Pathogenesis of non-familial colorectal carcinomas with high microsatellite instability

K Shitoh, F Konishi, M Miyaki, T Iijima, T Furukawa, T Tsukamoto, H Nagai

Abstract

***Aims*—Microsatellite instability (MSI) was first observed in hereditary non-polyposis colorectal carcinoma (HNPCC) and was subsequently seen in non-familial colorectal carcinoma. The relation between MSI and cancer associated genes in non-familial colorectal carcinomas has yet to be evaluated. To clarify this matter, changes in cancer associated genes were examined in non-familial colorectal carcinomas.**

***Methods*—Alterations in the adenomatous polyposis coli (APC), p53, and Ki-ras genes were analysed in 24 MSI high (alterations in four to seven of seven loci), nine MSI low (alterations in one to three of seven loci), and 31 MSI negative non-familial carcinomas. The hMSH2 and hMLH1 genes were also analysed in 24 MSI high carcinomas.**

***Results*—Both the frequencies and types of alterations in the APC and p53 genes in MSI high carcinomas were the same as those in MSI low and MSI negative carcinomas; however, they were different from those seen in HNPCC. The frequency of Ki-ras mutation was significantly lower in the MSI high cases (two of 24; 8%) than in the others (15 of 38; 39%). Somatic mutation of hMSH2 or hMLH1 was detected in six of 24 (25%) of the MSI high cases.**

***Conclusions*—These results suggest that APC and p53 alterations occur irrespective of microsatellite instability status in non-familial colorectal carcinomas, and that Ki-ras mutation is not involved in MSI high non-familial colorectal carcinoma. The pathogenesis of these carcinomas may differ from both the usual adenoma–carcinoma sequence and HNPCC carcinogenesis.**

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Keywords: microsatellite instability; non-familial colorectal carcinoma; cancer associated genes

Department of Surgery, Jichi Medical School, 3311-1 Yakushiji, Minamikawachimachi, Tochigi 324-0498, Japan
K Shitoh
F Konishi
T Furukawa
H Nagai

Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome Bunkyo-ku, Tokyo 113-8697, Japan
M Miyaki
T Iijima

Department of Pharmacology, Kitasato University, 5-9-1 Shirogane, Minato-ku, Tokyo 108-8641, Japan
T Tsukamoto

Correspondence to:
Dr Shitoh
kshitoh@jichi.ac.jp

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Microsatellite instability (MSI) has been observed in hereditary non-polyposis colorectal carcinoma (HNPCC) and non-familial colorectal carcinoma.[1–6] In HNPCC, this phenomenon is thought to be caused by two allelic mutations at one of the mismatch repair genes and the frequency of mutations in the APC (adenomatous polyposis coli), p53, and Ki-ras genes has been reported to be low.[6] Thus, MSI is thought to be important in carcinogenesis in HNPCC.[6 7] In familial adenomatous polyposis and non-familial colorectal carcinomas without MSI, it is thought that the first mutations occur in the APC gene and that chromosomal instability (CI) is the main mechanism.[8] However, HNPCC results from MSI rather than APC mutations. In addition, the development of most non-familial colon cancers occurs after CI.[9 10] In a previous report, it was proposed that the spectrum of gene mutation in MSI associated cancers (HNPCC) was different from that in CI associated cancers.[8] In our study, we investigated whether the spectrum of mutations differs between non-familial colorectal carcinomas with MSI and CI associated cancers.[1 11–13] Therefore, we compared genetic changes in non-familial colorectal carcinomas with MSI with those without MSI. We compared mutations of the APC, p53, and Ki-ras genes,[10 11] and also analysed somatic mutations of the hMSH2 and hMLH1 genes in tumours with MSI.

Materials and methods

Specimens of non-familial colorectal carcinoma and matched normal tissue were obtained from the files of the Jichi Medical School Hospital, Japan. We selected 75 proximal colon carcinomas and 77 distal and rectal carcinomas, and none of the patients had any family history of colorectal carcinoma. Genomic DNA was prepared from fresh carcinomas and matched normal tissue specimens and was then treated by the guanidium/phenol/chloroform method. Twenty four cases (16 proximal, eight distal and rectal) were identified as MSI high carcinomas (defined as alterations in four to seven of seven loci), and nine cases (seven proximal, two distal and rectal) were MSI low carcinomas (alterations in one to three of seven loci). Of the remaining 119 carcinomas without MSI, 31 cases (21 proximal, 10 distal and rectal) were analysed at random for genetic changes. MSI high carcinomas, MSI low carcinomas, and those without MSI were also compared regarding genetic changes of cancer associated genes. Seven microsatellite markers—D2S123, D2S72, D3S1611, D3S1029, TP53, Mfd26, and BAT26—were used to determine the MSI status by means of the polymerase chain reaction (PCR). These markers have all been reported in previous studies.[14–17] The MSI phenotype was determined when at least one band, which was not found in those of the normal mucosa, at one or more loci was present in the PCR products of the carcinomas (fig 1). Clinicopathological features were compared among different grades of MSI status. We classified peritumoral Crohn's like lymphocytic infiltration into three grades (−, +, ++). Conspicuous infiltration corresponded to ++ status, and inconspicuous infiltration included − and + status. Lymphatic invasion and venous invasion

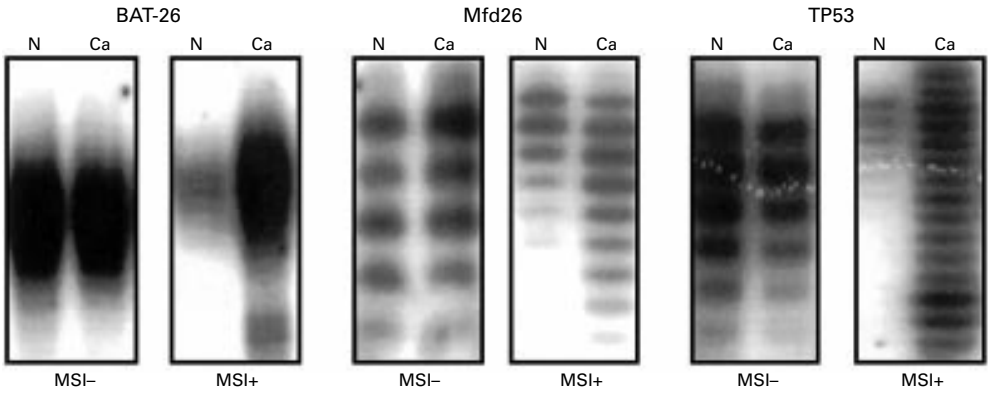

Figure 1 MSI negative and positive cases determined using the BAT-26, Mfd26, and TP53 microsatellite markers. N, normal sample; Ca, carcinoma sample; MSI−, MSI negative; MSI+, MSI positive.

were classified into four grades according to the Japanese classification of colorectal carcinomas.[18] Genomic DNA samples of 64 non-familial colorectal carcinomas were amplified for single strand conformation polymorphism (SSCP) in APC genes using PCR.[19] The analysed exons of the APC gene comprised 15A, 15C, 15D, 15G, 15H, and 15I, including the hot spots of somatic mutations. We did not analyse 15B, 15E, and 15F because of the low frequency of mutations at these sites.[20] The primers used for APC were the same as those reported previously.[20 21] For the p53 gene, exons 5–8 were amplified using previously described primers.[22] Exons 1 and 2 of Ki-ras were analysed to detect mutations in codons 12, 13, and 61. Aberrant single strand DNA fragments were extracted with distilled water from the corresponding bands of a PCR-SSCP gel. The DNA fragments were amplified through the asymmetrical PCR (100 : 1 or 1 : 100) using the same primers as those used for PCR-SSCP. The amplified DNAs were purified by the QIAquick spin purification kit (Qiagen, Catsworth, California, USA) and sequenced with the dideoxy chain termination reaction using Sequenase Version 2.0 (United States Biochemical Co, Cleveland, Ohio, USA) and primers used in PCR-SSCP. Loss of heterozygosity (LOH) on 17p was detected using the TP53 microsatellite marker and the same PCR and electrophoresis conditions as those used previously for the analysis of microsatellite instability (as described earlier). Some cases were categorised as uninformative because of the nature of MSI—only unequivocal allele patterns were regarded as LOH. Somatic mutations in the hMSH2 and hMLH1 genes among 24 MSI high cases were analysed by PCR-SSCP using the same method as described previously.[6] After PCR-SSCP, direct sequencing was performed for DNA fragments from aberrant bands as stated earlier. Each exon from 1 to 16 of the hMSH2 gene and each exon from 1 to 19 of hMLH1 was amplified from genomic DNA by PCR using the previously reported primers.[23 24]

Table 1 Clinicopathological features of microsatellite instability (MSI) high carcinomas and the other cases

Feature	*MSI high (24)*	*MSI low (9)*	*MSI negative (31)*
Age (years)	67.5 (13.8)	67.3 (9.9)	65.1 (12.4)
Dukes's class			
A	1	1	4
B	12	0	8
C	7	6	13
D	4	2	6
Differentiation			
Well	9	8	18
Moderate	10	1	9
Poor	2	0	1
Mucinous	3	0	3
Lymphatic invasion			
0–1	18	5	21
2–3	6	4	9
Venous invasion			
0–1	21	7	22
2–3	3	2	8
Lymphocytic infiltration			
Conspicuous	6	1	2
Inconspicuous	13	6	17

Results are mean (SD).
Lymphatic invasion: 0, no invasion; 1, mild invasion; 2, moderate invasion; 3, pronounced invasion.
Venous invasion: 0, no invasion; 1, mild invasion; 2, moderate invasion; 3, pronounced invasion (Japanese classification of colorectal carcinomas).
Lymphocytic infiltration, peritumoral Crohn's like reaction: conspicuous, ++ status; inconspicuous, − and + status.

Results

No obvious difference was seen in the clinicopathological features between MSI high cases and other cases (table 1). Using PCR-SSCP, somatic mutation of the APC gene was analysed in 24 MSI high non-familial colorectal carcinomas, nine MSI low carcinomas, and 31 without MSI. When mutant bands were detected in the SSCP gel, DNA fragments extracted from mutant bands were analysed by direct sequencing (fig 2A). Table 2 summarised the results obtained from these analyses. No germline mutation on the APC gene was found. Somatic APC mutations were found in 46% (11 of 24) of the MSI high, 44% of the MSI low, and 32% of the MSI negative carcinomas, and no obvious differences were observed. In addition, the patterns and distributions did not differ between the MSI high carcinomas and others, and no obvious tendency for mutation was seen in the MSI high carcinomas occurring at the oligonucleotide repeated sequences of the APC gene.

We also analysed the somatic changes at the p53 locus (table 3; fig 2B). The frequencies of p53 somatic mutation and 17p LOH in MSI high cases were similar to the other cases. In addition, the distribution and pattern of p53 mutation was similar as was the frequency of carcinomas having 17p LOH with p53 somatic mutations. However, the frequency of Ki-ras mutation was significantly lower in the MSI high cases than in the others (table 4; fig 2C).

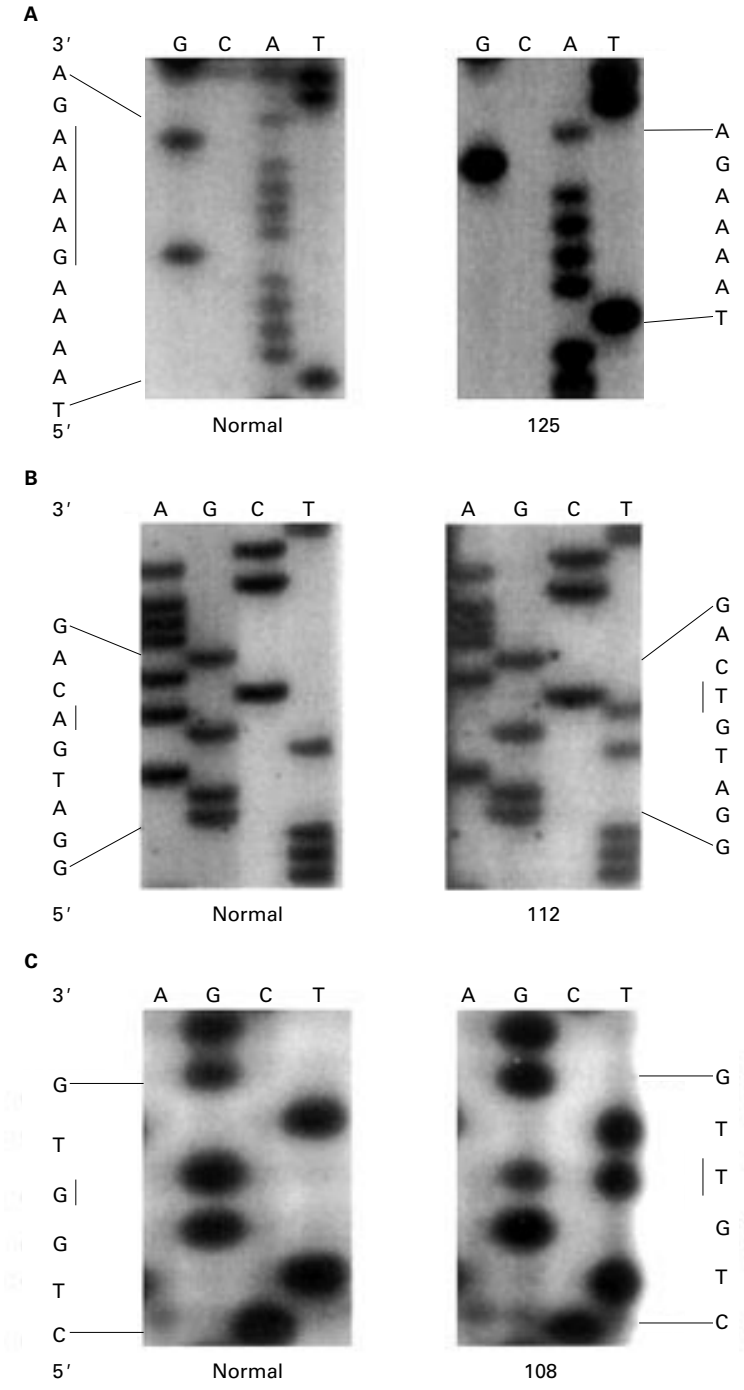

Figure 2 DNA sequencing of the APC (adenomatous polyposis coli), p53, and Ki-ras genes. Sequencing of DNA fragments eluted from normal and mutant bands. (A) In number 125, 5 bp (GAAAA) are deleted at codon 1309 in exon 15 of the APC gene. (B) In number 112, A is replaced by T at codon 208 in exon 6 of the p53 gene (amino acid changed from Asp to Val). (C) In number 108, G is replaced by T at codon 12 in exon 1 of the Ki-ras gene (amino acid changed from Gly to Val).

The twenty four MSI high non-familial colorectal carcinomas were analysed for hMSH2 and hMLH1 gene mutations because they were suspected to have defects in the DNA mismatch repair genes. Somatic mutations of hMSH2 or hMLH1 were found in six cases. Of these six cases, hMSH2 was mutated in one whereas hMLH1 was mutated in the other five (table 5). All six cases with these somatic mutations had no germline mutations in the hMSH2 or hMLH1 genes.

Discussion

MSI was analysed in 152 non-familial colorectal carcinomas. In many previous reports, the criteria for the selection of non-familial colorectal carcinomas has not been stringent.[11–13] We excluded all cases with colorectal carcinoma in first degree relatives. We classified MSI into MSI high (alterations in four to seven of seven loci), and MSI low (alterations in one to three of seven loci), and focused only on carcinomas showing high frequency MSI. Such a separation into high and low frequency MSI seems to be important for assessing the contribution of "true" MSI in the carcinogenesis of non-familial cases. The clinicopathological features of our MSI high cases were not different from those of MSI low and MSI negative cases. The nature of MSI high non-familial colorectal carcinomas might be considered to be different from that of HNPCC (table 1).

We have previously found the frequencies of mutations among APC, p53, and Ki-ras in HNPCC tumours with MSI to be lower than in non-familial colorectal carcinomas.[6] However, it was not clear whether these mutation frequencies were lower in MSI high non-familial colorectal carcinoma. Our results revealed that APC mutations in MSI high non-familial colorectal carcinoma were not substantially different from MSI low and MSI negative carcinomas with regard to frequency, type, and distribution within the gene. Although our results are consistent with those reported by Homfray *et al*,[13] the cases in their study included carcinomas with MSI at one locus only. There have been other reports on the association between APC mutation and MSI status in non-familial cases. Olschwang *et al* and Jass *et al* reported the frequency of mutations or LOH in APC genes to be less in replication error (RER) positive than in RER negative carcinomas.[11 25] Konishi *et al* reported that no APC mutations were present in four cases of sporadic carcinoma with severe RER[6] and Salahshor *et al* mentioned the low frequency of APC mutations in MSI high carcinomas.[26] It is unclear why our present results are different from those of previous reports. The contamination of HNPCC cases in previous reports might be one possible reason for these discrepancies. However, this is unlikely because of the small proportion of HNPCC among colorectal carcinoma in general. Our results suggest that APC mutation initiates tumorigenesis irrespective of MSI status in non-familial cases, and thus closely correlate with the assumptions of Tomlinson *et al*.[27]

Our study also revealed that the frequency of p53 mutation did not substantially differ between MSI high, MSI low, and MSI negative non-familial colorectal carcinomas. Lleonart *et al* also reported no correlation between MSI and p53 mutations in sporadic colon carcinomas.[28] Olschwang *et al* found no difference in the frequency of p53 mutation between RER positive and RER negative carcinomas, but the frequency of 17p LOH in RER positive carcinomas was significantly lower than that in RER negative carcinomas.[11] Simms *et al* and Salahshor *et al* mentioned the low frequency of p53 mutation and Jass *et al* reported the low

frequency of 17p LOH in MSI high cancers.[25 26 29] In previous reports, the frequency of abnormal p53 immunohistochemistry in MSI high cancers was low.[26 30–34] We thought that such differences might result from the inadvertent inclusion of familial cases; however, the reasons for the differences are still unclear. In our cases, the frequency of 17p LOH was as high in MSI high carcinomas as in MSI low and MSI negative carcinomas, thus suggesting that alterations in two alleles of the p53 gene contribute to carcinogenesis in non-familial cases, irrespective of MSI status. Ki-ras mutations were much less frequent in MSI high non-familial colorectal carcinomas than in MSI low and MSI negative carcinomas. In some cases, high MSI might have

Table 2 List of somatic mutations at the adenomatous polyposis coli (APC) gene

Case	*MSI*	*Codon*	*Type*	*Nomenclature*	*Base change*
1	++	1449	DEL1	Frame	AAG → AG
18	++	1465	DEL1	Frame	AGAGT → AGGT
39	++	1336	DEL7	Frame	AGACTGCAG → AG
53	++	1465	DEL1	Frame	AGAGT → AGGT
72	++	889	NS	NS	AAA → TAA
		1450	NS	NS	CGA → TGA
73	++	665	DEL2	Frame	TTA → A
75	++	665	DEL2	Frame	TTA → A
78	++	1336	DEL7	Frame	AGACTGCAG → AG
82	++	1557	INS1	Frame	ACT → ACCT
125	++	1309	DEL5	Frame	GAAAGATT → GATT
148	++	1308	DEL8	Frame	ATAAAAGAAAA → ATA
27	+	1450	NS	NS	CGA → TGA
31	+	1450	NS	NS	CGA → TGA
64	+	1453	DEL1	Frame	CCT → CT
83	+	1450	INS1	Frame	CGA → CCGA
12	−	1320	DEL5	Frame	AAGATTG → AA
15	−	1469	NS	NS	CAA → TAA
24	−	1309	DEL5	Frame	GAAAAGATT → GATT
26	−	917	DEL1	Frame	GAT → GT
		1464	DEL4	Frame	GAGAGT → GT
66	−	1451	INS2	Frame	GAA → GGGAA
68	−	1336	DEL7	Frame	AGACTGCAG → AG
77	−	1336	DEL7	Frame	AGACTGCAG → AG
106	−	736	NS	NS	AAG → TAG
108	−	895	NS	NS	TCA → TAA
109	−	1557	INS1	Frame	ACT → ACCT

MSI, microsatellite instability; MSI++, MSI high; MSI+, MSI low; MSI−, MSI negative; DEL, deletion; INS, insertion; NS, nonsense mutation; Frame, frameshift mutation.

Table 3 Details of cases with p53 mutations

			p53 mutation				
Case	*MSI*	*p53 LOH*	*Exon*	*Codon*	*Base change*	*Nomenclature*	*Amino acid change*
29	++	NI	6	198	GAA → CAA	Mis	Arg → His
39	++	+	5	175	CGC → CAC	Mis	Arg → His
72	++	+	5	132–136	16 bp del	Frame	Stop at 164
82	++	−	7	248	CGG → TGG	Mis	Arg → Trp
102	++	+	5	175	CGC → CAC	Mis	Arg → His
151	++	+	5	146	TGG → TCG	Mis	Trp → Ser
27	+	+	5	147	1 bp ins	Frame	Stop at 148
31	+	+	6	205	TAA → AAT	Mis	Tyr → Asn
15	−	+	8	273	CGT → CAT	Mis	Arg → His
26	−	−	5	170	ACG → TCG	Mis	Thr → Ser
30	−	+	5	175	CGC → CAC	Mis	Arg → His
67	−	NI	6	221	GAG → CAG	Mis	Glu → Gln
69	−	−	5	170	ACG → TCG	Mis	Thr → Ser
77	−	+	5	175	CGC → CAC	Mis	Arg → His
109	−	+	5	175	CGC → CAC	Mis	Arg → His
110	−	−	7	248	CGG → CTG	Mis	Arg → Leu
111	−	+	8	273	CGT → CAT	Mis	Arg → His
112	−	+	6	208	GAC → GTC	Mis	Asp → Val
136	−	+	8	282	CGG → TGG	Mis	Arg → Trp

MSI, microsatellite instability; MSI++, MSI high; MSI+, MSI low; MSI−, MSI negative; NI, not informative; bp, base pair; del, deletion; ins, insertion; LOH, loss of heterozygosity; LOH+, LOH positive; LOH−, LOH negative; Mis, Missense mutation; Frame, Frameshift mutation.

Table 4 Summary of frequency of somatic changes in microsatellite instability (MSI) high, MSI low, and MSI negative non-familial colorectal carcinomas

		No. of tumours with somatic changes				
		Mutation/informative tumours (%)			*LOH/informative tumours (%)*	
MSI type	*No. of tumours analysed*	*APC*	*p53*	*Ki-ras*	*17p*	*LOH of 17p/p53 mutation (%)*
MSI high	24	11/24 (46)	6/22 (27)	2/24 (8)	9/23 (39)	4/6 (67)
MSI low	9	4/9 (44)	2/7 (28)	4/9 (44)	4/8 (50)	1/2 (50)
MSI negative	31	10/31 (32)	11/29 (38)	11/29 (38)	11/26 (42)	7/11 (64)
p Value, MSI high *v* MSI low and MSI negative		0.4	0.4	0.008	0.68	>0.999

Sequence accession numbers by Genebank (APC, M74088; p53, M14694; Ki-ras, M34904).
LOH, loss of heterozygosity.

Table 5 Somatic mutations of hMSH2 and hMLH1 genes in microsatellite instability (MSI) high cases

Case	*Gene*	*Affected codon*	*DNA change*	*Nomenclature of mutation*
72	hMSH2	754	ins G	Frameshift to stop at 786
78	hMLH1	163	TAC-TAA	Nonsense
148	hMLH1	179	ins G	Frameshift to stop at 197
151	hMLH1	291	TTG-TAG	Nonsense
121	hMLH1	425	GAT-GGT	Missense from Asp to Gly
102	hMLH1	320	ins C	Frameshift to stop at 361

Sequence accession numbers by Genebank (hMLH1, U07418; hMSH2, U03911).
ins, insertion.

been induced by mutations in mismatch repair genes because six of the 24 MSI high carcinomas had somatic mutations in the hMSH2 or hMLH1 gene. However, another mechanism must be responsible for high MSI in the remaining cases. MLH1 promoter methylation could be an alternative mechanism and this should be investigated in future studies.

Our results suggest another possible pathway for the development of MSI high non-familial colorectal carcinoma, which is different from both the usual adenoma–carcinoma sequence and HNPCC carcinogenesis. In these cases, APC and p53 mutation occurs commonly, but Ki-ras mutation does not. A previous report found a low frequency of K-ras mutation in MSI high colorectal carcinoma,[25] and the reasons for this are unclear. A low Ki-ras mutation frequency has been reported by Kojima *et al* in flat-type colorectal carcinomas compared with that seen in polypoid colorectal carcinomas in early stages.[34] Furthermore, MSI was also reported to be more frequent in flat-type non-familial proximal colon carcinomas than in polypoid carcinomas in early stages,[35] indicating that the frequency of Ki-ras mutation was lower but MSI was higher in flat type colorectal carcinoma. However, in these studies, sample preparation and analytical methods were different from ours. Nonetheless, it is possible that MSI high proximal non-familial colorectal carcinoma might originate from flat type tumours. Jass *et al* suggested the so called serrated pathway as a pathway of non-familial MSI cancers.[25] We would propose the flat–adenoma–carcinoma sequence as another possible pathway. This might be one mechanism for carcinogenesis in MSI high non-familial colorectal carcinoma in the proximal colon. However, further studies on early colorectal carcinomas regarding both MSI and cancer associated genes are still needed to confirm this assumption.

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