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ORIGINAL ARTICLE

IGF2 differentially methylated region hypomethylation in relation to pathological and molecular features of serrated lesions

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Abstract

AIM: To investigate *insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation in relation to clinicopathological and molecular features in colorectal serrated lesions.

METHODS: To accurately analyze the association between the histological types and molecular features of each type of serrated lesion, we consecutively collected 1386 formalin-fixed paraffin-embedded tissue specimens that comprised all histological types [hyperplastic polyps (HPs, *n* = 121), sessile serrated adenomas (SSAs, *n* = 132), traditional serrated adenomas (TSAs, *n* = 111), non-serrated adenomas (*n* = 195), and colorectal cancers (CRCs, *n* = 827)]. We evaluated the methylation levels of *IGF2* DMR0 and long interspersed nucleotide element-1 (LINE-1) in HPs (*n* = 115), SSAs (*n* = 120), SSAs with cytological dysplasia (*n* = 10), TSAs (*n* = 91), TSAs with high-grade dysplasia (HGD) (*n* = 15), non-serrated adenomas (*n* = 80), non-serrated adeno-

mas with HGD (n = 105), and CRCs (n = 794). For the accurate quantification of the relative methylation levels (scale 0%-100%) of *IGF2* DMR0 and LINE-1, we used bisulfite pyrosequencing method. Tumor specimens were analyzed for microsatellite instability, *KRAS* (codons 12 and 13), *BRAF* (*V600E*), and *PIK3CA* (exons 9 and 20) mutations; *MLH1* and *MGMT* methylation; and IGF2 expression by immunohistochemistry.

RESULTS: The distribution of the *IGF2* DMR0 methylation level in 351 serrated lesions and 185 non-serrated adenomas (with or without HGD) was as follows: mean 61.7, median 62.5, SD 18.0, range 5.0-99.0, interquartile range 49.5-74.4. The *IGF2* DMR0 methylation level was divided into quartiles (Q1 $\geq$ 74.5, Q2 62.6-74.4, Q3 49.6-62.5, Q4 $\leq$ 49.5) for further analysis. With regard to the histological type, the *IGF2* DMR0 methylation levels of SSAs (mean ± SD, 73.1 ± 12.3) were significantly higher than those of HPs (61.9 ± 20.5), TSAs (61.6 ± 19.6), and non-serrated adenomas (59.0 ± 15.8) (P < 0.0001). The *IGF2* DMR0 methylation level was inversely correlated with the IGF2 expression level (r = -0.21, P = 0.0051). *IGF2* DMR0 hypomethylation was less frequently detected in SSAs compared with HPs, TSAs, and non-serrated adenomas (P < 0.0001). Multivariate logistic regression analysis also showed that *IGF2* DMR0 hypomethylation was inversely associated with SSAs (P < 0.0001). The methylation levels of *IGF2* DMR0 and LINE-1 in TSAs with HGD (50.2 ± 18.7 and 55.7 ± 5.4, respectively) were significantly lower than those in TSAs (61.6 ± 19.6 and 58.8 ± 4.7, respectively) (*IGF2* DMR0, P = 0.038; LINE-1, P = 0.024).

CONCLUSION: *IGF2* DMR0 hypomethylation may be an infrequent epigenetic alteration in the SSA pathway. Hypomethylation of *IGF2* DMR0 and LINE-1 may play a role in TSA pathway progression.

Core tip: The serrated pathway attracts considerable attention as an alternative colorectal cancer (CRC) pathway. We previously reported the association of *insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation with prognosis and its link to LINE-1 hypomethylation in CRC; however, there have been no studies describing its role in the serrated pathway. Therefore, we evaluated the methylation levels of *IGF2* DMR0 and long interspersed nucleotide element-1 (LINE-1) in 351 serrated lesions and 185 non-serrated adenomas. Our results suggest that the *IGF2* DMR0 may be an infrequent epigenetic alteration in the sessile serrated adenoma pathway. Moreover, we found that the hypomethylation of *IGF2* DMR0 and LINE-1 may play an important role in the progression of traditional serrated adenoma.

INTRODUCTION

The serrated neoplasia pathway has attracted considerable attention as an alternative pathway of colorectal cancer (CRC) development, and serrated lesions exhibit unique clinicopathological or molecular features[1-23]. According to the World Health Organization (WHO) classification[24], colorectal premalignant (or non-malignant) neoplastic lesions with serrated morphology currently encompass three major categories: hyperplastic polyp (HP), sessile serrated adenoma (SSA), and traditional serrated adenoma (TSA).

SSA and TSA are premalignant lesions, but SSA is the principal serrated precursor of CRCs[15]. In particular, there are many clinicopathological and molecular similarities between SSA and microsatellite instability (MSI)-high CRC, for example, right-sided predilection, *MLH1* hypermethylation, and frequent *BRAF* mutation[7,15,17-19,25-28]. Therefore, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon[7,15,17,25,26,28,29].

In contrast, TSAs are much less common than SSAs, and thus, there are fewer data on their molecular profile[15,25]. TSAs typically do not show *MLH1* hypermethylation or develop to MSI-high CRCs, but they do commonly have *MGMT* hypermethylation[15,25,26]. With regard to the *PIK3CA* gene, a previous study reported that no mutation was found in serrated lesions, and that mutations were uncommonly, but exclusively, observed in non-serrated adenomas (1.4%)[30]. Because some HPs do share molecular features with TSAs (*e.g.*, *KRAS* mutation)[3,25,26,31], it has been suggested that the TSA pathway (HP-TSA-carcinoma sequence) may diverge from the SSA pathway (HP-SSA-SSA with cytological dysplasia-carcinoma sequence) on the basis of *KRAS vs BRAF* mutations and/or *MLH1 vs MGMT* hypermethylation within subsets of HPs[15]. However, a definite precursor of TSA has not been established. In addition, the key carcinogenic mechanism involved in this TSA pathway remains largely unknown.

Loss of imprinting (LOI) of *insulin-like growth factor 2* (*IGF2*) has been shown to be associated with an increased risk of CRC[32,33], suggesting that it may play a role in colorectal carcinogenesis. The imprinting and expression of *IGF2* are controlled by CpG-rich regions known as differentially methylated regions (DMRs)[34-37]. In particular, *IGF2* DMR0 hypomethylation has been

suggested as a surrogate-biomarker for *IGF2* LOI[38]. Previously, we reported that *IGF2* DMR0 hypomethylation in CRC was associated with poor prognosis and might be linked to global DNA hypomethylation [long interspersed nucleotide element-1 (LINE-1) hypomethylation][38]. However, to date, there have been no studies describing the role of *IGF2* DMR0 hypomethylation in the early stage of colorectal carcinogenesis.

To investigate the role of *IGF2* DMR0 hypomethylation in serrated lesions we examined *IGF2* DMR0 and LINE-1 methylation levels as well as other molecular alterations using a large sample of 1330 colorectal tumors (351 serrated lesions, 185 non-serrated adenomas, and 794 CRCs).

MATERIALS AND METHODS

Histopathological evaluation of tissue specimens of colorectal serrated lesions

Histological findings related to all colorectal serrated lesion specimens were evaluated by a pathologist (Fujita M) who was blinded to the clinical and molecular information. Serrated lesions (HPs, SSAs, and TSAs) were classified on the basis of the current WHO criteria[24]. HPs were further subdivided into microvesicular HPs and goblet cell HPs.

SSAs are characterized by the presence of a disorganized and distorted crypt growth pattern that is usually easily identifiable upon low-power microscopic examination. Crypts, particularly at the basal portion of the polyp, may appear architecturally distorted, dilated, and/or branched, particularly in the horizontal plane, which leads to the formation of boot, L, or anchor-shaped crypts. The cytology is typically quite bland, but a minor degree of nuclear atypia is allowable, particularly in the crypt bases[15,25,26].

To accurately analyze the association between the histological types and molecular features of each type of serrated lesion we consecutively collected more than 100 formalin-fixed paraffin-embedded (FFPE) tissue specimens of each histological type (HP, SSA, and TSA). In total, 364 tissue specimens of serrated lesions [121 HPs, 122 SSAs, 10 SSAs with cytological dysplasia, 96 TSAs, and 15 TSAs with high-grade dysplasia (HGD)] from patients who underwent endoscopic resection or other surgical treatment at Sapporo Medical University Hospital, Keiyukai Sapporo Hospital or Teine-Keijinkai Hospital between 2001 and 2012 were available for assessment. All of HPs were microvesicular HPs.

The serrated lesions were classified by location: the proximal colon (cecum, ascending and transverse colon), distal colon (splenic flexure, descending, sigmoid colon) and rectum. Informed consent was obtained from all the patients before specimen collection. This study was approved by the institutional review boards of the participating institutions. The term "prognostic marker" is used throughout this article according to the REMARK Guidelines[39].

Tissue specimens of CRC and non-serrated adenomas

FFPE tissue specimens of 827 CRCs (stages I -IV), 85 non-serrated adenomas (*i.e.*, tubular or tubulovillous adenomas), and 110 non-serrated adenomas with HGD from patients who underwent surgical treatment or endoscopic resection at the above hospitals were also collected. The criterion for diagnosing cancer was invasion of malignant cells beyond the muscularis mucosa.

DNA extraction and pyrosequencing for KRAS, BRAF, and PIK3CA and MSI analysis

Genomic DNA was extracted from the FFPE tissue specimens of the colorectal tumors using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, United States). PCR and targeted pyrosequencing were then performed using the extracted genomic DNA to determine the presence of *KRAS* (codons 12 and 13), *BRAF* (*V600E*) and *PIK3CA* (exons 9 and 20) mutations[40,41]. MSI analysis was performed as previously described using 10 microsatellite markers[14]. MSI-high was defined as instability in ≥ 30% of the markers and MSI-low/microsatellite stable (MSS) as instability in < 30% of the markers[14].

Sodium bisulfite treatment and pyrosequencing to measure IGF2 DMR0 and LINE-1 methylation levels

Bisulfite modification of genomic DNA was performed using a BisulFlash™ DNA Modification Kit (Epigentek, Brooklyn, NY, United States).

We measured the relative methylation level at the *IGF2* DMR0 using a bisulfite-pyrosequencing assay as previously described[38]. The amount of C relative to the sum of the amounts of C and T at each CpG site was calculated as percentage (scale 0%-100%). We calculated the average of the first and second CpG sites in the *IGF2* DMR0 as the *IGF2* DMR0 methylation level. Likewise, to accurately quantify the LINE-1 methylation levels we utilized a pyrosequencing assay, as previously described[42].

Pyrosequencing to measure MGMT and MLH1 promoter methylation

Pyrosequencing for *MGMT* and *MLH1* methylation was performed using the PyroMark kit (Qiagen). We used a previously defined cut-off of ≥ 8% methylated alleles for *MGMT* and *MLH1* hypermethylated tumors[43].

Immunohistochemistry for IGF2 expression

For IGF2 staining, we used anti-IGF2 antibody (Rabbit polyclonal to IGF2; Abcam, Cambridge, MA, United States) with a subsequent reaction performed using Target Retrieval Solution, Citrate pH 6 (Dako Cytomation, Carpinteria, CA, United States). In each case, we recorded cytoplasmic IGF2 expression as no expression, weak expression, moderate expression, or strong expression relative to normal colorectal epithelial cells. IGF2 expression was visually interpreted by Nosho K, who was unaware of the other data. For the agreement study of IGF2 expression, 128 randomly selected cases were examined by a second pathologist (by Naito T), who was also unaware
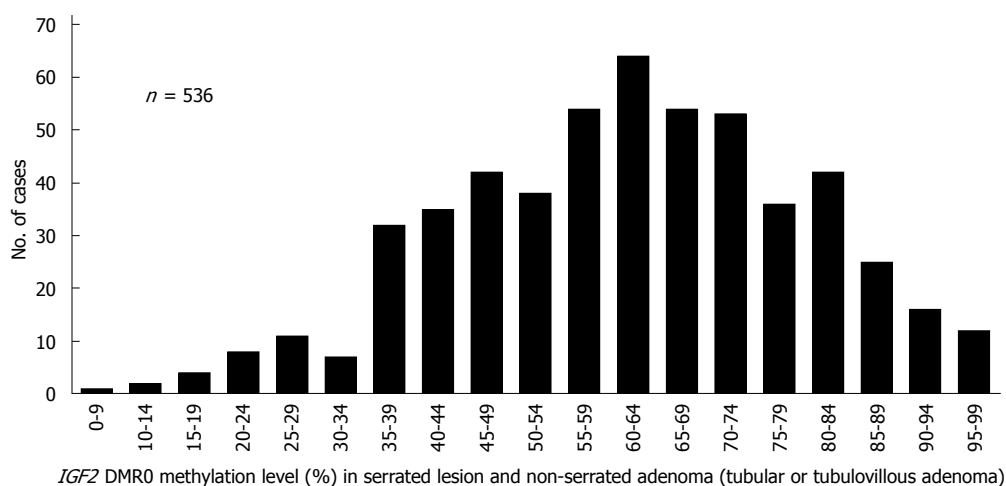
Figure 1 Distribution of *IGF2* differentially methylated region 0 methylation levels in 351 serrated lesions. Hyperplastic polyp, sessile serrated adenoma (SSA), SSA with cytological dysplasia, traditional serrated adenoma (TSA) and TSA with high-grade dysplasia (HGD) and 185 non-serrated adenomas (tubular adenoma, tubular adenoma with HGD, tubulovillous adenoma and tubulovillous adenoma with HGD). DMR: Differentially methylated region; *IGF2*: *Insulin-like growth factor 2*.

of the other data. The concordance between the two pathologists ($P < 0.0001$) was 0.84 ($\kappa = 0.69$), indicating substantial agreement.

Statistical analysis

JMP (version 10) software was used for all statistical analyses (SAS Institute, Cary, NC, United States). All *P* values were two-sided. Univariate analyses were performed to investigate the clinicopathological and molecular characteristics including *IGF2* DMR0 and LINE-1 hypomethylation, according to histological type, classified as serrated lesion, non-serrated adenoma, and CRC. *P* values were calculated by analysis of variance for age, tumor size, and the methylation levels of *IGF2* DMR0 and LINE-1 and by χ^2 or Fisher's exact test for all other variables. A multivariate logistic regression analysis was employed to examine associations with *IGF2* DMR0 hypomethylation (as an outcome variable), adjusting for potential confounders. The model initially included sex, age, tumor size, tumor location, histological type, and the LINE-1 methylation level, and MSI, *BRAF*, *KRAS*, and *PIK3CA* mutations. In the CRC-specific survival analysis, the Kaplan-Meier method and log-rank test were used to assess the survival time distribution. The Spearman correlation coefficient was used to assess the correlation of the *IGF2* DMR0 methylation level and IGF2 expression.

RESULTS

The IGF2 DMR0 methylation level in serrated lesion and non-serrated adenomas

We assessed 559 FFPE tissue specimens of serrated lesions and non-serrated adenomas in the *IGF2* DMR0 methylation assay and obtained 536 (96%) valid results. The distribution of the *IGF2* DMR0 methylation level in 351 serrated lesions and 185 non-serrated adenomas (with or without HGD) was as follows: mean 61.7, median 62.5, SD 18.0, range 5.0-99.0, interquartile range

49.5-74.4 (all on a 0-100 scale) (Figure 1). The *IGF2* DMR0 methylation level was divided into quartiles (Q1 $\geqslant$ 74.5, Q2 62.6-74.4, Q3 49.6-62.5, Q4 $\leqslant$ 49.5) for further analysis.

We evaluated the *IGF2* DMR0 methylation level in serrated lesions (HP, SSA, and TSA) and non-serrated adenomas according to their histological type. The *IGF2* DMR0 methylation levels of SSAs (n = 120, mean ± SD, 73.1 ± 12.3) were significantly higher than those of HPs (n = 115, 61.9 ± 20.5, $P < 0.0001$), TSAs (n = 91, 61.6 ± 19.6, $P < 0.0001$), and non-serrated adenomas (n = 80, 59.0 ± 15.8, $P < 0.0001$) (Figure 2).

IGF2 DMR0 hypomethylation was associated with larger tumor size in serrated lesions and non-serrated adenomas (Table 1). With regard to the histological type, *IGF2* DMR0 hypomethylation was less frequently detected in SSAs than in HPs, TSAs, and non-serrated adenomas ($P < 0.0001$) (Table 1). Multivariate logistic regression analysis also showed the *IGF2* DMR0 hypomethylation was inversely associated with SSAs ($P < 0.0001$).

Association of IGF2 expression and IGF2 DMR0 methylation level in serrated lesions and non-serrated adenomas

We examined IGF2 overexpression in 168 colorectal serrated lesions and non-serrated adenomas. The *IGF2* DMR0 methylation level was inversely correlated with the IGF2 expression level (r = -0.21, P = 0.0051).

IGF2 DMR0 methylation level in colorectal cancer

A total of 827 paraffin-embedded CRCs (stages Ⅰ-Ⅳ) were subjected to an *IGF2* DMR0 methylation assay with 794 (96%) valid results. The distribution of the *IGF2* DMR0 methylation level in these 794 CRCs was as follows: mean 54.7, median 55.0, SD 13.7, range 7.5-98.0, interquartile range 46.1-63.0 (all on a 0-100 scale). The *IGF2* DMR0 methylation level was divided into quartiles (Q1 $\geqslant$ 63.0, Q2 55.0-62.9, Q3 46.1-54.9, Q4 $\leqslant$ 46.0) for
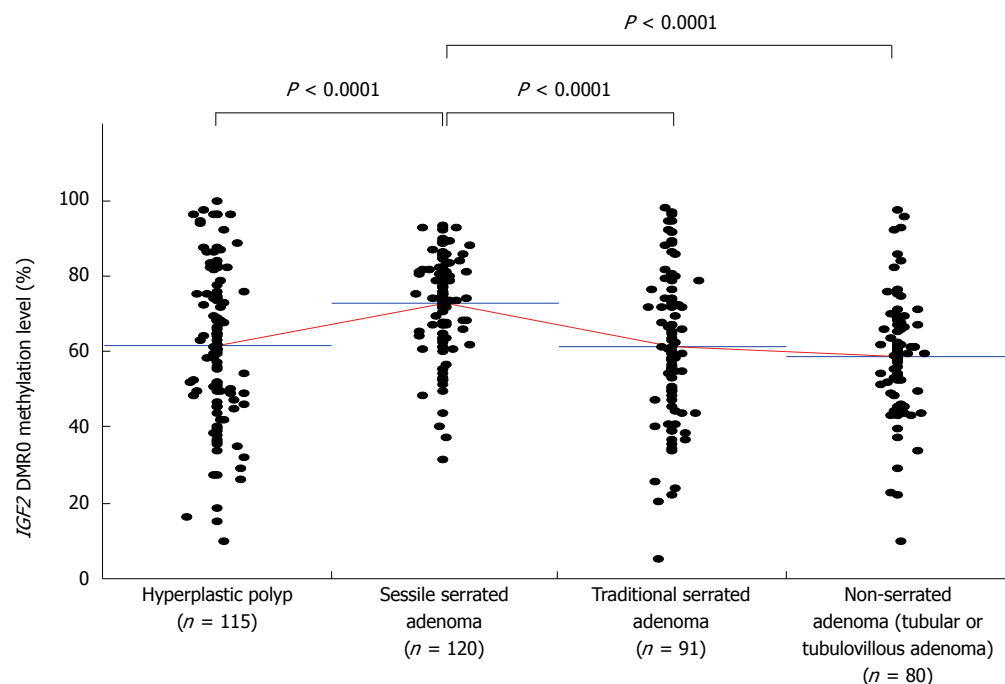
Figure 2 *IGF2* differentially methylated region 0 methylation level according to histological type. *Insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 methylation levels of sessile serrated adenoma (mean ± SD; 73.1 ± 12.3) were significantly higher compared with those of hyperplastic polyp (61.9 ± 20.5, $P < 0.0001$), traditional serrated adenoma (61.6 ± 19.6, $P < 0.0001$), and non-serrated adenoma (59.0 ± 15.8, $P < 0.0001$). *P*-values were calculated by analysis of variance.

Table 1 *IGF2* differentially methylated region 0 hypomethylation in serrated lesions and non-serrated adenomas *n* (%)

Clinicopathological feature	Total *n*	*IGF2* DMR0 methylation (quartile)				*P* value
		Q1 (⩾ 74.5)	Q2 (62.6-74.4)	Q3 (49.6-62.5)	Q4 (⩽ 49.5)	
All cases	536	134	130	131	141	
Sex						
Male	326 (61)	78 (58)	80 (62)	92 (70)	76 (54)	0.041
Female	210 (39)	56 (42)	50 (38)	39 (30)	65 (46)	
Age (mean ± SD)	61.5 ± 12.2	59.9 ± 12.3	60.8 ± 12.0	63.1 ± 11.6	62.3 ± 13.0	0.150
Tumor size (mm) (mean ± SD)	14.3 ± 11.4	9.9 ± 4.0	13.4 ± 7.4	14.7 ± 11.1	19.1 ± 17.6	< 0.0001
Tumor location						
Rectum	70 (13)	11 (8.5)	14 (11)	18 (14)	27 (20)	0.061
Distal colon	161 (31)	35 (27)	43 (33)	37 (29)	46 (33)	
Proximal colon	296 (56)	84 (65)	72 (56)	75 (58)	65 (47)	
Histological type						
Hyperplastic polyp (HP)	115 (21)	33 (25)	25 (19)	23 (18)	34 (24)	< 0.0001
Sessile serrated adenoma (SSA) without cytological dysplasia	120 (22)	60 (45)	39 (30)	15 (11)	6 (4.3)	
SSA with cytological dysplasia	10 (1.9)	1 (0.8)	3 (2.3)	6 (4.6)	0 (0)	
Traditional serrated adenoma (TSA) without high-grade dysplasia (HGD)	91 (17)	22 (16)	21 (16)	23 (18)	25 (18)	
TSA with HGD	15 (2.8)	2 (1.5)	2 (1.5)	2 (1.5)	9 (6.4)	
Non-serrated adenoma (tubular or tubulovillous adenoma) without HGD	80 (15)	11 (8.2)	17 (13)	32 (24)	20 (14)	
Non-serrated adenoma with HGD	105 (20)	5 (3.7)	23 (18)	30 (23)	47 (33)	

Percentage indicates the proportion of patients of each histological type who met the criteria for a specific clinical or molecular feature. *P* values were calculated by analysis of variance for age and tumor size and by χ^2 or Fisher's exact test for all other variables. The *P* value for significance was adjusted by Bonferroni correction to 0.010 (= 0.05/5).

further analysis.

Colorectal cancer patient survival and IGF2 DMR0 methylation level

The influence of the *IGF2* DMR0 methylation level on clinical outcome was assessed in CRC patients. During the follow-up of 398 patients with metastatic CRC (stages Ⅲ-Ⅳ) who were eligible for survival analysis, mortality occurred in 134, including 118 deaths confirmed to be attributable to CRC. The median follow-up period for

IGF2 DMR0 methylation level and cancer-specific survival

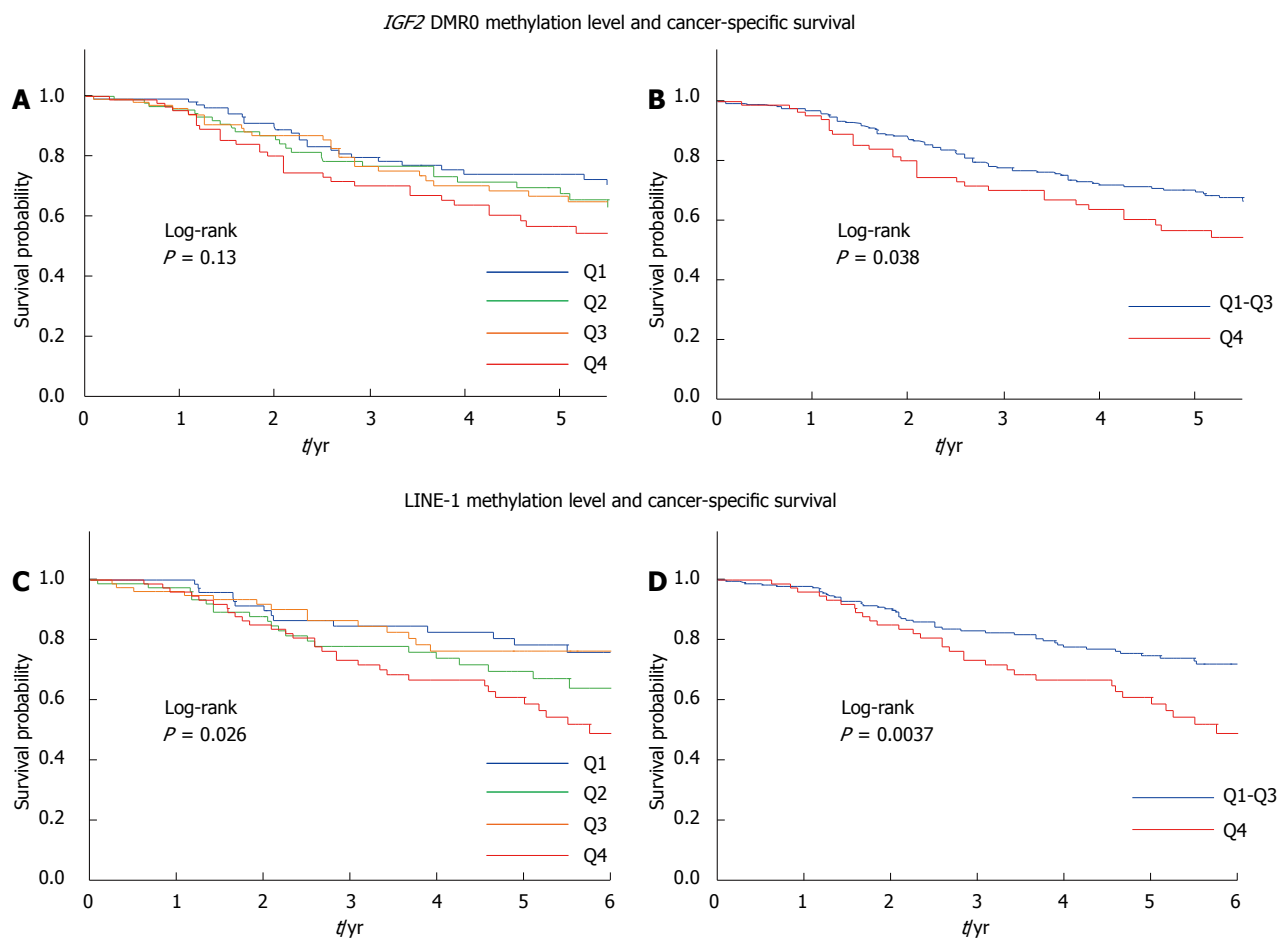LINE-1 methylation level and cancer-specific survival

Figure 3 Kaplan-Meier survival curves for colorectal cancer according to the *IGF2* differentially methylated region 0 and long interspersed nucleotide element-1 methylation levels in metastatic colorectal cancers. A: Patients with *Insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation had a slightly higher mortality rate than those with *IGF2* DMR0 hypermethylation, but this difference was not significant (log-rank test: $P = 0.13$); B: *IGF2* DMR0 hypomethylation (Q4 cases) was significantly associated with unfavorable cancer-specific survival (log-rank test: $P = 0.038$); C: Significantly higher mortality was observed in patients with long interspersed nucleotide element-1 (LINE-1) hypomethylation compared with those with LINE-1 hypermethylation (log-rank test: $P = 0.026$); D: LINE-1 hypomethylation (Q4 cases) was significantly associated with unfavorable cancer-specific survival (log-rank test: $P = 0.0037$).

censored patients was 3.3 years. Kaplan-Meier analysis was performed using categorical variables (Q1, Q2, Q3, and Q4). Slightly but insignificantly higher mortality was observed in patients with *IGF2* DMR0 hypomethylation compared with those without hypomethylation in terms of cancer-specific survival (log-rank test: $P = 0.13$) (Figure 3A). In another Kaplan-Meier analysis, Q4 cases were defined as the "hypomethylated group" and the Q1, Q2, and Q3 cases were combined into a "non-hypomethylated group"; the hypomethylated group (log-rank test: $P = 0.038$) was found to have significantly higher mortality (Figure 3B). Similar results were observed in terms of overall survival (log-rank test: $P = 0.040$) (data not shown).

LINE-1 methylation level and CRC patient survival
The LINE-1 methylation level in CRC was also divided into quartiles (Q1 $\geq$ 58.7, Q2 54.8-58.6, Q3 50.8-54.7, and Q4 $\leq$ 50.7). A significantly higher mortality rate was observed among Q4 cases (log-rank test: $P = 0.0037$) in

the Kaplan-Meier analysis (Figure 3C, D).

Association of histological type and IGF2 DMR0 and LINE-1 methylation levels as well as other molecular features of serrated lesions and non-serrated adenomas
Table 2 shows the clinicopathological and molecular features of serrated lesions and non-serrated adenomas. No significant difference was observed between SSAs (69.0 ± 10.8) with cytological dysplasia and SSAs without (73.1 ± 12.3) in *IGF2* DMR0 methylation levels ($P = 0.32$). In contrast, MSI-high was more frequently ($P < 0.0001$) found in SSAs with cytological dysplasia [40% (4/10)] than in SSAs [0.8% (1/120)]. With regard to the LINE-1 methylation level, no significant difference was observed between the methylation level and histological type in serrated lesions and non-serrated adenomas ($P = 0.59$).

Mutations of *BRAF*, *KRAS*, and *PIK3CA* were detected in 49%, 19%, and 0.9% of HPs, 87%, 2.5%, and 0% of SSAs, 69%, 17%, and 0% of TSAs and 2.6%, 19%, and 1.3% of non-serrated adenomas, respectively (Table 2).

Table 2 Clinical and molecular features of serrated lesions and non-serrated adenomas (tubular or tubulovillous adenoma) according to histological type *n* (%)

Clinical or molecular feature	Total *n*	Histological type						*P* value
		Serrated lesion				Non-serrated adenoma		
		HP	SSA without cytological dysplasia	SSA with cytological dysplasia	TSA without high-grade dysplasia (HGD)	Tubular adenoma without HGD	Tubulovillous adenoma without HGD	
All cases	416	115	120	10	91	77	3	
Sex								
Male	263 (63)	78 (68)	72 (60)	5 (50)	55 (60)	50 (65)	3 (100)	0.36
Female	153 (37)	37 (32)	48 (40)	5 (50)	36 (40)	27 (35)	0 (0)	
Age (mean ± SD)	60.3 ± 11.8	57.5 ± 12.1	57.2 ± 11.6	74.1 ± 4.7	60.9 ± 12.3	66.6 ± 11.4	66.0 ± 8.9	< 0.0001
Tumor size (mm) (mean ± SD)	10.5 ± 5.4	9.3 ± 3.7	11.6 ± 5.4	12.3 ± 6.4	9.7 ± 4.7	10.9 ± 7.2	15.7 ± 13.2	0.0069
Tumor location								
Rectum	42 (10)	15 (13)	0 (0)	0 (0)	16 (18)	10 (14)	1 (33)	< 0.0001
Distal colon	127 (31)	44 (39)	17 (14)	1 (10)	39 (44)	25 (34)	1 (33)	
Proximal colon	239 (59)	54 (48)	103 (86)	9 (90)	34 (38)	38 (52)	1 (33)	
BRAF mutation								
Wild-type	183 (44)	59 (51)	16 (13)	2 (20)	28 (31)	75 (97)	3 (100)	< 0.0001
Mutant	231 (55)	56 (49)	104 (87)	8 (80)	61 (69)	2 (2.6)	0 (0)	
KRAS mutation								
Wild-type	357 (87)	92 (81)	117 (98)	10 (100)	74 (83)	62 (81)	2 (67)	< 0.0001
Mutant	55 (13)	21 (19)	3 (2.5)	0 (0)	15 (17)	15 (19)	1 (33)	
PIK3CA mutation								
Wild-type	406 (99)	113 (99)	117 (100)	10 (100)	89 (100)	74 (99)	3 (100)	0.67
Mutant	2 (0.5)	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (1.3)	0 (0)	
MSI status								
MSS/MSI-low	408 (98)	113 (98)	119 (99)	6 (60)	90 (99)	77 (100)	3 (100)	0.0004
MSI-high	8 (1.9)	2 (1.7)	1 (0.8)	4 (40)	1 (1.1)	0 (0)	0 (0)	
IGF2 DMR0 methylation level (mean ± SD)	64.5 ± 17.2	61.9 ± 20.5	73.1 ± 12.3	69.0 ± 10.8	61.6 ± 19.6	58.9 ± 16.1	61.0 ± 7.1	< 0.0001
LINE-1 methylation level (mean ± SD)	58.7 ± 5.0	58.6 ± 3.4	58.1 ± 5.4	58.3 ± 8.4	58.8 ± 4.7	59.4 ± 6.0	60.9 ± 1.4	0.59

Percentage indicates the proportion of patients of each histological type who met the criteria for a specific clinical or molecular feature. *P* values were calculated by analysis of variance for age, tumor size, methylation levels of *IGF2* DMR0 and LINE-1 and by χ^2 or Fisher's exact test for all other variables. The *P* value for significance was adjusted by Bonferroni correction to 0.0050 (= 0.05/10). HGD: High-grade dysplasia; HP: Hyperplastic polyp; MSI: Microsatellite instability; MSS: Microsatellite stable; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; *IGF2*: *Insulin-like growth factor 2*.

IGF2 DMR0 and LINE-1 hypomethylation in TSAs and non-serrated adenomas with high-grade dysplasia

Tables 3 and 4 show the clinicopathological and molecular features of the TSAs (with or without HGD), non-serrated adenomas (with or without HGD), and CRCs (stages I -IV). The *IGF2* DMR0 methylation levels in TSAs with HGD (50.2 ± 18.7) were significantly lower than those in TSAs without (61.6 ± 19.6, *P* = 0.038) (Table 3). With regard to LINE-1, the methylation levels in TSAs with HGD (55.7 ± 5.4) were significantly lower than those in TSAs without (58.8 ± 4.7) (*P* = 0.024).

Similarly, the methylation levels of *IGF2* DMR0 (52.0 ± 13.6) and LINE-1 (56.9 ± 5.5) in non-serrated adenomas with HGD were significantly lower than those in non-serrated adenomas without (59.0 ± 15.8, *P* = 0.0016 and 59.5 ± 5.9, *P* = 0.0027, respectively) (Table 3).

DISCUSSION

In this study, we examined the *IGF2* DMR0 and LINE-1 methylation levels as well as other molecular alterations in

351 serrated lesions, 185 non-serrated adenomas, and 794 CRCs. *IGF2* DMR0 hypomethylation was less frequently detected in SSAs than in HPs, TSAs, and non-serrated adenomas. We also found that *IGF2* DMR0 and LINE-1 hypomethylation in TSAs and non-serrated adenomas with HGD were more frequently detected in TSAs and non-serrated adenomas without HGD, suggesting that hypomethylation may play an important role in the progression of these tumors.

In the current study, we confirmed that *IGF2* DMR0 hypomethylation was associated with poor CRC prognosis, suggesting its oncogenic role and malignant potential. In addition, our data showed that the *IGF2* DMR0 methylation level was inversely correlated with the IGF2 expression level. Therefore, our findings support the validity of the quantitative DNA methylation assay (bisulfite-pyrosequencing) for examining the *IGF2* DMR0 methylation level.

HPs are classified into three subtypes, namely microvesicular HPs, goblet cell HPs, and mucin-poor HPs. Microvesicular and goblet cell HPs are the most com-

Table 3 Clinical and molecular features of sessile serrated adenomas with cytological dysplasia, traditional serrated adenomas, non-serrated adenomas (tubular or tubulovillous adenoma), and colorectal carcinomas according to disease stage *n* (%)

| Clinical or molecular feature | Histological type | | | | | | | | | P value |
| | SSA with cytological dysplasia | Colorectal adenoma | | | | Colorectal carcinoma | | | | |
		TSA without HGD	TSA with HGD	Non-serrated adenoma without HGD	Non-serrated adenoma with HGD	Stage I	Stage II	Stage III	Stage IV	
All cases	10	91	15	80	105	171	217	292	114	
Sex										
Male	5 (50)	55 (60)	9 (60)	53 (66)	54 (51)	107 (63)	123 (57)	168 (58)	73 (64)	0.50
Female	5 (50)	36 (40)	6 (40)	27 (34)	51 (49)	64 (37)	94 (43)	124 (42)	41 (36)	
Age (mean ± SD)	74.1 ± 4.7	60.9 ± 12.3	62.7 ± 13.6	66.6 ± 11.2	66.3 ± 10.5	65.1 ± 11.0	67.4 ± 11.5	66.6 ± 12.5	63.4 ± 9.5	0.0016
Tumor size (mm) (mean ± SD)	12.3 ± 6.4	9.7 ± 4.7	12.8 ± 4.3	11.0 ± 7.4	29.3 ± 17.3	26.3 ± 15.8	53.1 ± 23.5	50.5 ± 22.7	50.9 ± 19.6	< 0.0001
Tumor location										
Rectum	0 (0)	16 (18)	5 (33)	11 (14)	23 (22)	65 (38)	73 (34)	135 (46)	37 (33)	< 0.0001
Distal colon	1 (10)	39 (44)	7 (47)	26 (34)	27 (26)	44 (25)	64 (29)	59 (20)	42 (37)	
Proximal colon	9 (90)	34 (38)	3 (20)	39 (51)	54 (52)	62 (36)	80 (37)	98 (34)	34 (30)	
BRAF mutation										
Wild-type	2 (20)	28 (31)	7 (47)	78 (98)	102 (98)	161 (95)	204 (94)	282 (97)	103 (95)	< 0.0001
Mutant	8 (80)	61 (69)	8 (53)	2 (2.5)	2 (1.9)	9 (5.3)	13 (6.0)	9 (3.0)	6 (5.5)	
KRAS mutation										
Wild-type	10 (100)	74 (83)	11 (73)	64 (80)	48 (46)	108 (64)	145 (69)	202 (70)	84 (74)	< 0.0001
Mutant	0 (0)	15 (17)	4 (27)	16 (20)	57 (54)	62 (36)	66 (31)	88 (30)	29 (26)	
PIK3CA mutation										
Wild-type	10 (100)	89 (100)	14 (93)	77 (99)	99 (94)	161 (94)	194 (89)	249 (85)	103 (90)	< 0.0001
Mutant	0 (0)	0 (0)	1 (6.7)	1 (1.3)	6 (5.7)	10 (5.9)	23 (11)	43 (15)	11 (9.7)	
MSI status										
MSS/MSI-low	6 (60)	90 (99)	15 (100)	80 (100)	105 (100)	163 (95)	198 (91)	276 (95)	110 (96)	< 0.0001
MSI-high	4 (40)	1 (1.1)	0 (0)	0 (0)	0 (0)	8 (4.7)	19 (8.8)	16 (5.5)	4 (3.5)	
IGF2 DMR0 methylation level (mean ± SD)	69.0 ± 10.8	61.6 ± 19.6	50.2 ± 18.7	59.0 ± 15.8	52.0 ± 13.6	55.7 ± 15.8	53.4 ± 13.3	55.5 ± 12.9	53.1 ± 12.9	< 0.0001
LINE-1 methylation level (mean ± SD)	58.3 ± 8.4	58.8 ± 4.7	55.7 ± 5.4	59.5 ± 5.9	56.9 ± 5.5	55.8 ± 7.2	53.1 ± 6.2	55.1 ± 6.5	54.1 ± 7.6	< 0.0001

Percentage indicates the proportion of patients of each histological type who met the criteria for a specific clinical or molecular feature. *P* values were calculated by analysis of variance for age, tumor size, methylation levels of *IGF2* DMR0 and LINE-1 and by χ^2 or Fisher's exact test for all other variables. The *P* value for significance was adjusted by Bonferroni correction to 0.0050 (= 0.05/10). HGD: High-grade dysplasia; HP: Hyperplastic polyp; MSI: Microsatellite instability; MSS: Microsatellite stable; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; *IGF2*: *Insulin-like growth factor 2*.

mon, whereas mucin-poor HPs are rare[44]. Recent studies have reported that microvesicular HPs may be a precursor lesion of SSAs and that borderline lesions between microvesicular HPs and SSAs can occur[25,26,28]. In the current study, we found that the *IGF2* DMR0 methylation levels of SSAs were significantly higher compared with those of HPs (microvesicular HPs), TSAs, and non-serrated adenomas. Our data also showed that *IGF2* DMR0 hypomethylation was less frequently detected in SSAs compared with HPs, TSAs, and non-serrated adenomas.

Our current study had some limitations due to its cross-sectional nature and the fact that unknown bias (*i.e.*, selection bias) may confound the results. Nevertheless, our multivariate regression analysis was adjusted for potential confounders including age, tumor size, tumor location, LINE-1 methylation level, and *BRAF* and *KRAS* mutation. The results demonstrate that *IGF2* DMR0 hypomethylation is inversely associated with SSAs. Moreover, our data have shown that the *IGF2* DMR0 methylation levels of SSAs with cytological dysplasia were higher than those of HPs, suggesting that HPs (microvesicular

HPs) or SSAs with *IGF2* DMR0 hypomethylation may tend not to progress to the typical SSA pathway [HP-SSA-SSA with cytological dysplasia-carcinoma (MSI-high) sequence] but to the alternate pathway. Thus, our finding of differential patterns of *IGF2* DMR0 hypomethylation in serrated lesions may be a clue for elucidating the differentiation of serrated lesions.

In the current study, *IGF2* DMR0 hypomethylation was found in TSAs and hypomethylation was more frequently detected in TSAs with HGD when compared with TSAs without HGD. These results may imply that *IGF2* DMR0 hypomethylation can occur in the early stage of the TSA pathway and that TSAs with *IGF2* DMR0 hypomethylation are precursor lesions that progress to TSAs with HGD or CRCs with hypomethylation. In other words, TSAs without *IGF2* DMR0 hypomethylation may tend not to progress to TSAs with HGD. Otherwise, TSAs without *IGF2* DMR0 hypomethylation may tend to rapidly develop to CRCs; therefore, they are infrequently detected in the stage of TSA with HGD. However, because the number of TSA with HGD sam-

Table 4 Clinicopathological and molecular features of fifteen traditional serrated adenomas with high-grade dysplasia

No.	Age/sex	Size (mm)	Location	KRAS mutation	BRAF mutation	PIK3CA mutation	MGMT methylation	MLH1 methylation	MSI status	LINE-1 methylation level	IGF2 DMR0 methylation level	IGF2 expression
1	75/M	8	Rectum	c.35G>A (p.G12D)	Wild	Wild	(-)	(-)	MSS/MSI-low	58	70	Weak
2	54/F	20	Sigmoid colon	c.35G>A (p.G12D)	Wild	Wild	(+)	(-)	MSS/MSI-low	53.7	39.5	Strong
3	62/F	15	Transverse colon	c.38G>A (p.G13D)	Wild	Wild	(+)	(-)	MSS/MSI-low	53.3	72.0	No expression
4	84/M	5	Rectum	c.35G>A (p.G12D)	Wild	Wild	(-)	(-)	MSS/MSI-low	65.0	26.5	Moderate
5	85/M	12	Sigmoid colon	Wild	c.1799T>A (p.V600E)	Wild	(-)	(-)	MSS/MSI-low	58.0	45.5	Strong
6	48/M	20	Sigmoid colon	Wild	c.1799T>A (p.V600E)	Wild	(-)	(-)	MSS/MSI-low	53.7	40.5	Moderate
7	69/M	10	Sigmoid colon	Wild	c.1799T>A (p.V600E)	Wild	(-)	(-)	MSS/MSI-low	58.7	52.0	No expression
8	60/M	9	Descending colon	Wild	c.1799T>A (p.V600E)	Wild	(-)	(-)	MSS/MSI-low	59.0	41.5	Moderate
9	34/M	18	Sigmoid colon	Wild	c.1799T>A (p.V600E)	Wild	(+)	(-)	MSS/MSI-low	57.3	42.0	Strong
10	61/M	10	Rectum	Wild	c.1799T>A (p.V600E)	Wild	(-)	(-)	MSS/MSI-low	56.7	29.0	Strong
11	52/F	15	Ascending colon	Wild	c.1799T>A (p.V600E)	Wild	(+)	(+)	MSS/MSI-low	57.0	57.0	Moderate
12	70/F	13	Rectum	Wild	c.1799T>A (p.V600E)	Wild	(+)	(+)	MSS/MSI-low	63.0	84.5	Weak
13	66/F	12	Ascending colon	Wild	Wild	c.1624G>A (p.E542K)	(-)	(-)	MSS/MSI-low	49.7	28.0	Moderate
14	52/M	12	Sigmoid colon	Wild	Wild	Wild	(-)	(-)	MSS/MSI-low	48.0	44.5	Moderate
15	69/F	13	Rectum	Wild	Wild	Wild	(+)	(-)	MSS/MSI-low	44.3	80.0	Weak

HGD: High-grade dysplasia; MSI: Microsatellite instability; MSS: Microsatellite stable; TSA: Traditional serrated adenoma.

ples was small (n = 15), our findings require further confirmation from future independent studies.

Global DNA hypomethylation is associated with genomic instability, which leads to cancer[45-50]. As the LINE-1 or L1 retrotransposon constitutes a substantial portion (ca. 17%) of the human genome, the level of LINE-1 methylation is regarded as a surrogate marker of global DNA methylation[46,51]. We previously reported that LINE-1 methylation is highly variable but is strongly associated with a poor prognosis in CRC[45]. However, no previous study has reported the role of LINE-1 hypomethylation in serrated lesions. In serrated lesions, unlike the *IGF2* DMR0 methylation level, no significant difference was observed between the LINE-1 methylation level and histological type. We also found that the LINE-1 methylation levels in TSAs with HGD were significantly lower than those in TSAs. These results suggest that both *IGF2* DMR0 hypomethylation and LINE-1 hypomethylation are important epigenetic alterations in the progression of TSAs. Because the carcinogenic mechanism remains unclear, further analyses are needed to clarify the role in the TSA pathway of the hypomethylation of these locations.

Previous studies have reported that SSAs with cytological dysplasia have accumulated genetic abnormalities and are at a high risk of progression to colorectal carcinoma[7,26,28]. Loss of staining for MLH1 leads to MSI and repeat tract mutation in genes such as *TGFβRII* is restricted to lesions with cytological dysplasia in SSAs[26,27,52,53]. In the current study, MSI-high was more frequently detected in SSAs with cytological dysplasia than in SSAs without. Our data indicate that in SSAs with cytological dysplasia, it is not hypomethylation of *IGF2* DMR0 or LINE-1 but rather MSI due to *MLH1* hypermethylation that plays an important role in the evolution to colorectal carcinoma.

The RAS-RAF-MEK-ERK signaling pathway is commonly altered in CRC and serrated lesions through oncogenic mutation of either *BRAF* or *KRAS*[15,21,25]. Moreover, CRCs with serrated morphology are particularly prone to mutations targeted by anti-epidermal growth factor receptor therapy. Therefore, as the variety of molecularly targeted agents for CRC increases, understanding of molecular alterations is becoming increasingly important[21,40]. *BRAF* and *KRAS* mutations are mutually exclusive and demonstrate a subtype specificity in serrated lesions[10,15,17-19,28]; they are most likely initiating events in the majority of HPs[54]. Previous studies have reported that *BRAF* is mutated with increasing frequency in SSAs (60%-100%)[3-5,9,11,16]. In the current study, *BRAF* mutations were detected in 49% of HPs and 87% of SSAs, respectively. Therefore, our data relating to the frequency of *BRAF* mutations in SSAs are consistent with previous reports. The activation of the RAS-RAF-MEK-ERK signaling pathway by *BRAF* or *KRAS* mutation is also common in TSAs. Previous studies have reported *BRAF* mutation rates of 29%-46%[68] in TSAs ranging from 27% to 55%[6,8,16,55], compared to *KRAS* mutation rates in TSAs. In the cur-

rent study, *BRAF* and *KRAS* mutations were detected in 69% and 17% of TSAs, respectively. Thus, the wide variation in the relative proportion of *BRAF vs KRAS* mutations in different studies reflects differences in histological classification or small sample size.

In conclusion, we found that *IGF2* DMR0 hypomethylation can occur in the early stage of any histological types of serrated lesions; however, hypomethylation may be an infrequent epigenetic alteration in SSAs. These results imply that *IGF2* DMR0 hypomethylation may be a key epigenetic event that affects the progression of HPs. Our data also suggest that the hypomethylation of *IGF2* DMR0 and LINE-1 may play an important role in the progression of the TSA pathway.

COMMENTS

Background
The serrated pathway attracts considerable attention as an alternative colorectal cancer (CRC) pathway. Authors previously reported the association of *insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation with poor prognosis and its link to global DNA hypomethylation [long interspersed nucleotide element-1 (LINE-1) hypomethylation] in CRC; however, to date, there have been no studies describing its role in the serrated pathway.

Research frontiers
Sessile serrated adenoma (SSA) and traditional serrated adenoma (TSA) are premalignant lesions, but SSA is the principal serrated precursor of CRC. In particular, there are many clinicopathological and molecular similarities between SSA and microsatellite instability (MSI)-high CRC, for example, right-sided predilection, *MLH1* hypermethylation, and frequent *BRAF* mutation. Therefore, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon. In contrast, a definite precursor of TSA has not been established. In addition, the key carcinogenic mechanism involved in this TSA pathway remains largely unknown. To investigate the role of *IGF2* DMR0 hypomethylation in serrated lesions they examined *IGF2* DMR0 methylation levels as well as other molecular alterations.

Innovations and breakthroughs
This is the first report of an association between histopathological findings and *IGF2* DMR0 hypomethylation in serrated lesions. *IGF2* DMR0 hypomethylation was less frequently detected in SSAs than in hyperplastic polyps (HPs), TSAs, and non-serrated adenomas. They also found that *IGF2* DMR0 and LINE-1 hypomethylations in TSAs and non-serrated adenomas with high-grade dysplasia were more frequently detected in TSAs and non-serrated adenomas, suggesting that such hypomethylation may play an important role in the progression of those tumors. Thus, their finding of differential patterns of *IGF2* DMR0 hypomethylation in serrated lesions may be a clue for elucidating the progression of serrated lesions.

Applications
In the current study, authors found that the *IGF2* DMR0 methylation levels of SSAs were significantly higher compared with those of HPs (microvesicular HPs), TSAs, and non-serrated adenomas. They also showed that *IGF2* DMR0 hypomethylation was less frequently detected in SSAs compared with HPs, TSAs, and non-serrated adenomas. Therefore, their data challenge the common conception of discrete molecular features of SSAs *vs* other serrated lesions (TSAs and HPs) and may have a substantial impact on clinical and translational research, which has typically been performed with the dichotomous classification of SSAs.

Terminology
IGF2 DMR: IGF2 expression is controlled by CpG-rich regions known as *IGF2* DMRs in CRC. In particular, *IGF2* DMR0 hypomethylation has been suggested as a surrogate-biomarker for *IGF2* loss of imprinting. LINE-1: Global DNA hypomethylation is associated with genomic instability, which leads to cancer. As the long interspersed nucleotide element-1 or L1 retrotransposon constitutes a substantial portion of the human genome, the level of LINE-1 methylation is regarded as a surrogate marker of global DNA methylation. Serrated pathway: The serrated neoplasia pathway has attracted considerable attention as an

alternative pathway of CRC development, and serrated lesions exhibit unique clinicopathological or molecular features. Of the serrated lesions, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon.

Peer review
The authors investigated the hypomethylations of *IGF2* DMR0 and LINE-1; MSI; and mutations of *KRAS*, *BRAF*, and *PIK3CA* in patients with serrated lesions and non-serrated adenomas. The results demonstrated that *IGF2* DMR0 hypomethylation can occur in the early stage of any histological types of serrated lesions; however, hypomethylation may be an infrequent epigenetic alteration in SSAs. The authors also revealed that the hypomethylation of *IGF2* DMR0 and LINE-1 may play an important role in the progression of the TSA pathway. This article may have a substantial impact on clinical and translational research in the progression of serrated lesions related to malignant transformation.

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