

MDM4 as a Prognostic Factor for Patients With Gastric Cancer With Low Expression of p53

XIAOCHEN ZHANG¹, YOSHIYUKI YAMAMOTO¹, XIAOXUAN WANG¹,
MASASHI SATO¹, MAMIKO IMANISHI¹, AKINORI SUGAYA², MITSUAKI HIROSE^{1,3},
SHINJI ENDO^{1,4}, TOSHIKAZU MORIWAKI¹, KENJI YAMATO¹ and ICHINOSUKE HYODO^{1,5}

¹Department of Gastroenterology, Institute of Clinical Medicine,
Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan;

²Division of Gastroenterology, Ibaraki Prefectural Central Hospital, Kasama, Japan;

³Department of Gastroenterology, Tsuchiura Clinical Education and Training Center,
University of Tsukuba Hospital, Tsuchiura, Japan;

⁴Department of Gastroenterology and Hepatology, Shinmatsudo Central General Hospital, Matsudo, Japan;

⁵Department of Gastrointestinal Medical Oncology, NHO Shikoku Cancer Center, Matsuyama, Japan

Abstract. *Background/Aim: The oncoproteins murine double minute (MDM) 2 and MDM4 inactivate tumor-suppressor protein p53. Their mutual relationship with the prognosis of gastric cancer (GC) remains unknown. Patients and Methods: Expression of MDM2, MDM4, and p53 in tumors of 241 patients with GC were evaluated immunohistochemically. Effects of overexpression of MDM4 on tumor-growth properties and sensitivity to cytotoxic drugs were investigated using NUGC4 human GC cell line. Results: High expression of p53 was associated with poor overall survival in the whole population. Among 173 patients with low expression of p53 (implying nonmutation), high expression of MDM4 was an independent factor of poor prognosis in both stage I-III and IV, but of MDM2 was not. MDM4-transduced NUGC4 cells formed twice as many colonies and had a higher 50% inhibitory concentration for 5-fluorouracil and oxaliplatin than did the control cells. Conclusion: MDM4 expression is a factor conferring poor prognosis in patients with GC with low expression of p53 and may confer drug resistance.*

The gene *TP53*, which encodes the tumor-suppressor *p53* protein in humans, is inactivated by mutations in various cancer types. The mutation rate of *TP53* in gastric cancer (GC) is approximately 40% to 50% (1). In *TP53* wild-type (WT) cancer, the gene function is often suppressed by enhanced expression of oncogenes such as murine double minute 2 (*MDM2*) and *MDM4* (2-4).

In normal cells, cellular *p53* is maintained at a very low level via regulation by *MDM2* through an autoregulatory feedback loop (5-7). As a structural homolog of *MDM2*, *MDM4* has gradually attracted attention in recent years. *MDM4* not only inhibits *p53* transcriptional ability by directly binding to the transactivation domain of *p53* (8), but also promotes the ubiquitin-dependent degradation of *p53* by forming a complex with *MDM2* (9, 10).

In cancer cells, the complex and precise regulatory relationship among these three proteins is disrupted by mutations of *TP53*, and by overexpression of *MDM2* and *MDM4*, leading to tumor growth. *MDM2* overexpression is suggested to be a factor indicating a poor prognosis in patients with GC (11). According to the Human Protein Atlas (<https://www.proteinatlas.org/ENSG00000198625-MDM4>), the prognostic value of *MDM4* has been controversial for some types of cancer. In GC, the association between the expression levels of *p53*, *MDM2*, and *MDM4* and cancer prognosis remains unknown. It is important to clarify the roles of *MDM2* and *MDM4* in patients with WT *TP53* GC.

In this study, we investigated the regulatory relationship among these proteins using clinical data and tumor tissue samples. Moreover, we evaluated the effect of *MDM4* overexpression on tumor-growth properties and sensitivity of cancer cells to cytotoxic drugs in an *in vitro* study.

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Correspondence to: Yoshiyuki Yamamoto, Department of Gastroenterology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. Tel: +81 298533218, Fax: +81 298533218, e-mail: y-yamamoto@md.tsukuba.ac.jp

Key Words: MDM2, MDM4, p53, gastric cancer, immunohistochemical study.

Patients and Methods

Patients. We retrospectively collected the clinical data and tumor tissue samples of patients with stage I-IV (12) GC who had undergone surgery or received chemotherapy at the University of Tsukuba Hospital between January 2006 and December 2018. This study was conducted according to Japanese ethical guidelines proposed by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare (http://www.lifescience.mext.go.jp/files/pdf/n1443_01.pdf) and obtained the approval of the Ethics Review Committee (R01-197) of the University of Tsukuba Hospital. The requirement for informed consent was waived owing to the retrospective nature of the study.

Immunohistochemical staining. The expression of p53, MDM2, and MDM4 in the formalin-fixed paraffin-embedded tumor tissue samples were examined using immunohistochemistry (IHC) staining with anti-p53 (DO-7, ready-to-use; Dako, Glostrup, Denmark), anti-MDM2 (2A10, diluted 1:80; Abcam, Cambridge, UK), anti-MDM4 (2D10F4, diluted 1:500; Thermo Fisher Scientific, Waltham, MA, USA) and EnVision+ System-HRP secondary antibody (K4001, ready-to-use; Dako). For the positive control for p53, MDM2, and MDM4, we used lung cancer, retinoblastoma, and colon cancer tissues, respectively. Negative controls were obtained by replacing the primary antibody with phosphate-buffered saline.

Based on previous reports (13, 14), we determined the expression status as follows: p53 was defined as high expression of when more than 25% of the tumor nuclei were obviously stained, and MDM2 and MDM4 were defined as high expression of when more than 50% of the tumor nuclei were stained (Figure 1). Expression status was independently judged by three investigators (X.Z., A.S., M.H.) blinded to the clinical data. Finally, we confirmed whether all of their judgments matched.

Study design. The primary endpoint was overall survival (OS), and secondary endpoints were disease-free (DFS) and progression-free (PFS) survival. OS was defined as the time from the date of curative resection in patients with stage I-III or the initiation of first-line chemotherapy to the date of death from any cause in those with stage IV. DFS was defined as the time from the date of curative resection to the date of recurrence or death. PFS was defined as the time from the date of initiation of first-line chemotherapy to the date of disease progression or death. Patients without OS events were censored at the date of the last follow-up. Patients without DFS or PFS events were censored at the date of the last tumor evaluation by use of computed tomographic scans.

Experiments using an MDM4-overexpressing cell line. Cell line and lentiviral transduction: NUGC4 human GC cell line with WT *TP53* was obtained from the Riken BioResource Center Cell Bank (Tsukuba, Japan), and was cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (Nichirei Biosciences, Tokyo, Japan). MDM4 cDNA was isolated using the 3× FLAG-MdmX/pcDNA3.1 plasmid (a kind gift from Dr. M. Kitagawa) (15) and subcloned in a lentivirus expression plasmid (pLenti6.3/V5-DEST; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Enhanced green fluorescent protein (EGFP)- and MDM4-infectious recombinant viruses were produced using a ViraPower Bsd Lentiviral Support

Kit and 293FT cells (Invitrogen) and transduced in NUGC4 cells according to the manufacturer's instructions.

Immunoblot analysis: Immunoblot analysis was performed as described previously (16). To circumvent the low linear dynamic range of immunoblot analysis, serially diluted total protein extracts from MDM4-NUGC4 cells and undiluted extracts from EGFP-NUGC4 cells were electrophoresed and probed for MDM4 levels. Mouse monoclonal antibody to MDM4 (D-4) and rabbit polyclonal antibody to β -actin (PM053) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Medical & Biological Laboratories (Nagoya, Japan), respectively.

Growth curves: Cells were seeded in 24-well plates with 500 μ l complete medium at a density of 2,000 cells per well, and cultured for 7 days. Relative viable cell numbers were determined by crystal violet staining every 24 h after seeding as described elsewhere (17).

Soft-agar colony-formation assay: A soft-agar colony-formation assay was performed as described elsewhere (18). Colonies consisting of more than 50 cells were counted. All assays were performed in triplicate and repeated three times.

Drug sensitivity analysis: 5-Fluorouracil (5-FU), cisplatin, and oxaliplatin were chosen because these are the most widely accepted first-line regimen drugs for patients with advanced GC. 5-FU was purchased from Kyowa Hakko Kirin (Tokyo, Japan); cisplatin and oxaliplatin were purchased from Wako (Osaka, Japan). The relative cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Dojindo, Kumamoto, Japan) as described elsewhere (19).

Statistical analysis. OS, DFS, and PFS were estimated using the Kaplan–Meier method, and compared using the log-rank test. Multivariable regression analyses were performed using the Cox proportional hazards model to examine the association between patient background factors and survival using the forward stepwise selection (likelihood ratio) method. A propensity score-matched analysis was performed to reduce bias due to confounding variables. The propensity score was calculated using a multivariable logistic regression model. A 1:1 propensity score matching was performed using the nearest-neighbor matching method. Patient backgrounds were compared between groups using the chi-squared test. The *t*-test was used to evaluate the effect of MDM4 expression on the drug sensitivity. Two-sided *p*-values of less than 0.05 were considered significant. Statistical analyses were performed using the IBM SPSS software, version 25 (IBM, Armonk, NY, USA).

Results

Background factors and survival of all patients. Clinical data and tumor tissue samples were collected from 241 patients with stage I-IV GC. The specimens of 146 patients with stage I-III GC were obtained from surgical resections of GC; the specimens of 95 patients with stage IV disease were mainly obtained from endoscopic biopsies and all these patients had received systemic chemotherapies. The first-line regimens were S-1 plus cisplatin in 45, S-1 in 25, S-1 plus oxaliplatin in eight, FOLFOX (5-FU plus levofolinate plus oxaliplatin) in eight, docetaxel plus cisplatin plus S-1 in three, and other types in six.

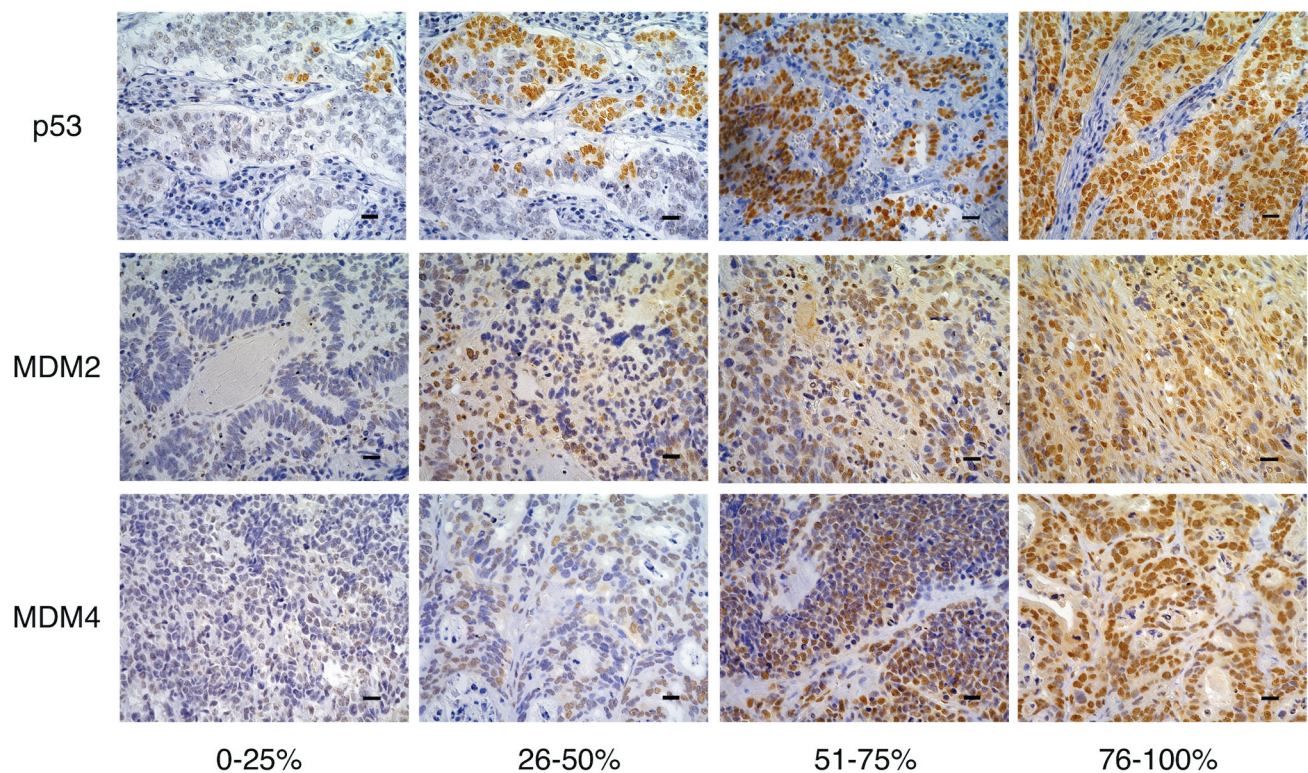


Figure 1. Representative immunohistochemical staining images for p53, murine double minute (MDM) 2, and MDM4 with 0-25%, 26-50%, 51-75% and 76-100% positive tumor nuclei, respectively. Scale bars indicate 20 μ m.

Survival of patients with stage I-III GC. The median duration of follow-up for patients with stage I-III GC was 5.2 years (range=0.1-11.5 years). The 5-year OS rate [95% confidence interval (CI)] was 74% (66-81%). The 5-year DFS rate was 68% (60-76%). The multivariable analysis for OS showed that high stage [hazard ratio (HR)=4.5, 95% CI=2.0-10.4; $p<0.001$] and p53 expression (HR=2.3, 95% CI=1.0-5.2; $p=0.047$) were independent prognostic factors (Table I). Similarly, high stage (HR=5.0, 95% CI=2.3-10.7; $p<0.001$) and p53 expression (HR=2.3, 95% CI=1.1-4.9; $p=0.025$) were independent prognostic factors for DFS.

Survival of patients with stage IV GC. The median duration of follow-up of patients with stage IV GC was 21 months (range=1.3-78.2 months). The median OS was 18 (95% CI=13-21) months, while the median PFS was 6.5 (95% CI=5.5-7.5) months. The multivariable analysis for OS showed that only high p53 expression (HR=2.3, 95% CI=1.3-4.1; $p=0.006$) was an independent prognostic factor (Table II). The Eastern Cooperative Oncology Group performance status (HR=1.8, 95% CI=1.1-2.8; $p=0.014$) and p53 expression (HR=1.9, 95% CI=1.2-3.0; $p=0.007$) were independent prognostic factors for PFS.

Background factors and survival of patients with GC with low expression of p53. The number of patients with GC with low expression of p53 was 119 in the group with stage I-III and 54 in that the stage IV disease. The first-line chemotherapy regimens were S-1 plus cisplatin in 25, S-1 in 14, FOLFOX in five, S-1 plus oxaliplatin in four, S-1 plus cisplatin plus trastuzumab in three, docetaxel plus cisplatin plus S-1 in two, and irinotecan plus cisplatin in one. The proportion of patients with high expression of MDM2 increased gradually with disease progression, while that of patients with high expression of MDM4 decreased (Figure 2).

Survival of patients with stage I-III disease with low expression of p53. The 5-year OS rate was 77 (95% CI=67-85%), while the 5-year DFS rate was 73 (95% CI=63-81%). Although MDM4 was not a significant factor in the univariable analysis for OS ($p=0.72$), the multivariable analysis for OS showed that MDM4 was an independent prognostic factor (HR=2.7, 95% CI=1.1-6.4; $p=0.027$) in addition to stage (HR=10, 95% CI=3.3-32.2; $p<0.001$) (Table III). Stage was also an independent significant prognostic factor (HR=7.0, 95% CI=2.6-18.8; $p<0.001$) for DFS.

Table I. Background factors and survival analysis for 146 patients with stage I-III gastric cancer.

Variable	Comparison	Number of patients	Overall survival						Disease-free survival					
			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
			HR	p-Value	HR	95% CI	p-Value		HR	p-Value	HR	95% CI	p-Value	
Gender	Male vs. female	103/43	0.95	0.89					1.15	0.69				
Age	>65 vs. ≤65 years	64/82	1.05	0.89					1.01	0.99				
Tumor size	>50 vs. ≤50 mm	70/76	1.66	0.12					1.89	0.03				
Adjuvant chemotherapy	Yes vs. no	76/70	3.03	0.002					2.74	0.001				
Histology	Diffuse vs. intestinal	75/71	1.82	0.08					1.61	0.12				
ECOG PS	1/2 vs. 0	61/70	1.21	0.59					1.35	0.35				
Stage	III vs. I/II	79/67	4.35	<0.001	4.54	1.98-10.41	<0.001		4.96	<0.001	4.98	2.30-10.74	<0.001	
CEA	Abnormal vs. normal	25/115	1.93	0.08					1.71	0.13				
p53	High vs. low	27/119	2.42	0.01	2.30	1.01-5.21	0.047		2.61	0.003	2.33	1.11-4.88	0.025	
MDM2	High vs. low	73/73	1.87	0.06					1.93	0.03				
MDM4	High vs. low	59/87	0.82	0.57					0.86	0.62				

CEA: Carcinoembryonic antigen; ECOG PS: Eastern Cooperative Oncology Group performance status; MDM2/4: murine double minute 2/4.

Table II. Background factors and survival analysis for 95 gastric cancer patients with stage IV cancer.

Variable	Comparison	Number of patients	Overall survival						Progression-free survival					
			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
			HR	p-Value	HR	95% CI	p-Value		HR	p-Value	HR	95% CI	p-Value	
Gender	Male vs. female	74/21	1.08	0.82					1.48	0.14				
Age	>65 vs. ≤65 years	42/53	0.99	0.98					1.16	0.51				
Histology	Diffuse vs. intestinal	61/34	1.35	0.33					1.28	0.28				
ECOG PS	1/2 vs. 0	44/51	1.75	0.06					1.91	0.005	1.78	1.12-2.83	0.014	
CEA	Abnormal vs. normal	44/51	1.12	0.70					1.05	0.83				
Sites of metastasis	>1 vs. 1	62/33	1.10	0.75					1.13	0.59				
p53	High vs. low	41/54	2.29	0.005	2.29	1.26-4.14	0.006		1.20	0.002	1.88	1.19-2.97	0.007	
MDM2	High vs. low	67/28	1.50	0.24					1.28	0.32				
MDM4	High vs. low	24/71	1.48	0.26					1.45	0.16				

CEA: Carcinoembryonic antigen; ECOG PS: Eastern Cooperative Oncology Group performance status; MDM2/4: murine double minute 2/4.

The patients were divided into two groups with high and low expression of MDM4, and a propensity score-matched analysis was performed to reduce the bias, especially the influence of stage. After 1:1 propensity score matching, 62 patients were selected: 31 patients with high expression and 31 patients with low expression of MDM4. The patient backgrounds of the two matched groups were well-balanced, including stage ($p=0.41$) (Table IV). OS was significantly shorter in the group with high expression of MDM4 than in that with low expression (HR=5.1, 95% CI=1.1-23.7; $p=0.02$) (Figure 3).

Survival of patients with stage IV disease with low expression of p53. The median OS was 24 (95% CI=14-34) months, and the median PFS was 7.9 (95% CI=6.3-9.5) months. The multivariable analysis for OS showed that MDM4 was the only significant independent prognostic factor (HR=6.2, 95% CI= 2.2-17.6; $p=0.001$) (Table V). For PFS, the Eastern Cooperative Oncology Group performance states was a significant independent prognostic factor (HR=2.0, 95% CI=1.1-3.9; $p=0.03$). Figure 4 shows the Kaplan–Meier curves of OS for the three groups with: A: Low expression of p53 and low expression of MDM4; B:

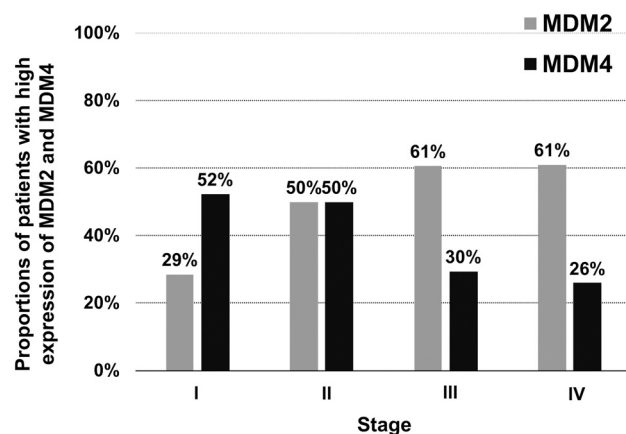


Figure 2. Expression status of murine double minute (MDM) 2 and MDM4 in patients with gastric cancer with low expression of p53. The proportion of patients with high expression of MDM2 increased gradually from stage I to stage IV, while that of patients with high expression of MDM4 decreased.

low expression of p53 and high expression of MDM4; and C: high expression of p53. The OS of group B was significantly shorter than that of group A (HR=6.2, 95% CI=2.2-17.6; $p<0.001$). The OS durations of groups B and C were similar (HR=1.3, 95% CI=0.6-3.1; $p=0.54$).

Cell proliferation and drug resistance of MDM4-transduced NUGC4 cells. MDM4-NUGC4 cells expressed a nearly 24-fold higher level of MDM4 than EGFP-NUGC4 cells (control cells) (Figure 5). In the conventional plate culture, both control and MDM4-NUGC4 cells grew similarly, with a doubling time of 48 h. In the soft-agar culture, MDM4-NUGC4 cells formed twofold more colonies than the control cells (193 ± 37 vs. 101 ± 13 , $p<0.02$).

In the drug-sensitivity analysis (Figure 6), the 50% inhibitory concentration (IC_{50}) of 5-FU and oxaliplatin in the MDM4-NUGC4 cells were 5.3-fold and 3.5-fold higher than those for the control cells, respectively. The IC_{50} for cisplatin was similar in MDM4-NUGC4 and control cells.

Discussion

Since p53 IHC positivity is a prognostic marker in various cancer types including GC, we examined whether expression of MDM2 and MDM4 might also be useful markers in GC, especially in WT *TP53* GC. We confirmed that p53 was a prognostic factor for our patients with GC, but did not find any relationship between MDM4 expression and prognosis when all the patients were analyzed. However, among the patients with low expression of p53, those with high MDM4 expression had significantly shorter OS than those with low expression of MDM4. To our knowledge, this is the first

report to show that MDM4 is a poor prognostic marker in patients with GC with low expression of p53.

The multivariable analysis including all the patients showed that those with high expression of p53 had significantly shorter survival than those with low expression of p53, suggesting that p53-positive staining indicates poor prognosis. This result is consistent with the findings of other reports (20-22). Without any stresses – such as genotoxicity, nutrient deficiency, and hypoxia – normal p53 is degraded by MDM2 to levels undetectable by IHC (23). Therefore, the state of low expression of p53 could be considered a surrogate for WT *TP53*.

Among patients with stage IV disease, those with low expression of p53 and high expression of MDM4 had similar OS to those with high p53 expression. This might suggest that normal p53 function was almost suppressed by high expression of MDM4. However, MDM4 expression has not yet been explicitly reported to be a poor prognostic marker for GC. To date, only one study has shown MDM4 expression to be associated with lymph node metastasis, which is an independent prognostic factor of GC regardless of p53 staining pattern (24). In our study, we focused on the biological role of MDM4 and found direct evidence to show that MDM4 was an independent poor prognostic factor in patients with low p53 expression in GC.

In such patients, high expression of MDM2 was associated with advanced tumor stage. This finding was comparable to those of other reports (11, 21, 22). In contrast, high expression of MDM4 was inversely associated with tumor stage. A previous study reported results opposite to ours on the association between MDM4 expression and stage (24). The reason for this discrepancy is unclear; however, our finding might be explained by the loss of phosphatase and tensin homolog (PTEN) function. PTEN is a tumor-suppressor protein that negatively regulates MDM2 (25, 26). Loss of PTEN function induces MDM2 accumulation, and MDM2 can degrade not only p53 but also MDM4 (27). The mutations of *PTEN* and *TP53* are mutually exclusive, and loss of PTEN expression has been frequently reported in advanced GC (28). MDM4-positive patients with GC may lose PTEN function, resulting in MDM2 accumulation and reduced MDM4 expression with disease progression. Future studies are warranted to investigate the relationship between PTEN and MDM4 in patients with GC with low p53 expression to understand the exact mechanism of MDM4 expression.

We previously reported that knockdown of *MDM4* weakly increased p53 activity and caused marginal growth suppression in the NUGC4 cell line with relatively low expression of MDM4, but caused intense p53 activation and potent growth inhibition in the *MDM4*-amplified MCF-7 breast cancer cell line (29). This suggests that MDM4 may have an expression level-dependent negative effect on p53 activity. To strengthen our clinical findings in this study, we evaluated the effects of MDM4 overexpression on tumor cell growth and sensitivity to cytotoxic drugs using NUGC4 cells. *MDM4* transduction

Table III. Background factors and survival analysis for 119 patients with stage I-III gastric cancer and low expression of p53.

Variable	Comparison	Number of patients	Overall survival					Disease-free survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			HR	p-Value	HR	95% CI	p-Value	HR	p-Value	HR	95% CI	p-Value
Gender	Male vs. female	81/38	0.73	0.42				0.84	0.64			
Age	>65 vs. ≤65 Years	50/69	1.14	0.73				0.95	0.89			
Tumor size	> 50 vs. ≤50 mm	56/63	1.94	0.08				1.92	0.06			
Adjuvant chemotherapy	Yes vs. no	61/58	5.07	<0.001				3.42	0.001			
Histology	Diffuse vs. intestinal	64/55	1.83	0.14				1.75	0.13			
ECOG PS	1/2 vs. 0	51/58	1.01	0.99				1.20	0.63			
Stage	III vs. I/II	61/58	6.25	<0.001	10.27	3.28-32.16	<0.001	6.18	<0.001	7.04	2.63-18.83	<0.001
CEA	Abnormal vs. normal	20/93	1.76	0.22				1.37	0.48			
MDM2	High vs. low	57/62	1.73	0.16				1.69	0.14			
MDM4	High vs. low	48/71	1.15	0.72	2.68	1.12-6.44	0.027	0.99	0.98			

CEA: Carcinoembryonic antigen; ECOG PS: Eastern Cooperative Oncology Group performance status; MDM2/4: murine double minute 2/4.

Table IV. Characteristics of patients with stage I-III gastric cancer and low expression of p53 in a propensity score-matched analysis.

Variable	Comparison	Before matching (n=119)			After matching (n=62)		
		MDM4 expression, n (%)		p-Value	MDM4 expression, n (%)		p-Value
		High	Low		High	Low	
Gender	Male vs. female	34 (71)/14 (29)	47 (66)/24 (34)	0.60	18 (58)/13 (42)	19 (61)/12 (39)	0.80
Age	>65 vs. ≤65 years	25 (52)/23 (48)	44 (62)/27 (38)	0.28	18 (58)/13 (42)	19 (61)/12 (39)	0.80
Tumor size	>50 vs. ≤50 mm	26 (54)/22 (46)	37 (52)/34 (48)	0.83	18 (58)/13 (42)	20 (65)/11 (36)	0.60
Adjuvant chemotherapy	Yes vs. no	18 (38)/30 (62)	43 (61)/28 (39)	0.014	12 (39)/19 (61)	11 (36)/20 (64)	0.79
Stage	I, II vs. III	30 (63)/18 (38)	28 (39)/43 (61)	0.014	17 (55)/14 (45)	22 (71)/9 (29)	0.41
ECOG PS	0 vs. 1, 2	19 (40)/28 (60)	39 (63)/23 (37)	0.02	30 (97)/1 (3)	30 (97)/1 (3)	0.75
CEA	Abnormal vs. normal	11 (23)/36 (77)	9 (14)/57 (86)	0.18	6 (19)/25 (81)	4 (13)/27 (87)	0.49
Histology	Intestinal vs. diffuse	23 (48)/25 (52)	32 (45)/39 (55)	0.76	13 (42)/18 (58)	16 (52)/15 (48)	0.45
MDM2 expression	High vs. low	26 (54)/22 (46)	32 (45)/39 (55)	0.26	13 (42)/18 (58)	11 (36)/20 (64)	0.60

CEA: Carcinoembryonic antigen; ECOG PS: Eastern Cooperative Oncology Group performance status; MDM2/4: murine double minute 2/4. For comparison of patient backgrounds between groups p-Value was conducted using the chi-square test.

enhanced anchorage-independent cell growth as compared to the control cells, showing that MDM4 overexpression induced more potent tumor-forming activity. Moreover, MDM4 overexpression provided NUGC4 cells with resistance to 5-FU and oxaliplatin but not to cisplatin. Oxaliplatin contains the 1,2-diaminocyclohexane carrier ligand, whereas cisplatin does not. Oxaliplatin represses transcription of genes involved in thymidylate synthesis using the 1,2-diaminocyclohexane carrier ligand in a p53 expression level-dependent manner (30). Resistance to oxaliplatin and 5-FU is most likely mediated by MDM4-induced p53 inactivation (31). Since these two drugs are frequently used as standard first-line chemotherapy, this resistance may partly explain why patients with stage IV disease

with high MDM4 expression had significantly shorter OS compared to those with low MDM4 expression. Interestingly, MDM4 overexpression had no effect on sensitivity to cisplatin. This may indicate that chemotherapy for GC including cisplatin benefits patients with low p53 and high MDM4 expression. Therefore, we tried to analyze differences in PFS between patients administered oxaliplatin-based and cisplatin-based chemotherapies. However, this comparison was not possible because of the small number of patients.

This study had some methodological limitations. Firstly, there would have been unexpected bias due to the retrospective nature of the study. Secondly, because of a small number of patients, we were unable to judge whether survival was

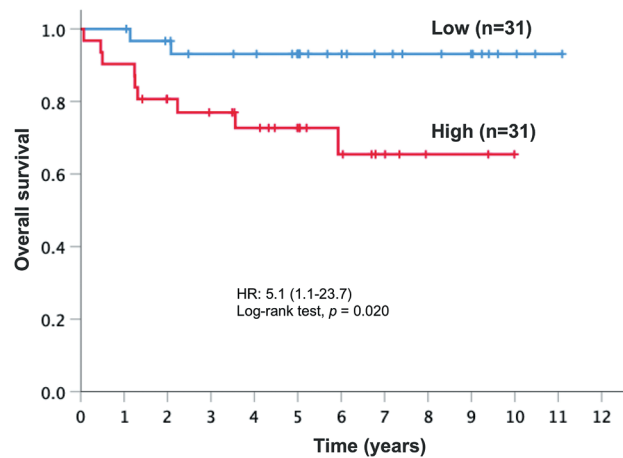


Figure 3. Overall survival (OS) of patients with stage I-III gastric cancer with low expression of p53, adjusted using the propensity score-matched method. Patients were divided into two groups according to murine double minute (MDM) 4 expression. The 5-year OS rate of patients with high MDM4 expression was 73% [95% confidence interval (CI)= 63-81%], and that of patients with low MDM4 expression was 93% (95% CI=86-97%). Patients with high MDM4 expression had shorter OS than those with low MDM4 expression (hazard ratio=5.1, 95% CI=1.1-23.7; $p=0.020$).

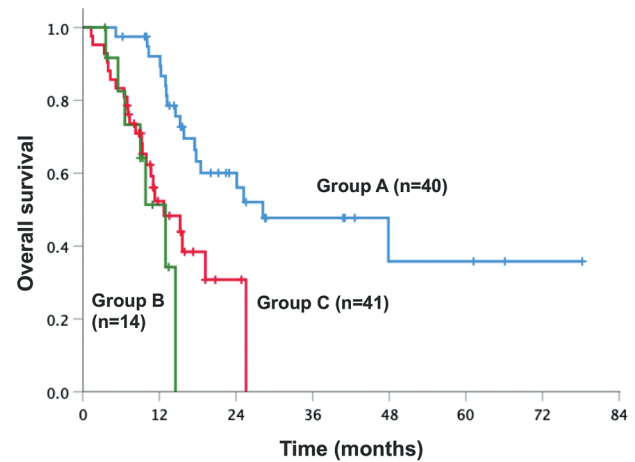


Figure 4. Overall survival (OS) of patients with stage IV disease. Patients were divided into groups by expression pattern: Group A, patients with low p53 and low murine double minute (MDM) 4 expression, median OS was 28 (95% confidence interval [CI]=7.0-49.4) months; group B, patients with low p53 expression and high MDM4 expression, median OS was 13 (95% CI=6.9-19.2) months; and group C, patients with high p53 expression, median OS was 13 (95% CI=6.7-18.7) months. Group B had shorter OS than group A (hazard ratio=6.2, 95% CI=2.2-17.6; $p<0.001$). Survival curves were similar for groups B and C (hazard ratio=1.3, 95% CI=0.6-3.1; $p=0.538$).

Table V. Background factors and survival analysis for 54 patients with stage IV gastric cancer and low expression of p53.

Variable			Overall survival						Progression-free survival					
			Univariable analysis			Multivariable analysis			Univariable analysis			Multivariable analysis		
			HR	p-Value	HR	95% CI	p-Value		HR	p-Value	HR	95% CI	p-Value	
	Comparison	Number of patients												
Gender	Male vs. female	39/15	0.87	0.76					1.28	0.47				
Age	>65 vs. ≤65 years	21/33	0.75	0.50					1.13	0.70				
Histology	Diffuse vs. intestinal	30/24	1.01	0.99					0.98	0.95				
ECOG PS	1/2 vs. 0	22/32	1.63	0.25					2.02	0.03	2.02	1.06-3.86	0.033	
CEA	Abnormal vs. normal	23/31	1.29	0.54					0.89	0.70				
Sites of metastasis	>1 vs. 1	31/23	1.06	0.89					0.98	0.95				
MDM2	High vs. low	33/21	0.94	0.88					1.23	0.52				
MDM4	High vs. low	14/40	6.21	<0.001	6.21	2.20-17.58	0.001		1.90	0.09				

CEA: Carcinoembryonic antigen; CI: confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status; HR: hazard ratio; MDM2/4: murine double minute 2/4.

influenced by factors such as chemotherapy regimens in addition to the variables discussed in this article. Thirdly, we did not perform gene sequencing to determine the *TP53* genotype. However, we divided p53 status into two categories by IHC and obtained significant prognostic results. We consider that the IHC method can be used as an alternative to the gene sequencing method of *TP53*. Finally, an optimal cutoff percentage of positively stained cells was not fully established

in this study. We set candidate cutoff percentages of 25%, 50%, and 75% for each marker studied and tried to analyze survival (data not shown). As a result, the cutoff percentage in the present study seemed appropriate for our analysis.

In conclusion, we demonstrated the role of MDM4 as a factor indicating poor prognosis for patients with GC with low expression of p53. MDM4 may be a promising drug target in patients with GC with low p53 and high MDM4 expression.

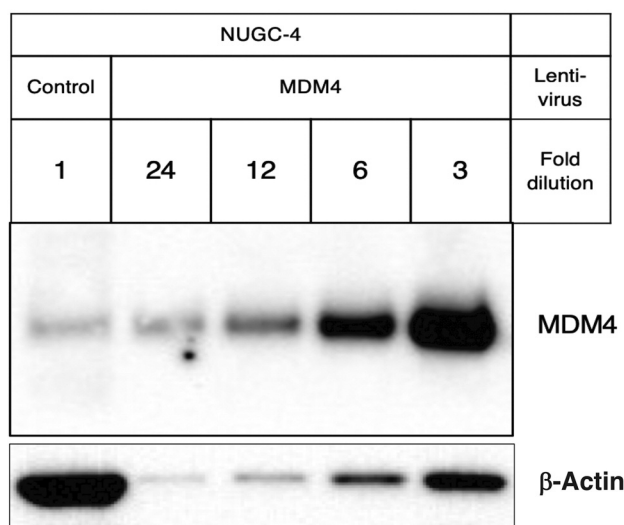


Figure 5. Expression of murine double minute (MDM) 4 in MDM4-transduced NUGC4 cells. MDM4-transfected cells expressed a level of MDM4 nearly 24-fold higher than the control cells.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

All Authors contributed to the study conception and design. XZ, YY, XW, MS, MI, AS, MH, SE, and TM collected clinical materials and data. XZ, YY, XW, MI, and KY performed laboratory experiments and analysis. XZ analyzed statistics and wrote the article. YY, TM, KY and IH reviewed the data and analysis, and revised the article. All Authors have read and approved the final article.

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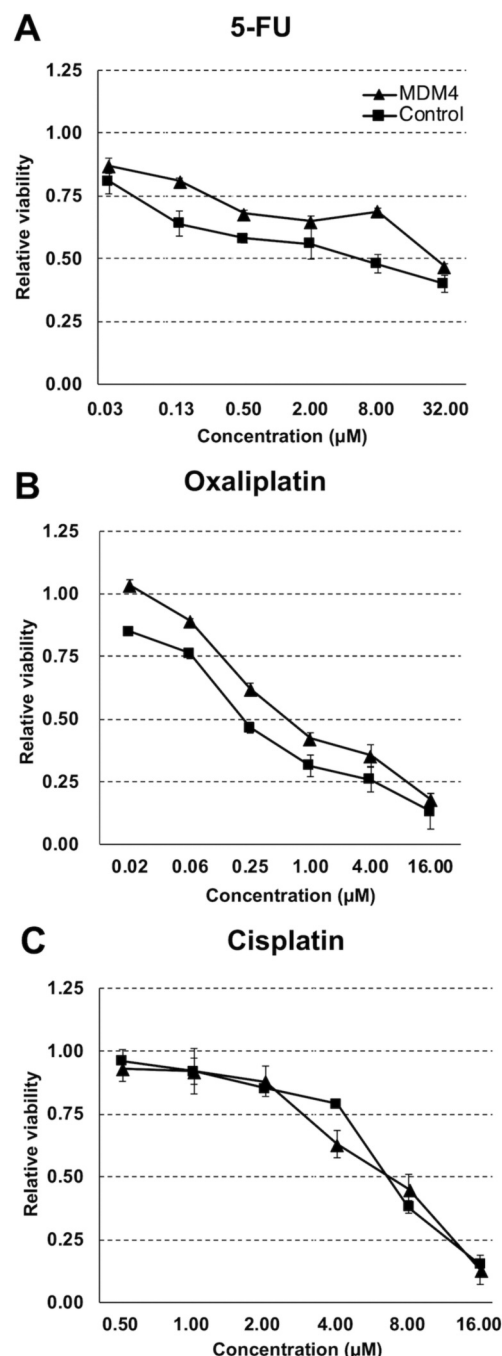


Figure 6. Cell viability assay and effect of murine double minute (MDM) 4 overexpression on sensitivity to different cytotoxic drugs. A: 5-Fluorouracil; B: oxaliplatin; C: cisplatin.

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