Online Submissions: wjg.wjgnet.com
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2007 August 7; 13(29): 3932-3938
World Journal of Gastroenterology ISSN 1007-9327

COLORECTAL CANCER

Involvement of Krüppel-like factor 6 (*KLF6*) mutation in the development of nonpolypoid colorectal carcinoma

Shinichi Mukai, Toru Hiyama, Shinji Tanaka, Masaharu Yoshihara, Koji Arihiro, Kazuaki Chayama

Shinichi Mukai, Kazuaki Chayama, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Program for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
Toru Hiyama, Masaharu Yoshihara, Health Service Center, Hiroshima University, Higashihiroshima, Japan
Shinji Tanaka, Department of Endoscopy, Hiroshima University Hospital, Hiroshima, Japan
Koji Arihiro, Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan
Supported in part by a Grant from the Japanese Society of Gastrointestinal Endoscopy, Chugoku Branch
Correspondence to: Dr. Shinji Tanaka, Department of Endoscopy, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. colon@hiroshima-u.ac.jp
Telephone: +81-82-2575538 Fax: +81-82-2575538
Received: 2007-01-16 Accepted: 2007-04-04

Abstract

AIM: To examine *Krüppel-like factor 6* (*KLF6*) mutations in nonpolypoid-type tumors and alterations of K-*ras*, *p53*, and B-*raf* in relation between mutation and morphologic type, particularly nonpolypoid-type colorectal carcinomas.

METHODS: Fifty-five early nonpolypoid colorectal carcinomas were analyzed. Loss of heterozygosity (LOH) of *KLF6* and *p53* was determined by microsatellite assay. Mutations of *KLF6*, K-*ras*, and B-*raf* were examined by polymerase chain reaction-single-strand conformation polymorphism followed by direct sequencing. In LOH-positive and/or mutation-positive tumors, multiple (4-7) samples in each tumor were microdissected and examined for genetic alterations. *p53* expression was evaluated by immunohistochemistry.

RESULTS: LOH of *KLF6* and *p53* was found in 14 of 29 (48.3%) and 14 of 31 (45.2%) tumors, respectively. In 10 of the 14 (71.4%) *KLF6* LOH-positive tumors and 9 of the 14 (64.3%) *p53* LOH-positive tumors, LOH was found in all of the microdissected samples. In 1 of the 10 (10.0%) *KLF6* LOH-positive tumors, a single missense mutation was identified. K-*ras* and B-*raf* mutations were found in 5 of 55 (9.1%) and 6 of 55 (10.9%) tumors, respectively. However, these mutations were detected only in subsets of microdissected tumor samples.

CONCLUSION: These data suggest that *KLF6* and *p53* mutations are involved in the development of nonpolypoid colorectal carcinoma, whereas K-*ras* and B-*raf* mutations are not.

Key words: Nonpolypoid colorectal carcinoma; *KLF6*; *p53*; K-*ras*; B-*raf*

Mukai S, Hiyama T, Tanaka S, Yoshihara M, Arihiro K, Chayama K. Involvement of Krüppel-like factor 6 (*KLF6*) mutation in the development of nonpolypoid colorectal carcinoma. *World J Gastroenterol* 2007; 13(29): 3932-3938

http://www.wjgnet.com/1007-9327/13/3932.asp

INTRODUCTION

Two distinct types of colorectal carcinomas have been described, polypoid and nonpolypoid[1-4]. The concept of a precursor lesion led to the adenoma-carcinoma sequence hypothesis, based on the polypoid type, whereby a benign adenoma progresses to malignant adenocarcinoma. Nonpolypoid-type colorectal carcinoma was first described in detail by Muto *et al*[5]. These lesions show subtle morphologic differentiation and are often undetectable, posing diagnostic problems. Nonpolypoid-type tumors frequently invade the submucosal layer, even though they are less than 10 mm in diameter[6]. Because of the high malignancy potential of nonpolypoid-type tumors, these are considered important lesions. Nonpolypoid-type carcinomas are thought to be *de novo* tumors that develop directly from normal colorectal mucosa.

It has been reported that K-*ras* mutations and loss of heterozygosity (LOH) at chromosome 3p are more frequent in early polypoid colorectal carcinomas than in nonpolypoid-type tumors[2,3]. In addition, *APC* mutations are less frequent in nonpolypoid-type tumors than in polypoid-type tumors[2]. Thus, these two types of colorectal tumors may possess different genetic backgrounds.

Krüppel-like factors (KLFs) are core transcription factors that regulate numerous mammalian genes[7]. The *KLF6* gene encodes one of these transcription factors, which has been identified as a tumor-suppressor gene involved in the regulation of cell proliferation and differentiation[7]. *KLF6* mediates the inhibition of proliferation by upregulating the cell-cycle inhibitor CDKN1A ($p21^{WAF1/CIP1}$) through an interaction with cyclin D1 in a *p53*-independent manner[8]. Recently, a high frequency (44%) of *KLF6* somatic mutations has been reported in colorectal cancers[9]. However, the relation between these genetic changes and morphologic type,

particularly nonpolypoid type, is unclear. In this study, examined *KLF6* mutations in early nonpolypoid-type tumors. We also examined alterations of K-*ras*, *p53*, and B-*raf* genes. In addition, we examined genetic alterations in multiple samples from a given tumor obtained by microdissection.

MATERIALS AND METHODS

Tumor samples

Samples from early nonpolypoid colorectal tumors resected surgically or endoscopically were collected at Hiroshima University Hospital (Hiroshima, Japan) during the period 2000 to 2002. Nonpolypoid-type colorectal carcinomas were diagnosed according to the criteria of Shimoda *et al*[10]. In brief, nonpolypoid-type tumors were tumors without intramucosal protuberant growth. Ten samples were mucosal adenocarcinomas, and 45 were adenocarcinomas that had invaded the submucosal layer. The present study was approved by the local ethical committee (No. I-Rin-Hi-107).

Histologic examination

Tissue sections (4-μm thick) were prepared from formalin-fixed, paraffin-embedded colorectal tissues. The sections were stained with hematoxylin and eosin (HE) for histologic examination. Depth of invasion was classified as intramucosal (m) or submucosal (sm).

DNA extraction

Tissue sections (10-μm thick) were stained with HE, dehydrated in a graded ethanol series, and then dried without a cover glass. Tissue samples from 55 tumors and corresponding normal tissues were cut with sterile needles, and the DNA was extracted with 20 μL extraction buffer (100 mmol/L Tris-HCl, pH 8.0, 2 mmol/L EDTA, 400 μg/mL proteinase K) at 55℃ overnight. The tubes were boiled for 5 min to inactivate proteinase K, and 1-2 μL of each extract was used for polymerase chain reaction (PCR) amplification.

LOH analysis of KLF6 and p53

LOH analysis of *KLF6* and *p53* was performed by microsatellite assay. Primer pairs specific for microsatellites *KLF6* (D10S591 and D10S594) and *p53* (TP53) are shown in Table 1[11,12]. The microsatellite assay was performed as described previously[12]. In brief, each 15-μL reaction mixture containing 10-20 ng genomic DNA, 6.7 mmol/L Tris-HCl, pH 8.8, 6.7 mmol/L EDTA, 6.7 mmol/L $MgCl_2$, 0.33 μmol/L primer labeled with [γ^{32}-P]dATP, 0.175 μmol/L unlabeled primer, 1.5 mmol/L of each deoxynucleotide triphosphate, and 0.75 units AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ) was amplified with 40 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and elongation at 72℃ for 30 s. PCR products were separated by electrophoresis on 6% polyacrylamide-8 M urea-32% formamide gels and subjected to autoradiography overnight at -80℃ on Fuji RX film. LOH was identified when only one major band was detected in DNA isolated from cancerous tissue; two major bands were present in the normal tissue specimen from the same sample.

Mutational screening and direct sequencing of KLF6, K-ras, and B-raf

DNA samples were screened for mutations of *KLF6*, K-*ras*, and B-*raf* by PCR-single-strand conformation polymorphism (SSCP) analysis. The PCR primers were designed to amplify the exons including mutational hot spots of *KLF6*, K-*ras*, and B-*raf* and are listed in Table 1[11,13,14]. PCR-SSCP analysis was performed as described previously[12]. In brief, each 25 μL reaction mixture contained 1 × AmpliTaq Gold Buffer (8.0 mmol/L Tris-HCl, pH 8.3, 40 mmol/L KCl; Perkin-Elmer), 4 mmol/L $MgCl_2$, 0.3 mmol/L of each deoxynucleotide triphosphate, 100 pmol of each primer, 10-20 ng genomic DNA, 2.5 mCi [γ^{32}-P]dCTP (3000 Ci/mmol/L, 10 mCi/mL), and 1.25 U AmpliTaq Gold DNA polymerase. The reaction mixtures were heated to 95℃ for 10 min, followed by 45 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 2 min, and strand elongation at 72℃ for 2 min. After PCR, the samples were electrophoresed on 6% polyacrylamide gels (ratio of acrylamide:bis-acrylamide, 19:1) with 10% glycerol at 4℃. The gels were then subjected to autoradiography overnight at -80℃.

To confirm *KLF6*, K-*ras*, and B-*raf* mutations, direct sequencing was performed as described previously[15]. The aberrantly migrating band on the SSCP gel was removed, reamplified, and directly sequenced on both strands with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA). For the sequencing reaction, a PRISM AmpliTaq DNA polymerase FS Ready Reaction Dye Terminator Sequencing Kit (Perkin-Elmer Applied Biosystems) was used.

Microdissection

For mutation- or LOH-positive tumors, multiple (4-7) samples of each tumor were cut out by microdissection with sterile needles. DNA was then extracted and examined for mutations of *KLF6*, K-*ras*, and B-*raf* and for LOH of *KLF6* and *p53*.

Immunohistochemistry

p53 expression was evaluated by immunohistochemistry (IHC) using a monoclonal antibody (DO7, diluted 1:50; Novocastra, Newcastle, United Kingdom). Immunohistochemical staining was performed with the labeled streptavidin-biotin method (LSAB kit; Dako, Denmark). After deparaffinization and rehydration, the tissue sections were subjected to microwave treatment for 15 min. Endogenous peroxidase activity was blocked with hydrogen peroxide, and sections were incubated with goat serum at room temperature for 20 min to block nonspecific binding. Primary antibody was applied overnight at 4℃. This was followed by incubation with biotinylated secondary antibody for 20 min and then with a streptavidin-biotin-peroxidase complex reagent for 20 min at room temperature. The sections were washed thoroughly with phosphate-buffered saline between incubation steps. Diaminobenzidine tetrahydrochloride

Table 1 PCR primer sets

Priming region	Primer sequence
Loss of heterozygosity analysis	
p53 (TP53)	5′-AGGGATACTATTCAGCCCGAGGTG-3′
	5′-ACTGCCACTCCTTGCCCCATTC-3′
KLF6 (D10S591)	5′-ACCTCGAAGGTCTGTTCTCC-3′
	5′-GGCTTTATGGATCATATTAATCCAC-3′
KLF6 (D10S594)	5′-GGGCAGCGTTGCTGAGA-3′
	5′-GCACCCAGATAGGCATAGAGA-3′
Mutation analysis	
K-*ras* exon 1:	5′-GGCCTGCTGAAAATGACTGA-3′
	5′-GGTGCAGGACCATTCTTTGAT-3′
B-*raf* exon 11:	5′-AAACACTTGGTAGACGGGAC-3′
	5′-AATGTGGTGACATTGTGACAAGT-3′
B-*raf* exon 15:	5′-CTTCATGAAGACCTCACAGT-3′
	5′-TCCACTGATTAAATTTTTGGCC-3′
KLF6 exon 1:	5′-GCCTCGCCGCCCTCGC-3′
	5′-AACTCCAAACAGCCGACCC-3′
exon 2:	5′-CAATCACGTGCCTTCTCTGG-3′
	5′-GAGAAAGTGAGGATTTGTCTG-3′
exon 3	5′-GTCTGCGGGTCAGTGAAGTC-3′
	5′-GTCATCACATTCCCAAGGCC-3′

was used as the chromogen, and sections were counterstained with hematoxylin. Cases with sporadic or no *p53* staining were considered negative, whereas focal or diffuse immunoreactivity was considered positive. Immunohistochemical evaluations were performed without any knowledge of clinicopathologic data.

Statistical analysis

Chi-square and Fischer's exact probability tests were used for comparisons of clinicopathologic and genetic factors. $P < 0.05$ was regarded as significant.

RESULTS

Clinicopathologic features of the 55 colorectal tumors are shown in Table 2. We first examined genetic alterations in tissue samples without microdissection. Individual data and summary of genetic analyses are shown in Tables 3 and 4, respectively. LOH of *KLF6* was found in 14 of 29 (48.3%) nonpolypoid tumors, including 2 of 6 (33.3%) mucosal tumors and 12 of 23 (52.2%) submucosal tumors. Mutations of *KLF6* were detected in 2 of 34 (5.9%) tumors, including none of 6 (0%) mucosal tumors and 2 of 34 (5.9%) submucosal tumors. LOH of *p53* was found in 14 of 31 (45.2%) tumors, including 4 of 8 (50.0%) mucosal tumors and 10 of 23 (43.5%) submucosal tumors. *p53* overexpression was found in 19 of 55 (34.5%) tumors, including 2 of 10 (20.0%) mucosal tumors and 17 of 45 (37.8%) submucosal tumors. K-*ras* mutations were found in 5 of 55 (9.1%) tumors, including 1 of 10 (10.0%) mucosal tumors and 4 of 45 (8.9%) submucosal tumors. All of the detected mutations were G13C. B-*raf* mutations were found in 6 of 55 (10.9%) tumors, including 1 of 10 (10.0%) mucosal tumors and 5 of 45 (11.1%) submucosal tumors. Three of the 6 B-*raf* mutations were A560C, and the others were A608C, A396T, and G378A. Two samples showed both K-*ras* and B-*raf* mutations. In each genetic alteration, there were no significant difference between the frequency in mucosal carcinomas and that in submucosal carcinomas. There were no significant associations between genetic alterations and clinicopathologic characteristics including patients' sex, tumor location, histology and depth.

Table 2 Summary of clinicopathologic features of 55 colorectal tumors

Characteristics	Number (%)
Sex	
Male	39 (71)
Female	16 (29)
Tumor location[1]	
Right-sided	20 (36)
Left-sided	35 (64)
Tumor histology[2]	
Well-differentiated adenocarcinoma	46 (84)
Moderately differentiated adenocarcinoma	9 (16)
Tumor depth	
Mucosal	10 (18)
Submucosal	45 (82)

[1]Right-sided: from the cecum to the transverse colon; Left-sided: from the splenic flexure to the rectum. [2]According to the Japanese Classification of Colorectal Carcinoma.

In mutation- or LOH-positive tumors, we next examined genetic alterations by microdissection. In the 10 of the 14 (71.4%) *KLF6* LOH-positive tumors, LOH was found in all of the microdissected tumor samples. In 1 of 14 *KLF6* LOH-positive tumors, case 22, a missense mutation (exon 3, codon 735, CTG to GCT) was detected (Figure 1). In 9 of the 14 (64.3%) *p53* LOH-positive tumors, LOH was found in all of the microdissected tumor samples (Figure 2). In both of the K-*ras* and B-*raf* mutation-positive cases, the mutations were detected in only a subset of microdissected tumor samples (Figure 3).

DISCUSSION

Results of this study suggest that *KLF6* and *p53* mutations are involved in the development of nonpolypoid colorectal carcinoma, whereas K-*ras* and B-*raf* mutations are not. *KLF6* encodes a nuclear protein with three zinc fingers at the C terminus a serine/threonine-rich central region, and an acidic domain in the N-terminal region. The zinc fingers are responsible for specific binding to guanine-rich core promoter elements. The central region may be involved in activation or posttranslational regulatory pathways, and the acidic N-terminal domain may be involved in transcriptional activation. The DNA binding and transcriptional activity of this protein, in conjunction with its expression pattern, suggests that it may participate in the regulation and/or maintenance of the basal expression of pregnancy-specific glycoprotein genes and possibly other TATA box-less genes[7-9].

K-*ras* encodes a 21-kD plasma membrane-bound guanosine triphosphate-binding protein that is a key regulatory component of signal transduction pathways that transmit growth stimulatory signals from cell surface receptors to intracellular targets[16]. K-*ras* mutations are found in approximately 30% of colorectal cancers[17].

Table 3 Individual data for genetic analysis of 55 nonpolypoid colorectal tumors

Case	*KLF6*		*p53*		K-*ras*	B-*raf*
	LOH[1]	Mutation	IHC[2]	LOH[4]	Mutation	Mutation
Intramucosal cancer						
16	NI[3]	Wild	(-)	NI	GGC to GCC [13, Gly to Ala (1/4)]	Wild
17	(-)		(-)		Wild	Wild
29	LOH (3/3)	Wild	(-)		Wild	Wild
30	LOH (2/4)	Wild	(-)	(-)	Wild	Wild
31		Wild	(-)	(-)	Wild	Wild
32		Wild	(++)	LOH (3/3)	Wild	Wild
33	(-)		(+)	LOH (1/3)	Wild	AAA to CAA [560, Lys to Gln (1/3)]
34	(-)		(+++)		Wild	Wild
35		Wild	(-)	LOH (1/3)	Wild	Wild
36	(-)		(-)	LOH (1/4)	Wild	Wild
Submucosal cancer						
1	LOH (1/4)	Wild	(-)		Wild	Wild
2		Wild	(-)	(-)	Wild	Wild
3	NI	Wild	(+)		GGC to GCC [13, Gly to Ala (1/4)]	CAT to CTT [396, His to Leu (1/4)]
4	(-)		(+++)	LOH (1/5)	Wild	Wild
5		Wild	(+++)	(-)	Wild	Wild
6	NI	Wild	(-)	LOH (2/3)	Wild	Wild
7	NI		(+++)	(-)	Wild	Wild
8	LOH (2/3)	Wild	(++)	(-)	Wild	Wild
9	NI	Wild	(+++)		Wild	Wild
10	NI	Wild	(-)	LOH (2/3)	Wild	Wild
11	NI		(+++)	(-)	Wild	Wild
12	NI		(+++)	LOH (2/4)	Wild	Wild
13	LOH (3/7)	Wild	(+++)	(-)	Wild	Wild
14	NI	Wild	(-)	(-)	Wild	Wild
15	LOH (3/4)	Wild	(+++)		Wild	Wild
18	(-)		(+)		Wild	Wild
19	NI		(-)		Wild	Wild
20		Wild	(-)		GGC to GCC [13, Gly to Ala (1/4)]	CAG to CCG [608, Glu to Pro (1/4)]
21	NI	Wild	(++)	(-)	Wild	GTG to ATG [378, Val to Met (1/3)]
22	LOH (2/4)	CTG to GCT [735, Leu to Ala (2/4)]	(++)		Wild	Wild
23	NI	CTG to GCT [735, Leu to Ala (2/4)]	(+)	(-)	Wild	Wild
24	NI	Wild	(-)	(-)	GGC to GCC [13, Gly to Ala (1/5)]	Wild
25			(+)		Wild	AAA to CAA [560, Lys to Gln (1/5)]
26	(-)	Wild	(-)		Wild	AAA to CAA [560, Lys to Gln (2/6)]
27	(-)		(-)	NI	Wild	Wild
28		Wild	(-)		Wild	Wild
37	(-)		(-)	LOH (4/4)	Wild	Wild
38	(-)		(+++)	LOH (4/4)	Wild	Wild
39	(-)		(-)		Wild	Wild
40	(-)		(++)	(-)	Wild	Wild
41		Wild	(-)		Wild	Wild
42			(-)		Wild	Wild
43	(-)		(++)		Wild	Wild
44	LOH (3/3)	Wild	(-)	NI	Wild	Wild
45	LOH (1/5)	Wild	(+++)	LOH (5/5)	Wild	Wild
46	(-)		(+++)	(-)	Wild	Wild
47	LOH (3/3)	Wild	(-)	(-)	Wild	Wild
48	LOH (2/4)	Wild	(-)		Wild	Wild
49		Wild	(-)	(-)	GGC to GCC [13, Gly to Ala (1/4)]	Wild
50		Wild	(-)	LOH (3/5)	Wild	Wild
51	(-)		(-)	(-)	Wild	Wild
52	LOH (3/4)	Wild	(-)		Wild	Wild
53			(-)	LOH (1/4)	Wild	Wild
54	LOH (1/5)	Wild	(-)		Wild	Wild
55	LOH (1/4)	Wild	(+++)	LOH (3/4)	Wild	Wild

[1]LOH: loss of heterozygosity; [2]IHC: immunohistochemistry; [3]NI: not informative; blank space, not examined; [4]Number of LOH or mutation-positive foci per number of microdissected tumor foci is indicated in parenthesis.

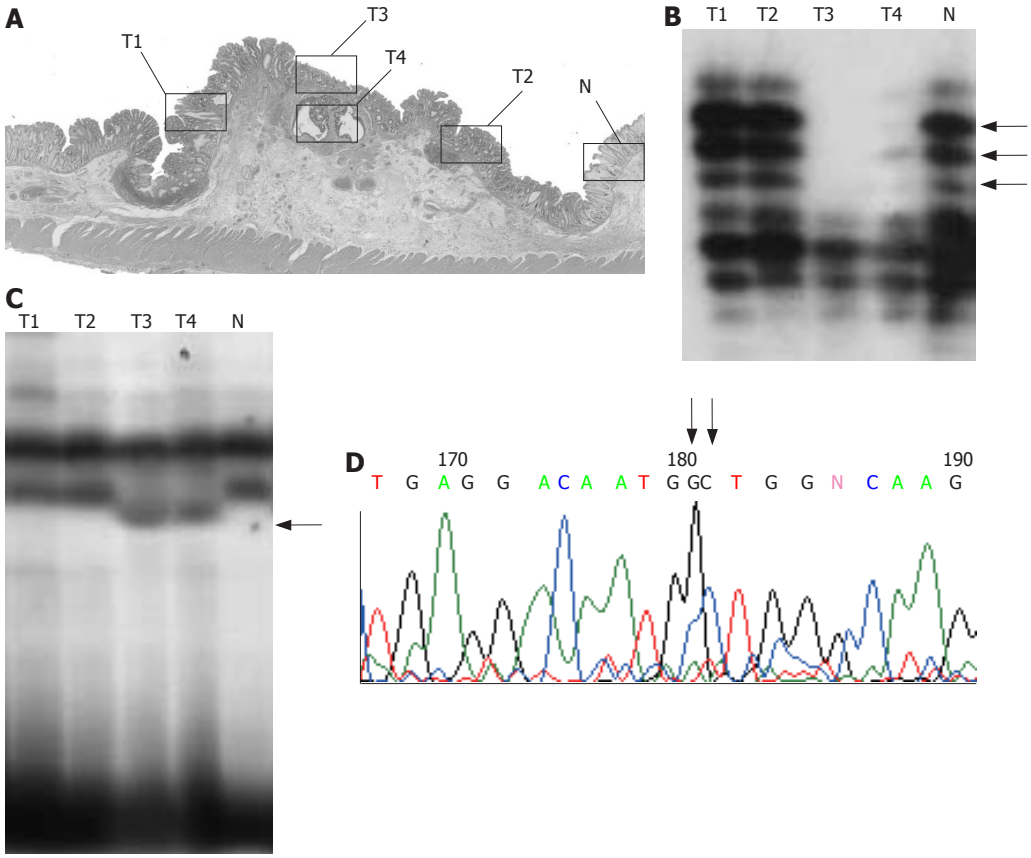

Figure 1 Representative example (case 22). **A**: Histologic findings. Open squares indicate microdissected areas. T1-T4: tumor samples; N: normal sample. **B**: Microsatellite assay of D10S591. T3 and T4 show allelic loss of the locus (arrow). **C**: polymerase chain reaction-single-strand conformation polymorphism analysis of KLF6. T3 and T4 show mobility shift (arrow). **D**: Direct sequencing analysis of KLF6. A missense mutation at codon 735 (CTG to GCT) is confirmed.

Table 4 Summary for genetic analysis of 55 nonpolypoid colorectal tumors

Genetic alteration	Frequency		
	Mucosal cancer	Submucosal cancer	Total
KLF6, LOH[1] (+)	2/6 (33.3%)	12/23 (52.2%)	14/29 (48.3%)
KLF6, mutation	0/6 (0%)	2/28 (7.1%)	2/34 (5.9%)
p53, LOH (+)	4/8 (50.0%)	10/23 (43.5%)	14/31 (45.2%)
p53, overexpression	2/10 (20.0%)	17/45 (37.8%)	19/55 (34.5%)
K-*ras*, mutation	1/10 (10.0%)	4/45 (8.9%)	5/45 (9.1%)
B-*raf*, mutation	1/10 (10.0%)	5/45 (11.1%)	6/55 (10.9%)

[1]LOH, Loss of heterozygosity.

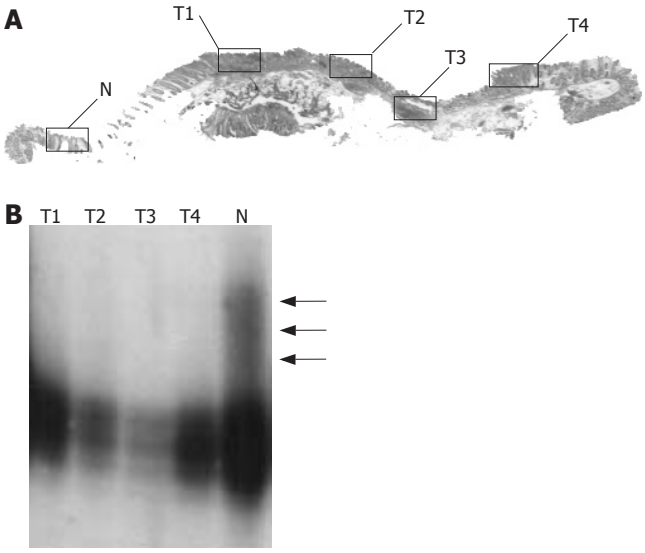

Figure 2 Representative example (case 38). **A**: Histologic findings. T1-T4: tumor samples; N: normal sample. **B**: Microsatellite assay of TP53. All of the microdissected tumor foci (T1-T4) show allelic loss of the locus (arrow).

The majority of the mutations involve a single amino acid substitution at codon 12 or 13, which decreases the intrinsic guanosine triphosphatase activity, which leads to the constitutive activation of the K-*ras* signaling pathway. Interestingly, K-*ras* mutations are associated with polypoid-type colorectal tumors.

The raf family, which includes A-*raf*, B-*raf*, and *raf*-1, comprises serine/threonine kinases that are regulated by binding to ras. K-*ras* mutations lead to activation of this signaling pathway and to malignant transformation[18]. B-*raf* mutations are observed in malignant melanoma, colorectal cancers, and ovarian borderline tumors. They are mutually exclusive with ras mutations[14,19]. B-*raf* mutations are observed in approximately 10% of colorectal carcinomas[19]. Mutations occur in two regions of the kinase domain, in exon 15 (the activation segment, which protects the substrate-binding site), and less commonly, in exon 11 (the G loop, which mediates ATP binding). Mutated B-*raf* protein shows increased kinase activity and can transform NIH3T3 cells[14]. B-*raf* mutations are also associated with polypoid-type colorectal tumors.

In the present study of nonpolypoid tumors, frequent alterations of *KLF6* and *p53* were found. In more than 60% of the cases, these alterations were found in all of the microdissected tumor samples. On the contrary, the frequencies of K-*ras* and B-*raf* mutations were low, and these mutations were detected only in subsets of

microdissected tumor samples. Alterations were detected homogeneously or heterogeneously in the microdissected samples for each gene. Homogeneous alterations were considered to be the result of expansion of a single clone. Heterogeneous alterations were considered to occur as the tumor progressed or diverged genetically. During genetic progression, in addition to the early homogeneous genetic events, alterations are detected only in subsets of microdissected tumor samples, indicating linear genetic progression of a single neoplastic clone. During genetic divergence, in addition to early homogeneous genetic events, different alterations of several genes are detected in different parts of the tumor, indicating that a single neoplastic clone has diverged in two or more directions[20]. Thus, alterations of *KLF6* and *p53* may be early and important events in the development of nonpolypoid-type tumors, whereas K-*ras* and B-*raf* mutations may not. However, morphologically mixed-type tumors, such as polypoid lesions in depressed tumors, are occasionally found. Tumors with mixed morphology may be the result of recently mutated K-*ras*. Microdissection is useful in the examination of genetic heterogeneity of tumors. Use of this method to examine genetic heterogeneity in tumors of mixed morphology will provide information regarding the genetic events involved.

Although LOH of *KLF6* was detected in 14 of 31 (45.2%) tumors, only a single *KLF6* missense mutation was detected in the present study. We examined the reported hot spot region of the gene. Current reports are all from Western countries. There are no studies on the frequency of *KLF6* mutation in Asian countries. The mutation spectrum may differ between Western countries and Asian countries, including Japan. Further examination is necessary to clarify this issue.

Recently, Konishi *et al*[21] reported B-*raf* mutation in 9% of nonpolypoid tumors, and Noda *et al*[22] reported that 10% of tumors showed this mutation. They concluded that B-*raf* mutation may be involved in the development of nonpolypoid colorectal carcinoma. The mutation frequency of this gene was similar to that in the present study. However, mutation was detected only in subsets of microdissected tumor samples, indicating little or no role in the development of nonpolypoid tumors. Microdissection was not used in previous studies. Thus, genetic clonality was not assessed.

In conclusion, results of the present study suggest that *KLF6* and *p53* mutations are involved in the development of nonpolypoid colorectal carcinoma, whereas K-*ras* and B-*raf* mutations are not. Polypoid-type tumors were not examined in the present study; therefore, the present study had limitations. The frequency of *KLF6* mutation in polypoid-type tumors is of great interest. A high frequency of *KLF6* somatic mutation has been reported in colorectal carcinoma, and the adenoma-carcinoma sequence is predominant in the development of colorectal carcinoma. Therefore, *KLF6* mutation in polypoid-type tumors may be frequent. In this relation, the frequency of *KLF-6* mutation in adenomatous polyps is of great interest. However, there were no reports on the frequency. Future research will examine mutations in polypoid-type tumors and adenomatous polyps.

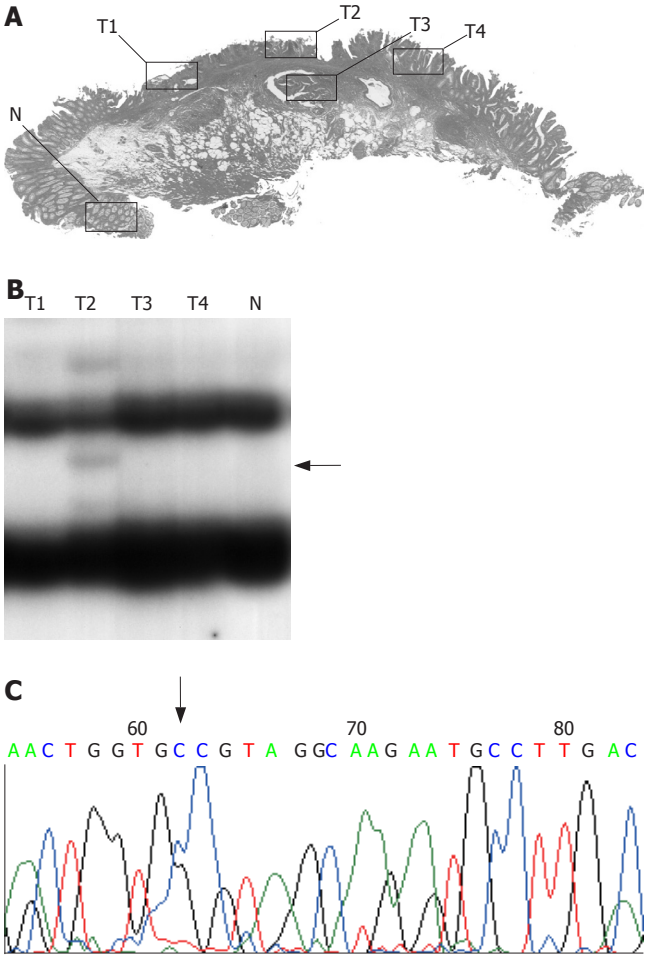

Figure 3 Representative example (case 20). **A**: Histologic findings. T1-T4: tumor samples; N: normal sample. **B**: polymerase chain reaction-single-strand conformation polymorphism analysis of K-*ras*. T2 shows a mobility shift. **C**: Direct sequencing analysis of K-*ras*. A misssense mutation at codon 13 (GGC to GCC) is confirmed.

ACKNOWLEDGMENTS

This study was supported in part by a Grant from the Japanese Society of Gastrointestinal Endoscopy, Chugoku Branch.

REFERENCES

1 **Watanabe T**, Muto T. Colorectal carcinogenesis based on molecular biology of early colorectal cancer, with special reference to nonpolypoid (superficial) lesions. *World J Surg* 2000; **24**: 1091-1097
2 **Umetani N**, Sasaki S, Masaki T, Watanabe T, Matsuda K, Muto T. Involvement of APC and K-ras mutation in non-polypoid colorectal tumorigenesis. *Br J Cancer* 2000; **82**: 9-15
3 **Sakamoto N**, Terai T, Ajioka Y, Abe S, Kobayasi O, Hirai S, Hino O, Watanabe H, Sato N, Shimoda T, Fujii H. Frequent hypermethylation of RASSF1A in early flat-type colorectal tumors. *Oncogene* 2004; **23**: 8900-8907
4 **Akiyama Y**, Arai T, Nagasaki H, Yagi OK, Nakahata A, Nakajima T, Ohkura Y, Iwai T, Saitoh K, Yuasa Y. Frequent allelic imbalance on chromosome 18q21 in early superficial colorectal cancers. *Jpn J Cancer Res* 1999; **90**: 1329-1337
5 **Muto T**, Kamiya J, Sawada T, Konishi F, Sugihara K, Kubota Y, Adachi M, Agawa S, Saito Y, Morioka Y. Small "flat adenoma" of the large bowel with special reference to its clinicopathologic features. *Dis Colon Rectum* 1985; **28**: 847-851

6 **Kudo S**, Tamura S, Hirota S, Sano Y, Yamano H, Serizawa M, Fukuoka T, Mitsuoka H, Nakajima T, Kusaka H. The problem of de novo colorectal carcinoma. *Eur J Cancer* 1995; **31A**: 1118-1120

7 **Narla G**, Heath KE, Reeves HL, Li D, Giono LE, Kimmelman AC, Glucksman MJ, Narla J, Eng FJ, Chan AM, Ferrari AC, Martignetti JA, Friedman SL. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science* 2001; **294**: 2563-2566

8 **Chen C**, Hyytinen ER, Sun X, Helin HJ, Koivisto PA, Frierson HF, Vessella RL, Dong JT. Deletion, mutation, and loss of expression of KLF6 in human prostate cancer. *Am J Pathol* 2003; **162**: 1349-1354

9 **Reeves HL**, Narla G, Ogunbiyi O, Haq AI, Katz A, Benzeno S, Hod E, Harpaz N, Goldberg S, Tal-Kremer S, Eng FJ, Arthur MJ, Martignetti JA, Friedman SL. Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer. *Gastroenterology* 2004; **126**: 1090-1103

10 **Shimoda T**, Ikegami M, Fujisaki J, Matsui T, Aizawa S, Ishikawa E. Early colorectal carcinoma with special reference to its development de novo. *Cancer* 1989; **64**: 1138-1146

11 **Jeng YM**, Hsu HC. KLF6, a putative tumor suppressor gene, is mutated in astrocytic gliomas. *Int J Cancer* 2003; **105**: 625-629

12 **Hiyama T**, Yokozaki H, Shimamoto F, Haruma K, Yasui W, Kajiyama G, Tahara E. Frequent p53 gene mutations in serrated adenomas of the colorectum. *J Pathol* 1998; **186**: 131-139

13 **Bos JL**. The ras gene family and human carcinogenesis. *Mutat Res* 1988; **195**: 255-271

14 **Davies H**, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature* 2002; **417**: 949-954

15 **Yokozaki H**, Shitara Y, Fujimoto J, Hiyama T, Yasui W, Tahara E. Alterations of p73 preferentially occur in gastric adenocarcinomas with foveolar epithelial phenotype. *Int J Cancer* 1999; **83**: 192-196

16 **Hoffman M**. Getting a handle on Ras activity. *Science* 1992; **255**: 159

17 **Andreyev HJ**, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, Young J, Walsh T, Ward R, Hawkins N, Beranek M, Jandik P, Benamouzig R, Jullian E, Laurent-Puig P, Olschwang S, Muller O, Hoffmann I, Rabes HM, Zietz C, Troungos C, Valavanis C, Yuen ST, Ho JW, Croke CT, O'Donoghue DP, Giaretti W, Rapallo A, Russo A, Bazan V, Tanaka M, Omura K, Azuma T, Ohkusa T, Fujimori T, Ono Y, Pauly M, Faber C, Glaesener R, de Goeij AF, Arends JW, Andersen SN, Lövig T, Breivik J, Gaudernack G, Clausen OP, De Angelis PD, Meling GI, Rognum TO, Smith R, Goh HS, Font A, Rosell R, Sun XF, Zhang H, Benhattar J, Losi L, Lee JQ, Wang ST, Clarke PA, Bell S, Quirke P, Bubb VJ, Piris J, Cruickshank NR, Morton D, Fox JC, Al-Mulla F, Lees N, Hall CN, Snary D, Wilkinson K, Dillon D, Costa J, Pricolo VE, Finkelstein SD, Thebo JS, Senagore AJ, Halter SA, Wadler S, Malik S, Krtolica K, Urosevic N. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 2001; **85**: 692-696

18 **Kolch W**. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J* 2000; **351** Pt 2: 289-305

19 **Chan TL**, Zhao W, Leung SY, Yuen ST. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. *Cancer Res* 2003; **63**: 4878-4881

20 **Shima H**, Hiyama T, Tanaka S, Yoshihara M, Arihiro K, Chayama K. Genetic progression and divergence in superficial esophageal squamous cell carcinoma by loss of heterozygosity analysis. *Oncol Rep* 2006; **16**: 685-691

21 **Konishi K**, Takimoto M, Kaneko K, Makino R, Hirayama Y, Nozawa H, Kurahashi T, Kumekawa Y, Yamamoto T, Ito H, Yoshikawa N, Kusano M, Nakayama K, Rembacken BJ, Ota H, Imawari M. BRAF mutations and phosphorylation status of mitogen-activated protein kinases in the development of flat and depressed-type colorectal neoplasias. *Br J Cancer* 2006; **94**: 311-317

22 **Noda H**, Kato Y, Yoshikawa H, Arai M, Togashi K, Nagai H, Konishi F, Miki Y. Frequent involvement of ras-signalling pathways in both polypoid-type and flat-type early-stage colorectal cancers. *J Exp Clin Cancer Res* 2006; **25**: 235-242

S- Editor Liu Y **L- Editor** Rippe RA **E- Editor** Lu W