

Validation of the histone methyltransferase EZH2 as a therapeutic target for various types of human cancer and as a prognostic marker

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The emphasis in anticancer drug discovery has always been on finding a drug with great antitumor potential but few side-effects. This can be achieved if the drug is specific for a molecular site found only in tumor cells. Here, we find the enhancer of zeste homolog 2 (EZH2) to be highly overexpressed in lung and other cancers, and show that EZH2 is integral to proliferation in cancer cells. Quantitative real-time PCR analysis revealed higher expression of *EZH2* in clinical bladder cancer tissues than in corresponding non-neoplastic tissues ($P < 0.0001$), and we confirmed that a wide range of cancers also overexpress *EZH2*, using cDNA microarray analysis. Immunohistochemical analysis showed positive staining for EZH2 in 14 of 29 cases of bladder cancer, 135 of 292 cases of non-small-cell lung cancer (NSCLC), and 214 of 245 cases of colorectal cancer, whereas no significant staining was observed in various normal tissues. We found elevated expression of EZH2 to be associated with poor prognosis for patients with NSCLC ($P = 0.0239$). In lung and bladder cancer cells overexpressing EZH2, suppression of EZH2 using specific siRNAs inhibited incorporation of BrdU and resulted in significant suppression of cell growth, even though no significant effect was observed in the normal cell strain CCD-18Co, which has undetectable EZH2. Because EZH2 expression was scarcely detectable in all normal tissues we examined, EZH2 shows promise as a tumor-specific therapeutic target. Furthermore, as elevated levels of EZH2 are associated with poor prognosis of patients with NSCLC, its overexpression in resected specimens could prove a useful molecular marker, indicating the necessity for a more extensive follow-up in some lung cancer patients after surgical treatment. (*Cancer Sci* 2011; 102: 1298–1305)

The emergence of effective cancer chemotherapy is one of the major medical advances of recent years. Adjuvant chemotherapy for lung, breast, or colon cancer can augment the survival benefit afforded by surgical management. Even in patients with advanced solid tumors or recurrences after surgery, chemotherapy can offer lengthened survival of worthwhile quality.⁽¹⁾ In the latter patients, the therapeutic index is narrow: responses are usually partial, and often disappointingly brief.⁽²⁾ Most anti-tumor agents have unexpected detrimental side-effects, therefore, it is critical to discover novel therapeutic targets to extend the capability of cancer chemotherapy and improve patient care.

The N-terminal tails of histones are subjected to post-translational modifications, including methylation, acetylation and phosphorylation, which generate an extensive repertoire of chro-

matin structures. We previously reported that SMYD3, a histone lysine methyltransferase, stimulates proliferation of cells and plays an important role in human carcinogenesis through its methyltransferase activity.^(3–7) With the exception of Dot1/DOT1L, all histone lysine methyltransferases (HKMTs) contain a SET domain of approximately 130 amino acids.⁽⁸⁾ The SET domain was originally identified as a domain shared by three *Drosophila* proteins involved in epigenetic processes: the suppressor of position-effect variegation [Su(var)3-9]; an enhancer of the eye colour mutant *zeste* which belongs to the polycomb group (PcG) of proteins [*E(Z)*]; and the homeobox gene regulator *trithorax* (*TRX*).⁽⁹⁾ Mammalian HKMTs can be classified into several families according to sequence similarities within their SET domain and adjacent sequences, and according to structural features such as the presence of other defined protein domains.⁽⁸⁾ Although our knowledge of the physiological functions of histone methyltransferases is growing, their involvement in human disease remains largely unclear.

In order to investigate the roles of HKMTs in human carcinogenesis, we examined expression profiles of human HKMTs in clinical tissues, and found that in various types of cancer, expression levels of the enhancer of zeste homolog 2 (*EZH2*) were significantly upregulated, compared with their corresponding normal tissues. *EZH2* is a PcG protein homologous to *Drosophila* enhancer of zeste, a histone methyltransferase associated with transcriptional repression. *EZH2* has a SET domain and catalyses the addition of methyl groups to histone H3 at lysine 27 (H3K27). This polycomb group protein is conserved during evolution and is required for early mouse development.⁽¹⁰⁾ *EZH2* acts as the catalytic subunit of the PRC2 core complex, which also contains the two PcG proteins SUZ12 and EED. The PRC2 complex is responsible for repressing of a large number of genes essential for development and differentiation.^(11–13) PcG proteins specify positional information such as antero-posterior patterning, through activating or repressing the stable state of *Hox* gene expression.^(14,15) In addition to these well-established functions in embryonic development, a series of studies suggest that PcG proteins may influence both Hox-dependent and independent downstream pathways that control cell proliferation. In this study, we show that deregulation of *EZH2* expression is observed in various types of cancers, and correlates with a negative outcome in patients with non-small-cell lung cancer (NSCLC) after surgical resection. This work

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forms the basis for further functional studies, which will explore EZH2 as a potential therapeutic and prognostic target in lung and other cancers.

Materials and Methods

Lung tissue samples for tissue microarray. Primary NSCLC tissue samples, and corresponding normal tissue adjacent to resection margins, were obtained from patients who had no anti-cancer treatment before tumor resection, with their informed consent.^(16–18) All tumors were staged on the basis of the pathological TNM classification of the International Union Against Cancer. Formalin-fixed primary lung tumors and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 292 patients undergoing curative surgery at the Saitama Cancer Center (Saitama, Japan).^(19,20) To be eligible for this study, tumor samples were selected from patients who fulfilled all of the following criteria: (i) patients suffered primary NSCLC with a histologically confirmed stage (only pT1–pT3, pN0–pN2, and pM0); (ii) patients had undergone curative surgery, but did not receive any preoperative treatment; (iii) among them, NSCLC patients with positive lymph node metastasis (pN1, pN2) had been treated with platinum-based adjuvant chemotherapies after surgical resection, whereas patients with pN0 did not receive adjuvant chemotherapies; and (iv) clinical follow-up data were available. This study and the use of all clinical materials mentioned were approved by the relevant institutional ethics committees.

Colorectal tissue samples for immunohistochemistry. For Japanese cases, 172 primary human colorectal adenocarcinomas (ADC) were studied by immunohistochemistry. All tumors were obtained from patients who had undergone endoscopic resection or surgery at Nagasaki University Hospital (Nagasaki, Japan) between 2000 and 2004. Of the 172 patients with colorectal carcinoma, there were 91 men and 81 women. The median age was 63.5 years (range, 16–90 years). Each tumor was assigned a histological type according to the World Health Organisation classification: well differentiated ADC; moderately differentiated ADC; poorly differentiated ADC; and mucinous ADC.⁽²¹⁾

Immunohistochemical staining and tissue microarray. Immunohistochemical analysis was carried out using a specific mouse-EZH2 antibody (NCL-L-EZH2; Leica Microsystems, Wetzlar, Germany) as described previously.^(18,22–25) Tumor tissue microarrays were constructed with 292 primary NSCLCs obtained by a single institutional group (as above) with one protocol for collection, fixation, and preservation of tissues after resection.^(26–28) Considering the histologic heterogeneity of individual tumors, a tissue area for sampling was selected based on visual alignment with the corresponding H&E-stained section on a slide. Three, four, or five tissue cores (diameter, 0.6 mm; depth, 3–4 mm) taken from a donor tumor block were placed into a recipient paraffin block with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). A core of normal tissue was punched from each case, and 5- μ m sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators semiquantitatively assessed EZH2 positivity without prior knowledge of clinicopathologic data. Because the intensity of staining within each tumor tissue core was mostly homogeneous, EZH2 staining was semiquantitatively evaluated using the following criteria: negative (no appreciable staining in tumor cells); and positive (brown staining appreciable in the nucleus of tumor cells). Cases were accepted as positive only if all reviewers independently defined them as such.

Quantitative real-time PCR. Specific primers for human *GAPDH*, *B2M* (housekeeping genes), and *EZH2* were designed (primer sequences are given in Table S1). Polymerase chain reactions were carried out using the LightCycler 480 System

(Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol.

siRNA transfection and cell growth assay. siRNA oligonucleotide duplexes were purchased from Sigma-Aldrich (St. Louis, MO, USA) for targeting the human *EZH2* transcripts. siEGFP and siNegative control (siNC, B-Bridge), which consists of three different oligonucleotide duplexes, were used as control siRNAs. The siRNA sequences are described in Table S2. siRNA duplexes (100 nM final concentration) were transfected into lung and bladder cancer cell lines with Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) for 72 h, and cell growth was examined using the Cell Counting kit-8 (Dojindo, Kumamoto, Japan).⁽³⁾

Statistical analysis. Student's *t*-test or Mann–Whitney's *U*-test was used to analyze the difference between two independent subgroups. Survival curves were calculated from the date of surgery to either the time of death relating to only cancer, or to the last follow-up observation. Kaplan–Meier curves were calculated for each relevant variable and for EZH2 expression; differences in survival times among patient subgroups were analyzed using the log–rank test. Univariate and multivariate analyses were done with the Cox proportional hazard regression model to determine associations between clinicopathological variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors including age, gender, histology, pT classification, and pN classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong EZH2 expression into the model, along with any and all variables that satisfied an entry level of $P < 0.05$. As the model continued to add factors, independent factors did not exceed an exit level of $P < 0.05$.

Results

EZH2 expression upregulated in clinical bladder cancer tissue but barely detectable in normal tissue. We examined the level of histone lysine methyltransferase gene products in a small subset of British clinical bladder cancer samples by quantitative real-time PCR, and found significant overexpression of *EZH2* in the cancer samples compared with non-cancerous samples (data not shown). We then analyzed 119 bladder cancer samples and 25 normal control samples (also British), and confirmed a significant elevation of *EZH2* expression levels in tumor cells compared with normal cells (Fig. 1A; $P < 0.0001$, Mann–Whitney *U*-test). Importantly, quantitative real-time PCR analysis showed that expression levels of *EZH2* in bladder tumor tissues are notably higher than those in any other normal tissues we examined (Fig. 1B). We then analyzed the expression patterns of *EZH2* in a number of clinical samples derived from Japanese bladder cancer subjects examined by cDNA microarray (Fig. 1C), and again identified significant overexpression ($P < 0.0001$, Mann–Whitney *U*-test). To evaluate protein expression levels of EZH2 in bladder tissues, we carried out immunohistochemical analyses using an anti-EZH2 antibody, and observed strong staining in the nucleus of malignant cells, but weak or absent staining in non-neoplastic tissues (Fig. 1D). Tissue microarray analysis indicated that EZH2 was positively stained in 14 of 29 bladder cancer cases (48.3%), whereas no staining was observed in normal bladder tissues (Table S3). Subclassification of tumors according to tumor grade identified that Grade III tumors showed significantly higher EZH2 protein expression than Grade I and Grade II tumors, implying that EZH2 expression is more elevated in advanced bladder tumors (Table S4). Importantly, specific EZH2 signals were not detected in normal heart, lung, liver, colon, kidney, or bladder tissues (Fig. 1E), consistent with our real-time PCR result.

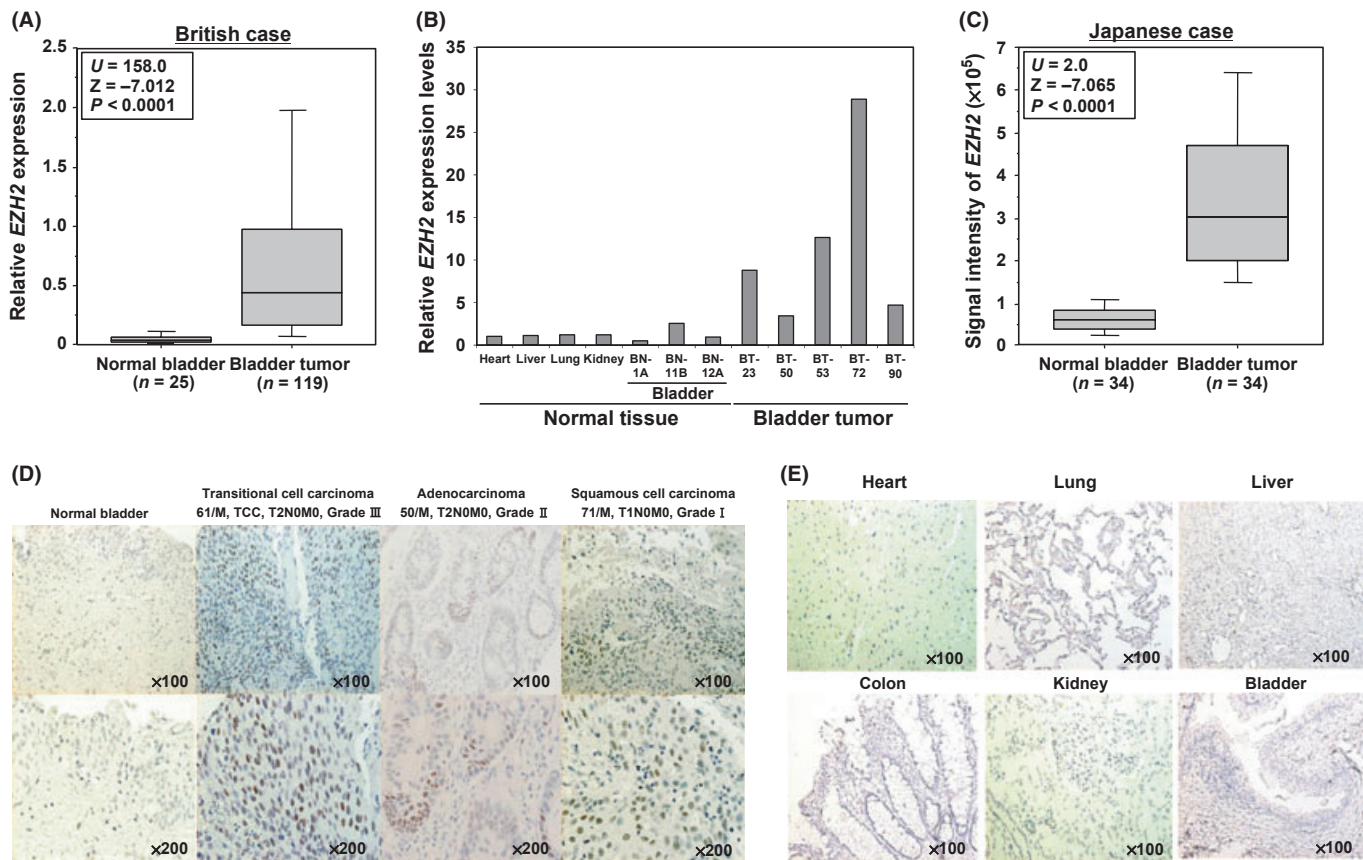


Fig. 1. Elevated expression of the histone methyltransferase EZH2 in human bladder cancer. (A) *EZH2* gene expression in normal and tumor bladder tissues in British cases. Expression levels of *EZH2* were analyzed by quantitative real-time PCR, and the result is shown by box-whisker plot (median 50% boxed). Relative mRNA expression shows the value normalized by *GAPDH* and *SDH* expressions. The Mann-Whitney *U*-test was used for statistical analysis ($***P < 0.0001$). (B) Quantitative real-time PCR analysis was carried out in normal heart, liver, lung, and kidney tissues and randomly selected normal and tumor bladder tissues (three cases and five cases, respectively). (C) Comparison of *EZH2* expression between normal and tumor bladder tissues in Japanese patients. Signal intensity for each sample was analyzed by cDNA microarray, and the result is shown by a box and whisker plot (median 50% boxed). The Mann-Whitney *U*-test was used for statistical analysis. (D) Immunohistochemical staining of EZH2 in bladder tissues. Clinical information for each section is represented above histological pictures. Original magnification, $\times 100$, $\times 200$. (E) Immunohistochemical analysis of EZH2 in various normal tissues. No significant staining was observed. Original magnification, $\times 100$.

EZH2 expression significantly high in lung and colorectal cancer tissues, and correlates with poor prognosis in NSCLC. cDNA microarray experiments showed that *EZH2* expression was similarly elevated in lung tumor tissues compared with corresponding non-neoplastic tissues (Fig. 2A; $P < 0.0001$, Mann-Whitney *U*-test). We then examined *EZH2* protein expression levels in lung tissue by immunohistochemistry and observed strong *EZH2* staining in cancer tissues but no significant staining in non-neoplastic tissues (Fig. 2B). To analyze the significance of *EZH2* protein expression in lung cancer tissues in more detail, we carried out immunohistochemical analysis on a tissue microarray containing tissue sections from 292 NSCLC patients who had undergone surgical resection (Fig. S1). *EZH2* stained positively in 135 out of 292 cases (46.2%) and negatively in 157 cases (53.8%). Subsequently, we analyzed the association of *EZH2* expression with clinical outcome, and found that expression of *EZH2* in NSCLC patients was significantly associated with male gender ($P = 0.0002$, Fisher's exact test; Table 1), non-ADC histology ($P < 0.0001$), smoking status (smoker; $P < 0.0001$), pN factor (N1 + N2; $P = 0.0269$), and tumor-specific 5-year survival after the resection of primary tumors ($P = 0.0239$ by log-rank test; Fig. 2C). Univariate analysis revealed associations between poor prognosis in NSCLC patients and several factors, including *EZH2*

expression, age, gender, histologic type (non-ADC versus ADC), pT stage (tumor size, T1 versus T2 + T3), and pN stage (node status, N0 versus N1 + N2; Table 2). In contrast, multivariate analysis revealed that *EZH2* status did not show statistical significance as an independent prognostic factor for surgically treated NSCLC patients enrolled in this study, whereas age and pT and pN factors did (Table 2). This result might be due to the preponderance of *EZH2* expression up to the pN factor.

Quantitative real-time PCR analysis revealed that *EZH2* expression levels in cancer cells were significantly higher than those in normal human cells, and intriguingly, expression levels of *EZH2* in colorectal cancer cell lines are remarkably high (Fig. 2D). Therefore, we also examined *EZH2* expression levels in colorectal clinical tissues by immunohistochemistry and observed that elevated expression of *EZH2* was restricted to cancer tissues (Fig. 2E). Tissue microarray analysis showed that overexpression of *EZH2* protein could be observed in 55 out of 72 colorectal carcinoma cases (76.4%; Table S5). To clarify the association between *EZH2* overexpression and clinical outcomes in colorectal cancer, we carried out additional immunohistochemical analysis using a number of Japanese cases, who had undergone endoscopic resection or surgery at Nagasaki University Hospital (Fig. S2). *EZH2* stained positively in 158

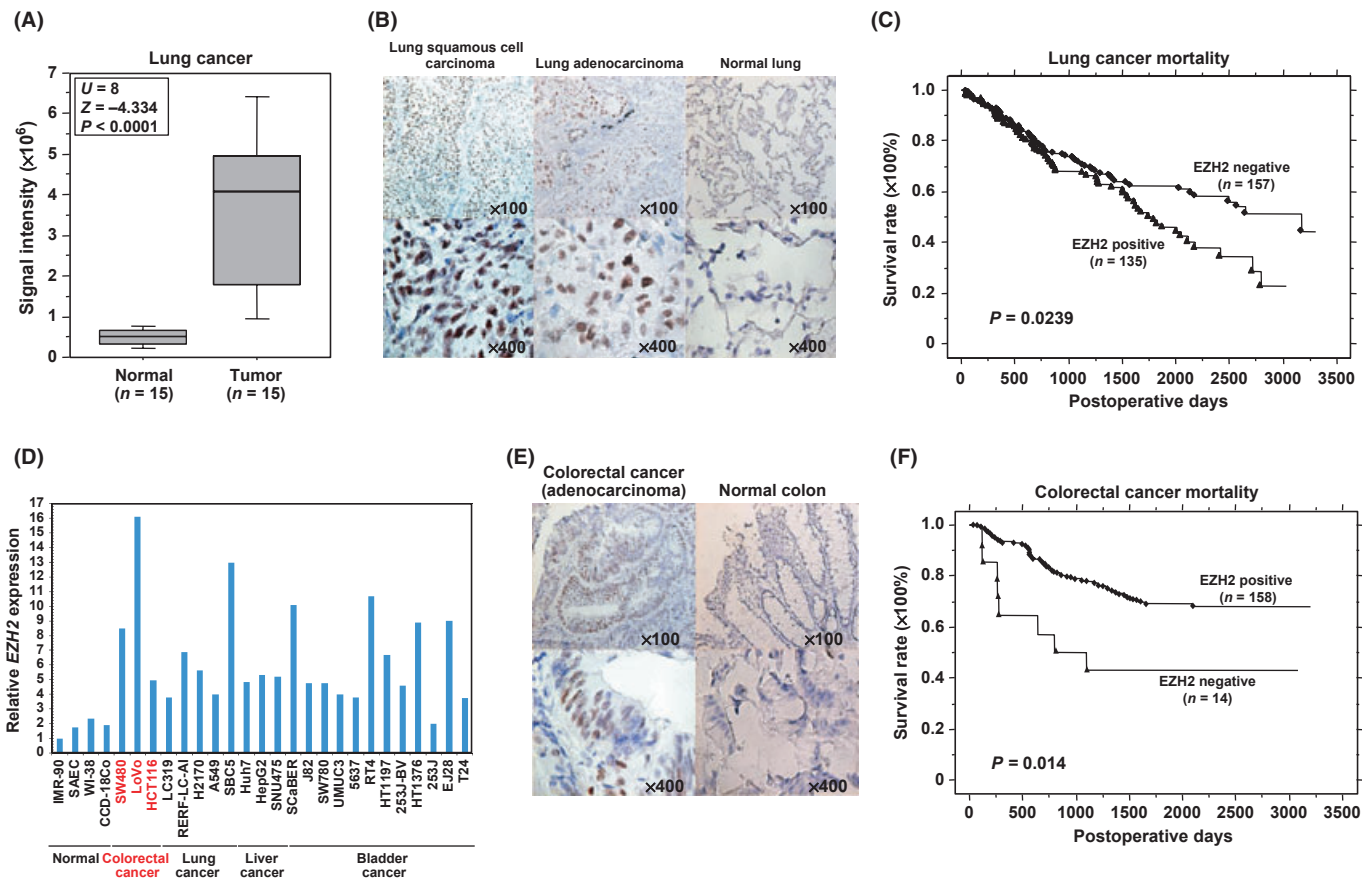


Fig. 2. Histone methyltransferase EZH2 was overexpressed in lung and colorectal cancers, and appears to be a prognostic marker of non-small-cell lung cancer (NSCLC). (A) Comparison of *EZH2* expression between normal and tumor (SCLC) lung tissues in Japanese patients. Signal intensity for each sample was analyzed by cDNA microarray, and the result is shown by a box and whisker plot (median 50% boxed). The Mann-Whitney *U*-test was used for statistical analysis. (B) Immunohistochemical staining of EZH2 in lung tissues. Clinical information for each section is represented above histological pictures. Original magnification, $\times 100$, $\times 400$. (C) Kaplan–Meier estimates of overall survival time of patients with NSCLC ($P = 0.0239$, log–rank test). (D) Expression of *EZH2* in four normal cell lines, three colorectal cancer cell lines, five lung cancer cell lines, three liver cancer cell lines, and 12 bladder cancer cell lines. Expression levels were analyzed by quantitative real-time PCR, and relative mRNA expression shows the value normalized by *GAPDH* and *SDH* expressions. (E) Immunohistochemical staining of EZH2 in colorectal tissues. Original magnification, $\times 100$, $\times 400$. (F) Kaplan–Meier estimates of overall survival time of patients with NSCLC ($P = 0.014$, log–rank test).

out of 174 cases (91.4%) and negatively in 14 cases (8.6%; Table 3). Next, we examined the association of EZH2 expression with clinical outcomes, and found that expression of EZH2 in colorectal cancer patients was significantly associated with P-factor negative cases ($P = 0.019$, Fisher's exact test; Table 3), M-factor negative ($P = 0.011$), and also tumor-specific 5-year survival after resection of primary tumors ($P = 0.014$, log–rank test; Fig. 2F). Univariate analysis of association between patient prognosis and several factors, including EZH2 expression, age, gender, serum carcinoembryonic antigen level, serum CA19-9 level, pT factor, pN factor, and M factor, was examined. All factors except age and gender were associated with poor prognosis (Table 4). In addition, multivariate analysis revealed that EZH2 status and M factor for colorectal cancer did show statistical significance as independent prognostic factors for surgically treated colorectal cancer patients enrolled in this study. These results imply that EZH2 protein is markedly overexpressed at the protein level in colorectal cancer, and EZH2 overexpression indicates a good prognosis, in contrast to the poor prognosis associated with EZH2 overexpression in lung cancer.

EZH2 required for cancer cell proliferation. In order to examine whether elevated expression of EZH2 plays a critical role in the proliferation of cancer cells, we prepared two independent

siRNA oligonucleotide duplexes to specifically suppress the expression of *EZH2* (siEZH2#1, #2), and transfected each of them into cancer cells. Knockdown of EZH2 in cancer cells was confirmed by real-time PCR and Western blotting as shown (Fig. 3A). We carried out cell growth assays on cancer cells transfected with these siRNAs, and observed significant growth suppression in two bladder cancer cell lines (SW780 and RT4), two lung cancer cell lines (A549 and SBC5) and one colorectal cancer cell line (HCT116) (Fig. 3B). When the EZH2 siRNAs were transfected into the normal cell line CCD-18Co, expressing undetectable levels of EZH2 (Fig. S3), they had no effect on CCD-18Co cells (Fig. 3B). This implies that suppression of cancer cell growth by treatment with these specific siRNAs does not deploy a mechanism used by normal cells, and thus directly acts on a growth mechanism specific to cancer cells. To further assess the mechanism of growth suppression induced by the siRNA, we carried out BrdU and 7-amino-actinomycin D staining and examined cell cycle status (Fig. 3C). The proportion of cancer cells at the G_1 phase was significantly higher in the cells treated with siEZH2 than those treated with control siRNAs, whereas the proportion of cancer cells at the S phase was remarkably decreased (Fig. 3C). The data revealed that EZH2 plays a crucial role in the cell cycle progression of cancer cells.

Table 1. Association between EZH2-positive status in non-small-cell lung carcinomas and patients' characteristics

	Total (n = 292)	EZH2 positive (n = 135)	EZH2 negative (n = 157)	P-value
Gender				
Male	204	109	95	0.0002*
Female	88	26	62	
Age (years)				
<65	129	59	70	NS (0.9063)
≥65	163	76	87	
Histological type				
ADC	180	63	117	<0.0001***
SCC	79	50	29	
Others	33	22	11	
Smoking status				
Smoker	208	114	94	<0.0001*
Never	84	21	63	
pT factor				
T1	124	49	75	NS (0.0575)
T2 + T3	168	86	82	
pN factor				
N0	189	78	111	0.0269*
N1 + N2	103	57	46	

*P < 0.05 (Fisher's exact test); **ADC versus non-ADC. ADC, adenocarcinoma; NS, not significant; Others, large-cell carcinoma plus adenosquamous cell carcinoma; SCC, squamous cell carcinoma.

Table 2. Cox's proportional hazards model analysis of prognostic factors in patients with non-small-cell lung carcinoma

Variables	Hazards ratio	95% CI	Unfavorable/ favorable	P-value
Univariate analysis				
EZH2	1.482	1.051–2.091	Positive/negative	0.0248*
Age (years)	1.677	1.171–2.401	≥65/<65	0.0048*
Gender	1.620	1.089–2.415	Male/female	0.0173*
Histological type	1.551	1.103–2.181	Non-ADC/ADC	0.0117*
Smoking status	1.308	0.882–1.939	Smoker/never	0.1821
pT factor	2.755	1.862–4.065	T2 + T3/T1	<0.0001*
pN factor	2.427	1.724–3.413	N1 + N2/N0	<0.0001*
Multivariate analysis				
EZH2	1.142	0.790–1.652	Positive/ negative	NS (0.4791)
Age (years)	1.821	1.266–2.620	≥65/<65	0.0012*
Gender	1.234	0.794–1.919	Male/female	NS (0.3498)
Histological type	1.056	0.718–1.554	Non-ADC/ADC	NS (0.6896)
pT factor	2.155	1.429–3.257	T2 + T3/T1	0.0003*
pN factor	2.227	1.565–3.165	N1 + N2/N0	<0.0001*

*P < 0.05 (Fisher's exact test). ADC, adenocarcinoma; CI, confidence interval; NS, not significant.

Discussion

Cancer-related death is on the rise in most countries. Although molecular-targeting agents such as cetuximab and bevacizumab have been developed and proven efficacious, their adverse effects and limited application for some patients makes it important to continue to search for novel molecular-targeting agents. Recent tumor genomics data will be used to develop rationally selected drugs that target proteins expressed exclusively or at

Table 3. Association between EZH2-positive status in colorectal cancer and patients' characteristics (n = 172)

	Total (n = 172)	EZH2 positive (n = 158)	EZH2 negative (n = 14)	P-value
Gender				
Male	91	82	9	0.416
Female	81	76	5	
Age (years)				
<65	84	75	9	0.272
≥65	88	83	5	
Serum CEA level				
<5.0	121	114	7	0.123
≥5.0	51	44	7	
Serum CA19-9 level				
<37	131	123	8	0.102
≥37	41	35	6	
pT factor				
T1≥	21	19	2	0.682
T2≤	151	139	12	
pN factor				
N ⁻	97	87	10	0.274
N ⁺	75	71	4	
H factor				
H ⁻	150	140	10	0.085
H ⁺	22	18	4	
P factor				
P ⁻	164	153	11	0.019*
P ⁺	8	5	3	
M factor				
N ⁻	145	137	8	0.011*
N ⁺	27	21	6	

*P < 0.05 (Fisher's exact test). CEA, carcinoembryonic antigen; NS, not significant.

Table 4. Cox's proportional hazards model analysis of prognostic factors in patients with colorectal cancer

Variables	Hazards ratio	95% CI	Unfavorable/ favorable	P-value
Univariate analysis				
EZH2	0.391	0.185–0.827	Positive/ negative	0.014*
Age(years)	0.958	0.409–1.157	≥65/<65	0.158
Gender	1.022	0.611–1.710	Male/ female	0.934
Serum CEA level	3.862	2.294–6.500	5.0>/5.0≤	<0.001*
Serum CA19-9 level	2.534	1.495–4.293	37>/37≤	0.001*
pT factor	4.517	1.102–18.516	T1≥/T2≤	0.036*
pN factor	2.450	1.441–4.167	N ⁻ /N ⁺	0.001*
M factor	8.570	4.986–14.731	M0/M1	<0.001*
Multivariate analysis				
EZH2	0.423	0.185–0.967	Positive/ negative	0.041*
Age(years)	0.958	0.548–1.675	≥65/<65	0.881
Gender	1.196	0.688–2.078	Male/ female	0.526
Serum CEA level	1.445	0.731–2.858	5.0>/5.0≤	0.29
Serum CA19-9 level	1.169	0.604–2.262	37>/37≤	0.643
pT factor	2.089	0.483–9.031	T1≥/T2≤	0.324
pN factor	1.447	0.783–2.673	N ⁻ /N ⁺	0.238
M factor	4.620	2.380–8.969	M0/M1	<0.001*

*P < 0.05 (Fisher's exact test). CEA, carcinoembryonic antigen; CI, confidence interval.

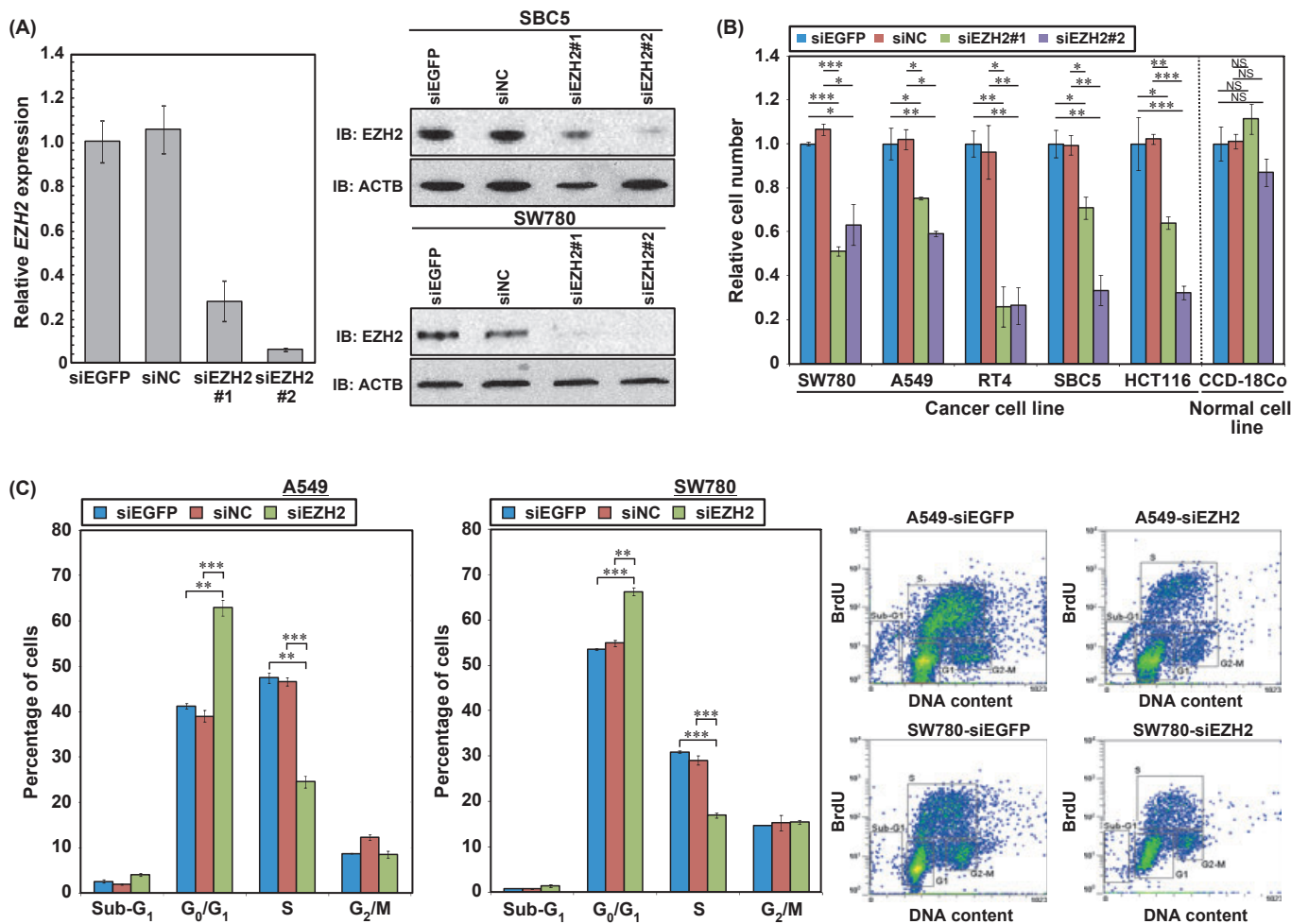


Fig. 3. Involvement of histone methyltransferase EZH2 in the cell cycle regulation of cancer cells. (A) Knockdown effects of siRNAs targeting EZH2 on cancer cells. Left, quantitative real-time PCR showing suppression of endogenous expression of *EZH2* by two EZH2-specific siRNAs (siEZH2#1 and #2) in SBC5 cells. Right, Western blot analysis of EZH2 in SBC5 and SW780 cells after treatment with two EZH2 siRNAs (siEZH2#1 and #2) and two control siRNAs (siEGFP and siNC). β -actin was used as an internal control. (B) Effect of *EZH2* siRNA knockdown on the viability of bladder cancer cell lines (SW780, RT4), lung cancer cell lines (A549, LC319), and a colorectal cancer cell line (HCT116). Relative cell number shows the value normalized to siEGFP-treated cells. Results are the mean \pm SD of three independent experiments. *P*-values were calculated using Student's *t*-test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). (C) Effect of EZH2 knockdown on cell cycle kinetics in A549 and SW780 cells. Cells were collected 72 h after treatment with siRNAs, and cell cycle distribution was analyzed by flow cytometry after coupled staining with FITC-conjugated anti-BrdU and 7-amino-actinomycin D. We show numerical analysis of the FACS results (left) and representative histograms (right) for each experiment. Results are the mean \pm SD of three independent experiments. *P*-values were calculated using Student's *t*-test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

particularly high levels in tumor compared with essential normal adult cells. Specific pharmaceutical targeting of such proteins could result in a new generation of highly active drugs having minimal collateral host toxicity.⁽²⁹⁾ In this study, we showed that, in various cancer tissues, expression levels of EZH2 were significantly high at both RNA and protein levels whereas EZH2 expression in various normal tissues was barely detected (see also Fig. S4 and Table S6). We also found that EZH2 plays a crucial role in the cell cycle progression of cancer cells. Although knockdown of EZH2 expression by specific siRNAs significantly suppressed the growth of cancer cells, no growth suppression was observed in the normal colon fibroblast CCD-18Co cells, with undetectable EZH2. These data clearly indicate EZH2 as an ideal molecular target for cancer therapy. Indeed, other researchers have been actively analyzing the involvement of EZH2 in the progression of human cancer.^(30–34) Development of methyltransferase inhibitors began recently,^(35–37) and a specific inhibitor targeting EZH2 could have great potential as

an anticancer agent. In addition, as we have been successfully developing therapeutic cancer vaccines,^(38,39) EZH2 could be a promising target for cancer vaccine therapy.

Our data suggest that EZH2 could also be a good marker, enabling us to predict the prognosis of NSCLC patients, and to carry out a more intensive follow-up according to EZH2 expression status in resected specimens. In contrast, EZH2 overexpression indicated a good prognosis in colorectal cancer. In this case, EZH2 was overexpressed in the majority of cancer cases (91.4%), and the number of EZH2-negative cases was small (*n* = 14). Therefore, although indicative of cancer, it appears difficult to use EZH2 expression as a prognostic marker in colorectal cancer. It is also possible that EZH2 overexpression might affect the prognosis differently, depending on the type of cancer. For instance, it was reported that a high EZH2 index was significantly associated with better relapse-free survival, and with a trend for better cancer-specific survival, in colon cancer stages II and III derived from Norwegian subjects,⁽³¹⁾ consistent with

our results. In contrast, in breast cancer and melanomas, EZH2 expression in tumor cells was linked to an aggressive clinical behavior and worse prognosis.^(40,41) In addition, in prostate cancer cells, EZH2 was found to promote invasiveness and proliferation.⁽⁴²⁾ With regard to Japanese colorectal cancer cases in this study, the EZH2 positive ratio is statistically low in P-factor positive and M-factor positive cases ($P = 0.019$ and $P = 0.011$, respectively), and there is a possibility that EZH2 could repress peritoneal and distant metastases from colorectal cancers. Large-scale validation of the present results, and further functional analyses of this protein in human carcinogenesis, may assist in comprehending the various modes of EZH2 expression in different cancer types. Furthermore, we need to develop anticancer therapies targeting EZH2 with due attention to cancer type.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Representative cases for positive and negative EZH2 staining in lung cancer and normal lung tissues on the tissue microarray.

Fig. S2. Representative cases for positive and negative EZH2 staining in colorectal cancer and normal colorectal tissues.

Fig. S3. Validation of EZH2 protein expression in various cell lines.

Fig. S4. Expression levels of *EZH2* in 78 normal tissues.

Table S1. Primer sequences for quantitative real-time PCR.

Table S2. siRNA sequences used for siRNA transfection and cell growth assay.

Table S3. Clinicopathological characteristics of bladder tissues on tissue microarray.

Table S4. Statistical significance between EZH2 expression and tumor grade.

Table S5. Clinicopathological characteristics of colorectal tissues on tissue microarray.

Table S6. Expression profile of *EZH2* analyzed by cDNA microarray.

Data S1. Materials and Methods.

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