

Loss of *COP1* expression determines poor prognosis in patients with gastric cancer

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Abstract. Previous studies have suggested conflicting roles for the E3 ubiquitin ligase COP1 in tumorigenesis, providing evidence that both the oncoprotein c-Jun and the tumor suppressor p53 may be COP1 targets. In the present study, we focused on the clinical significance of *COP1* expression in gastric cancer cases and analyzed the malignant behavior of *COP1*-knockdown gastric cancer cells *in vitro*. We analyzed *COP1* expression in cancer lesions and the corresponding normal mucosa to demonstrate the clinical significance of *COP1* expression in 133 cases of gastric cancer. We also investigated the relationship between COP1 expression and cell proliferation and the association of COP1 with c-Jun transcriptional target genes, such as *MMP1*, *MMP7* and *MMP10*. The expression of *COP1* mRNA was significantly lower in gastric cancer tissues compared to the corresponding normal mucosa ($P=0.049$). In multivariate analysis for overall survival, we found that *COP1* expression was an independent prognostic factor in gastric cancer. Knockdown of *COP1* expression in the gastric cancer cell lines MKN-45 and NUGC4 promoted proliferation, and significant associations between *COP1* expression and *MMP1*, *MMP7* and *MMP10* were also observed in knockdown assays. In conclusion, the present study suggests that loss of *COP1* expression may be a novel indicator for the biological aggressiveness in gastric cancer.

Introduction

Gastric cancer is the second leading cause of cancer-related mortality worldwide and may become one of the leading causes of all deaths in the near future (1-3). According to data from the National Cancer Institute (NCI), it is estimated that

more than 24,000 patients are diagnosed with gastric cancer each year in the United States (4). The development of adjuvant chemotherapies has improved clinical outcomes; however, advanced gastric cancer with lymph node metastasis still has a poor prognosis (5,6). Identification of genes responsible for the development and progression of gastric cancer and a clear understanding of the clinical significance of these genes are important for the diagnosis and treatment of this disease.

The evolutionarily conserved protein COP1 has been shown to operate as an E3 ubiquitin ligase complex, and a number of putative substrates have been identified, including the c-Jun oncoprotein and p53 tumor-suppressor protein (7-9). In mammalian cells, COP1 regulates various cellular functions, such as proliferation, cell cycle progression, apoptosis, and DNA repair (7,10); however, the definitive role of COP1 has not yet been determined among diverse cancer types. COP1 has been found to be overexpressed in ovarian and breast adenocarcinomas, and its expression has been shown to correlate with the unstable state of p53 protein in cancers that retain wild-type p53 gene status (11). COP1 was also found to be frequently overexpressed in human hepatocellular carcinoma (HCC), and transfection of HCC cell lines with COP1 siRNA in the context of wild-type p53 inhibited growth; in contrast, p53-null Hep3B cells were resistant to the effects of transfection with COP1 siRNA (12). The results of these studies indicate that COP1 has significant functions as an oncogene by degradation of p53 and could be a novel therapeutic target in ovarian cancer and HCC. On the other hand, several intriguing studies have also demonstrated that COP1 has a tumor-suppressor role. Vitari *et al* (13) found that COP1 negatively regulates the proto-oncogenes *ETV1*, *ETV4* and *ETV5*, which encode transcription factors in the E26 transformation-specific (ETS) family, and that combined loss of COP1 and PTEN enhances the invasiveness of mouse prostate adenocarcinomas. Another study showed that COP1 specifically binds basic leucine zipper factors of the Jun family and that expression of COP1 downregulates c-Jun-dependent transcription as a functional consequence of COP1-zipper factor interactions (14). Thus, it is difficult to determine whether COP1 serves primarily as an oncogene or

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as a tumor-suppressor gene among diverse malignancies and circumstances.

In the current study, we analyzed *COP1* mRNA expression using clinical samples from 133 patients diagnosed with primary gastric cancer. We then examined the relationships between *COP1* mRNA expression and clinicopathological factors and determined the clinical significance of aberrant *COP1* expression. Moreover, we confirmed the biological significance of *COP1* in gastric cancer cells using *in vitro* assays.

Materials and methods

Clinical sample and cell lines. A total of 133 gastric cancer patients were enrolled in the present study. All patients underwent surgery without pre-operative treatments such as chemotherapy and radiotherapy. Tumor and adjacent normal tissue were obtained. Total RNAs were extracted using a QIAamp DNA Micro kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Patients were closely observed each month after surgery, and the mean post-operative follow-up period was 3 years. Histopathological evaluation was assessed according to the Japanese Classification of Gastric Cancer, 3rd English edition.

MKN-45 and NUGC4 cell lines were provided by the American Type Culture Collection and were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured in a humidified 5% CO₂ incubator at 37°C. The study protocol was reviewed and approved by Kyushu University.

Real-time quantitative reverse transcription (RT)-PCR. The primer sequences were as follows: *COP1*, forward 5'-TGCAAAGTTTGTGAGTGGTGA-3' and reverse 5'-GAACGTAGGCAGTATGGTTCC-3'; *MMP1*, forward 5'-GCTAACCTTTGATGCTATAACTACGA-3' and reverse 5'-TTTGTGCGCATGTAGAATCTG-3'; *MMP7*, forward 5'-CTGACATCATGATTGGCTTTG-3' and reverse 5'-ATCTCCTCCGAGACCTGTCC-3'; *MMP10*, forward 5'-CAAAGAGGAGGACTCCAACA-3' and reverse 5'-TTCACATCCTTTTCAGGTTG-3'; and *GAPDH*, forward 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse 5'-AGTCTTCTGGGTGGCAGTGAT-3'. *COP1*, *MMP1*, *MMP7* and *MMP10* levels were normalized to that of *GAPDH*. The real-time quantitative monitoring of PCR reactions was performed using a LightCycler® System and a LightCycler® 480 Probes Master kit (both from Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol.

***COP1* RNA interference.** For the siRNA knockdown experiment, double-stranded RNA duplexes targeting human *COP1* (5'-AGGAGCGUCCAGUAGAUGAACACGC-3'/5'-GCGUGUUCAUCUACUGGACGCUCCU-3', 5'-UUCAAUGCUUAAACUUUGGGAUUG-3'/5'-CAAUCCCAAAGUUUAAGCAUUUGAA-3', and 5'-UGGAAAGGAAGAUGAAGUCGCGAGG-3'/5'-CCCUGCGACUUGAUCUCCUUUCCA-3') were purchased (Stealth RNAi; Invitrogen, Carlsbad, CA, USA). Negative control siRNA was also purchased from Invitrogen. MKN-45 and NUGC4 cells were transfected with siRNA at a concentration of 20 μmol/l using Lipofectamine (RNAiMAX) and were incubated in glucose-free Opti-MEM (both from

Invitrogen). Total RNA from transfected cell lines was extracted using a QIAamp DNA Micro kit following the manufacturer's protocol.

Proliferation assay. MKN-45 and NUGC4 cells transfected with specific siRNAs or negative control siRNA were seeded at 8x10³ cells/well in 96-well flat-bottomed microtiter plates in a final volume of 100 μl of culture medium/well. Cells were incubated in a humidified atmosphere (37°C and 5% CO₂) for 48 or 96 h after initiation of transfection. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics) was used to measure cell growth inhibition. After incubation, 10 μl of MTT labeling reagent (final concentration of 0.5 mg/ml) was added to each well, and the plate was incubated for 4 h in a humidified atmosphere. Solubilization solution (100 μl) was added to each well and the plate was incubated overnight in a humidified atmosphere. After confirming that the purple formazan crystals were completely solubilized, the absorbance of each well was measured by a Model 550 series microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), at a wavelength of 570 nm corrected to 655 nm. The assay was performed using 6 replicates.

Statistical analysis. The significance of differences between 2 groups was estimated with the Student's t-test and χ^2 test. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Variables with a P-value of <0.05 by univariate analysis were used in subsequent multivariate analysis on the basis of the Cox proportional hazards model. All differences were considered statistically significant at the level of P<0.05. Statistical analyses were conducted using JMP 5 software (SAS Institute).

Results

***COP1* expression in gastric cancer tissues.** A significant difference in *COP1* mRNA expression between tumor tissue and the corresponding normal mucosa was observed in 133 gastric cancer cases by quantitative real-time PCR. *COP1* was significantly downregulated in tumor tissues compared to the corresponding normal mucosa (median *COP1*/*GAPDH* ratio in tumor tissues, 1.30; median *COP1*/*GAPDH* ratio in the corresponding normal mucosa, 1.90; P = 0.049) (Fig. 1).

Relationship between *COP1* mRNA expression and clinicopathological factors. To evaluate the relationship between *COP1* mRNA expression and clinicopathological factors, we divided the 133 patients with gastric cancer into a high *COP1* expression group (n=67) and a low *COP1* expression group (n=66), according to the *COP1*/*GAPDH* ratio of 0.80 in cancerous tissue. There were no significant differences between the low and high *COP1* expression groups in terms of clinicopathological factors (Table I). However, the low *COP1* expression group showed an increased tendency to be associated with greater tumor size (P=0.099; Table I).

Low *COP1* expression resulted in poor prognoses in patients with gastric cancer. The low *COP1* expression group

Table I. Relationship between *COPI* mRNA expression and clinicopathologic factors.

| Factors | Low expression (n=67) | High expression (n=66) | P-value |
|-----------------------------|-----------------------|------------------------|---------|
| Age, mean ± SD | 65.2±11.1 | 66.4±12.5 | |
| Gender, n | | | 0.433 |
| Male | 44 | 39 | |
| Female | 23 | 27 | |
| Histological grade, n | | | 0.187 |
| Well and mod | 34 | 26 | |
| Por and sig | 33 | 40 | |
| Tumor size (mm), n | | | 0.099 |
| <30 | 13 | 21 | |
| >31 | 54 | 45 | |
| Depth, n | | | 0.581 |
| T1 | 18 | 15 | |
| T2-4 | 49 | 51 | |
| Lymph node metastasis, n | | | 0.949 |
| Positive | 44 | 43 | |
| Negative | 23 | 23 | |
| Lymphatic invasion, n | | | 0.667 |
| Positive | 46 | 43 | |
| Negative | 21 | 23 | |
| Venous invasion, n | | | 0.8 |
| Positive | 15 | 16 | |
| Negative | 52 | 50 | |
| Liver metastasis, n | | | 0.477 |
| Positive | 5 | 3 | |
| Negative | 62 | 63 | |
| Peritoneal dissemination, n | | | 0.325 |
| Positive | 11 | 7 | |
| Negative | 56 | 59 | |

SD, standard deviation; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet ring cell adenocarcinoma.

showed significantly poorer prognosis than the high *COPI* expression group ($P=0.026$) (Fig. 2). Univariate analysis of overall survival revealed significant associations with *COPI* expression, depth of tumor invasion, tumor size, lymph node metastasis, lymphatic invasion and venous invasion in patients with gastric cancer (Table II). Thus, we applied these factors to multivariate analysis and found that *COPI* expression was an independent prognostic indicator for overall survival in patients with gastric cancer ($P=0.0085$; Table II).

Inhibition of *COPI* expression with siRNA in gastric cancer cell lines. For proliferation assays, *COPI* siRNA was transfected into MKN-45 and NUGC4 cells (expressing wild-type p53). A significant reduction in *COPI* expression by siRNA

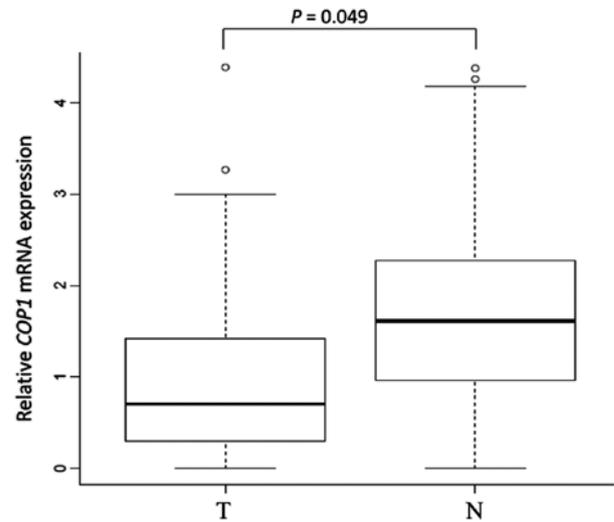


Figure 1. Analysis of *COPI* mRNA expression in gastric tumor tissues and the corresponding normal mucosa by real-time RT-PCR. *COPI* mRNA was significantly downregulated in tumor tissues compared with normal mucosa ($P=0.049$). T, gastric tumor tissues; N, normal mucosa.

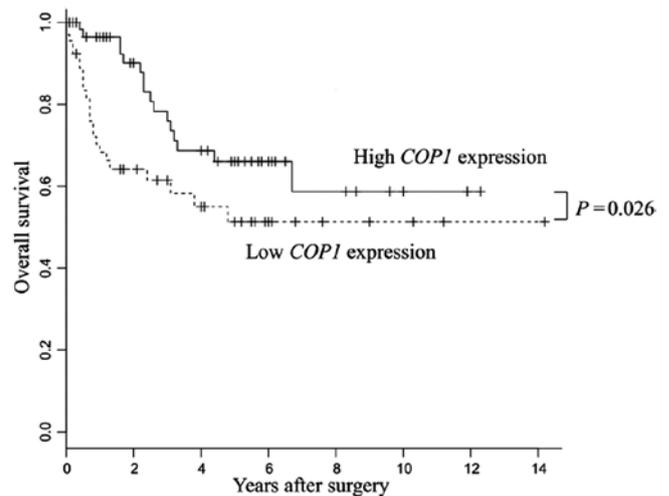


Figure 2. Kaplan-Meier survival curves for gastric cancer patients according to *COPI* mRNA expression. Patients in the low *COPI* expression group had poorer survival than those in the high *COPI* expression group ($P=0.026$).

transfection was confirmed by quantitative real-time RT-PCR. Using MTT assays, we found that *COPI* knockdown by siRNA significantly increased the numbers of MKN-45 and NUGC4 cancer cells as compared with the corresponding control cells (MKN-45, $P=0.041$; NUGC4, $P=0.023$) (Fig. 3).

These results indicated the possibility that *COPI* may function as a tumor suppressor. Therefore, we investigated the association between *COPI* expression and the expression of *MMP1*, *MMP7* and *MMP10*, transcriptional targets of c-Jun and ETV1. We found that *COPI* knockdown by siRNA significantly increased the expression of *MMP1*, *MMP7* and *MMP10* as compared to the negative control (Fig. 4).

Discussion

The ubiquitin ligase complex, which targets proteins for proteasome-mediated degradation, plays important roles

Table II. Univariate and multivariate analysis for overall survival using the Cox proportional hazards regression model.

| Factor | Univariate analysis | | | Multivariate analysis | | |
|--|---------------------|--------------|---------|-----------------------|-------------|---------|
| | RR | 95% CI | P-value | RR | 95% CI | P-value |
| Age | 0.7611 | 0.551-1.039 | 0.086 | - | - | - |
| Gender | 0.9837 | 0.715-1.381 | 0.9215 | - | - | - |
| Histology grade (well and mod/por and sig) | 1.2632 | 0.919-1.772 | 0.151 | - | - | - |
| Tumor size >30 mm (negative/positive) | 1.9167 | 1.212-3.504 | 0.0035 | 1.37539 | 0.850-2.546 | 0.2093 |
| Depth (T1/T2-4) | 4.095 | 1.908-17.252 | <0.0001 | 2.114491 | 0.857-9.353 | 0.1143 |
| Lymph node metastasis (negative/positive) | 3.076 | 1.844-6.272 | <0.0001 | 2.177924 | 1.245-4.611 | 0.0042 |
| Lymphatic invasion (negative/positive) | 2.221 | 1.405-4.063 | 0.0002 | 1.172472 | 0.690-2.239 | 0.577 |
| Venous invasion (negative/positive) | 1.7543 | 1.263-2.403 | 0.0011 | 1.392089 | 0.983-1.955 | 0.0617 |
| <i>COP1</i> mRNA expression (low/high) | 0.7019 | 0.506-0.960 | 0.0269 | 0.642875 | 0.455-0.894 | 0.0085 |

RR, relative risk; CI, confidence interval; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet ring cell adenocarcinoma.

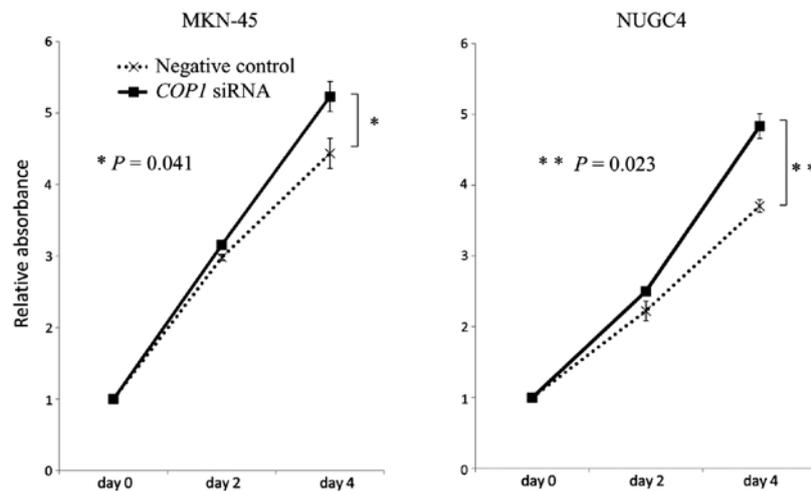


Figure 3. Knockdown of *COP1* promotes the proliferation of MKN-45 and NUGC4 cells. Cell growth was measured on day 2 (48 h) and day 4 (96 h) by MTT assay. The absorbance at day 0 was assigned a value of 1. The results are the mean ± SD of 6 replicates.

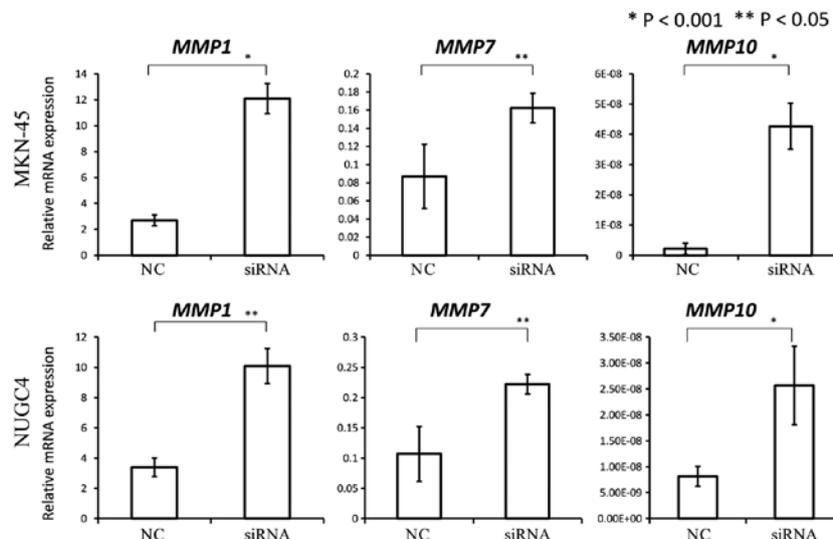


Figure 4. Association between *COP1* expression and *MMP1*, *MMP7* and *MMP10* expression. The expression of *MMP1*, *MMP7* and *MMP10* was significantly higher in MKN-45 and NUGC4 cells transfected with *COP1* siRNA than in the negative control cells. NC, negative control.

in maintaining cellular homeostasis (15). Accordingly, the dysregulation of ubiquitin ligase has been implicated in a variety of diseases, including cancer. Overexpression of COP1 was observed in ovarian cancer, breast cancer, and HCC, and COP1 has been recognized as an oncogene by targeting p53 for degradation in a ubiquitin-dependent fashion (11,12). Li *et al* (16) also found that COP1 was overexpressed in gastric cancer and observed a negative correlation between COP1 protein expression and p53 protein expression. In contrast, in a mouse model of prostate cancer, COP1 was identified as a tumor suppressor that negatively regulates ETV1, ETV4 and ETV5 (13). Moreover, identification of well-known oncoproteins, such as c-Jun (14) and MTA1 (17), as potential COP1 substrates has indicated the possibility that COP1 may function as a tumor suppressor.

In the present study, we revealed that low *COP1* expression was an independent factor predicting poor prognosis in patients with gastric cancer (Table II). These results, in contrast to those of Li *et al* (16), suggest that COP1 acted as a tumor suppressor in gastric cancer. The major factor that may account for the observed differences between our results and the results published by Li *et al* was the p53 mutation status of clinical samples; indeed, p53 mutation status was unknown in both studies. The mutation rate of p53 has been reported to be 41 and 73% in 2 exome sequencing studies of gastric cancer (18,19), suggesting that COP1-p53 interactions should not affect tumor biology in about half of all gastric cancer cases. Thus, in order to properly evaluate the clinical significance of COP1 expression, it is important to investigate COP1 expression in gastric cancer samples expressing wild-type p53.

Migliorini *et al* (20) utilized a genetic approach to generate an allelic series of *Cop1*-mutant mice. This study firmly established that COP1 is a ubiquitin ligase for c-Jun and that COP1 acts as a tumor suppressor *in vivo*. In the present study, we observed that *COP1* expression was significantly correlated with the expression of *MMP1*, *MMP7* and *MMP10* and that knockdown of *COP1* by siRNA increased cell proliferation (Figs. 3 and 4). Therefore, we speculated that the poorer prognosis in patients exhibiting low expression of *COP1* within tumor tissues may be caused by the activation of c-Jun (AP-1) and MMP pathways.

In conclusion, while the role of COP1 in malignancies is controversial, our current data support that COP1 acts as a tumor suppressor in gastric cancer. Further studies in the near future will be required to clarify how the role of COP1 is determined in various malignancies and to elucidate the mechanisms that regulate COP1 expression.

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