Mismatch repair deficiency commonly precedes adenoma formation in Lynch Syndrome-Associated colorectal tumorigenesis

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Lynch syndrome is a cancer predisposition syndrome caused by germline mutations in mismatch repair (MMR) genes. MMR deficiency is a ubiquitous feature of Lynch syndrome-associated colorectal adenocarcinomas; however, it remains unclear when the MMR-deficient phenotype is acquired during tumorigenesis. To probe this issue, the present study examined genetic alterations and MMR statuses in Lynch syndrome-associated colorectal adenomas and adenocarcinomas, in comparison with sporadic adenomas. Among the Lynch syndrome-associated colorectal tumors, 68 of 86 adenomas (79%) and all adenocarcinomas were MMR-deficient, whereas all the sporadic adenomas were MMR-proficient, as determined by microsatellite instability testing and immunohistochemistry for MMR proteins. Sequencing analyses identified *APC* or *CTNNB1* mutations in the majority of sporadic adenomas (58/84, 69%) and MMR-proficient Lynch syndrome-associated adenomas (13/18, 72%). However, MMR-deficient Lynch syndrome-associated adenomas had less *APC* or *CTNNB1* mutations (25/68, 37%) and frequent frameshift *RNF43* mutations involving mononucleotide repeats (45/68, 66%). Furthermore, frameshift mutations affecting repeat sequences constituted 14 of 26 *APC* mutations (54%) in MMR-deficient adenomas whereas these frameshift mutations were rare in MMR-proficient adenomas in patients with Lynch syndrome (1/12, 8%) and in sporadic adenomas (3/52, 6%). Lynch syndrome-associated adenocarcinomas exhibited mutation profiles similar to those of MMR-deficient adenomas. Considering that WNT pathway activation sufficiently drives colorectal adenoma formation, the distinct mutation profiles of WNT pathway genes in Lynch syndrome-associated adenomas suggest that MMR deficiency commonly precedes adenoma formation.
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Lynch syndrome is one of the most common inherited cancer predisposition syndromes, and is caused by germline mutations in mismatch repair (MMR) genes, including *MLH1*, *MSH2*, *PMS2,* and *MSH6*.[1,2] Patients with Lynch syndrome have increased risks for cancers of multiple organs, including the colon, stomach, small intestine, and urinary bladder, with colorectal cancer being the most prevalent.[3,4] Although both Lynch syndrome-associated and sporadic colorectal cancers predominantly develop through the adenoma-carcinoma pathway, tumorigenesis in these two different clinical settings involves different sets of molecular abnormalities.[1,2] Most importantly, MMR deficiency resulting from the biallelic inactivation of MMR

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genes is a consistent feature of Lynch syndrome-associated colorectal cancers, but does not play a role in the conventional pathway of sporadic tumorigenesis.[5–10]

MMR deficiency is a virtually ubiquitous feature of advanced adenomas and adenocarcinomas associated with Lynch syndrome, whereas a subset of small, low-grade adenomas is MMR-proficient.[6–10] These observations led to the suggestion that MMR deficiency is acquired after adenoma formation during colorectal tumorigenesis in Lynch syndrome.[2,9] However, a recent study identified MMR-deficient crypt foci, characterized by MMR protein loss and microsatellite instability in the absence of adenomatous morphology, as a potential precursor of colorectal tumors in patients with Lynch syndrome.[11] This implies that MMR protein loss might precede adenoma formation at least in some instances.

Activation of the WNT signaling pathway due to *APC* or *CTNNB1* mutations is the most common molecular abnormality in sporadic colorectal cancers.[12–14] These genetic alterations are regarded as the initial molecular event in sporadic colorectal carcinogenesis via the conventional adenoma-carcinoma pathway and are sufficient to induce adenoma formation by themselves.[15–18] However, the prevalence of *APC* mutations in Lynch syndrome-associated adenocarcinomas is significantly lower than that in sporadic cases.[19] *RNF43* is another recently identified tumor suppressor gene related to WNT signaling.[20] RNF43 is a transmembrane E3 ligase that ubiquitinates and downregulates WNT receptors, Frizzled and LRP; therefore, RNF43 inactivation potentiates WNT signaling by enhancing ligand-dependent activation of this pathway.[21] *RNF43* has two mononucleotide repeats in its coding sequence and frameshift mutations affecting these repeat sequences are frequent in sporadic MMR-deficient colorectal cancers,[20] however, their involvement in Lynch syndrome-associated tumorigenesis remains elusive.

The present study aimed to characterize the relationship between MMR deficiency and genetic alterations in colorectal tumorigenesis in Lynch syndrome. We examined genetic alterations involved in early colorectal tumorigenesis, including those related to the WNT signaling pathway, and MMR statuses in Lynch syndrome-associated colorectal adenomas and adenocarcinomas in comparison with sporadic colorectal adenomas.

Materials and methods

Cases

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. In the present study, we analyzed 86 adenomas and 36 adenocarcinomas of the colorectum obtained from 44 patients with genetically confirmed Lynch syndrome (Table 1 and Supplementary Table 1). Among these, 62 adenomas and 10 adenocarcinomas were analyzed for MMR protein expression in our previous study.[10] Lesions included in the present study were selected based on the availability of sufficient tissue samples for DNA extraction. Newly added cases were selected by the availability of small adenomas or adenocarcinomas to increase the variability of lesions to be analyzed. In addition, 84 sporadic colorectal tubular adenomas were analyzed for comparison. All tissue samples were obtained by endoscopic or surgical resection at the National Cancer Center Hospital, Tokyo, Japan, fixed in 10% formalin and embedded in paraffin. Histological diagnoses of the lesions were made based on the World Health Organization classification. All the germline mutations in patients with Lynch syndrome were categorized as classes IV or V by InSiGHT classification.[22]

Table 1 Summary of Lynch syndrome-associated lesions

	Adenoma (n = *86*)			*Adenocarcinoma* (n = *36*)	
Age, year-old, median (range)	62 (34–84)			51 (20–74)	
Sex					
Male	45			16	
Female	41			20	
Location					
Proximal	34			22	
Distal	52			14	
Polyp size, mm/T factor	≤ 5	32	T1		8
	6–10	31	T2		8
	> 10	23	T3		18
			T4		2
Germline mutation					
MLH1	25			11	
MSH2	60			24	
MSH6	1			1	

Proximal colon, cecum to transverse colon; Distal colon, descending colon to rectum.

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded specimens, as described previously.[10] Antigen retrieval was performed by autoclaving in 10 mM citrate buffer (pH 6.0) for 10 min. Primary antibodies used were anti-MLH1 (ES05, 1:200 dilution; Dako, Glostrup, Denmark), anti-MSH2 (FE11, 1:200 dilution; Calbiochem, La Jolla, CA, USA), anti-PMS2 (A16-4, 1:200 dilution; Biocare Medical, Concord, CA, USA), and anti-MSH6 antibodies (SP93, 1:200 dilution; Spring Bioscience, Pleasanton, CA, USA). We used an automated stainer (Dako) and EnVision Detection System (Dako) according to the vendor's protocol. Normal colonic epithelial and stromal cells served as internal positive controls. Tumors showing significantly reduced or loss of expression of any MMR proteins were deemed to be MMR-deficient, and those retaining all four MMR proteins were regarded as MMR-proficient.

DNA Extraction

Deparaffinized 10-μm-thick sections from each paraffin block were microdissected using sterilized toothpicks under a microscope to enrich tumor content. The microdissected samples were subjected to DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany).

Microsatellite Instability Testing

Microsatellite instability testing was performed using five mononucleotide repeat markers, as described previously.[23] Tumors in which two or more of the five markers exhibited instability were regarded as microsatellite instability-high.

Next-Generation Sequencing

Next-generation sequencing libraries were prepared by two-step tailed PCR.[24] The first round PCR was performed using two pools of primers consisting of a gene-specific sequence and a consensus primer-binding sequence. Each pool contains 12 pairs of primers targeting frequently mutated regions of *APC*, *BRAF*, *CTNNB1*, *KRAS*, *NRAS*, and *RNF43* (Supplementary Table 2). Each PCR was performed using 10 ng genomic DNA with Kapa HiFi Hotstart Ready Mix (Kapa Biosystems, Boston, MA, USA). Amplification conditions were as follows: initial denaturation for 3 min at 95 °C, 23 cycles of 20 s at 98 °C, 20 s at 64 °C, and 20 s at 72 °C, and final extension of 3 min at 72 °C. The PCR products were purified using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA) and eluted in 30 μl low TE buffer.

The second round PCR was performed using 5 μl of purified first round PCR products to incorporate sample-specific indexes and P5/P7 flow-cell binding sequences (Supplementary Table 2). Amplification conditions of the second round PCR were as follows: initial denaturation for 3 min at 95 °C, 10 cycles of 20 s at 98 °C, 20 s at 65 °C, and 20 s at 72 °C, and final extension of 3 min at 72 °C. The products were purified and eluted in 30 μl low TE buffer as mentioned above.

Sequencing was performed by using MiSeq (Illumina, San Diego, CA, USA) and MiSeq Reagent Kit v3 (150 cycles) (Illumina) according to the manufacturer's protocol. The resulting sequences were mapped onto the human reference genome hg19, following removal of primer sequences on each end of the amplicons by using TruSeq Amplicon application (Illumina). Sequence variations with variant allele frequencies of more than 10% were identified as candidate mutations. Synonymous mutations and common single nucleotide polymorphism, based on the Single Nucleotide Polymorphism Database build 137, were excluded. A subset of mutations detected by next-generation sequencing was subjected to Sanger sequencing for confirmation. The primers and methods for Sanger sequencing were, as described previously.[25]

Statistical Analysis

Fisher's exact test was used to analyze each 2-by-2 table. P values < 0.05 were considered to indicate statistical significance.

Results

Immunohistochemically, 68 adenomas and all the adenocarcinomas from patients with Lynch syndrome showed a homogeneous loss of at least one MMR protein and were deemed MMR-deficient (Figure 1a and Supplementary Table 1). The MMR proteins lost were always consistent with the underlying germline mutation. The remaining 18 Lynch syndrome-associated adenomas and all the sporadic adenomas retained all four MMR proteins, and were regarded as MMR-proficient. Lynch syndrome-associated adenomas and adenocarcinomas underwent further microsatellite instability testing. Consistent with the results of immunohistochemistry, all the MMR-deficient adenomas and adenocarcinomas were microsatellite instability-high, and all the adenomas with intact MMR protein expression were microsatellite-stable (Figure 1b and Supplementary Table 1). At least one of the tumors in each patients with Lynch syndrome was MMR-deficient, supporting the diagnosis of Lynch syndrome.

Next-generation sequencing resulted in the median total coverage per sample of 272,666 reads (range, 97 301 to 391 887). Median coverage per amplicon across the all samples was 8 182 reads (range, 128 to 97 301). After exclusion of synonymous variants and common single nucleotide polymorphism, 238 non-

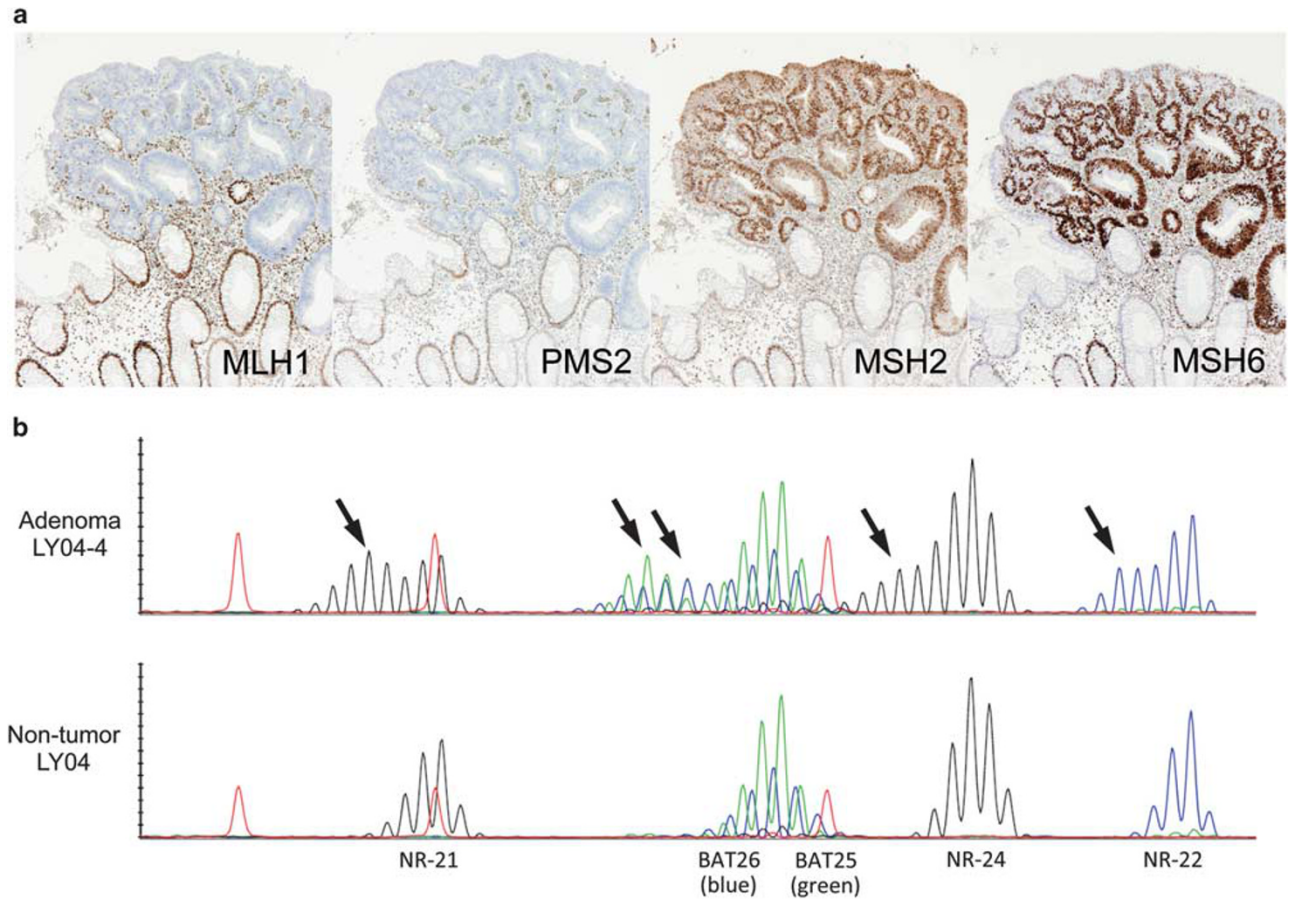

Figure 1 Immunohistochemistry for mismatch repair (MMR) proteins and microsatellite instability testing. (**a**) Immunohistochemical analysis of a Lynch syndrome-associated adenoma, showing loss of MLH1 and PMS2 and retention of MSH2 and MSH6 expression. (**b**) Microsatellite instability testing using five mononucleotide repeat markers. All the five markers show instability in this adenoma sample (arrows). Red peaks represent size markers.

synonymous variants were identified (Figure 2 and Supplementary Table 1). We analyzed 102 of the detected mutations by Sanger sequencing and confirmed all of them. For *APC* mutations, only nonsense and frameshift mutations were included in the following analyses, as most missense mutations are considered non-pathogenic.[26]

Protein-truncating *APC* mutations were significantly less frequent in Lynch syndrome-associated adenomas (34/86, 40%) than in sporadic adenomas (50/84, 60%; $P=0.014$). *CTNNB1* mutations were present in minor subsets in both Lynch syndrome-associated (4/86, 5%) and sporadic adenomas (8/84, 10%). *RNF43* was exclusively and frequently altered in Lynch syndrome-associated adenomas (45/86, 52%). *KRAS*, *NRAS*, and *BRAF* mutations were uncommon in both groups of adenomas. Adenocarcinomas in patients with Lynch syndrome frequently had *RNF43* mutations similar to adenomas (20/36, 56%), and in addition, *KRAS* mutations (16/36, 44%). *APC* mutations were infrequent (10/36, 28%).

Lynch syndrome-associated adenomas showed clearly different mutation profiles depending on the MMR statuses. *RNF43* mutations were exclusive to MMR-deficient adenomas (45/68, 66%; Figure 3a) and were mostly frameshift mutations affecting mononucleotide repeats. *APC* or *CTNNB1* mutations were more frequent in MMR-proficient adenomas, compared with MMR-deficient adenomas (13/18, 72 *vs* 25/68, 37%; $P=0.0086$). Furthermore, MMR-deficient adenomas showed a characteristic *APC* mutation spectrum. The mutation cluster region of *APC* contains short repeat sequences: an A5-repeat at codon 1455, an AG5-repeat at codon 1465, and an A6-repeat at codon 1554. More than half of *APC* mutations in MMR-deficient adenomas were insertions or deletions involving these repeat sequences (14/26, 54%; Figures 3b and c). These frameshift mutations were also common in Lynch syndrome-associated adenocarcinomas (6/11, 55%; $P=1.0$), but were significantly less frequent in Lynch syndrome-associated MMR-proficient adenomas (1/12, 8%; $P=0.0080$) and in sporadic adenomas (3/52, 6%; $P=3.6\times10^{-6}$). *RNF43* mutations often coexisted with *APC* or *CTNNB1* mutations in Lynch syndrome-associated adenomas and adenocarcinomas. These mutations tended to be mutually exclusive of each other, but did not show statistically

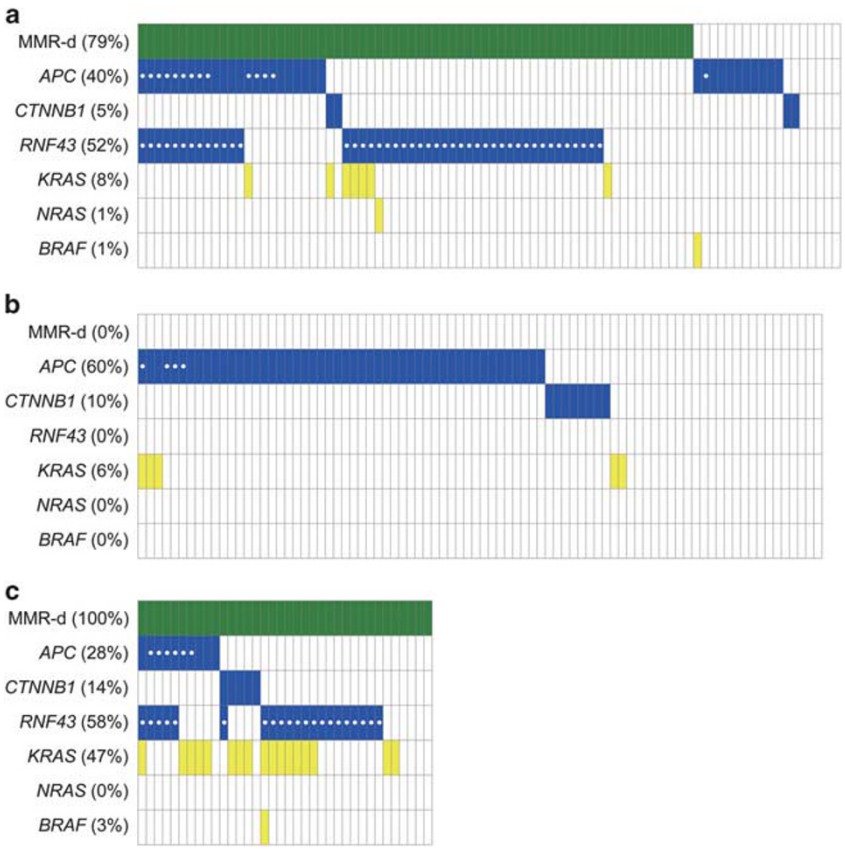

Figure 2 Mismatch repair status and genetic alterations in Lynch syndrome-associated (**a**) and sporadic adenomas (**b**) and Lynch syndrome-associated adenocarcinomas (**c**). The majority of Lynch syndrome-associated adenomas and all adenocarcinomas are mismatch repair-deficient (MMR-d, green), whereas all sporadic adenomas are MMR-proficient. *RNF43* mutations are exclusive to MMR-deficient tumors. Among the WNT pathway genes (blue), *APC* and *CTNNB1* mutations are always mutually exclusive, but *RNF43* mutations often coexist with *APC* or *CTNNB1* mutations. *KRAS* mutations are rare in Lynch syndrome-associated and sporadic adenomas but are common in Lynch syndrome-associated adenocarcinomas. Only protein-truncating mutations are indicated for *APC*. White circles indicate frameshift mutations affecting repeat sequences.

significant correlation (MMR-deficient adenoma, $P = 0.069$; adenocarcinoma, $P = 0.090$).

Discussion

Adenoma is a major precursor of both Lynch syndrome-associated and sporadic colorectal cancers. However, the present study showed that adenomas that develop on these two different backgrounds have distinct molecular features. Consistent with previous studies, we found that MMR deficiency was common in Lynch syndrome-associated adenomas, but was absent in sporadic adenomas.[5–10] In addition, our study showed that Lynch syndrome-associated adenomas had less *APC* mutations and frequent *RNF43* mutations, indicating distinct modes of WNT pathway activation. Furthermore, Lynch syndrome-associated colorectal adenomas have clearly different genetic profiles depending on MMR status. MMR-deficient adenomas showed mutation profiles characteristic to Lynch syndrome-associated adenomas, as described above. In contrast, mutation profiles of MMR-proficient adenomas are indistinguishable from those of sporadic adenomas.

Two-thirds of Lynch syndrome-associated MMR-deficient adenomas had frameshift *RNF43* mutations affecting mononucleotide repeats. Furthermore, *APC* mutations in Lynch syndrome-associated MMR-deficient adenomas were predominantly frameshift mutations affecting repeat sequences, which were rare in Lynch syndrome-associated MMR-proficient adenomas and sporadic adenomas. Increased prevalence of frameshift mutations is consistent with the consequence of MMR deficiency; in fact, these *RNF43* and *APC* mutation patterns have been reported to be also common in sporadic MMR-deficient colorectal cancers.[20,27]

Considering that a subset of small, low-grade Lynch syndrome-associated adenomas is MMR-proficient,[6–10] MMR deficiency has been thought to be acquired during the progression from early to advanced adenomas.[2,9] However, this model contradicts the present results. As WNT signaling pathway activation sufficiently drives adenoma formation,[15,16] the characteristic mutation spectra of *RNF43* and *APC* in

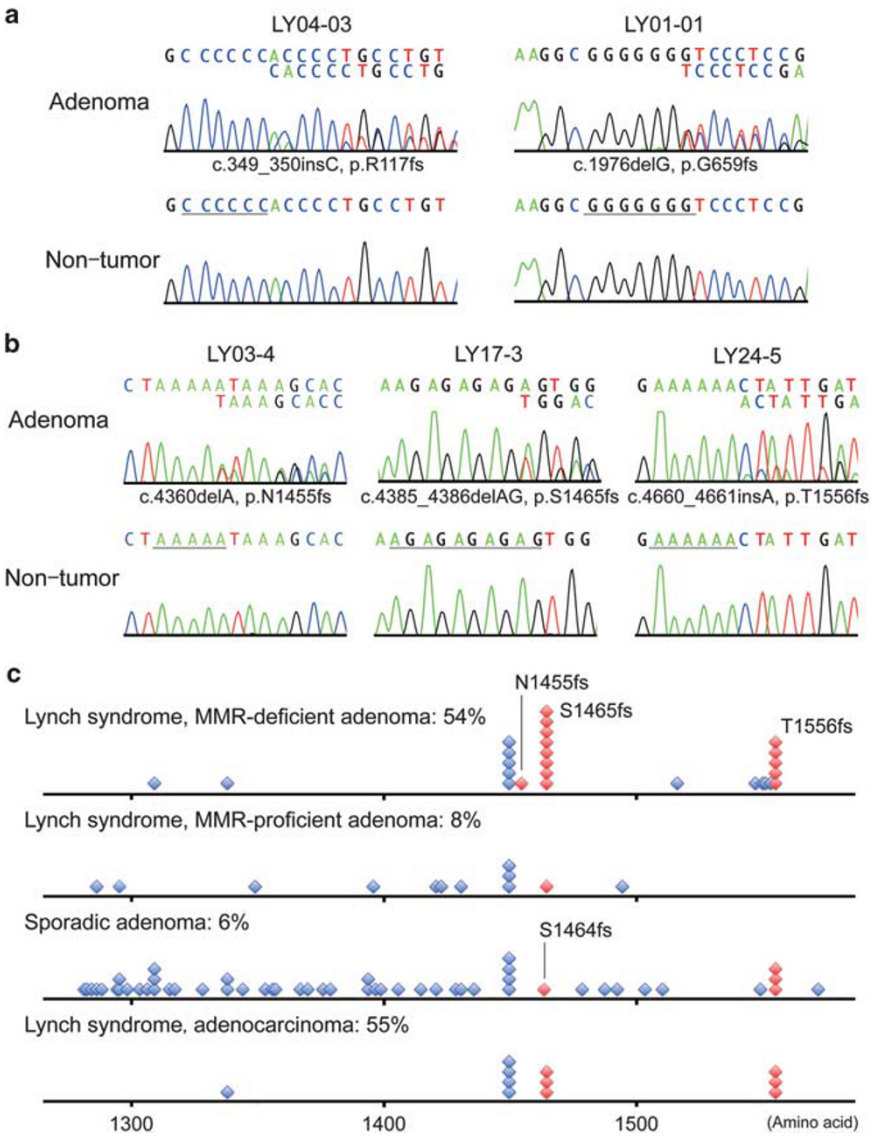

Figure 3 Mismatch repair (MMR)-deficient adenomas in patients with Lynch syndrome frequently show frameshift mutations of *RNF43* and *APC*. (**a**) Sanger sequencing results showing frameshift *RNF43* mutations affecting repeat sequences (underlined). (**b**) Frameshift *APC* mutations affecting repeat sequences within the mutation cluster region (underlined). (**c**) Distribution of protein-truncating *APC* mutations in the mutation cluster region. Prevalence of frameshift mutations affecting repeat sequences is indicated. Red markers indicate frameshift mutations affecting repeat sequences.

MMR-deficient adenomas suggest that biallelic MMR gene inactivation commonly precedes adenoma formation in these lesions. It is still conceivable that MMR-proficient adenomas acquire MMR deficiency before progression to adenocarcinomas. However, the *APC* mutation profile of Lynch syndrome-associated adenocarcinomas, simulating that of MMR-deficient adenomas, indicates that carcinogenesis via MMR-proficient adenomas is not a major pathway. In addition, a previous study showed that focal loss of MMR proteins is rarely observed in Lynch syndrome-associated adenomas,[10] further implying that the transition from an MMR-proficient to MMR-deficient adenoma is an uncommon event.

Recent studies identified MMR-deficient crypt foci as a potential precursor of colorectal cancers in patients with Lynch syndrome.[11,28] These lesions are predominantly mono- or oligocryptic and lack adenomatous morphology, suggesting that they represent the earliest lesion in the Lynch syndrome-associated colorectal tumorigenesis. Obviously, MMR-deficient crypt foci do not give rise to MMR-proficient adenomas; therefore, these lesions are related to distinct tumorigenic pathways. Moreover, it is unclear what proportions of adenocarcinomas in Lynch syndrome are derived from MMR-deficient foci. However, considering that the majority of the Lynch syndrome-associated adenocarcinomas acquire MMR-deficiency

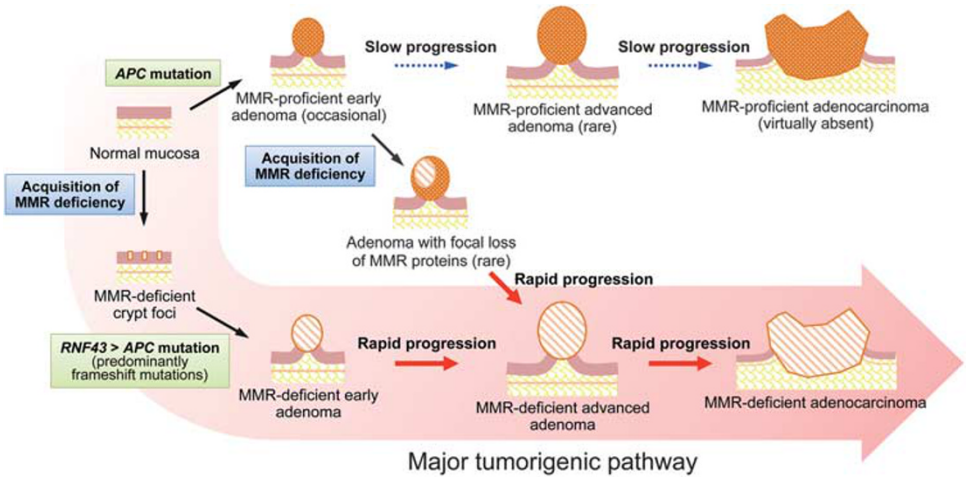

Figure 4 Model of Lynch syndrome-associated colorectal tumorigenesis. A subset of early colorectal adenomas in patients with Lynch syndrome are mismatch repair (MMR)-proficient, whereas most advanced adenomas are MMR-deficient, leading to the suggestion that acquisition of MMR deficiency occurs after adenoma formation. However, adenomas with focal loss of MMR proteins are rare and mutation profiles of *RNF43* and *APC* in MMR-deficient and -proficient adenomas are highly different from each other; thus, transition from MMR-proficient to -deficient adenoma is unlikely to be a common phenomenon. Importantly, *RNF43* and *APC* mutations in MMR-deficient adenomas are predominantly frameshift mutations affecting repeat sequences, implying that MMR-deficiency usually precedes the adenoma formation and that MMR-deficient crypt foci are major precursors.

before adenoma formation, MMR-deficient crypt foci may be the major precursor of Lynch syndrome-associated colorectal cancers (Figure 4).

In sporadic colorectal polyps and cancers, *RNF43* mutations show strong mutual exclusivity to other WNT pathway gene mutations.[20,25] However, significant proportions of *RNF43*-mutated adenomas and adenocarcinomas had concurrent *APC* mutations in patients with Lynch syndrome. *RNF43* and *APC* mutations activate the WNT signaling pathway through distinct mechanisms. RNF43 antagonize WNT signaling by targeting WNT receptors for proteasomal degradation; consequently, *RNF43* mutations enhance ligand-dependent pathway activation.[21] In contrast, APC inactivation constitutively stabilizes β-catenin and upregulates the WNT pathway in a cell autonomous and ligand-independent manner.[29] Thus, although it remains elusive why *RNF43* and *APC* mutations often coexisted only in Lynch syndrome-associated colorectal tumors, theoretically, the acquisition of *APC* mutations might further activate WNT signaling even in the presence of *RNF43* mutations.

This study revealed the frequent presence of *RNF43* mutations and a unique *APC* mutation profile, which are likely consequences of MMR deficiency, in Lynch syndrome-associated MMR-deficient adenomas and adenocarcinomas. The characteristic mutation profiles of WNT pathway genes suggest that MMR deficiency is commonly acquired before adenoma formation during tumorigenesis. This further implies that Lynch syndrome-associated colorectal tumors already have a considerable mutation burden at the time of adenoma formation, potentially providing an additional explanation for their rapid progression.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)