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BRAF MUTATION ASSOCIATED WITH DYSREGULATION OF APOPTOSIS IN HUMAN COLORECTAL NEOPLASMS

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Abbreviations: PCR-SSCP, polymerase chain reaction–single strand conformation polymorphism; FTI, farnesyltransferase inhibitor; MEK, extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase;

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ABSTRACT

To understand the role of BRAF dysfunction in the carcinogenesis and progression/development of colorectal tumors, the authors investigated genetic alterations in the *BRAF* gene in human colorectal neoplasms as well as the effects of an RAS inhibitor in *BRAF*-mutant cells. Seven colon cancer cell lines and 116 colorectal tumors (34 adenomas and 82 adenocarcinomas) were analyzed. Genetic alterations in the *BRAF* and K-*ras* genes were examined using PCR-SSCP and direct sequencing analyses. The growth-inhibitory and apoptosis-inducing effects of the FTI-277 RAS inhibitor in colon cancer cell lines were analyzed as well. An immunohistochemical study was also performed to investigate the correlations between the clinicopathologic parameters involved in the Ki-67 labeling index and the number of apoptotic bodies in tumor cells. FTI-277 did not suppress the proliferation of *BRAF*-mutant cells (WiDr and TCO), but remarkably inhibited the growth of K-*ras* mutant cells (LoVo). Interestingly, LoVo cells underwent apoptosis by FTI-277 in a dose-dependent manner, whereas WiDr cells were resistant to this agent. In tumor samples, *BRAF* mutations were found in 1 (3.0%) of 33 adenomas and 6 (7.2%) of 83 adenocarcinomas. No tumor exhibited mutations in both the *BRAF* and K-*ras* genes. Neither *BRAF* nor K-*ras* mutations correlated with the Ki-67 labeling index immunohistochemically. However, the number

of apoptotic bodies was significantly decreased in the *BRAF*-mutant tumors. Mutation in the *BRAF* gene may contribute to colorectal carcinogenesis by up-regulating the anti-apoptotic role of the RAS/RAF/MEK/ERK pathway.

INTRODUCTION

The development of colorectal cancer is a multi-step process characterized by the accumulation of genetic alterations.[1] The *RAS* proto-oncogene is mutated to an oncogenic form in about 30% of human cancers, and frequent K-*ras* mutations have been reported in pancreatic, colorectal, and lung cancers.[1-4] Especially, alterations in K-*ras* at codons 12, 13, and 61 lead to increased unregulated cellular proliferation and malignant transformation.[5] In human colorectal cancers, K-*ras* mutation has been considered an early event in the development of adenomas. This genetic event is more common in large adenomas than small ones, suggesting that it may be required for the activation of adenoma progression in the RAS/RAF/MEK/ERK pathway.[1,6] Therefore, up-regulation of RAS-regulated intracellular signaling is necessary for colorectal tumorigenesis.

Genes of the RAF family encode RAS-regulated kinases and mediate cellular responses to growth signals.[7] Recently, the activation of BRAF, one of the three RAF members, has been reported to occur by somatic mutation in many human cancers, particularly in human malignant melanoma (over 60%),[8,9] human colorectal cancers (5-15%),[8,10,11] and a small fraction of other cancers.[8] Frequent mutations in the *BRAF*

gene have been detected in the two regions of the *BRAF* kinase domain: the G-loop in exon 11 and the activation segment in exon 15.[8] The majority of the *BRAF* mutations each represent a single nucleotide change of T-A at nucleotide 1796, resulting in the change of valine to glutamic acid at codon 599 within the activation segment of BRAF.[8] Moreover, a previous report showed that overexpression of BRAF could act as a potent inhibitor of apoptosis by activating the BRAF/MEK/ERK pathway, which interferes with apoptosis at the level of cytosolic caspase activation and consequently inhibits the release of cytochrome *c* from mitochondria.[12] Although *BRAF* mutations were found in about 5-15% of colorectal carcinomas, colorectal carcinomas with *BRAF* mutations tended to be in lower clinical tumor stages.[11] However, it has been suggested that alteration in the *BRAF* gene may cause the activation of the RAS/RAF/MEK/ERK pathway, consequently increasing cell proliferation but suppressing the inhibition of apoptosis. Furthermore, although the acquisition of a *BRAF* mutation have been associated with the progression of hyperplastic polyp to serrated adenoma,[13] further investigations are needed to clarify the role of mutant *BRAF* in human colorectal tumorigenesis.

To elucidate the roles of K-*ras* and *BRAF* oncogenic mutations in colorectal tumorigenesis, we analyzed the effects of an RAS inhibitory agent, FTI-277, on colon cancer cell lines. We investigated *BRAF* mutations in human colorectal tumors and

correlation relationships among clinicopathologic parameters. An immunohistochemical study was also performed to evaluate the correlation with the Ki-67 labeling index and the number of apoptotic bodies in this series of colorectal tumors.

MATERIALS AND METHODS

Cell lines and tissue samples

Seven colon cancer cell lines (WiDr, LoVo, TCO, DLD-1, SW480, HCT-15, and Colo320) were used in this study. The cells were routinely maintained in RPMI-1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 1 mM L-glutamine, 10% FBS (Life Technologies), and 1% antibiotic-antimycotic solution (Life Technologies). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The RAS-specific inhibitory agent FTI-277 was purchased from Calbiochem Co. (La Jolla, CA). A stock solution of this compound was prepared in 100% dimethyl sulfoxide (DMSO) and stored at -20°C.

A total of 116 sporadic colorectal neoplasms (carcinoma, 82 cases; adenoma, 34 cases) were employed in this study. Tumor samples with matched adjacent normal colorectal mucosae were surgically and endoscopically removed at Kobe University Hospital and affiliated hospitals between October 1995 and July 1999. The

clinicopathological data on these colorectal tumors are shown in Table I. Histological typing, depth of invasion, and clinical stage were determined according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (1998)[14] along with the International Union Against Cancer classification.[15] For molecular analyses, tissue samples were frozen immediately and stored at –80°C until extraction of genomic DNAs as described previously.[16]

WST-1 cell proliferation assay

Cell growth was determined using the Premix WST-1 Cell Proliferation Assay System (Takara Biochemicals Co., Tokyo, Japan). Briefly, 1.0 **x** 10^5 cells (100 μl volume/well) in RPMI-1640 supplemented with 2% FBS was inoculated into a 96-well microtiter plate and incubated for 24 hours at 37°C. FTI-277 (5-20 μM) was added to the wells and cultured in the absence of FBS for 24 hours at 37°C. Premix WST-1 was added to each microculture well. The plates were incubated for 30 min at 37°C, after which absorbance at 450 nm was measured with a microplate reader.

Western blotting

The cells were treated with 5-10 μM FTI-277 in the absence of FBS for 24 hours at 37°C. The cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA containing both 1% protease inhibitor (Sigma Chemical Co., St. Louis, MO) and 1% phosphatase inhibitor cocktail II (Sigma Chemical Co.). Cell lysates were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and were then electrotransferred onto an Immunobilon (Millipore, Chelmsford, MA). Phosphorylation-specific rabbit polyclonal antibodies for Erk1/2 (Thr202/Tyr204) and MEK1/2 (Ser217/221) were purchased from Cell Signaling Technology (Beverly, MA). Peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences Co., Buckinghamshire, UK) was used as a secondary antibody for enhanced chemiluminescence (Wako, Osaka, Japan).

Apoptotic bodies and DNA fragmentation

After treatment with FTI-277, apoptotic (sub-G1 phase) LoVo and WiDr cells were counted after staining with propidium iodide (Molecular Probes, Eugene, OR). The number of apoptotic bodies was determined by counting the apoptotic cells (%) in a total of more than 300 cells. To detect apoptotic DNA fragmentation, genomic DNA

was extracted from members of both cell lines as described elsewhere.[17] Cells treated with FTI-277 and GW5074 were lysed in a buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, and 0.5% sodium *N*-lauroylsarcosinate. Next, the lysates were incubated with 1 μg RNase A and then 1 μg of proteinase K at 50 °C for 30 minutes each, and were then electrophoresed in 1% agarose gels.

PCR-SSCP and direct sequencing analyses

Mutations in exons 11 and 15 of the *BRAF* gene and exon 2 of the K-*ras* gene were examined by PCR-SSCP analysis using the following primer sets: *BRAF*-exon 11, 5'-CACTTGGTAGACGGGACTCG-3' (sense)/ 5'-CATGCCACTTTCCCTTGTAG-3' (antisense), *BRAF*-exon 15, 5'-CTTCATGAAGACCTCACAGT-3' (sense)/ 5'-CATCC ACAAAATGGATCCAG-3' (antisense), K-*ras*-exon 2, 5'-GACTGAATATAAACTTGT GG-3' (sense)/ 5'-CTATTGTTGGATCATATTCG-3' (antisense). PCR was performed in a total 15 μl reaction volume containing 1 μl template DNA, 0.56 μM of each primer, 0.12 mM each of dATP, dGTP, dCTP, dTTP, 4.5 mM of $MgCl_2$, 0.15 unit of Taq DNA polymerase. Reaction conditions were first 95°C at 10 min, 30 sec at 58°C, 30 sec at 55°C, 30 sec at 72°C for 40 cycles, followed by a final extension for 10 min at 72°C. PCR-SSCP analysis of amplified exons was performed as a screening step before

sequencing. PCR products of *BRAF* and K-*ras* exons were separated by 30% polyacrylamide gels with 5% glycerol, and the gels were stained and visualized with the Silver Staining Kit (ATTO, Osaka, Japan). Mutations detected by PCR-SSCP in different exons of the *BRAF* and K-*ras* genes were identified and confirmed by direct sequencing using the ABI PRISM BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA) and an automated ABI PRISM 310 DNA sequencer (Applied Biosystems).

Immunohistochemistry

Immunohistochemistry was investigated to study the proliferative activity and induction of apoptosis in colorectal neoplastic tissues. A mouse monoclonal antibody against Ki-67 (Immunotech, Marseille, France) and a rabbit polyclonal antibody to single-stranded DNA (ssDNA; DAKO, Glostrup, Denmark) were used as primary antibodies. A modified version of the immunoglobulin enzyme bridge technique was used as described elsewhere.[18] Briefly, deparaffinized tissue sections were immersed in 10 mM sodium citrate buffer, and pretreatment autoclaving was performed to retrieve antigenicity, except for ssDNA sections. Endogenous peroxidase activity and nonspecific binding were blocked by hydrogen peroxide and a blocking reagent in the

LSAB2 Kit (DAKO), respectively. The slides were incubated at 4°C overnight with the primary monoclonal antibody (ssDNA, 3 hours at 4°C) and then incubated sequentially at room temperature with a biotinylated secondary antibody for 30 min, streptavidin peroxidase for 15-20 min, and DAKO DAB for 10 minutes in the LSAB2 Kit. The slides were then counterstained with Mayer's hematoxylin.

The Ki-67 labeling index (mean ± standard deviation) was determined by counting the positive cells (%) in a total of 1000 cells in 10 representative high-power fields (**x** 400). Apoptotic body index was determined by counting the ssDNA-positive cells in a total of 10 high-power fields (**x** 400).

Statistical analysis

The efficacies of FTI-277 in the growth and induction of apoptosis in human colon cancer cell lines, as well as the relationships among clinicopathological parameters, were determined using the χ^2 test, one-factor ANOVA and Fisher's PLSD test. A level of $P < 0.05$ was taken to be statistically significant.

RESULTS

The growth-inhibitory and apoptosis-inducing effects of FTI-277 in colon cancer cell lines

To investigate the roles of K-*ras* and *BRAF* in human colon cancer cell lines, we first examined mutations of the *BRAF* and K-*ras* genes in 7 human colon cancer cell lines. Of these, 2 cell lines contained alterations in the *BRAF* gene (WiDr and TCO, 28%), while 4 cell lines (LoVo, DLD-1, SW480, and HCT-15, 57%) contained alterations in the K-*ras* gene (Fig. 1). Missense mutations at codon 599 (V599E) were detected in both the WiDr and TCO *BRAF*-mutant cell lines, and missense mutations at codon 12 (G12V) or codon 13 (G13D) were detected in the K-*ras*-mutant cell lines, LoVo, DLD-1, SW480, and HCT-15. None of the cell lines showed neither *BRAF* mutation nor K-*ras* mutation (Table II).

Next, the growth-inhibitory effects of FTI-277 in these *BRAF*-mutant and K-*ras*-mutant cell lines were analyzed to investigate the role of the RAS/RAF/MEK/ERK pathway in these cells. As shown in Fig. 2A, when the LoVo cells were treated with 10 μM or 20 μM FTI-277 after serum starvation for 24 hours, cell proliferation decreased significantly, to 33% or 19%, respectively, in a concentration-dependent manner. In contrast, FTI-277 did not affect cell growth in

WiDr or TCO *BRAF*-mutant cells ($P < 0.0001$). However, in LoVo and WiDr cell lines, treatment with FTI-277 did not interfere with the levels of phosphorylated MEK and ERK expression (Fig. 2B). Moreover, we counted the apoptotic bodies in the current series of human colon cancer cell lines to show the relationship between FTI-277 and the induction of apoptosis. Although FTI-277 did not affect the induction of apoptosis in WiDr cells (< 2%), the number of apoptotic bodies increased significantly ($P = 0.002$) in LoVo cells (8-12%) in a concentration-dependent manner (Fig. 2C). We confirmed the induction of apoptosis in LoVo cells by detecting an apoptotic DNA ladder (Fig. 2D).

Mutation analyses of* BRAF *and* K-ras *genes in human colorectal cancers and relationship with clinicopathological parameters

To detect mutations in *BRAF* and K-*ras* genes, we performed PCR-SSCP analysis and direct sequencing in the same of 116 colorectal tumors samples. *BRAF* mutations were found in 1 (3.0%) of 33 colorectal adenomas and 6 (7.2%) of 83 colorectal adenocarcinomas (Table III). Interestingly, the adenoma with the *BRAF* mutation showed a serrated glandular structure (serrated adenoma), but no *BRAF* alteration was found in the tubular adenomas. Most of the tumors demonstrated

missense mutations at codon 599 (V599E), and a mutation at codon 468 (G468V) was detected in one adenocarcinoma (Fig. 3). Overall, mutations in the K-*ras* gene were detected in 2 (6.0%) of 33 colorectal adenomas and 22 (26.5%) of 83 colorectal adenocarcinomas. We found that none of the tumors contained mutations in both the *BRAF* and K-*ras* genes.

Next, we analyzed the relationship among clinicopathologic findings in this series of colorectal tumors. Of the 6 colorectal cancers with *BRAF* mutations, 2 (33.3%) were early colorectal cancers and 4 (66.7%) were advanced. Colorectal tumors with K-*ras* and *BRAF* mutations were larger than wild-type colorectal tumors ($p = 0.0042$). Moreover, the incidence of lymph node metastasis was more frequent in K-*ras* and *BRAF*-mutant colorectal cancers ($p = 0.0459$), and colorectal cancers with K-*ras* or *BRAF* mutations tended to be in more advanced clinical stages ($p = 0.0076$) than colorectal tumors with wild-type K-*ras* and *BRAF* (Table IV). To clarify the differences in proliferation and apoptosis in colorectal neoplasms with alterations in the *BRAF* or K-*ras* genes, we investigated Ki-67 labeling index and the numbers of apoptotic bodies in tumor cells (Fig. 4A). Interestingly, although the proliferative activity was not statistically significant between *BRAF* and K-*ras* mutations (Fig. 4B), the number of apoptotic bodies was significantly suppressed in colorectal neoplasms with *BRAF*

mutations (p = 0.0285) (Figure 4C). Furthermore, the index values of apoptotic bodies by the TUNEL method was significantly reduced in colorectal tumors with *BRAF* mutations as well as by using a polyclonal antibody specific to ss-DNA (data not shown).

DISCUSSION

The RAS/RAF/MEK/ERK pathway is at the heart of signaling networks that govern proliferation, differentiation, and cell survival.[19] In the development of human colorectal carcinomas, alterations in K-*ras* gene are frequently the cause of dysfunction in this intracellular signaling. In this study, we detected genetic changes in the *BRAF* gene in 7.2% of colorectal cancers, a rate nearly congruent with that in a previous report.[11] In addition, most *BRAF* mutations in colorectal tumors were detected at a mutation hot spot, codon 599 (V599E).[9,10,11] V599E mutations have been reported to show greatly increased activity in the BRAF/MEK/ERK pathway, both *in vitro* and *in vivo*,[8] and mutation at codon 599 was shown to activate NFκB-dependent signaling.[20] Moreover, suppression of $BRAF^{V599E}$ expression by RNA interference in cultured human

melanoma inhibited the mitogen-activated protein kinase cascade, consequently arresting growth and the promoting apoptosis.[21] Interestingly, we found a novel mutation at codon 468 (G468V), located in the glycine residues of the GXGXXG motif within the glycine-rich loop of the kinase domain. A previous report showed that mutation of the glycine residues in the GXGXXG motif of the ATP-binding domain could activate kinases.[8] To our knowledge, this is the first report of a *BRAF* mutation with a single nucleotide change of G-T at nucleotide 1403 in human malignancies.

RAS has been shown to regulate several pathways that contribute to cellular transformation, such as the Rac, Rho, and RAS/RAF/MEK/ERK pathways.[22] Since a single-point mutation of the *RAS* gene can lead to its constitutive activation of RAS protein, knocking out the activated K-*ras* gene in the human colon cancer cell lines DLD-1 and HCT-116 resulted in cell lines incapable of clone formation on soft agar and lacking tumorigenicity in nude mice.[23] Mutations of the *RAS* gene in colon and pancreatic cancers were found only in K-*ras*, especially in the early stage of carcinogenesis. In this study, we detected genetic changes in the K-*ras* gene in 6.0% and 26.5% of colorectal adenomas and cancers, which is in conflict with previous reports.[1-4] Moreover, we found that none of the tumors contained mutations in both the *BRAF* and K-*ras* genes, indicating that the coincidence of *BRAF* and K-*ras* mutations in the same

colorectal sample would be a rare event.[11] Our data suggest that an alteration in the *BRAF* gene may contribute to activation of the RAS/RAF/MEK/ERK pathway signaling in human colorectal tumorigenesis, independent of K-*ras* mutation.

As described above, clinicopathological similarities in colorectal tumors with K-*ras* or *BRAF* mutations may reflect a role for the RAS/RAF/MEK/ERK pathway in colorectal tumorigenesis. In our analysis, colorectal cancers with altered *BRAF* or K-*ras* genes showed frequent lymph node metastasis with advanced clinical stages. However, our results were in disagreement with a previous report, which exhibited that colorectal carcinomas with *BRAF* mutations tend to be at a lower clinical stage.[11] Our observations suggested that colorectal neoplasms with *BRAF* or K-*ras* mutations may have almost the same clinicopathological features, whereas *BRAF* gene alterations of colorectal tissues were associated with significant decreases in rates of cell apoptosis in tumor cells. In contrast, colorectal tumors of *BRAF* or K-*ras* mutations were not associated with a significant increase in the rates of cell proliferative activities. These findings suggest that the development and progression of *BRAF*-mutant colorectal neoplasms may be closely associated with the dysregulation of apoptosis induction.

Chan et al.[13] found that the high frequencies of *BRAF* mutations in hyperplastic polyp (30%) and serrated adenoma (100%) contrasted sharply with the low incidence

observed in classical adenomas. In our samples, *BRAF* mutations were found in 50% of serrated adenomas. Furthermore, we analyzed alterations in the *BRAF* and K-*ras* genes in flat-typed colorectal cancer[24,25] without an adenomatous component, and found alterations in *BRAF* and K-*ras* genes were in 15% and 0% of the tumors, respectively (data not shown). These data may suggest that alteration in the *BRAF* gene is, like that in the K-*ras* gene, an early event in the adenoma-carcinoma sequence of the colorectum, and that alteration in the *BRAF* gene may be related to the progression/development of colorectal cancer arising *de novo*. Further investigation will be needed to clarify the roles of *BRAF* in the multistep carcinogenesis of the colorectum.

In vivo, the enzyme farnesyltransferase inhibitors (FTI) have shown antiproliferative activity, apoptosis induction, and the blockade of morphological alterations associated with RAS transformation.[26,27] FTIs have emerged as important targets for anti-cancer therapies and have been introduced in clinical trials of subjects with colorectal cancer.[28] In this study, although FTI-277 influenced neither cell proliferation nor the induction of apoptosis by *BRAF*-mutant cells, it did inhibit the proliferation of K-*ras* mutant cells and induced apoptosis in them. These results support the hypothesis that a mutation of the *BRAF* gene leads to the activation of RAF independent of RAS in the common role of the RAS/RAF/MEK/ERK pathway

signaling in colorectal tumorigenesis. On the other hand, Erhardt et al. demonstrated that overexpression of BRAF confers resistance against apoptosis induced by growth factor withdrawal or PI3-kinase inhibition, and that the BRAF/MEK/ERK pathway confers protection against apoptosis at the level of cytosolic caspase activation, downstream from the release of cytochrome *c* by mitochondoria.[12] Moreover, the knockdown of *BRAF* expression and the inhibition of downstream signaling in WM793 human melanoma cells cause growth arrest and promote apoptosis, and these consequences prevent colony formation in suspension.[21] Our *in vitro* studies using FTI-277 RAS inhibitor did not reveal the effects as was previously observed in the specific knockdown of *BRAF* or K-*ras* expression by siRNA method or oncogene-targeted oligodeoxynucleotides.

The results of our clinical study may indicate that cell growth in the *BRAF*-mutant colon tumor is closely related to the inhibition of apoptosis. Detection of *BRAF* gene mutations may promote a colorectal cancer treatment targeting apoptosis.

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TABLE I - Clinicopathological data of the cases with adenoma and adenocarcinoma of the colorectum

		Adenoma		Adenocarcinoma	
	n	**n**	**(%)**	**n**	**(%)**
Total	116	34	(29.3)	82	(70.7)
Location[a]					
proximal	46	17	(37)	29	(63.0)
distal	70	17	(24.3)	53	(75.7)
Gender					
male	74	21	(28.4)	53	(71.6)
female	42	13	(31.0)	29	(69.0)
Age (mean)	62.1 (1.0)	62.8 (2.0)		61.8 (1.3)	
Tumor size (mm)	26.0 ± 2.1	7.6 ± 0.8		34.9 ± 2.4	
typing[a]				Tis, T1	34 (45.3)
tubular adenoma	29 (85.3)			T2	9 (12.0)
tubulovillous adenoma	3 (8.8)			T3, T4	32 (42.7)
serrated adenoma	2 (5.9)			**Invasion and metastasis**[a]	
Adenocarcinoma				Venous vessels	23 (34.3)
Histological typing[a]				Lymph nodes	13 (23.6)
wel	49 (59.8)			**Clinical stage**[a]	
mod	20 (24.4)			0	8 (13.3)
por	3 (3.7)			I	19 (31.7)
G-5 (biopsy specimen)	7 (8.5)			II	17 (28.3)
				III	12 (20.0)
				IV	4 (6.7)

[a] Histological typing, depth of invasion and clinical stage were determined according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (1998)[14] along with the International Union Against Cancer classification.[15]

TABLE II - Results of mutational analysis of *BRAF* and K-*ras* genes in human colon cancer cell lines

	BRAF mutation			K-*ras* mutation		
	Codon	Nucleotide change	Substitution	Codon	Nucleotide change	Substitution
WiDr	599	GTG to GAG	Val to Glu		Wild type	
LoVo		Wild type		13	GGC to GAC	Gly to Asp
TCO	599	GTG to GAG	Val to Glu		Wild type	
DLD-1		Wild type		13	GGC to GAC	Gly to Asp
SW480		Wild type		12	GGT to GTT	Gly to Val
HCT-15		Wild type		13	GGC to GAC	Gly to Asp

TABLE III - Results of somatic mutation of *BRAF* and K-*ras* genes in colorectal neoplasms

***BRAF* mutations**		**Gender**	**Location**[a]	**Size**	**Morphology**[a]	**Histology**[a]	**Depth**[a]	**Stage**[a]
Nucleotide	amino acidT1796A		V599E	F	P	28	2	wel T2 III
T1796A	V599E	M	P	80	2	por	T3	II
T1796A	V599E	M	D	80	2	por*	-	IV
T1796A	V599E	M	D	13	0-Isp	wel	Tis	0
T1796A	V599E	M	P	12	0-IIa+IIc	mod	T1	I
G1403T	G468V	F	P	35	2	mod	T3	III
T1796A	V599E	F	D	5	Is	SA[b]	-	-
K-*ras* mutations		**Gender**	**Location**[a]	**Size**	**Morphology**[a]	**Histology**[a]	**Depth**[a]	**Stage**[a]
Nucleotide	amino acid							
G35A	G12D	M	P	40	1	wel	T2	I
G35A	G12D	M	P	50	1	wel*	-	-
G35A	G12D	F	D	22	2	mod	T2	I
G35A	G12D	M	D	40	2	por	T3	III
G35A	G12D	F	P	80	2	mod	T3	III
G35A	G12D	M	D	38	2	wel	T3	II
G35A	G12D	M	D	50	2	wel	T3	III
G35A	G12D	M	P	50	0-Isp	wel	Tis	0
G35A	G12D	M	D	12	0-Isp	wel	Tis	0
G35A	G12D	M	P	30	0-Isp	wel	T1	I
G35A	G12D	F	P	45	0-IIa	wel	Tis	0
G38A	G13D	M	P	80	2	wel	T3	II
G38A	G13D	F	P	35	2	mod	T3	III
G38A	G13D	F	D	60	2	mod	T3	III
G38A	G13D	F	D	45	2	mod	T3	III
G38A	G13D	M	D	8	0-Isp	wel	Tis	0
G38A	G13D	F	D	32	0-IIa	wel	Tis	0
G38A	G13D	M	P	34	0-IIa	wel	Tis	0
G34T	G12C	M	D	30	2	wel	T3	II
G34T	G12C	F	D	50	2	mod	T3	IV
G34A	G12S	F	D	45	2	mod	T3	IV
G34A	G12S	M	P	26	0-IIa	wel	T1	I
G37T	G13C	F	D	25	IIa	TA[c]	-	-
G34C	G12R	M	D	11	Ip	TVA[d]	-	-

[a] Histological typing, depth of invasion and clinical stage were determined according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (1998)[14] along with the International Union Against Cancer classification.[15]

[b] SA, serrated adenoma. [c] TA, tubular adenoma. [d] TVA, tubulovillous adenoma.

TABLE IV - Relationship of *BRAF* and K-*ras* mutations with clinicopathological parameters

	BRAF mutation	K-*ras* mutation	Wild type	*P*-value[b]
Total	7	24	85	
Location[a]	Proximal	4	10	32
Distal	3	14	53	0.5073
Gender				
male	4	14	56	
female	3	10	29	0.5794
Age	62.4 ± 3.2	60.8 ± 2.5	62.5 ± 1.3	0.8072
Tumor size (mm)	28.8 ± 11.2	35.7 ± 3.6	22.6 ± 2.4	0.0042
Histology[a]	adenoma	1	2	31
adenocarcinoma	6	22	54	0.0066
Depth of invasion[a]	Tis, T1	3	7	22
T2, T3, T4	3	13	25	0.6642
Invasion metastasis[a]	Lymphatic vessels	2	8	22
Venous vessels	2	8	13	0.6770
Lymph nodes	2	7	4	0.0459
Clinical stage[a]	0, I, II	3	12	41
III, IV	3	8	5	0.0076

[a] Histological typing, depth of invasion and clinical stage were determined according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus (1998)[14] along with the International Union Against Cancer classification.[15] [b] A level of $P < 0.05$ was taken to be statistically significant.

FIGURE LEGENDS

FIGURE 1

DNA sequence analysis of colon cancer cell lines with *BRAF* or K-*ras* mutations. *A*, WiDr colon cancer cell line with V599E (T1796A) mutation of *BRAF* gene. *B*, LoVo colon cancer cell line with G13D (G38A) mutation of K-*ras* gene. The normal sequences of *BRAF* and K-*ras* are shown on the right.

FIGURE 2

The growth-inhibitory and apoptosis-inducing effects of FTI-277 in human colon cancer cells. *A*, WST-1 absorbance of each colon cancer cell line treated in the absence of FBS (24 hours) was regarded as 100 %. * Significant difference between WiDr and LoVo was observed ($p < 0.0001$). *B*, Western blot analysis of the levels of phosphorylated MEK and ERK in LoVo and WiDr cells. The cells were treated with FTI-277 (0–20 μM) in the absence of FBS for 24 hours at 37°C. *C*, Induction of apoptosis by FTI-277 in LoVo and WiDr cells. Apoptotic bodies in LoVo cells were increased in a concentration–dependent manner. *D*, Apoptotic DNA fragmentation in LoVo and WiDr cells. An apoptotic DNA ladder was detected only in LoVo cells by FTI-277.

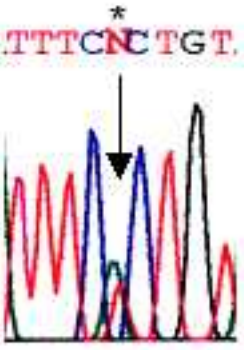
FIGURE 3

Results of PCR-SSCP and direct sequencing analysis of exons 11 and 15 of the *BRAF* gene. *A*, Representative result of PCR-SSCP analysis in tumors with *BRAF* mutation at codon 599. In direct sequencing analysis, a T-to-A transition at nucleotide 1796 of the *BRAF* gene in the tumor sample was detected. *B*, Representative result of PCR-SSCP analysis in tumors with *BRAF* mutation at codon 468. In direct sequencing analysis, a G-to-T transition at nucleotide 1403 of the *BRAF* gene in the tumor sample was detected.

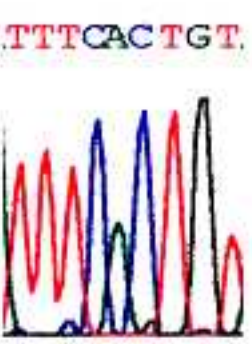
FIGURE 4

Relationship between *BRAF* or K-*ras* mutations, proliferative activity, and induction of apoptosis. *A*, Apoptosis was confirmed by immunohistochemical staining of single-stranded DNA (ssDNA). a, Colorectal carcinoma with *BRAF* mutation. b, Colorectal carcinoma with K-*ras* mutation. c, Wild type. *B*, Ki-67 labeling index (mean ± SD) was determined by counting the positive cells (%) in a total of 1000 in 10 representative high-power fields (**x** 400). *C*, Apoptotic body index was determined by counting the ssDNA-positive cells in a total of 10 high-power fields (**x** 400).

A

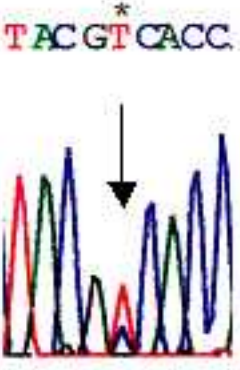


T*1796* A
(V599E)

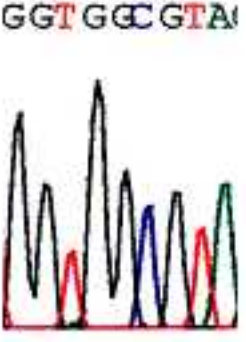


Wild-type

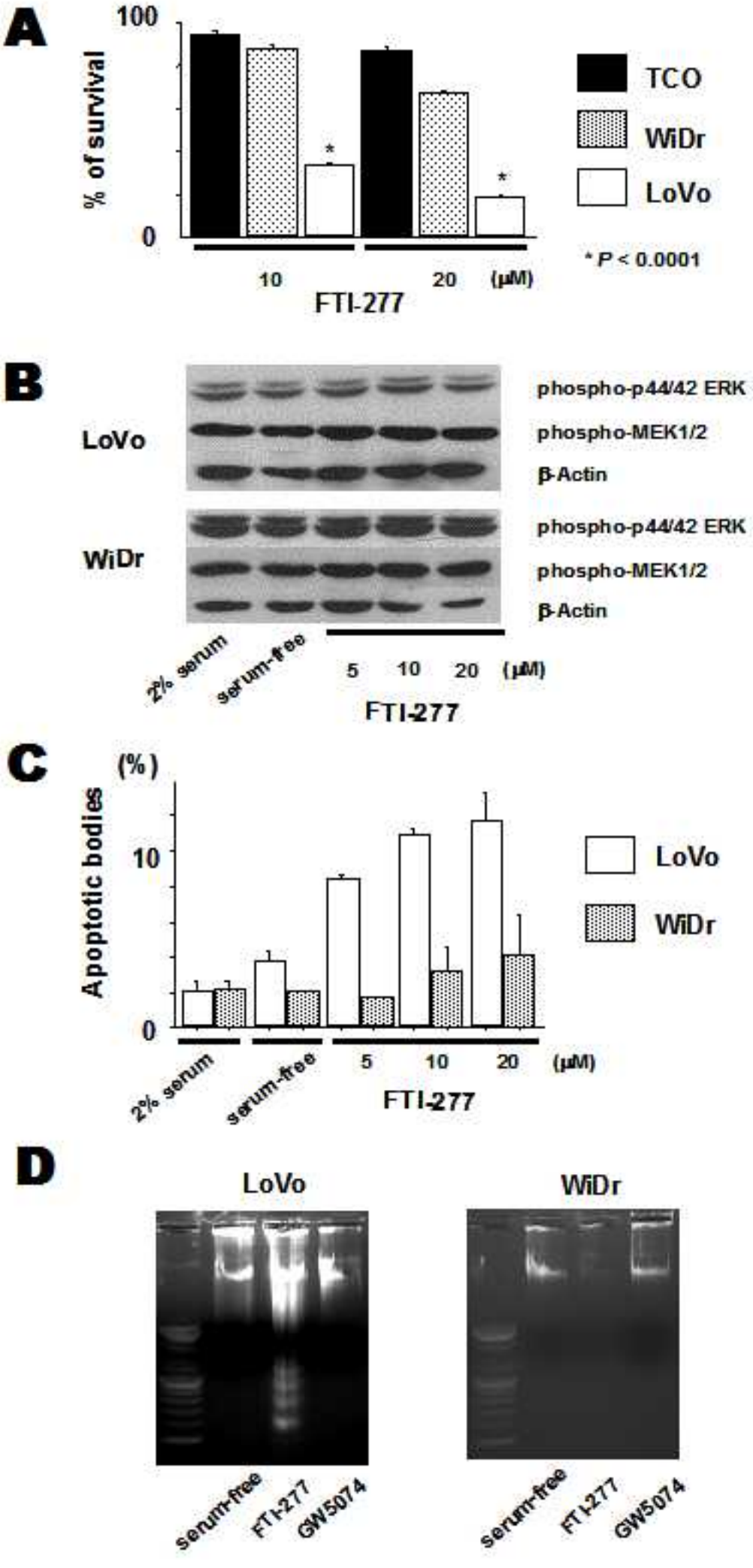
B

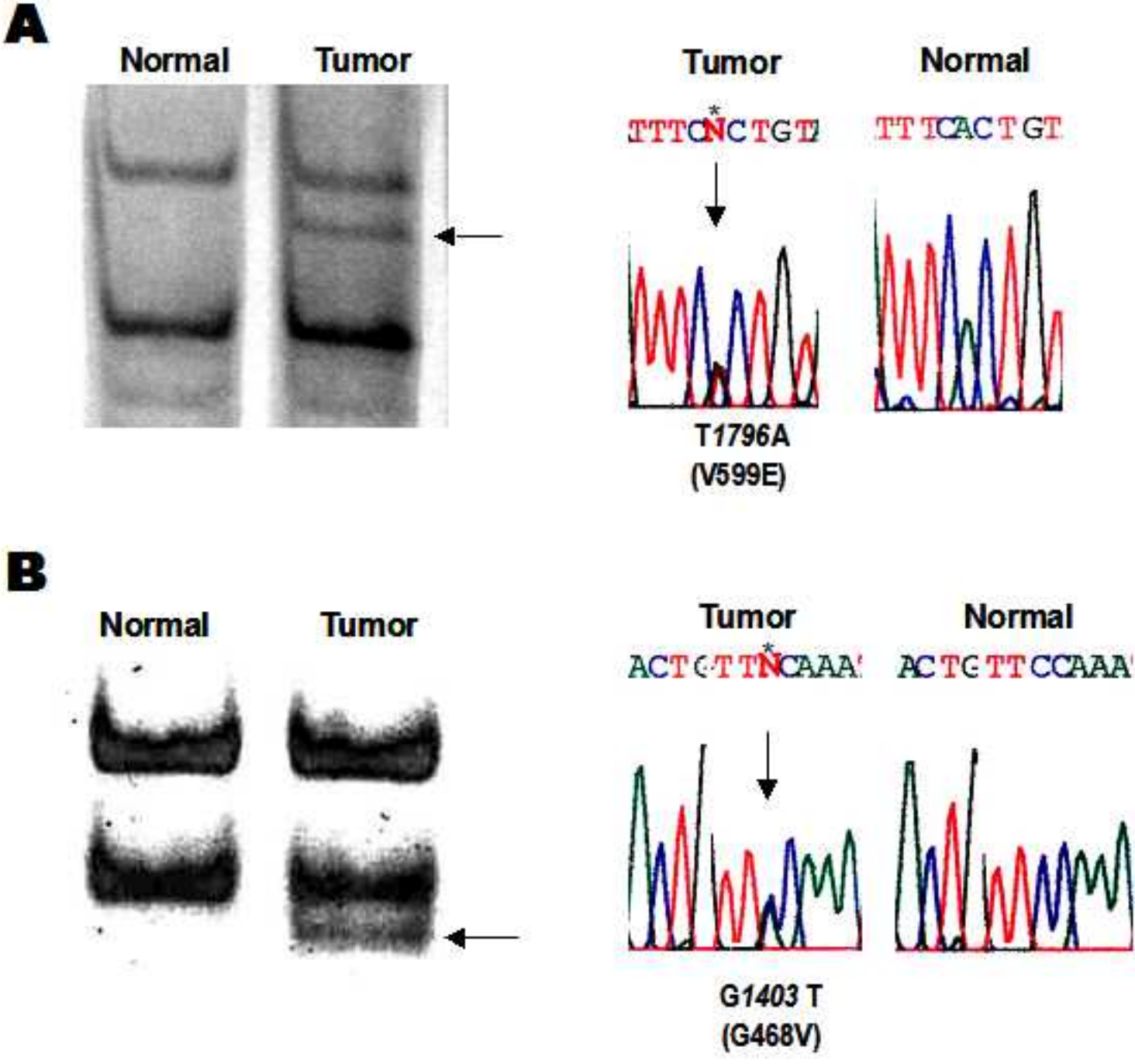


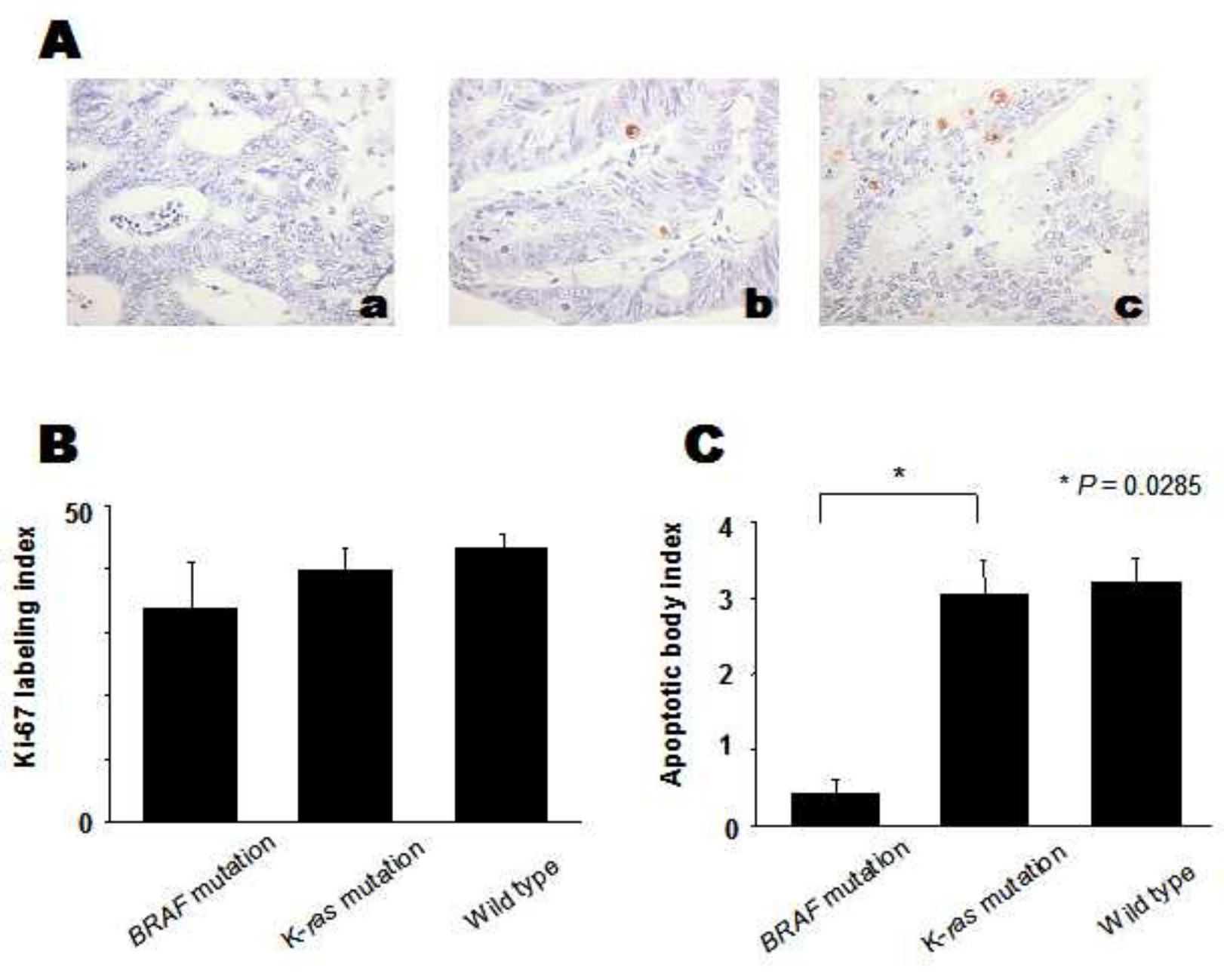
G*38*A
(G13D)



Wild-type


A
100
0
% of survival
*
*
TCO
WiDr
LoVo
* P < 0.0001
10
20
(μM)
FTI-277
B
LoVo
phospho-p44/42 ERK
phospho-MEK1/2
β-Actin
WiDr
phospho-p44/42 ERK
phospho-MEK1/2
β-Actin
2% serum
serum-free
5
10
20
(μM)
FTI-277
C
(%)
Apoptotic bodies
10
0
LoVo
WiDr
2% serum
serum-free
5
10
20
(μM)
FTI-277
D
LoVo
WiDr
serum-free
FTI-277
GW5074
serum-free
FTI-277
GW5074


A
Normal
Tumor
Tumor
Normal
T1796A
(V599E)
B
Normal
Tumor
Tumor
Normal
G1403 T
(G468V)


A
a
b
c
B
Ki-67 labeling index
50
0
BRAF mutation
K-ras mutation
Wild type
C
* P = 0.0285
*
Apoptotic body index
4
3
2
1
0
BRAF mutation
K-ras mutation
Wild type