a quantitative method using pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden). A detailed pyrosequencing protocol was described previously[18 20] and was used here. The methylation levels at different CpG sites, as measured by pyrosequencing, were averaged to represent the degree of methylation in each sample for each gene. Genes with methylation levels greater than 15% were considered methylation positive, because lower values could not be easily distinguished from background.[18] Primer sequences are summarised in supplementary table 1, available online only.

MCAM analysis

Forty GIST of the training set (average age 60.2 years, range 37–81 years) were analysed using MCAM technology. This panel consisted of nine small GIST, 13 malignant-prone GIST and 18 malignant GIST. A detailed protocol for MCAM analysis was described previously[18] and was used here. Briefly, amplicons from individual GIST were labelled with Cy5 dye and cohybridised against amplicons from normal controls labelled with Cy3 dye on 15K custom-promoter microarrays from Agilent Technologies (G4497A; Agilent Technologies, Santa Clara, California, USA) containing 6157 unique genes, which we had initially validated in a previous study.[18] In the present study, eight randomly selected genes (*LIMD1*, *NME1*, *RASSF1A*, *IGF2BP1*, *REC8*, *PAX3*, *MGMT* and *NR2E1*) from the MCAM analysis were subsequently assessed by pyrosequencing analysis of GIST samples. A good concordance was observed between the methylation status by MCAM (a signal ratio >2.0 in MCAM considered methylation positive) and pyrosequencing analyses (specificity, 80%; sensitivity, 74%, data not shown) as was also demonstrated in previous studies.[16 21] We thus considered a signal ratio greater than 2.0 in MCAM as methylation positive. The data of our microarray are available in the Array express (http://www.ebi.ac.uk/arrayexpress) with accession codes: E-TABM-1022.

KIT and *PDGFRA* gene mutation

Mutations in the *KIT* (exons 9, 11, 13 and 17) and *PDGFRA* (exons 12, 14 and 18) genes were determined by direct sequencing. All sequencing reactions were carried out in forward and reverse direction. Primer sequences are summarised in supplementary table 1 (available online only).

Detection of homozygous deletion in *p16* locus

Homozygous deletions were identified by real-time PCR using primers within the *p16* and *GAPDH* loci (supplementary table 1, available online only). Samples that displayed a relative ratio of *p16:GAPDH* less than 0.1 were defined has having a homozygous deletion.

Reverse transcription PCR analyses

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). Two micrograms of RNA was reverse transcribed with MPMLV (Promega, Madison, Wisconsin, USA). Reverse transcription PCR was carried out in triplicate for the target genes (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are shown in supplementary table 1 (available online only).

Data analysis and statistics

All statistical analyses were performed using JMP statistical software version 5.1. Fisher's exact test was used to determine non-random associations between two categorical variables. Kruskal–Wallis analysis was used to evaluate the extent of differences among more than three groups. All reported p values are two sided, with $p<0.05$ taken as statistically significant. Patients were followed until incidence of death or until September in 2010, whichever came first. Survival information was available for 69 of the 115 cases. Overall survival was calculated from the date of diagnosis until the date of death or the date the patient was last known to be alive (censored). Median follow-up times are 24.5 months. Overall survival curves were generated using the Kaplan–Meier method, and the log-rank test was used for statistical analysis. A multivariate analysis using the Cox proportional hazards model was performed to estimate the HR. All variables for the multivariate analysis were categorical variables. The factors considered in the multivariate model included tumour origin, prognosis marker and mitotic index, which were marginally significant ($p<0.1$) or statistically significant ($p<0.05$) by univariate Cox regression analysis, in addition to tumour size.

RESULTS

Genetic and epigenetic analyses of candidate genes in the training set of GIST samples

First, we examined mutations in the *KIT* and *PDGFRA* genes in the training set, finding that 36 of 40 (90%) GIST harbour mutations at one or other of these loci (see supplementary table 2, available online only). The majority of mutations were found in exon 9 (five GIST, 13%) and exon 11 (28 GIST, 70%) of the *KIT* gene, the frequency of which is similar to that observed in previous studies.[22] There was no correlation between the type of mutation and frequencies of disease recurrence or metastasis (data not shown).

Next, we examined the DNA methylation status of four tumour suppressor genes, *p16*, *RASSF1A*, *O-6-methylguanine-DNA methyltransferase (MGMT) and E-cadherin* (*CDH1*) (figure 1 and supplementary table 3, available online only) in 40 GIST. The methylation status of the examined regions of *p16*, *RASSF1A* and *MGMT* correlated well with gene expression, although they were not within nucleosome-free regions.[23 24] A lower level of DNA methylation of *RASSF1A* was found in small GIST and increased progressively from small to malignant GIST. *p16* was specifically methylated in malignant-prone and malignant GIST. DNA methylation was less frequently found in *MGMT* and *CDH1* compared with *RASSF1A* and *p16*. These data suggest that DNA methylation of *RASSF1A* is an early event in tumorigenesis and evolves progressively, while *p16* methylation was a specific event associated with more advanced (malignant-prone and malignant) GIST (figure 1 and supplementary table 3, available online only). While aberrant hypermethylation of the gene promoters has been demonstrated in tumours, methylation of the *LINE-1* gene, an indicator of global methylation status, has been shown to be inversely associated with tumour transformation.[11 25 26] Our analysis showed that *LINE-1* methylation was decreased in malignant GIST (63.4±6.1%) compared with malignant-prone GIST (68.8±3.1%) and small GIST (67.6±1.8%; $p=0.02$). These results raise the possibility that genome-wide DNA methylation profiles are different between small, malignant-prone and malignant GIST, which may reflect the clinicopathological differences between these tumour types. To examine this possibility, we conducted genome-wide DNA methylation analysis in small, malignant-prone and malignant GIST.

Genome-wide DNA methylation profiling of training set of GIST samples by MCAM analysis

MCAM analysis was performed on 40 GIST. We selected these samples based on both the quality and quantity of DNA

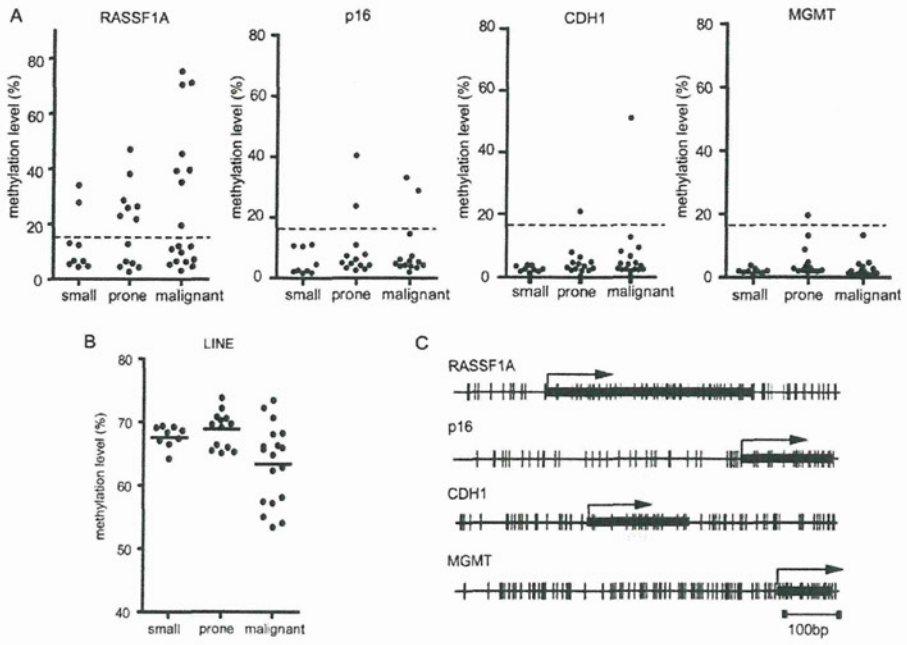

Figure 1 DNA methylation status of the candidate genes *p16, RASSF1A, CDH1, MGMT* (A) and *LINE-1* repetitive elements (B) in gastrointestinal stromal tumours (GIST). Levels of methylation were measured by bisulfite-pyrosequencing analysis in small, malignant-prone (prone), and malignant GIST. The Y-axis indicates the level of DNA methylation of each gene. Dots represent the methylation level of the indicated gene within individual samples. Broken lines indicate a methylation level of 15%. Genes with methylation levels greater than 15% were considered methylation positive. (C) Diagrams of promoters of the *RASSF1A, p16, CDH1* and *MGMT*. Each vertical line represents a single CpG site. The transcription start site (arrow) and the location of exon1 (black box) are indicated. Thick grey bars denote the regions analysed by pyrosequencing.

available for this type of analysis. The characteristics of the samples used are shown in supplementary table 2 (available online only). Dissected muscle tissue served as a normal control. As ICC in gastrointestinal muscles are thought to be the cell of origin for GIST, it would be appropriate to use them as a normal counterpart for the study. However, our preliminary experiments showed that DNA from microscopic dissection did not yield stable results in MCAM analysis; it might be due to the quality of DNA (see supplementary figure 1, available online only, data not shown). To evaluate differences in DNA methylation status of the candidate loci between microscopically dissected ICC and normal muscle layer tissues, we examined DNA methylation levels of 16 genes and *LINE-1* in these two tissues (see supplementary table 4, available online only). The DNA methylation levels of these genes as well as *LINE-1* were found to be identical in these two tissues. Therefore, we used normal muscle layers as a control in this study, although we still cannot exclude the possibility that DNA methylation in some genes is not identical between these two tissues based on the analysis of this limited set of genes.

The number of methylated genes identified was not significantly associated with any other clinicopathological factors apart from clinical classification (see supplementary table 2, available online only and figure 2A). More genes were methylated in malignant-prone and malignant GIST than small GIST (473 genes±182 genes vs 360 genes±82 genes, p=0.012, figure 2A). This is also true when the number of methylated genes was compared between small and malignant-prone GIST (360 genes±82 genes vs 462 genes±139 genes, p=0.043). These data indicate that clear differences exist with respect to methylation profiles between small GIST and more advanced GIST.

We next analysed to what degree the DNA methylation target genes identified in small, malignant-prone and malignant GIST overlapped with each other. A heat-map overview of 766 methylated genes, which were commonly methylated in more than 30% of the cases in any of the three groups, showed that the majority of DNA methylation target genes in small GIST appeared to be methylated in malignant-prone GIST and/or malignant GIST (figure 2B). Schematic representation of these data in a Venn diagram showed that, although the majority of DNA methylation target genes (482 genes) were commonly methylated in small, malignant-prone and malignant GIST, a number of genes were specifically methylated in malignant-prone GIST and/or malignant GIST (172 genes and 155 genes in malignant-prone and malignant GIST, respectively; 136 genes commonly methylated in both malignant-prone and malignant GIST; figure 2B). These data suggest that DNA methylation in a subset of genes occurs progressively during the malignant process in GIST.

DNA methylation profiling identifies clinically or biologically distinct subgroups of cancers. Recent studies of aberrant DNA methylation in human cancers demonstrated high rates of aberrant promoter methylation in a subset of cancers, termed the CpG island methylator phenotype.[27 28] The number of methylated genes in malignant-prone GIST and malignant GIST showed the existence of a bimodal distribution pattern of methylation; three GIST were extensively methylated, as demonstrated previously in CpG island methylator phenotype-positive tumours[29] (see supplementary figure 2, available online only). These three cases were all malignant GIST; however, no other characteristic features were identified in these cases.

Principal components analysis in gastric GIST and small intestinal GIST

The DNA methylation pattern of these 155 genes in small, malignant-prone and malignant GIST was further investigated in a principal components analysis. The first three principal components accounted for 81.7% (75.5%, 3.9% and 2.3% of the first, second and third components, respectively) of the total variance in terms of DNA methylation status observed with these 155 genes, showing characteristic patterns for each subgroup (figure 2C). In relation to gastric GIST, the variance of malignant-prone GIST was encompassed by that of malignant GIST. This relationship between malignant-prone and malignant GIST was also true for small-intestinal GIST. However, the variance of gastric GIST is different from small-intestinal GIST. We further conducted multivariate two-sample tests to quantify the statistical significance of the difference between samples of stomach and small intestine origin in these 155 methylation

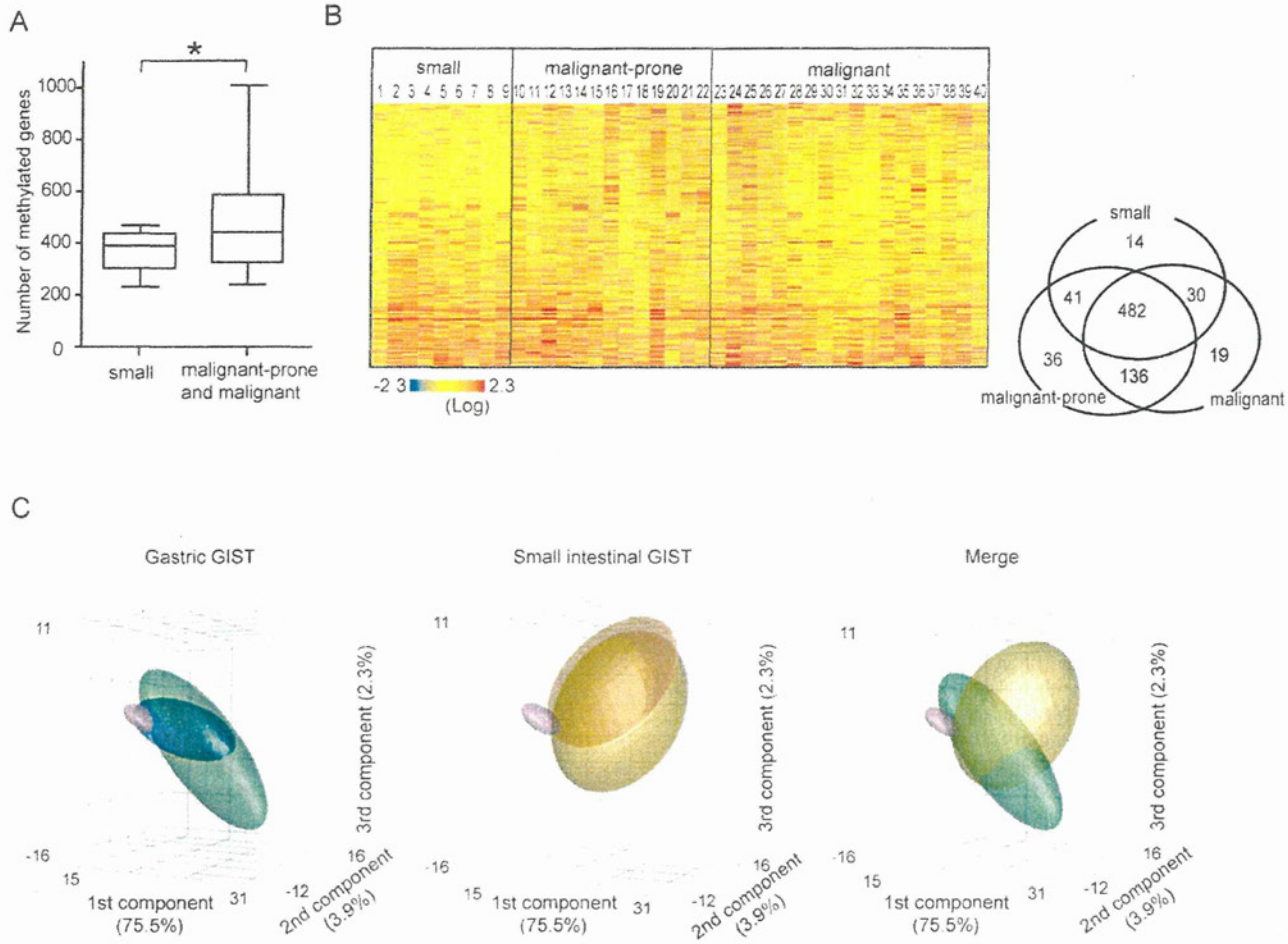

Figure 2 Methylated CpG island amplification microarrays (MCAM) analysis in gastrointestinal stromal tumours (GIST). (A) Box and whisker plots of the number of methylated genes in small GIST, as well as advanced GIST (malignant-prone and malignant GIST). The mean is marked by a horizontal line inside the box whose ends denote the upper and lower quartiles. Error bars represent 5th and 95th percentile values, *p=0.012. (B) Heat-map overview of 766 genes, which are commonly methylated in more than 30% of the cases in either small GIST, malignant-prone GIST or malignant GIST (left). Venn diagram of this set of 766 genes showing the relationship of methylation target genes in the three classified groups (right). A number of genes were specifically methylated in advanced GIST; malignant-prone GIST (172 genes) and malignant GIST (155 genes, highlighted in blue). (C) Principal components analysis of the 155 genes associated with gastric GIST (left) and small intestinal GIST (middle), along with merged image of gastric malignant GIST and small intestinal malignant GIST (right). The first three principal components accounted for 81.7% of the total variance. Ellipsoids of standard deviations (75% probability) are drawn with respect to each group. Magenta, small GIST; deep green, malignant-prone GIST from stomach; green, malignant GIST from stomach; orange, malignant-prone GIST from small intestine; yellow, malignant GIST from small intestine.

patterns. A non-parametric multivariate two-sample test (nearest-neighbour test) revealed that there was a statistically significant difference in the methylation pattern between gastric GIST and small-intestinal GIST (p=0.0478, using its default options with k=1 and correlation similarity measure).[30] These data suggest that, in epigenetic terms, GIST is not a uniform disease, which may be linked to the observed variable outcome of patients with GIST from different locations.[1]

Identification of prognostic markers in GIST

DNA methylation has been proposed as a diagnostic and/or prognostic marker for malignancies.[14] We next investigated whether DNA methylation status could be a prognostic marker for GIST. We examined the MCAM data relating to the above-mentioned set of 155 genes using a statistical model based on recursive descent partition analysis in order to distinguish the malignant GIST from small GIST (figure 3A, supplementary figure 3, available online only).[31] In the training set, two genes, *REC8* and *paired box 3 (PAX3)*, were found to be potent markers for identifying the malignant GIST (see supplementary figure 3, available online only). Of the 27 cases in the training set (malignant GIST, n=18; small GIST, n=9), 17/18 (94%) of malignant GIST were methylation positive (signal intensity of GIST/control >2.0 by MCAM) in either of the two genes, while eight out of nine (89%) small GIST were methylation negative (signal intensity of GIST/control ≤2.0 by MCAM) in both of the two genes (see supplementary figure 3, available online only and figure 3A). The methylation status of these two genes in the 40 GIST was confirmed by pyrosequencing analysis. The *REC8* and *PAX3* genes were found to be significantly more methylated in malignant GIST compared with small GIST (p=0.003 and p=0.026, respectively). A subset of malignant-prone GIST also showed substantial DNA methylation of these two genes (figure 3B and supplementary table 3, available online only). Interestingly, neither of these genes was observed to be expressed in the normal muscle layer, without evidence of substantial DNA methylation (see supplementary figure 4, available online only).

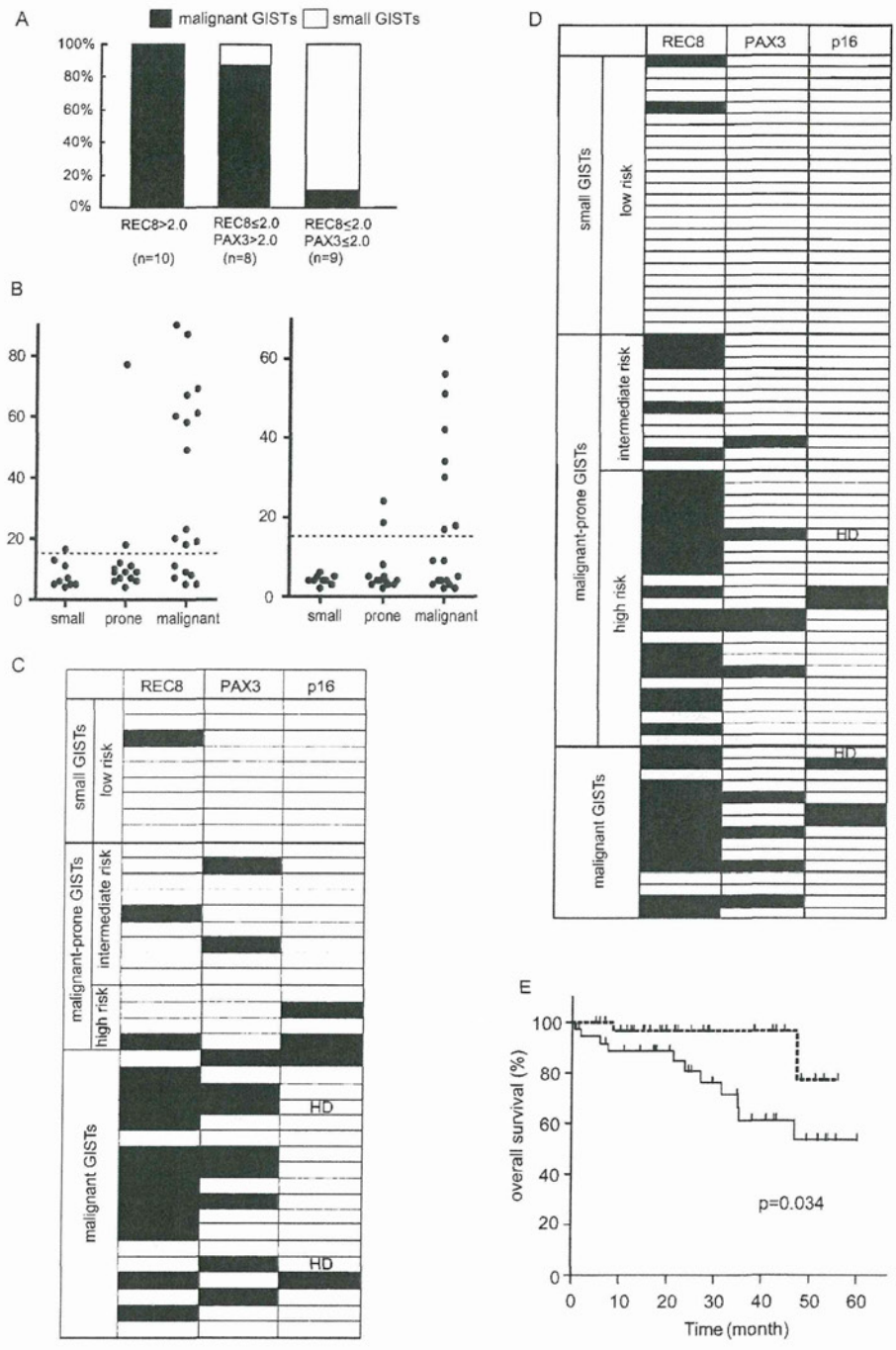

Figure 3 Identification of diagnostic and prognostic markers for advanced gastrointestinal stromal tumours (GIST). (A) *REC8* and *PAX3* are effective markers to distinguish malignant from small GIST on the basis of methylated CpG island amplification microarrays (MCAM) data in the training set (see supplementary figure 3, available online only). (B) DNA methylation status of *REC8* and *PAX3* in the test set (n=40) (nine small GIST, 13 malignant-prone GIST and 18 malignant GIST). The format of the scatter plot is the same as figure 1. Broken lines indicate a methylation level of 15%. (C), (D) Methylation frequencies of GIST patients. Each column represents the methylation status of three genes of interest, namely *REC8*, *PAX3* and *p16*, within individual GIST, either small, malignant-prone or malignant. Black boxes denote methylation positive, whereas white boxes indicate no evidence of methylation. Homozygous deletions (HD) were detected, on occasion at the *p16* locus (C), in the test set (n=40) and (D) in the validation set (n=75). (E) Kaplan–Meier analysis of overall survival in cases of GIST with and without DNA methylation in at least one of the *REC8*, *PAX3* or *p16* genes. Broken and solid lines indicate GIST without (n=32) and with methylation (n=37), respectively (p=0.034).

Although our statistical model showed that *REC8* and *PAX3* might be potent markers for the prediction of malignant GIST, we added *p16* to further analyses, because we found that methylation of this gene was also specific to advanced GIST (figure 1 and supplementary table 3, available online only). Indeed, a relationship between genetic alterations or DNA methylation of *p16* and the prognosis of GIST has been suggested previously.[32 33] The frequency of aberrant DNA methylation in at least one of the three markers (*REC8*, *PAX3* or *p16*) was found to be one out of nine (11%), five out of 13(38%) and 15/18 (83%) in small, malignant-prone and malignant GIST, respectively (figure 3C).

Validation analysis of the potential prognostic markers identified

The relationships between clinical stratifications and the three putative prognostic markers, *REC8*, *PAX3* and *p16*, were independently confirmed in the validation set.

The sample in the training set provided adequate statistical power (96.4%) to detect a between-groups difference (ie, DNA methylation frequencies of the three genes were one out of nine (11%) and 15/18 (83%) in small GIST and malignant GIST, respectively; α=0.05 for two-sided test). Consequently, we calculated the sample size of the validation set necessary to detect a between-groups difference as was found in the training set. Our assumptions were a statistical power of 0.9, an α level

of 0.05 for a two-sided test and a similar proportion of the malignant GIST group and the small GIST group to the training set. According to these assumptions, the calculated results indicated that we required a minimum of eight, 12 and 16 cases of the small GIST, the malignant-prone GIST and the malignant GIST, respectively. Our validation set (n=75) fulfils these criteria and provided statistical power of 99.8% to detect the between-groups difference (α=0.05 for two-sided test).

We investigated the methylation status of these three markers by pyrosequencing analysis. DNA methylation of the three markers in small, malignant-prone and malignant GIST in the validation set progressively increased from small to malignant GIST (8%, 69% and 80% in small, malignant-prone and malignant GIST, respectively, as shown in figure 3D and supplementary table 5, available online only). The methylation status of these three markers could effectively distinguish malignant and small GIST in the validation set (sensitivity 80%; specificity 92%; positive predictive value 86%; negative predictive value 88%), confirming the validity of these markers. Malignant-prone GIST in our classification consist of two risk groups,[3] namely intermediate and high risk (table 1). Intriguingly, in the validation set, the frequency of aberrant DNA methylation in at least one of the three markers in the high-risk group (19/24, 79%) was higher than in the intermediate-risk group (six out of 12, 50%, p=0.125). This trend is significant in the total 115 GIST cohort (nine out of 21, 43% in the intermediate-risk group vs 21/28, 75% in the high-risk group, p=0.038). The methylation status of the three markers could effectively distinguish malignant and small GIST in the total cohort of 115 samples (sensitivity 82%; specificity 91%; positive predictive value 90%; negative predictive value 83%).

As *p16* was identified using different methods, we evaluated whether adding *p16* to the two markers (*REC8* and *PAX3*) was advantageous for the prediction of malignant GIST. Specificity, sensitivity, positive predictive value and negative predictive value for the prediction of malignant GIST determined by the three markers including *p16* were identical with those determined by *REC8* and *PAX3* in both the training and validation sets (figure 3C,D). However, the frequency of DNA methylation in at least one of the three markers was higher than that in either *REC8* or *PAX3* in the high-risk group GIST, which may have a high chance of progression to malignant GIST (21/28, 75% vs 19/28, 67%; figure 3C,D). In addition, DNA methylation of *p16* was only found in the high-risk group or malignant GIST. Therefore, aberrant DNA methylation of the three markers including *p16* was significantly associated with clinical classification in both the training set and the validation set (table 2).

Relationship between DNA methylation status of the three markers identified and disease outcome in GIST

To investigate whether the status of DNA methylation in these three markers impacts on overall survival, Kaplan–Meier survival analysis was performed in 69 patients from either training set or validation set whose survival information was available. Log-rank tests revealed that patients with GIST displaying no methylation (n=32) survived significantly longer than those with at least one gene methylated (n=37; p=0.034) (figure 3E). We evaluated the statistical power for the survival analysis comparing survival rate at the median follow-up time of the groups with and without methylation (76.1%, n=37 and 96.6%, n=32) and found that our sample provided 74.4% statistical power (one-sided, 5% log-rank test).

Table 2 Relationship between DNA methylation status of three genes and clinical background

	Training set (n=40)			Validation set (n=75)		
		No of cases with specific methylation			No of cases with specific methylation	
Feature	No of cases	No of cases with specific methylation (%)	p Value	No of cases	No of cases with specific methylation (%)	p Value
Sex						
Male	19	12 (63)	0.2248	43	26 (60)	0.1513
Female	21	9 (43)		32	14 (44)	
Age, years						
<60	19	11 (58)	0.5158	22	13 (59)	0.5196
≥60	21	10 (48)		53	27 (51)	
Tumour origin						
Stomach	23	9 (39)	0.0149	43	18 (42)	0.2590
Small intestine	15	12 (80)		25	19 (76)	
Rectum	2	0 (0)		6	2 (33)	
Oesophagus	0	0 (0)		1	1 (100)	
Size, cm*						
<5	15	4 (27)	0.0189	31	8 (26)	0.1564
≥5	23	16 (70)		44	32 (73)	
Mitotic index/50 HPF*						
<5	18	6 (33)	0.0732	42	14 (33)	0.0006
≥5	14	10 (71)		28	21 (75)	
Classification						
Small			0.0024			0.0001
Low risk	9	1 (11)		24	2 (8)	
Malignant-prone						
Intermediate risk	9	3 (33)		12	6 (50)	
High risk	4	2 (50)		24	19 (79)	
Malignant						
Metastasis	18	15 (83)		15	12 (77)	

*Clinical data of some patients were unavailable.
HPF, high-power fields.

Table 3 Univariate and multivariate Cox regression analysis for overall survival (n=69)

	Univariate Cox regression analysis		Multivariate Cox regression analysis	
Factor	**HR (95% CI)**	**p Value**	**HR (95% CI)**	**p Value**
Tumour origin*	3.381 (1.487 to 8.004)	0.004	5.045 (1.721 to 18.266)	0.003
Marker†	4.448 (1.192 to 28.771)	0.024	4.579 (1.053 to 33.517)	0.042
Mitotic index (>5/50 HPF)	3.723 (1.134 to 16.645)	0.029	3.046 (0.849 to 14.906)	0.090
Tumour size (>5 cm)	2.470 (0.804 to 9.118)	0.116	1.136 (0.351 to 4.394)	0.838
Sex	0.809 (0.244 to 2.432)	0.709		
Age (≥60 years)	1.163 (0.376 to 4.309)	0.801		

*Gastrointestinal stromal tumours in the small intestine have a worse prognosis than the others.
†Aberrant DNA methylation in at least one of the three genes (*REC8*, *PAX3* or *p16*).
HPF, high-power field.

Using univariate Cox regression analysis, overall survival was correlated with tumour origin, methylation status of the three markers and mitotic index (p=0.004, p=0.024 and p=0.029, respectively; table 3). In addition to tumour size, these factors were entered into the multivariate Cox regression analysis, proving to be valid as independent prognostic factors in predicting overall survival. Tumour origin and methylation status were defined as independent prognostic factors in this analysis (HR 5.045, p=0.003 and 4.579, p=0.042, respectively). It has been suggested that small-intestinal/rectal GIST are more aggressive than gastric GIST of equal size.[1] We also performed univariate and multivariate Cox regression analyses using different size criteria for the categorical values of small-intestinal/rectal GIST (≥2 cm) and gastric GIST (≥5 cm) (see supplementary table 6, available online only). This analysis also defined tumour origin and methylation status as independent prognostic factors (HR 7.414, p=0.003 and 5.990, p=0.023, respectively).

DISCUSSION

This study reports, for the first time, the global DNA methylation profiles of GIST and associated correlations with clinical features. Although the impact of DNA methylation on tumour behaviour has been demonstrated in many human cancers,[13] its association with the malignant progression of GIST has not been well understood. We examined the DNA methylation status of *p16*, *RASSF1A*, *CDH1* and *MGMT*, each of which have a tumour-suppressor function and have been studied previously in GIST samples.[34 35] Among these markers, DNA methylation of *RASSF1A* increased progressively from small to malignant GIST, while *p16* was specifically methylated in malignant-prone and malignant GIST, implying that *p16* methylation can be a specific marker for advanced GIST. Consistent with our data, inactivation of p16 has been suggested as a poor prognosis marker in GIST.[32 33]

We found that DNA methylation accumulates genome wide during GIST progression. This finding was also supported by data reflecting the overall global DNA methylation status, showing that aberrant *LINE-1* methylation was most apparent in malignant GIST as was also reported very recently by others.[26] In contrast to all the small GIST, which showed less DNA methylation than malignant GIST, a subset of malignant-prone GIST shows a similar methylation profile to that displayed by malignant GIST. The outcome of these malignant-prone GIST is now under investigation; indeed, planned longer-term observation will reveal whether such malignant-prone GIST displaying frequent evidence of DNA methylation may progress to invasive or metastatic GIST.

The anatomical location of GIST has been reported to affect the clinical outcome of this tumour. Small intestinal GIST are known to be more aggressive than gastric GIST of equal size, with this being factored into the risk assessment of a primary tumour.[1] In the current study, principal components analysis showed that the DNA methylation profiles of gastric and small intestinal GIST were consistently different. In addition, multivariate Cox regression analysis proved tumour location to be an independent prognostic factor. Taken together, these data suggest that GIST represent a family of closely related but distinct tumour subtypes, rather than a single pathological entity,[36] in which characteristic targets are epigenetically regulated and, therefore, may show different clinical outcomes.

In addition to the pathogenic implications, DNA methylation profiles represent a chemically and biologically stable source of molecular diagnostic information. Recent technology has been developed to perform genome-wide screening for altered DNA methylation profiles, which can identify new candidate biomarkers for use in diagnosis and determining prognosis.[13 14] In the current study, we identified three potent methylation-based markers, namely *REC8* and *PAX3* from MCAM analysis and *p16* from pyrosequencing analysis. PAX3 is a member of the paired box family of transcription factors.[37] REC8, a key component of the meiotic cohesion complex, limits synapsis between homologous chromosomes during meiosis.[38] The methylation status of these three markers could effectively distinguish malignant and small GIST. In addition, the methylation status of GIST is closely associated with prognosis. Therefore, we propose here that the methylation status of these markers may potentially serve as a biomarker predicting aggressive GIST with poor survivability. Notably, the negative predictive value in the total cases is 83%, suggesting that 17% of the GIST patients who are diagnosed as being negative based on these three markers actually have malignant potential. Validation studies of the relevance of these methylation marker genes using larger independent cohorts is necessary for a further evaluation of their promise as diagnostic and prognostic markers for malignant GIST. Nevertheless, a multidisciplinary effort is desired to elucidate better ways to overcome the current limitations of prediction for malignant GIST.

Interestingly, the expression of *REC8* and *PAX3* was found to be silenced within the normal muscle layer, without evidence of substantial DNA methylation. However, this is not surprising, because many methylation events in malignant cells occur at the promoters of genes that are not tumour suppressors, and the majority of these genes are actually already repressed in normal tissues.[39] It seems that CpG islands targeted by aberrant DNA methylation in malignant cells are determined by a pre-programmed targeting mechanism.[40] Studies have demonstrated that the existence of stalled or active RNA polymerase II (Pol II) on promoter regions might be a cue to determine the epigenetic fate of promoter DNA methylation.[40–42] The

presence of Pol II in normal cells or embryonic stem cells may be associated with resistance to DNA methylation in cancer cells. Indeed, the difference in embryonic stem cell Pol II marks (data were obtained from a previous paper)[41] between the 766 methylated genes (see figure 2B) and 5391 unmethylated genes in GIST is highly significant (235/766 genes, 30% vs 2002/5391 genes, 37%; Pearson's test, p=0.0005). The susceptible genes (non-Pol II targets) can be a target of DNA methylation in malignant cells in which the precise control of epigenetic regulation is highly disrupted.[43] The identified genes may thus not be tumour suppressors; rather, they may be sensitive markers for malignant GIST. Similar results have been demonstrated showing the usefulness of marker genes, which were unlikely to confer growth advantage, for characterisation of tumours.[44 45]

In conclusion, here we decipher the epigenetic abnormalities contributing to the malignant progression of GIST. We show that DNA methylation profiles may be a potential new biomarker of risk prediction in GIST. The detection of methylation markers in easily accessible biological materials such as serum or sputum has potential usefulness for the diagnosis of human malignancies [13 14] The endoscopic ultrasound-guided fine-needle aspiration technique is accurate and efficient for adequate sampling of GIST. We initially examined the DNA methylation status of samples obtained by fine-needle aspiration in comparison with the corresponding surgical samples of GIST. DNA methylation status in those paired samples was quite consistent (see supplementary table 7, available online only). As pyrosequencing analysis is a convenient and reproducible technique to evaluate DNA methylation levels, it might be possible for the hospital laboratory to perform this technique as a new diagnostic tool for risk assessment of GIST. Our findings in the current study combined with the endoscopic ultrasound-guided fine-needle aspiration technique may be capable of evaluating the risk of progression of GIST, and would be of great help for determining the appropriate therapeutic management of this disease.[46]

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Aberrant DNA methylation associated with aggressiveness of gastrointestinal stromal tumour

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RESEARCH ARTICLE

Hypermethylation of Sox17 gene is useful as a molecular diagnostic application in early gastric cancer

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Abstract Although minimal invasive treatment is widely accepted in the early stages of gastric cancer (GCa), we still do not have any appropriate risk markers to detect residual neoplasia and the potential for recurrence. We previously reported that aberrant DNA methylation is an early and frequent process in gastric carcinogenesis and could be useful for the detection of gastric neoplasia. Our goal is to find and identify some candidate genes, using genome-wide DNA methylation analysis, as a treatment marker for early gastric cancer (EGC). We performed methylated CpG island amplification microarray analysis using 12 gastric washes (six each of pre- and post-endoscopic treatment in each of the same patients). We finally focused on Sox17 gene. We examined the DNA methylation status of Sox17 in a validation set consisting of 128 wash samples (pre, 64; post, 64) at EGC. We next carried out functional studies to identify Sox17. Sox17 showed significant differential methylation between pre- and post-treatments in EGC patients (Sox17, $p<0.0001$). Moreover, treating GCa cells that lacked Sox17 expression with a methyltransferase inhibitor, 5-aza-2′-deoxycytidine, restored the gene's expression. Additionally, the introduction of exogenous Sox17 into silenced cells suppressed colony formation. Gastric wash-based DNA methylation analysis could be useful for early detection of recurrence following endoscopic resection in EGC patients. Our data suggest that the silencing of Sox17 occurs frequently in EGC and may play a key role in the development and progression of the disease.

Keywords Sox17 · DNA methylation · Gastric cancer · Gastric washes

Abbreviations

GCa	Gastric cancer
EGC	Early gastric cancer
5-aza-dC	5-aza-2′-Deoxycytidine
Sox17	SRY (sex determining region Y)-box 17
MCAM	Methylated CpG island amplification microarray
ER	Endoscopic resection

Introduction

Gastric cancer (GCa) is the second leading cause of cancer death in the world. Its prognosis is determined by clinical

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staging at diagnosis and treatment [1–3]. Diagnostic tools such as gastrointestinal endoscopy followed by pathological analysis and/or fluoroscopy have proven useful; however, the mortality rate has remained high throughout the world. The sensitivity and specificity of gastrointestinal (GI) endoscopy is high, but its diagnostic accuracy depends on the technical skill of the endoscopist—especially with artificial stomachs which have been partially resected by endoscopic or surgical treatment. Endoscopic biopsy is a topical procedure whereby only a small portion of abnormal tissue is removed. It can be difficult to determine which tissue layer to remove, thus occasionally leading to a misdiagnosis. Moreover, GI endoscopy is neither comfortable nor risk-free for patients, and it is associated with frequent morbidity. Furthermore, gastric cancer is more prevalent among elderly patients, who are likely to be taking medications such as antiplatelet or anticoagulant drugs, which further complicate the procedure.

The need for less invasive and more efficient diagnostic tools has led to a search for GCa antigens [4, 5]. However, we now know that common biomarkers such as CEA are not found frequently enough to yield high specificity or sensitivity assays. Molecular markers that distinguish benign from clinically silent malignant diseases are needed to reduce the number of unnecessary endoscopic biopsies and to improve detection of gastric dysplasia and gastric cancer at an early stage.

Endoscopists obtain gastric washes for analysis by washing around the stomach mucosa with a saline solution during routine endoscopic examination. Given that an abundant amount of cells are exfoliated into the washes and undamaged DNA recovered from the washes can be assayed with sensitive and quantitative techniques, there is a strong biological rationale to pursue this emerging technology [6].

Cytosine DNA methylation is an important epigenetic change which leads to the recruitment of transcription repressors and chromatin changes. During the development and progression of GCa, many genes are silenced by aberrant methylation of CpG islands, which are CpG dinucleotide-rich areas located within the promoters of approximately 60% of human genes [7]. Aberrant DNA methylation occurs more frequently than mutations in GCa [8–14]. Studies have detected cancer-specific DNA methylation in stool, blood plasma, urine, and pancreatic juice in several different cancers [15, 16]. Furthermore, concordant promoter hypermethylation of multiple genes, which is described as the "CpG island methylator phenotype", has been found in both gastric and colorectal carcinomas [17–22]. Therefore, these methylation markers could be useful for detecting field cancerization in this disease [23–25].

Recently, methylated CpG island amplification microarray (MCAM)-based genome-wide DNA methylation profiles have been available for analyzing primary neoplasms [26–30]. Although some candidate genes have been reported to undergo alterations in DNA methylation in GCa, such known genes are still limited for early gastric cancer (EGC) [18, 31–39]. We reported that gastric wash-based methylation analysis is useful for the detection of primary gastric neoplasia [6]. We hypothesized that gastric wash-based genome-wide methylation analysis could potentially be useful for selecting candidate genes in EGC. Additionally, gastric washes include large amounts of DNA recovered from cells on the surface of the stomach, making it simple to collect DNAs from patients endoscopically both pre- and post-minimal surgery treatment such as endoscopic resection (ER) [40, 41]. Comparing DNA methylation levels in each of the patients pre- and post-treatment is significant, as the selection of non-biased (i.e., aging, chronic inflammations, and *Helicobacter pylori* infections) EGC-specific genes, with alterations in DNA methylation, may become easier. Our goal is to find and identify some candidate genes as a treatment marker using genome-wide DNA methylation analysis for EGC.

Materials and methods

Cell lines

Seven GCa cell lines (MKN1, MKN7, MKN45, MKN74, NUGC3, KatoIII, and AZ521) were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Japanese Collection of Research Bioresources (Tokyo, Japan). All cell lines were maintained in appropriate media containing 10% fetal bovine serum in plastic tissue culture plates.

Patient characteristics

Twelve test sets and 128 validation sets obtained from 140 gastric washes were collected from patients who underwent endoscopic resection for EGC at St. Marianna University School of Medicine Hospital (Kanagawa, Japan) from March 2005 to February 2010. In addition to the tumor samples, non-neoplastic gastric washes were collected from 32 age-matched patients who underwent endoscopic examination and were diagnosed with gastritis (Table 1). The study was conducted in accordance with all rules and regulations of the St. Marianna University School of Medicine Institutional Review Board (#1498), and informed consent was obtained from each patient.

Sample collection of gastric washes

To obtain gastric washes, patients were required to swallow a liquid solution (100 ml of water containing 80 mg of

Table 1 Clinical features of patients (cancer $n=70$, non-neoplastic $n=32$)

	Test set	Validation set			
	$n=6$	$n=64$	Average	Sox17 gene methylation (%)	p value
Age ($n=70$)			71.3±8.4		$p=0.70$
Male ($n=50$)	3	47	70.2±8.3	20.2±9.8	
Female ($n=20$)	3	17	74.4±8.0	22.0±11.2	
Endoscopic appearance					$p=0.67$
Polypoid	0	5		18.9±7.6	
Slightly elevated	6	29		20.4±9.3	
Flat	0	1		11.5±0.0	
Slightly depressed	0	29		21.5±11.5	
Histology (adenocarcinoma)					$p=0.66$
Well differentiated	6	44		20.3±10.1	
Moderately differentiated	0	20		21.4±10.4	
Stage					N/A
I	6	64		20.6±10.1	
II/III/IV	0	0		N/A	
Helicobacter pylori infection					$p=0.76$
Positive	3	43		20.7±10.5	
Negative	3	21		20.6±9.7	
Locations (stomach)					$p=0.35$
Upper body	0	11		17.6±7.8	
Middle/lower body	6	53		21.3±10.5	
Atrophy					$p=0.80$
Closed type	0	18		20.4±10.5	
Open type	6	46		20.8±10.1	
Intestinal metaplasia					$p=0.23$
Positive	6	54		21.3±10.5	
Negative	0	10		17.1±7.6	
Tumor size (square measure)			341.3±611.0		$p=0.51$
<341.3	6	48	108.4±96.9	20.9±10.1	
□341.3	0	16	1,040.0±919.1	20.0±10.6	
Control set					
Gastritis	0	32		13.6±6.8	

dimethylpolysiloxane [Gascon: Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan], 1 g of sodium bicarbonate, and 20,000 U of pronase [Pronase MS: Kaken Pharmaceutical Co., Ltd., Tokyo, Japan]) approximately 10 min prior to endoscopic examination. Gastric washes were aspirated through the suction channel of the endoscope into specimen collection containers (No. 16200BZZ00045: Nippon Sherwood, Tokyo, Japan). The containers were directly connected to the endoscope modulator, and the washes were vacuumed manually. The samples were then immediately centrifuged and the pellets were frozen at −80°C. DNA was extracted using the standard phenol–chloroform method. The concentration and quantity of all DNA extracted from gastric washes were measured using the NanoDrop spectrophotometer (ND-1000 Spectrophotometer; Nano Drop Technologies, Wilmington, DE, USA).

After the collection of gastric washes, biopsies were carried out using biopsy forceps (Radial Jaw: Boston Scientific Corp., Natick, MA, USA) under endoscopic guidance with a GIF-Q260 endoscope using the EVIS LUCERA system (Olympus, Inc., Tokyo, Japan). Mucosal samples (approximately 5 mm in diameter each) of the gastric body and antrum were collected by biopsy. The rapid urease test was performed on two biopsy specimens using the PyloriTek test kit for *H. pylori* detection in the control set (Serim Research Corp. Elkhart, IN, USA). We used disposable sample collection tubes, connector tubes, and endoscopic devices. The endoscope

was washed with an automatic washing machine and disinfectant (DISOPA Solution 0.55%, Johnson and Johnson, Langhorne, PA, USA) after each patient according to the guidelines.

DNA and RNA preparation

DNA was extracted using the standard phenol–chloroform method from gastric cancer cell lines, gastric washes, and microdissected formalin-fixed paraffin-embedded (FFPE) tissues. Total RNA was extracted from the harvested cells using the Trizol (Invitrogen, Carlsbad, CA, USA) from gastric cancer cell lines. We also extracted an RNA sample from each of the 10 patients using Trizol (Invitrogen, Carlsbad, CA, USA) from endoscopically resected FFPE tissues in the validation set of EGC samples.

Methylated CpG island amplification microarray

For MCAM analysis, we analyzed 12 samples of primary EGC samples (six each of pre- and post-endoscopic resection in each of the same patients) using a test set. A detailed protocol of methylated CpG island amplification (MCA) was described previously [42]. We used a custom human promoter array (G4426A-02212; Agilent Technologies, Santa Clara, CA, USA) containing 36,579 probes corresponding to 9,021 unique genes. The probes on the array were selected to recognize *SmaI*/*XmaI* fragments, mostly around gene transcription start sites.

Five micrograms of genomic DNA was digested with 100 U of methylation-sensitive restriction endonuclease *SmaI* (New England Biolabs, Ipswich, MA, USA) for 24 h at 25°C, which cuts unmethylated DNA and leaves blunt ends (CCC/GGG). Subsequently, the DNA was digested with 20 U of methylation-insensitive restriction endonuclease *XmaI* for 6 h at 37°C, creating sticky ends (C/CCGGG). Five hundred milligrams of digested DNA was ligated using 50 μl of RMCA12 (5′-CCGGGCAGAAAG-3′) / RMCA24 (5′-CCACCGCCATCCGAGCCTTTCTGC-3′) primer and T4 DNA ligase (TaKaRa Bio Inc., Shiga, Japan) for 16 h at 16°C. After filling in the overhanging ends of the ligated DNA fragments at 72°C, the DNA was amplified at 95°C for 5 min followed by 25 cycles of 1 min at 95°C and 3 min at 77°C using 100 pmol of RMCA24 primer. MCA products were labeled with Cy5 (red) for DNA from pre-ER (both adjacent normal and tumor cells found in DNA from gastric washes) and Cy3 (green) for DNA from post-ER (only adjacent normal cells found in DNA from gastric washes) using a random primed Klenow polymerase reaction (Invitrogen) at 37°C for 3 h; 4×44 K human CpG island arrays were purchased from Agilent Technologies. Microarray protocols, including labeling, hybridization, and post-hybridization washing procedures, can be found at http://www.agilent.com/. Labeled samples were then hybridized to arrays in the presence of human Cot-1 DNA for 24 h at 65°C. After the washing procedures, arrays were scanned on an Agilent DNA Microarray Scanner and analyzed using Agilent Feature Extraction software (FE version 9.5.1.1, Agilent Technologies) at St. Marianna University School of Medicine. We used GeneSpring software (Agilent) for choosing candidate genes after normalization of the raw data.

Hierarchical clustering analysis

Unsupervised hierarchical clustering was done using an agglomerative hierarchical clustering algorithm following Lowess normalization. For specimen clustering, pairwise similarity measures among specimens were calculated using GeneSpring GX software based on the DNA methylation intensity measurements across all genes (version 11, Agilent Technologies).

DNA methylation analysis

Bisulfite polymerase chain reaction (PCR) was performed using an EpiTect Bisulfite Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. One microliter of bisulfite-treated DNA was used as a template. All of the primers used for amplifying promoter CpG DNA fragments of the gene are described in Supplementary Table 1. After PCR, the biotinylated strand was captured on steptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers (Supplementary Table 1). Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given promoter. These adjacent sites usually show highly concordant methylation. Therefore, the mean percentage of methylation at detected sites was used as a representative value for each gene promoter.

Trichostatin A and 5-aza-2′-deoxycytidine treatment of cells

To analyze restoration of each gene expression, cell lines were incubated for 96 h with 1 or 5 μM of 5-aza-2′-deoxycytidine (5-aza-dC) and/or 200 nM of trichostatin A (TSA) after which they were harvested and their RNA was extracted for further analysis.

Reverse transcription-polymerase chain reaction

First-strand cDNA was prepared by reverse transcription of 1-μg samples of total RNA using Superscript III Reverse Transcriptase (Invitrogen). Real-time quantitative reverse transcription-PCR was carried out using Taqman Gene Expression Assays [Sox17, Hs00751752_s1, and glyceraldehyde-3-phosphate dehydrogenase, Hs_00266705_g1 (Applied

Biosystems)] with an ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS2.1 software (Applied Biosystems) was used to do comparative delta-Ct analysis. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control.

Colony formation assays

Cells (0.5×10^5) were plated in 2-cm^2 culture dishes for 24 h before transfection with the expression vector Myc-DDK-tagged pCMV6-Sox17 expression vector or empty vector (RC220888 and PS100001, OriGene Technologies, Rockville, MD, USA) using 2 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, cells were preserved for 14 days in a medium containing 0.2 mg/ml G418 for MKN74, 0.6 mg/ml G418 for MKN45 and stained with Giemsa. The resultant colonies were then stained with crystal violet and cells were counted in triplicate cultures using NIH Image software. Western blotting was carried out using anti-Sox17 antibody (AF1924, R&D Systems, Minneapolis, MN, USA) and anti-DDK monoclonal antibody (TA50011, OriGene Technologies).

Statistical analysis

Methylation levels (percentage) were analyzed as a continuous variable for comparison of Sox17 genes. The mean and 95% confidential intervals were calculated for clinicopathologic features. The correlation of DNA methylation between gastric wash samples and formalin-fixed, paraffin-embedded tissues were analyzed using the Mann–Whitney test for continuous variables and $p<0.05$ was considered significant. All statistical analyses were performed using PRISM software for Windows, version 4 (GraphPad Prism, Inc., San Diego, CA, USA).

Results

Clinicopathologic characteristics of patients with EGC

A total of 140 gastric wash samples from 70 primary EGC patients were analyzed including 5 polypoid type, 35 (6+29) slightly elevated types, 1 flat type, and 29 slightly depressed types. The samples were analyzed histologically including 50 (6+44) well-differentiated adenocarcinoma and 20 moderately differentiated adenocarcinoma at the St. Marianna University School of Medicine (50 (3+47) men and 20 (3+17) women; average age, 71.3±8.4 years) (Table 1). All samples were reviewed by an expert gastrointestinal pathologist. Thirty of the 140 FFPE slides were obtained from the pathology archives of St. Marianna University School of Medicine. The 140 test samples and validation sets collected both included stage I, differentiated adenocarcinoma samples that are approved in standard ER therapy by The Japanese Gastric Cancer Association gastric cancer treatment guidelines [43]. A control set of non-neoplastic gastric washes from 32 age-matched patients who underwent endoscopic examination had gastritis (20 men and 12 women; average age, 70.1±4.2 years) (Table 1). Only one case was diagnosed to have residual cancer due to tumor cells found in the vertical margin of the resected specimen. Otherwise, we did not find any residual/recurrent disease, even in short-term follow-up.

Sox17 selected as a candidate gene for EGC detection by MCAM analysis

To compare the gastric wash-based global DNA methylation profiles of pre- and post-ER, we analyzed 12 test set samples from six patients (two samples/patients) using MCAM. A Cy5/Cy3 (pre-ER/post-ER) signal in excess of 1.0 (Log2 ratio) in MCAM was considered methylation-positive in three out of six cases (Fig. 1a). Eighteen probes (18/36,579) corresponding to 11 unique genes (11/9,021) were selected as candidate genes after calculations were made using GeneSpring GX software (version 11, Agilent Technologies) based on the DNA methylation intensity measurements (data not shown) (Fig. 1b). One of the probe is the Sox17 (SRY (sex determining region Y)-box 17) gene (Fig. 1b).

We next used Ingenuity Pathway Analysis to analyze all of the data (IPA version 8.0, Ingenuity Systems, Inc. Redwood, CA, USA). All of our microarray data (36,579 probes) were uploaded to IPA software to identify significantly related biological networks (31,125 probes were mapped). Functional classification of differentially methylated genes indicated that a cluster of genes were involved in EGC. In "biological functions from statistical analysis", these top ranking categories suggested interesting functions, such as "Cancer", "Developmental Disorder", and "Genetic Disorder" in the Disease and Disorders category; "Tissue Development", "Organ Development", "Organismal Development", and "Embryonic Development" in Physiological System Development and Function category; and "Molecular Mechanisms of Cancer", "Ephrin Receptor Signaling", "Human Embryonic Stem Cell Pluripotency", and "Wnt/β-catenin Signaling" in the Canonical Pathways category (Supplementary Table 2).

In the end, we focused on the Sox17 gene because it has a CpG island in its promoter region and is known to be associated with both "Cancer" and "Wnt/ β-catenin signaling pathway" in gastric neoplasia (Fig. 4a) [44]. We next made Sox17 pyrosequencing primer to confirming MCAM data using 12 test sets from the EGC samples. High concordance was observed between the methylation status of genes

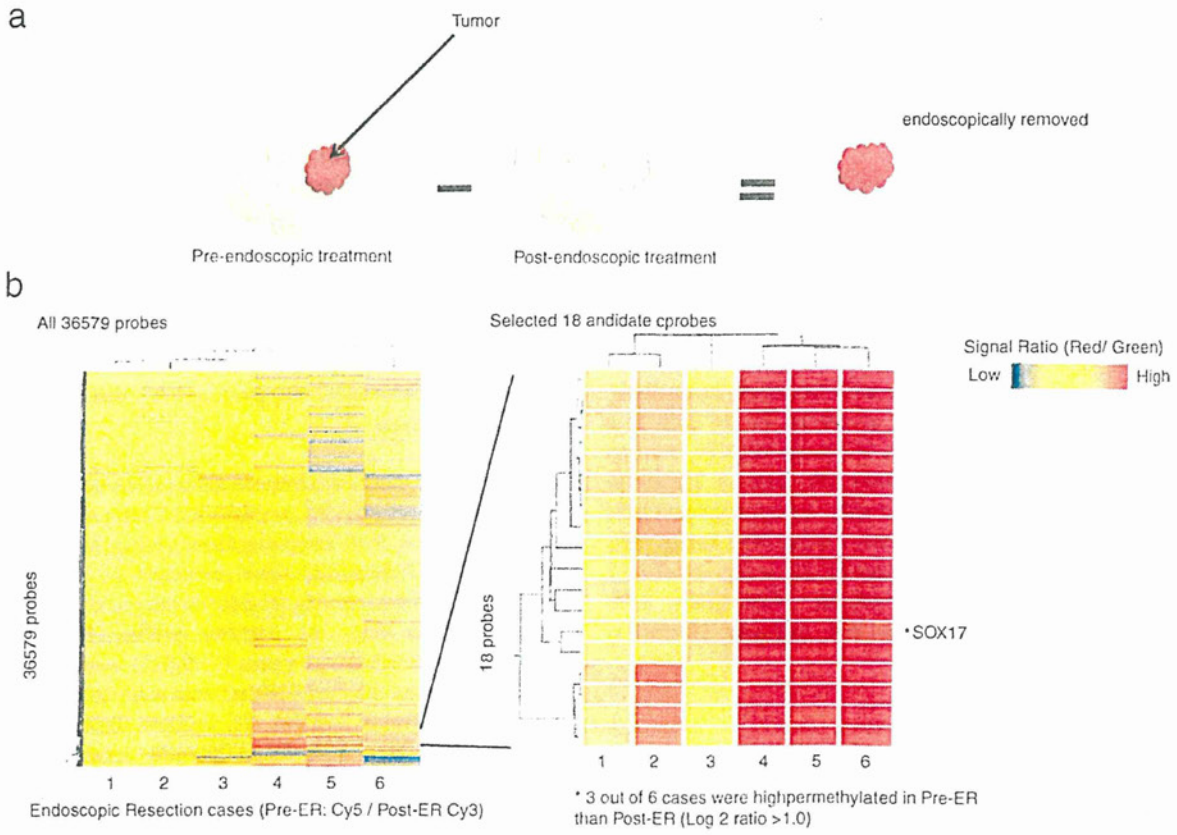

Fig. 1 Schema of our strategy and hierarchical clustering view. a We hypothesized that by comparing methylation levels of gastric washes from pre- and post-ER, we could more efficiently extract cancer-specific genes into the whole genome. b Sox17 gene was selected after hierarchical clustering by MCAM analysis. Three out of six patients were more highly methylated in pre-ER than in post-ER

identified by MCAM and pyrosequencing analysis in the test set ($p<0.05$, $r=0.81$).

Sox17 methylation may be useful as therapeutic marker for EGC

To further evaluate gastric wash-based DNA methylation of Sox17 genes, we next carried out quantitative bisulfite-pyrosequencing analysis using a larger panel of validation sets (128 samples). The Sox17 gene showed significantly higher methylation levels in pre-ER than post-ER (pre, $n=64$; post, $n=63$; excluded, $n=1$; Fig. 2, example in Fig. 3a). Pathological findings were diagnosed by an expert gastrointestinal pathologist. In the incomplete ER case, gastric wash-based Sox17 methylation showed no decrease in a level, and the pathologist also found that additional

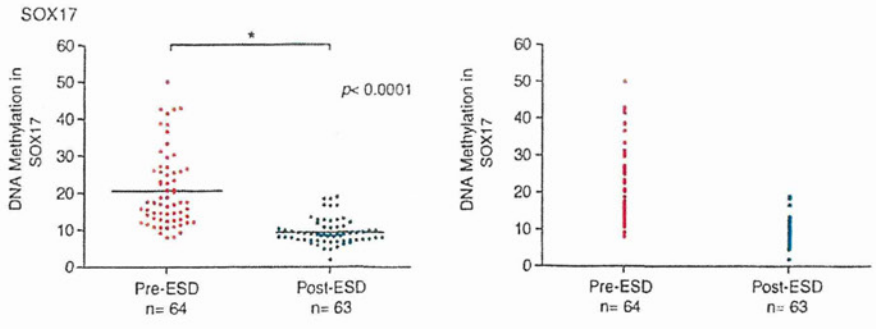

Fig. 2 DNA methylation in the validation set of EGC. Methylation levels of Sox17 measured by quantitative bisulfite pyrosequencing (pre- and post-ER). Results are of individual genes in the validation set. There is a significant difference in methylation levels between pre- and post-ER ($p<0.0001$)

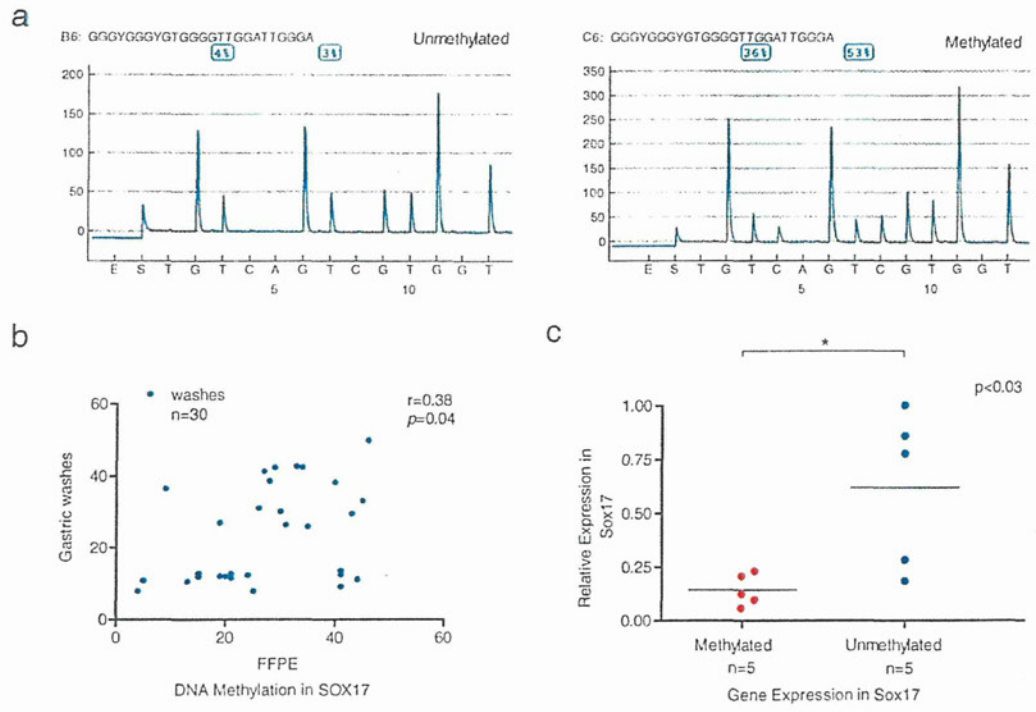

Fig. 3 Sox17 silenced by DNA methylation in EGC. **a** Gastric wash-based pyrogram of Sox17 gene in EGC (*left*, unmethylated; *right*, methylated). **b** Correlation of methylation levels between gastric washes and FFPE samples in the same patient. **c** Expression of Sox17 in EGC with or without DNA methylation ($p<0.03$). Real-time PCR was carried out using cDNA from extracted tumor tissue by laser capture microdissection after ER

resection resulted in the proof of residual cancer (Supplementary Fig. 1b, c). Additionally, to confirm correlations in Sox17 methylation levels between gastric washes and FFPE, 30 DNAs were extracted from 30 endoscopically resected FFPE tissues in conjunction with 30 wash samples in a validation set of 128 EGC patients are shown in Fig. 3b. Methylation levels of the Sox17 gene were approximately correlated between washes and FFPE by Spearman's correlation coefficient analysis ($r=0.38$, $p=0.04$) (Fig. 3b). To compare the Sox17 methylation levels between EGC and the gastritis samples (control samples), we analyzed the data using DNA from gastric washes of pre-ER ($n=64$)/post-ER ($n=63$) endoscopic treatment and 32 gastritis samples. The results show a significant difference between not only pre-ER and post-ER, but also between pre-ER and gastritis samples (Supplementary Fig. 1a). Interestingly, methylation levels were widely spread among gastritis samples, and we successfully divided them into two methylation groups based on the status of *H. pylori* infection (Supplementary Fig. 1a). On the other hand, there were no significant differences of Sox17 methylation levels between *H. pylori* positive and negative in EGC samples (gastric washes in pre-ER) (Table 1, Supplementary Fig. 1a).

Silencing of Sox17 associated with its promoter CpG island hypermethylation in gastric cancer cell lines

Human BLAT search sequence analysis of Sox17 5′ regulatory regions shows that there is a CpG island encompassing its transcription start site (UCSC Genome Bioinformatics Group, Santa Cruz, CA, USA). We designed primers for bisulfite-pyrosequencing analysis in a region downstream of the transcription start site (Fig. 4a). Although two of the gastric cell lines showed low methylation (MKN1, 57%; KatoIII, 35%), hypermethylation was detected in four of them (MKN7, 82%; MKN45, 92%; MKN74, 75%; AZ521, 86%). These four cell lines also expressed low levels of Sox17 (Fig. 4b). There were good correlations between DNA methylation levels and gene expression levels using 10 primary EGC samples (total RNA was extracted from 10 endoscopically resected FFPE tissues) (Fig. 3c).

To assess restoration of Sox17 expression, two hypermethylated cell lines (MKN45, MKN74) were incubated for 72 h with 1 and 5 μmol/l 5-aza-2′-deoxycytidine (Sigma-Aldrich, St. Louis, MO, USA), a methyltransferase inhibitor. The cells were then harvested, and total RNA was extracted for further analysis. Treating the two cell lines

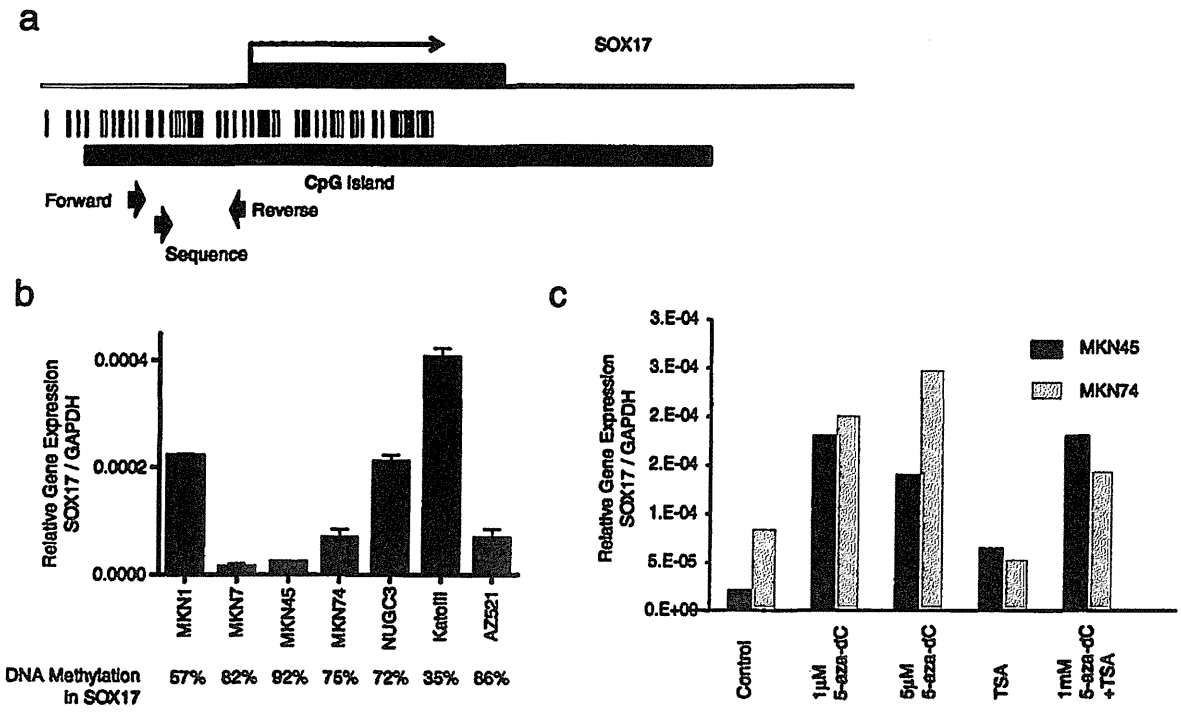

Fig. 4 CpG island of Sox17 and gene expressions. a Schema of the promoter region of Sox17 gene and CpG island (*black bar*). *Three arrows* were used to describe pyrosequencing primers for methylation analysis. b Relative expression levels of Sox17 gene in seven gastric cancer cell lines. Levels of Sox17 gene expression are normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). c Using real-time PCR, expression of Sox17 was analyzed in two gastric cancer cell lines (MKN74 and MKN45) that were highly methylated in Sox17. In two gastric cancer cell lines that expressed Sox17 at barely detectable levels, expression levels were restored after treatment with 5-aza-dC

(MKN45, MKN74) with the methyltransferase inhibitor 5-aza-dC, they showed hypermethylation in Sox17, and expression levels were restored (Fig. 4c).

Expression of exogenous Sox17 suppresses cell growth in gastric cancer cell lines

We next used colony formation assays to determine whether Sox17 has some potential for tumor suppressor activities (Fig. 5). When Sox17 was introduced to two gastric cancer cell lines that do not otherwise express the gene, there was a significant reduction in colony formation.

Discussion

Aberrant DNA methylation plays a crucial role in the development and progression of human cancers and is now recognized as a third mechanism by which inactivation of tumor suppressor genes occurs [45]. Aberrant CpG island hypermethylation is also frequently observed in chronic inflammation and precancerous lesions, which suggests that it is an early occurrence in tumorigenesis that could serve as a useful tumor marker [46, 47]. A strategy was put in place to compare methylation levels between tumor and adjacent normal site in order to select candidate genes that were specifically methylated in neoplasia. However, it is difficult to eliminate some hypermethylated genes that were associated with age and inflammations (e.g., chronic gastritis by *H. pylori*). Our gastric wash-based MCAM analysis is an innovative approach to evaluating the DNA in gastric washes of cancer patients before and after ER of EGC (pre-ER gastric washes include both the tumor site and normal site; post-ER gastric washes only include the normal site due to the curative removal of a tumor site by endoscopic treatment). In the present study, we successfully selected 11 candidate genes out of 9,021 genes by means of gastric wash-based MCAM analysis.

It was reported previously that DNA methylation of the region around the transcription start site of Sox17 is involved in silencing the gene in colorectal cancer cells [48]. Consistent with that report, we also found that gene silencing by Sox17 hypermethylation was seen in MKN74 and MKN45 of gastric cancer cell lines (Fig. 4). The molecular mechanism by which Sox17 suppresses cell growth remains unknown. However, Zhang et al. [48] reported that

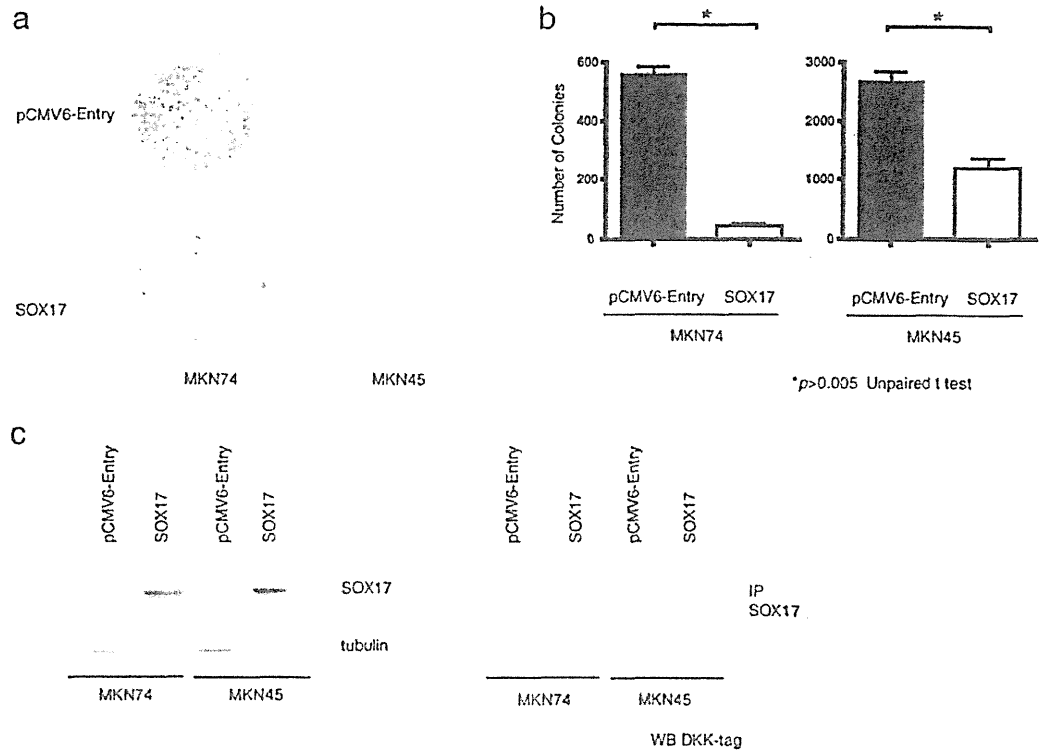

Fig. 5 Suppression of colorectal cancer cell growth by Sox17. a MKN74 and MKN45 cells were transfected in the presence of G418. b Cell counts were obtained 28 days after transfecting MKN74 and MKN45 with pCMV6 (empty vector) or pCMV6-Sox17 (Sox17). c To confirm Sox17 protein levels after transfection, we used pCMV6-Sox17 plasmid with DKK-tag by western blotting analysis

introducing Sox17 into colorectal cancer cells using a pcDNA3.1/V5-His vector represses β-catenin/TCF activity in cells, suggesting that the Sox17 is involved in the Wnt/β-catenin pathway. We also confirmed that introduction of exogenous Sox17 into silenced cells suppressed colony formation in gastric cancer cell lines (MKN74 and MKN45) (Fig. 5).

Molecular markers that distinguish benign from clinically silent malignant disease are needed to reduce the number of unnecessary endoscopic biopsies and to improve detection of gastric dysplasia and EGC at an early stage. We previously reported that gastric washes closely mirrored biopsy results in DNA methylation level [6]. We also found an approximately good correlation of DNA methylation levels from FFPE and gastric washes (Fig. 3b). In our present clinical study, Sox17 showed significantly different methylation levels between pre- and post-treatment in EGC patients using gastric washes ($p<0.0001$) (Fig. 2). The dot plots results of Sox17 show significant differences in methylation levels between not only pre- and post-ER ($p<0.0001$) but also between pre-ER and gastritis samples. Additionally, there is a significant difference in methylation levels between gastritis with and without *H. pylori* infection (Supplementary Fig. 1a). Moreover, we could successfully identify the incomplete ER case based on the Sox17 methylation status in gastric washes (without decreasing Sox17 methylation levels), and additional resection resulted in the successful removal of residual cancer (Supplementary Fig. 1b, c). However, without follow-up studies, it is still uncertain how methylation levels affect precancerous regions (which have not been viewed endoscopically) remaining in the stomach after ER. Moreover, it may be more useful to detect EGC with high sensitivity by a combination of markers including those we previously reported [6].

In summary, we have shown that Sox17 expression is often epigenetically silenced in gastric cancer. Such silencing of Sox17 was mediated by DNA methylation and the introduction of Sox17 into cancer cells suppressed cell growth. Understanding the precise role played by Sox17 in gene transcription will not only increase our understanding of the biology of gastric cancer but it may also enable epigenetic silencing of Sox17 to serve as a useful molecular target for diagnosis. These data raise hope for the possibility of obtaining gastric washes without requiring an endoscope in EGC detection, an approach that should be tested in future trials.

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Conflicts of interest None.

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