

Correlation of LINE-1 Hypomethylation With Size and Pathologic Extent of Dysplasia in Colorectal Tubular Adenomas

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INTRODUCTION: Conventional adenomas (tubular adenoma [TA] or tubulovillous adenoma) and sessile serrated lesions (SSLs) are neoplastic precancerous lesions frequently detected in patients undergoing average risk screening colonoscopy and polyp surveillance. Metachronous risk stratification of adenomas is currently limited to histologic features and size of polyps. We report long interspersed nucleotide element-1 (LINE-1) methylation levels in SSL in comparison to TA and the impact of TA size and presence of high-grade dysplasia (HGD) on LINE-1 methylation.

METHODS: LINE-1 methylation was assessed by pyrosequencing of bisulfite-converted DNA. We compared LINE-1 methylation between TA and SSL, among varying sizes of TA, and between TA with HGD and low-grade dysplasia (LGD).

RESULTS: LINE-1 methylation declined with increasing polyp size in TA when comparing those <5 mm (72.31 ± 6.11), 5 to <10 mm (67.50 ± 7.00), and ≥ 10 mm (66.75 ± 11.89). There were lower LINE-1 methylation levels in TA with LGD ($n = 119$) compared with SSLs ($n = 29$) (69.11 ± 8.62 vs 81.41 ± 2.43 , $P < 0.001$). TA containing HGD ($n = 26$) had lower LINE-1 methylation levels than those with LGD ($n = 119$) (59.86 ± 7.93 vs 69.11 ± 8.62 , $P < 0.001$).

DISCUSSION: HGD and increasing size of TA/tubulovillous adenoma were associated with lower LINE-1 methylation. This supports a hypothesis that LINE-1 hypomethylation in TAs indicates advancement along the CRC tumorigenesis pathway. Lower LINE-1 methylation and greater variance of global DNA methylation was seen in TA compared with SSL. LINE-1 methylation in adenomas correlates with polyp size and degree of dysplasia and deserves further study as a predictor of metachronous colorectal cancer risk.

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INTRODUCTION

Patients who have high-risk adenomas based on polyp features of size and anatomical features are associated with increased risk of future colorectal cancer (CRC) and related death, whereas those with low-risk adenomas are not associated with increased risk of CRC and related death, suggesting that a less invasive surveillance interval may be appropriate (1,2). Improved prognostic indicators beyond conventional anatomic pathology markers of size, histology, and the presence of dysplasia to better target patients in timing of surveillance colonoscopy would apply colonoscopy resources most efficiently to those who will benefit from polyp detection and resection. Molecular markers in colorectal polyps offer promise for clinical application to prognosticate the risk for metachronous advanced neoplasia but are not currently used in clinical practice. Epigenetic

markers are an emerging way in which the presence of CRC and its precursors, adenomatous neoplastic polyps, can be characterized.

Two general alterations in normal DNA methylation processes occur in cancer cells. Hypermethylation of gene promoters, especially tumor suppressor genes, silences their expression (loss of function), promoting unregulated cell division. Intergenic global DNA hypomethylation can activate normally suppressed gene expression, particularly in retrotransposons (gain of function), resulting in chromosomal instability (3). In contrast to specific gene methylation, global hypomethylation status is not currently used in available diagnostic tests for CRC screening (4). DNA global hypomethylation is known as an early event in colorectal carcinogenesis and has been shown to have a linear correlation between the status of demethylation and disease stage from early adenoma to adenocarcinoma to metastasis (5,6).

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DNA methylation plays a crucial role in regulating epigenetic changes. Long interspersed nucleotide element-1 (LINE-1) is a repetitive DNA retrotransposon. A type of epigenetic alteration, hypomethylation of repetitive DNA sequences—i.e., short, interspersed elements (SINEs or Alu elements) or long interspersed nuclear elements (LINEs)—may predispose cells to chromosomal defects and rearrangements that result in genetic instability and elevated mutational rates (7). The large quantity of LINE-1 sequences in the genome makes it a potentially strong comparative biomarker to define neoplasia status. Normal tissue of the colon and rectum has highly methylated LINE-1 elements, whereas hypomethylation can be observed in early stages of precancerous adenomas (5).

Preferential LINE-1 hypomethylation in tubular adenomas (TAs) found with synchronous CRC versus those in patients without CRC suggest that LINE-1 hypomethylation may be found at various levels in specific adenomatous polyps (5). In addition, greater hypomethylation of normal colonic tissue in patients with CRC compared with those without CRC is suggestive of LINE-1 serving as a marker of synchronous dysplasia (8). These results support LINE-1 hypomethylation as having a possible role in describing either synchronous associated dysplasia or as a marker of a lesion extent of dysplasia progression. Development of a molecular biomarker to further risk stratify patients with findings of adenomatous polyps could serve as a more objective marker for metachronous advanced neoplasia compared with the currently used criteria of size, number, and histology with endoscopist and pathologist variability and with modest predictive values.

Our aim was to evaluate LINE-1 methylation status in conventional TA compared with sessile serrated polyps and LINE-1 methylation status in TAs of increasing size and with or without high-grade dysplasia (HGD). We hypothesized that LINE-1 methylation would decrease with anatomical histopathology

characteristics of dysplasia severity, such as increased size of adenoma and the presence of HGD.

METHODS

Patients

We selected patients from 2005 to 2014 from a natural language search on pathology reports for “tubular adenoma,” “tubulovillous adenoma,” and “sessile serrated polyps” from screening and surveillance colonoscopies collected in a database. The methylation tests were performed on tissue which had been collected as part of a research biorepository. Inclusion criteria were age older than 18 years of age. Exclusion criteria were patients with a history of previous CRC, history of colonic resections, inflammatory bowel disease, or the presence of 5 or more SSL or TA. The patients had no previous colonoscopy within the past 3 years, and those with poor colonoscopy preps were excluded (Figure 1). Poor colonoscopy prep was defined as inability to exclude lesions >5 mm in size. Polyps ≥ 10 mm were removed by hot snare and those measuring 6–9 mm in size were resected by cold or hot snare. Polyps 5 mm or less were resected by cold snare or cold forceps based on endoscopists preference. Formalin-fixed specimens of single representative TAs or sessile serrated lesions (SSLs) from each patient were included in the analysis. Colorectal tumor tissue from patients with diagnosed CRC were also included in the analysis. LINE-1 methylation status was contrasted between all groups. As male gender, tobacco use, and advanced age have all been associated with increased risk for advanced neoplasia and CRC, they were examined for an association with LINE-1 status of neoplastic polyps (9–12).

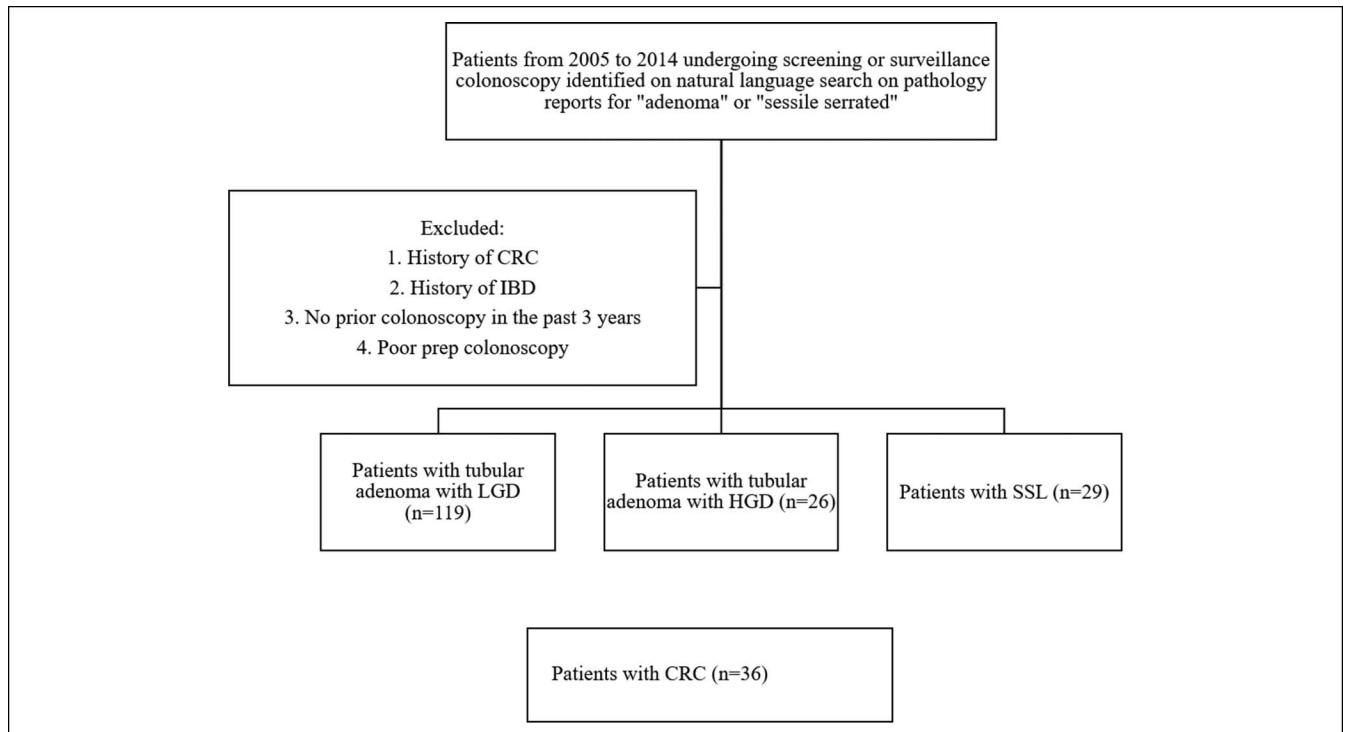


Figure 1. Flow chart of patient selection. CRC, colorectal cancer; HGD, high-grade dysplasia; IBD, inflammatory bowel disease; LGD, low-grade dysplasia; SSL, sessile serrated lesion.

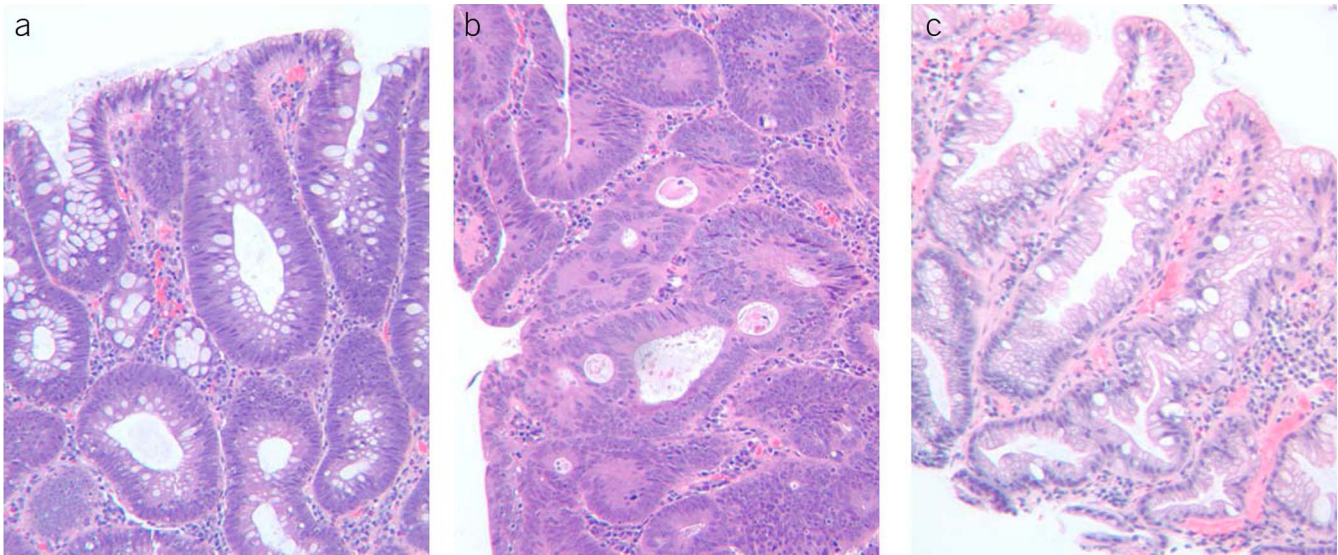


Figure 2. (a) Hematoxylin and eosin stain of a conventional tubular adenoma showing stratification of nuclei which do not reach the luminal surface and apical mucin. (b) Tubular adenoma with high-grade dysplasia showing complex glandular crowding and irregularity, a cribriform appearance, and cytologic features of loss of cell polarity and markedly enlarged nuclei. (c) Sessile serrated lesion with sawtooth serrated glands with narrower luminal openings, gland branching with wider bases, and excessive cytoplasmic mucin and goblet cell population.

The protocol was approved by the Internal Review Board of Rush University Medical Center (14080703-IRB01), and informed written consent was obtained from all patients. All authors had access to the study data and had reviewed and approved the final manuscript.

Specimen collection

Three 4- μ m sections were cut from formalin-fixed tumor or polyp tissue sections (Figure 2a–c). One slide for each specimen was stained with hematoxylin and eosin. The stained slides were reviewed by a pathologist to confirm the adequacy (at least 3 mm²) and type of polyp/tumor/nonmalignant tissue on each thin section. Collected data included gender, race, genotype, age at diagnosis, date of diagnostic colonoscopy, additional polyps, focal HGD, cancer arising from adenoma, location (right or left), tumor size (cm), and tumor stage. The presence of HGD was determined by pathologists using a combination of architectural features such as complex glandular crowding and irregularity, a cribriform appearance, and prominent intraluminal papillary tufting, as well as cytologic features including loss of cell polarity, markedly enlarged nuclei, atypical mitotic features, and prominent apoptosis as described by the World Health Organization and UK National Health Service bowel cancer screening program (13,14).

DNA isolation

Using the reviewed hematoxylin and eosin-stained slide as a guide, tumor, polyp, or nonmalignant tissue was scraped from 1 or 2 adjacent unstained sections (macrodissection) and placed in 60–200 μ L lysis buffer (10 mM Tris 50 mM KC, pH 8.3, 1.0 mg/mL proteinase K). The volume used depended on the amount of tissue available from the slides. Macrodissection of tissue samples yielded >90% dysplastic material in the samples. The samples were incubated for at least 6 hours before methylation analysis. Proteinase activity was eliminated at the end of the digestion by a 5-minute incubation at 95 $^{\circ}$ C.

LINE-1 methylation

LINE-1 methylation was assessed by pyrosequencing of bisulfite-converted DNA (15). Precision of the pyrosequencing assay for LINE-1 methylation levels has previously been validated for colon cancer tissue and normal colonic mucosa (16). Ten microliters of DNA lysate from macrodissected polyp, tumor, or nonmalignant tissue were bisulfite converted using the Zymo EZ DNA Methylation TM Kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. The converted DNA was amplified using forward primer TTTTGAGTTAGGTGTGGGATATA and biotinylated reverse primer (Biotin)-AAAATCAAAAATTCCTTTC. The LINE-1 retrotransposon targeted was located on 22q11-q12; genomic coordinates (GRCh38): 22:15,000,000–37,200,000; and the primer sequences were based on repeat elements (locus X58075:111-358). After amplification, 15 μ L PCR product was subjected to pyrosequencing. Sequencing was performed on a PyroMark Q24 pyrosequencer (Qiagen) programmed with the following sequence to analyze TYGATTTTTAGGTGYGTTYGTTA. The average of the relative percent C (methylated) vs T (unmethylated) at each of 3 CpG sites was reported (Figure 3). Non-CpG cytosines, which should be 100% converted, were included in each sequence to confirm complete bisulfite conversion.

Statistical analysis

Basic summary statistics were calculated for global methylation levels and other continuous variables. Binary and categorical variables were tabulated. The comparison of means in methylation levels among groups was assessed by independent *t* test. The association of global methylation and single gene promoter methylation with patient characteristic groups was assessed by Mann-Whitney *U* test. Analyses were performed in SPSS statistical software. Based on a preliminary comparison of LINE-1, the relative percent methylation of 72% in diminutive polyps (<6 mm) with a SD of 6%, a desired power of 80%, and a type 1 error rate of 5%, and the assumption that $\geq 4\%$ difference in percent methylation could be considered significant among an

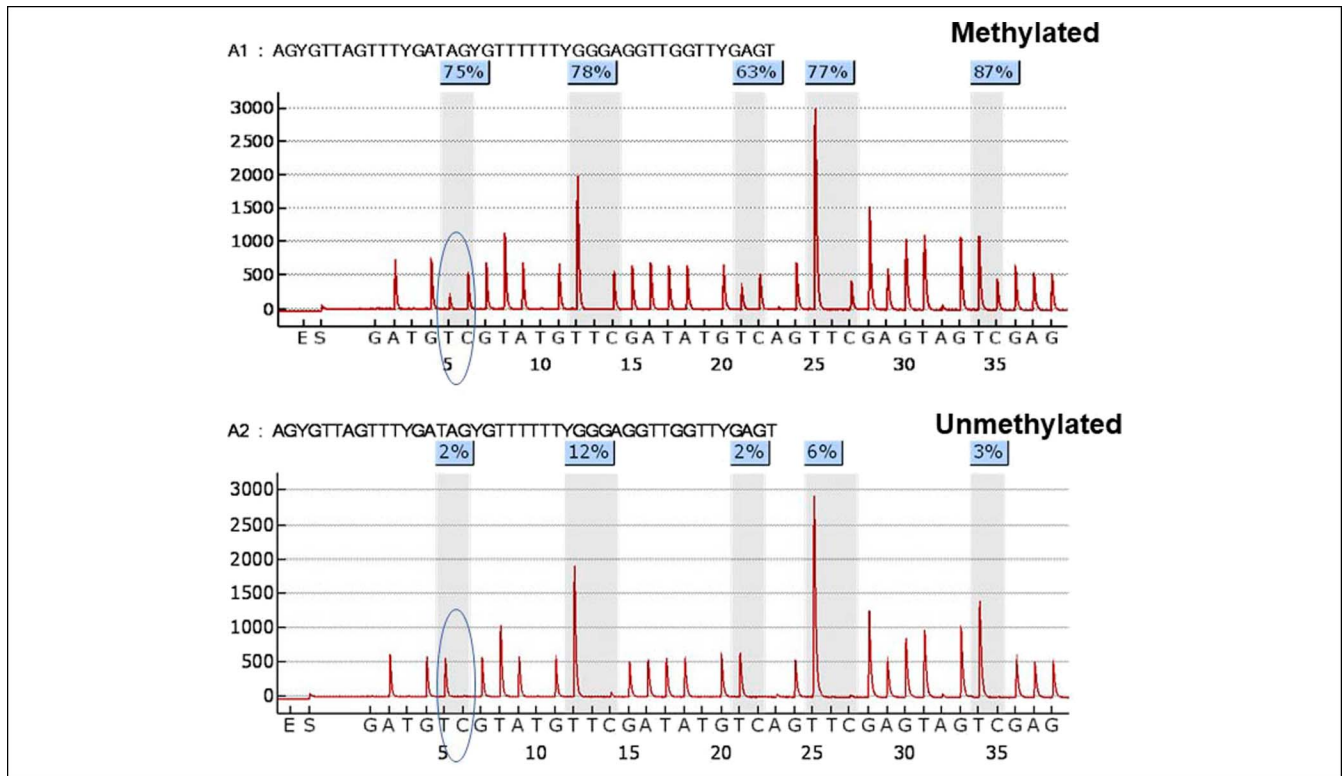


Figure 3. Example of pyrosequencing to measure long interspersed nucleotide element-1 (LINE-1) methylation. The percent methylation level was measured as a proportion of peak C height and peak T height at each CpG site after bisulfite conversion (encircled). The average of the relative percent C (methylated) vs T (unmethylated) at each of 3 CpG sites was reported as the LINE-1 methylation level.

alternative sized adenoma group (polyps >6 mm); a sample size ≥ 36 per group was derived to compare polyps across size (<6 mm vs larger in size vs 6–9 mm in size vs larger).

RESULTS

Demographics and polyp characteristics

A total of 145 patients were found to have TA on colonoscopy, 29 patients had SSL, and 36 patients had CRC. In each case, the most advanced polyp by size and histology was analyzed for methylation per patient. Of the 145 patients with TA, 26 of these patients were found to have HGD. Polyp size greater than 10 mm was present in 58 of 145 of the patients (Table 1).

Among TAs with low-grade dysplasia (LGD), there was no difference in LINE-1 methylation among patients older than 60 years old compared with patients younger than 60 years old (68.36 ± 8.73 vs 69.67 ± 8.57 , $P = 0.342$) or in male compared with female patients (68.53 ± 8.39 vs 69.87 ± 8.94 , $P = 0.342$). Previous smoking history also did not affect polyp LINE-1 methylation (68.60 ± 9.00 vs 70.77 ± 7.81 , $P = 0.235$). There was no significant difference in LINE-1 methylation between proximal compared to distal location of the polyp (70.15 ± 7.80 vs 67.83 ± 9.47 , $P = 0.221$).

LINE-1 methylation correlation with adenoma histology and size

Comparison of LINE-1 across polyp type groups and with tumor tissue found progressively decreasing levels of LINE-1 methylation in TA with LGD of increasing size. TA equal to or less than 5 mm in size ($n = 45$) had higher LINE-1 methylation levels compared with TAs 6–9 mm in size ($n = 42$) and equal to or

Table 1. Characteristics of neoplastic lesions analyzed by LINE-1 methylation

Group	TA (n = 145)	SSP (n = 29)
Age, mean \pm SD	61.80 \pm 8.41	61.34 \pm 6.34
Gender (F/M), n (%)	62/83 (43)	14/15 (48)
Polyp location, n (%)		
Right	83 (57)	20 (69)
Left	62 (43)	9 (31)
Polyp size, n (%)		
<5 mm	45 (31)	4 (14)
6–9 mm	42 (29)	2 (7)
≥ 10 mm	58 (40)	23 (79)
Low-grade dysplasia, n (%)	119 (82)	
Advanced neoplasia, n (%)	63 (43)	0
High-grade dysplasia or villous in polyp <10 mm, n (%)	26 (18)	
10 mm or more plus villous or high-grade dysplasia morphology, n (%)	22 (15)	
10 mm or more by size alone, n (%)	37 (25)	
LINE-1, long interspersed nucleotide element-1; SSP, sessile serrated polyp; TA, tubular adenoma.		

Table 2. LINE-1 methylation level by polyp or tissue type

Polyp type (n)	LINE-1 methylation level
Sessile serrated polyp (29)	81.41 ± 2.43
Tubular adenomas with low-grade dysplasia (119)	69.11 ± 8.62
Tubular adenoma ≤5 mm (45)	72.31 ± 6.11
Tubular adenoma 6–9 mm (42)	67.50 ± 7.00
Tubular adenoma ≥1 cm (32)	66.75 ± 11.89
Tubular adenoma with high-grade dysplasia (26)	59.86 ± 7.93
Colorectal cancer tumor tissue (36)	50.36 ± 8.40

LINE-1, long interspersed nucleotide element-1.

greater than 10 mm (n = 32) (72.31 ± 6.11 vs 67.50 ± 7.00 vs 66.75 ± 11.89, $P < 0.001$, Table 2).

In addition, lower levels of LINE-1 methylation were found sequentially in those TA with only LGD, TA with HGD, and CRC (Figure 4). TA containing HGD (n = 26) had lower LINE-1 methylation levels compared with TA with LGD (n = 119) (59.86 ± 7.93 vs 69.11 ± 8.62, $P < 0.001$). CRC tumor tissue (n = 36) had the lowest levels of LINE-1 methylation at 50.36 ± 8.40 (Table 2).

Comparison of LINE 1 in SSL vs TA with LGD

There was lower LINE-1 methylation in TA with LGD (n = 119) compared with SSL (n = 29) (69.11 ± 8.62 vs 81.41 ± 2.43, $P < 0.001$; Figure 5). In addition, there was greater variation of LINE-1 methylation levels in TA vs SSL. Variance of 20% from the mean level of LINE-1 hypomethylation was present in 13 of 119 (10.9%) in TA with LGD compared with 0 of 29 SSL.

DISCUSSION

Our findings of lower LINE-1 methylation in TA of greater size and with the presence of HGD compared with LGD show that LINE-1 methylation could be a promising marker for progression of adenomatous polyp neoplasia. There was a smaller degree of variance in LINE-1 methylation among SSL compared with TA and lower LINE-1 methylation in TA than SSL. It should be noted that we did not analyze SSL with dysplasia or traditional serrated adenomas that are associated with potential greater malignant progression (17). Because LINE-1 methylation did not vary with size or histologic features in our series of SSL, our findings suggest that LINE-1 methylation has a more limited role in the sessile serrated pathway. Alternative molecular markers which associate with dysplasia or other conventional high-risk features in SSL should be further investigated.

There are multiple ways in which molecular markers of adenomatous polyps and markers of dysplasia progression may be helpful to clinical endoscopic practice. Multivariate analyses have shown that villous features and polyp size have only modest increased risk for metachronous advanced neoplasia (18). A pooled analysis found that patients deemed high risk at baseline were found to have metachronous advanced neoplasia detected in 15.5% compared with 6.9% deemed low risk at baseline (18). recurrent advanced adenoma findings are present in only approximately 1 of 5 surveillance colonoscopy cases. One idea would be to use molecular markers to assess future metachronous

neoplastic risk. Given our findings of lower LINE-1 methylation in TA with HGD and of greater size, LINE-1 methylation of TA could be evaluated in prospective studies as a potential predictor of metachronous CRC risk in the surveillance of patients with TA. A second theoretical way to integrate molecular markers could be using the measurement of LINE-1 methylation of a resected TA and the postpolypectomy margin to assess for complete eradication of a dysplastic lesion. Although we did not directly evaluate these in our study, future directions should address the potential usefulness of LINE-1 methylation as a marker for metachronous risk assessment and resection completeness.

There are several limitations to our study. The sample size is relatively modest, and the extent to which LINE-1 methylation might have been affected by the presence of synchronous neoplasia burden was not evaluated. However, patients who had more than 5 adenomas or SSL were excluded. The study is dependent on interobserver variability in pathology diagnosis to correlate LINE 1 status with neoplasia, and some reports have shown significant variation in dysplasia degree among pathologists (19,20). Because patients were not followed over time, the metachronous implications of the extent to which LINE-1 can impact risk assessment of future risk is not yet defined.

In summary, we found that LINE-1 methylation decreased with markers of more advanced neoplasia, including size and degree of dysplasia. LINE-1 holds promise for a marker that characterizes the extent of dysplasia in adenomatous polyps and deserves further study as a potential marker for metachronous. Thus, when surveillance colonoscopy is performed on patients with advanced adenomas deemed high risk by current guidelines based on pathology review of their polyp features, recurrent advanced adenomas are present in only approximately 1 of 5

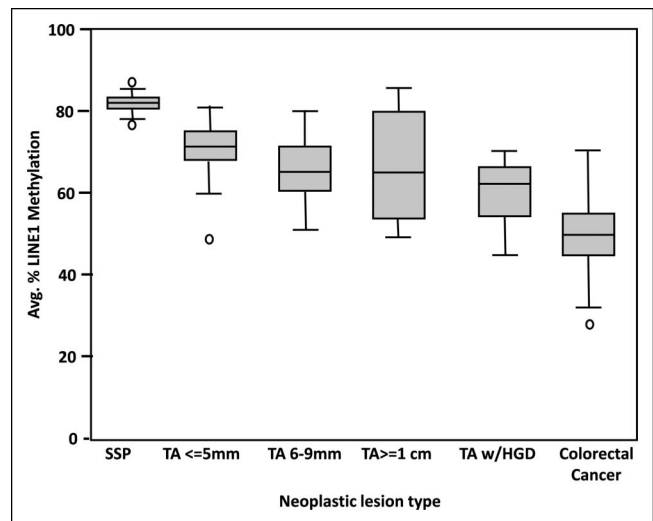


Figure 4. Greater long interspersed nucleotide element-1 (LINE-1) hypomethylation was found in tubular adenomas (TAs) of increasing size and with the presence of high-grade dysplasia (HGD). LINE-1 methylation levels decreased from TAs equal to or less than 5 mm in size (72.31 ± 6.11, n = 45) compared with TAs 6–9 mm in size (67.50 ± 7.00, n = 42) and equal to or greater than 10 mm in size (66.75 ± 11.89, n = 32, $P < 0.001$). Lower LINE-1 methylation levels were found in TAs with high-grade dysplasia (n = 26) compared with TAs with low-grade dysplasia (n = 119) (59.86 ± 7.93 vs 69.11 ± 8.62, $P < 0.001$). Colorectal cancer tumor tissue (n = 36) had the lowest levels of LINE-1 methylation at 50.36 ± 8.40. SSP, sessile serrated polyp.

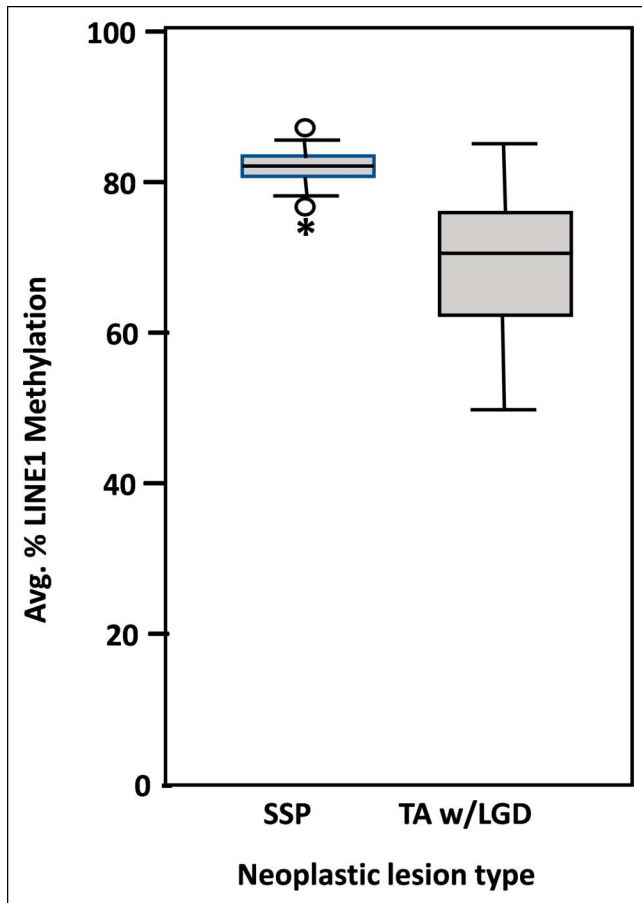


Figure 5. Greater long interspersed nucleotide element-1 (LINE-1) hypomethylation in tubular adenomas (TA) with low-grade dysplasia (LGD; $n = 119$) compared with sessile serrated lesions (SSLs; $n = 29$) (69.11 ± 8.62 vs 81.41 ± 2.43 , $P < 0.001$).

surveillance colonoscopy cases. Current methodologies based on adenoma size, multiplicity, and type to determine interval surveillance colonoscopy are imperfect tools for risk assessment of colorectal adenomas. Combining these features potentially with a marker of global hypomethylation deserves further study in efforts to develop more targeted surveillance approaches.

CONFLICTS OF INTEREST

Guarantor of the article: Joshua E. Melson, MD, MPH.

Specific author contributions: Alice C. Jiang, MD, and Lela Buckingham, PhD, contributed equally to this manuscript. A.C.J. and S.S. collected and interpreted the data, drafted the manuscript, data analysis, and approved the final draft. L.B., K.M., and J.E.M. contributed to planning and conducting the study, collecting and interpreting the data, data analysis, drafting and editing the manuscript, and approval of the final draft. F.B. contributed to planning and conducting the study, collecting and interpreting the data, editing the manuscript, and approval of the final draft.

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Study Highlights

WHAT IS KNOWN

- ✓ Interval surveillance colonoscopy is currently based on adenoma size and histology, which may be inaccurate prognostic features for development of colorectal cancer.
- ✓ Molecular biomarkers of colorectal adenomas are lacking.

WHAT IS NEW HERE

- ✓ Lower long interspersed nucleotide element-1 (LINE-1) methylation levels were found in tubular adenomas (TAs) of increasing size and with high-grade dysplasia.
- ✓ There is greater variation of LINE-1 methylation in TAs compared with sessile serrated lesions.

TRANSLATIONAL IMPACT

- ✓ Lower LINE-1 methylation was found in colorectal TAs of increasing size and high-grade dysplasia.
- ✓ This epigenetic change holds promise as a prognostic biomarker for progression along the colorectal cancer carcinogenesis pathway.

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