expression of ABCC2 was observed in various human solid tumors, including GC, and it was associated with multichemoresistance inhibiting drug excretion. Thus, these findings suggest that TSPAN31 might be a key molecule for predicting chemoresistance. Furthermore, TSPAN31 could be the pivotal inhibiting target for improving the chemosensitivity of CDDP in prospective GC patients with overexpression of TSPAN31, through its interaction with ABCC2. Detailed clinical analyses are currently being evaluated.

Regarding the clinical application of TSPANs as a target molecular therapy, there have been several studies showing that the inhibitors decrease tumor growth in vitro and induce partial or complete tumor remission in vivo. In GC, ALB6, which is an anti-CD9 mAb, reportedly induces apoptosis, decreases tumor growth, or inhibits invasion, migration, and metastasis properties in human GC cell xenografts. However, the side effects on normal tissues were not fully evaluated because this mAb directly affects human CD9. Moreover, other anti-TSPAN Abs have been examined in xenograft models of various human solid cancers. In acute lymphoblastic leukemia patients with autologous bone marrow transplantation, a cocktail treatment using three Abs (BA-1/CD24, BA-2/CD9, and BA-3/CD10) to suppress the malignant bone marrow did not show toxicity against hematological stem cells. Also, in patients with chronic lymphocytic leukemia, a phase 1 trial using BI 836826, a chimeric mouse–human mAb against CD37, was undertaken in 2019. Thus, the promising biological functions of TSPAN inhibitors in clinical settings are being studied widely, suggesting that inhibitors of TSPANs might be therapeutic agents in GC.

In conclusion, we clearly showed frequent overexpression of the TSPAN31 protein and its prognostic value in patients with GC. Although studies involving larger cohorts and in vivo analyses using TSPAN31 inhibition will be necessary to validate these findings before exploring their application in clinical settings, our results provide pivotal evidence that TSPAN31 could be a crucial molecular marker for determining the malignant properties of GC cells and also that it could be a target for molecular therapy in patients with GC.

CONFLICT OF INTEREST
Hitoshi Tsuda is Editor of Cancer Science. The other authors have no conflict of interest.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
This study was designed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. The animal protocol was approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine (M2021-571).

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Additional supporting information may be found in the online version of the article at the publisher’s website.

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