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ORIGINAL ARTICLE

Genetic alterations related to endoscopic treatment of colorectal tumors

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Key words
copy number alteration, early colorectal carcinoma, genetic mutation, magnifying endoscopy, next-generation sequencing.

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Abstract

Background and Aim: Genetic indicators of endoscopic resection for colorectal carcinoma remain inconclusive. This study analyzed genetic changes in early colorectal tumors that could inform decisions for endoscopic procedures.

Methods: A total of 83 colorectal tumors from 81 patients, including adenoma (n = 7), Tis–T1a (n = 22), T1b (n = 14), and advanced carcinoma (n = 40), were analyzed. Tis tumors (n = 16) and some T1 carcinomas (n = 11) were analyzed as mixed adenomas and carcinomas. Lesions were laser-capture microdissected for DNA extraction, and targeted sequencing of 50 cancer-related genes was performed. Genetic data were then correlated with clinical records, including magnifying endoscopic findings.

Results: Numbers of gene alteration rates in *TP53* and *SMAD4* increased with tumor progression from adenoma to carcinoma. Frequencies of mutant variants in *TP53* (P = 0.004) and rates of copy number loss in *SMAD4* (P = 0.006) increased in carcinoma components of mixed tumors compared to adenoma components. Moreover, adenoma components of T1b carcinomas had higher *TP53* mutation rates than Tis or T1a carcinomas (P = 0.011) and pure adenomas (P = 0.026). Gene alterations in *TP53* (P = 0.0055) and *SMAD4* (P = 0.0055) increased in cases with irregular surface patterns of magnifying endoscopic findings.

Conclusions: Numbers of copy number variations and *TP53* and *SMAD4* alterations were related to colorectal tumor progression. *TP53* alteration rates in adenoma components were high in T1b carcinomas, warranting complete treatment with en bloc resection. Magnifying endoscopic findings might reflect the genetic status of colorectal tumors.

Introduction

Colorectal cancers (CRCs) are the fourth and third leading global causes of cancer death in men and women, respectively,[1] and are the second leading cause of cancer death in Japan.[2] Surveillance by fecal occult blood[3] and sigmoidoscopy[4] can reduce CRC mortality rates, and early colorectal tumors can be resected endoscopically. Endoscopic resection of colorectal tumors is indicated for adenomas and early colorectal carcinomas with less than 1000-μm deep invasions into mucosal layers and differentiated carcinomas into submucosal layers, which have been shown to lack lymph node metastasis.[5–7] These indications are usually easily examined by narrow-band imaging (NBI) magnifying endoscopy,[8] and en bloc resection is generally the preferred treatment for CRCs because incomplete resection increases the rates of local recurrence and additional surgery, even when the histology of positive margins indicates adenoma.[9]

Although some CRCs develop directly from normal mucosa without adenoma components (de novo cancer), most arise from adenomas that develop into carcinomas after accumulating various gene alterations (adenoma–carcinoma sequences).[10] De novo cancer is characterized by *TP53* mutations and, rarely, *KRAS* mutations.[11,12] Mutations in *APC* and *KRAS* are associated with early tumor development and enlargement, as well as increased atypicality of adenoma–carcinoma sequences. Mutations in *TP53* and *SMAD4* occur during the transformation to carcinoma,[13–15] but *SMAD4* mutations reportedly occur late during cancer development and are related to malignant behaviors, such as metastasis, rather than transformation from adenoma to carcinoma.[16,17] These

conflicting data need to be resolved to better understand the nature of CRCs. In addition, genetic indications for endoscopic treatments of CRC remain poorly defined but could be used to inform treatments.

Recent advances in next-generation sequencing have enabled rapid and comprehensive gene sequencing, resulting in accumulated evidence of gene alterations in numerous tumors, including colorectal carcinoma.[18] Multiple gene mutations and copy number variations (CNVs) can be detected, simultaneously and with high sensitivity, using targeted deep sequencing, even with low amounts of DNA from clinical samples such as formalin-fixed paraffin-embedded (FFPE) tissues.[19,20] Heterogeneous tumor samples can also be cut separately using laser-capture microdissection (LCM), allowing DNA from adenoma and carcinoma components of the same colorectal tumor to be isolated from each other. These methods enable analyses of tumor heterogeneity and clarification of accumulated genetic changes during tumor development.

In this study, we conducted targeted deep sequencing of colorectal tumors and analyzed accumulations of mutations during tumor development, especially during early stages for which endoscopic treatments are appropriate. Furthermore, we analyzed genetic changes upon the transition from adenoma to carcinoma using LCM and subsequent targeted deep sequencing.

Methods

Patients and treatments. We retrospectively reviewed the medical records of 81 patients with 83 tumors who received endoscopic treatments or surgical resections of colorectal tumors at Yamanashi University Hospital between February 2009 and August 2016. Endoscopic treatments were conducted with endoscopic mucosal resection (EMR)[21] or with endoscopic submucosal dissection (ESD)[22] as described previously. CRCs at stages of T2 or more and those of the T1 stage with invasion depths of more than 1000 μm were resected in en bloc surgical operations. We conducted a magnifying endoscopy with NBI before treatment in 19 cases with adenoma or early CRCs and classified magnifying endoscopic findings into "Type 2A," "Type 2B," and "Type 3" based on the Japan NBI Expert Team (JNET) classification.[23] JNET "Type 2B" findings were further classified into two groups depending on the presence or absence of surface irregularity. This study was approved by the Human Ethics Review Committee of Yamanashi University Hospital (Receipt number: 921 and 1326).

Histological assessment and DNA extraction. Histological diagnoses were made by an experienced pathologist using HE-stained slides. Tumor depths were defined according to the TNM Classification of Malignant Tumors published in 2010 as follows: Tis, carcinoma in situ (M); T1, tumor invades submucosa (SM); and T2–4, tumor invades beyond SM. In addition, T1 tumors were subclassified according to submucosal invasion depths of less than (T1a) or greater than or equal to 1000 μm (T1b) according to the Japanese Society for Cancer of the Colon and Rectum Guidelines 2016.[24]

Mixed tumors of adenoma and carcinoma were carefully assessed by a pathologist (Kunio Mochizuki) who specializes in gastroenterology, and the adenoma and carcinoma components were separated by LCM using an ArcturusXT Laser Capture Microdissection System (Life Technologies, Carlsbad, CA, USA) from 8-μm-thick sections of FFPE samples. DNA extraction from LCM specimens was performed as previously reported.[19] DNA from biopsied specimens was extracted using GeneRead DNA FFPE Kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Quantities and qualities of extracted DNA were assessed using a NanoDrop (Thermo Fisher, Waltham, MA, USA) instrument with the Qubit (Thermo Fisher) platform.

Genetic mutational analysis of colorectal tumor samples. Genetic analyses of tumor specimens were performed as described previously.[19,20] Briefly, extracted DNA (10 ng) was amplified using barcode adaptors (Ion Xpress Barcode Adapters 1-96 Kit, Life Technologies) with the Ion AmpliSeq Cancer Hotspot panel v.2 (Thermo Fisher), which contains 207 primer pairs and targets approximately 2800 hotspot mutations in the following 50 cancer-related genes from the COSMIC database[25]: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *HNF1A*, *HRAS*, *IDH1*, *JAK2*, *JAK3*, *IDH2*, *KDR/VEGFR2*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*,

Table 1 Patient characteristics

	Adenoma	Early cancer		Advanced cancer
		Tis–T1a	T1b	T2–T4
	(*n* = 7)	(*n* = 22)	(*n* = 14)	(*n* = 40)
Age, median (range)	59 (43–80)	71 (35–82)	68.5 (44–82)	67.5 (46–87)
Gender				
Male	4	12	9	24
Female	3	10	5	16
Location				
C/A	0	1	3	11
T	3	3	2	3
D	1	3	1	2
S	2	6	2	11
R	1	9	6	13
Size (mm), median (range)	13 (7–22)	15.5 (8–58)	22.5 (10–45)	NA
Type				
0-Ip, 0-Isp, 0-Is, 0-IIa	5	16	7	—
0-IIc, 0-IIa+IIc	0	0	0	—
LST-G	2	4	4	—
LST-NG	0	2	3	—
Histological differentiation				
Well-differentiated	—	22	13	39
Un-differentiated	—	0	1	1

C/A, cecum and ascending colon; D, descending; LST-G, granular type of laterally spreading tumor; LST-NG, nongranular type of laterally spreading tumor; NA, not available; R, rectum; S, sigmoid; T, transverse.

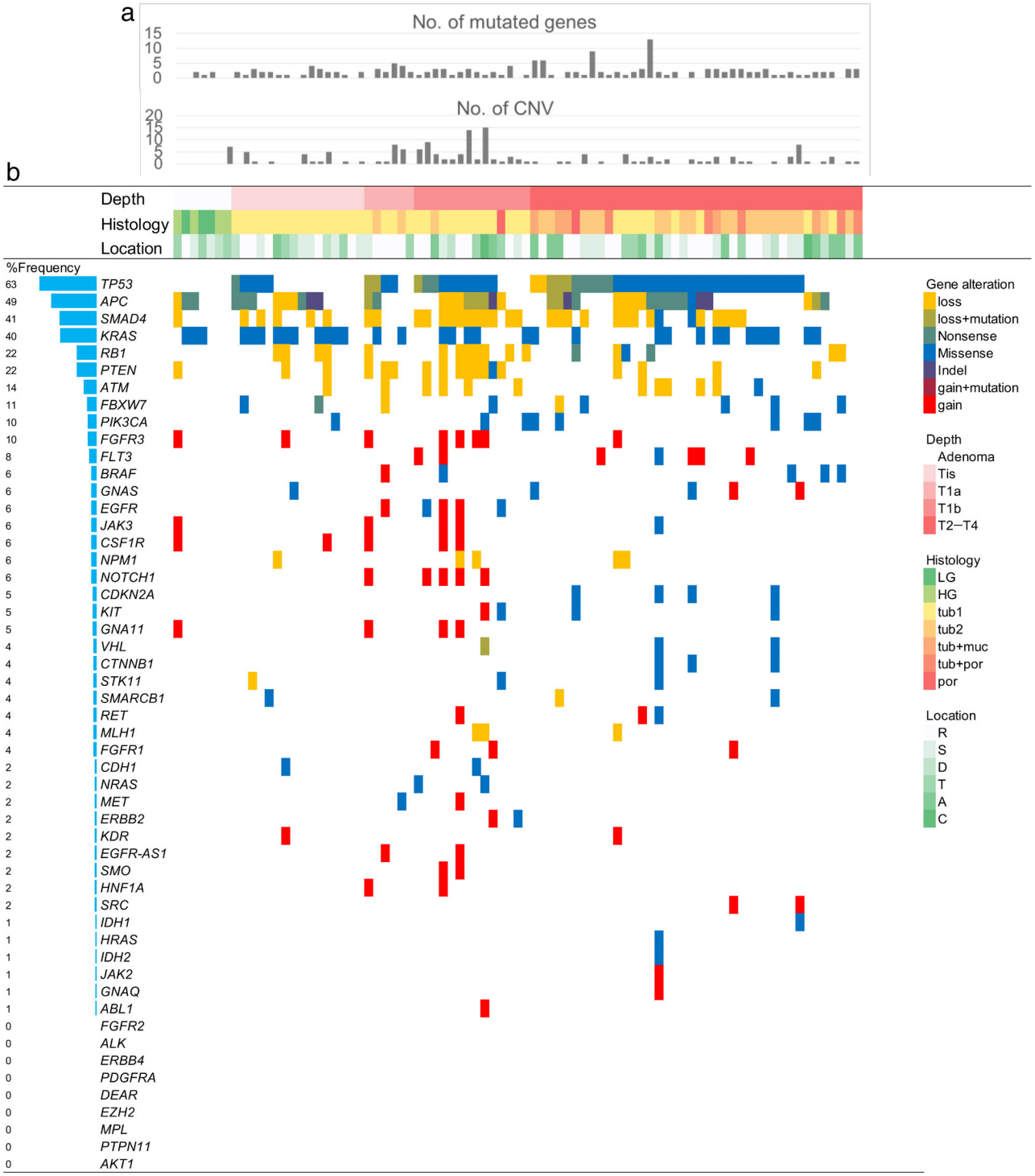

Figure 1 Genomic landscape of colorectal adenomas and carcinomas. (a) Numbers of mutated genes and CNVs per case; (b) overview of genetic changes in all cases; the columns in the central panel represent the gene status of one patient, and the left panel shows the percentage of cases with each gene alteration. The upper panel shows clinical information for each patient. Color indicators are defined in the right part of the figure. A, ascending colon; C, cecum; CNV, copy number variation; D, descending; HG, high-grade dysplasia; LG, low-grade dysplasia; muc, mucinous; por, poorly differentiated; R, rectum; S, sigmoid; T, transverse; tub, tubular.

SMAD4, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL*. Barcoded libraries were amplified using emulsion polymerase chain reaction on Ion Sphere particles, and sequencing was performed on an Ion Chef System and an Ion Proton Sequencer (Life Technologies) using an Ion PI Hi-Q Chef Kit (Life Technologies) in accordance with the manufacturer's instructions. Variants were identified using Ion Reporter software version 5.10 (Thermo Fisher), and to avoid false positive variants due to sequencing errors, only those with a variant frequency of >2% (with a sequence read depth of >100) were considered true variants.

Statistical analysis. Associations between mutations and clinical variables were evaluated using χ^2, Fisher's exact, Cochran–Armitage trend, Steel–Dwass, or Wilcoxon signed rank tests and were considered significant when $P < 0.05$. All statistical analyses of recorded data were performed using the Excel statistical software package (Ekuseru-Toukei 2012; Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Patient characteristics and qualitative assessments of extracted DNA. A total of 81 patients with 83 colorectal tumors were enrolled in the study, and their clinical characteristics were recorded (Table 1). Among subjects, 7 had adenomas, and 22, 14, and 40 had Tis–T1a carcinoma, T1b carcinoma, and advanced carcinoma, respectively.

Median quantities and concentrations of extracted DNA were 110 ng (range, 4.9–618 ng) and 3.7 ng/μL (range, 0.16–20.6), respectively. Target regions of 50 cancer-related genes included 22 027 bases; the average (±standard deviation) sequenced read depth was 5088 (±16 079), and its median (range) was 3305 (8–232 224).

Genetic landscape of colorectal tumors and numbers of gene mutations. In studies of the reported 50 cancer-related genes, two cases had more than eight mutations per sample of advanced colorectal carcinoma tissues (Fig. 1a). Numbers of CNVs per sample are shown in the bar graph in Figure 1a. The four most frequent mutations were observed in *TP53* (63%), *APC* (49%), *SMAD4* (41%), and *KRAS* (40%) genes, followed by those in *RB1* (22%) and *PTEN* (22%; Fig. 1b).

Through further analyses, we determined relationships between numbers of gene alterations and various clinical variables. Numbers of CNVs in T1b colorectal carcinomas were greater than in Tis–T1a carcinomas ($P = 0.010$) and adenomas ($P = 0.016$), and mutations tended to increase with tumor progression, although there was no statistical significance (Fig. 2a,b).

Associations between tumor depths and genetic alterations. Because *APC*, *KRAS*, *TP53*, and *SMAD4* have been identified as major contributors to the adenoma–carcinoma sequence of colorectal tumors, we investigated changes in the frequencies of these genes with tumor progression. Gene alteration rates in *TP53* increased with progression ($P < 0.001$), and those in *SMAD4* increased from adenoma to T1b carcinoma stages ($P = 0.002$), whereas those in *KRAS* and *APC* genes did not differ between tumor stages (Fig. 3a). Because colorectal carcinomas are treated after predicting invasion depth by magnifying endoscopy, we further analyzed the association between magnifying endoscopic findings of adenoma and early carcinoma and their gene alterations, especially in *TP53* and *SMAD4*, which were related to tumor progression (Fig. 3b). The gene alterations in *TP53* (Fig. 3c, $P = 0.0055$) and *SMAD4* (Fig. 3d, $P = 0.0055$) increased in cases with irregular surface patterns of magnifying endoscopic finding, suggesting that endoscopic findings reflect the genetic status of colorectal tumors at least in part.

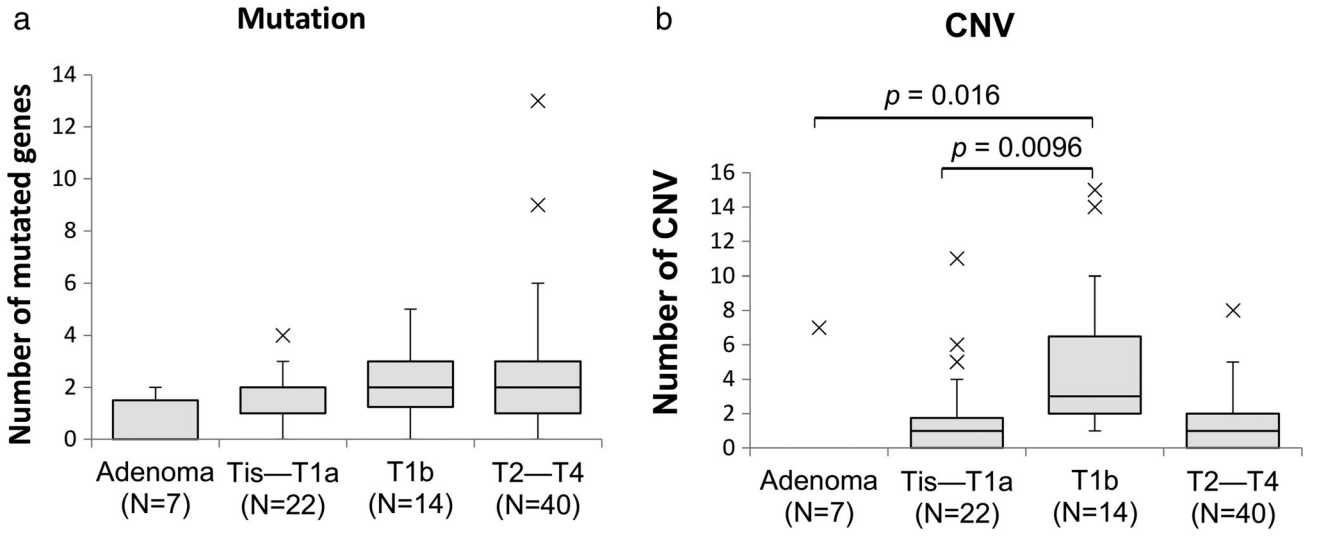

Figure 2 Analyses of numbers of mutations and CNVs. The number of mutations did not increase with tumor progression (a), whereas that of CNVs increased significantly from adenoma to T1b stage carcinoma (b). CNVs, copy number variations (Steel–Dwass test).

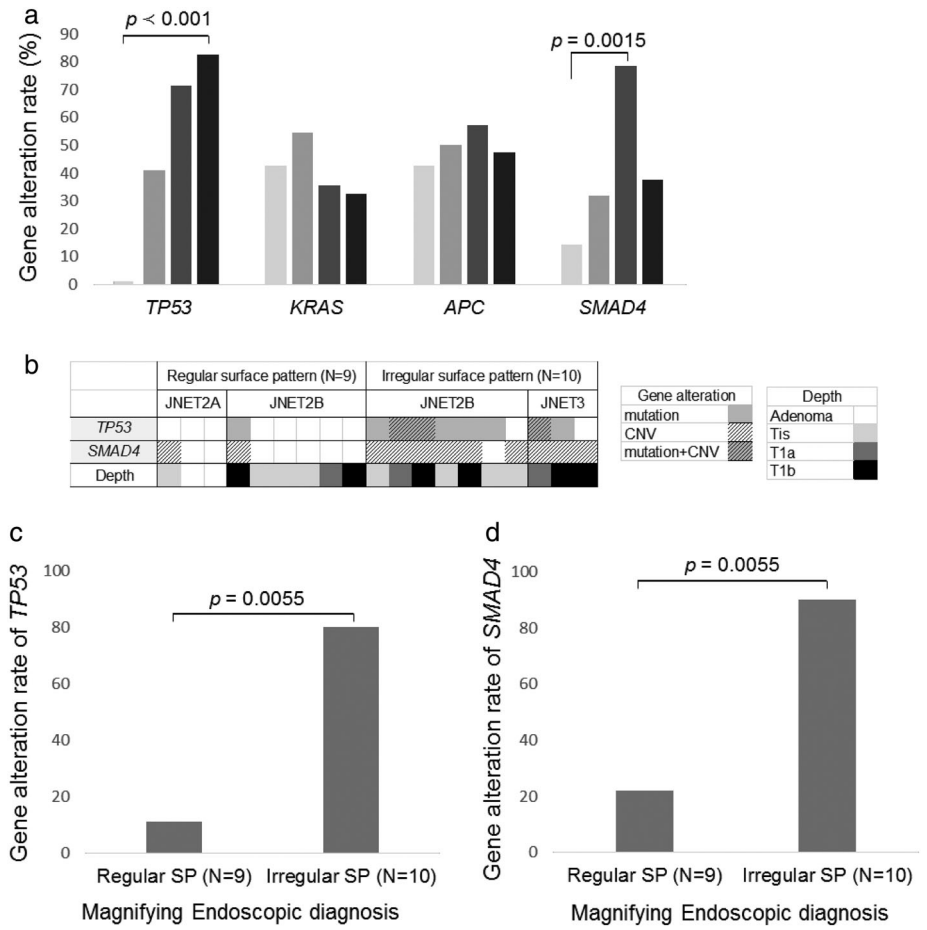

Figure 3 Accumulation of alterations in major genes with tumor progression. Alteration rates in *TP53* and *SMAD4* genes increased significantly with tumor progression (Cochran–Armitage test), whereas *KRAS* and *APC* alteration rates did not (a). The relation between magnifying endoscopic findings and gene alterations are shown. Magnifying endoscopic findings were classified according to the JNET classification, and "Type 2B" findings were further classified depending on the presence and absence of surface irregularity (b). The gene alteration rates in *TP53* (c) and *SMAD4* (d) were stratified with surface irregularity by magnifying endoscopic findings (Fisher's exact test). CNVs, copy number variations; JNET, Japan NBI Expert Team; SP, surface pattern.

Associations between genetic alterations in adenoma and carcinoma components of the same tumor. Although *TP53* gene alteration rates were previously compared between adenoma and carcinoma components in mixed tumors,[26] no analyses of mutant allele frequencies of *TP53* or copy number changes of *SMAD4* have been reported between adenoma and carcinoma components of mixed tumors. Therefore, we analyzed mutant allele frequencies of *TP53* and copy number changes of *SMAD4* between adenoma and carcinoma components of the same mixed tumor after precisely separating the tissues using LCM (Fig. 4a). Among 27 tumor tissues that had both adenoma and carcinoma components, variant frequencies of *TP53* mutations were greater ($P = 0.004$) and copy numbers of *SMAD4* were fewer ($P = 0.006$) in carcinomas than in adenomas. Finally, we analyzed gene mutation rates of pure adenomas and adenoma components from a mixed tumor and found higher rates of *TP53* mutation in adenoma components from T1b carcinomas than in those from pure adenomas ($P = 0.026$) and adenoma components from Tis or T1b carcinomas ($P = 0.011$).

Discussion

In this study, we performed targeted deep sequencing of 50 cancer-related genes in 83 colorectal tumors and found that genetic alterations, especially in *TP53* and *SMAD4*, accumulate during tumor progression. Numbers of CNV also increased with tumor progression, especially in tissues with *TP53* mutations. In unprecedented experiments, we identified *SMAD4* mutations in carcinoma components of mixed tumors using precisely separated tissue specimens, and we showed that adenoma components of T1 carcinomas possessed high numbers of mutated genes. Finally, the irregular surface pattern of tumors by magnifying endoscopy was related to the existence of gene alterations in *TP53* and *SMAD4*. These observations have important clinical

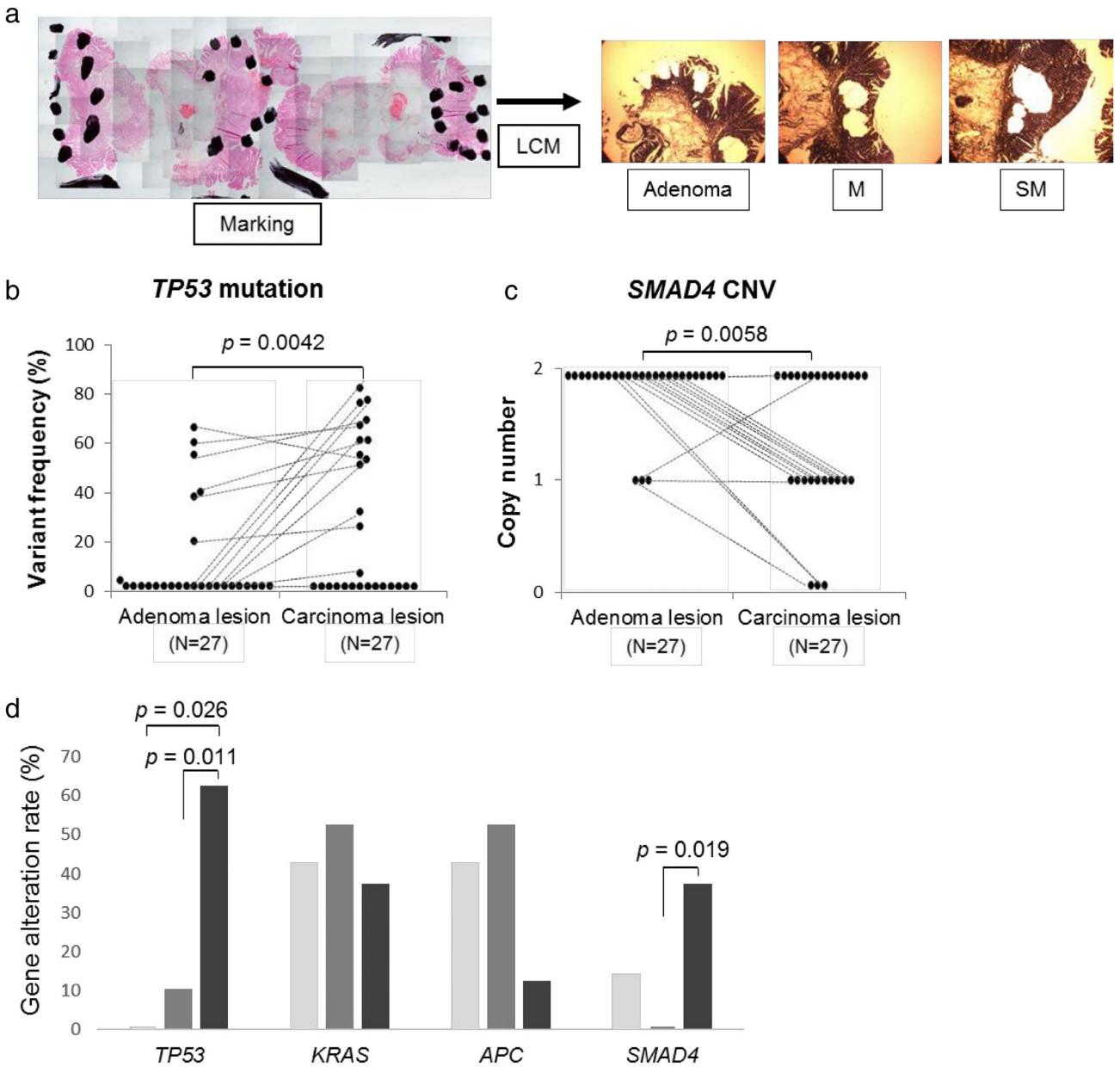

Figure 4 Comparison of genetic status between adenoma and carcinoma components of a mixed tumor. Adenoma components and adjacent carcinomas of colorectal tumors were stained on slides with hematoxylin and eosin by an experienced pathologist. Tissue components were precisely separated using LCM (a). Mutant variant frequencies of *TP53* in carcinoma components were higher than those in adenoma components (b), and there were fewer copy numbers of *SMAD4* in carcinoma components than in adenoma components (c, Wilcoxon signed-rank test). (d) Gene alteration rates in the adenoma component of a mixed tumor; among four major genes, *TP53* alteration rates in adenoma components of T1b carcinomas were higher than in adenoma components of Tis–T1a carcinomas or pure adenomas (Fisher's exact test). LCM, laser-capture microdissection; SM, submucosa.

implications relating to the use of en bloc surgery or endoscopic resection.

Recent advances in next-generation sequencing have demonstrated comprehensive gene profiles of numerous cancers, including colorectal carcinomas, which have been shown to have frequent gene mutations in *APC*, *TP53*, *KRAS*, *TTN*, *SYNE1*, and *PIK3CA* and CNVs in *BCL2L1*, *POFUT1*, *ASXL1*, *DNMT3B*, and *SEMG1*.[18,27] *APC*, *TP53*, *KRAS*, *PIK3CA*, *FBXW7*, and *SMAD4* are reportedly the most frequently affected genes in CRCs,[18,27] and we observed similar tendencies of mutation rates in these genes. Most colorectal carcinomas follow an adenoma–carcinoma sequence in which mutations in *APC* and *KRAS* occur early and are associated with tumor development, enlargement, and increased atypicality, and *TP53* and *SMAD4* mutations occur

during transformation into carcinoma.[13–15] In accordance with this oncogenic pathway, our data show similar mutation rates in *APC* and *KRAS* genes during tumor progression and increased *TP53* and *SMAD4* mutation rates with progression of carcinoma. We also demonstrate relationships between numbers of mutations and CNVs and tumor progression. Specifically, numbers of mutations were stable after initial tumor progression into carcinoma, whereas those of CNVs increased during tumor progression from carcinoma, suggesting that numbers of CNV increase after sufficient accumulation of various gene mutations. Although recent reports show associations between mutations and numbers of CNVs in stage II and III colon cancers, suggesting that mutations in *APC* or *TP53* are associated with increased CNVs,[28] these tendencies were observed with *TP53* mutations ($P = 0.017$) but not with *APC* mutations ($P = 0.972$) in our study. We could conclude that *TP53* mutations in adenoma or early carcinoma lead to increased numbers of CNVs.

Here, we report the first comparison of gene status between adenoma and carcinoma components of a mixed tumor and show that *SMAD4* mutations occurred during carcinogenesis from adenoma instead of occurring in advanced cancers. *SMAD4* encodes the Smad4 protein, which is an intracellular signaling mediator of the transforming growth factor-β (TGF-β) superfamily of cytokines[29,30] and was originally identified as a tumor suppressor gene (deleted in pancreatic cancer, locus 4 [DPC4]) that was functionally inactivated in one-half of a pancreatic adenocarcinoma.[31] In CRC patients, *SMAD4* alterations in advanced stage cancers reportedly contributed to tumor metastasis.[16,17] Other studies show that *SMAD4* mutations contribute to the enlargement of adenomas and promote carcinogenesis.[32] In our study, *SMAD4* alterations occurred during carcinogenesis, but the frequency of *SMAD4* alterations was not high in advanced CRCs. We also associated *SMAD4* status with overall survival in advanced CRC patients but found no significant differences between groups with and without *SMAD4* alterations (data not shown).

TP53 mutations occurred during progression from adenoma to carcinoma, with increases in mutation rates and variant frequencies. *TP53* is also a tumor suppressor gene that encodes functional tetramers of the p53 protein. Mutations in *TP53* inhibit tetramerization of p53 and thus block tumor suppressor functions. Although the clinical implications of these data may depend on the tissue of origin, *TP53* mutations have been found in many cancers, with an overall prevalence of 42%.[33] In a previous study, *TP53* mutations occurred during progression from adenoma to carcinoma in mixed tumors of adenoma and carcinoma.[26] However, we showed, for the first time, that mutant variant frequencies of *TP53* increased during progression from adenoma to carcinoma in mixed tumors and that *TP53* mutation rates increased with further progression. Moreover, *TP53* alterations were significantly associated with poor prognosis among our patients with advanced CRCs, whereas *SMAD4* alterations were not significantly associated (data not shown).

Multiple clinical implications arise from the present study. First, adenoma components of mixed tumors with submucosal invasions, especially T1b carcinomas, had higher frequencies of gene alterations than mixed tumors with Tis–T1a carcinomas or adenomas alone, suggesting that adenomas with invasive submucosal carcinomas (T1b) have the potential to progress rapidly toward carcinoma. These findings were also supported by a previous report which described that aberrant p53 and SMAD4 were all increased in the adenoma fractions of carcinoma cases compared with pure adenomas.[34] Therefore, EMR, which is sometimes incomplete and has been related to frequent local recurrences,[9] should be avoided, especially if the tumor has invaded the submucosal layer. Second, mutations and CNVs increased in submucosal invasions with depths of greater than 1000 μm. Because this depth is considered the adaptive limit for endoscopic therapy, further increases in mutations and CNVs might be related to lymph node metastasis.[6]

This study is the first report to show the relationship between magnifying endoscopic findings and gene alterations. JNET developed the NBI magnifying endoscopic classification of colorectal tumors, which consists of four categories (i.e. Types 1, 2A, 2B, and 3) according to irregular vessel and surface patterns.[8] JNET Types 1, 2A, 2B, and 3 correspond with hyperplastic polyp, adenoma–Tis, Tis–T1a, and T1b–advanced cancer, respectively. We further divided Type 2B into two groups according to the presence and absence of surface irregularity and analyzed the relation between genetic alterations and surface irregularities of magnifying endoscopic findings. Surprisingly, surface irregularities of magnifying endoscopic findings were more closely related to genetic alterations in *TP53* and *SMAD4* than histopathological depth, suggesting that the surface pattern of magnifying endoscopic findings reflect the genetic status of colorectal tumors. Although the pit pattern classification,[35] another classification of magnifying endoscopy, may reflect genetic status more precisely due to its more detailed representation of the surface pattern, we could not assess this classification due to lack of adequate cases.

This study has several limitations. First, the design is retrospective, and only a small number of cases were recruited from a single center. Second, the *SMAD4* alteration rates in advanced carcinoma were lower than in less advanced tumors. Potentially, most alterations in *SMAD4* were CNVs that could not be detected in advanced tumors because they are abundant in stromal and inflammatory cells and have low tumor cellularity. Third, we should discuss pathological and molecular subtypes of colorectal tumors[14,36] because certain subtype gathers attention for use of newly developed immunotherapy. We could not classify subtypes well in this study because it was not possible to pursue the genetic status of microsatellite instability or mismatch repair deficiency with our 50-gene analysis.

In conclusion, we performed a comprehensive genetic analysis of colorectal adenomas and carcinomas and found evidence that could be used to inform endoscopic treatments of colorectal tumors. Furthermore, we showed that magnifying endoscopic findings might reflect the genetic status of colorectal tumors. We believe that these findings will help improve future decisions to perform endoscopic treatments.

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