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TP53 mutation at early stage of colorectal cancer progression from two types of laterally spreading tumors

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Key words

APC, colon cancer, DNA methylation, laterally spreading tumor, *TP53*

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Although most sporadic colorectal cancers (CRC) are thought to develop from protruded adenomas through the adenoma–carcinoma sequence, some CRC develop through flat lesions, so-called laterally spreading tumors (LST). We previously analyzed epigenetic aberrations in LST and found that LST are clearly classified into two molecular subtypes: intermediate-methylation with *KRAS* mutation and low-methylation with absence of oncogene mutation. Intermediate-methylation LST were mostly granular type LST (LST-G) and low-methylation LST were mostly non-granular LST (LST-NG). In the present study, we conducted a targeted exon sequencing study including 38 candidate CRC driver genes to gain insight into how these genes modulate the development of LST. We identified a mean of 11.5 suspected nonpolymorphic variants per sample, including indels and non-synonymous mutations, although there was no significant difference in the frequency of total mutations between LST-G and LST-NG. Genes associated with RTK/RAS signaling pathway were mutated more frequently in LST-G than LST-NG ($P = 0.004$), especially *KRAS* mutation occurring at 70% (30/43) of LST-G but 26% (13/50) of LST-NG ($P < 0.0001$). Both LST showed high frequency of *APC* mutation, even at adenoma stage, suggesting its involvement in the initiation stage of LST, as it is involved at early stage of colorectal carcinogenesis via adenoma-carcinoma sequence. *TP53* mutation was never observed in adenomas, but was specifically detected in cancer samples. *TP53* mutation occurred during development of intramucosal cancer in LST-NG, but during development of cancer with submucosal invasion in LST-G. It is suggested that *TP53* mutation occurs in the early stages of cancer development from adenoma in both LST-G and LST-NG, but is involved at an earlier stage in LST-NG.

Colorectal cancers (CRC) can be divided into molecular subtypes. The major subtypes of CRC are those with microsatellite instability (MSI) characterized by DNA replication and repair defects, and those with chromosomal instability (CIN) characterized by aneuploidy, multiple chromosomal rearrangements and accumulation of somatic mutations in oncogenes. [(1)] According to a report by the Cancer Genome Atlas (TCGA) group, CRC with MSI has frequent *BRAF* mutation, frequent DNA hypermethylation and *MLH1* silencing,[(2)] which are molecular features of sessile serrated adenoma,[(3–5)] and shows a significantly higher number of genetic mutations.[(2,6,7)] In contrast, microsatellite-stable (MSS) CRC have shown frequent mutations of well-known key driver genes (e.g. *APC*, *KRAS*, *TP53* and *PIK3CA*),[(2)] which are thought to be associated with the adenoma–carcinoma sequence.[(8,9)]

While frequent DNA hypermethylation, also referred to as CpG island methylator phenotype (CIMP),[(10,11)] is observed in CRC with MSI, these high-methylation CRC are significantly associated with *BRAF* mutations.[(11,12)] In contrast, we and others clearly stratified MSS CRC into two distinct molecular subgroups using comprehensive DNA methylation data:[(12–14)] intermediate-methylation CRC correlating with *KRAS* mutation, and low-methylation CRC correlating with the absence of *BRAF* and *KRAS* mutations.[(12)] Regarding classifier genes, there are two types of methylation markers to classify CRC into these three methylation epigenotypes: Group-1 markers and Group-2 markers. High-methylation/CIMP-high CRC showed methylation of both Group-1 and Group-2 markers, intermediate-methylation/CIMP-low CRC showed methylation of Group-2 markers but not Group-1 markers, and low-methylation/CIMP-negative CRC showed no methylation of either Group-1 or Group-2 markers.[(12,15)] Using these markers, protruded adenomas were classified into the intermediate-methylation and low-methylation epigenotypes,[(16)] suggesting that they could be considered as precursors of MSS CRC.

Recently, we further investigated the epigenetic characteristics of flat, early lesions of CRC, so-called laterally spreading tumors (LST). Although the majority of sporadic CRC are

thought to develop from protruded adenomas through the adenoma–carcinoma sequence, some CRC develop through these flat lesions through the *de novo* pathway.[17,18] We demonstrated that LST can be classified into intermediate-methylation cases with *KRAS* mutation, and low-methylation cases with absence of oncogene mutations, as observed in MSS CRC and protruded adenomas. Interestingly, intermediate-methylation LST mostly represented granular morphology (LST-G), and low-methylation LST mostly represented non-granular morphology (LST-NG), suggesting that the two distinct molecular characteristics of LST mostly reflect two different macroscopic morphologies of these lesions.[19] Although LST are regarded as part of precursors of MSS CRC, developing through these different molecular pathways, genetic alterations involved in the development of LST are largely unknown.

In this study, we conducted a targeted exon sequencing study including 38 candidate CRC driver genes (e.g. *APC*, *PIK3CA* and *TP53*) to gain insight into how these driver genes modulate the genesis and progression of LST. LST showed high frequency of *APC* mutation even at adenoma stage, suggesting that *APC* mutation is involved in the initiation stage of LST, as it is involved at early stage of colorectal carcinogenesis via the adenoma-carcinoma sequence.[20] *TP53* mutation was never detected at adenoma stage, but was specifically detected in cancer samples in both LST-G and LST-NG, and was suggested to contribute to tumorigenesis of LST-NG at an earlier stage than was the case for LST-G.

Material and Methods

Clinical samples. Laterally spreading tumor tissue samples were obtained from patients who underwent endoscopic submucosal dissection at the Yokohama City University Hospital and Kanto Medical Center, NTT East, between May 2010 and December 2013. Among the 125 colorectal LST samples which had been examined in our previous study,[19] 32 samples were excluded because of insufficient quality or quantity of DNA for targeted exon sequencing as described below. The clinicopathological characteristics of the 93 patients (43 LST-G and 50 LST-NG), including age, sex, tumor size and tumor location, were evaluated at the time of endoscopic or surgical resection. Histopahological examinations were performed by two experienced pathologists. The diagnosis of carcinoma was based on cytological (enlarged/rounded nuclei, prominent nucleoli and loss of polarity) and architectural (complex budding, branching and back to back glands) changes. According to Vienna classification,[21] carcinoma *in situ* (intraepithelial non-invasive carcinoma) were considered as cancer, as well as invasive carcinoma (invading beyond the lamina propria). When LST samples contained both adenoma and carcinoma components, they were classified into cancer groups and only carcinoma components were microdissected. Their intraobserver agreement was good (kappa value was 0.94). If discrepancies occurred, the findings were reviewed simultaneously by both pathologists and a consensus was reached. Written informed consent was obtained from each of the enrolled patients. The present study was approved by the ethics committee of Yokohama City University, Chiba University and Kanto Medical Center, NTT East.

DNA extraction. From formalin-fixed, paraffin-embedded (FFPE) tissue specimens, laser capture microdissection (Carl Zeiss, Oberkochen, Germany) was performed to dissect tumor cells, and DNA was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA).

DNA methylation analysis. The bisulfite conversion of 500 ng of genomic DNA was performed using the EZ DNA Methylation Kit (ZymoResearch, Irvine, CA, USA). The methylation levels of six Group-1 markers and 14 Group-2 markers were analyzed quantitatively by pyrosequencing using PyroMark Q96 (Qiagen) in our previous study of methylation epigenotypes of LST,[19] and adopted in this report to show methylation levels of LST-G and LST-NG (Fig. S1). The methylation levels of *SFRP1* and *SFRP2* were additionally examined in the present study. Primer information is shown in Table S1. Further details of the analytic methods have been described in previous reports.[16,19]

Targeted exon sequence. Quantity and quality of the DNA from the FFPE samples were analyzed using a GeneRead DNA QuantiMIZE Array Kit (Qiagen), to exclude 32 and select 93 out of 125 samples for the subsequent targeted exon sequence. Targeted exon sequencing was performed using Colorectal Cancer GeneRead DNAseq Targeted Panels V2 (Qiagen), which targets 38 candidate CRC driver genes, suits short amplicon design, and enables detection of low level mutations, reducing the amount of DNA required. For each sample, 40 ng of genomic DNA was amplified and enriched for all coding exons of these genes. Subsequently, we prepared libraries of amplified DNA with a GeneRead DNA Library I Core Kit (Qiagen). All libraries were quantified with GeneRead DNAseq Library Quant Array (Qiagen) using Bio-Rad CFX96, and diluted to the designed range for cluster generation for Illumina platform. We then performed deep-sequencing using MiSeq (Illumina, San Diego, CA, USA) following a 150-bp paired-end protocol, and the results were analyzed using GeneRead Panel Calling software (Qiagen). Exon sequence data was submitted to the NCBI BioSample database (http://www.ncbi.nlm.nih.gov/biosample), and the accession numbers of 93 samples were SAMN04492029–SAMN04492121.

Among variants identified by the GeneRead DNAseq panel system, we analyzed those with an allele frequency between 0.1 and 0.9, for fear of sequence error due to DNA modification by formalin fixation in the analysis of FFPE samples. Considering that matched normal tissues were not analyzed in the target exon sequence, and that the minimum read depth covering 92% of the target bases was 30 (Fig. S2), we decided to analyze variants with allele frequency >0.1, not >0.05, to avoid the sequence error. Known variants reported in the dbSNP (http://www.ncbi.nlm.nih.gov/SNP) and JSNP database (http://snp.ims.u-tokyo.ac.jp/map/Dump/) were filtered out. Synonymous mutations were also excluded. We then used MutationTaster classification tools[22] to predict the functional consequences of amino acid changes or frame-shift mutations. The mutations found to be "disease causing" were defined as significant. Each significant mutation was reviewed by Integrative Genomica Viewer (http://www.broadinstitute.org/igv/), including allele frequency and location. When the detected variants were on the COSMIC database, COSMIC ID was also shown (Table S2).

Statistical analysis. The correlations between LST morphologies and clinicopathological factors were analyzed by Fisher's exact test, except for age and tumor size, which were analyzed by Student's *t*-test. The differences of methylation levels were analyzed by Student's *t*-test. Unless otherwise specified, *P*-values of <0.05 were considered to denote statistical signifi-

cance. All the statistical analyses were performed using SPSS, version 11.0 (SPSS, Chicago, IL, USA).

Results

Comparison of clinicopathological characteristics. The clinicopathological data of the 93 analyzed LST cases are summarized in Table 1. No significant differences in sex or age were observed between LST-G and LST-NG patients. Tumor size of LST-G was significantly larger than that of LST-NG (41.4 ± 20.9 *vs* 26.4 ± 8.6, $P < 0.0001$). There was no correlation between LST morphologies and tumor locations. Although there was no significant difference in the frequency of carcinoma components (20/43, 47% *vs* 34/50, 68%, $P = 0.06$), submucosal invasion was detected in LST-NG more frequently (6/43, 14% *vs* 19/50, 38%, $P = 0.01$).

DNA methylation analysis. Methylation epigenotypes were decided through unsupervised hierarchical clustering analysis using DNA methylation information in our previous study.[19] It was confirmed that intermediate-methylation epigenotype strongly correlated with LST-G, and low-methylation epigenotype strongly correlated with LST-NG ($P < 0.0001$) in the 93 analyzed samples (Table 1). When methylation levels in LST-G and LST-NG were compared, all Group-2 markers showed substantially higher methylation in LST-G than in LST-NG (Fig. S1a). When methylation levels in adenoma and cancer cases were compared for LST-G, there were no significant differences in both Group-1 markers and Group-2 markers (Fig. S1b). When compared for LST-NG, however, the methylation levels of *MLH1*, *RASSF2*, *UCLH1* and *NEUROG1* were significantly higher in LST-NG cancer cases than in LST-NG adenoma cases ($P = 0.05$, $P = 0.001$, $P = 0.05$ and $P = 0.02$, respectively) (Fig. S1c).

As for the *SFRP* genes, which act as stroma-derived Wnt signal inhibitors, there were no significant differences in the methylation levels between LST-G and LST-NG for both *SFRP1* (34.7 ± 24.0% *vs* 31.2 ± 19.7%, $P = 0.5$) and *SFRP2* (8.1 ± 14.1% *vs* 6.2 ± 9.0%, $P = 0.4$) (Fig. S1). When compared between adenoma and cancer, there were also no significant differences for both *SFRP1* (31.6 ± 23.8% *vs* 33.6 ± 20.2%, $P = 0.7$) and *SFRP2* (8.4 ± 15.0% *vs* 6.1 ± 8.3%, $P = 0.4$).

Table 1. Clinicopatholpgical characteristics of LST according to macroscopic morphology

	LTS-G (%)	LST-NG (%)	*P*-value
Number	43	50	
Sex			
Male	20 (47)	32 (64)	0.10
Female	23 (53)	18 (36)	
Age			
Mean ± SD (year)	66.7 ± 11.9	65.6 ± 10.4	0.65
Tumor location			
Proximal	20 (47)	26 (52)	0.81
Distal	10 (23)	12 (24)	
Rectum	13 (30)	12 (24)	
Tumor size			
Mean ± SD (mm)	41.4 ± 20.9	26.4 ± 8.6	<0.0001*
Adenoma/cancer			
Adenoma	23 (53)	16 (32)	0.06
Cancer	20 (47)	34 (68)	
Invasion status			
Lymph invasion	1 (2)	3 (6)	0.62
Vascular invasion	1 (2)	4 (8)	0.37
Submucosal invasion	6 (14)	19 (38)	0.01*
Methylation epigenotypes			
Intermediate-methylation epigenotype	33 (77)	12 (24)	<0.0001*
Low-methylation epigenotype	10 (23)	38 (76)	

Tumor locations were classified into three groups: proximal (cecum, ascending and transverse colon), distal (descending and sigmoid colon) and rectum. Tumor size was recorded as the maximum diameters of the extirpated specimen. Intermediate-/low-methylation epigenotypes were decided through unsupervised hierarchical clustering analysis using DNA methylation information in our previous study.[19] *P*-values were analyzed by Fisher's exact test, or Student's *t*-test for age and tumor size. **P* value < 0.05.

Performance of targeted exon sequence. Coverage data for exon sequencing are presented in Supplementary Figure S2. On average, approximately 2.4 million purity-filtered reads were generated for each sample. The median read depth in the target region was 1179, ranging from 491 to 1771. Of the target bases, 95%, 92% and 82% were covered by at least 10, 30 and 100 reads, respectively (Fig. S2).

Differences of mutation frequency. The average mutation frequency is summarized in Figure S3. A total of 2860 somatic mutations were identified in 93 LST samples analyzed. Among them, 1513 mutations were identified in coding regions, and 179, 813 and 75 somatic mutations were identified as nonsense mutations, missense mutations and indels, respectively.

The comparison of mutation numbers identified in the coding region is summarized in Figure 1. There was no significant difference in the mutation frequencies between LST-G and LST-NG (16.7 ± 19.7 *vs* 15.9 ± 19.1, $P = 0.9$), or between adenoma and cancer (12.6 ± 12.4 *vs* 21.4 ± 25.2, $P = 0.2$ for LST-G; 15.4 ± 22.7 *vs* 16.2 ± 17.5, $P = 0.9$ for LST-NG) (Fig. 1a). Per sample, 11.5 suspected nonpolymorphic variants were detected, including non-synonymous mutations and indels. The frequencies of these variants were not significantly different between LST-G and LST-NG ($P = 0.8$), or between adenoma and cancer ($P = 0.3$).

When the mutation frequencies were compared between samples with/without mutation of mismatch repair (MMR) genes, the total number of mutations was significantly higher in samples with MMR gene mutation (22.4 ± 23.8 *vs* 9.6 ± 9.0, $P = 0.001$). Non-synonymous mutation was also more frequently observed (14.9 ± 16.2 *vs* 6.2 ± 6.4, $P = 0.001$) (Fig. 1b).

Mutation spectra. The patterns of mutation spectra were similar between LST-G and LST-NG, and also between adenoma and cancer. The predominant type of substitution was a C:G to T:A transition, followed by a T:A to C:G transition (Fig. S4). In the samples with MMR gene mutation, the frequency of a C:G to T:A transition was especially high (74%).

Somatic mutations identified in LST. Significant mutations of all the genes detected by the targeted exon sequencing are shown in Table S2. *APC*, *KRAS*, *ERBB2*, *DMD*, *MSH2*, *EP300*, *DCC*, *TP53* and *FBXW7* were identified as frequently mutated genes in LST. The mutation frequencies of key CRC-driver genes are shown in Figure 2. *APC* mutation was frequently identified both in LST-G and LST-NG, with no statistical significance (38/43, 88% *vs* 41/50, 82%, $P = 0.6$). As we previously reported,[19] targeted exon sequencing in this study also revealed that frequency of *KRAS* mutation in LST-G was substantially and significantly higher than LST-NG (30/43, 70% *vs* 13/50, 26%, $P < 0.0001$). *BRAF* mutation was rarely identified in LST-G and LST-NG (7% and 0%, respectively),

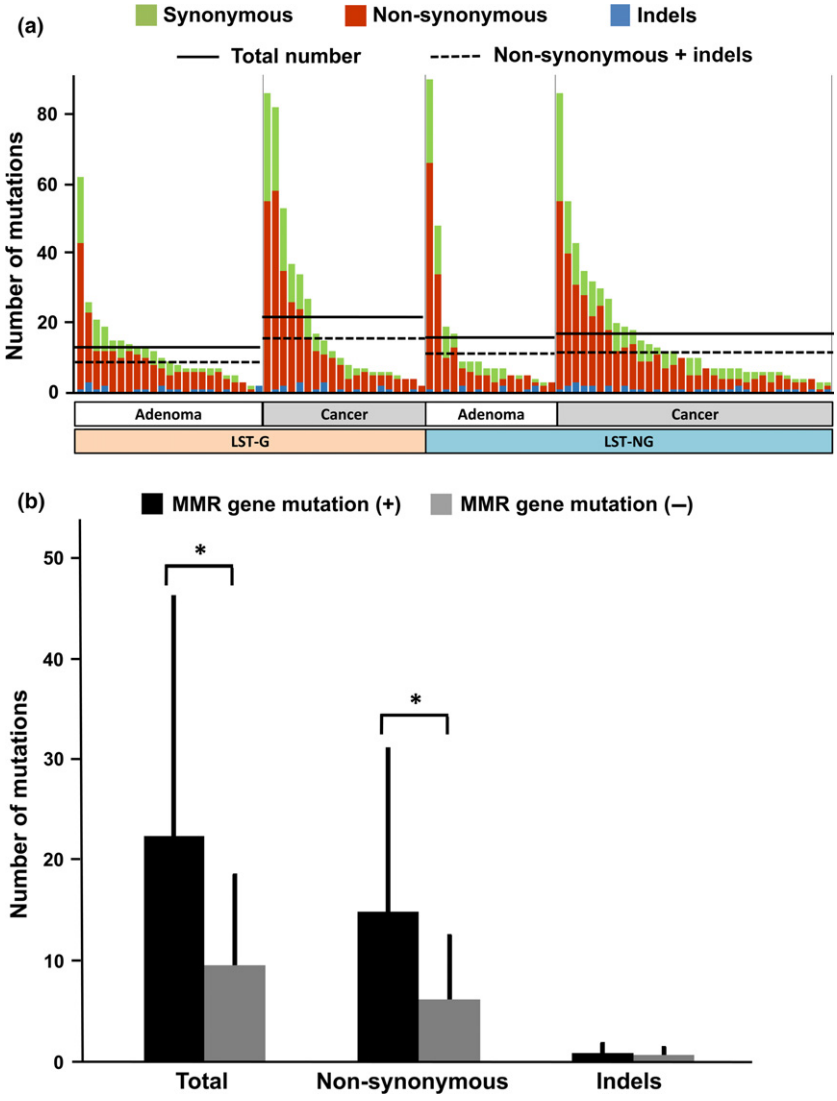

Fig. 1. Comparison of mutation numbers. (a) Comparison between LST-G and LST-NG. The frequency of somatic mutations were not significantly different between LST-G and LST-NG both for all variants and suspected nonpolymorphic variants (non-synonymous mutations and indels) ($P = 0.9$ and $P = 0.8$, respectively). When compared between adenoma and cancer cases, the frequency of somatic mutations were also not significantly different both for all variants and suspected nonpolymorphic variants ($P = 0.2$ and $P = 0.2$ for LST-G; $P = 0.9$ and $P = 0.9$ for LST-NG). (b) Comparison between samples with/without MMR gene mutations. The total number of mutations was significantly higher in the samples with MMR gene mutation ($P = 0.001$), as well as non-synonymous mutation ($P = 0.001$).

but occurred in a mutually exclusive manner to *KRAS* mutation. *PIK3CA* mutation was identified in LST-G and LST-NG at similar frequencies (8/43, 19% *vs* 9/50, 18%, $P = 1$). Frequency of *CTNNB1* mutation was not significantly different between LST-G and LST-NG (8/43, 19% *vs* 5/50, 10%, $P = 0.3$). When adenoma and cancer cases were compared, *TP53* mutation was never detected in adenoma cases, but frequently observed in cancer cases (0/23, 0% *vs* 2/20, 10%, $P = 0.2$ for LST-G; 0/16, 0% *vs* 21/34, 62%, $P < 0.0001$, for LST-NG), with statistical significance for LST-NG.

Alterations in well-defined signals. To evaluate how some well-defined carcinogenic pathways are associated with the development of LST, we compared alterations in RTK/RAS, PI3K, WNT, TGF-ß and TP53 signaling between LST-G and LST-NG (Fig. 3). WNT signaling was frequently altered both in LST-G and LST-NG (39/43, 91% and 46/50, 92%). Genes involved in RTK/RAS signaling were altered in LST-G more frequently than LST-NG (38/43, 88% *vs* 31/50 62%, $P = 0.004$), whereas those involved in TP53 signaling were altered in LST-NG more frequently than LST-G (10/43, 23% *vs* 26/50 52%, $P = 0.006$). Genes involved in PI3K and TGF-ß signaling were not frequently altered. Mutations of MMR genes were identified in approximately half of LST samples, and their frequencies were not different between LST-G and LST-NG (22/43, 51% *vs* 26/50, 52%, $P = 1$).

***APC* mutation occurred in adenoma stage of laterally spreading tumors.** *APC* mutations were detected in most samples, and the frequency of mutation was not different between adenoma and cancer cases (21/23, 91% *vs* 17/20, 85%, $P = 0.7$ for LST-G; 13/16, 81% *vs* 28/34, 82%, $P = 1$ for LST-NG). The types of *APC* mutations are summarized in Figure 4. When the frequency of frameshift/truncating mutations was compared, there was no significant difference between LST-G and LST-NG (36/38, 95% *vs* 38/41, 93%, $P = 1$). The frequency of biallelic mutations was significantly higher in LST-G than LST-NG (33/38, 87% *vs* 25/41, 61%, $P = 0.01$). Compared with LST-G, however, the frequency of CTNNB1 nuclear expression was significantly higher in LST-NG (4/43, 9% *vs* 25/50, 50%, $P < 0.0001$), according to our previous immunohistochemistry (IHC) data.[19]

***TP53* mutation in early cancer of laterally spreading tumors.** While *TP53* mutation was never involved at adenoma stage and was frequently observed in cancer, *TP53* mutation predominantly occurred in cancer samples of LST-NG (Fig. 5).

In cancer with submucosal invasion, the frequency of *TP53* mutation was detected at 33% (2/6) for LST-G and 74% (14/

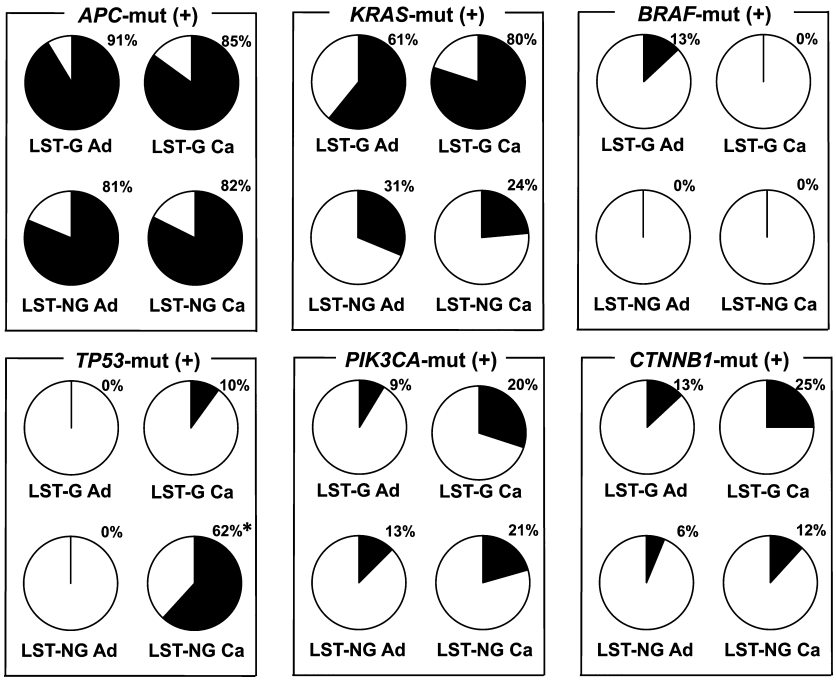

Fig. 2. Frequency of genetic alterations. When compared between LST-G and LST-NG, *APC* mutation was frequently identified both in LST-G and LST-NG, with no statistical significance ($P = 0.6$). Frequency of *KRAS* mutation in LST-G was substantially and significantly higher than LST-NG (30/43, 70% *vs* 13/50, 26%, $P < 0.0001$). *BRAF*, *PIK3CA* and *CTNNB1* mutation were not frequently identified in LST-G and LST-NG. When adenoma and cancer were compared, mutation frequencies were not significantly different, except *TP53*. *TP53* mutation was never observed in adenoma, but identified frequently in cancer cases (2/20, 10% for LST-G and 21/34, 62% for LST-NG). *$P < 0.0001$, for comparison between LST-NG adenoma and LST-NG cancer.

19) for LST-NG without significant difference ($P = 0.1$). While *TP53* mutation was never detected in intraepithelial cancer of LST-G (0/14, 0%), *TP53* mutation occurred in 40% (6/15) of intraepithelial cancer of LST-NG ($P = 0.01$). Such tendency was also observed when intermediate-methylation LST and low-methylation LST were compared. These results indicated that the role of *TP53* mutation in the early stage of cancer development from adenoma to cancer is apparent in LST-NG (=mostly low-methylation) rather than LST-G (=mostly intermediate-methylation).

Finally, the result of *TP53* mutation detected in the present study was compared with IHC results in our previous study.[19] TP53 IHC showed a good negative predictive value of *TP53* mutations, 92% (54/59), but a rather low positive predictive value, 53% (18/34) (Fig. 5).

Discussion

Sporadic CRC can be clustered into three DNA methylation epigenotypes with distinct oncogene mutation.[12–14] High-methylation CRC mostly occurs at the proximal colon, and showed strong correlation with presence of the *BRAF* mutation and MSI-high. These high-methylation CRC are considered to be developed from sessile serrated adenoma,[3,23,24] and our recent analysis of serrated adenoma and high-methylation CRC by targeted exon sequencing revealed that mutations of mismatch repair genes, genes in PI3K, WNT, TGF-β and BMP signaling, but not in TP53 signaling, were significantly involved in development from serrated adenoma to CRC.[24] To gain insight into the carcinogenic process of two other subtypes of CRC, we previously investigated epigenetic characteristics of conventional protruded adenomas[16] and LST.[19] We demonstrated that these tumors are classified into intermediate-methylation tumors with *KRAS* mutation and low-methylation tumors with no oncogene mutation,[16,19] suggesting that these tumors could be precursors of MSS CRC with these molecular features.

Luo *et al.*[25] report through comprehensive methylation analysis of conventional protruded adenomas that subclass of frequently methylated adenomas with *KRAS* mutation might be the precursors for MSS CRC, while low-methylation protruded adenoma might have rather low potential to progress to CRC.[25] While LST were also classified into intermediate-methylation and low-methylation tumors, molecular aberrations to modulate their tumorigenesis and progression have been largely unknown. Therefore, we conducted targeted exon sequencing analysis of 38 candidate CRC driver genes to evaluate their involvement in LST.

Among 1513 mutations identified in coding regions, 179, 813 and 75 mutations were identified as nonsense mutations, missense mutations and indels, respectively. In mutation frequencies, there was no significant difference between LST-G and LST-NG ($P = 0.9$), or between adenoma and cancer ($P = 0.2$ for LST-G, $P = 0.9$ for LST-NG). However, when the mutation frequencies were compared between samples with/without mutation of MMR genes, the total number of mutations was significantly higher in samples with MMR gene mutation ($P = 0.001$). Non-synonymous mutation was also more frequently observed ($P = 0.001$). The frequency of a C:G to T:A transition was especially high. These tendencies were also reported in previous exome analyses of advanced CRC[2] and other cancers.[26] The increase of mutation frequency caused by MMR gene abrogation is suggested to be due to impaired recognition/repair of G:T mismatches, at least partly. Although there was no significant difference between LST-G and LST-NG (22/43, 51% *vs* 26/50, 52%, $P = 1$), mutations of MMR genes were identified in half of LST samples, suggesting that these mutations and subsequent hypermutation might play an important role in tumorigenesis of LST, as well as genesis of high-methylation CRC through the serrated pathway.[2,24]

As for mutations of individual genes, genes involved in WNT signaling (e.g. *APC* mutation) was altered even at adenoma stages in both LST-G and LST-NG. Genes involved in RTK/RAS signaling (e.g. *KRAS* mutation) were altered in LST-G more frequently than LST-NG. As for TP53 signaling, *TP53* mutation occurred at an early stage of cancer development from adenoma in both LST-G and LST-NG, but

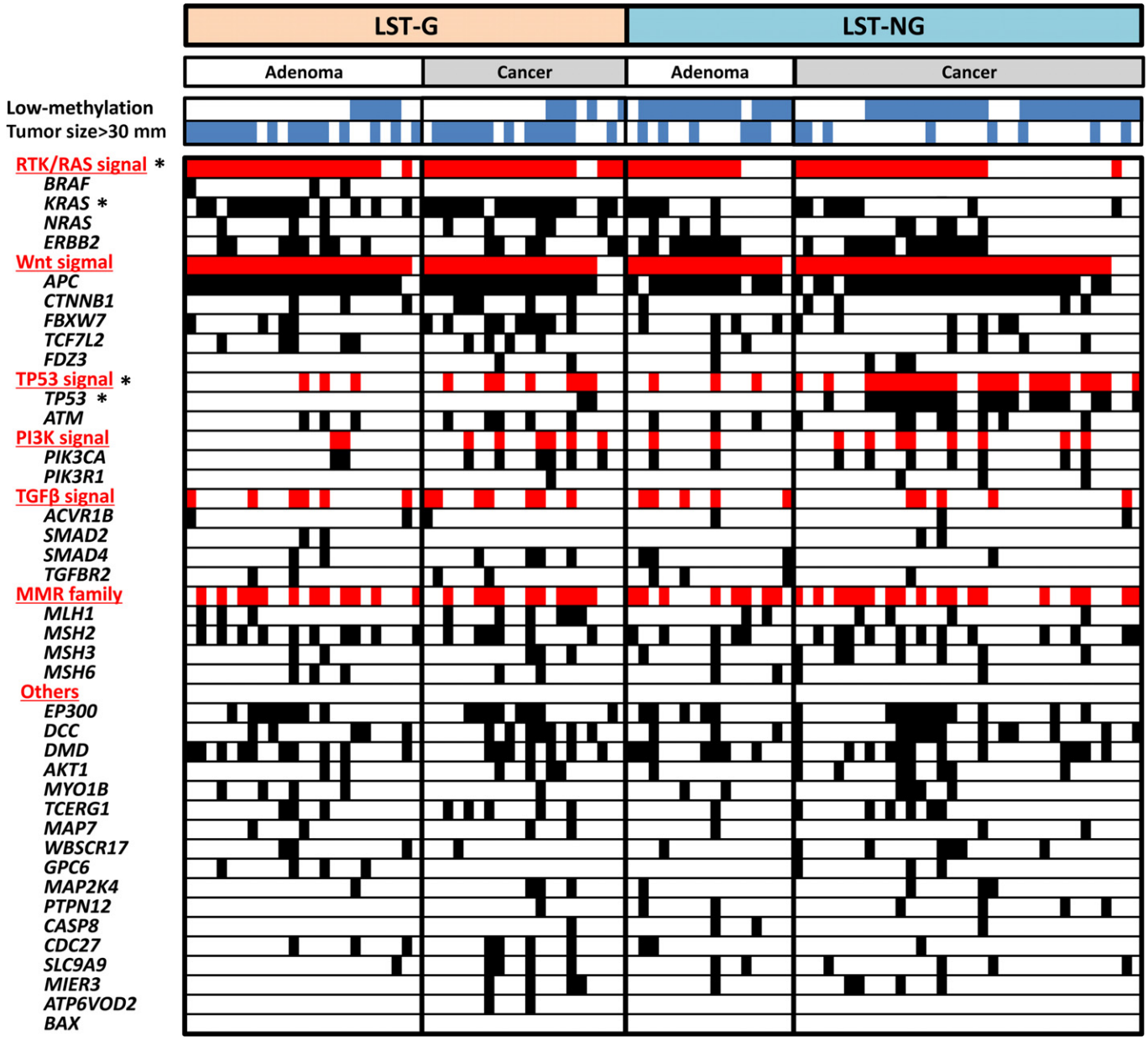

Fig. 3. Genetic alterations in individual genes and carcinogenic signaling pathways. Low-methylation: intermediate-/low-methylation epigenotypes were decided through unsupervised hierarchical clustering analysis using DNA methylation information in our previous study,[19] and low-methylation LST cases are shown by blue boxes. Black boxes for an individual gene: cases with one or more mutations of the gene that were predicted to be "disease causing" by MutationTaster.[22] Red boxes for a signal: cases with one or more mutated genes involved in a signal. *$P < 0.05$, in comparison between LST-G and LST-NG.

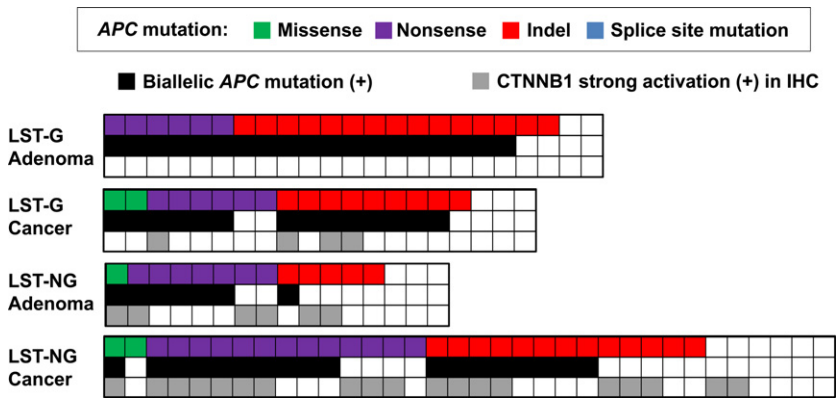

Fig. 4. Details of the *APC* mutation. Green: missense mutation. Purple: nonsense mutation. Red: indel. APC mutations were detected in most of LST samples, for both LST-G and LST-NG. Grey: samples with CTNNB1 strong activation in immunohistochemistry (IHC); that is, CTNNB1 nuclear staining (+) in the previous IHC analysis.[19] Whereas biallelic mutation (black) was observed more frequently in LST-G than LST-NG ($P = 0.01$), the strong activation of CTNNB1 was significantly higher in LST-NG than LST-G ($P < 0.0001$).

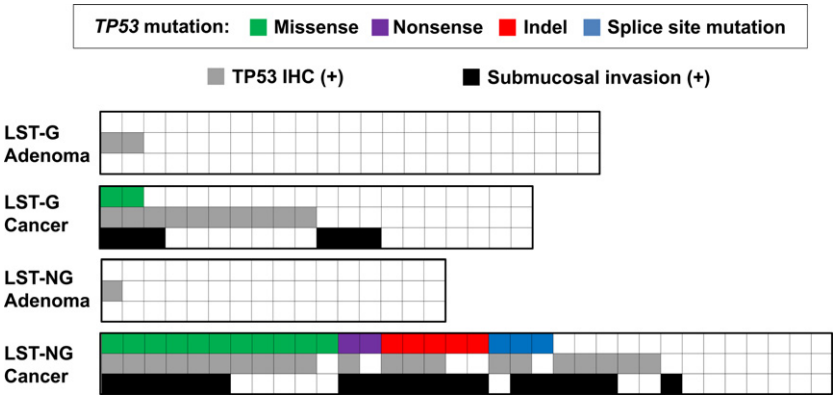

Fig. 5. Details of the TP53 mutation. Green: missense mutation. Purple: nonsense mutation. Red: indel. Blue: splice site mutation. *TP53* mutation did not occur by adenoma stage, but was frequently observed in cancer. Black, cancer samples with submucosal invasion. *TP53* mutation was never observed in 14 intraepithelial cancer samples of LST-G (0%), but occurred in 6 of 15 (40%) intraepithelial cancer cases of LST-NG ($P = 0.01$). Grey: samples with TP53 nuclear staining in the previous immunohistochemistry (IHC) analysis.[19] IHC were not so reliable in detection of *TP53* mutation; the positive and negative predictive values of *TP53* mutations were 53% (18/34) and 92% (54/59).

was involved at an earlier stage in LST-NG than LST-G. Genes involved in MMR were mutated in half of LST samples as described above. Genes involved in PI3K and TGF-ß signaling were not frequently altered, suggesting that their contribution to LST initiation and progression is rather low compared with WNT, RTK/RAS, and TP53 signaling and MMR.

In the adenoma–carcinoma sequence, mutations occur frequently in gate-keeper genes (e.g. the *APC* gene at early stage).[9] In good agreement, recent exome sequencing study conducted by Zhou *et al.*[27] revealed that *APC* was mutated in both colorectal adenoma and cancer from the same patients. The small adenoma that results from *APC* mutation grows slowly, but a second mutation in another gene, such as *KRAS*, unleashes a second round of clonal growth that allows an expansion of cell number.[28] This process of mutation is reported to be followed by clonal expansion of malignant tumors that can invade submucosa and metastasize to lymph nodes and distant organs, with accumulation of mutations in genes such as *TP53* and *PIK3CA*.[8,29] In LST, *APC* mutations were detected in most samples, and the frequency of mutation was not different between adenoma and cancer, for both LST-G and LST-NG. It is suggested that *APC* mutation is involved in the initiation step of LST development in both LST-G and LST-NG, as observed in protruded adenoma in the adenoma–carcinoma sequence.

In our previous immunostaining analysis of CTNNB1 in LST, CTNNB1 activation (e.g. loss of CTNNB1 expression at cellular membrane) was frequently detected in LST, but strong CTNNB1 activation (e.g. nuclear expression) was predominantly identified in samples of LST-NG.[19] Whereas *APC* mutation was detected in most of the samples, both for LST-G and LST-NG, only the mutations that truncate the encoded protein within its N-terminal 1600 amino acids should be considered to act as driver mutations.[30] When such driver mutations were compared, there was also no significant difference in the mutation frequencies between LST-G and LST-NG (33/43, 77% *vs* 38/50, 76%, $P = 1$). The frequency of frameshift/truncating mutations was not different either. SFRP family proteins are known to inhibit proliferation and to induce apoptosis by direct binding to Wnt ligands and to prevent the activation of Wnt signal. As promotor methylation is the major mechanism to inactivate SFRP family genes in early CRC,[31] we analyzed methylation levels of *SFRP1* and *SFRP2* in LST samples. Although *SFRP1* methylation was frequently found in LST samples, there was no significant difference between LST-G and LST-NG. While these alterations might contribute to genesis of LST, they are suggested to be associated little with the different molecular basis between LST-G and LST-NG. To reveal how CTNNB1 is strongly activated in LST-NG, additional studies are necessary.

TP53 mutations in CRC have been reported to occur at approximately 50%.[32] While *TP53* mutation was absent in high-methylation CRC, it was significantly detected in the other subtypes of CRC.[12,24] In the adenoma–carcinoma sequence of CRC, *TP53* mutation is involved during cancer development from adenoma, and heralds the transition from pre-invasive to invasive disease.[30] Although TP53 overexpression was detected somewhat at 9% of LST-G adenoma and 6% of LST-NG adenoma in our previous IHC analysis,[9] *TP53* mutation was never detected by adenoma stage in the present study. This might be due to the difference between IHC and sequence analyses. While positivity by IHC was reported to correlate with the presence of *TP53* mutation,[33] it was also reported that IHC was not a reliable technique for detecting *TP53* mutation.[34] Indeed, our TP53 IHC showed good negative predictive value of *TP53* mutations, 92% (54/59), but rather low positive predictive value, 53% (18/34). Using exome sequence technique in this study, it was clearly demonstrated that *TP53* mutation is never involved at adenoma stage, but occurs in the early stages of cancer development from adenoma, in both LST-NG and LST-G.

TP53 mutation occurs at an earlier stage in LST-NG than in LST-G. While LST-G (=mostly low-methylation LST) showed *TP53* mutation in cancer with submucosal invasion, LST-NG (=mostly intermediate-methylation LST) accompanied with *TP53* mutation was already at the stage of intramucosal cancer. *TP53* is a key regulator gene reducing the risk of malignant transformation through apoptosis in cells with oncogenic activation, and *TP53* mutation can, thus, provide a proliferative advantage or an increased risk of cancer progression.[35] Although LST-NG are rather difficult to detect when using optical or virtual colonoscopy because of their smaller size and flat shape,[36] caution should be paid in resection of these lesions because of their risk of progression at the very early stage.

In summary, *APC* mutation is observed in most of adenoma and cancer samples with no significant difference at frequencies, indicating the involvement of *APC* mutation in tumor initiation in LST in both LST-G and LST-NG. *TP53* mutation occurs at an early stage of cancer development from adenoma for both LST-G and LST-NG, but is involved at an earlier stage in LST-NG.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Comparison of the methylation levels of each marker.

Fig. S2. Sequence coverage of targeted bases.

Fig. S3. Average number of somatic mutations per each sample.

Fig. S4. Pattern of mutation spectra.

Table S1. Primer sequences for pyrosequencing.

Table S2. List of mutation detected by targeted exon sequencing.