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Long interspersed nuclear element-1 hypomethylation is a potential biomarker for the prediction of response to oral fluoropyrimidines in microsatellite stable and CpG island methylator phenotype-negative colorectal cancer

Kazuyuki Kawakami,[1,3] Aika Matsunoki,[1,2] Mami Kaneko,[1,2] Kenichiro Saito,[1,2] Go Watanabe[2] and Toshinari Minamoto[1]

[1]Division of Translational and Clinical Oncology, Cancer Research Institute, Kanazawa University; [2]Department of General and Cardiothoracic Surgery, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

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We investigated the clinical value of methylation of long interspersed nuclear element-1 (LINE-1) for the prognosis of colorectal cancer (CRC) and for the survival benefit from adjuvant chemotherapy with oral fluoropyrimidines. LINE-1 methylation in tumor DNA was measured by quantitative methylation-specific PCR in 155 samples of stage II and stage III CRC. The presence of microsatellite instability and CpG island methylator phenotype (CIMP) were assessed and 131 microsatellite stable/CIMP– cases were selected for survival analysis, of which 77 patients had received postoperative adjuvant chemotherapy with oral fluoropyrimidines. The CRC cell lines were used to investigate possible mechanistic links between LINE-1 methylation and effects of 5-fluorouracil (5-FU). High LINE-1 methylation was a marker for better prognosis in patients treated by surgery alone. Patients with low LINE-1 methylation who were treated with adjuvant chemotherapy survived longer than those treated by surgery alone, suggestive of a survival benefit from the use of oral fluoropyrimidines. In contrast, a survival benefit from chemotherapy was not observed for patients with high LINE-1 methylation. The CRC cell lines treated with 5-FU showed increased expression of LINE-1 mRNA. This was associated with upregulation of the phospho-histone H2A.X in cells with low LINE-1 methylation, but not in cells with high LINE-1 methylation. The 5-FU-mediated induction of phospho-histone H2A.X, a marker of DNA damage, was inhibited by knockdown of LINE-1. These results suggest that LINE-1 methylation is a novel predictive marker for survival benefit from adjuvant chemotherapy with oral fluoropyrimidines in CRC patients. This finding could be important for achieving personalized chemotherapy. (*Cancer Sci* 2011; 102: 166–174)

Epigenetic alterations including CpG island hypermethylation and global hypomethylation of DNA are commonly observed in colorectal cancer (CRC).[1] The aberrant hypermethylation of CpG islands is associated with distinctive clinical features of CRC and shows promise as a diagnostic marker.[2,3] Global hypomethylation, on the other hand, has attracted much less attention and its clinical significance in cancer has not been extensively investigated.

Long interspersed nuclear element-1 (LINE-1) is a non-long-terminal-repeat class of retroposon that is the most successfully integrated mobile element and accounts for approximately 18% of the human genome.[4–6] The LINE-1 sequence is 6 kb in length and contains a 5′ untranslated region (UTR), two open reading frames and a 3′UTR. The 5′UTR has internal promoter activity and the open reading frame encodes for nuclease and reverse transcriptase activities that are necessary for transposition. Although the majority of retroposons no longer have the ability to transpose due to mutations and deletions in their sequence, approximately 100 full-length copies of LINE-1 in the human genome retain this ability.[7,8] Because of the abundance and functional ability of LINE-1, the level of its methylation is a surrogate of global gene methylation[9] and its hypomethylation has been suggested to cause chromosomal instability.

Hypomethylation of LINE-1 is predicted to be a factor for unfavorable prognosis in CRC patients because of the association between global hypomethylation and chromosomal instability,[10] a known marker of poor prognosis.[11,12] Consistent with this, our preliminary analysis of stage II and III CRC patients who underwent curative surgery found that LINE-1 hypomethylation was associated with a worse patient outcome.[13] Another recent study also found that LINE-1 hypomethylation was independently associated with shorter survival of colon cancer patients.[14] However, these studies did not take into account the use of postoperative adjuvant chemotherapy in their patient cohorts and its possible influence on the observed prognostic value of LINE-1 hypomethylation.

In this study, we investigated the prognostic value of LINE-1 methylation in stage II and III CRC patients who underwent curative surgery and in whom the postoperative adjuvant chemotherapy status was known. Our preliminary investigations suggested that LINE-1 hypomethylation could be an indicator of survival benefit from postoperative adjuvant chemotherapy with oral fluoropyrimidines. However, this marker is associated with the microsatellite instability (MSI) and CpG island methylator (CIMP) phenotypes,[15,16] both of which have been implicated in the response to 5-fluorouracil (5-FU).[2,17–19] To avoid a possible confounding influence from these phenotypes, in the present study we evaluated the prognostic and predictive significance of LINE-1 methylation in a cohort of CRC patients with microsatellite stable (MSS) and CIMP-negative (CIMP–) tumors.

Materials and Methods

Patients and specimens. The present study included 155 patients with stage II or III CRC who underwent surgery with

[3]To whom correspondence should be addressed.
E-mail: kawakami@med.kanazawa-u.ac.jp

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curative intent at Kanazawa University Hospital. The patients comprised 90 males and 65 females and ranged in age from 33 to 93 years (mean, 65.6 years). The site of tumor was classified as proximal or distal to the splenic flexure. A total of 49 patients were thus defined as having proximal CRC and 106 with distal CRC. Tumor stage was defined according to the International Union Against Cancer (UICC) TNM system.[20] The postoperative course of patients treated with and without adjuvant chemotherapy was retrospectively assessed by referring to their medical records. In all, 94 patients received adjuvant chemotherapy with oral fluoropyrimidines and 61 were treated with surgery alone. None of the patients received pre-operative radio-chemotherapy or postoperative radiation therapy. The oral fluoropyrimidines used were 5-FU, UFT (a combination of tegafur and uracil), doxifluridine and carmofur in 14, 52, 17 and 11 patients, respectively. The median period of adjuvant chemotherapy use was 105 weeks (range, 21–275 weeks). The median follow-up time was 58 months (range, 11–128 months) and 49 patients died of disease recurrence during the course of follow up. Survival data for the 13 patients who died of other causes was censored at the time of death.

Tissue samples for methylation analysis were obtained from formalin-fixed and paraffin-embedded tumor blocks selected on the basis of tumor cell content. The tumor tissue was dissected manually from 10 μm paraffin sections. After deparaffinization using xylene and ethanol, genomic DNA was extracted from the tissue using QIAamp DNA mini kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA samples from adjacent normal tissues were prepared using the same method. This project was approved by the Kanazawa University Medical Ethics Committee.

Cell lines and culture conditions. The CRC cell lines SW48, SW480, HCT116, HT-29, COLO205, LS411N and Caco-2 were obtained from the American Type Culture Collection (Manassas, VA, USA). Other cell lines were provided by Health Science Research Resources Bank (Osaka, Japan). The cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle (SW48, SW480, SW837, HCT116, HT-29, Caco-2, CaR-1, CCK81, LoVo) or RPMI 1640 (COLO205, CW2, DLD-1, LS411N) medium supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin; Gibco, Grand Island, NY, USA). Cells were harvested during the exponential growth phase for extraction of DNA, RNA and protein.

DNA methylation analysis. Genomic DNA extracted from tissues and cultured cell lines was treated with bisulfite as described previously.[21] Following the bisulfite treatment, LINE-1 methylation was analyzed using a methylation-specific real-time PCR assay as previously described.[22]

Methylation levels of CpG islands within promoter regions were evaluated by MethyLight assay for the CIMP panel of markers comprising CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1, where the percentage methylated reference (PMR) values were derived using the ALU normalization control reaction.[23] Simultaneous hypermethylation (PMR ≥ 10) of three or more of these five markers was used to define a tumor as CIMP+.

Microsatellite analysis for MSI and LOH. The MSI status was determined using a three-marker panel of mononucleotide repeats (BAT26, NR21 and NR27) reported to be highly sensitive for the identification of MSI in CRC.[24] Primer sequences and PCR conditions were described previously[25] and sense primers were end-labeled with 6-carboxyfluorescein (BAT26 and NR-21) or VIC (NR-27). The PCR amplified fragments were analyzed by capillary electrophoresis using the ABI-PRISM 310 Sequence Detection System (Applied Biosystems, Foster, CA, USA). GeneMapper software version 4.0 (Applied Biosystems) was used to detect the fragment signal and estimate the allelic size of each marker. Matched DNA samples from tumor and adjacent normal tissues were analyzed in 139 cases, and cases showing new alleles in the tumor DNA compared with normal DNA were classified as having MSI. Microsatellite instability was determined without reference to matching normal DNA in 16 cases. For these cases, an allelic size difference of >3 bp for BAT26 and >2 bp for the NR markers relative to the quasi-monomorphic variation range was considered to represent MSI, as described previously.[24] The quasi-monomorphic variation range of each marker was determined from investigation of 139 normal DNA samples. Tumors were classified as MSI if they showed instability at one or more markers. The MSI status of CRC cell lines was also determined using the quasi-monomorphic variation range and the three-marker panel described above.

The LOH status was determined by screening three microsatellite loci (D18S58, D18S61, D18S64) on chromosome 18q. DNA samples from matched tumor and normal tissues were amplified with sets of primers (Table S1) in which the forward primer was labeled with 6-carboxyfluorescein. The PCR conditions and determination of LOH were described previously.[26] Tumors that showed LOH at one or more loci were classified as LOH positive.

Northern blot analysis. The RNA probes to detect LINE-1 and β-actin mRNA were made by cloning into pGEM-T easy Vector (Promega, Madison, WI, USA) of the RT-PCR fragment of each gene from SW480 using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), random hexamers and primers listed in Table S1. This was followed by confirmation of the sequence using BigDye Terminator Cycle Sequencing kit Version 1.1 (Applied Biosystems). The RNA probes were synthesized by SP6 RNA polymerase using the DIG Northern Starter kit (Roche, Mannheim, Germany).

Total RNA was prepared by the single-step guanidinium isothiocyanate method using ISOGEN (Nippon Gene, Toyama, Japan). Polyadenylated (polyA) RNA was then purified using oligotex-dT30 (TaKaRa Bio, Otsu, Japan). Total RNA (50 μg) was denatured for 5 min at 65°C in a mixture of 200 μL binding buffer (10 mM Tris–HCl at pH 8.0, 1 mM EDTA, 0.1% SDS) and 15 μL of oligotex-dT30 followed by the addition of 40 μL of 5 M NaCl and incubation for 10 min at 37°C. The solution was centrifuged at 18 000*g* for 3 min. The pellet of oligotex-dT30-binding polyA RNA was washed in 1 mL buffer (10 mM Tris–HCl at pH 8.0, 1 mM EDTA, 0.1% SDS, 0.5 M NaCl) and then centrifuged at 18 000*g* for 3 min. The pellet was suspended in 200 μL TE (10 mM Tris–HCl at pH 8.0, 1 mM EDTA) and denatured for 5 min at 65°C followed by rapid chilling on ice and centrifugation at 18 000*g* for 3 min. The supernatants were collected and polyA RNA was precipitated in 440 μL of ethanol containing 20 μL of 3 M sodium acetate and 20 μg of glycogen. After centrifugation at 18 000*g* for 15 min at 4°C, the precipitated RNA was washed with 70% ethanol, air dried and suspended in 10 μL of nuclease-free water.

Purified polyA RNA was electrophoresed in formaldehyde-denatured 1% agarose gels and blotted onto Hybond N+ (Amersham, Little Chalfont, Buckinghamshire, UK). The blotted membrane was prehybridized in DIG Easy Hyb buffer (Roche) at 68°C for 30 min. Heat-denatured LINE-1 probe (95°C for 5 min) was then added to fresh DIG Easy Hyb buffer and incubated with the membrane in a plastic bag at 68°C overnight. The hybridized probe signal was developed using DIG Wash and Block Buffer Set (Roche), alkaline phosphatase-labeled anti-DIG antibody (Roche) and CDP-Star (New England BioLabs, Beverly, MA, USA) following the manufacturer's protocol. The β-actin probe was used as an internal control.

Western blot analysis. Histone proteins were extracted from cultured cells using EpiQuik Total Histone Extraction kit (Epigentek, Brooklyn, NY, USA) following the manufacturer's

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protocol. A 10 μg aliquot of histone protein extract was separated in 12% NuPAGE Novex Bis–Tris gel (Invitrogen), electrotransferred to a nitrocellulose membrane and analyzed by western immunoblotting for the proteins of interest. Primary antibodies to phospho-histone H2A.X^{Ser139} and total histone H2A.X were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and used at dilutions of 1:2000 and 1:3000, respectively. Signals were developed using enhanced chemiluminescence (ECL; Amersham). Immunoblotting signals were measured by the CS analyzer version 2.0 (ATTO, Tokyo, Japan).

RNA interference. Small interfering RNA (siRNA) specific to the LINE-1 sequence and non-specific siRNA were synthesized by Nippon EGT (Toyama, Japan); the sequences are listed in Table S1. The SW480 cells were transiently transfected with 2.5 nM of LINE-1 siRNA or non-specfic siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection with the respective siRNA, the cells were treated with DMSO or 5-FU for 6 days and then harvested for examination.

Statistical analysis. The level of LINE-1 methylation was expressed as a median value (25th–75th percentile). The Mann–Whitney *U*-test or the Kruskal–Wallis test was used to compare the LINE-1 methylation levels between two or three variables, respectively. Kaplan–Meier analysis and the log-rank test were used to evaluate differences in survival between patient groups. Clinicopathological characteristics of the respective patient groups were compared by Mann–Whitney *U*-test for age and Chi-squared test for other variables. The prognostic significance of multiple variables was evaluated using the Cox proportional hazard regression model. Correlation between LINE-1 methylation and mRNA expression level was analyzed by Spearman's rank test. All *P*-values shown are two tailed with $P < 0.05$ taken as statistically significant. Statistical analyses were carried out using the R software package version 2.7.2.[27]

Table 1. Association between the LINE-1 methylation level and clinicopathological features

	n	LINE-1 methylation	*P*-value
Age (years)			
>66	73	84.3 (78.5–88.1)	0.91
≤66	82	84.7 (78.7–88.5)	
Gender			
Male	90	85.4 (79.2–89.0)	0.18
Female	65	82.9 (76.5–88.0)	
Site			
Proximal	49	86.7 (81.6–89.8)	0.041
Distal	106	84.1 (77.3–87.7)	
Stage			
II	80	84.0 (77.3–88.1)	0.23
III	75	85.8 (81.2–88.7)	
Histology			
Well	71	85.3 (79.3–88.9)	0.020
Moderately	67	82.3 (76.1–86.5)	
Poorly	10	88.1 (86.4–91.1)	
Mucinous	7	87.0 (80.4–89.3)	
MSI			
MSI	15	88.1 (85.7–90.6)	0.0085
MSS	140	84.3 (78.1–88.2)	
CIMP			
+	18	87.5 (82.2–89.6)	0.053
–	137	84.3 (77.3–88.4)	

LINE-1 methylation levels are shown as the median (25th–75th percentile). Histology of adenocarcinoma was sub-classified into well-, moderately- and poorly-differentiated adenocarcinoma according to their grading. CIMP, CpG island methylator phenotype; LINE-1, long interspersed nuclear element-1; MSI, microsatellite instability; MSS, microsatellite stable; mucinous, mucinous adenocarcinoma; *n*, number of patients.

Results

LINE-1 hypomethylation is an independent factor for poor prognosis in CRC and is associated with LOH on chromosome 18q. The median level of LINE-1 methylation in primary tumors as determined by quantitative methylation-specific PCR was 84.7% (range, 27.8–94.0%). The LINE-1 methylation was significantly higher in proximal colon tumors compared with those arising in the distal colon and rectum (Table 1). Moderately differentiated adenocarcinomas showed less LINE-1 methylation than tumors with other histological subtypes (well- and poorly-differentiated adenocarcinoma, mucinous carcinoma). Consistent with previous reports,[15,16] LINE-1 methylation was higher in tumors that were MSI ($P = 0.0085$) or CIMP+ ($P = 0.053$).

In order to investigate the prognostic significance of LINE-1 methylation independently of MSI and CIMP status, survival analyses were performed on 131 CRC patients whose tumors were MSS and CIMP− according to the criteria described in the Materials and Methods. The median LINE-1 methylation level in tumor DNA from these patients was 84.3% and no association was observed with the site of tumor origin (Table S2). The LINE-1 methylation level was significantly lower in tumor compared with adjacent normal tissue ($P < 0.0001$, Fig. 1). Patients who received adjuvant chemotherapy showed better outcome compared with those treated by surgery alone ($P = 0.024$, Fig. 2a), suggesting a positive influence of postoperative therapy on patient prognosis.

The LINE-1 methylation level of tumors was classified as high or low relative to the median value of 84.3%. This value is <10 percentile (85.6%) in the distribution of LINE-1 methylation of normal tissues (Fig. 1). High LINE-1 methylation was associated with a trend for longer survival in the overall patient group ($P = 0.055$, Fig. 2b). This reached statistical significance in patients treated by surgery alone ($P = 0.018$, Fig. 2c) and multivariate analysis revealed it to be an independent marker of good prognosis in such patients ($P = 0.022$, Table 2). Consistent with this observation, high LINE-1 methylation was not associated with any of the standard clinicopathological features in patients treated by surgery alone (Table S3). High LINE-1 methylation showed no prognostic significance in patients who received adjuvant chemotherapy (Fig. 2d). A trend of association between good prognosis and high LINE-1 methylation level in the patients treated by surgery alone but not in those who received adjuvant chemotherapy was also observed when the LINE-1 level was classified into low (1–33 percentile), medium (34–66 percentile) and high (67–100 percentile; Fig. S1).

To test for possible association between LINE-1 hypomethylation and genetic abnormality in our CRC cases, we analyzed the LOH status of chromosome 18q by microsatellite analysis and capillary electrophoresis in 37 randomly selected MSS/CIMP− cases. LINE-1 methylation was significantly lower in tumors with 18q LOH compared with those without LOH (Fig. S2). This observation is consistent with a previous report showing the link between LINE-1 hypomethylation and chromosomal instability in CRC.[28]

LINE-1 hypomethylation is a potential biomarker for the prediction of response to oral fluoropyrimidines in CRC. The survival benefit from adjuvant chemotherapy with oral fluoropyrimidines was assessed in patient sub-groups stratified according to the median value of LINE-1 methylation level. Patients with low LINE-1 methylation who received adjuvant chemotherapy lived significantly longer than those treated by surgery alone ($P = 0.0079$, Fig. 3a). In contrast, patients with high LINE-1 methylation showed no difference in survival between

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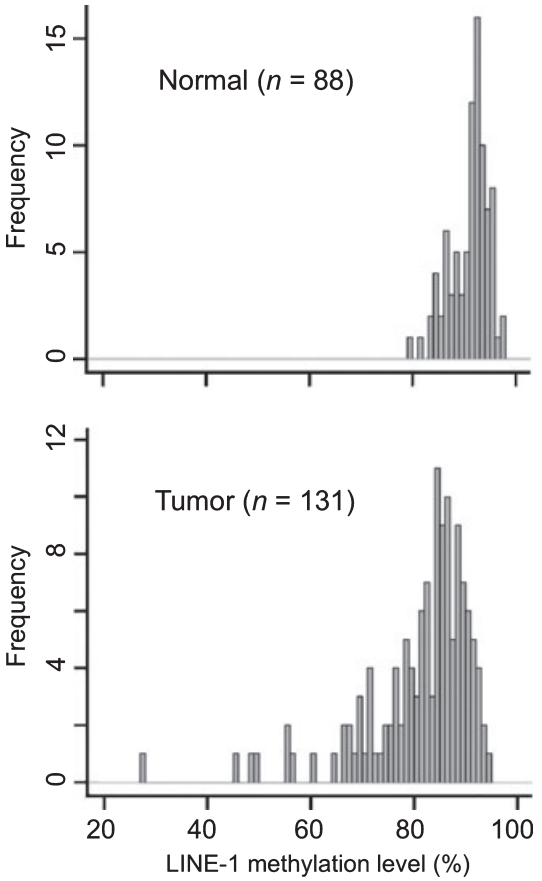

Fig. 1. Distribution of the long interspersed nuclear element-1 (LINE-1) methylation level in normal and tumor tissues. The LINE-1 methylation is significantly lower in tumor compared with normal tissue ($P < 0.0001$).

those treated with or without adjuvant chemotherapy ($P = 0.68$, Fig. 3b). When the LINE-1 level was classified into low (1–33 percentile), medium (34–66 percentile) and high (67–100 percentile), the patients who received adjuvant chemotherapy showed better survival in the low ($P = 0.030$) and medium ($P = 0.068$) but not in the high ($P = 0.98$) LINE-1 methylation group (Fig. S3). These results suggest that low levels of LINE-1 methylation are predictive of response to oral fluoropyrimidines, but not high levels.

The clinicopathological features of the four patient subgroups shown in Figure 3 are outlined in Table S4. In both the high and low methylation groups, patients who received adjuvant chemotherapy were significantly younger than those who did not. While this could potentially account for the survival difference observed between treatment groups, it does not explain why only patients with low LINE-1 methylation appeared to benefit from adjuvant chemotherapy (Fig. 3). Apart from age, no other significant differences in clinicopathological characteristics

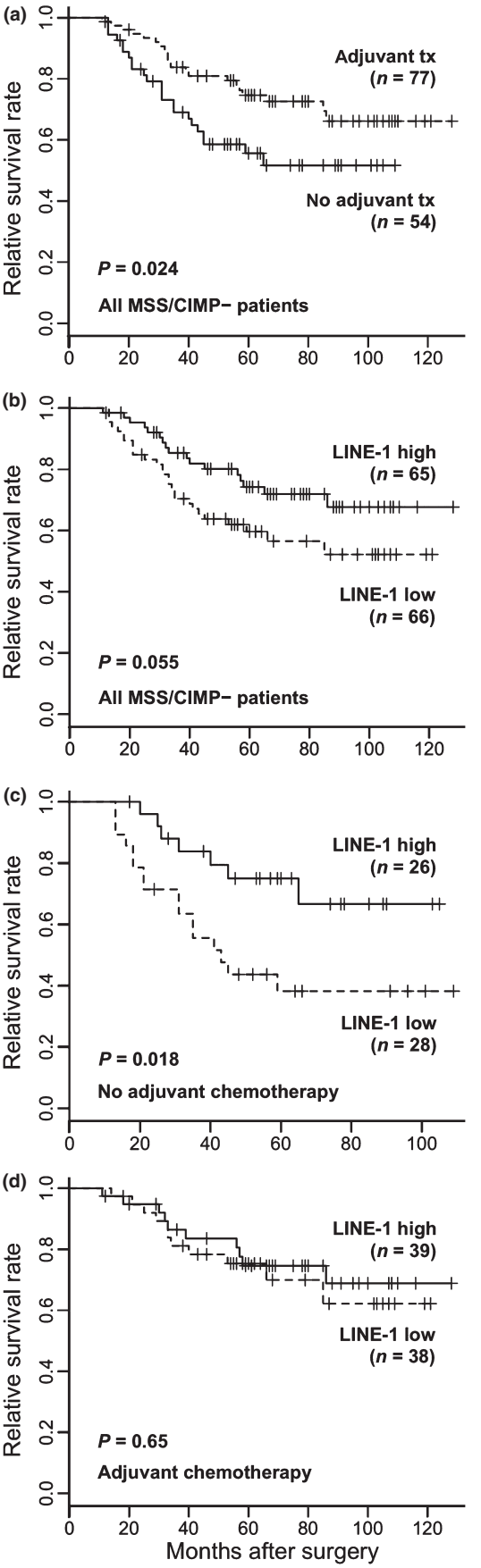

Fig. 2. Kaplan–Meier survival analysis of patient groups stratified according to use of postoperative adjuvant chemotherapy (a) and/or long interspersed nuclear element-1 (LINE-1) methylation (b–d). The prognosis of LINE-1 low group (dashed line) and those of LINE-1 high group (solid line) were compared in the overall MSS/CIMP− cohort (b) and in patients treated by surgery alone (c) or with adjuvant chemotherapy (d). The log-rank test was used for each comparison and *P*-values are shown. CIMP, CpG island methylator phenotype; MSS, microsatellite stable. tx, chemotherapy.

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Table 2. Multivariate analysis for the prognostic significance of clinicopathological factors and LINE-1 methylation in MSS/CIMP- CRC treated with surgery alone

Variables	*n*	Hazard ratio (95% CI)	*P*-value
Age (years)			
>66	40	1.00	0.10
≤66	14	0.41 (0.14–1.19)	
Gender			
Male	29	1.00	0.46
Female	25	0.72 (0.30–1.72)	
Site			
Proximal	14	1.00	0.56
Distal	40	1.41 (0.44–4.55)	
Stage			
II	30	1.00	0.77
III	24	0.88 (0.37–2.11)	
Histology			
Others	28	1.00	0.10
Well	26	0.44 (0.16–1.19)	
LINE-1 methylation			
High	26	1.00	0.022
Low	28	3.02 (1.17–7.80)	

Histology of adenocarcinoma was sub-classified into well-, moderately- and poorly-differentiated adenocarcinoma according to their grading. Cases of moderately- (*n* = 26) and poorly-differentiated adenocarcinoma (*n* = 1) and mucinous adenocarcinoma (*n* = 1) were combined for the analysis. CI, confidence interval; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; LINE-1, long interspersed nuclear element-1; MSS, microsatellite stable; *n*, number of patients.

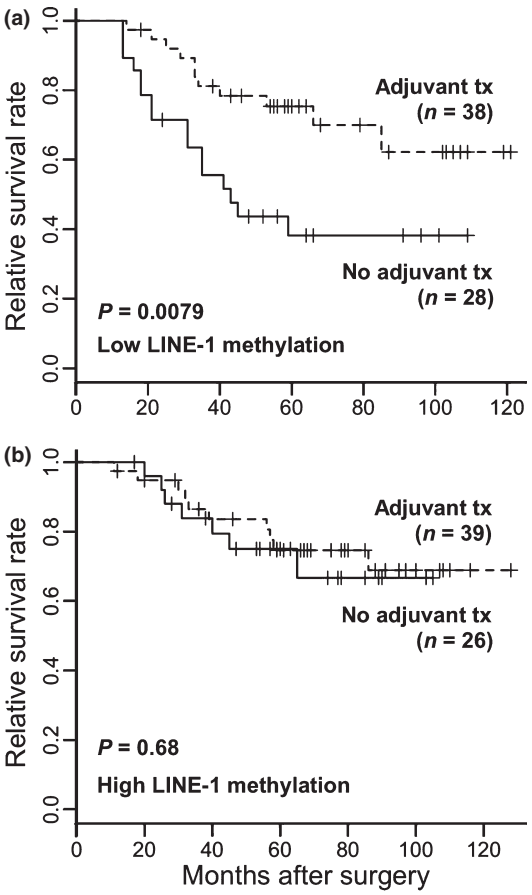

Fig. 3. Kaplan–Meier survival analysis of patients stratified according to the use of postoperative adjuvant chemotherapy in patients with low long interspersed nuclear element-1 (LINE-1) methylation (a) and in patients with high LINE-1 methylation (b). Solid lines indicate patients treated with surgery alone and dashed lines indicate patients treated with adjuvant chemotherapy. The log-rank test was used for each comparison and *P*-values are shown. tx, chemotherapy.

were apparent between patients treated with or without adjuvant chemotherapy. Multivariate analysis revealed that adjuvant chemotherapy was an independent prognostic factor for better survival in patients with low LINE-1 methylation, but not in patients with high LINE-1 methylation (Table 3). These results suggest that LINE-1 hypomethylation is a potential biomarker for the response of CRC patients to oral fluoropyrimidines in the adjuvant setting.

5-FU increases LINE-1 mRNA expression and induces DNA damage in CRC cells with LINE-1 hypomethylation. *In vitro* studies were performed to investigate the possible mechanism by which LINE-1 hypomethylation was associated with a good clinical response to oral fluoropyrimidines. Of the 13 CRC cell lines screened for MSI and CIMP status, three (SW480, Caco-2, CaR-1) were found to be MSS/CIMP− (Fig. 4a). The relationship between LINE-1 methylation and expression of its full-length transcript was examined by northern blot analysis in these MSS/CIMP− cell lines and in HT-29, HCT116 and SW48 cells (Fig. 4b). LINE-1 mRNA was detected as a single band of approximately 6 kb in size, representing its known full-length transcript (Fig. 4b, upper panel). The LINE-1 methylation level was inversely correlated with the expression of its full-length mRNA (Fig. 4b, lower panel).

Since LINE-1 mRNA was highly expressed in CRC cells with LINE-1 hypomethylation, we hypothesized that it may play a role in the therapeutic effect of 5-FU. To investigate this hypothesis, we looked for changes in the expression of LINE-1 mRNA in CRC cells following treatment with 5-FU. The 5-FU treatment had the effect of increasing LINE-1 mRNA expression in all three MSS/CIMP− cell lines, although the baseline expression level of the LINE-1 mRNA was less in CaR-1 cells than SW480 and Caco-2 cells (Fig. 4c, upper panels). The LINE-1 sequence contains an open reading frame that encodes a protein with nuclease activity,[29] suggesting that increased LINE-1 expression may be responsible for the DNA double strand breaks (DSB) observed in cells following treatment with 5-FU. To test this possibility, the phosphorylation of histone H2A.X at serine 139 (p-histone H2A.X^{Ser139}) was analyzed by western immunoblotting. Phospho-histone H2A.X^{Ser139} is a well-established marker of DSB[30] and have been used to assess the effect of 5-FU.[31,32] In SW480 and Caco-2 cells, the level of p-histone H2A.X^{Ser139} increased in parallel with the upregulation of LINE-1 mRNA after 5-FU treatment (Fig. 4c, lower panels). In contrast, the level of p-histone H2A.X^{Ser139} showed no change in CaR-1 cells following 5-FU treatment. The CaR-1 cells express less LINE-1 mRNA and have more LINE-1 methylation compared with SW480 and Caco-2 cells (Fig. 4b,c).

To further investigate whether increased LINE-1 expression is responsible for DNA damage following 5-FU treatment, RNA interference was used to deplete LINE-1 prior to treatment of the SW480 cells. When the cells were pretreated with non-specific control siRNA, p-histone H2A.X^{Ser139} expression was significantly induced following 5-FU treatment. However, when cells were pretreated with LINE-1-specific siRNA this induction of p-histone H2A.X^{Ser139} by 5-FU was suppressed (Fig. 4d). These results suggest that in CRC cells with low LINE-1 methylation, augmented high expression of LINE-1 mRNA by 5-FU subsequently leads to an increase in DSB and eventually to cell death. 5-FU may be ineffective in cells with high LINE-1

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Table 3. Multivariate analysis for the prognostic significance of clinicopathological factors and postoperative therapy in MSS/CIMP− CRC with a low LINE-1 methylation level and in those with a high LINE-1 methylation level

Variables	Low LINE-1 methylation			High LINE-1 methylation		
	n	Hazard ratio (95% CI)	*P*-value	*n*	Hazard ratio (95% CI)	*P*-value
Age (years)						
>66	33	1.00	0.21	29	1.00	0.67
≤66	33	0.60 (0.27–1.34)		36	1.29 (0.40–4.16)	
Gender						
Male	34	1.00	0.49	46	1.00	0.58
Female	32	0.76 (0.35–1.67)		19	0.67 (0.16–2.78)	
Site						
Proximal	16	1.00	0.42	17	1.00	0.058
Distal	50	1.52 (0.56–4.00)		48	5.88 (0.94–33.3)	
Stage						
II	37	1.00	0.32	34	1.00	0.023
III	29	1.48 (0.67–3.20)		31	4.19 (1.22–14.4)	
Histology						
Others	38	1.00	0.48	28	1.00	0.14
Well	28	0.75 (0.33–1.68)		37	2.61 (0.74–9.26)	
Adjuvant therapy						
Surgery alone	28	1.00	0.021	26	1.00	0.27
Chemotherapy	38	0.38 (0.17–0.87)		39	0.49 (0.14–1.74)	

Histology of adenocarcinoma was sub-classified into well-, moderately- and poorly-differentiated adenocarcinoma according to their grading. Cases of moderately- and poorly-differentiated adenocarcinoma and mucinous adenocarcinoma were combined for the analysis in the low LINE-1 methylation group (*n* = 35, 1 and 2, respectively) and in the high LINE-1 methylation group (*n* = 23, 4 and 1, respectively). CI, confidence interval; LINE-1, long interspersed nuclear element-1; *n*, number of patients.

methylation because the low baseline expression of LINE-1 mRNA in these cells is insufficient to increase DSB.

Discussion

In the present study, we explored the prognostic and predictive significance of LINE-1 methylation in CRC with the MSS/CIMP− phenotype. The MSI phenotype has been studied extensively in CRC and is associated with a good prognosis.[33] In addition, several studies have shown that CRC patients with an MSS/CIMP+ tumor phenotype have a worse survival compared with those with the MSS/CIMP− phenotype.[34–36] LINE-1 methylation was shown in the present (Table 1) and previous studies[15,16] to be associated with both the MSI and CIMP phenotypes. Because this could complicate the interpretation of survival data for LINE-1 methylation, we restricted the current study to MSS/CIMP− CRC.

Consistent with a previous study on a large CRC cohort,[14] patients with low LINE-1 methylation had shorter survival than those with high LINE-1 methylation (Fig. 2b). However, the present study also included information on postoperative adjuvant chemotherapy and analysis of this data showed that the prognostic significance of LINE-1 hypomethylation was restricted to patients treated by surgery alone (Fig. 2c,d). This result demonstrates that LINE-1 hypomethylation is a marker of poor prognosis in non-adjuvant treated CRC patients independently of the MSI and CIMP+ phenotypes. The association between LINE-1 hypomethylation and poor prognosis in CRC is not surprising because previous reports demonstrated that LOH-positive CRC has a lower LINE-1 methylation level[28] and the LOH on chromosome 18q is a poor prognostic marker.[11,37] To test whether the LINE-1 methylation level measured by our method is indeed associated with the 18q LOH we analyzed three microsatellite locus on chromosome 18q. The result showed that the LINE-1 methylation level was significantly lower in tumors with 18q LOH compared with those without LOH, suggesting links between LINE-1 hypomethylation, LOH on 18q and poor prognosis in CRC. These results warrant further study of LINE-1 methylation as a potentially useful prognostic marker for predicting the clinical course of early stage CRC patients.

The major and unexpected finding from this study was the predictive value of LINE-1 hypomethylation for survival benefit from chemotherapy with oral fluoropyrimidines in CRC patients (Fig. 3a). This was not apparent for patients with high LINE-1 methylation levels (Fig. 3b). A previous clinical trial has demonstrated that CRC patients with stage III disease who underwent a curative operation obtain a survival benefit from adjuvant chemotherapy with 5-FU-based regimens.[38] However, adjuvant chemotherapy is not effective for all patients and a substantial number suffer from disease recurrence and/or unpredictable serious adverse effects. Many strategies have therefore been explored to achieve optimal selection of patients who will benefit from adjuvant chemotherapy.[39] Although a number of candidate markers that predict survival benefit from adjuvant chemotherapy with 5-FU-based regimens have been proposed,[18,40,41] to date none have been validated in prospective trials or introduced into clinical practice. Our results suggest the LINE-1 methylation level in tumor DNA is a promising predictive marker for benefit from adjuvant treatment with oral fluoropyrimidines after curative surgery of CRC. Orally bioavailable fluoropyrimidines can improve the quality of life for cancer patients compared with intravenous administration of 5-FU.[42] The LINE-1 methylation marker in CRC could potentially optimize the clinical use of oral fluoropyrimidines and improve patient quality of life. Prospective randomized studies are required to clarify the clinical significance of LINE-1 methylation status as a predictive factor for response to adjuvant chemotherapy with oral fluoropyrimidines in CRC.

Our observation of an apparent survival benefit from oral fluoropyrimidines in patients with low tumor levels of LINE-1 methylation was supported by mechanistic studies linking LINE-1 methylation, 5-FU treatment and DNA damage in CRC cells. We observed a close relationship between the levels of LINE-1 methylation and full-length LINE-1 mRNA expression in CRC cells (Fig. 4b). This observation led us to hypothesize that cells with low LINE-1 methylation had a greater potential

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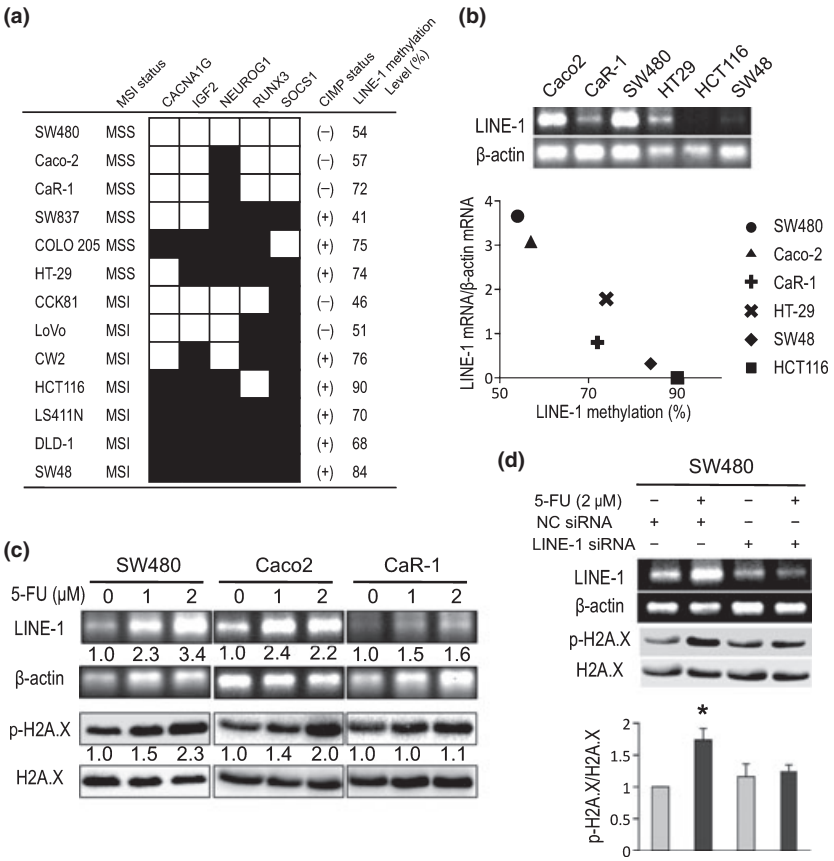

Fig. 4. *In vitro* mechanistic studies linking long interspersed nuclear element-1 (LINE-1) methylation, 5-fluorouracil (5-FU) treatment and DNA damage in colorectal cancer (CRC) cells. (a) Results for 13 CRC cell lines screened for microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and LINE-1 methylation level. The methylation status of five CIMP-defining CpG island markers was determined by MethyLight assay in which a reading of the percentage of methylated reference (PMR) ≥10 was defined as hypermethylated (black boxes). White boxes indicate PMR <10. (b) The relationship between the LINE-1 methylation level and expression of LINE-1 mRNA. Northern blot analysis of full-length LINE-1 mRNA and β-actin expression is shown in the upper panel. The LINE-1 methylation level correlated inversely with the relative amount of LINE-1 mRNA quantified by densitometry and normalized to β-actin with statistical significance (Spearman's $\rho = -0.94$, $P = 0.017$, lower panel). (c) Microsatellite stable (MSS)/CIMP− cells were treated with the indicated 5-FU concentration for 6 days followed by cell harvesting to isolate RNA and histone protein. LINE-1 and β-actin mRNA were detected by northern blot analysis and are shown in the upper panel with numbers reflecting the relative amount of LINE-1 mRNA quantified by densitometry and normalized to β-actin. Phospho-histone H2A.X^{Ser139} (p-H2A.X) and total histone H2A.X (H2A.X) proteins were detected by western blot analysis and are shown in the lower panel with numbers reflecting the relative amount of p-H2A.X protein normalized to H2A.X. (d) SW480 cells were transfected with either non-specific (NC siRNA) or LINE-1-specific siRNA (LINE-1 siRNA) followed by treatment with either DMSO or 5-FU (2 μM) for 6 days. The expression of p-H2A.X and H2A.X was quantified using densitometry and the relative expression of p-H2A.X to H2A.X (p-H2A.X/H2A.X) is shown in the bar graph at the bottom of the panel. The SW480 cells treated with non-specific siRNA and DMSO was set as 1. Values shown are the mean and the error bars represent standard deviation from three separate experiments. The asterisk indicates statistical significance ($P < 0.05$) for comparison of DMSO and 5-FU-treated cells in the same experimental conditions for transfected siRNA.

to undergo DSB following 5-FU treatment because the full-length LINE-1 mRNA encodes for a protein with nuclease activity.[29] Our *in vitro* study demonstrated that 5-FU treatment increases the expression of LINE-1 mRNA in MSS/CIMP− CRC cells (Fig. 4c). This was paralleled by increased DNA damage (assessed by p-histone H2A.X^{Ser139} expression) in SW480 and Caco-2 cells with low LINE-1 methylation (54% and 57%), but not in CaR-1 cells with high LINE-1 methylation (72%). Therefore, the increase in LINE-1 expression may be causally linked to 5-FU-induced DNA damage in cases where tumor cells have low LINE-1 methylation and express high baseline levels of LINE-1 mRNA (in case of SW480 and Caco-2). A causal link between increased LINE-1 mRNA and DNA damage induced by 5-FU treatment was further suggested by knockdown experiments of LINE-1 expression (Fig. 4d). These results could explain why tumors with LINE-1 hypomethylation appear to be responsive to adjuvant chemotherapy using oral fluoropyrimidines. On the other hand, we postulate that 5-FU may be ineffective in CRC cells with high LINE-1 methylation because their low baseline expression of LINE-1 mRNA is insufficient to induce DSB following 5-FU treatment (in case of CaR-1). This mechanism could explain our observation that CRC patients whose tumors show high LINE-1 methylation respond poorly to oral fluoropyrimidines (Fig. 3b). Our *in vitro* results suggest that further studies on the possible links between LINE-1 hypomethylation, LINE-1 expression, nuclease activity, DSB formation and sensitivity to 5-FU may lead to novel strategies for improving the cytotoxic effect of 5-FU.

In conclusion, we found that LINE-1 hypomethylation in MSS/CIMP− CRC had predictive value for benefit from adjuvant chemotherapy with oral fluoropyrimidines. Our *in vitro* studies have suggested a possible underlying mechanism for the link between LINE-1 hypomethylation in CRC cells and their susceptibility to fluoropyrimidines. These observations could have important implications for the future development of personalized chemotherapy and novel 5-FU-based regimens.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

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

Fig. S1. Kaplan–Meier survival analysis of patient groups stratified according to LINE-1 methylation.

Fig. S2. Association between LINE-1 methylation and LOH on chromosome 18q in CRC.

Fig. S3. Kaplan–Meier survival analysis of the patients stratified according to the use of post-operative adjuvant chemotherapy.

Table S1. Primer and siRNA sequences used in the present study.

Table S2. Association between the LINE-1 methylation level and clinicopathological features in MSS/CIMP− patients.

Table S3. Patient characteristics according to LINE-1 methylation in the subgroup treated with surgery alone.

Table S4. Patient characteristics in the subgroups analyzed for associations between LINE-1 methylation, prognosis and benefit from adjuvant chemotherapy.

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