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Overexpression of SMYD2 relates to tumor cell proliferation and malignant outcome of esophageal squamous cell carcinoma

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Although we have identified two putative targets, *ATF3* and *CENPF*, for a frequently gained/amplified region around 1q32–q41 in esophageal squamous cell carcinoma (ESCC), it is possible that other amplification targets remain to be identified. In this study, we tested whether *SET* and *MYND domain-containing protein 2* (*SMYD2*), located between those two genes and encoding a lysine methyltransferase for histone H3K36 and p53K370 that regulates transcription and inhibits transactivation activity, respectively, acts as a cancer-promoting gene through activation/overexpression in ESCC. Frequent overexpression of SMYD2 messenger RNA and protein was observed in KYSE150 cells with remarkable amplification at 1q32–41.1 and other ESCC cell lines (11/43 lines, 25.6%). Overexpression of SMYD2 protein was frequently detected in primary tumor samples of ESCC (117/153 cases, 76.5%) as well and significantly correlated with gender, venous invasion, the pT category in the tumor–lymph node–metastases classification and status of recurrence. Patients with SMYD2-overexpressing tumors had a worse overall rate of survival than those with non-expressing tumors, and SMYD2 positivity was independently associated with a worse outcome in the multivariate analysis. Knockdown of SMYD2 expression inhibited and ectopic overexpression of SMYD2 promoted the proliferation of ESCC cells in a *TP53* mutation-independent but SMYD2 expression-dependent manner. These findings suggest that SMYD2 plays an important role in tumor cell proliferation through its activation/overexpression and highlight its usefulness as a prognosticator and potential therapeutic target in ESCC.

Abbreviations: BAC, bacterial artificial chromosome; ESCC, esophageal squamous cell carcinoma; FACS, fluorescence-activated cell sorting; MD, methylation defective; mRNA, messenger RNA; RT–PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA; SMYD2, SET and MYND domain-containing protein 2; TNM, tumor–lymph node–metastases.

Introduction

Esophageal cancer is the eighth most common cancer in the world (1) and esophageal squamous cell carcinoma (ESCC) accounts for ~90% of esophageal carcinomas diagnosed in Asian countries. Although surgical techniques and perioperative management have progressed, ESCC remains one of the most aggressive carcinomas of the gastrointestinal tract. Since finding molecular targets for ESCC treatment might help to improve the survival of patients with this lethal disease, studies have attempted to identify biological factors involved in the malignant potential of ESCC. However, few genes have been demonstrated to be associated with biological or pathological features of ESCC, suggesting that novel genes associated with the progression of ESCC need to be identified.

Gene amplification and overexpression are among the major genomic aberrations involved in the pathogenesis of ESCC (2). To identify genes activated and subsequently involved in promoting ESCC, we have searched regions with amplification/copy number gains and their targets using chromosome or bacterial artificial chromosome (BAC) array-based comparative genomic hybridization (3–5), although it is possible that the amplification of identified genes is a rare event and other mechanisms contribute to their transcriptional overexpression/functional activation. Among this program, we firstly identified a copy number gain at 1q32, reported as the second most common gain in various solid tumors (6) in ESCC through a chromosomal comparative genomic hybridization-assisted approach (3). Although we reported *ATF3* and *CENPF* as possible targets for 1q32 amplification (3), progress in studies of the human genome prompted us to search for additional target genes located around the amplified region in ESCC because amplicons often contain several syntenic genes providing a selective growth advantage to the cell, such as amplifications of the 11q13 and 12q13 loci (7,8), resulting in their stabilization in the cancer cell's genome.

Recently, SET and MYND domain-containing protein 2 (SMYD2) was identified as a lysine methyltransferase for histone H3K36 and K370 of p53, acting to regulate transcription and inhibit p53's transactivation activity, respectively (9,10). Since p53 and its target molecules, which regulate the cell cycle and trigger apoptosis following DNA damage, play a key role in a wide range of human cancers including ESCC (11–13), it is possible that SMYD2 promotes cell proliferation and/or survival through its overexpression/activation-induced inhibition of p53's transactivation activity. According to the human genome databases (http://www.ncbi.nlm.nih.gov/, http://genome.ucsc.edu/), the *SMYD2* gene is located between *ATF3* (1q32) and *CENPF* (1q41.1), making it a strong candidate for an additional target for 1q32–q41.1 amplification and prompting us to determine the biological and clinicopathological significance of SMYD2 overexpression/activation in ESCC.

In the study presented here, we examined (i) the expression status of SMYD2 in a panel of ESCC cell lines; (ii) the expression status of the SMYD2 protein and its clinicopathological significance in surgical specimens of ESCC and (iii) the proliferation-promoting effects of SMYD2 on ESCC cells to demonstrate whether SMYD2 expression is useful as a novel prognosticator and SMYD2 is a potential therapeutic target in patients with this lethal disease.

Materials and methods

Cell lines and primary tissue samples

A total of 43 ESCC cell lines were used, of which 31 belonged to the KYSE series established from surgically resected tumors (14) and 12 were TE series lines provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. All ESCC cells were maintained as described elsewhere. The status of the *TP53* gene (exons 5–8) mutation was determined as described previously (15).

Normal esophageal RNA purchased from Ambion (Austin, TX) and RNA isolated from non-tumorous esophageal mucosa, which were obtained during surgery from four ESCC patients undergoing tumor resection at the Tokyo Medical and Dental University Hospital (Tokyo, Japan), were used as controls for quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR). Additional 153 primary tumor samples of ESCC had been obtained from ESCC patients treated at the National Defense Medical College Hospital (Saitama, Japan) between 1981 and 1995 and 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 tumor–lymph node–metastases (TNM) classification (16). In this series, all the M_1 tumors had distant lymph node metastases and there was no organ metastasis. The median follow-up period for the surviving patients was 32.5 months (ranging from 1 to 124 months).

Fluorescence in situ hybridization

Metaphase chromosomes were prepared from normal male lymphocytes and from each ESCC cell line. FISH analyses were performed as described previously (17), using BACs located around the region of interest as probes with the control probe. Precise localization of each BAC was confirmed using normal metaphase chromosomes.

Quantitative real-time RT–PCR

Single-stranded complementary DNAs generated from total RNA were amplified with primers specific for each gene. Levels of messenger RNA (mRNA) expression were measured with a quantitative real-time fluorescence detection method (ABI PRISM 7500 Sequence Detection System; Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays (Hs00220210_m1, Hs00193201_m1 and Hs00231069_m1 for *SMYD2*, *CENPF* and *ATF3*, respectively; Applied Biosystems) according to the manufacturer's instruction. Gene expression values are expressed as ratios between the genes of interest and an internal reference gene (Hs99999903_m1 for *beta-actin*; Applied Biosystems) that provides a normalization factor for the amount of RNA isolated from a specimen and subsequently normalized with the value in the controls (relative expression level). Each assay was performed in duplicate for each sample.

Western blotting

Anti-SMYD2 rabbit polyclonal antibody was raised against a 14-amino acid peptide from human SMYD2 (HPYISEIKQEIESH; Operon Biotechnology, Tokyo, Japan) and purified through an affinity column. Anti-FLAG tag and anti-β-actin antibodies were purchased from Sigma (St Louis, MO), and anti-p53 (DO7) and anti-p21 antibodies were from Novocastra Laboratories (Newcastle upon Tyne, UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Cells were lysed in Tris buffer (50 mmol/l, pH 7.5) containing 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP-40, 10% glycerol, 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 2 mmol/l Na_2VO_3 and a protease inhibitor cocktail (Roche, Tokyo, Japan), and lysates were analyzed as described elsewhere (17).

Immunohistochemistry

Tumor samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, sectioned into 4 μm-thick slices and subjected to immunohistochemical staining of SMYD2 and p53 protein with the avidin–biotin–peroxidase method as described by Naoi *et al.* (18). 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 citrate buffer (pH 6.0) at 95°C for 40 min. After treatment with Block Ace (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 30 min at room temperature, the sections were incubated at 4°C overnight with an anti-SMYD2 (1:100) or anti-p53 (DO7, Novocastra Laboratories; 1:50) antibody. The avidin–biotin–peroxidase complex system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was used for color development with diaminobenzidine tetrahydrochloride. The slides were counterstained with Mayer's hematoxylin. A formalin-fixed ESCC cell line overexpressing SMYD2 (KYSE150) or p53 (KYSE1170), in which >50% of cells showed staining of each protein, was used as a positive control, whereas a formalin-fixed ESCC cell line with low expression of SMYD2 (HLE, liver cancer cell line) or p53 (TE-14, non-sense mutation) was included as a negative control. For scoring SMYD2 expression, the intensity (intensity score: 0 = negative, 1 = weak, 2 = moderate, 3 = strong) and percentage of the total cell population (proportion score: $0 < 10\%$, $10\% \leq 1 < 33.3\%$, $33\% \leq 2 < 66.7\%$, $66.7\% \leq 3$) that expressed SMYD2 was evaluated for each case. Expression of SMYD2 was graded as either positive (a total of intensity plus proportion scores ≥ 1) or negative (no staining, a total of intensity plus proportion scores = 0) using high-power (×200) microscopy. For p53, a distinct nuclear immunoreaction in ≥10% of the cancer cells was judged positive.

Loss of function by small interfering RNA and cell proliferation analysis

Loss-of-function screening was done by using small interfering RNAs (siRNAs) targeting the *SMYD2* (SMARTpool #M-020291-00; Dharmacon, Lafayette, CO) and control *luciferase* (*Luc*, 5′-CGUACGCGGAAUACUUCGA-3′; Sigma, Tokyo, Japan). Each siRNA (10 nmol/l) was transfected into ESCC cells using Lipofectamine RNAiMAX (Invitrogen, St Louis, MO) according to the manufacturer's instructions. The knockdown of a target gene was confirmed by quantitative real-time RT–PCR and western blotting. The numbers of viable cells at various time points after transfection were assessed by a colorimetric water-soluble tetrazolium salt assay as described elsewhere (17). The cell cycle was evaluated 72 h after transfection by fluorescence-activated cell sorting (FACS) as described elsewhere (15).

Colony-formation assay

Plasmids expressing FLAG-tagged wild-type SMYD2 (pCMV-3Tag1A-SMYD2) and methylation defective (MD) mutant of SMYD2 (pCMV-3Tag1A-SMYD2MD) were obtained by cloning the full coding sequences for wild-type SMYD2 and the MD mutant form, in which Histidine 207 was mutagenized to Alanine (10), respectively, in-frame into the vector pCMV-3Tag1A (Stratagene, La Jolla, CA) along with the FLAG epitope. pCMV-3Tag1A-SMYD2, pCMV-3Tag1A-SMYD2MD or the empty vector (pCMV-3Tag4A-mock) as a control was introduced into ESCC cells as described previously (17). The expression of SMYD2 protein in transfected cells was confirmed by western blotting. After 3 weeks of incubation with appropriate concentrations of G418, cells were fixed with 70% ethanol and stained with crystal violet.

Statistical analysis

The clinicopathological variables pertaining to the corresponding patients were analyzed for statistical significance by the χ^2 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 tested 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. For multiple group comparisons, one-way analysis of variance, followed by the Scheffé's post hoc test, was used. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

Amplification and overexpression of SMYD2 in ESCC cell lines

As the KYSE150 cell line was shown to harbor the most remarkable amplification around 1q32 as a homozygously staining pattern in our previous study (3), we determined whether *SMYD2* is located within this amplicon. As expected based on information obtained from human genome databases (http://www.ncbi.nlm.nih.gov/, http://genome.ucsc.edu/), a FISH analysis using BAC clone containing the *SMYD2* gene (RP11-74E6) at 1q41 showed a clear homozygously staining pattern that indicates remarkable amplification in KYSE150 cells (Figure 1A). We then performed a quantitative RT–PCR analysis to test whether *SMYD2* is overexpressed in KYSE150 cells and another 42 ESCC lines compared with normal esophagus. *SMYD2* mRNA overexpression was observed in the KYSE150 cells as well as other ESCC lines (11/43 lines, 25.6%) compared with normal esophagus ($n = 5$, mean + 2SD = 1.42), suggesting this gene to be a target for activation in ESCC cell lines (Figure 1B). Among previously reported possible target genes (3), *CENPF* was also overexpressed in KYSE150 cells as well as other ESCC lines compared with normal esophagus, whereas *ATF3* was highly expressed in KYSE150 cells compared with the other cells without amplification but not overexpressed in most ESCC cell lines including KYSE150 compared with normal esophagus (supplementary Figure S1A and B is available at *Carcinogenesis* Online). mRNA expression pattern seems to be different among *SMYD2*, *CENPF* and *ATF3*, suggesting that transcription of these genes to be differentially regulated by various mechanisms other than genomic copy number. SMYD2 protein

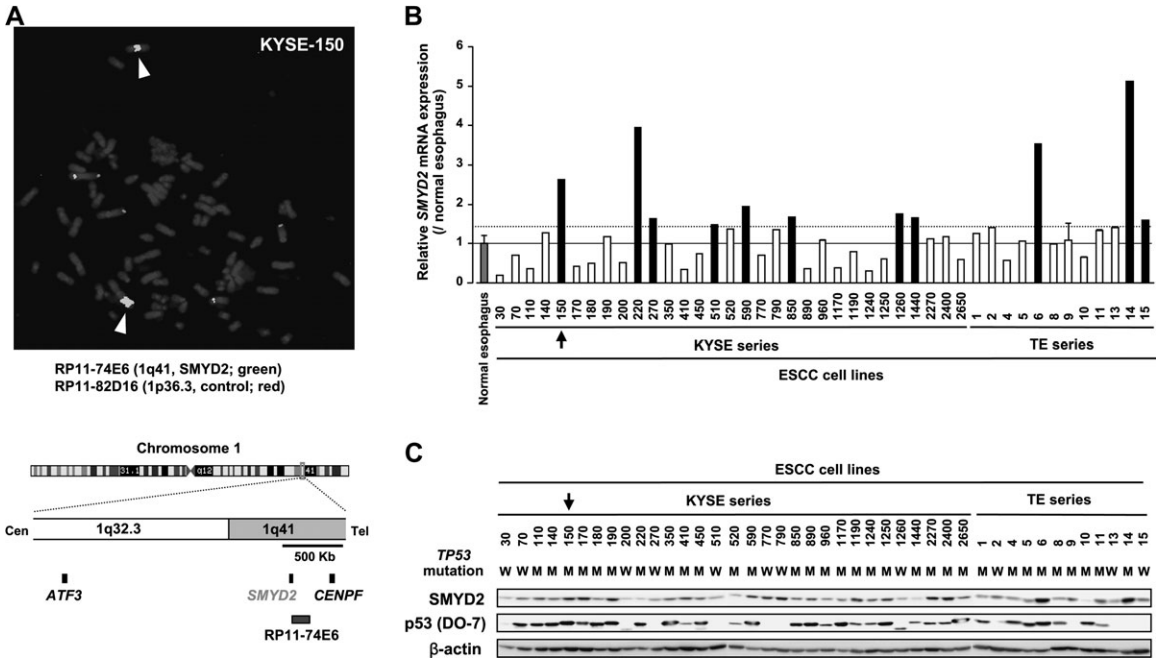

Fig. 1. (**A**) Upper, representative images of fluorescence *in situ* hybridization (FISH) with the BAC clone RP11-74E6 containing part of the SMYD2 gene at 1q41 (green signal) and the control BAC clone RP11-82D16 at 1p36 (red signals) hybridized to metaphase chromosomes from KYSE150 cells, which showed a remarkably increased copy number of *SMYD2* with a homogeneously staining region pattern (arrowheads). Precise localization of each BAC was confirmed using normal metaphase chromosomes. Lower, location of the *ATF3*, *CENPF* and *SMYD2* genes around 1q32.3–1q41 and BAC RP11-74E6 used for FISH. (**B**) Level of SMYD2 mRNA determined by quantitative real-time RT–PCR in normal esophagus and a panel of ESCC cell lines. Results are shown with means ± SD (bars) relative to the value for normal esophagus ($n = 5$, gray bar). Black bars represent cell lines, in which a remarkable upregulation of SMYD2 mRNA expression (>mean + 2SD of normal esophagus, dotted line) was observed compared with that in normal esophagus. Arrow indicates KYSE150 cells with remarkable amplification of *SMYD2*. (**C**) Expression of the SMYD2 and p53 proteins in a panel of ESCC cell lines. The mutation status of *TP53* exons 5–8 determined by direct sequencing in each ESCC cell line is indicated by W (wild-type) or M (mutation). Note that, among *TP53*-mutated ESCC cell lines, TE-9 and TE-14 cells have a frameshift mutation and non-sense mutation, respectively. Arrow indicates KYSE150 cells with remarkable amplification of *SMYD2*.

expression seems to be correlated with the mRNA expression in ESCC cell lines by western blotting using SMYD2-specific antibody (Figure 1B and C and supplementary Figure S1C is available at *Carcinogenesis* Online), although there are at least some clear exceptions, *e.g.* KYSE170 and KYSE220 cell lines.

Since repression of p53 activity through SMYD2-mediated methylation at K370 was reported recently (10), we examined the status of *TP53* mutation by directly sequencing exons 5–8 and from p53 protein expression, which is positively associated with *TP53* mutations (19,20), by western blotting in a panel of ESCC cell lines. Although wild-type p53 is expected to be a more suitable substrate than mutant p53 for SMYD2, the levels of SMYD2 mRNA and protein were not correlated with the mutation status and protein level of p53 (Figure 1B and C).

Immunohistochemical analysis of SMYD2 expression in primary tumors of ESCC

Since SMYD2 mRNA and protein were overexpressed in cells with remarkable amplification of this gene (KYSE150) as well as those without remarkable amplification, *SMYD2* amplification is assumed to be only part of the mechanism to induce the overexpression/activation. Therefore, we examined the clinicopathological significance of SMYD2 expression in primary tumor samples of ESCC based on the immunohistochemical staining pattern of this protein. Specific immunostaining of SMYD2 protein with anti-SMYD2 antibody was confirmed using cell lines showing high and low levels of the protein (supplementary Figure S2B is available at *Carcinogenesis* Online). We classified 153 ESCC tumors into positive and negative groups according to the intensity and proportion of SMYD2 staining among tumor cells as described in Materials and Methods. In primary cases, SMYD2 protein expression was negative (intensity score = 0) in most of the non-tumorous esophageal epithelial cell population (intensity plus proportion scores = 0, Figure 2A). On the other hand, the tumors frequently showed SMYD2 immunoreactivity (positive = 117/153 cases, 76.5%; Figure 2A, Table I). In addition, the tumors showed heterogenous expression of SMYD2 protein: *e.g.* immunoreactivity was sometimes greater in tumor cells in the invasive front than in those at the center of the tumor or in cells at the outer edge compared with those at the center of cancer peals (Figure 2A).

Association between level of SMYD2 protein and clinicopathological characteristics in primary cases of ESCC

The relationship between the expression of SMYD2 protein and the clinicopathological characteristics is summarized in Table I. The protein expression of SMYD2 was significantly associated with gender, venous invasion, pT category (depth of tumor invasion) in the TNM classification and the status of recurrence: ESCC tended to show SMYD2 expression in males ($P = 0.0338$), tumors with venous invasion (v1–3, $P = 0.0042$), tumors with a higher pT category ($P = 0.0203$) and cases involving recurrence ($P = 0.0049$). However, the SMYD2 protein expression in each tumor was not associated with other characteristics including lymphatic invasion and pN category, pM category and stage in the TNM classification.

Kaplan–Meier survival estimates (Figure 2A) showed that SMYD2 immunoreactivity in tumor cells was significantly associated with a worse overall survival in all cases ($P < 0.005$, log-rank test). In the Cox proportional hazard regression model (Table II), univariate analyses demonstrated that SMYD2 protein expression, gender, lymphatic invasion, venous invasion, pT category, pN category, pM category and stage of TNM classification were significantly associated with overall survival, whereas histological grading and p53 immunoreactivity were not. When the data were stratified for the multivariate

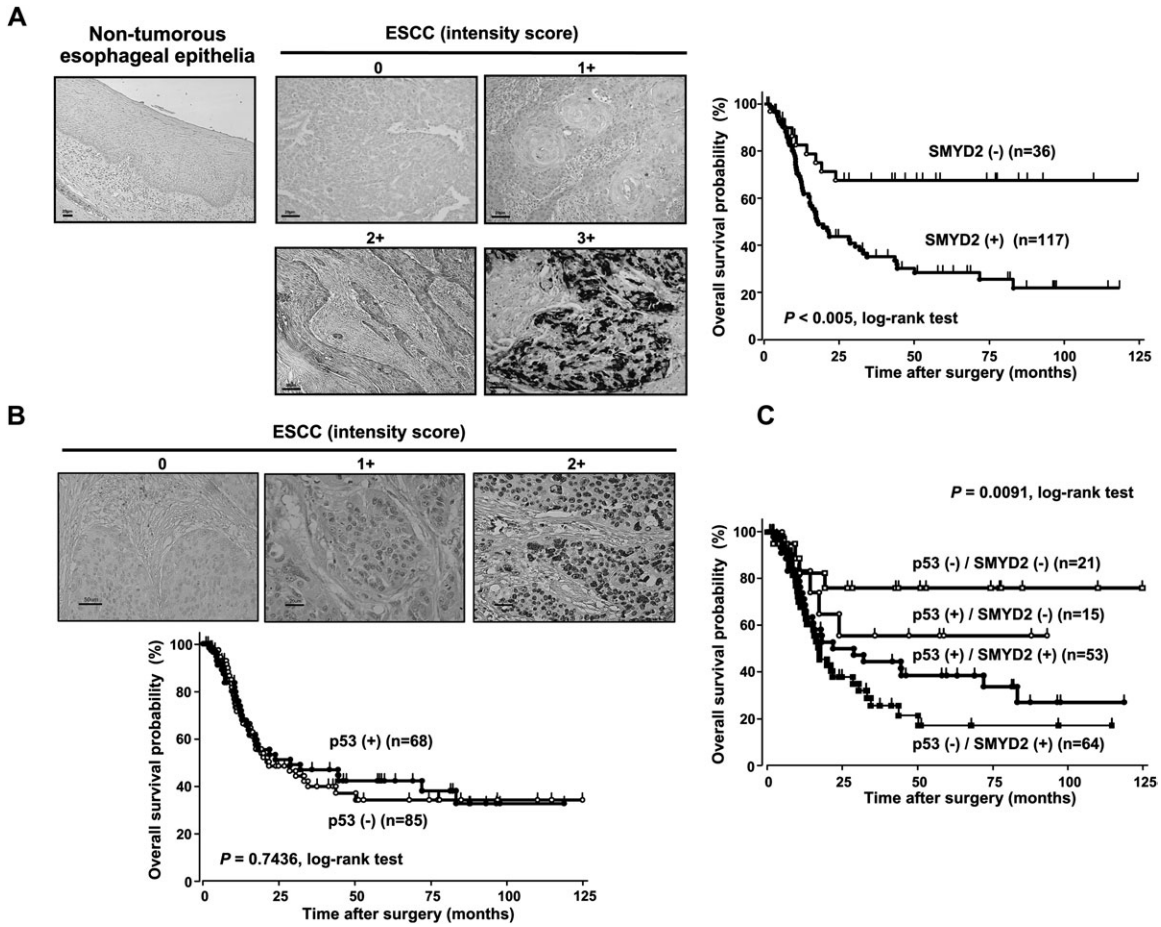

Fig. 2. (**A**) Left, representative results of immunohistochemical staining of SMYD2 protein in non-tumorous human esophageal epithelial and primary tumors of ESCC. SMYD2 expression was not observed in non-tumorous esophageal epithelial cells (original magnification, ×200). In primary tumors of ESCC, no (intensity score = 0), weak (1), moderate (2) or strong (3) staining of SMYD2 was observed (original magnification, ×400). Bars, 20 μm. Right, Kaplan–Meier curves for overall survival rates of patients at all stages according to the expression of SMYD2. SMYD2 immunoreactivity in tumor cells was significantly associated with a worse overall survival at all stages ($P < 0.0005$). (**B**) Upper, representative results of immunohistochemical staining of p53 protein in primary tumors of ESCC. p53 expression was not observed in non-tumorous esophageal epithelial cells. In primary tumors of ESCC, no (intensity score = 0), weak (1) or strong (2) staining of p53 was observed (original magnification, ×400). Bars, 20 μm. Lower, Kaplan–Meier curves for overall survival rates of patients at all stages according to the expression of p53. There was no difference (NS) in survival between the patients positive and negative for p53. (**C**) Postoperative overall survival curves according to a combination of the expression of SMYD2 and p53. A significant difference in survival was observed among the patients positive (+) or negative (−) for p53 and SMYD2 ($P = 0.0091$, log-rank test). Notably, in the p53-negative group, the patients with SMYD2 expression had a dramatically worse outcome than those with no SMYD2 expression.

analysis using both the forward and backward stepwise Cox regression procedures, SMYD2 immunoreactivity in tumor cells remained significant at $P = 0.0025$ (hazard ratio, 2.849) for overall survival in all patients, suggesting the immunoreactivity to be an independent predictor of overall survival. The multivariate analysis using the stepwise Cox regression procedure revealed that tumor stage ($P < 0.0001$) and gender ($P = 0.0265$) were also independently selected predictive factors for overall survival.

Relationship between protein expression of SMYD2 and p53 in primary cases of ESCC

In ESCC cell lines, neither *TP53* mutation nor p53 protein expression was correlated with SMYD2 expression, suggesting that p53 is unlikely to be a primary target for SMYD2 in ESCC. To test this hypothesis in primary cases of ESCC, we examined the relationship between the protein expression of SMYD2 and p53 in primary tumor cells. Since a good correlation is known to exist between p53 protein accumulation (p53 immunopositivity) and the presence of *TP53* gene mutations, especially missense mutations (19,20), in ESCC, we used immunohistochemistry to evaluate *TP53* mutation status. Of 153 cases of ESCC, tumors showing positive p53 immunoreactivity were detected in 74 cases (Figure 2B, 48.4%). No significant correlations were found between the p53 and SMYD2 immunohistochemical staining patterns ($P = 0.7013$, Table I). Kaplan–Meier survival estimates showed that the expression of p53 was not associated with overall survival ($P = 0.7436$, log-rank test; Figure 2B). In both the p53-negative and p53-positive groups, however, the overall survival rate tended to be worse in patients with SMYD2 expression than in those without ($P = 0.0091$, log-rank test in four groups; Figure 2C). Notably, a significant difference was found between patients positive and negative for SMYD2 in the p53-negative group: SMYD2-positive patients had a worse overall survival than SMYD2-negative patients ($P = 0.0015$, log-rank test). On the other hand, no significant difference was found between patients positive and negative for SMYD2 in the p53-positive group ($P = 0.2976$, log-rank test).

Suppression of cell proliferation by downregulation of SMYD2 expression and proliferation promotion by ectopic SMYD2 overexpression in ESCC cell lines

To gain further insight into the potential role of SMYD2 as an oncogene whose overexpression could be associated with esophageal carcinogenesis, we first performed a cell proliferation assay using siRNA

Table I. Association between clinicopathological characteristics and SMYD2 expression

	n	SMYD2 immunoreactivity		*P* value[a]
		Positive (%)	Negative (%)	
Total	153	117 (76.5)	36 (23.5)	
Gender				
Male	128	102 (79.7)	26 (20.3)	**0.0338**
Female	25	15 (60.0)	10 (40.0)	
Age (years)				
Mean	63.7			
≥60	102	76 (74.5)	26 (25.5)	0.4187
<60	51	41 (80.4)	10 (19.6)	
Location[b]				
Upper	25	21 (84.0)	4 (16.0)	0.2366
Middle	79	56 (70.9)	23 (29.1)	
Lower	49	40 (81.6)	9 (18.4)	
Histopathological grading				
Well moderately differentiated	137	107 (78.1)	30 (21.9)	0.2797
Poorly differentiated	16	10 (62.5)	6 (37.5)	
Venous invasion				
0	40	24 (60.0)	16 (40.0)	**0.0042**
1–3	113	93 (82.3)	20 (17.7)	
Lymphatic invasion				
0	18	11 (61.1)	7 (38.9)	0.1803
1–3	135	106 (78.5)	29 (21.5)	
TNM classification				
pT categories				
pT1	17	11 (64.7)	6 (35.3)	**0.0208**
pT2/3	97	70 (72.2)	27 (27.8)	
pT4	39	36 (92.3)	3 (7.7)	
pN categories				
0	34	26 (76.5)	8 (23.5)	0.8186
1	119	91 (76.5)	28 (23.5)	
pM categories				
0	126	97 (77.0)	29 (23.0)	0.9413
1	27	20 (74.1)	7 (25.9)	
pStage				
I	7	4 (57.1)	3 (42.9)	0.6702
II	37	29 (78.4)	8 (21.6)	
III	78	60 (76.9)	18 (23.1)	
IV	31	24 (77.4)	7 (22.6)	
Recurrence				
Absent	77	51 (66.2)	26 (33.8)	**0.0049**
Present	76	66 (86.8)	10 (13.2)	
p53 immunoreactivity				
Negative	79	64 (81.0)	15 (19.0)	0.7013
Positive	74	53 (71.6)	21 (28.4)	

Statistically significant values are in boldface type.
[a]*P* values are from χ^2 or Fisher's exact test and were statistically significant at <0.05.
[b]Upper, cervical + upper thoracic esophagus; Middle, mid-thoracic esophagus; lower, lower thoracic + abdominal esophagus.

Table II. Cox proportional hazard regression analysis for overall survival

Factor	Univariate			Multivariate[a]
	Hazard ratio	95% Confidence interval	*P* value[b]	*P* value[b]
Gender				
Male versus Female	2.03	1.006–4.115	**0.0481**	**0.0265**
Age (years)				
≥60 versus <60	0.81	0.497–1.302	0.376	X
Histopathological Grading				
Poor versus well-moderate	1.12	0.537–2.351	0.7575	X
Venous invasion				
1–3 versus 0	2.45	1.368–4.367	**0.0025**	X
Lymphatic invasion				
1–3 versus 0	2.79	1.116–6.944	**0.0282**	X
TNM classification				
pT categories				
pT2–4 versus pT1	4.51	1.410–14.286	**0.011**	X
pN categories				
pN1 versus pN0	3.24	1.546–6.803	**0.0019**	X
pM categories[c]				
pM1 versus pM0	1.9	1.049–3.425	**0.034**	X
pStage				
III + IV versus I + II	3.01	1.667–5.435	**0.0003**	**<0.0001**
SMYD2 expression[d]				
Positive versus negative	2.85	1.410–5.780	**0.0035**	**0.0025**
p53 immunoreactivity				
Positive versus negative	0.92	0.572–1.490	0.7438	X

Statistically significant values are in boldface type.
[a]Forward and backward stepwise analyses were used for multivariate analysis.
[b]*P* values are from two-sided tests and were statistically significant at <0.05.
[c]All the M1 tumors had distant lymph node metastases but none had organ metastases.
[d]SMYD2 expression was evaluated by immunohistochemical analysis as described in Materials and Methods.

specific to SMYD2 to investigate whether knockdown of SMYD2 expression would suppress proliferation of ESCC cells showing amplification and/or overexpression of the gene. In the KYSE150 cell line, the expression of SMYD2 mRNA and protein was efficiently knocked down 24–72 h after the transient introduction of a *SMYD2*-specific siRNA (siRNA-SMYD2) compared with a control siRNA (siRNA-Luc, Figure 3A). The proliferation of KYSE150 cells was decreased after the knockdown of endogenous SMYD2 expression (Figure 3A). In a FACS analysis of KYSE150, siRNA-SMYD2 treatment resulted in an accumulation of cells in G_0–G_1 phase and a slight increase in sub-G_1 phase cells compared with control siRNA-treated counterparts (Figure 3A). In addition, p21 expression was induced at both the mRNA and protein levels after treatment with siRNA-SMYD2 (Figure 3A), suggesting that the downregulation of SMYD2 expression directly or indirectly induced transcription of p21, resulting mainly in G_0–G_1 arrest with cell death in this cell line.

A proliferation inhibitory effect of SMYD2's downregulation by siRNA treatment was also observed in KYSE790 cells highly expressing SMYD2 and having wild-type *TP53*, but not observed in KYSE200 and KYSE220 cells with low levels of SMYD2 and having wild-type and mutated *TP53*, respectively (Figure 3A). p21 protein was also detected in KYSE790 cells, but not in KYSE200 or KYSE220 cells, after the knockdown of SMYD2 (Figure 3A). In addition, an FACS analysis demonstrated no alteration in each population on the knockdown of SMYD2 in KYSE200 and KYSE220 cells (Figure 3A), suggesting that the proliferation inhibitory effect of the knockdown to be associated not with p53 mutation/expression status in cell lines but with SMYD2 expression levels. Consistent effect of SMYD2's downregulation by siRNA treatment on cell proliferation of the ESCC cell lines was confirmed by various siRNA reagents (supplementary Figure S3 is available at *Carcinogenesis* Online), suggesting the effect of SMYD2 knockdown by siRNAs on cell proliferation is not a false phenotype due to off-target effects of siRNA reagents.

To examine the proliferation-promoting effect of the ectopic overexpression of SMYD2 in ESCC cells, we carried out a colony-formation assay by transiently transfecting with expression constructs of *SMYD2*, the KYSE200, KYSE510 and TE-10 cell lines which exhibit relatively low levels of SMYD2 (Figure 1C). Ectopic expression of wild-type and MD-mutant SMYD2 with a FLAG-tag in these cell lines was verified by western blotting using an epitope tag-specific antibody (Figure 3B). Wild-type SMYD2 produced remarkably more colonies than did the empty plasmid and MD-mutant form

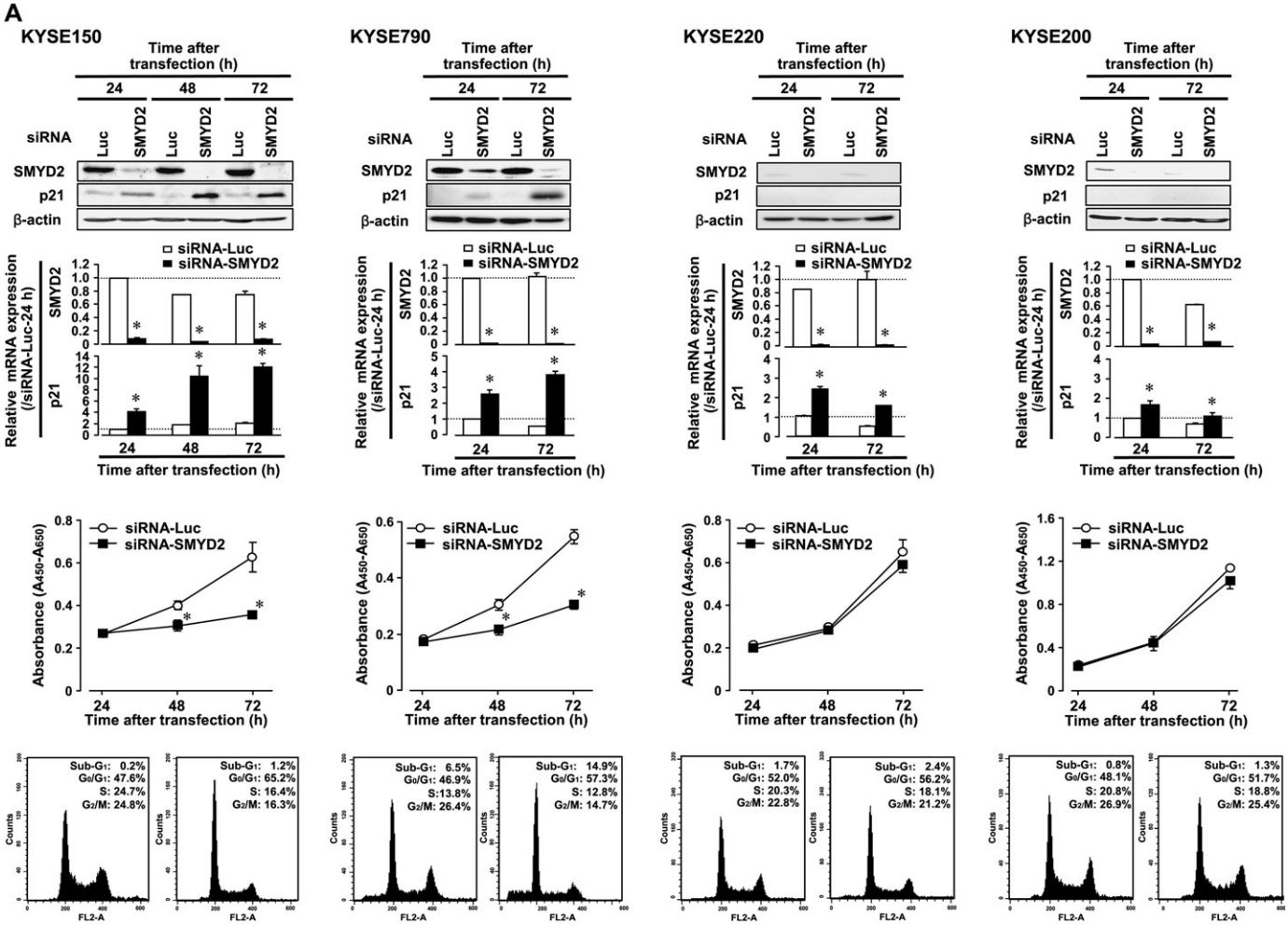

Fig. 3. (**A**) Effects of downregulation of SMYD2 expression caused by transfection of *SMYD2* siRNA (siRNA-SMYD2) compared with control siRNA for *luciferase* (siRNA-Luc) in KYSE150 (high SMYD2, mutant TP53), KYSE790 (high SMYD2, wild-type TP53), KYSE220 (low SMYD2, mutant TP53) and KYSE200 (low SMYD2, wild-type TP53) cell lines. Upper, the effect of knocking down endogenous SMYD2 expression on protein and/or mRNA levels of SMYD2 and p21 24–72 h after transfection in ESCC cell lines. Middle, the effects of knocking down endogenous SMYD2 expression with siRNA-SMYD2 in ESCC cell lines on cell proliferation determined by the water-soluble tetrazolium salt assay at the indicated times compared with the siRNA-Luc-transfected counterparts. Results are shown with means ± SD (bars) for quadruplicate experiments. The Mann–Whitney *U*-test was used for the statistical analysis: $^{*}P < 0.05$ versus siRNA-Luc-transfected counterpart. Lower, representative results of the population in each phase of the cell cycle in ESCC cell lines assessed by FACS 72 h after treatment with siRNA. (**B**) Colony-formation assays using the KYSE200, KYSE510 and TE-10 cell lines. Cells with relatively weak SMYD2 expression were transiently transfected with FLAG-tagged constructs containing empty vector (pCMV-3Tag1A-empty), wild-type SMYD2 (pCMV-3Tag1A-SMYD2) or MD mutant SMYD2 (pCMV-3Tag1A-SMYD2MD) and selected with appropriate concentrations of G418 for 3 weeks. Top, western blotting prepared with 20 μg of protein extract and anti-FLAG tag-specific antibody, demonstrating that cells transiently transfected with pCMV-3Tag1A-SMYD2 or pCMV-3Tag1A-SMYD2MD expressed FLAG-tagged wild-type or MD-mutant SMYD2, respectively. Middle, 3 weeks after transfection and subsequent selection of drug-resistant colonies, the colonies formed by *SMYD2*-transfected cells, but not by *SMYD2MD*-transfected cells, were more numerous than those formed by empty vector-transfected cells. Bottom, quantitative analysis of colony formation (colonies >2 mm were counted). Columns, means of three separate experiments, each performed in triplicate; bars, SD (histogram). Differences among multiple comparisons were analyzed by one-way analysis of variance with subsequent Scheffé's tests: a, $P < 0.05$ versus empty vector; b, $P < 0.05$ versus pCMV-3Tag1A-SMYD2MD. (**C**) Hypothetical model of the overexpression/activation of SMYD2 in ESCC cells. Overexpressed/activated SMYD2 possibly methylates various proteins, including p53 and histone H3 and inhibits p21 and other unknown targets indirectly, resulting in the promotion of cell proliferation.

of SMYD2 in all cell lines (Figure 3B). The results suggest that SMYD2 promotes cell proliferation at least partly dependent on its lysine methyltransferase activity in ESCC cells independently of *TP53* mutation status.

Discussion

SMYD2 was recently reported to be a lysine methyltransferase for K370 of p53, acting to repress p53's transactivation activity (10), suggesting that the aberrant activation of SMYD2 may inhibit p53 from functioning and contribute to the pathogenesis of human cancers. Until now, however, the role of SMYD2 and its expression status in human cancers have remained unknown. Although our previous study identified frequent copy number gains, including remarkable amplification, at 1q32–q41, which contains the *SMYD2* gene, in a panel of ESCC cell lines (3), *SMYD2* had not been mapped to this region in those days. In the present study, we hypothesized that overexpression/activation of *SMYD2* through some mechanisms including 1q32–q41.1 amplification may promote tumor cell proliferation and/or survival through mechanisms such as inhibition of the tumor suppressive function of p53 in ESCC. To test this hypothesis, we examined the expression status of SMYD2 and the clinicopathological as well as biological significance of its expression in cell lines and primary tumors of ESCC. We demonstrated that SMYD2 was frequently

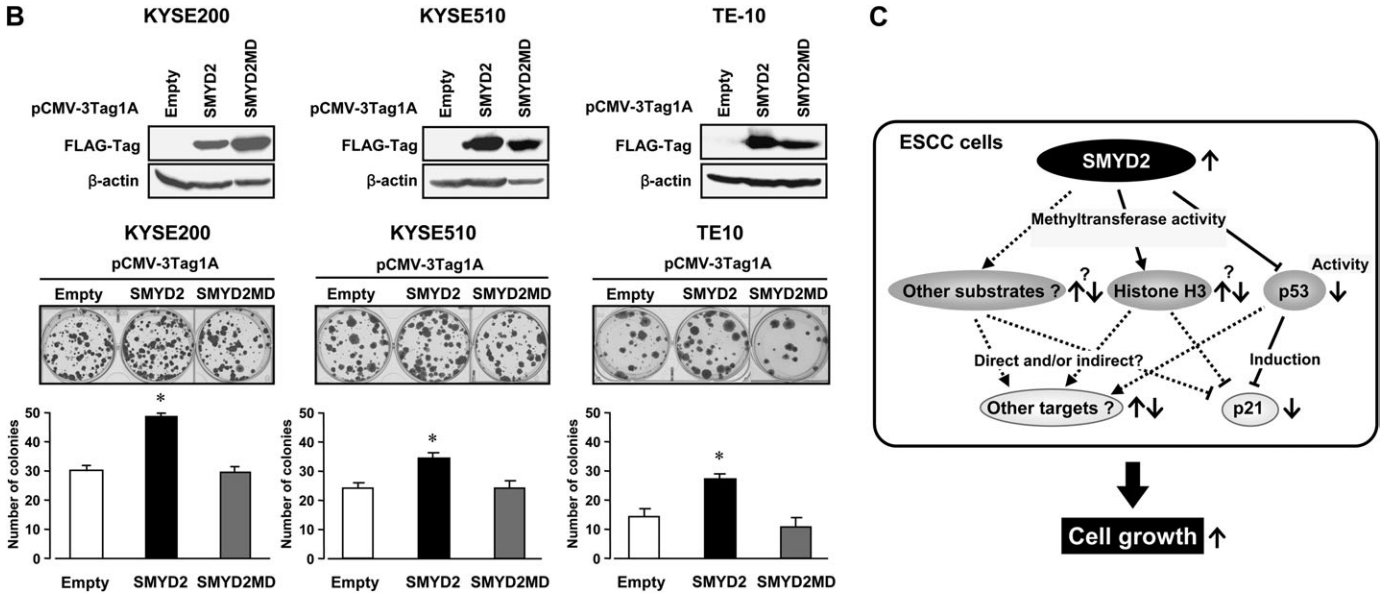

Fig. 3. Continued.

overexpressed in both the cell lines and primary tumors compared with normal esophageal epithelia, although gene amplification is probably to be one of the mechanisms for the overexpression. For overall survival in clinical cases of ESCC, overexpression of SMYD2 protein was a poor prognosticator independent of other prognostic factors. In addition, downregulation of SMYD2 expression suppressed cell proliferation mainly inducing G_0–G_1 arrest in ESCC cell lines overexpressing the gene, suggesting that SMYD2 works as a tumor promoter in esophageal carcinogenesis. However, an unexpected finding was that *TP53* mutation status and p53 protein expression status, which indicates the mutation status of this gene, are not associated with SMYD2 expression status in ESCC. Indeed, *TP53* mutation and protein expression of p53 were detected even in KYSE150 cells with remarkable amplification and moderate overexpression of SMYD2. Although wild-type p53 could be a good target for posttranslational modification by SMYD2 for its oncogenic function (10), it is possible that some substrates for the methyltransferase activity of SMYD2 other than p53 also contribute to the oncogenic function of SMYD2 in ESCC.

The most striking finding of our immunohistochemical analysis of SMYD2 using a panel of 153 primary tumor samples of ESCC was that immunoreactivity to the SMYD2 protein in each sample was significantly associated with a worse clinical outcome even after stratification with other clinicopathological characteristics in a multivariate analysis. Since the status of SMYD2 expression was associated with tumor depth (T categories) but not with tumor stage of the TNM classification, our result suggests the activation of SMYD2 to be involved in the malignant phenotype of ESCC at least partly in a stage-independent manner. On the other hand, p53 immunoreactivity was not correlated with prognosis in our series. This result was consistent with previous studies on ESCC using an immunohistochemical method in which no relationship between the overexpression of p53 and a poor prognosis was observed (21,22), although other studies found a close relationship (23,24). In combination with no relationship between expression levels of SMYD2 and p53 in primary tumors of ESCC, these results suggest immunoreactivity to SMYD2 to be useful as an independent prognosticator in patients with ESCC. In the p53-negative group, however, patients with tumors positive for SMYD2 expression showed significantly worse survival those with tumors lacking SMYD2 expression, suggesting that, in tumors with wild-type p53, p53 may be an important substrate for SMYD2, and a combination of p53 and SMYD2 immunoreactivity might be a more useful predictor for survival of patients with ESCC. Further examinations using cohorts of larger sizes will be needed to test this possibility and use SMYD2 as a novel biomarker in the clinical setting.

Knockdown of SMYD2 expression by specific siRNA treatment had a proliferation suppressive effect on ESCC cell lines overexpressing SMYD2 more or less independently of *TP53* mutation status. Cell cycle analysis using FACS demonstrated that the proliferation suppression caused by the knockdown was mainly due to G_0–G_1 arrest. Increases in p21 mRNA and protein levels after the knockdown of SMYD2 supported this result, although it remains unknown how SMYD2 inhibits the transcription of p21 in a p53-independent manner. These results were consistent with our additional findings of a similar proliferation-inhibiting effect upon the knockdown of SMYD2 in p53-null SaOS2 osteosarcoma cells (supplementary Figure S4 is available at *Carcinogenesis* Online). Proliferation suppression by the downregulation of SMYD2 expression was not significant in the cell lines expressing little SMYD2, suggesting that SMYD2 promotes the proliferation of ESCC cells in an expression level-dependent but at least partly p53-independent manner. This possibility was supported by the result of our colony formation assay of ectopic expression of SMYD2 in *TP53*-mutated cell line (TE-10), although the proliferation-promoting effect of SMYD2 may depend on its lysine methyltransferase activity. Since the immunohistochemical findings provide for the possibility that SMYD2 exerts its proliferation-promoting effect through both p53-dependent and -independent mechanisms, we hypothesized that overexpressed SMYD2 in ESCC cells promotes cell proliferation by inhibiting p21 expression through methylation of molecules other than p53 and/or p53, depending on the mutation status of *TP53*.

Although the proliferation-promoting effect of overexpressed SMYD2 on ESCC cells is inconsistent with recent reports that the transient transfection of NIH3T3 cells with SMYD2 led to a decrease in their proliferation (9), it is possible that the SMYD2-mediated pathway differs between epithelial cells and fibroblasts or among cell lineages. According to the recently reported phenomenon 'oncogene addiction' (25,26), the proliferation-promoting effect of SMYD2 on ESCC cells combined with its frequent overexpression in primary tumors of ESCC supports our contention that SMYD2 and/or SMYD2-dependent pathways could be attractive therapeutic targets for treatment purposes in ESCC.

One possible target for methylation by SMYD2 other than p53 may be histone H3 because SMYD2 was first identified as a split SET/MYND domain-containing histone H3K36-specific methyltransferase

(9). Methylation of H3K36 is known to be linked to transcriptional activity, although its mechanisms to activate genes and significance in carcinogenesis remain unclear (27). Very recently, methyltransferase activity of SMYD2 for histone H3K4 was also shown *in vitro* and several possible targets for transcriptional regulation through SMYD2-mediated histone H3K4 methylation were identified (28). SMYD3, another member of the SET/and MYND domain-containing methyltransferase family, was demonstrated to be a histone H3K4 methyltransferase and acts as an oncogene in various cancers including breast cancer (29,30). For SMYD3, indeed, vascular endothelial growth factor receptor 1 was reported as a possible lysine methylation target (31). Since both SMYD2 and SMYD3 have methyltransferase activity for histone H3K4 and this activity can be enhanced by heat shock protein 90α (HSP90α) (28,29), it is possible that the oncogenic function of these proteins is mediated through their methyltransferase activity for histone H3K4. However, methylation of histone H3K4 generally activates transcription, suggesting that p21 transcription to be indirectly repressed by histone H3K4-transactivated molecules or pathways/molecules other than p53 (Figure 3C). To test this hypothesis in esophageal carcinogenesis, further examination will be needed to clarify other targets for SMYD2.

In conclusion, this is the first report that *SMYD2* is a possible oncogene in human cancer. We showed the frequent overexpression of SMYD2 protein and its prognostic value in patients with ESCC. Although the functional involvement of SMYD2 and its association with p53, p21 and other molecules in ESCC cells remains unclear (Figure 3C), SMYD2 may be a useful marker for determining malignant properties and target for molecular therapy in patients with this lethal disease.

Supplementary material

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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