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Original

LINE-1 Expression is Associated with the Effectiveness of EGFR Inhibitors in Colorectal Cancer Cells

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Background: Colorectal cancer (CRC) sidedness is predictive of anti-epidermal growth factor receptor (anti-EGFR) antibody therapy effectiveness; however, the mechanism linking them is unclear. Long interspersed nuclear element-1 (LINE-1) methylation has been associated with sidedness. Here, we evaluated whether LINE-1 expression in CRC cell lines influenced the efficacy of EGFR inhibitors.
Methods: We analyzed LINE-1 methylation in 98 clinical CRC samples. We also treated *RAS*-wild type SW48 and Caco-2 and *RAS*-mutant SW480, HCT116, and DLD-1 CRC cell lines with EGFR inhibitors gefitinib or RG14620, and performed growth assays in LINE-1-suppressed Caco-2, SW480, and DLD-1 cells.
Results: Clinical CRC findings confirmed the association between LINE-1 methylation and sidedness. LINE-1 mRNA expression was high in SW480 and Caco-2 cells and low in HCT116 and SW48 cells. The half maximal inhibitory concentrations (IC_{50}) of gefitinib were lower for LINE-1-expressing Caco-2 cells than for non-LINE-1-expressing SW48 cells, revealing an association between LINE-1 expression and the efficacy of gefitinib in *RAS*-wild type cells. LINE-1 knockdown increased the IC_{50} of gefitinib in Caco-2 cells. There was trend of increase in RG14620 IC_{50} upon LINE-1 knockdown even in *RAS*-mutant SW480 and DLD-1 cells, suggesting other mechanisms of RG14620 than EGFR signal inhibition.
Conclusion: EGFR inhibitor gefitinib requires LINE-1 expression, and interventions targeting LINE-1 may increase its efficacy.

Key Words: LINE-1, EGFR inhibitor, colorectal cancer

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related deaths worldwide. Recent advances in anticancer drug therapy have improved the prognosis for patients with inoperable CRC. However, a substantial proportion of CRC patients receive anticancer drug therapy without benefit because the effects are unpredictable. To avoid

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ineffective therapy, predictive markers for anticancer drug therapy are required.

RAS mutational status (*RAS* status) is the most successful biomarker of the efficacy of anti-EGFR antibody therapy for CRC.[1, 2] In addition to the *RAS* status of CRC, recent clinical data have shown that CRC left-sidedness is associated with higher sensitivity to anti-epidermal growth factor receptor (anti-EGFR) antibody than right-sidedness.[3] Sidedness is already considered important in several clinical guidelines for decision-making for CRC therapy.[4] However, it is only a surrogate marker for the effectiveness of the anti-EGFR antibody. The biological mechanism underlying the association between sidedness and sensitivity to anti-EGFR antibody is still unclear.

Long interspersed nuclear element-1 (LINE-1) is the most abundant transposable element in the human genome, accounting for 17% of the entire human genome.[5-7] The methylation of LINE-1 is reportedly related to the localization of CRC, with higher LINE-1 methylation levels in right-sided than in left-sided CRC.[8-10] DNA methylation is known to regulate gene expression by influencing chromatin structure. Abundant DNA methylation leads to a tight chromatin structure that prevents gene expression.[11, 12]

Both the effects of EGFR inhibitors and LINE-1 expression correlate with CRC localization, which led us to hypothesize that LINE-1 expression might be associated with the effects of EGFR inhibitors. Therefore, in this study, we investigated the relationship between LINE-1 mRNA expression in CRC cells and their susceptibility to EGFR inhibitors.

Materials and Methods

Patients and specimens

This study included 98 patients with CRC who received chemotherapy at Tokyo Women's Medical University (Tokyo, Japan). The patients comprised 51 males and 47 females ranging in age from 48 to 88 years (mean, 67.8 years). The tumors were classified as right-sided or left-sided relative to the splenic flexure. A total of 43 patients were defined as having right-sided CRC and 55 as having left-sided CRC. Other clinicopathological information such as clinical stage and cancer histology was also obtained, although the stage in 4 and histology in 5 patients were not available at the time of this study. We obtained tissue samples for *RAS* mutation and LINE-1 methylation analysis from formalin-fixed, 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. This project was approved by the Tokyo Women's Medical University Medical Ethics Committee (approval number 260B).

Cell lines and culture conditions

The CRC cell lines SW48, SW480, HCT116, Caco-2, RKO, HT-29, DLD-1, LoVo, LS174T, HCT-15, and SW 620 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained at 37°C in a 5% CO_2 environment in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin; Gibco, Grand Island, NY, USA). Genomic DNA was extracted from the cell lines using QIAamp® DNA Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

RAS mutation analysis

Genomic DNA extracted from clinical samples and cultured cell lines was used for *RAS* mutation analysis. The DNA sequence was amplified by polymerase chain reaction (PCR) for *RAS* codons 12 and 13, followed by direct sequencing, as described previously.[2]

LINE-1 methylation analysis

Genomic DNA extracted from clinical samples and cultured cell lines was treated with bisulfite using EpiTect Bisulfite Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After bisulfite treatment, LINE-1 methylation levels were quantitatively measured using a methylation-specific real-time PCR assay as described previously.[13]

Northern blotting

Total RNA was prepared using TRIzol® (Invitrogen,

Table 1 Association between location of colorectal cancer and LINE-1 methylation level.

Case	Location	n	LINE-1 methylation	p-value
Total	Left	55	79.8 (69.8-86.3)	0.0025
	Right	43	84.5 (79.7-90.7)	
RAS-wild type	Left	37	77.3 (68.6-90.7)	0.012
	Right	23	83.4 (78.1-92.4)	
RAS-mutant type	Left	18	80.3 (71.5-89.3)	0.22
	Right	20	85.0 (82.1-88.8)	

LINE-1 methylation levels are shown as median (25th percentile-75th percentile).
n, number of patients.

Carlsbad, CA, USA) according to the manufacturer's instructions. Polyadenylated RNA was then purified and northern blotting was conducted as described previously.[14] RNA probes for LINE-1 mRNA detection were synthesized as described previously[14] and RNA probes for β-actin were included in the DIG Northern Starter Kit (Roche, Mannheim, Germany).

RNA interference

Small interfering RNAs (siRNAs) specific to the LINE-1 sequence and non-specific siRNAs were synthesized by NIPPON Gene (Toyama, Japan). The siRNA sequences have been described previously.[14] The cells were transiently transfected with LINE-1-specific or non-specific siRNAs using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 24 h after transfection with the respective siRNAs, the cells were treated with dimethyl sulfoxide or EGFR inhibitors for 72 h. The cell growth was analyzed by Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) assay.

CCK-8 assay

We determined the rate of cell proliferation with a CCK-8 kit according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate (10^4 cells/well). Then, the cells were treated with increasing concentrations of the EGFR inhibitors gefitinib or RG14620. The CCK-8 reagent was added, and the optical density at 450 nm was detected using a microplate reader (uQuant, BioTek, Winooski, VT). The assay was repeated at least three times and results are shown as mean and standard deviation.

Statistical analysis

The level of LINE-1 methylation was expressed as a median value (25th percentile to 75th percentile). The Mann-Whitney U test was used to compare LINE-1 methylation levels between two variables. Differences in clinical backgrounds by sidedness was analyzed by chi-squared test. Half maximal inhibitory concentrations (IC_{50}) was expressed as mean ± standard deviation. One-way analysis of variance and Tukey's HSD test were used to compare the IC_{50} of four CRC cells treated with gefitinib and RG14620. Paired t-test was used to compare the IC_{50} values between cells treated with LINE-1-specific and non-specific siRNAs. Statistical analyses were carried out using the R software package.[15]

Results

LINE-1 methylation is lower in left-sided CRC than in right-sided CRC

We measured LINE-1 methylation levels in clinical samples by methylation-specific PCR. **Table 1** shows the values for right- and left-sided CRC. Right-sided CRC samples had a significantly higher LINE-1 methylation level than left-sided CRC samples. The differences in LINE-1 methylation associated with the sidedness of CRC were also evident upon *RAS*-wild type sample analysis. Analysis of clinicopathological backgrounds showed that right-sided CRC was more frequent in elder and female patients, although the relationship was not observed when the analysis was limited to *RAS*-wild type sample (**Table 2**). Clinical stage and cancer histology were not associated with sidedness of CRC (Supplementary Table). As the results were consistent with previous reports,[8-10] we continued to test the hypothesis that LINE-1 expression is associated with the effectiveness of EGFR inhibitors.

LINE-1 methylation level and *RAS* status in CRC cell lines

We screened the *RAS* statuses and LINE-1 methylation levels of 11 commonly used CRC cell lines (**Table 3**). Most cell lines with LINE-1 methylation levels lower than 50% had *RAS* mutations. Only Caco-2 was *RAS*-

Table 2 Relation of patients' age and gender with location of colorectal cancer.

	n	Location		p-value
		Left	Right	
All cases	98	55	43	
Age, years				
< 69	50	34	16	0.027
69 ≤	48	21	27	
Gender				
Male	51	34	17	0.047
Female	47	21	26	
RAS-wild type	60	37	23	
Age, years				
< 69	33	23	10	0.25
69 ≤	27	14	13	
Gender				
Male	34	22	12	0.78
Female	26	15	11	
RAS-mutant type	38	18	20	
Age, years				
< 69	17	11	6	0.11
69 ≤	21	7	14	
Gender				
Male	17	12	5	0.024
Female	21	6	15	

n, number of patients.

Table 3 *RAS* status and LINE-1 methylation level in colorectal cancer cell lines.

Cell line	*RAS* status		LINE-1 methylation (%)
	K-*ras* codon 12	K-*ras* codon 13	
RKO	wt	wt	90.2
HCT116	wt	G13D	84.1
SW48	wt	wt	80.2
HT-29	wt	wt	69.5
DLD-1	wt	G13D	60.2
LoVo	wt	G13D	50.3
Caco-2	wt	wt	48.2
LS174T	G12D	wt	47.6
HCT-15	wt	G13D	47.5
SW480	G12V	wt	43.6
SW620	G12V	wt	30.3

Mutation was listed with 1 letter abbreviation of amino acid substitution.
wt, wild type.

wild type with less than 50% LINE-1 methylation. Based on the results, we selected 4 cell lines with varying *RAS* statuses and LINE-1 methylation levels (**Table 4**).

Effects of EGFR inhibitors in CRC cell lines

LINE-1 mRNA expression was high in SW480 and Caco-2 cells, but low in HCT116 and SW48 cells (**Figure 1A**), consistent with the LINE-1 methylation levels in those cells. IC_{50} of gefitinib was significantly lower in *RAS*-wild type, LINE-1-expressing Caco-2 (7.6 ± 0.46 μM) compared to SW480 (22.2 ± 1.8 μM), HCT116 (17.4 ± 0.36 μM), and SW48 (17.2 ± 0.52 μM), suggesting that LINE-1 expression correlates with the gefit-

Table 4 *RAS* status and LINE-1 methylation level of the colorectal cancer cell lines used in this study.

RAS mutation	LINE-1 methylation level	
	Low	High
wt	Caco-2	SW48
mt	SW480	HCT116

wt, wild type; mt, mutant type.

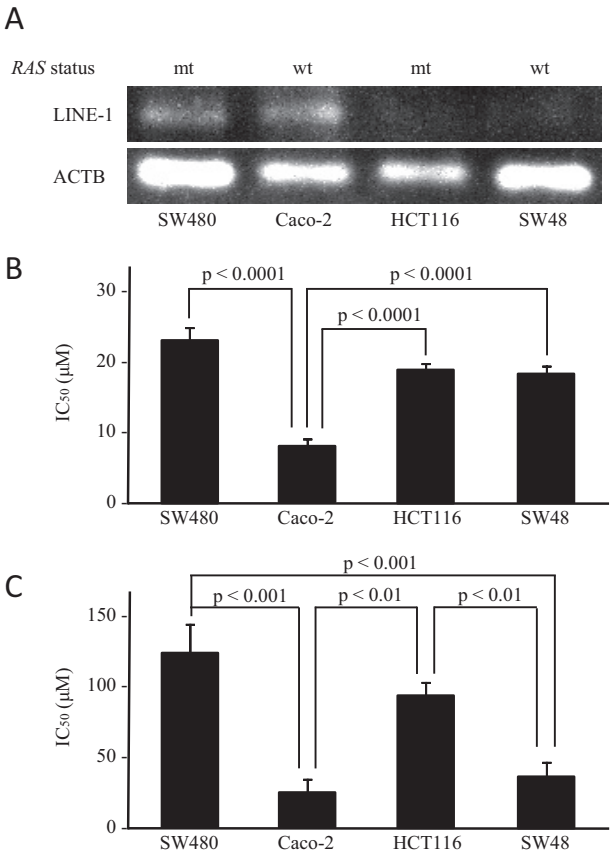

Figure 1 The association between LINE-1 mRNA expression and sensitivity to EGFR inhibitors in colorectal cancer (CRC) cell lines. (**A**) LINE-1 mRNA is expressed in SW480 and Caco-2, but not in HCT116 and SW48 cells. *RAS* status is indicated as wt for wild type and mt for mutant type. (**B**) Gefitinib IC_{50} determined by CCK-8 assay. (**C**) RG14620 IC_{50} determined by CCK-8 assay. P values calculated by statistical analysis are displayed in the figure. IC_{50}, half maximal inhibitory concentration.

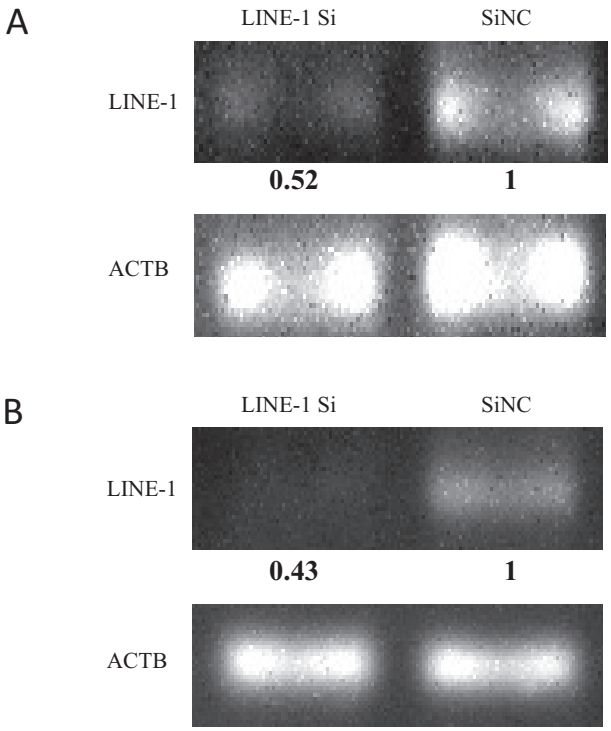

Figure 2 LINE-1 mRNA expression in (**A**) Caco-2 and (**B**) SW480 cells is suppressed by RNA interference. Northern blot analysis of LINE-1 mRNA are shown in the upper panel with numbers reflecting the relative amount of LINE-1 mRNA quantified by image analysis software, ImageJ, and normalized to β-actin. Northern blot analysis of β-actin mRNA are shown in the lower panel. LINE-1 Si is a LINE-1-specific 23-mer double-stranded RNA for RNA interference and SiNC is a non-specific sequence.

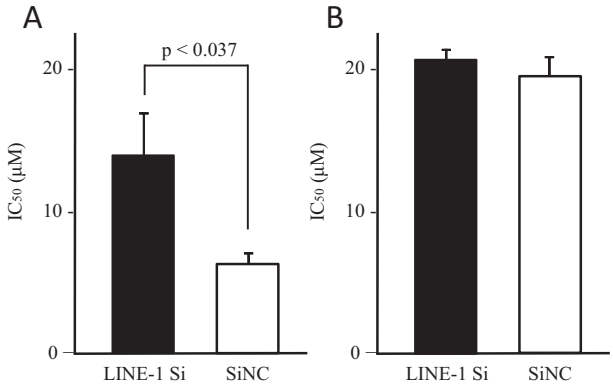

Figure 3 IC_{50} of gefitinib in (**A**) Caco-2 and (**B**) SW480 cells after LINE-1 suppression by RNA interference. P values calculated by statistical analysis are displayed in the figure. LINE-1 Si is a LINE-1-specific 23-mer double-stranded RNA for RNA interference and SiNC is a non-specific sequence.
IC_{50}, half maximal inhibitory concentration.

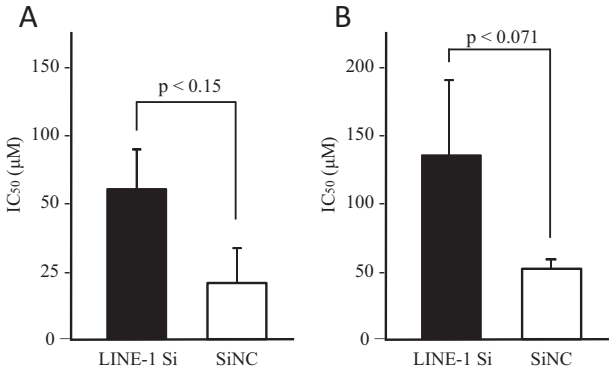

Figure 4 IC_{50} of RG14620 in (**A**) Caco-2 and (**B**) SW480 cells after LINE-1 suppression by RNA interference. P values calculated by statistical analysis are displayed in the figure. LINE-1 Si is a LINE-1-specific 23-mer double-stranded RNA for RNA interference and SiNC is a non-specific sequence.
IC_{50}, half maximal inhibitory concentration.

inib sensitivity of CRC cells without *RAS* mutations. In contrast, the IC_{50} of RG14620 was significantly lower in *RAS*-wildtype Caco-2 (20.8 ± 14.3 μM) and SW48 (30.4 ± 14.8 μM) compared to *RAS*-mutant SW480 (123.6 ± 22.4 μM) and HCT116 (92.3 ± 10.2 μM) independent of LINE-1 expression. These results suggest that gefitinib and RG14620 may have different anti-proliferating mechanism even though both are classified as EGFR inhibitors.

Effects of EGFR inhibitors in CRC cell lines are dependent on LINE-1 expression

We analyzed the effects of LINE-1 knockdown by RNA interference in the LINE-1-expressing cell lines Caco-2 and SW480 on their sensitivity to EGFR inhibitors. RNA interference successfully suppressed LINE-1 expression (**Figure 2**) after 48 h of siRNA treatment of the cells. Knockdown of LINE-1 significantly increased the IC_{50} of gefitinib in *RAS*-wild type Caco-2 cells (14.1 ± 3.0 vs. 5.6 ± 1.1 μM, **Figure 3A**) but had no effect on *RAS*-mutant SW480 cells (20.5 ± 1.3 vs. 19.9 ± 1.7 μM, **Figure 3B**). These results suggest that gefitinib requires LINE-1 expression for its anticancer effect in *RAS*-wild type cell.

Knockdown of LINE-1 increased the IC_{50} of RG14620 in Caco-2 (59.1 ± 32.0 vs. 23.5 ± 7.1 μM, **Figure 4A**) and SW480 cells (136.7 ± 53.1 vs. 47.0 ± 9.8 μM, **Figure 4B**) although it did not reach statistical significance. The statistical trend (p = 0.071) in *RAS*-mutant SW480 cells treated with RG14620 was unexpected, because EGFR inhibitor has generally no effect on *RAS*-mutant CRC, as observed by gefitinib treatment. Therefore, we

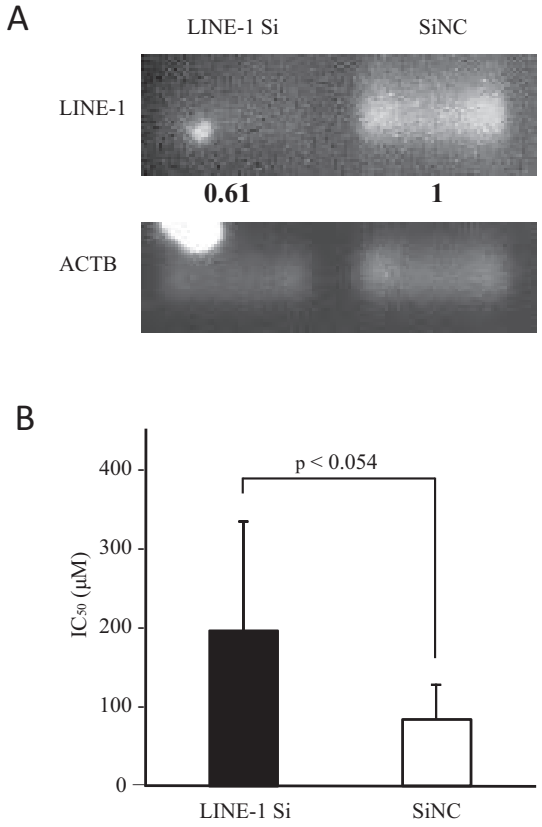

Figure 5 (**A**) LINE-1 mRNA expression in DLD-1 cells is suppressed by RNA interference. Northern blot analysis of LINE-1 mRNA are shown in the upper panel with numbers reflecting the relative amount of LINE-1 mRNA quantified by image analysis software, ImageJ, and normalized to β-actin. Northern blot analysis of β-actin mRNA are shown in the lower panel. LINE-1 Si is a LINE-1-specific 23-mer double-stranded RNA for RNA interference and SiNC is a non-specific sequence. (**B**) IC_{50} of RG14620 in DLD-1 cells after LINE-1 suppression by RNA interference. P values calculated by statistical analysis are displayed in the figure. LINE-1 Si is a LINE-1-specific 23-mer double-stranded RNA for RNA interference and SiNC is a non-specific sequence.
IC_{50}, half maximal inhibitory concentration.

repeated the experiment using another *RAS*-mutant cell line. We screened *RAS*-mutant cell lines and found that DLD-1 expresses LINE-1 mRNA and the expression was effectively suppressed by RNAi (**Figure 5A**). In *RAS*-mutant DLD-1 cells, knockdown of LINE-1 increased the IC_{50} of RG14620 (197.3 ± 126.5 vs. 76.6 ± 49.1 μM, **Figure 5B**), which was close to significance ($p = 0.054$). These results suggested that LINE-1 expression is associated with the effectiveness of RG14620 in *RAS*-mutant CRC cells.

Discussion

In the present study we explored the association between the expression of LINE-1 mRNA and the effectiveness of EGFR inhibitors in CRC cells. The results showed an association between LINE-1 expression and the effects of gefitinib in CRC cells without *RAS* mutations. However, Caco-2 was the only *RAS*-wild type, LINE-1-expressing line among our screened cell lines. Studies using additional *RAS*-wild type, LINE-1-expressing cells are needed to verify the reproducibility of and extend our findings.

Although our results are based on an in vitro study using cell lines, they are clinically important and warrant further investigation. The suggested relationship between LINE-1 expression and the efficacy of EGFR inhibitors may lead to novel therapeutic strategies. Controlling LINE-1 expression may enhance the effects of EGFR inhibitors and overcome therapeutic resistance. In addition, controlling LINE-1 expression in normal tissue may overcome the troublesome EGFR inhibitor side effect of skin rash. Our clinical investigation support the significance of LINE-1 expression. Right-sided CRC samples had a significantly higher LINE-1 methylation level than left-sided CRC samples when *RAS*-wild type CRC was analyzed. The clinical higher response to anti-EGFR antibody in left-sided CRC patients may be explained by the difference in LINE-1 methylation level. Further investigation is needed to clarify the mechanism underlying the relationship between LINE-1 expression and the effects of EGFR inhibitors to improve clinical strategies using EGFR inhibitors for CRC patients.

An unexpected result obtained in our study was the association between LINE-1 expression and the effectiveness of the EGFR inhibitor RG14620 in the *RAS*-mutant cell lines, SW480 and DLD-1. These results shed light on the role of RG14620 in patients with *RAS*-mutant CRC. RG14620 was synthesized as a member of tyrphostins, which are potent EGF receptor kinase inhibitors,[16] and has been used as an EGFR inhibitor in research. Recent study demonstrated that RG14620 also inhibits ABCG2 transporter, reversing multidrug resistance.[17] The difference between gefitinib and RG14620 in the current study may be due to an unknown mechanism of RG14620 other than EGFR inhibition. Further study of RG14620 is

required especially on its anti-proliferating effect on *RAS*-mutant CRC, because anti-EGFR antibody therapy is not allowed for *RAS*-mutant CRC patients, resulting in limitation of cancer therapy. LINE-1 expression may be clinically useful to select the patients with *RAS*-mutant CRC who could benefit from RG14620.

The development of a diagnostic method to evaluate LINE-1 expression in clinical samples will be essential to facilitate the use of LINE-1 expression as a predictive marker. We used northern blotting to analyze LINE-1 mRNA expression in CRC cell lines. As LINE-1 is a retroposon that is repeated genome-wide, it would be difficult to quantitate LINE-1 mRNA by PCR because of contamination by LINE-1 DNA. Detection of the full-length 6-Kb mRNA by northern blotting is the most reliable method; however, it is complex and, therefore, not appropriate for clinical diagnosis. One simple diagnostic method may be immunohistochemistry with a specific antibody that recognizes a protein translated from LINE-1 mRNA. LINE-1 mRNA is translated into two proteins from open reading frame 1 and open reading frame 2.[18] Development of a method for easy and reliable evaluation of LINE-1 protein levels may also highlight the importance of LINE-1 protein as a clinical marker. LINE-1 methylation has been investigated as a prognostic marker for CRC patients. Hypomethylation of LINE-1 has been associated with poor prognosis in a single large-scale study[19] and meta-analysis.[20] The association with hypomethylation of LINE-1 and poor prognosis is consistent with other cancers, such as esophageal cancer,[21] lung cancer,[22] gastric cancer,[23] hepatocellular carcinoma,[24] and cholangiocarcinoma.[25] These studies used methylation-specific PCR or pyrosequencing following bisulfite treatment of DNA; however, these method showed the disadvantage of normal tissue contamination. Methylation of LINE-1 in normal tissue is generally very high and the contamination of normal tissue in the sample tended to show higher than real LINE-methylation of tumor cells. This problem may be solved by immunohistochemistry using specific antibody to LINE-1 to analyze the LINE-1 protein expression rather than its methylation.

The mechanisms responsible for the relationship between LINE-1 expression and effectiveness of EGFR inhibitors are unknown. A study reported that LINE-1 ribonucleoprotein particles have a role in telomere stability.[26] It has also been reported that EGFR signaling has a role in telomere length regulation, and longer telomere length is a potential biomarker of the clinical outcome after anti-EGFR antibody therapy in patients with *RAS*-wild type CRC.[27] Telomere regulation may be a candidate mechanism underlying the association between LINE-1 expression and effects of EGFR inhibitors.

Growing evidence shows a pivotal role of LINE-1 expression in cancer development. Recent study reported somatic retrotransposition in 2,954 cancer genomes from 38 histological cancer subtypes.[28] As LINE-1 expression followed by reverse-transcription and insertion into genome is the most frequent type of retrotransposition, LINE-1 expression may influence cancer characteristics. Besides acting as a biomarker for the effects of EGFR inhibitors, cancer characteristics related to LINE-1-dependent retrotransposition also have potential clinical use for developing tailored medicine. A wide range of clinical implications associated with LINE-1 expression can be explored in future.

In conclusion, we showed an association between the expression of LINE-1 mRNA and the effects of EGFR inhibitors in CRC cells. Anti-EGFR antibodies and small-molecule EGFR inhibitors may be able to exert greater anticancer effects upon manipulation of LINE-1 expression. Further clarification of the mechanism underlying the association between the expression of LINE-1 mRNA and the effectiveness of EGFR inhibitors is required to develop more effective clinical therapies.

Conflicts of Interest: The authors declare no conflict of interest associated with this manuscript.

Disclaimer: Kazuhiko Hayashi is one of the Associate Editors of Tokyo Women's Medical University Journal and on the journal's Editorial Board. He was not involved in the editorial evaluation or decision to accept this article for publication at all.

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