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Original Article

Frequent CpG island methylation: a risk factor in the progression of traditional serrated adenoma of the colorectum

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Abstract: Background: Traditional serrated adenoma (TSA) features a unique serrated configuration because it involves two cell types: tall and short columnar cells. The serrated neoplasia pathway is related to the carcinogenesis of colorectal cancer. CpG island methylator phenotype-high (CIMP-high) is a unique genetic alteration in this pathway. Materials and Methods: This study investigated the prevalence and level of methylation and CIMP in 30 TSA cases. The tall and short cells in 28 TSAs were separated by microdissection. Methylation-specific PCR was performed to detect the methylation of MGMT, MLH1, P14, P16, MINT1, MINT2 and MINT31. Results: Overall, 30 cases presented CIMP-high, and the prevalence of CIMP-high was 100% (30/30) in tall cells and 93% (28/30) in short cells. Conclusions: No significant difference was found between tall and short columnar cells. The relationship between methylation and clinicopathological characters remains to be established.

Keywords: Traditional serrated adenoma, CpG island methylator phenotype-high

Introduction

Colorectal cancer (CRC) is a common gastrointestinal tumor and is the leading cause of cancer mortality worldwide [1-3]. Two well-studied molecular pathogenesis pathways contribute to the carcinogenesis of CRC: the adenoma-adenocarcinoma sequence and the serrated neoplasia pathway [4-9]. The adenoma-adenocarcinoma sequence considered the traditional pathogenic pathway, plays an important role in most CRC; in addition, cancers arise from tubular adenomas (TAs) through this sequence [10, 11]. Recent studies have demonstrated that Wnt/β-catenin signaling pathways contribute to the adenoma-adenocarcinoma sequence [12-14]. The serrated neoplasia pathway is distinct from the conventional adenoma-adenocarcinoma sequence pathway. It displays some unique former genetic alterations, such as *BRAF* mutation [5, 12, 15-17], DNA hypermethylation [5, 12, 15, 16] and microsatellite instability (MSI) [5, 8, 12, 18, 19].

The serrated glands of serrated polyps consist of two cell types: tall columnar cells of the intraluminal projecting portion and short columnar cells of the concave portion. Our previous studies considered the unique serrated epithelial architecture of serrated adenoma (SA) as a result of proliferation versus differentiation [14]. The Wnt signaling pathway contributes to SA and is related to serrated neoplasia [20]. However, the mechanism by which the serrated neoplasia pathway is activated in SA remains unclear.

Hypermethylation of CpG islands in the promoter region of tumor suppressor and tumor-related genes may cause the loss of gene expression in CRC [21-23]. Moreover, CpG island methylator phenotype (CIMP), which means a high degree of concurrent promoter methylation in multiple genes, has been described in a subtype of CRC [24-26]. Hypermethylation also occurs in precursor lesions such as aberrant crypt foci and colorectal polyps. A high frequency and level of methylation and CIMP have been observed in sessile serrated adenoma and traditional serrated adenoma (TSA) [15, 18, 27, 28]. However, studies rarely explored the difference in CIMP status between tall and short columnar cells in SA.

Table 1. Primer sequences used in CIMP analysis

Gene locus	Primers	Annealing temperature (°C)
MGMT		
Methylated		
Sense	5'-GTATCGGTCGAAGGGTTATTC-3'	58
Antisense	5'-TAAAACAATCTACGCATCCTCG-3'	58
Unmethylated		
Sense	5'-GTTTGTATTGGTTGAAGGGTTATTT-3'	57
Antisense	5'-CTAAAACAATCTACACATCCTCACT-3'	57
MLH1		
Methylated		
Sense	5'-TTTTTTTAGGAGTGAAGGAGGTTAC-3'	58
Antisense	5'-ACTAAACACGAATACTACGAACGAT-3'	58
Unmethylated		
Sense	5'-TTTTTTTAGGAGTGAAGGAGGTTAT-3'	55
Antisense	5'-AACTAAACACAAATACTACAAACAAT-3'	55
P14		
Methylated		
Sense	5'-GGTAGGTGGAAGTTTTTTAAAGAGC-3'	58
Antisense	5'-AAAAAAACAAACTACCCAAAACG-3'	58
Unmethylated		
Sense	5'-GTAGGTGGAAGTTTTTTAAAGAGTGG-3'	58
Antisense	5'-AAAAAAACAAACTACCCAAAACAC-3'	58
P16		
Methylated		
Sense	5'-GGGGAGTAGTATGGAGTTTTCG-3'	64
Antisense	5'-AACTATTCGATACGTTAAACAACGC-3'	64
Unmethylated		
Sense	5'-GGGAGTAGTATGGAGTTTTTGG-3'	60
Antisense	5'-AACTATTCAATACATTAAACAACACC-3'	60
MINT1		
Methylated		
Sense	5'-TCGTTAATTTTCGAATTTGAAGC-3'	59
Antisense	5'-ACTATAACCAACCTCTACGCGAA-3'	59
Unmethylated		
Sense	5'-TTGTTAATTTTTGAATTTGAAGTGT-3'	56
Antisense	5'-CAACTATAACCAACCTCTACACAAA-3'	56
MINT2		
Methylated		
Sense	5'-TTGATGTGTTAATTGGGGGTC-3'	58
Antisense	5'-ATAAATATCTATTCTTCCCCTTTCG-3'	58
Unmethylated		
Sense	5'-GTTGATGTGTTAATTGGGGGTT-3'	58
Antisense	5'-CTATAAATATCTATTCTTCCCCTTTCAC-3'	58
MINT31		
Methylated		
Sense	5'-TTTTTTCGTAGTGGCGTAAGC-3'	59
Antisense	5'-CTCCAAAAAAACTATAAATACCCGAA-3'	59

In the present study, we compared the frequencies of promoter methylation and CIMP in tall and short columnar cells in TSA. We also explored the relationship between CIMP status and clinical pathological characters. Considering previous findings, we elucidated the mechanism by which the Wnt signaling pathway and the serrated neoplasia pathway worked together to affect TSA. To analyze their methylation levels, tall and short columnar cells in TSA were separated through microdissection, and then detect the methylation frequencies of *MGMT*, *MLH1*, *P14*, *P16*, *MINT1*, *MINT2,* and *MINT31* were detected.

Materials and methods

Specimens

Thirty colorectal TSA specimens from 30 patients obtained endoscopically or surgically were selected from the pathology files of the Department of Pathology at the Second Hospital of Harbin Medical University from 2000 to 2011 in accordance with the World Health Organization criterion [17].

All procedures were performed in accordance with the university's ethical standards and hospital criteria. All participants provided informed consent.

Microdissection

Microdissection was assessed as previously described [20]. The tall and short cells of each case were placed in different tubes for DNA extraction.

DNA extraction

DNA extraction was assessed as previously described [20]. The extracted DNA was stored at -20°C until use.

Unmethylated		
Sense	5'-GTTTTTTTGTAGTGGTGTAAGTGT-3'	55
Antisense	5'-CTCCAAAAAACTATAAATACCCAAA-3'	55

Table 2. Clinicopathological findings of 30 serrated adenomas

	Serrated adenomas
Age ± SD (years)	56.6±13
Gender	
Female	8
Male	22
Site	
Right colon	7
Left colon	23
Diameter (mm) (average)	6.9±4
Dysplasia	
Mild	1
Moderate	27
Severe	1
Severe dysplasia with adenocarcinoma	1

Bisulfite treatment of DNA, methylation-specific PCR and determination of CIMP status

The methylation status of CpG sites in *MGMT*, *MLH1*, *P14*, *P16*, *MINT1*, *MINT2* and *MINT31* was determined by methylation-specific *PCR* (MSP) as previously described [29, 30]. Bisulfite-modified DNA templates were amplified by *PCR* using either methylation-specific primers or unmethylation-specific primers of seven loci (**Table 1**). CIMP status was classified as CIMP-negative if no locus was methylated, CIMP-low if one locus was methylated, and CIMP-high if two or more loci were methylated.

Statistical analysis

Chi-square test and Fisher's exact test were used for statistical analysis. Statistical significance was considered at $P<0.05$.

Results

Clinicopathological findings

In our study, the 30 SAs were chosen by strict pathological criteria and were all polypoid and nodular. The average age of patients was 56.6±13 (range, 32 to 75 years), and the ratio of male to female was 2.75:1. As shown in **Table 2**, the diameter of SAs ranged from 2 to 20 mm (mean, 6.9±4 mm). Lesions proximal to the splenic flexure were classified as right-sided, whereas lesions of the splenic flexure, descending colon, sigmoid colon, and rectum were classified as left-sided. Approximately 77% of SAs (23/30) occurred in the left colon and 23% cases (7/30) in the right colon. The level of dysplasia in most SAs (27/30) was moderate; the remaining cases respectively showed mild, severe dysplasia and severe dysplasia with adenocarcinoma.

Methylation at MGMT, MLH1, P14, P16, MINT1, MINT2 and MINT31

Examples of methylation at *MGMT*, *MLH1*, *P14*, *P16*, *MINT1*, *MINT2*, and *MINT31* are shown in **Figure 1**. In high columnar cells of serrated adenomas, methylation of *P16* and *MINT1* was identified in all 30 cases. However, the prevalence rates of *P16* and *MINT1* methylation in short columnar cells were 90% (27/30) and 87% (26/30), respectively. The frequency of methylation was 97% (29/30) at *MGMT*, *MLH1*, *P14*, and *MINT31* and 83% (25/30) at *MINT2*. However, in short columnar cells, the frequencies of methylation at *MGMT*, *MLH1*, *P14*, *MINT31*, and *MINT2* were 80% (24/30), 83% (25/30), 87% (26/30), 83% (25/30), and 73% (22/30), respectively (**Table 3**). Aside from these genes, no significant difference was detected between high and short columnar cells, but high columnar cells of serrated adenomas were more frequently methylated at multiple loci than short columnar cells.

CIMP in serrated adenoma and clinicopathological associations

The overall SAs showed CIMP-high after methylation analysis above all loci. Furthermore, we detected the CIMP level between high and short columnar cells. **Figure 2** summarizes the methylation status of high and short columnar cells in SAs. The prevalence rates of CIMP-high, CIMP-low, and CIMP-negative in short columnar cells of SAs were 3% (1/30), 7% (2/30), and 90% (27/30), respectively, and the CIMP level of high columnar cells was high in all cases. No significant relationship was found between them; however, the number of CIMP-high cases

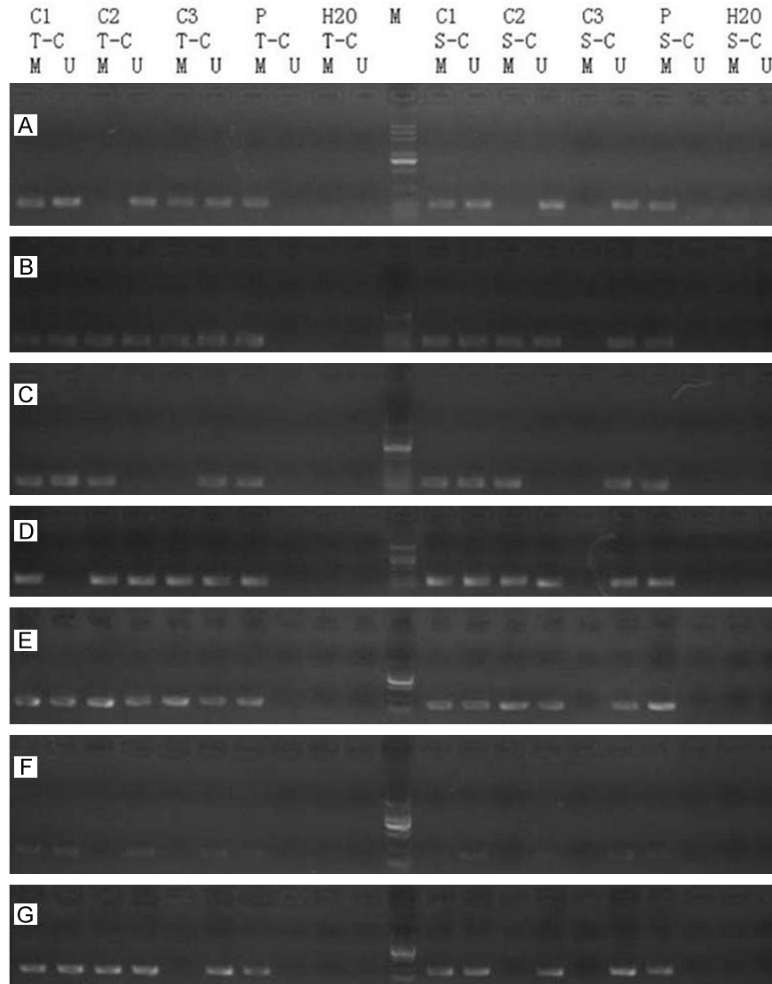

Figure 1. Methylation status. A: *MGMT*; B: *MLH1*; C: *P14*; D: *P16*; E: *MINT1*; F: *MINT2*; G: *MINT31*; C1: case1; C2: case2; C3: case3; P: Positive control; M: Marker; T-C: Tall columnar cell; S-C: Short columnar cell; M: Methylation; U: Unmethylation.

Table 3. Methylation of *MGMT*, *MLH1*, *P14*, P16, *MINT1*, *MINT2* and *MINT31*

		MGMT	*MLH1*	*P14*	*P16*	*MINT1*	*MINT2*	*MINT31*
Methylation	T-C	29/30	29/30	29/30	30/30	30/30	25/30	29/30
	S-C	24/30	25/30	26/30	27/30	26/30	22/30	25/30
P		0.1028	0.1945	0.3533	0.2373	0.1124	0.5321	0.1945

was greater in tall columnar cells than in short columnar cells.

We also detected the associations between CIMP and clinical pathological factors (**Table 4**). CIMP status and clinical pathological factors showed no association. Clinical pathological factors were also detected in two CIMP-high groups between tall and short columnar cells. The results showed no significant correlations between them as well.

Discussion

Our previous studies attempted to find the role of the serrated pathway in the colorectum. Results suggested that the serrated configuration of SAs is related to a proliferation versus differentiation process. In addition, BRAF mutation may be involved in the serrated morphology of SAs [14]. Furthermore, the genetic alteration in both the serrated pathway and the Wnt signaling pathway may contribute to SAs [20]. Considering that the BRAF mutation [5, 12, 15-17], DNA hypermethylation [5, 12, 15, 16], and MSI [5, 8, 12, 18, 19] have been defined as the unique genetic alteration for the serrated pathway, we further detected the frequency of methylation in short and tall columnar cells of SAs.

In the study of Park et al. [27], CIMP-high occurred in 68% (15/22) SAs, which was only 18% (6/34) in TAs. Thus, the frequency of methylation is much higher in SAs than in TAs. The epigenetic alterations in SAs may probably be due to the methylation of CpG islands. However, the study of Kim et al. [31] showed that the prevalence rates of methylation are 86% and 100% in SAs and TAs, respectively. Furthermore, the prevalence rates of CIMP-high are 39% and 28% in SAs and TAs, respectively. Thus, the effects of methylation of tumor-related genes in both SAs and TAs show no obvious difference. These opposite results were all detected by the sensitive methylation-specific PCR.

Figure 2. Methylation status of 7 loci in tradition serrated adenomas. Each column represents the methylation status of *MGMT*, *MLH1*, *P14*, P16, *MINT1*, *MINT2* and *MINT31*, respectively, or CIMP status. Horizontal rows represent individual polyp subgrouped as no, low and high methylation groups.

Table 4. Correlation of CIMP Status and Clinicopathological Features of serrated adenomas

Characteristics	Short columnar cells			P	Tall columnar cells	P
	CIMP-negative (*n*=1)	CIMP-low (*n*=2)	CIMP-high (*n*=27)		CIMP-high (*n*=30)	
	% (no.)	% (no.)	% (no.)		% (no.)	
Age ± SD (years)	70	62±13	56±13	ns	57±13	ns
Gender						
Female	0 (0)	50% (1)	26% (7)	ns	27% (8)	ns
Male	100% (1)	50% (1)	74% (20)		73% (22)	
Site						
Right colon	0 (0)	0 (0)	26% (7)	ns	23% (7)	ns
Left colon	100% (1)	100 (2)	74% (20)		77% (23)	
Size						
≤ ize c	100% (1)	100 (2)	89% (24)	ns	90% (27)	ns
>1.0 cm	0 (0)	0 (0)	11% (3)		10% (3)	

Considering that methylation also existed in normal tissues, we could not estimate how much the methylation level in normal tissues would affect the final results. We also speculated that the difference between their studies may be due to the methylation in normal tissues.

We separated the tall and short columnar cells from the SAs using a microdissection technique to prevent them from interfering with each other, together with pathological normal tissues, such as matrix and inflammatory cells. Most studies revealed that DNA-methylation usually occurs in SAs and contributes to the progression of SAs [15, 18, 32]. According to our results, the frequencies of methylation at *MGMT*, *MLH1*, *P14*, *P16*, *MINT1*, *MINT2*, and *MINT31* were 97%, 97%, 97%, 100%, 100%, 83%, and 97%, respectively, in tall columnar cells, and 80%, 83%, 87%, 90%, 87%, 73%, and 83%, respectively, in short columnar cells. No significant difference was found between them, but the frequency of methylation was higher in tall columnar cells than in short columnar cells. The level of CIMP in all 30 SAs cases was high. Moreover, CIMP-high occurred in 30 cases of tall columnar cells and in 27 cases of short columnar cells in SAs. No significant difference was detected between the two groups. These results indicate that methylation may contribute to the SAs but not to the unique formation of SAs. The high level of CIMP may be due to the sensitivity of methylation-specific PCR. Methylation could be detected through the amplification effect of PCR. Furthermore, our frequency of methylation (100%) was much higher than that in other studies (79.3%-91%). Some factors may be due to the high prevalence, as the specimens were all SAs without hyperplastic polyp and hybrid polyps; the cells were harvested by microdissection and avoided interference from other tissues.

The completeness of DNA and its copies is important to maintain the usual function of cells. Normally, the human body would recognize the errors in the copying process of DNA, and the cell cycle would stop to repair the segments of mismatch. If the DNA polymerase does not correct the errors in time, a backup system called the mismatch repair (MMR) system would adjust the mismatched DNA. However, if MMR failed, the mismatched DNAs would be the templates for the next copying, which may lead to the occurrence of diseases, such as cancers.

O-6-methylguanine-DNA methyltransferase (*MGMT*) is a DNA repair enzyme, and its variation may increase mutation rate and carcinogenesis [31]. Loss of *MGMT* expression is due to the methylation in its promoter region. Dong et al. [33] found that the methylation of *MGMT*, *P16*, *P14*, *TIMP3*, and *FHIT* plays a critical role in the carcinogenesis in the serrated pathway, and the methylation of these genes is also related to the histological progress. A report published on 2009 [34] revealed that the overall incidence rates of *MGMT* methylation in hyperplastic polyp, sessile SAs, and TSAs are 14%-22%, 23%-25%, and 16%-78%, respectively. In the present study, the frequencies of *MGMT* methylation in tall and short columnar cells of SAs were 97% and 80%, respectively, which are much higher than those in other reports. *MGMT* methylation and CIMP-low show a positive correlation in sporadic CRC [35]. However, such a relationship was not observed in SAs with *MGMT* methylation. CIMP-high correlated with *MGMT* methylation in the present study. The reason may be ascribed to the fact that our technique in collecting the cells could reduce the interference from tissues out of SAs. The results showed that MGMT methylation may be due to the occurrence of SAs.

hMLH1, *hMSH2*, *hMSH3*, *hMSH6*, *hPMS2*, and *hPMS1* are well-known MMR genes. Methylation always occurs in MMR genes, such as *hMLH1* and *hMSH2*, which could lead to MSI and loss of protein expression. Some studies pointed out that abnormal MMR gene expression is a specific marker for MSI-H [36], which is usually related to the methylation of the *MLH1* promoter region and the loss of *MLH1* expression [31]. The methylation of *MLH1* could be found in 70% of sessile SA and 90% of MSI-H sporadic CRC, and rarely occur in hyperplastic polyp and TSAs [5]. Thus, sessile SAs may be the origin of MSI-H CRC [37]. Our study revealed that *MLH1* methylation frequently occurred in both tall columnar cells (29/30) and short columnar cells (25/30) in TSAs. Noffsinger et al. [5] reported opposite results. The reason may be the difference in the technique of collecting cells and in the number of SA cases. The high prevalence of MLH1 methylation revealed that

MSI-H was related to the occurrence of TSAs. However, MSI status is determined by detecting the microsatellite markers recommended by most studies, such as *BAT25*, *BAT26*, *D5S346*, *D2S123*, and *D17S250* [38]. Lesions are defined as MSI-H when two or more markers show instability. Further studies should confirm the MSI status of the tall and short columnar cells of these 30 TSAs cases. Therefore, the relationship between the status of MSI and the occurrence of TSAs could be eventually confirmed.

One study pointed out that the characteristic molecular biological changes of both adenoma-adenocarcinoma sequence and the serrated neoplasia pathway could exist in some colorectal polyps showing the morphological features of SAs and TAs [39]. Therefore, some researchers introduced a new mechanism for the occurrence of CRC, which considered the two pathways combined to influence the carcinogenesis of CRC [40, 41]. On the basis of the molecular subtypes of CRC summarized by Noffsinger et al. [5], the cancer that originated origin from TAs and SAs with *KRAS* mutation, was caused by both adenoma-adenocarcinoma sequence and serrated neoplasia pathway. This carcinoma had some characteristics in common, such as CIMP-L, chromosome instability, *MGMT* methylation, *KRAS* mutation, MSI-L, or MSS. Furthermore, Jiao et al. [14] confirmed that the short columnar cells from SAs demonstrate a high expression level of Wnt-pathway-related proteins (β-catenin and *CD44*). Thus, they speculated that the Wnt signaling pathway contributes to SA formation. Our previous study proved that *BRAF* mutations appear in 22/28 (78.6%) TSAs and that all mutations occur in tall cells [20]. β-catenin-positive cells were also found in all SAs used in another previous research [14]. In the present study, the SAs all showed CIMP-H, which was another unique genetic alteration for the serrated neoplasia pathway. These results revealed that the Wnt signaling pathway and the serrated neoplasia pathway both contribute to TSAs but do not affect separately.

The formation of TSAs depended on complicated networks; in addition, the carcinogenesis of CRC that originated from TSAs needed multiple processes and involved multiple molecular events. Our previous studies and this study pointed out that both Wnt signaling pathway and serrated neoplasia pathway contribute to TSAs and possibly to CRC carcinogenesis. The DNA hypermethylation, *BRAF* mutation, mutations of proteins related to the Wnt signaling pathway and other molecular events worked in synergy to cause TSAs, even CRC. However, the status of MSI, difference in methylation level, and other types of polyps must be clarified to establish the relationship between CIMP and clinical pathological characters. Further research should be conducted to confirm these hypotheses.

Conclusions

In conclusion, methylation of *MGMT, MLH1*, *P14*, *P16*, *MINT1*, *MINT2*, and *MINT31* contributed to TSA. No significant difference was found between tall and short columnar cells. The relationship between methylation and clinical pathological characters was not established. The number of cases may need to be increased to establish this relationship. DNA hypermethylation was stable in the serrated neoplasia pathway.

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Disclosure of conflict of interest

None.

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