Figure 6 (...continued)

region of the elongated crypts. In such a crypt, a few Dll1+ve IECs (yellow arrowhead) were found at the lowest part of the crypt, which located adjacent to Hes1+ve IECs (white arrowhead). Dll4+ve IECs (yellow arrowhead) were frequently found in both lower and upper region of those crypts, which also located adjacent to Hes1+ve IECs (yellow arrowhead). Scale bar represents 20 μm. (D) Double immunostaining of ATOH1 (red) with either Dll1 or Dll4 (green). In the DSS-colitis mice, ATOH1+ve IECs were also increased in number, and distributed at both lower and upper region of the elongated crypts. In those crypts, Dll1+ve and Dll4+ve IECs were invariably positive for ATOH1 (yellow arrowhead). Scale bar represents 20 μm. The data shown in (C) and in (D) were acquired by confocal microscopy (FV10i).

indicate that Dll1+ve and Dll4+ve IECs develop along the same differentiation pathway, through which Dll1+ve IECs give rise to Dll4+ve IECs.

Also, the present difference in distribution of Dll1+ve and Dll4+ve IECs may provide an insight to the difference observed in the phenotype of Dll1- or Dll4-depletion in the intestinal epithelium. Conditional knockout studies have shown that depletion of Dll1 alone leads to a slight increase of secretory lineage cells, whereas depletion of Dll4 alone resulted in no distinguishable phenotype (*Pellegrinet et al., 2011*; *Stamataki et al., 2011*). Also, administration of the Dll4-neutralizing antibody had no effect on intestinal epithelial tissue homeostasis (*Ridgway et al., 2006*). Thus, Dll4+ve IECs may play a relatively limited role, compared to Dll1+ve IECs, in the normal intestinal epithelium. Given that a large number of Dll4+ve IECs were found at the villi or at the upper crypts, and did not appear to contribute to activation of Notch signaling in their adjacent cells, it may be quite easy to agree that depletion of Dll4 in such a cell population may result in only a minimal change. However, it remains possible that a difference in function may exist between Dll1 and Dll4, and therefore makes the Dll1+ve IECs functionally dominant over Dll4+ve IECs within the crypts. Further analysis of our Dll1 or Dll4 knockout mice (Fig. S1) may reveal how these ligands can compensate each other through the changes in their expression patterns.

Our present data clearly showed that colonic c-kit positive IECs could express Dll1 and Dll4 (Figs. 5C and 5D). Thus our data show that Dll1+ve and Dll4+ve IECs may constitute a part of the stem cell niche in the colonic crypts. In contrast, the low detection frequency of Dll1 or Dll4 expression in small intestinal Paneth cells might indicate that those cells are possible to activate Notch in adjacent stem cells at a relatively low level of Dll1 and Dll4 expression. Accordingly, studies using Dll1-LacZ mice have clearly demonstrated its reporter activity within Paneth cells (*Stamataki et al., 2011*).

We also found that the dominance of Dll1+ve or Dll4+ve IECs within the colonic crypt significantly shifts to Dll4+ve IECs in DSS-colitis mice. Those Dll4+ve IECs mostly located adjacent to Hes1+ve cells, thus indicating that they are contributing to activate Notch signaling in their neighboring IECs. In contrast, we frequently observed Dll4+ve IECs in the colonic crypts of the control mice, whose adjacent cells are clearly negative for Hes1. Thus Dll4+ve IECs in the normal intestinal crypts may serve as reserve cells that could immediately activate Notch signaling in adjacent cells upon tissue injury. The functional importance of Dll4+ve IECs in the colitic mucosa may be revealed by our forthcoming studies using intestinal-epithelial cell specific Dll4-knockout mice.

CONCLUSIONS

Through our present results, we show that Dll1 and Dll4 are expressed by distinct population of ATOH1+ve cells in the small intestinal and colonic epithelium. Upon tissue injury, the dominance within the crypt is shifted to Dll4+ve IECs over Dll1+ve IECs. Therefore, our results suggest that Dll1+ve and Dll4+ve IECs may play different roles in the normal and inflamed colonic mucosa.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Hiromichi Shimizu conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Ryuichi Okamoto conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Go Ito, Satoru Fujii and Toru Nakata performed the experiments, analyzed the data.
- Kohei Suzuki and Tatsuro Murano performed the experiments.
- Tomohiro Mizutani analyzed the data.
- Kiichiro Tsuchiya, Tetsuya Nakamura and Mamoru Watanabe conceived and designed the experiments.
- Katsuto Hozumi contributed reagents/materials/analysis tools.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Approval Number 0140053A).

Supplemental Information

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High Expression of SQSTM1/p62 Protein Is Associated with Poor Prognosis in Epithelial Ovarian Cancer

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High expression of SQSTM1/p62 (p62) protein, which functions as a hub for various cellular signaling pathways, has been detected in several human cancers. However, the clinicopathological impact of high p62 expression is largely unknown in epithelial ovarian cancer (EOC). Here, the expression level of p62 in primary EOCs (n=266) was assessed by immunohistochemistry, and its clinical significance was analyzed. Univariate and multivariate analyses were used to determine the impact of p62 expression on overall survival. p62 was expressed in the cytoplasm (Cyto) and/or nucleus (Nuc) in primary EOCs, and an expression subtype ($Cyto^{High}/Nuc^{Low}$), showing high expression in the cytoplasm but low expression in the nucleus, was significantly correlated with serous carcinoma (P<0.001), advanced stage (P=0.005), presence of residual tumor (P<0.001), and low overall survival rate (P=0.013). Furthermore, in serous carcinomas (n=107), the p62 $Cyto^{High}/Nuc^{Low}$ subtype was significantly correlated with low overall survival rate (P=0.019) as an independent factor (P=0.044). Thus, our findings suggest that high expression of cytoplasmic p62 may be a novel prognostic biomarker in EOC, particularly in serous carcinoma.

Key words: epithelial ovarian cancer, serous carcinoma, immunohistochemistry, p62, prognosis

I. Introduction

SQSTM1/p62 (hereafter referred to as p62) functions as a signaling hub for various cell survival or cell death pathways [15]. This protein is also known as one of the selective substrates for autophagy, a cellular degradation system by which cytoplasmic components, organelles, and incorporated p62 protein are degraded [1, 8, 15, 22]. Mice lacking *Atg5* or *Atg7*, which are essential components in autophagy pathway, show elevated rates of spontaneous tumor formation accompanied by p62 accumulation, and tumor size is reduced by deletion of p62, suggesting that p62 accumulation may contribute to tumor progression [9, 15, 26]. Furthermore, it has been demonstrated that excess p62 expression may be involved in the activation of various oncogenic signaling pathways, including the NF-κB [4, 15], Wnt [6], mTOR [17], or NRF2 [9, 15, 16] pathways. Moreover, abnormal expression of p62 has been indeed detected in several cancers including prostate [7, 13], kidney [17], liver [9], lung [10], breast [3, 21, 24], and oral [11, 18] cancer cases. Thus, accumulating evidence from previous studies has indicated that excess p62 expression may play an oncogenic role in human cancers.

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Ovarian cancer is the most lethal gynecological malignancy in developed countries [5]. Epithelial ovarian cancer (EOC), comprises 90% of all ovarian cancers, include serous, mucinous, clear cell, and endometrioid carcinomas [2]. Among them, serous carcinoma, particularly high-grade serous carcinoma (HGSC), usually occurs in the advanced stage and spreads beyond the ovary at diagnosis [23]. Therefore, it is necessary to identify novel and efficient biomarker to use for prognosis for human EOC. In the present study, we examined the expression of p62 protein in EOC patients and analyzed the clinicopathological implications of the p62 expression status. While p62 protein was detected in the cytoplasm (Cyto) and/or nucleus (Nuc) in primary EOCs, we found that an expression subtype ($Cyto^{High}/Nuc^{Low}$; high expression in the cytoplasm but low expression in the nucleus) was associated with aggressive phenotypes and poor clinical outcome. Furthermore, this expression subtype of p62 protein was an independent prognostic factor in serous carcinomas. Thus, our findings in the present study indicate that p62 may serve as a biomarker for the prognostic prediction of EOC, especially for patients with serous carcinoma.

II. Materials and Methods

Patients and tumor specimens

Formalin-fixed paraffin-embedded tissue blocks of primary epithelial ovarian cancers (EOCs) from 266 consecutive patients were used to construct tissue microarrays (TMAs). All patients underwent for in surgery at the National Defense Medical College (NDMC) Hospital (Saitama, Japan) from 1986 to 2006 with a ten year period of follow-up starting from the initial surgery. The average follow-up period was 59 months (range, 1–120 months). Of the 266 patients, 93 patients (35.0%) died due to their cancer, with a median follow-up period of 20 months (range, 2–108 months). The tumor histological types were classified according to the WHO criteria. Clinical stages of the disease were classified according to the International Federation of Gynecology and Obstetrics (FIGO) system in 1988. All patients gave their written informed consent in formal style before the study. This study was approved by the ethics committees of the National Defense Medical College, Keio University, and Tokyo Medical and Dental University.

Immunohistochemical analysis

We constructed TMAs from tissue blocks prepared from the 266 EOC tumors using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA) as previously described [14], and immunohistochemistry was performed on TMA sections. The tissue sections were deparaffinized in xylene, and rehydrated with graded ethanol (100%, 90%, 80%, 70%, and 50%) to water. After the retrieval of antigens by boiling in 10 mM citrate buffer (pH 6.0), the sections were treated with 0.3% hydrogen peroxide in methanol to inactivate the endogenous peroxidase activity. Non-specific binding was blocked by incubation in horse serum in PBS. Slides were incubated with mouse anti-p62 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilution: 1/2000) overnight at room temperature. The bound antibody was visualized using diaminobenzidine as a chromogen (VECTASTAIN Elute ABC kit, Vector Laboratories), and the sections were lightly counterstained with hematoxylin.

Evaluation of immunohistochemistry

Immunohistochemical evaluation was performed by three researchers (R.I., Ju.I., and H.T.), and cases with discrepant grades were re-evaluated by discussion until consensus was achieved. The intensity score of cytoplasmic p62 expression was defined as 0 (no expression), 1+ (weak), and 2+ (strong). Specimens with 10% or more immunoreactive tumor cells with an intensity score of 2+ were considered "high expression", and specimens with less than 10% of 2+ cells or specimens where almost all tumor cells had an intensity score of 1+ or 0 were considered "low expression". Nucleus staining of p62 was classified as either "high expression" or "low expression" when stained with greater or less than 5% of tumor cells, respectively. Based on the distribution (cytoplasm; Cyto or nucleus; Nuc) and the expression level (High or Low), specimens were classified into four subtypes: Type-A: $Cyto^{Low}/Nuc^{Low}$, Type-B: $Cyto^{Low}/Nuc^{High}$, Type-C: $Cyto^{High}/Nuc^{High}$, and Type-D: $Cyto^{High}/Nuc^{Low}$.

Statistical analysis

Correlation between p62 expression in primary EOCs and the clinicopathological variables were analyzed by the chi-square or Fisher's exact test. Survival data were analyzed by the Kaplan-Meier method, and compared with the expression status of p62 by log-rank (Cox-Mentel) test. Calculated P values lower than 0.05 were considered as statistically significant.

III. Results

Immunoreaction of p62 in epithelial ovarian cancer

To assess the clinical significance of p62 expression in EOC, we performed immunohistochemical analysis using TMAs from 266 primary EOCs. The expression of p62 protein was detected in the cytoplasm and/or nucleus of EOC tumor cells (Fig. 1). High cytoplasmic immunoreaction for p62 protein was found in 42 (15.8%) of 266 cases (Fig. 1D–F). Nuclear immunoreaction for p62 protein was shown in some population (5–90%) of tumor cells in 50 (18.8%) of 266 cases (Fig. 1G–I). Based on the expression level (Low or High) and the distribution (Cyto or Nuc), we classified tumors into four subtypes; $Cyto^{Low}/Nuc^{Low}$ as Type-A (n=178), $Cyto^{Low}/Nuc^{High}$ as Type-B (n=46), $Cyto^{High}/Nuc^{High}$ as Type-C (n=4), and $Cyto^{High}/Nuc^{Low}$ as Type-D (n=38). Interestingly, the staining intensity of cytoplasmic

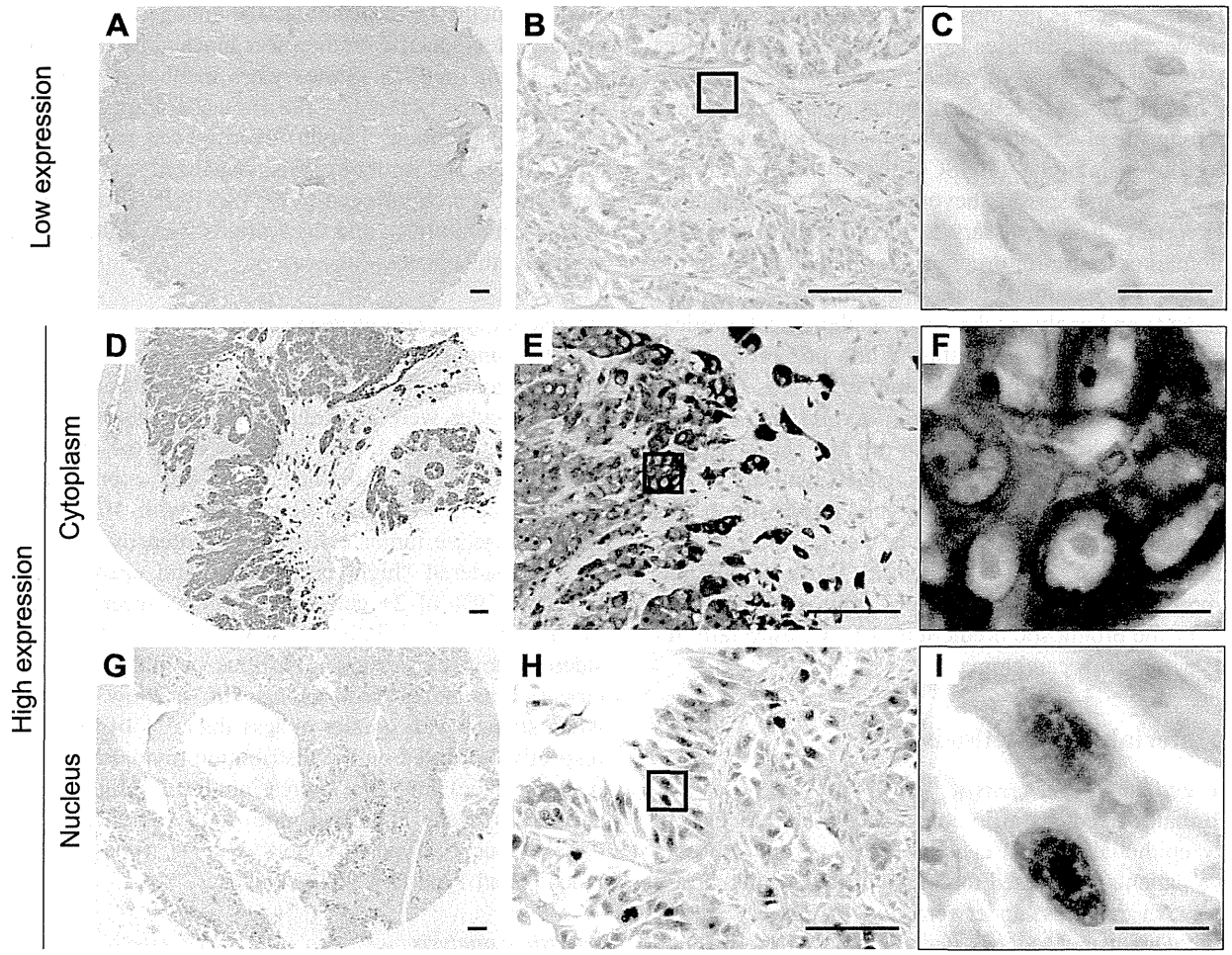

Fig. 1. Immunostaining of p62 expression in epithelial ovarian cancer. Representative images for tumors with low p62 expression (**A–C**), high cytoplasmic p62 expression (**D–F**), and high nuclear p62 expression (**G–I**). Bar=100 μm (**A, B, D, E, G,** and **H**) or 10 μm (**C, F,** and **I**).

p62 seemed to be stronger in the invasive front area than in the intratumoral area within p62-highly expressing tumors. Furthermore, in the invasive front area, EOC tumor cells with p62-positive aggregate-like structures were occasionally observed, but the frequency was very low (4 of 42 cytoplasmic p62-highly expressing cases, 9.5%; Fig. 1E and 1F).

Clinicopathological and prognostic implications of p62 expression for epithelial ovarian cancers

To examine the clinicopathological and prognostic implications of p62 expression status in EOC, we performed the chi-square or Fisher's exact test, as appropriate. High expression of cytoplasmic p62 and the Type-D ($Cyto^{High}/Nuc^{Low}$) expression subtype were significantly associated with aggressive phenotypes, including serous histology ($P=1.32\times10^{-6}$ and $P=1.46\times10^{-7}$, respectively), advanced FIGO stage ($P=7.85\times10^{-3}$ and $P=4.99\times10^{-3}$, respectively), and presence of residual tumor ($P=7.14\times10^{-4}$ and $P=3.74\times10^{-4}$, respectively) (Table 1). Overall survival time of EOC patients was significantly shorter in the high cytoplasmic p62 expression group compared with the group with low expression ($P=9.13\times10^{-3}$, Fig. 2A) or in the group with the expression subtype of Type-D compared with the group with other subtypes ($P=1.30\times10^{-2}$, Fig. 2B).

In the present cohort included 107 cases with serous carcinoma, among them 9 cases were low-grade, 65 were HGSC, and the others were not determined. Although there was no significant correlation between the clinicopathological characteristics and the expression status of p62 (Table 2), Kaplan-Meier survival curves revealed that overall survival time was significantly shorter in the Type-D expression subtype compared with the other subtypes ($P=1.91\times10^{-2}$, Fig. 2C). Additionally, even in the 65 patients with HGSC, the Type-D expression subtype was significantly correlated to shorter overall survival time ($P=1.20\times10^{-2}$, Fig. 2D).

Multivariate analysis for poor prognosis marker in epithelial ovarian cancers

In all 266 patients, univariate analysis showed that advanced FIGO stage, presence of residual tumor, and the Type-D expression subtype ($Cyto^{High}/Nuc^{Low}$) were significantly correlated with overall survival, however the p62

Table 1. *Correlation between p62 expression and clinicopathological variables in 266 patients with epithelial ovarian cancer*

	All	Expression level of Cytoplasmic p62 [a]				P [b]	Subtypes of p62 expression status [a]				P [b]
		Low		High			Type-A, -B, -C		Type-D		
	n	*n*	(%)	*n*	(%)		*n*	(%)	*n*	(%)	
Number	266	224	(84.2)	42	(15.8)		228	(85.7)	38	(14.3)	
Age, years [c]											
≤50	107	87	(81.3)	20	(18.7)	0.287	88	(82.2)	19	(17.8)	0.184
>50	159	137	(86.2)	22	(13.8)		140	(88.1)	19	(11.9)	
Histological type (serous vs. others)											
Serous	107	76	(71.0)	31	(29.0)	**<0.001**	77	(72.0)	30	(28.0)	**<0.001**
Mucinous	30	27	(90.0)	3	(10.0)		27	(90.0)	3	(10.0)	
Endometrioid	42	38	(90.5)	4	(9.5)		38	(90.5)	4	(9.5)	
Clear cell	87	83	(95.4)	4	(4.6)		86	(98.9)	1	(1.1)	
FIGO stage (I–II vs. III–IV)											
I	96	89	(92.7)	7	(7.3)	**0.008**	91	(94.8)	5	(5.2)	**0.005**
II	30	25	(83.3)	5	(16.7)		25	(83.3)	5	(16.7)	
III	104	86	(82.7)	18	(17.3)		88	(84.6)	16	(15.4)	
IV	36	24	(66.7)	12	(33.3)		24	(66.7)	12	(33.3)	
Residual tumor											
Absent	127	117	(92.1)	10	(7.9)	**<0.001**	119	(93.7)	8	(6.3)	**<0.001**
Present (>0 cm)	139	107	(77.0)	32	(23.0)		109	(78.4)	30	(21.6)	

[a] Expression status of p62 was assessed by IHC and classified into four subtypes based on expression level (Low or High) and distribution (cytoplasm; Cyto or nucleus; Nuc): Type-A ($Cyto^{Low}/Nuc^{Low}$), Type-B ($Cyto^{Low}/Nuc^{High}$), Type-C ($Cyto^{High}/Nuc^{High}$), and Type-D ($Cyto^{High}/Nuc^{Low}$).
[b] Chi-square or Fisher's exact test, as appropriate. Statistically significant values are in boldface type.
[c] Median age was 53 years (range, 20–81 years).

Table 2. *Correlation between p62 expression and clinicopathological variables in 107 patients with serous ovarian carcinoma*

	All	Expression level of Cytoplasmic p62 [a]				P [b]	Subtypes of p62 expression status [a]				P [b]
		Low		High			Type-A, -B, -C		Type-D		
	n	*n*	(%)	*n*	(%)		*n*	(%)	*n*	(%)	
Number	107	76	(71.0)	31	(29.0)		77	(72.0)	30	(28.0)	
Age, years [c]											
≤50	39	26	(66.7)	13	(33.3)	0.287	26	(66.7)	13	(33.3)	0.356
>50	68	50	(73.5)	18	(26.5)		51	(75.0)	17	(25.0)	
Histological grade (1 vs. 2 or 3)											
1	9	9	(100.0)	0	(0.0)	0.102	9	(100.0)	0	(0.0)	0.102
2 or 3	65	45	(69.2)	20	(30.8)		45	(69.2)	20	(30.8)	
Unknown	33	22	(66.7)	11	(33.3)		23	(69.7)	10	(30.3)	
FIGO stage (I–II vs. III–IV)											
I	11	8	(72.7)	3	(27.3)	0.655	8	(72.7)	3	(27.3)	0.583
II	7	4	(57.1)	3	(42.9)		4	(57.1)	3	(42.9)	
III	63	48	(76.2)	15	(23.8)		49	(77.8)	14	(22.2)	
IV	26	16	(61.5)	10	(38.5)		16	(61.5)	10	(38.5)	
Residual tumor											
Absent	19	15	(78.9)	4	(21.1)	0.401	15	(78.9)	4	(21.1)	0.455
Present (>0 cm)	88	61	(69.3)	27	(30.7)		62	(70.5)	26	(29.5)	

[a] Expression status of p62 was assessed by IHC and classified into four subtypes based on expression level (Low or High) and distribution (cytoplasm; Cyto or nucleus; Nuc): Type-A ($Cyto^{Low}/Nuc^{Low}$), Type-B ($Cyto^{Low}/Nuc^{High}$), Type-C ($Cyto^{High}/Nuc^{High}$), and Type-D ($Cyto^{High}/Nuc^{Low}$).
[b] Chi-square or Fisher's exact test, as appropriate. Statistically significant values are in boldface type.
[c] Median age was 54 years (range, 29–81 years).

expression status was not found to be an independent prognostic factor by multivariate analysis (Table 3). On the other hand, in 107 patients with serous cancer, multivariate analysis following univariate analysis revealed that the Type-D expression subtype was only an independent prognostic factor for overall survival ($P=4.36\times10^{-2}$, Table 3). These findings suggest that high expression of p62 protein in the cytoplasm may be a molecular marker of poor prog-

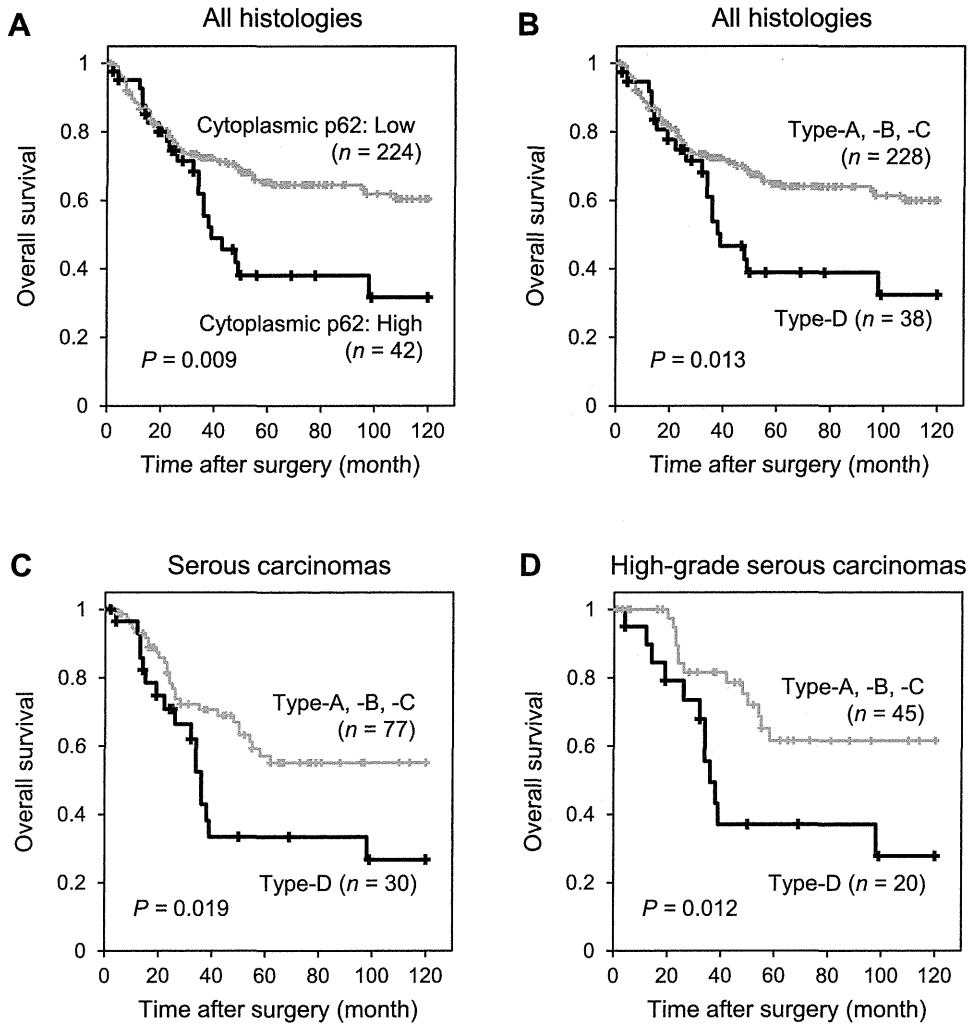

Fig. 2. Prognostic implication of the p62 expression status in epithelial ovarian cancer. **A.** Overall survival curves of all 266 patients with epithelial ovarian cancer (EOC) according to the expression level of cytoplasmic p62. This figure shows survival curves for 42 patients with high cytoplasmic p62-expressing tumors and for 224 patients with low cytoplasmic p62-expressing tumors. The two curves differ significantly. **B.** Overall survival curves of all 266 patients with EOC according to the p62 expression subtypes. This figure shows the survival curves for 38 patients with Type-D tumors (high expression of cytoplasmic p62 and low expression of nuclear p62; $Cyto^{High}/Nuc^{Low}$) and for 228 patients with Type-A, -B, or -C tumors ($Cyto^{Low}/Nuc^{Low}$, $Cyto^{Low}/Nuc^{High}$, or $Cyto^{High}/Nuc^{High}$, respectively). The two curves differ significantly. **C.** Overall survival curves of 107 patients with serous carcinoma according to the p62 expression subtypes. This figure shows the survival curves for 30 patients with Type-D tumors and 77 patients with Type-A, -B, or -C tumors. The two curves differ significantly. **D.** Overall survival curves of 65 patients with high-grade serous carcinoma according to the p62 expression subtypes. This figure shows the survival curves for 20 patients with Type-D tumors and 45 patients with Type-A, -B, or -C tumors. The two curves differ significantly.

nosis in patients with EOC, especially those with serous carcinomas.

IV. Discussions

In the present cohort, we found that the Type-D ($Cyto^{High}/Nuc^{Low}$) p62 expression subtype is correlated with poor prognosis in epithelial ovarian cancers (EOC), especially in serous histology. However, at present the mechanism underlying the change to high expression of p62 protein is unclear. While p62 protein is continuously degraded by the autophagy system, it has been demonstrated that this protein is highly accumulated in liver tumors that develop in mice lacking the *Atg5* or *Atg7* genes [9, 15, 26]. On the other hand, it has been known that p62 expression is regulated in a cellular context-dependent manner at the transcriptional level [12, 19, 25]. The p62 mRNA level is positively correlated with its protein level examined by immunohistochemistry in oral squamous cell carcinomas [18]. Thus, high level of p62 protein may be attributed to dysregulation at the transcription and/or translation levels.

Furthermore, we showed two characteristic distributions of p62 protein expression. We have observed localization of p62 in the nucleus and not only in the cytoplasm. It has been experimentally demonstrated that p62 protein can be shuttled between the nucleus and cytoplasm due to its signal for nuclear localization and export [20]. Furthermore, this distribution has been observed in human prostate

Table 3 *Cox proportional hazard regression analysis for overall survival*

	Univariate analyses			Multivariate analyses		
	HR	(95% CI) [a]	*P* [b]	HR	(95% CI) [a]	*P* [b]
All histologies (*n*=266)						
Age >50 years (vs. ≤50 years)	0.84	(0.56–1.26)	0.401			
Serous histology (vs. the others)	1.44	(0.96–2.16)	0.082			
FIGO stage III–IV (vs. I–II)	4.23	(2.58–6.95)	**<0.001**	2.76	(1.41–5.41)	**0.003**
Residual tumor >0 cm (vs. =0 cm)	3.68	(2.28–5.95)	**<0.001**	1.73	(0.90–3.33)	0.100
Type-D p62 expression (vs. Type-A, -B, -C) [c]	1.85	(1.13–3.04)	**0.015**	1.28	(0.77–2.12)	0.337
Serous carcinomas (*n*=107)						
Age >50 years (vs. ≤50 years)	0.75	(0.41–1.36)	0.341			
Histological grade 2 or 3 (vs. 1)	0.90	(0.31–2.60)	0.849			
FIGO stage III–IV (vs. I–II)	2.40	(0.86–6.72)	0.094			
Residual tumor >0 cm (vs. =0 cm)	3.29	(1.02–10.65)	**0.047**	3.00	(0.92–9.75)	0.068
Type-D p62 expression (vs. Type-A, -B, -C) [c]	2.03	(1.11–3.72)	**0.022**	1.87	(1.02–3.44)	**0.044**

[a] HR, hazard ratio; CI, confidence interval.
[b] Statistically significant values are in boldface type.
[c] Expression status of p62 was assessed by IHC and classified into four subtypes based on expression level (Low or High) and distribution (cytoplasm; Cyto or nucleus; Nuc): Type-A ($Cyto^{Low}/Nuc^{Low}$), Type-B ($Cyto^{Low}/Nuc^{High}$), Type-C ($Cyto^{High}/Nuc^{High}$), and Type-D ($Cyto^{High}/Nuc^{Low}$).

and oral cancers, and has been reported to be correlated with favorable prognosis at least in oral cancers [13, 18]. In the serous carcinomas of the present cohort, the patients with Type-D p62 expression ($Cyto^{High}/Nuc^{Low}$) had poorer prognosis than those with the other expression subtypes. This suggests that the high expression of p62 protein in the cytoplasm, not in the nucleus, may contribute to the malignancy of EOC cells, but the molecular mechanism is largely unknown. Expression of p62 was also stronger in the invasive front area than in the intratumoral area in primary EOC tumors with high expression of cytoplasmic p62. In this invasive front area, EOC tumor cells with p62-positive aggregate-like structures were occasionally observed. Many studies have suggested that excess p62 expression, notably formation of p62-positive aggregates, contributes to tumor growth and tolerance to cellular stress in tumor cells by the activation of oncogenic signals, including the NF-κB, Wnt, mTOR, and NRF2 pathways [4, 6, 9, 15–17]. This suggests that activation of these oncogenic signaling pathways may be involved in the migration and/or invasion of EOC cells within the invasive front area.

A pattern of cytoplasmic p62-expression was extremely infrequent in other histological types than serous carcinoma, such as mucinous, endometrioid, and clear cell, suggesting that p62 expression may be associated with tumor development only in the process of serous carcinoma, although its mechanism is largely unknown. Recently, it has been suggested that sub-classification of serous carcinomas by histological grade is important for proper prognosis; HGSCs are more malignant than low-grade serous carcinomas [23]. In patients with HGSCs from our cohort, patients with the Type-D subtype showed significantly shorter overall survival time than patients with other p62 expression subtypes. This suggests that determination of the expression status of p62, together with histological grade, may be helpful for diagnosis of patients with serous carcinomas. Thus, understanding the significance of the heterogeneous distribution of p62 protein and the mechanisms underlying the involvement of p62 in the malignancy of EOC cells is required to develop a better personalized therapeutic approach for EOC with high expression of cytoplasmic p62.

V. Disclosure

The authors declare no competing financial interests.

VI. Acknowledgments

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FULL PAPER

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Keywords: SMYD2; TP53; gastric cancer; prognosis

Overexpression of SMYD2 contributes to malignant outcome in gastric cancer

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Background: *SET and MYND domain-containing protein 2 (SMYD2)* is a lysine methyltransferase for histone H3, p53 and Rb and inhibits their transactivation activities. In this study, we tested whether SMYD2 (1q42) acts as a cancer-promoting factor by being overexpressed in gastric cancer.

Methods: We analysed 7 gastric cancer cell lines and 147 primary tumor samples of gastric cancer, which were curatively resected in our hospital.

Results: SET and MYND domain-containing protein 2 was detected in these cell lines (five out of seven cell lines; 71.4%) and primary tumor samples (fifty-six out of one hundred and forty-seven cases; 38.1%). Knockdown of SMYD2 using specific small interfering RNA inhibited proliferation, migration and invasion of SMYD2-overexpressing cells in a *TP53* mutation-independent manner. Overexpression of SMYD2 protein correlated with larger tumor size, more aggressive lymphatic invasion, deeper tumor invasion and higher rates of lymph node metastasis and recurrence. Patients with SMYD2-overexpressing tumours had a worse overall rate of survival than those with non-expressing tumours ($P=0.0073$, log-rank test) in an intensity and proportion score-dependent manner. Moreover, multivariate analysis demonstrated that SMYD2 was independently associated with worse outcome ($P=0.0021$, hazard ratio 4.25 (1.69–10.7)).

Conclusions: These findings suggest that SMYD2 has a crucial role in tumor cell proliferation by its overexpression and highlight its usefulness as a prognostic factor and potential therapeutic target in gastric cancer.

Gastric cancer is one of the most common causes of death from cancer worldwide (Siegel *et al*, 2013). Recent advances in diagnostic technologies and peri-operative management have increased early detection of gastric cancer and decreased the morbidity and mortality rates. However, patients with advanced gastric cancer still frequently experience recurrence despite extended radical resections, which results in an extremely poor survival rate (Martin *et al*, 2002).

Numerous genes have been analysed to understand molecular mechanisms of carcinogenesis and improve clinical outcomes for human gastric cancers; however, only a few genes with frequent alterations have been identified (Ushijima and Sasako, 2004) such as gene amplifications of *MET* and *ERBB2*, hypermethylation of *p16* (Oue *et al*, 2002; Ding *et al*, 2003), mutations of *TP53*, *APC* and *E-cadherin* (Becker *et al*, 1994; Maesawa *et al*, 1995; Lee *et al*, 2002), oncogenic activation of *β-catenin* and *K-ras* (Park *et al*, 1999)

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and inactivation of the mismatch repair gene *hMLH1*, which is associated with microsatellite instability (Fang *et al*, 2003). Including these reports, researchers have attempted to identify biological factors involved in the malignancy of gastric cancer. However, in clinical settings, only a few genes have been used as diagnostic biomarkers and/or therapeutic targets (Bang *et al*, 2010). We therefore wished to identify novel genes associated with the progression of gastric cancer.

SET and MYND domain-containing protein 2 (SMYD2) (1q42) was recently identified as a lysine methyltransferase for histone H3K36, H3K4 and K370 of p53, K860 and K810 of Rb and K528 of PARP1. It regulates transcription (Brown *et al*, 2006; Abu-Farha *et al*, 2008), inhibits tumour suppressor proteins p53 (Huang *et al*, 2006) and Rb (Saddic *et al*, 2010; Cho *et al*, 2012), and enhances the poly (ADP-ribose) activity of the oncogenic protein PARP1 in cancer cells (Piao *et al*, 2014). Moreover, SMYD2 is a clinically relevant prognostic marker for oesophageal squamous cell carcinoma (ESCC), bladder carcinoma and paediatric acute lymphoblastic leukaemia (Komatsu *et al*, 2009; Cho *et al*, 2012; Sakamoto *et al*, 2014) and it could be used to predict a potentially poor prognosis to plan more aggressive treatment for applicable patients. However, to date, there has been no report on the clinical and prognostic significance of SMYD2 in patients with primary gastric cancer. These findings prompted us to investigate the effects of SMYD2 overexpression and activation in primary gastric cancer.

Consequently, in this study, we demonstrated that SMYD2 was frequently overexpressed in gastric cancer cell lines and primary gastric cancers. Overexpression of SMYD2 was an indicator of poor prognosis independent of other prognostic factors. We also demonstrated that knockdown of SMYD2 suppressed cell proliferation, migration and invasion in gastric cancer cell lines. Our results provide evidence that SMYD2 could be an important molecular marker to determine malignant properties and a target for molecular therapy in patients with gastric cancer.

MATERIALS AND METHODS

Cell lines and primary tissue samples. A total of seven gastric cancer cell lines (MKN7, MKN74, HGC27, MKN45, KatoIII, NUGC4 and MKN28 cells) and a fibroblast cell line (WI-38) were used in this study. HGC27 and WI-38 cells were cultured in mixture of Dulbecco's Minimum Essential Medium and F12 medium, and the other cell lines were cultured in RPMI-1640 medium. All culture media were purchased from Sigma (St. Louis, MO, USA) and supplemented with 100 ml l^{-1} foetal bovine serum (Trace Scientific, Melbourne, VIC, Australia). All cell lines were cultured in 5% carbon dioxide at 37 °C in a humidified chamber. Primary tumour samples of gastric cancer were obtained from 147 gastric cancer patients, who underwent curative gastrectomy (R0) at the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine (Kyoto, Japan) between 2001 and 2003. These samples were embedded in paraffin after 24 h of formalin fixation. Relevant clinical and survival data were available for all patients. Written consent was always obtained in the formal style and after approval by the local ethics committee. None of these patients underwent endoscopic mucosal resection, palliative resection, preoperative chemotherapy or radiotherapy, and none of them had synchronous or metachronous multiple cancers in other organs. Disease stage was defined in accordance with the International Union against Cancer tumour-lymph node-metastases classification (7th edition (Sobin and Wittekind, 2009)). The median follow-up period for surviving patients was 54.5 months (ranging from 0.5 to 84.2 months).

Quantitative real-time RT–PCR. Single-stranded complementary DNA generated from total RNA was amplified with primers specific for each gene, as described below. Abundance of messenger RNA (mRNA) was measured with a quantitative real-time fluorescence detection method (ABI StepOnePlus Sequence Detection System; Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Assays (Hs00220210_m1 for SMYD2; Applied Biosystems) according to the manufacturer's instructions. Results of gene expression were calculated as a ratio between SMYD2 and an internal reference gene (Hs99999903_m1 for β-actin; Applied Biosystems) that provides a normalisation factor for the amount of RNA isolated from a specimen, and the ratio was subsequently normalised by that of the control fibroblast cell line (relative expression). This assay was performed in duplicate for each sample.

Western blotting. Anti-SMYD2 rabbit polyclonal antibody was generated against a 14-amino acid peptide from human SMYD2 (HPYISEIKQEIESH; Operon Biotechnology, Tokyo, Japan) and purified through an affinity column. Anti-β-actin antibody was purchased from Sigma, and anti-p53 (DO-7) and anti-p21 antibodies were from Novocastra Laboratories (Newcastle upon Tyne, UK) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Cells were lysed and their proteins were extracted by M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Twenty micrograms of proteins per lane were loaded for electrophoresis.

Knockdown of SMYD2 by siRNA and analysis of cell proliferation. We knocked down SMYD2 with small interfering RNA (siRNA) targeting SMYD2 (SMARTpool #M-020291-00; Dharmacon, Lafayette, CO, USA) and we used siRNA targeting luciferase as a negative control (Luc, 5′-CGUACGCGGAAUACUUCGA-3′; Sigma, Tokyo, Japan). Gastric cancer cell lines were transfected with each siRNA (10 nmol l^{-1}) with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Effects of SMYD2 knockdown on protein abundance was confirmed by Western blotting. To measure cell proliferation, the number of viable cells 24, 48 and 72 h after siRNA transfection was assessed by the colorimetric water-soluble tetrazolium salt assay (Cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan). Cell cycle was evaluated 72 h after transfection by fluorescence-activated cell sorting (FACS) as described elsewhere (Komatsu *et al*, 2009).

Transwell migration and invasion assays. Transwell migration and invasion assays were carried out in 24-well modified Boyden chambers (transwell-chamber, BD Transduction, Franklin Lakes, NJ, USA). For invasion assays, the upper surfaces of the 6.4-mm diameter filters with 8-μm pores that were used for migration assays were precoated with Matrigel (BD Transduction). siRNA transfectants (2×10^4 cells per well) were transferred into the upper chamber. Following 48 h of incubation, migrated or invasive cells on the lower surface of the filters were fixed and stained with the Diff-Quik stain (Sysmex, Kobe, Japan), and stained cell nuclei were counted directly in triplicate. We assessed the ability of the cells to move through extracellular matrices by calculating the number of cells, which is the ratio of the percentage invasion through the matrigel-coated filters relative to migration through the uncoated filters of test cells over that in the control cells as described elsewhere (Kashimoto *et al*, 2012; Nishimura *et al*, 2013).

Immunohistochemistry. Primary tumour samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, sectioned into 5-μm-thick slices and subjected to immunohistochemical staining of SMYD2 with the avidin–biotin–peroxidase method as described by Naoi *et al* (2008). In brief, after deparaffinization, endogenous peroxidases were quenched by incubating the sections for 20 min in 3% H_2O_2. Antigen retrieval was performed by heating the samples in 10 mmol l^{-1} citrate buffer (pH 6.0) at 95 °C for 60 min. After treatment with Block Ace (Dainippon Sumitomo

Pharmaceutical, Osaka, Japan) for 30 min at room temperature, sections were incubated at 4 °C overnight with the anti-SMYD2 (1 : 200) antibody. The avidin–biotin–peroxidase complex system (Vectastain Elite ABC universal kit; Vector Laboratories Inc., Burlingame, CA, USA) was used for colour development with diaminobenzidine tetrahydrochloride. Tissues were counterstained with Mayer's haematoxylin. To confirm the specificity of the anti-SMYD2 antibody, a formalin-fixed oesophageal cancer cell line overexpressing SMYD2 (KYSE170 cells), in which >50% of cells showed staining of SMYD2 protein, was used as a positive control, whereas KYSE170 cells incubated without the SMYD2 antibody were used as a negative control (Supplementary Figure S1; Komatsu *et al*, 2009). For scoring SMYD2 abundance, the intensity (intensity score 0 = negative, score 1 = weak, score 2 = moderate, score 3 = strong, as described below) and percentage of the SMYD2-expressing cells in the total population (proportion score 0, <10%; score 1, 10 to 33%; score 2, 34 to 66%; score 3, 67 to 100%) were evaluated for each tumour sample using high-power (×200) microscopy. In primary tumour samples, SMYD2 protein was not detected in most of the non-tumorous gastric mucosa and stroma. Primary tumours with no detectable SMYD2 (similar to non-tumorous gastric mucosa and stroma) were given an intensity score of 0, whereas those with the greatest SMYD2 abundance were given an intensity score of 3. The remaining tumours were categorised with intensity scores of 1 or 2 according to the intensity of immunohistochemical staining for SMYD2. Finally, expression of SMYD2 was graded based on the sum of the intensity score and the proportion score: high expression (intensity plus proportion scores ⩾4) or low expression (intensity plus proportion scores ⩽3) (Tsuda, 2008).

Statistical analysis. Clinicopathological variables pertaining to the corresponding patients were analysed for statistical significance by the χ^2-test or Fisher's exact test. For the analysis of survival, Kaplan–Meier survival curves were constructed for groups based on univariate predictors, and differences between the groups were analysed with the log-rank test. Univariate and multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox proportional hazards model. Differences between subgroups were tested with the non-parametric Mann–Whitney *U*-test. Differences were assessed with a two-sided test and considered statistically significant at $P<0.05$.

RESULTS

Overexpression of SMYD2 in gastric cancer cell lines. Quantitative RT–PCR and Western blotting analyses were performed to test whether SMYD2 is overexpressed in gastric cancer cell lines compared with the fibroblast cell line WI-38 (Figure 1A). Expression of SMYD2 protein detected by the SMYD2-specific antibody correlated with that of SMYD2 mRNA in gastric cancer cell lines. SMYD2 was overexpressed in almost all gastric cancer cell lines (five out of seven cell lines, 71.4%), suggesting this gene to be a target for activation in gastric cancer cell lines (Figure 1A). Because repression of p53 activity through SMYD2-mediated methylation at K370 was reported previously (Huang *et al*, 2006), we examined the status of *TP53* mutation in the gastric cancer cell lines by Western blotting. These statuses of *TP53* mutation in various cell lines are positively associated with their reported status of *TP53* mutation in the database (http://p53.free.fr/index.html) (M = mutant *TP53*; W = wild-type *TP*53.). Although wild-type p53 is expected to be a more suitable substrate than mutant p53 for SMYD2 (Huang *et al*, 2006; Komatsu *et al*, 2009), expression of SMYD2 mRNA and protein was not correlated with the mutation status and expression of p53 (Figure 1A).

Suppression of cell proliferation by knockdown of SMYD2. To gain an insight into the potential role of SMYD2 as an oncogene whose overexpression could be associated with gastric carcinogenesis, we first performed cell proliferation assays using siRNA specific for SMYD2 and investigated whether knockdown of SMYD2 would suppress proliferation of gastric cancer cell lines that overexpress SMYD2. We chose the HGC27 cell line for these assays, because it had the highest amount of SMYD2 protein (Figure 1A). Expression of SMYD2 protein in this cell line was

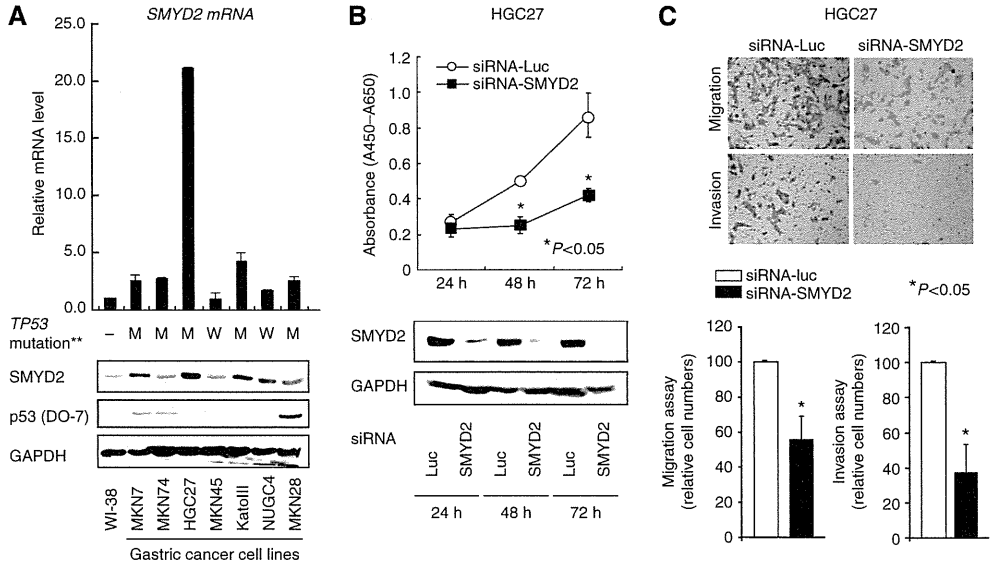

Figure 1. (A) Expression of SMYD2 mRNA (top) and protein (bottom) in seven gastric cancer cell lines compared with that in a fibroblast cell line WI-38. Also, the status of *TP53* mutation in each gastric cancer cell line was evaluated by Western blotting. The status of *TP53* mutation was positively associated with the reported status of *TP53* mutation in the database (http://p53.free.fr/index.html, M = mutant *TP53*; W = wild-type *TP*53.)**. (**B**) HGC27 cells were transfected with siRNA targeting SMYD2 or control luciferase (Luc) for the indicated times. Cell proliferation (top) and SMYD2 protein expression (bottom) were compared between these cells. (**C**) Migration and invasion of HGC27 cells transfected with siRNA targeting SMYD2. The graphs on the bottom show mean ± s.d. (bars) of $n=4$. The Mann–Whitney *U*-test was used for statistical analysis. $P<0.05$ is considered statistically significant.

efficiently knocked down 24–72 h after the transient introduction of SMYD2-specific siRNA (siRNA-SMYD2) (Figure 1B) than with the control luciferase siRNA (siRNA-Luc). We measured the proliferation of these siRNA-transfected HGC27 cells. The proliferation of the cells transfected with siRNA-SMYD2 was 51.6% lower than that of cells transfected with control siRNA (siRNA-Luc) 72 h after transfection (Figure 1B).

Suppression of cell migration and invasion by knockdown of SMYD2. Next, Transwell migration and invasion assays were performed to examine the ability of HGC27 cells transfected with siRNA-SMYD2 to move through pores under different conditions. Uncoated membrane was used for migration assays, whereas Matrigel-coated membrane was used for invasion assays. In Figure 1C, the number of cells that migrated into the lower chamber was significantly lower for siRNA-SMYD2-transfected cells than for siRNA-Luc-transfected cells under both conditions, suggesting that SMYD2 may increase the ability of gastric cancer cells to migrate.

Correlation between suppression of cell proliferation by knockdown of SMYD2 and TP53 mutation status. To gain further insight into the potential association of SMYD2 with *TP53* mutation status, we performed cell proliferation assays with the NUGC4 cell line, which has wild-type *TP53*, and the MKN28 cell line, which has mutant *TP53*. In both NUGC4 and MKN28 cell lines, endogenous SMYD2 protein was efficiently knocked down 24–72 h after the transient introduction of SMYD2-specific siRNA (Figure 2A and B, middle panels). An inhibitory effect of knockdown of SMYD2 on cell proliferation was also observed in both cell lines (Figure 2A and B. top panels), suggesting that this inhibitory effect is not associated with *TP53* mutation/expression status. Fluorescence-activated cell sorting analysis demonstrated that transfection of both NUGC4 and MKN28 cell lines with siRNA-SMYD2 resulted in an accumulation of cells in the G0–G1 phase compared with transfection with control siRNA. In addition, p21 protein abundance was also increased at the protein level in siRNA-SMYD2-transfected cells (Figure 2A and B, middle panels), suggesting that the knockdown of SMYD2 directly or indirectly induced the production of p21, which results mainly in G0–G1 arrest.

Immunohistochemical analysis of SMYD2 expression in primary gastric cancer. Because SMYD2 protein was overexpressed in almost all gastric cancer cell lines, we hypothesised that SMYD2 would also be highly expressed in primary gastric cancer tissues and would be involved in carcinogenesis and malignant outcomes. We examined the clinicopathological significance of SMYD2 in primary tumour samples based on the immunohistochemical staining pattern of this protein. SMYD2 protein was observed in both the cytoplasm and the nucleus of cancer cells regardless of tumour differentiation (right and left panels, respectively, of Supplementary Figure S2). We classified 147 gastric cancer samples into positive or negative groups according to the intensity and proportion scores of SMYD2 staining as described in the Materials and Methods section. In primary tumours, SMYD2 protein was not detected in most of the non-tumorous gastric mucosal cell population (intensity score 0) (Figure 3A), and it was weakly detected in the gastric fundic gland. Supplementary Table 1 shows the distribution of patients based on the intensity and proportion scores of SMYD2 immunoreactivity of tumour samples. A group of high expression with the combined scores of >4 was considered immunopositive and is shown in grey in this table. Kaplan–Meier survival estimates showed that SMYD2 immunoreactivity in tumour cells was statistically significantly associated with a worse overall survival based on the intensity and proportion scores (Figure 3C and D, respectively). Based on the combined scores, patients in the high expression group had statistically significantly poorer prognoses than did patients in the low expression group ($P = 0.0073$, log-rank test) (Figure 3B).

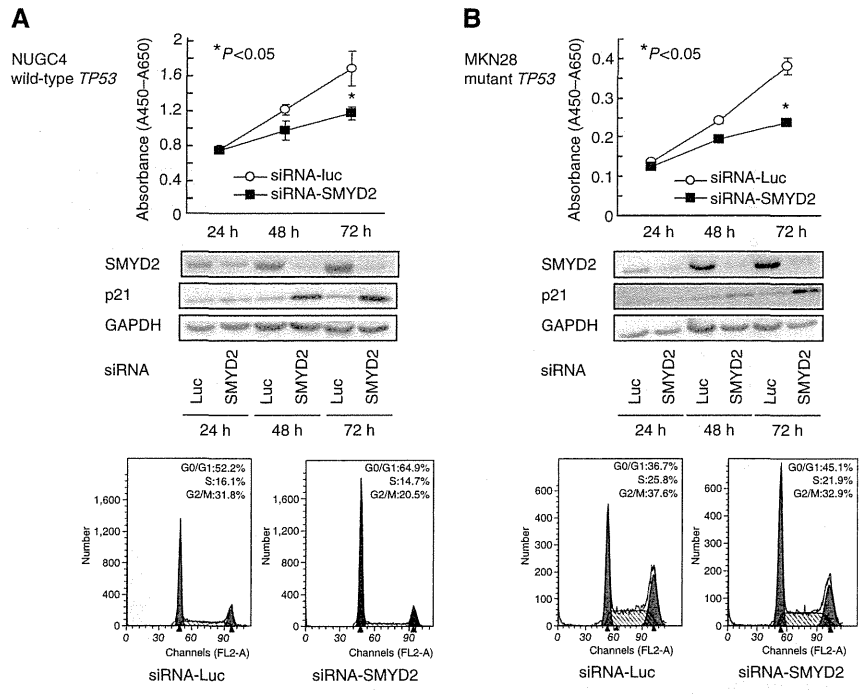

Figure 2. Effects of SMYD2 knockdown by siRNA (siRNA-SMYD2) compared with those of control siRNA (siRNA-Luc) in NUGC4 (wild-type *TP53*, A) and MKN28 (mutant *TP53*, B) cell lines. Top: effects of knocking down endogenous SMYD2 on cell proliferation at the indicated times. Results are mean ± s.d. (bars) for quadruplicate experiments. The Mann–Whitney *U*-test was used for the statistical analysis: $*P<0.05$ *vs* siRNA-Luc-transfected cells. Middle: effects of siRNA-SMYD2 on the abundance of endogenous SMYD2 and p21 proteins 24–72 h after transfection of gastric cancer cell lines. Bottom: representative results of the population in each phase of the cell cycle in gastric cancer cell lines assessed by FACS 72 h after treatment with siRNA.

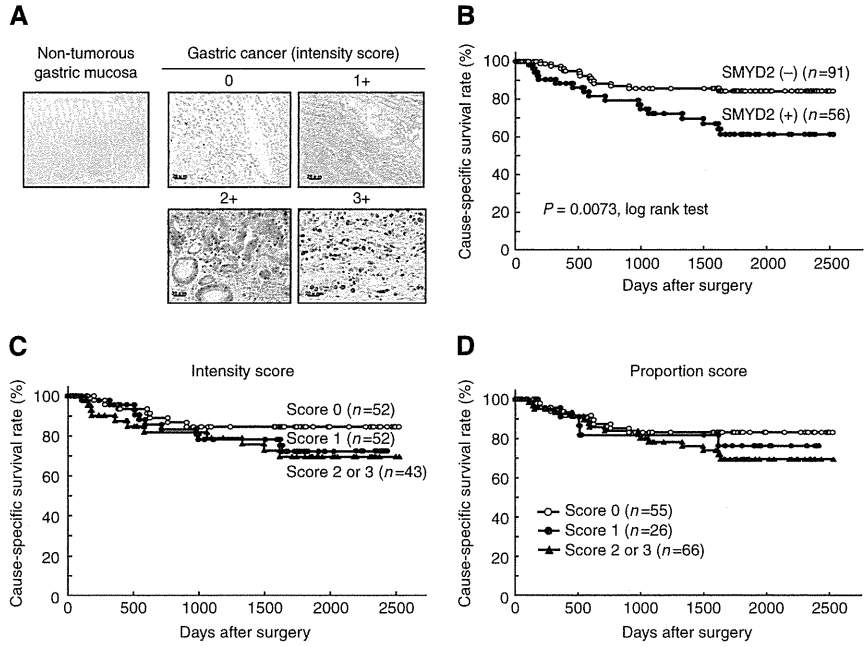

Figure 3. (**A**) Specific immunostaining of SMYD2 protein in a representative primary tumor sample. Based on this result, the intensity scores for SMYD2 staining were determined as follows; 0 = negative, 1 = weak, 2 = moderate, 3 = strong). Scale bar: 20 μm. (**B–D**) Cause-specific survival rates of gastric cancer patients (as determined by Kaplan–Meier plots) depending on the scores of the intensity (**C**), proportion (**D**) and in combination (**B**) based on the immunostaining analysis of their cancers. The log-rank test was used for statistical analysis. $P<0.05$ is considered statistically significant.

Correlation between SMYD2 protein abundance and clinicopathological characteristics in primary gastric cancer. The association between SMYD2 protein abundance and clinicopathological characteristics is summarised in Table 1. SMYD2 protein abundance was statistically significantly associated with larger tumour size, higher incidence of lymphatic invasion and lymph node metastasis, deeper invasion and higher recurrence rate, and tended to be associated with higher incidence of venous invasion.

In the Cox proportional hazard regression model (Table 2), univariate analyses demonstrated that SMYD2 protein abundance, age, location, tumour size, venous and lymphatic invasion, pT category and pN category were statistically significantly associated with cause-specific survival. When data were stratified for multivariate analysis using both the forward and backward stepwise Cox regression procedures, SMYD2 immunoreactivity in tumour cells remained significant, with $P<0.05$ (hazard ratio, 4.26 (1.69–10.72)) for overall survival in all patients, suggesting that SMYD2 immunoreactivity can be an independent predictor of overall survival.

DISCUSSION

Lysine methyltransferases for histone or non-histone proteins have emerged as attractive targets for disease treatments (Kelly *et al*, 2010), and intensive ongoing efforts have been made to clarify their molecular mechanisms and clinical outcomes in cancers (Chang and Hung, 2012; Anglin and Song, 2013; Shankar *et al*, 2013; Sarris *et al*, 2014) and to develop novel drugs for this class of enzymes (Wagner and Jung, 2012). The human SMYD family of lysine methyltransferases is composed of five members (SMYD1–5) (Brown *et al*, 2006), which have various roles in development and cancer. Disruption of SMYD1 leads to perturbed cardiac morphogenesis and embryonic lethality (Gottlieb *et al*, 2002). SMYD3 is associated with cancer cell proliferation, and it is overexpressed in hepatocellular, colorectal and breast carcinomas (Hamamoto *et al*, 2004, 2006). SMYD4, as a potential tumour suppressor, has a critical role in breast carcinogenesis at least partly through inhibiting the expression of PDGFRA (Hu *et al*, 2009). SMYD5, as a negative regulator of inflammatory response genes, is recruited to a subset of TLR4-responsive promoters through its association with NCoR corepressor complexes, where it trimethylates histone H4 K20 (Stender *et al*, 2012).

SMYD2 methylates both histones (H2B, H3 and H4) and non-histone proteins, including the tumour suppressor proteins, p53 and Rb, and the oncogenic protein, PARP1 (Brown *et al*, 2006; Huang *et al*, 2006; Abu-Farha *et al*, 2008; Wu *et al*, 2011). Methylation of K370 of p53 impairs its ability to bind to the promoters of target genes (Chuikov *et al*, 2004; Huang *et al*, 2006). Methylation of Rb at K860 generates an epitope that is selectively recognised by the transcriptional repressor L3MBTL1, providing a mechanism for recruiting L3MBTL1 to the promoters of specific Rb/E2F target genes, thereby repressing their activities (Saddic *et al*, 2010). In addition, SMYD2-dependent methylation of Rb at K810 promotes cell cycle progression of cancer cells (Cho *et al*, 2012). Moreover, methylation of K528 on PARP1 enhances its poly(ADP-ribose) activity in cancer cells (Piao *et al*, 2014). In agreement with these observations, overexpression of SMYD2 is associated with various human malignancies, such as ESCC (Komatsu *et al*, 2009), bladder carcinoma (Cho *et al*, 2012) and paediatric acute lymphoblastic leukaemia (Sakamoto *et al*, 2014), indicating that SMYD2 acts as a cancer-promoting factor. Moreover, SMYD2 is involved in maintaining the self-renewal activity of MLL-AF9-induced acute myeloid leukaemia (Abu-Farha *et al*, 2011) and might be related to the response to chemotherapy in breast cancer (Barros Filho *et al*, 2010). These findings prompted us to determine the clinicopathological and prognostic significance of SMYD2 overexpression activation in primary gastric cancer.

Here, we hypothesised that overexpression/activation of SMYD2 may promote tumour cell proliferation and/or poor survival of gastric cancer patients. To test this hypothesis, we examined the expression status of SMYD2 and clinicopathological and biological

Table 1. Association between clinicopathologic characteristics and SMYD2 expression

		SMYD2 immunoreactivity		
	n	High expression	Low expression	*P*-value[a]
Total	147	56	91	
Sex				
Male	100	41 (73%)	59 (65%)	
Female	47	15 (27%)	32 (35%)	0.2901
Age (years)				
Mean	63 (range:27–89)			
<60	56	23 (41%)	33 (36%)	
⩾60	91	33 (59%)	58 (64%)	0.5036
Location				
Upper	20	10 (18%)	10 (11%)	
Middle	75	23 (41%)	52 (57%)	
Lower	52	23 (41%)	29 (32%)	0.1505
Histopathological grading				
Differentiated	73	32 (57%)	41 (45%)	
Undifferentiated	74	24 (43%)	50 (55%)	0.1546
Tumor size (mm)				
<40	84	26 (46%)	58 (64%)	
⩾40	63	30 (54%)	33 (36%)	**0.0394**
Venous invasion				
0	104	35 (63%)	69 (76%)	
1–3	43	21 (38%)	22 (24%)	0.0846
Lymphatic invasion				
0	80	20 (36%)	60 (66%)	
1–3	67	36 (64%)	31 (34%)	**0.0003**
TNM classification				
pT categories				
pT1	83	24 (43%)	59 (65%)	
pT2/3	34	16 (29%)	18 (20%)	
pT4	30	16 (29%)	14 (15%)	**0.0290**
pN categories				
N0	96	34 (61%)	62 (68%)	
N1/2	28	7 (13%)	21 (23%)	
N3	23	15 (27%)	8 (9%)	**0.0204**
pStage				
I	94	31 (55%)	63 (69%)	
II	16	6 (11%)	10 (11%)	
III	37	19 (34%)	18 (20%)	0.2117
Rucurrence				
Absent	117	39 (70%)	78 (86%)	
Present	30	17 (30%)	13 (14%)	**0.0188**

Abbreviations: SMYD2 = SET and MYND domain-containing protein 2; TNM = tumour-lymph node-metastases. Statistically significant values are in bold.
[a]*P*-values are from χ^2- or Fisher's exact test and were statistically significant at <0.05.

Table 2. Multivariate analysis using the stepwise Cox regression procedures

	Univariate[a]	Multivariate[b]		
Variables	*P*-value	HR	95% CI	*P*-value
Sex				
Male *vs* female	0.9583	2.749	1.134–6.665	**0.0252**
Age				
⩾60 *vs* <60	**0.0391**	5.649	1.992–16.12	**0.0011**
Location				
U *vs* ML	**0.0262**		—	
Histological type				
Undifferentiated *vs* differentiated	0.8468		—	
Tumor size (mm)				
⩾40 *vs* <40	**<0.0001**		—	
Venous invasion				
Positive *vs* negative	**<0.0001**	5.681	2.173–14.92	**0.0004**
Lymphatic invasion				
Positive *vs* negative	**<0.0001**		—	
pT-stage				
T3-4 *vs* T1-2	**<0.0001**	6.493	1.385–27.77	**0.0177**
pN-stage				
N2-3 *vs* N0-1	**<0.0001**	17.54	4.672–66.66	**<0.0001**
SMYD2 expression				
High *vs* low	**0.0073**	4.258	1.690–10.72	**0.0021**

Abbreviations: CI = confidence interval; HR = hazard ratio; ML = middle and lower; SMYD2 = SET and MYND domain-containing protein 2; U = upper. Statistically significant values are in bold.
[a]Kaplan–Meier method, and the statistical significance was determined by log-rank test.
[b]Multivariate survival analysis was performed using Cox's proportional hazard model.

significance of its expression in cell lines and primary tumours of gastric cancer. Consequently, we demonstrated that SMYD2 was overexpressed in 38.1% (56/147) of primary gastric cancers as well as in 71.4% (5/7) of gastric cancer cell lines and this overexpression was an indicator of poor prognosis independent of other prognostic factors. The intensity and proportion of SMYD2 abundance detected by immunohistochemical tissue staining was correlated with the prognosis of gastric cancer patients. In addition, knockdown of SMYD2 suppressed cell proliferation, migration and invasion in the gastric cancer cell lines.

Because p53 and its target molecules, which regulate cell cycle and trigger apoptosis after DNA damage, have a key role in a wide range of human cancers, including gastric cancer (Caelles *et al*, 1994; Fenoglio-Preiser *et al*, 2003; Bellini *et al*, 2012), it is possible that overexpression of SMYD2 promotes cell proliferation and/or survival by inhibiting the transactivation activity of p53. Based on this hypothesis, we performed siRNA-mediated knockdown of SMYD2 which had an inhibitory effect on cell proliferation in gastric cancer cells, which overexpress SMYD2, more or less independently of *TP53* mutation status. Cell cycle analysis by FACS demonstrated that the inhibition of cell proliferation caused by SMYD2 knockdown occurred mainly because of G0–G1 arrest. This result was supported by our finding that increases in expression of p21 protein were caused by SMYD2 knockdown, although it remains unknown how SMYD2 inhibits the transcription of p21 in a p53-independent manner. These results are consistent with our previous findings of an inhibitory effect on cell proliferation upon SMYD2 knockdown in *TP53*-mutant ESCC cells and p53-null SaOS2 osteosarcoma cells (Komatsu *et al*, 2009).

More recently, the first SMYD2 inhibitor AZ505 (AstraZeneca, London, UK) was developed, which is highly selective and shows an activity at submicromolar concentrations *in vitro* (Ferguson *et al*, 2011). The IC50 of AZ505 for SMYD2 is 0.12 μM, which is >600-fold greater than the IC50s of AZ505 for other histone methyltransferases, such as SMYD3 (IC50 > 83.3 μM), DOT1L (IC50 > 83.3 μM) and EZH2 (IC50 > 83.3 μM), although functional data on the consequences of chemical inhibition of SMYD2 have not been reported (Ferguson *et al*, 2011). Moreover, anticancer effects of similar lysine methyltransferase inhibitors have already been proven in animals (Schenk *et al*, 2012; Willmann *et al*, 2012). Overexpression of SMYD2 protein was detected in 38.1% of patients with gastric cancer and 76.5% of patients with ESCC (Komatsu *et al*, 2009). These findings suggest that the SMYD2 inhibitor AZ505 may be a key molecule for the treatment of patients with these cancers. This drug is currently being evaluated *in vitro* and *vivo*.

In conclusion, this is the first report to show that SMYD2 has a crucial oncogenic role and is a potential therapeutic target in gastric cancer. We demonstrated the frequent overexpression of SMYD2 protein and its prognostic value in patients with gastric cancer. Although studies of larger cohorts are needed to validate these findings before moving to clinical settings, our results provide evidence that SMYD2 could be a pivotal molecular marker to determine malignant properties of gastric cancer cells and that it could be a target for molecular therapy in patients with this cancer.

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