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ORIGINAL ARTICLE

Aberrant DNA methylation associated with aggressiveness of gastrointestinal stromal tumour

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ABSTRACT

Background and aims The majority of gastrointestinal stromal tumors (GISTs) have *KIT* mutations; however, epigenetic abnormalities that could conceivably potentiate the aggressiveness of GISTs are largely unidentified. Our aim was to establish epigenetic profiles associated with the malignant transformation of GISTs.

Methods Methylation of four tumor suppressor genes, *RASSF1A, p16, CDH1, and MGMT* was analyzed in GISTs. Additionally, genome-wide DNA methylation profiles were compared between small, malignant-prone, and malignant GISTs using methylated GpG island amplification microarrays (MCAM) in a training set (n=40). Relationships between the methylation status of genes identified by MCAM and clinical features of the disease were tested in a validation set (n=75).

Results Methylation of *RASSF1A* progressively increased from small to malignant GISTs. *p16* was specifically methylated in malignant-prone and malignant GISTs. MCAM analysis showed that more genes were methylated in advanced than in small GISTs (average of 473 genes vs 360 genes, respectively, $P=0.012$). Interestingly, the methylation profile of malignant GISTs was prominently affected by their location. Two genes, *REC8* and *PAX3*, which were newly-identified via MCAM analysis, were differentially methylated in small and malignant GISTs in the training and validation sets. Patients with methylation of at least *REC8*, *PAX3*, or *p16* had a significantly poorer prognosis ($P=0.034$).

Conclusion Our results suggest that GIST is not, in epigenetic terms, a uniform disease and that DNA methylation in a set of genes is associated with aggressive clinical behavior and unfavorable prognosis. The genes identified may potentially serve as biomarkers for predicting aggressive GISTs with poor survivability.

Significance of this study

What is already known about this subject?

- Approximately 90% of gastrointestinal stromal tumours (GIST) have activating mutations in the *KIT* gene (~85%) or *platelet-derived growth factor receptor alpha* gene (~5%), which were detected even in small GIST (<10 mm), suggesting that these mutations are early initiation steps in GIST formation.
- Although two key prognostic features (tumour size and mitotic index) of primary GIST appear to be of use for risk stratification, a persistent problem is that a subset of cases classified as low risk nonetheless occasionally metastasize.
- Location may also be considered as a factor for the risk assessment of GIST, because a small intestinal GIST is more aggressive than a gastric GIST of equal size.
- Elucidating the underlying mechanism that potentiates the aggressiveness of GIST is key to facilitating the development of a new strategy for the treatment of malignant GIST.

What are the new findings?

- Genome-wide DNA methylation analysis revealed that more genes were methylated in advanced GIST than small GIST, suggesting a link between the accumulation of DNA methylation and disease progression.
- Among the hypermethylated genes, we identified potent methylation markers, *REC8*, *PAX3* and *p16*, of which DNA methylation status is significantly correlated with a worse prognosis of GIST.
- The DNA methylation profile of malignant GIST was prominently affected by its location in the gastrointestinal system; gastric and small-intestinal GIST displayed distinctive DNA methylation profiles, supporting the idea that the anatomical location of GIST affects the clinical significance of the respective disease.

Gastrointestinal stromal tumours (GIST) are the most common mesenchymal tumours of the digestive tract. Risk assessment at initial diagnosis is recognised as quite important, because a subset of GIST relapses after surgery.[1 2] Two key prognostic features of a primary GIST are its size and mitotic index.[3] These two features appeared to be practically useful for a consensus approach to risk stratification (low, intermediate, or high) of GIST.[4] However, a persistent problem is that a subset of cases exist that, having low mitotic rates and being classified as low risk, occasionally metastasise to or invade other organs. In addition to these two prognostic features, location may be considered as a factor for the risk assessment of a primary

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Significance of this study

How might it impact on clinical practice in the foreseeable future?

- Our findings provide a better understanding of the role of aberrant DNA methylation in GIST, and may provide new molecular diagnostic tools for this disease subtype.
- Examination of three methylation markers could be informative for diagnostic and prognostic assessment of GIST and could be elucidated using samples obtained from minimally invasive procedures, such as biopsy specimens obtained by the endoscopic ultrasound-guided fine-needle aspiration technique.

tumour, because a small intestinal GIST is more aggressive than a gastric GIST of equal size.[1]

GIST are known to originate from interstitial cells of Cajal (ICC) or their precursors. It has been suggested that the acquisition of *KIT* and platelet-derived growth factor receptor alpha (*PDGFRA*) mutations in ICC is a possible early initiation step in GIST tumorigenesis.[3 5 6] In addition to *KIT* and *PDGFRA* mutations, the majority of GIST also display other genetic abnormalities as they progress to a malignant phenotype.[7] One particular study demonstrated that other genetic abnormalities, such as losses of 13q, 10q and 22q, contribute to the progression and malignant transformation of GIST.[8] Inactivation of the cell cycle regulators p16 and/or p27 has also been shown to correlate with malignancy in GIST.[9 10] However, these genetic abnormalities do not completely explain the underlying mechanisms that potentiate the aggressiveness of this tumour type.

Aberrant DNA methylation has been observed in many human malignancies, sometimes correlated with potentiated aggressiveness of the tumour.[11 12] As DNA methylation of particular genes is known to be associated with patient outcome, the detection of aberrant DNA methylation in clinical specimens could be a useful biomarker for malignancies.[13 14] Recent advances in technologies for high-throughput genome-wide DNA methylation analyses have facilitated epigenetic profiling of human malignancies.[15] To investigate epigenetic events in GIST, we performed genome-wide screening for genes with aberrant DNA methylation using a methylated CpG island amplification microarray (MCAM) approach, which provides reproducible results with a high validation rate.[16–18] We further evaluated correlations between DNA methylation status and clinicopathological features of GIST including gene mutations.

MATERIALS AND METHODS

Tissue samples

We collected 115 GIST samples in accordance with institutional policy from patients who underwent surgical resection at the Aichi Cancer Center Central Hospital, Nagoya, at the Osaka University Hospital, Osaka, at the Osaka Police Hospital, Osaka, or at Sapporo Medical University Hospital, Sapporo in Japan. Specimens showing a high proportion of tumour cells (>80%) without definite evidence of necrosis were analysed. We divided GIST samples into two sets, a training set (n=40) and a validation set (n=75) without any bias (table 1). GIST were classified into three groups: small GIST (n=33), malignant-prone GIST (n=49), and malignant GIST (n=33), based on the modification of 2002 consensus criteria (low, intermediate and high-risk groups; table 1).[3] Small GIST refers to small tumours (≤5 cm) with low mitotic frequency (≤5 mitosis per 50 high-power fields) and without any evidence of metastasis or invasion (low-risk group). Malignant-prone GIST refers to larger tumours (>5 cm) or those with more than five mitoses per 50 high-power fields and without any evidence of metastasis or invasion (intermediate and high-risk groups). Malignant GIST refers to tumours from patients with a history of metastasis or invasion. Metastatic disease has been diagnosed using imaging studies and clinical analysis when primary tumours were surgically treated. Patients in the training set and in the validation set had similar features, except more patients in the validation cohort were classified as having malignant-prone GIST (table 1). We also collected 18 muscle layers from six stomachs, six small intestines and six colons. Samples and clinical data were collected after the approval of the institutional review board of Aichi Cancer Center was received and written informed consent had been obtained from all patients.

Microscopic dissection of ICC

ICC were identified as c-Kit-positive cells among the intestinal cells. A higher density of ICC is observed at the level of the myenteric plexus between circular and longitudinal muscle layers in the small intestine.[19] To obtain ICC as a normal counterpart of GIST cells, we prepared serial sections (10 μm thick), one of which was stained with anti-c-Kit (A4502; DAKO, Glostrup, Denmark) as an indicator, and we carefully dissected an unstained section under stereoscopic microscope observation (see supplementary figure 1, available online only).

Bisulfite-pyrosequencing for DNA methylation analysis

We performed bisulfite treatment on genomic DNA as previously described.[18] DNA methylation levels were measured by

Table 1 Clinical features of the GIST in this study

Feature	Total (n=115) No of patients (%)	Training set (n=40) No of patients (%)	Validation set (n=75) No of patients (%)	p Value
Sex				
Male	62 (54)	19 (48)	43 (57)	0.314
Female	53 (46)	21 (52)	32 (43)	
Age, years				
<60	41 (35)	19 (48)	22 (29)	0.067
≥60	74 (65)	21 (52)	53 (71)	
Tumour origin				
Stomach	66 (57)	23 (57)	43 (57)	0.801
Small intestine	40 (35)	15 (38)	25 (33)	
Rectum	8 (7)	2 (5)	6 (8)	
Oesophagus	1 (1)	0 (0)	1 (2)	
Size, cm*				
<5	46 (41)	15 (40)	31 (41)	0.216
≥5	67 (59)	23 (60)	44 (59)	
Mitotic index/50 HPF*				
<5	60 (59)	18 (56)	42 (60)	0.139
≥5	42 (41)	14 (44)	28 (40)	
Classification				
Small				
Low risk†	33 (29)	9 (23)	24 (32)	0.006
Malignant-prone				
Intermediate risk†	21 (18)	9 (23)	12 (16)	
High risk†	28 (24)	4 (10)	24 (32)	
Malignant				
Metastasis	33 (29)	18 (44)	15 (20)	

*Clinical data of some patients were unavailable.
†Low risk, size less than 5 cm and mitotic index less than 5/50 high-power fields (HPF); intermediate risk, size less than 5 cm and mitotic index 5–10/50 HPF, or size 5–10 cm and mitotic index less than 5/50 HPF; high risk, size 5–10 cm and mitotic index 5–10/50HPF, or size 10 cm or greater or mitotic index 10/50 HPF or greater.
GIST, gastrointestinal stromal tumour.

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.

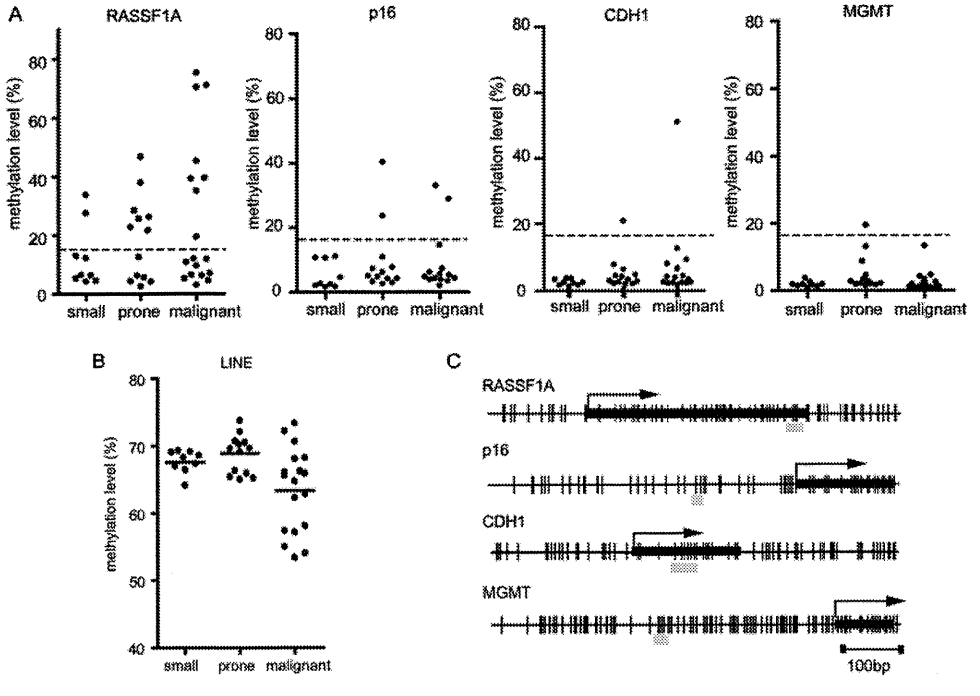

Figure 1 DNA methylation status of the candidate genes *p16*, *RASSF1A*, *CDH1*, *MGMT* (A) and *LINE-1* repetitive elements (B) in gastrointestinal stromal tumours (GIST). Levels of methylation were measured by bisulfite-pyrosequencing analysis in small, malignant-prone (prone), and malignant GIST. 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.

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

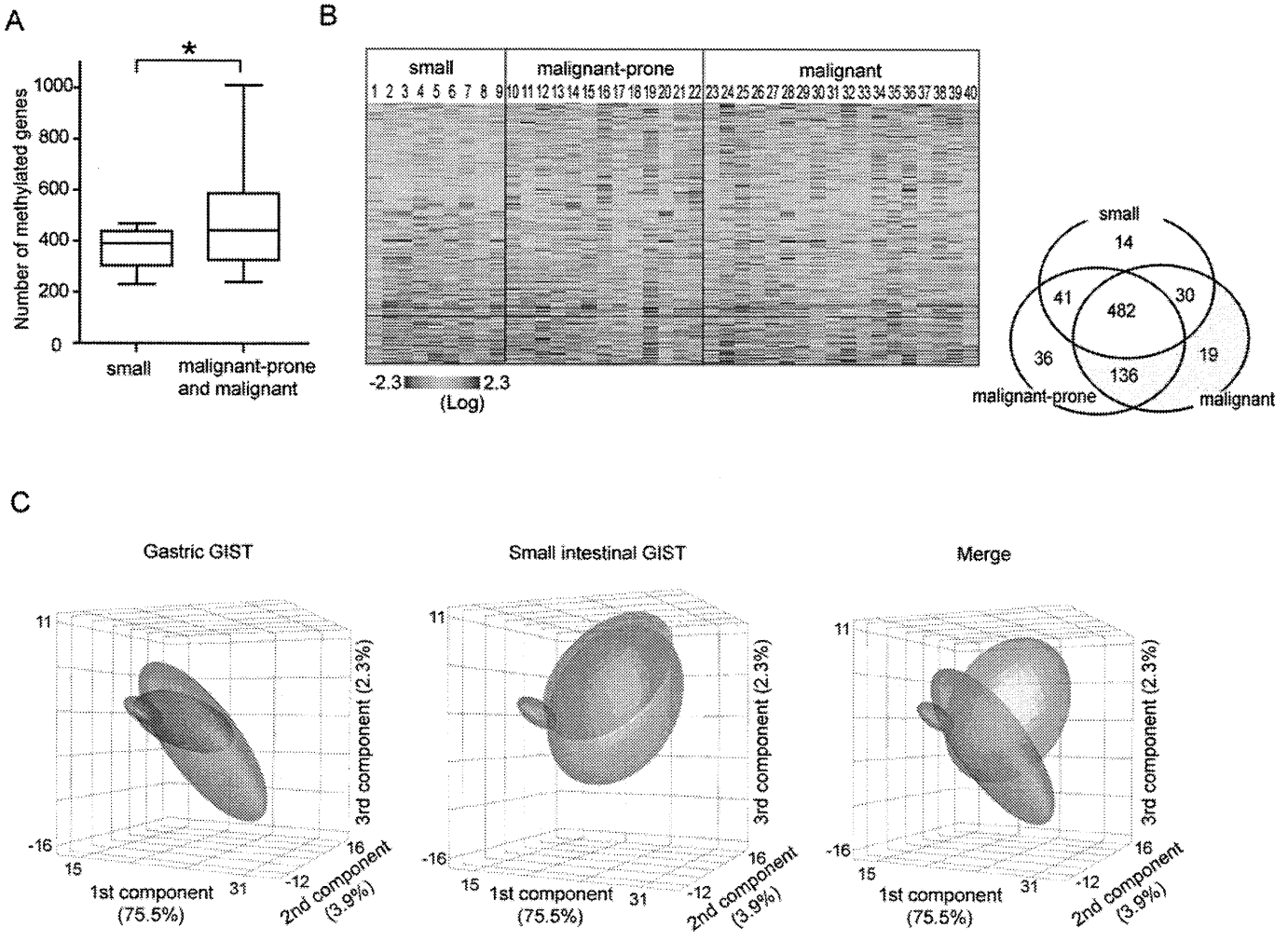

Figure 2 Methylated CpG island amplification microarrays (MCAM) analysis in gastrointestinal stromal tumours (GIST). (A) Box and whisker plots of the number of methylated genes in small GIST, as well as advanced GIST (malignant-prone and malignant GIST). The mean is marked by a horizontal line inside 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.

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 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

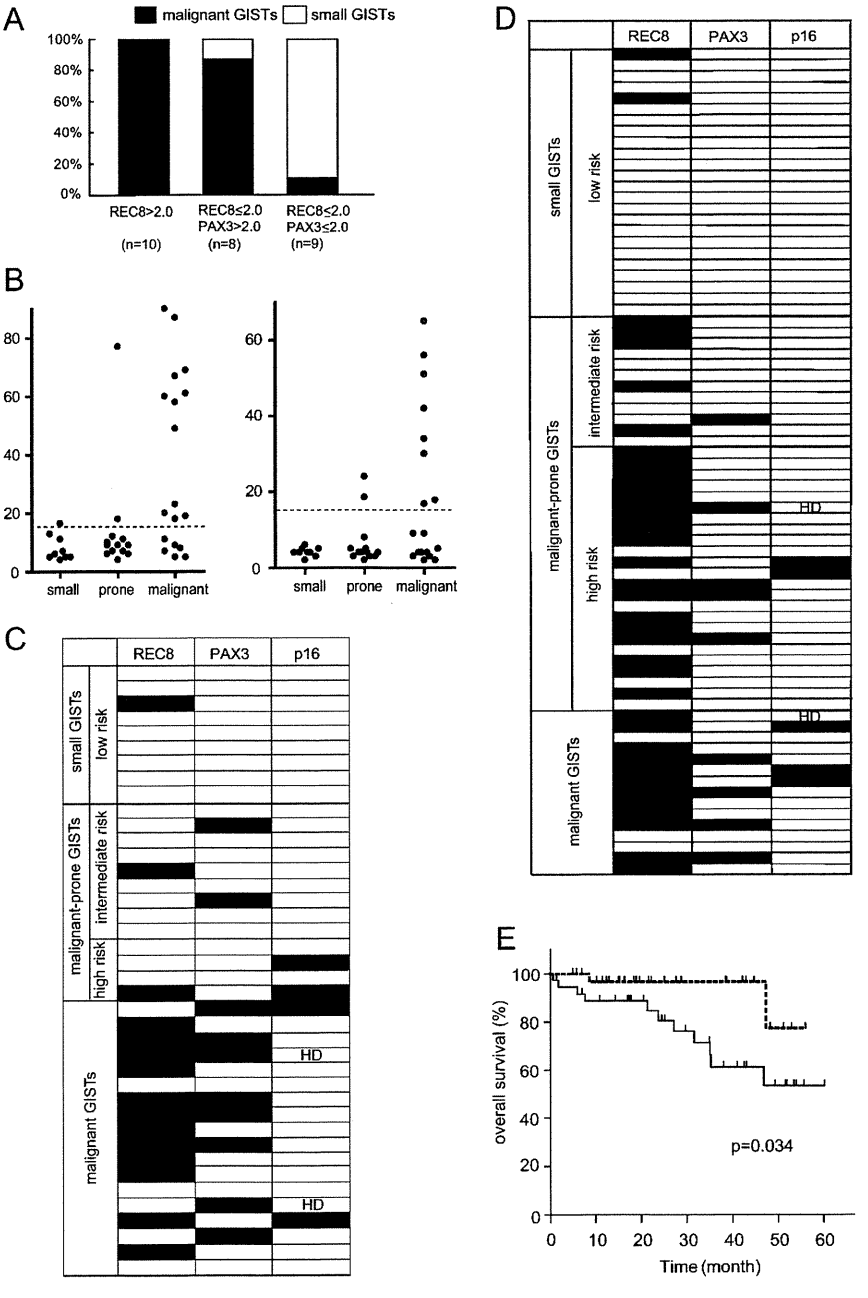

Figure 3 Identification of diagnostic and prognostic markers for advanced gastrointestinal stromal tumours (GIST). (A) *REC8* and *PAX3* are effective markers to distinguish malignant from small GIST on the basis of methylated CpG island amplification microarrays (MCAM) data in the training set (see 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).

(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 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

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.

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).

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

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.

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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).

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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Editor's quiz: GI snapshot

A rare gastrointestinal presentation of a common malignancy

CLINICAL PRESENTATION

A 61-year-old man presented with a 4-week history of rectal bleeding, constipation, bloating, abdominal distension and low back pain. His bowels had not opened for 4 days prior to admission. Physical examination revealed a distended, tympanic abdomen. Routine laboratory tests confirmed renal failure (urea 13 mmol/l, creatinine 200 μmol/l), hypercalcaemia (3.7 mmol/l), albumin 32 g/l and C-reactive protein (CRP) 25 mg/l. Parathormone was suppressed (7 pg/l (15–65)). Abdominal x-ray suggested an ileus. CT abdomen revealed mural thickening of the left hemi-colon along with collapse of the third lumbar vertebra. An isotope bone scan was unremarkable, while MRI of the spine confirmed the presence of multiple lytic lesions. Immunoglobulin G (IgG) was 27.39 g/l (6.0–16.0) and electrophoresis showed a band in the fast γ region. λ Light chains were elevated 6653.5 mg/l (5.7–26.3).

He was managed with intravenous fluids and bisphosphonate with improvement in both renal function and corrected calcium. Over the course of admission he developed anorexia and vomiting. Gastroscopy revealed nodularity of gastric and duodenal mucosa. Flexible sigmoidoscopy showed congested, erythematous, friable mucosa (figure 1). Gastric biopsies are displayed (figures 2 and 3).

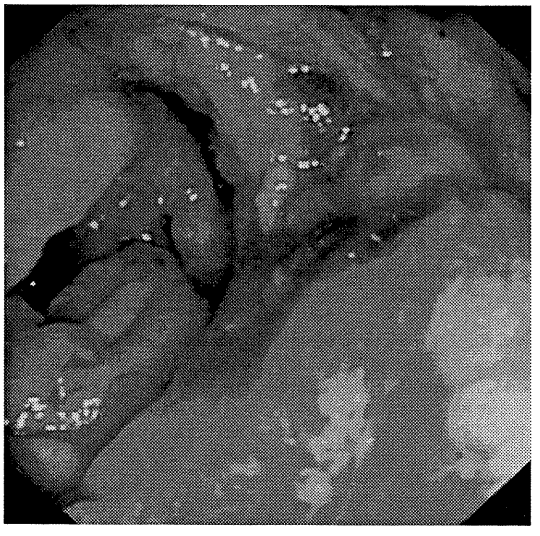

Figure 1 Mucosal appearances sigmoid colon.

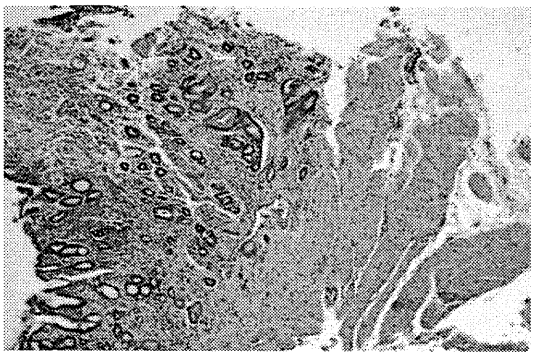

Figure 2 Congo red stain of gastric mucosa.

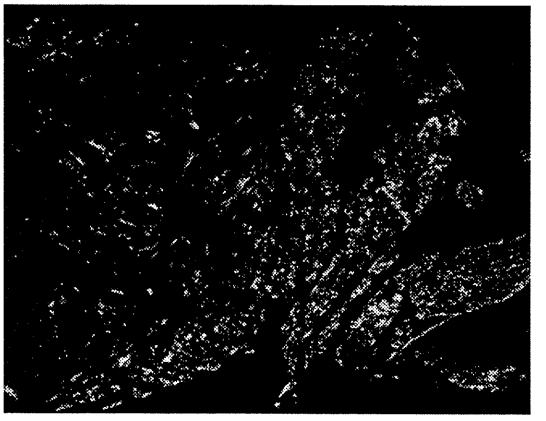

Figure 3 Congo red stain of gastric mucosa under polarised light.

QUESTION

What are the histological changes seen and what is the unifying diagnosis for this man's presentation with gastrointestinal symptoms?

See page 438 for the answer

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Aberrant DNA methylation associated with aggressiveness of gastrointestinal stromal tumour

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