

Expression of DBC1 and SIRT1 Is Associated with Poor Prognosis of Gastric Carcinoma

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Abstract Purpose: SIRT1 (silent mating-type information regulation 2 homologue 1) expression has been reported to predict poor survival in some cancers. We therefore investigated the expression levels of SIRT1 and its negative regulator, DBC1 (deleted in breast cancer 1), in gastric cancer patients.

Experimental Design: We evaluated immunohistochemical expression of DBC1, SIRT1, and p53 using 3-mm tumor cores from 177 gastric cancer patients for tissue microarray.

Results: Positive expressions of DBC1 and SIRT1 were seen in 62% (109 of 177) and in 73% (130 of 177) of patients, respectively. Expression of DBC1 was significantly correlated with tumor stage ($P = 0.007$), lymph node metastasis ($P < 0.001$), tumor invasion ($P = 0.001$), venous invasion ($P = 0.001$), histologic types ($P < 0.001$), p53 expression ($P < 0.001$), and SIRT1 expression ($P < 0.001$). SIRT1 expression was also significantly correlated with tumor stage ($P < 0.001$), lymph node metastasis ($P < 0.001$), tumor invasion ($P < 0.001$), histologic types ($P < 0.001$), and p53 expression ($P = 0.001$). In addition, expression of DBC1 was significantly associated with shorter overall survival and relapse-free survival by univariate analysis ($P < 0.001$ and $P < 0.001$, respectively). SIRT1 expression was also significantly associated with shorter overall survival and relapse-free survival by univariate analysis ($P = 0.001$ and $P = 0.001$, respectively). Multivariate analysis showed that tumor stage and expression of DBC1 were independent prognostic factors significantly associated with overall survival and relapse-free survival.

Conclusion: This study shows that expression of DBC1 and SIRT1 is a significant prognostic indicator for gastric carcinoma patients.

SIRT1 (silent mating-type information regulation 2 homologue 1) is the closest mammalian homologue of yeast Sir2, which functions as an NAD⁺-dependent histone deacetylase and belongs to the class III histone deacetylases (1–3). SIRT1 plays an important role in cell survival under genotoxic and oxidative stress through deacetylation of key cell cycle molecules and apoptosis regulatory proteins, including p53

(4, 5), FOXO family proteins (6), nuclear factor κ B (7), and Ku70 (8). Recent reports have revealed that SIRT1 is up-regulated in various human and mouse malignant tumors (9–12), and it has been suggested that SIRT1 may be involved in tumorigenesis through its antiapoptotic activity (13). Up-regulated SIRT1 inactivates p53 by deacetylation (12, 14), and this allows cells to proliferate in the presence of damaged DNA and subsequently promotes tumor progression (12, 14, 15). Recently, we have reported that a high proportion (74%) of diffuse large B-cell lymphomas express SIRT1 and that is associated with poor prognosis of diffuse large B-cell lymphoma, suggesting that SIRT1 is involved in the development and progression of diffuse large B-cell lymphoma (16). In addition, it has been suggested that SIRT1 may be involved in the development of malignant tumors, including human intraepidermal cancer (9), human breast cancer (10), human colon cancer (10), human prostate cancer (11), mouse pulmonary adenocarcinoma (12), mouse sarcoma (12), and mouse lymphoma (12).

Recent studies have shown that SIRT1 is negatively regulated by DBC1 (deleted in breast cancer 1; refs. 17, 18). DBC1 was initially cloned from a region 8p21 that was homozygously deleted in breast cancer (19). DBC1 specifically inhibits the deacetylation activity of SIRT1 by directly binding to the catalytic domain of SIRT1 (17, 18). However, despite the

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Translational Relevance

This is the first report that has examined the immunohistochemical expression and prognostic significance of DBC1 (deleted in breast cancer 1) in human malignant tumors and SIRT1 (silent mating-type information regulation 2 homologue 1) in human gastric carcinoma. This study shows that (a) a high proportion of gastric carcinomas express DBC1 and SIRT1; (b) expression of DBC1 and SIRT1 is significantly associated with advanced clinicopathologic parameters such as an elevated serum level of carcinoembryonic antigen, an advanced clinical stage (stage III or IV), lymph node metastasis, and advanced tumor invasion; and (c) expression of DBC1 and SIRT1 is associated with a significantly shorter survival in gastric carcinoma patients. Taken together, our findings indicate that DBC1 and SIRT1 may be involved in the progression of gastric carcinomas and can be used as clinically significant prognostic indicators for gastric carcinoma patients. Moreover, DBC1 and SIRT1 may also provide a target for a novel therapeutic approach for gastric carcinomas.

possible role of DBC1 as a tumor suppressor, recent gene microarray data in the Oncomine database of published cancer microarray data⁶ indicate that many human malignant tumors show increased expression of DBC1 compared with normal counterpart cells (20–23).

Gastric carcinoma is one of the most common carcinomas in the world. Although its incidence has been declining, it remains the second leading cause of cancer death worldwide (24–26). However, the clinical relevance of the expression of SIRT1 and DBC1 in gastric carcinomas has not been reported previously. In this study, we have used immunohistochemical staining to examine the prevalence and prognostic impact of SIRT1 and DBC1 expression in gastric carcinoma patients.

Materials and Methods

Patients and samples. Six hundred forty-three cases of gastric carcinoma patients who had radical gastrectomy in Chonbuk National University Hospital between January 1997 and December 2005 were included in the present study. To minimize confounding effects, we conducted a matching process. All of the patients who were in stage IV ($n = 50$) with complete clinical information were incorporated in this analysis. Pathologic staging was reviewed based on the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer (27). Thereafter, patients who were in stage I, II, or III were matched with them on the following variables: gender, age (± 2 y), and calendar year of surgery (± 2 y). Among the 200 selected cases of gastric cancer, paraffin-embedded tissue blocks from 23 cases were unavailable. Therefore, 177 cases of gastric cancer were included in the present study. In addition, 10 cases of noncancerous gastric mucosa were also included for the evaluation of the DBC1 and SIRT1 expression in the noncancerous gastric

mucosa. All of the cases were reviewed and reclassified according to the criteria of the WHO classification (28). This study had local ethics committee approval from the institutional review board of Chonbuk National University Hospital. Informed consent was provided according to the Declaration of Helsinki. The patients were grouped according to their age, sex, serum carcinoembryonic antigen (CEA) levels, serum CA19-9 levels, stage (I and II versus III and IV), presence of lymph node metastasis, presence of distant metastasis, presence of venous invasion, histologic types by WHO classification, histologic grade of tubular type carcinoma, Lauren classification, and tumor invasion (early gastric carcinoma versus advanced gastric carcinoma).

Immunohistochemical staining and scoring. Immunohistochemistry was done using 3.0-mm tumor cores for tissue microarray. One core per case was arrayed. The tissue sections were treated with a microwave antigen retrieval procedure in sodium citrate buffer for 12 min. The following markers were used: DBC1 (1:100; Bethyl Lab), SIRT1 (1:50; Santa Cruz Biotechnology; clone H-300), and p53 (1:50; Novocastra; clone DO-7). Immunohistochemical analysis was done by three authors (K.Y. Jang, E.J. Cha, and S.J. Noh) by consensus without knowledge of the clinicopathologic information. Each case was evaluated by estimating the percentages of tumor cells that stained positively for each marker. Immunostaining for DBC1, SIRT1, or p53 was considered positive if $\geq 30\%$ of the tumor cells were stained with an antibody; a uniform cutoff of 30% was chosen because it had been used by ourselves and others evaluating tissue microarray material (16, 29).

In situ hybridization. To determine the localization of EBV in gastric carcinomas, we did EBV-encoded small RNA *in situ* hybridization using a fluorescein-conjugated EBV probe for detection of EBV-encoded small RNA transcripts (NCL-EBV-K, Novocastra). The negative control section was processed identical to the above, except that EBV probe solution was replaced by negative control probe solution provided in the kit. Specimen from a patient with known EBV-positive nasal natural killer/T-cell lymphoma was used as positive control.

Statistical analysis. The endpoints of interest were relapse-free survival and overall survival. The endpoint of follow-up was the date of the last contact or the date of death through March 2008. Overall survival was calculated as the time from diagnosis to the date of death or last contact. Patients who were alive at last contact were treated as censored for overall survival analysis. Relapse-free survival was calculated from the time of diagnosis to the date of recurrence, death, or last contact. Patients who were alive at last contact and who had not recurred were treated as censored for relapse-free survival analysis. The associations between staining index and other categorical factors potentially predictive of prognosis were analyzed using Pearson's χ^2 test. Univariate, and multivariate Cox proportional hazards regression analysis was done to estimate the impact of clinicopathologic factors and expression of each marker on relapse-free survival and overall survival. Kaplan-Meier survival curves were constructed to further illustrate the impact of overall survival when indicated. SPSS software (version 15.0) was used throughout, and $P < 0.05$ was considered statistically significant.

Results

Association of DBC1 and SIRT1 expression with clinicopathologic characteristics of gastric carcinoma patients. The clinicopathologic features are summarized in Table 1. Immunohistochemical staining of DBC1, SIRT1, and p53, and *in situ* hybridization for EBV-encoded small RNA in normal gastric mucosa and gastric carcinoma tissues are shown in Fig. 1. Immunoreactivity for DBC1 and p53 was found primarily in the nuclei. Although SIRT1 was expressed in the nuclei and cytoplasm, we evaluated nuclear SIRT1

⁶ www.oncomine.org

Table 1. Clinicopathologic variables and the expression status of DBC1 and SIRT1

Characteristics	No. of patients	DBC1		SIRT1		
		Positive	P	Positive	P	
Age (y)	<60 y	53	26 (49%)	0.025	32 (60%)	0.01
	≥60 y	124	83 (67%)		98 (79%)	
Sex	Female	42	28 (67%)	0.438	30 (71%)	0.735
	Male	135	81 (60%)		100 (74%)	
CEA*	Normal	114	62 (54%)	<0.001	79 (69%)	0.018
	Elevated	31	28 (90%)		28 (90%)	
CA19-9*	Normal	128	77 (60%)	0.249	93 (73%)	0.2
	Elevated	16	12 (75%)		14 (88%)	
TNM stage	I and II	84	43 (51%)	0.007	51 (61%)	<0.001
	III and IV	93	66 (71%)		79 (85%)	
LN metastasis	Absence	64	27 (42%)	<0.001	35 (55%)	<0.001
	Presence	113	82 (73%)		95 (84%)	
Distant metastasis	Absence	173	106 (61%)	0.577	127 (73%)	0.943
	Presence	4	3 (75%)		3 (75%)	
Venous invasion	Absence	146	82 (56%)	0.001	104 (71%)	0.148
	Presence	31	27 (87%)		26 (84%)	
WHO classification	Tubular	139	97 (70%)	<0.001	113 (81%)	<0.001
	SRC	19	6 (32%)		8 (42%)	
	Mucinous	17	5 (29%)		7 (41%)	
	Papillary	2	1 (50%)		2 (100%)	
Histologic grade †	WD	10	8 (80%)	0.38	8 (80%)	0.484
	MD	68	50 (74%)		58 (85%)	
	PD	61	39 (64%)		47 (77%)	
Lauren classification	Intestinal	76	52 (68%)	0.018	61 (80%)	0.008
	Diffuse	76	38 (50%)		47 (62%)	
	Mixed	25	19 (76%)		22 (88%)	
Tumor invasion	EGC	39	15 (38%)	0.001	18 (46%)	<0.001
	AGC	138	94 (68%)		112 (81%)	
EBV	Negative	163	99 (61%)	0.43	120 (74%)	0.859
	Positive	14	10 (71%)		10 (71%)	
p53	Negative	150	84 (56%)	<0.001	103 (69%)	0.001
	Positive	27	25 (93%)		27 (100%)	
SIRT1	Negative	47	4 (9%)	<0.001		
	Positive	130	105 (81%)			

Abbreviations: LN, lymph node; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; SRC, signet ring cell carcinoma; EGC, early gastric cancer; AGC, advanced gastric cancer.

*Preoperative serum CEA and CA19-9 level were not measured in 32 and 33 patients, respectively.

† Histologic grade was applied primarily to tubular adenocarcinoma according to the WHO histologic classification of gastric tumors.

expression only. In 10 cases of normal gastric mucosa, very weak nuclear staining was identified in two cases for DBC1 and three cases for SIRT1. However, strong expression of DBC1 or SIRT1 was not identified in the normal mucosa. All 10 normal cases were negative for p53 immunostaining and *in situ* hybridization for EBV-encoded small RNA. Positive expression of DBC1 was seen in 62% (109 of 177) and of SIRT1 in 73% (130 of 177) of gastric carcinoma patients. EBV infection was seen in 8% (14 of 177) of gastric carcinoma patients. Expression of DBC1 was significantly correlated with patient age ($P = 0.025$), serum CEA level ($P < 0.001$), tumor stage ($P = 0.007$), lymph node metastasis ($P < 0.001$), classification according to tumor invasion ($P = 0.001$), venous invasion ($P = 0.001$), histologic types ($P < 0.001$), p53 expression ($P < 0.001$), and SIRT1 expression ($P < 0.001$). SIRT1 expression was also significantly correlated with the age of patients ($P = 0.01$), serum CEA level ($P = 0.018$), tumor stage ($P < 0.001$), lymph node metastasis ($P < 0.001$), classification according to the tumor invasion ($P < 0.001$), histologic types ($P < 0.001$), and p53 expression ($P = 0.001$). There was no correlation between EBV infection and DBC1 or SIRT1 expression.

Expression of DBC1 and SIRT1 in gastric carcinoma correlates with reduced relapse-free survival and overall survival. Univariate Cox proportional hazard analysis of the expression of each protein and its relationship to relapse-free survival and overall survival are shown in Table 2. Elevated serum CEA and CA19-9, high TNM stage, the presence of lymph node metastasis, the presence of venous invasion, and advanced gastric carcinoma predicted shorter overall survival and relapse-free survival (Fig. 2A). Expression of DBC1 was significantly associated with shorter overall survival and relapse-free survival by univariate analysis ($P < 0.001$ and $P < 0.001$, respectively; Fig. 2B). SIRT1 expression was also significantly associated with shorter overall survival and relapse-free survival by univariate analysis ($P = 0.001$ and $P = 0.001$, respectively; Fig. 2C). However, the expression of p53 or EBV infection did not predict overall survival or relapse-free survival.

Expression of DBC1 in gastric carcinoma is an independent prognostic factor for disease recurrence and poor survival outcome. Multivariate analysis was done using 143 patients with complete information for all variables (Table 3). Variables considered in the analysis were the serum level of CEA and

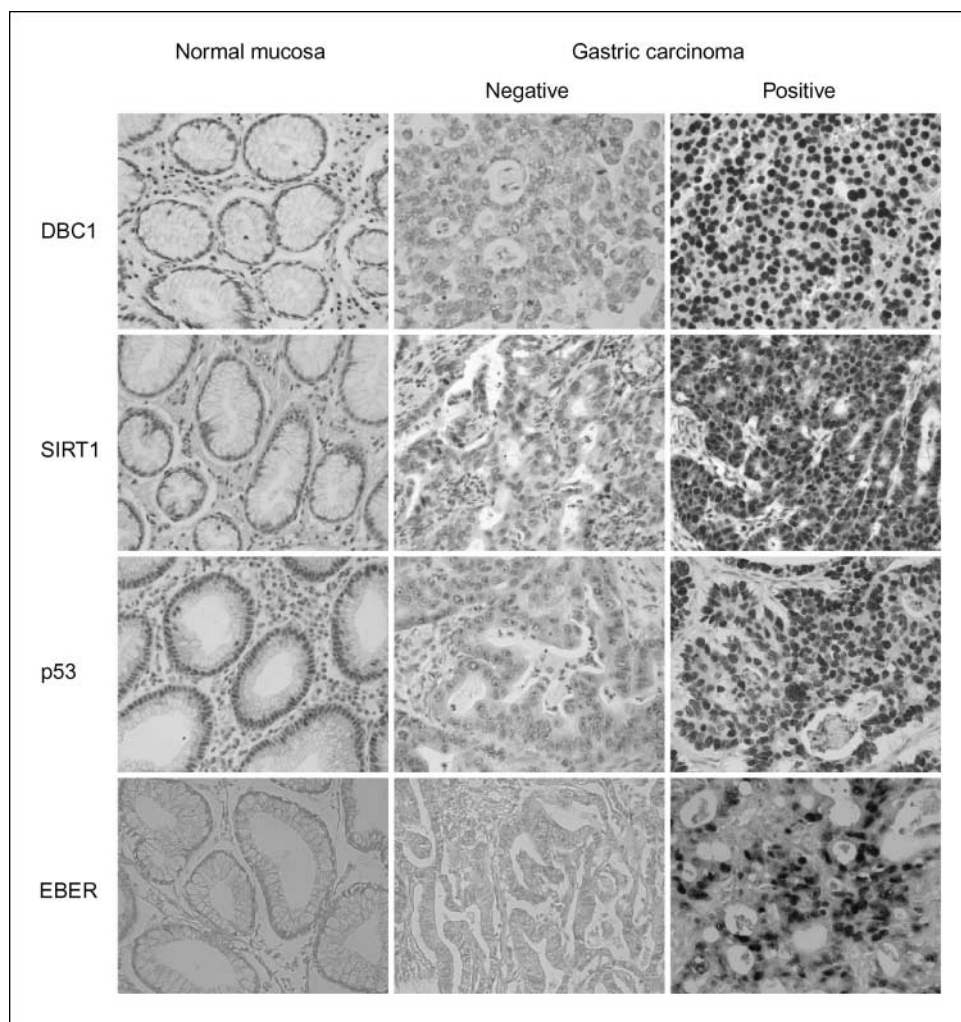


Fig. 1. Immunohistochemical staining and *in situ* hybridization for EBV-encoded small RNA in normal gastric mucosa and gastric carcinoma. Normal gastric mucosa is negative for DBC1, SIRT1, p53, and EBV-encoded small RNA. In gastric carcinoma specimens, DBC1, p53, and EBV-encoded small RNA show nuclear positivity. On the other hand, SIRT1 is positive in the nuclei and cytoplasm. Original magnification, $\times 400$.

CA19-9, tumor stage, presence of lymph node metastasis, tumor invasion (early gastric carcinoma versus advanced gastric carcinoma), venous invasion, EBV infection, DBC1 expression, and SIRT1 expression. From the multivariate analysis, only tumor stage and expression of DBC1 were independent prognostic factors significantly associated with overall survival and relapse-free survival. Patients with DBC1-expressing gastric carcinoma had a 3.334-fold [95% confidence interval (95% CI), 1.557-7.139] greater risk for death and a 4.180-fold (95% CI, 1.549-11.276) greater risk for recurrence of disease. However, SIRT1 was not an independent prognostic factor associated with overall survival and relapse-free survival by multivariate analysis in the gastric carcinoma patients.

Discussion

In this study, we have examined the immunohistochemical expression of DBC1 and SIRT1 in human gastric cancer and its prognostic significance. This study has shown for the first time that (a) a high proportion of gastric carcinomas expressed DBC1 and SIRT1; (b) expression of DBC1 and SIRT1 was significantly associated with unfavorable clinicopathologic

variables such as high serum level of CEA, high clinical stage (stage III and IV), lymph node metastasis, and advanced tumor invasion (early gastric carcinoma versus advanced gastric carcinoma); and (c) expression of DBC1 was associated with a significantly shorter survival in gastric carcinoma patients. Expression of SIRT1 was also poor prognostic factor of gastric carcinoma by univariate analysis. However, SIRT1 was not an independent prognostic indicator by multivariate analysis. It may be due to high correlation between DBC1 and SIRT1 expression. Taken together, our findings indicate that DBC1 and SIRT1 may be involved in the progression of gastric carcinomas and are significant prognostic indicators for gastric carcinoma patients.

Several previous reports have shown that SIRT1 is involved in cancer resistance to chemotherapy and ionizing radiation (30, 31). Our recent report also showed that SIRT1-expressing diffuse large B-cell lymphoma showed a significantly low complete response rate to chemotherapy compared with that of SIRT1-negative diffuse large B-cell lymphoma, suggesting that SIRT1 is involved in the resistance to chemotherapy (16). Moreover, SIRT1-deficient cells are more sensitive to chemotherapeutic agents (31), and inhibition of SIRT1 function induces growth arrest or apoptosis of human cancer cells (32–35). Therefore, SIRT1, in addition to serving as a

prognostic marker, may also provide a target for a novel therapeutic approach for gastric carcinomas.

SIRT1 was initially been thought to represent an exclusively nuclear protein (36). However, partial or temporary cytoplasmic localization was observed (37), and a recent report has shown that the cytoplasmic localization of SIRT1 seems to sensitize cells to oxidative stress-mediated apoptosis (38). Therefore, when we separately evaluated cytoplasmic SIRT1 expression, 74 of 177 cases showed cytoplasmic SIRT1 expression (weakly positive, 40 cases; moderately positive, 27 cases; strongly positive, 7 cases). However, there was no prognostic significance of cytoplasmic SIRT1 expression (overall survival; log-rank $P = 0.712$).

DBC1 promotes p53-mediated apoptosis through specific inhibition of SIRT1 by directly binding to the catalytic domain of SIRT1 (17, 18). When considering the possible roles of SIRT1 in tumor development and progression, DBC1 may play an antioncogenic role. Therefore, it has been suggested that loss of DBC1 would result in the inhibition of cell death and promote tumorigenesis (17). However, our study showed that DBC1 expression was positively correlated with SIRT1 expression and that DBC1 and SIRT1 expression were significantly correlated with advanced clinicopathologic factors of gastric carcinoma and poor prognosis for gastric carcinoma patients. In agreement with our findings, recent DNA microarray data sets revealed that DBC1 is up-regulated in urothelial carcinoma of the bladder (20), uterine cervical carcinoma (22), head and neck carcinoma (22), renal cell carcinoma (21), and breast cancers (23) compared with normal counterpart tissues. Among the malignant tumors, endometrial carcinoma, gastrointestinal carcinoid tumor, colon carcinoma, prostate carcinoma, ovarian carcinoma, and uterine cervical carcinoma show higher expression of the *DBC1* gene according to the Oncomine database. DBC1 expression was also higher in

metastatic breast carcinoma than in breast carcinoma at the primary site (23).

The reason why the expression of DBC1, suggested as a tumor suppressor, is associated with advanced cancer characteristics is still an enigma. It may be due to an accumulation of immunohistochemically detectable mutant DBC1 or to downstream functional defects despite the presence of normal DBC1 protein. Alternatively, because DBC1 directly binds the catalytic domain of SIRT1 and inhibits its deacetylation activity (17, 18), DBC1 expression might be increased to compensate for up-regulated SIRT1 activity. The results of our study that SIRT1 expression was significantly correlated with DBC1 expression supports the contention that DBC1 is up-regulated to modulate SIRT1 activity. Another possible explanation of our results is that DBC1 may have a role in tumorigenesis, in contrast to the other studies that say DBC1 is a tumor suppressor. This hypothesis may be supported by the observation that DBC1 collaborates with the estrogen receptor to suppress apoptosis and promote hormone-independent breast cancer cell growth (39). However, studies on the exact role of DBC1 have been very limited, and therefore, further analysis of SIRT1 and DBC1 expression in cancers is required to identify their mechanism of action and to determine whether SIRT1 and DBC1 have important roles in carcinogenesis.

The p53 protein is a key regulator of cell cycle progression and apoptosis. During tumor development, up-regulated SIRT1 allows cells to bypass apoptosis and survive DNA damage by deacetylation and inactivation of p53 (12, 14, 15). Therefore, we have examined the possible correlation among SIRT1, DBC1, and p53 expression status. In this study, expression of p53 was significantly associated with SIRT1 and DBC1 expression. Of p53-positive cases, 100% and 93% showed SIRT1 and DBC1 expression, respectively. Therefore, we suggest that SIRT1 and DBC1 may have a role in the

Table 2. Clinicopathologic factors and their effect on relapse-free survival and overall survival by univariate Cox proportional hazards regression analysis

Characteristics	No. of patients	OS		RFS	
		HR (95% CI)	P	HR (95% CI)	P
CEA	Normal	114	1	1	
	Elevated	31	1.953 (1.068-3.569)	2.117 (1.214-3.691)	0.008
CA19-9	Normal	128	1	1	
	Elevated	16	2.711 (1.313-5.595)	2.78 (1.436-5.381)	0.002
TNM stage	I and II	84	1	1	
	III and IV	93	7.473 (3.794-14.720)	8.038 (4.211-15.344)	<0.001
LN metastasis	Absence	64	1	1	
	Presence	113	7.76 (3.346-17.996)	9.201 (3.984-21.249)	<0.001
Tumor invasion	EGC	39	1	1	
	AGC	138	5.3 (1.926-14.581)	6.248 (2.278-17.133)	<0.001
Venous invasion	Absence	146	1	1	
	Presence	31	3.427 (2.015-5.831)	3.412 (2.059-5.652)	<0.001
EBV	Negative	163	1	1	
	Positive	14	1.852 (0.845-4.061)	1.671 (0.766-3.648)	0.197
DBC1	Negative	68	1	1	
	Positive	109	3.914 (2.044-7.494)	3.45 (1.923-6.187)	<0.001
SIRT1	Negative	47	1	1	
	Positive	130	4.318 (1.863-10.010)	3.607 (1.729-7.526)	0.001

Abbreviations: OS, overall survival; RFS, relapse-free survival; HR, hazard ratio.

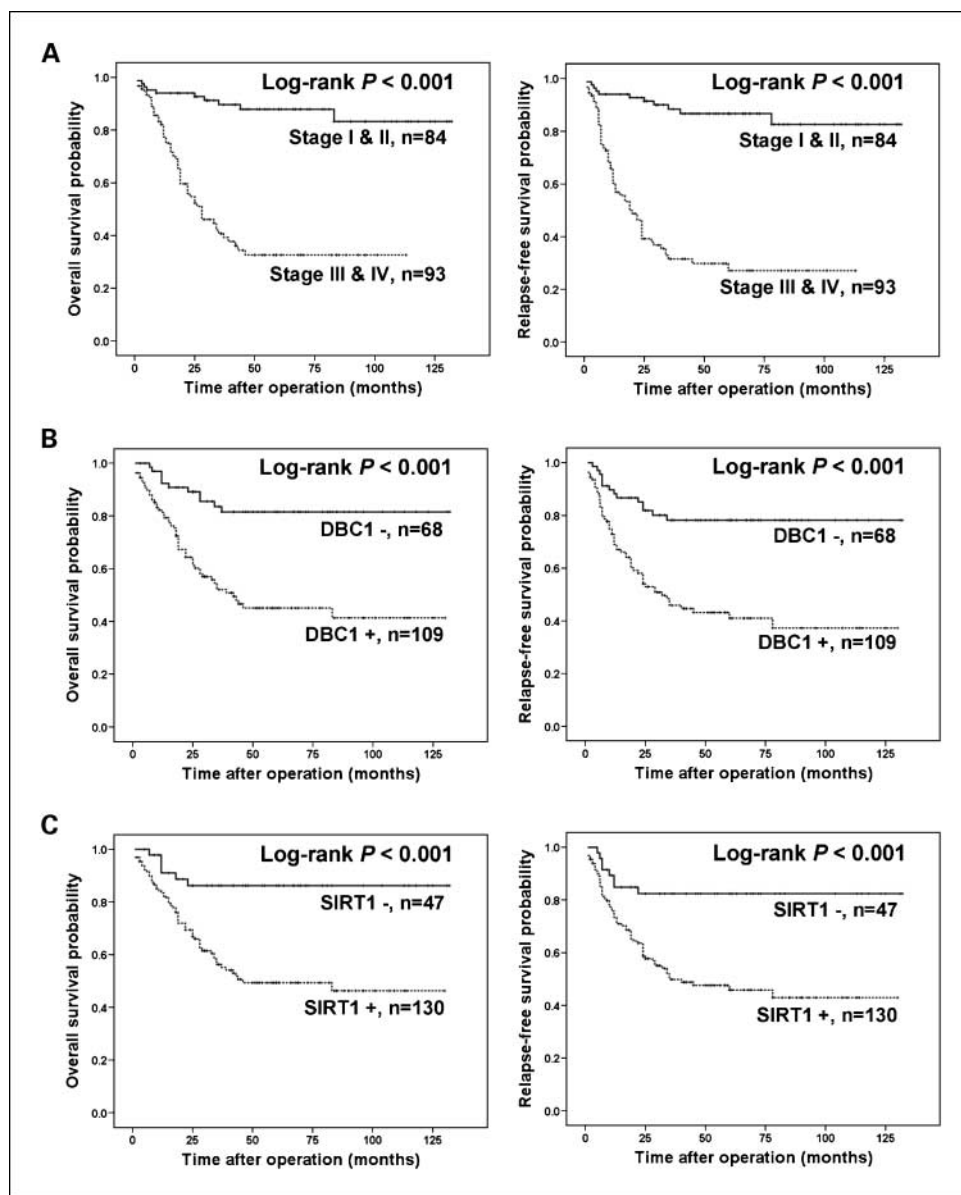


Fig. 2. Survival analysis in gastric carcinoma. A, overall survival and relapse-free survival in high – and low – clinical stage groups. B, relationship of DBC1 expression to overall survival and relapse-free survival. C, relationship of SIRT1 expression to overall survival and relapse-free survival. P values were determined by comparing survival distributions using the log-rank test.

development and progression of gastric carcinoma that is closely related to p53.

Recently, EBV-associated gastric carcinomas have been shown to comprise a distinct clinicopathologic entity of gastric

carcinoma with a better survival (40, 41). However, our result did not show prognostic significance of EBV infection. In addition, there was no correlation of EBV infection with DBC1 or SIRT1 expression.

Table 3. Multivariate Cox regression analysis for relapse-free survival and overall survival

Characteristics		OS		RFS	
		HR (95% CI)	P	HR (95% CI)	P
DBC1	Negative	1		1	
	Positive	3.334 (1.557-7.139)	0.002	2.321 (1.182-4.558)	0.014
TNM stage	I and II	1		1	
	III and IV	5.769 (2.687-12.389)	<0.001	3.181 (1.303-7.766)	0.011

NOTE: Variables considered in the analysis were the pretreatment serum level of CEA and CA19-9, tumor stage, presence of lymph node metastasis, tumor invasion (early gastric carcinoma versus advanced gastric carcinoma), venous invasion, EBV infection, DBC1 expression, and SIRT1 expression.

In conclusion, a high percentage of gastric carcinomas showed expression of SIRT1 and DBC1, and SIRT1 or DBC1 expression was associated with unfavorable gastric carcinoma characteristics and poor prognosis of gastric carcinoma patients. Although the role of DBC1 and SIRT1 in tumorigenesis is not clear, our data suggest that their expression is a

clinically significant prognostic indicator for gastric carcinoma patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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