Original article

***BRAF/K-ras* mutation, microsatellite instability, and promoter hypermethylation of *hMLH1/MGMT* in human gastric carcinomas**

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Abstract

Background. The *BRAF* and *K-ras* genes are the most frequently mutated oncogenes in various human malignancies. We examined *BRAF* and *K-ras* mutations in human gastric cancer, and investigated their relationship with microsatellite instability (MSI) and the hypermethylation of promoter regions in *hMLH1* and *O*⁶-methylguanine DNA methyltransferase (*MGMT*).

Methods. Sixteen gastric cancer cell lines and 62 gastric cancer tissue samples were screened for *BRAF* and *K-ras* mutations by direct sequencing. We also performed a microsatellite assay and investigated methylation status in the promoter regions of *hMLH1* and *MGMT*.

Results. *BRAF* mutation was not found in any of the cancer cell lines examined. One (1.6%) cancer tissue sample showed a point mutation in the *BRAF* gene (GTG → GAG; V599E). *K-ras* mutation (GGT → GAT, G12D) was detected in five (31%) gastric cancer cell lines and in 1 (1.6%) gastric cancer tissue sample. In the gastric cancer tissue samples examined, MSI was detected in 23 (37%) samples. Hypermethylated promoter regions in *hMLH1* and *MGMT*, respectively, were detected in 6 (10%) and 13 (21%) gastric cancer tissue samples. Microsatellite stable (MSS) tumors showed frequent lymphatic invasion ($P = 0.050$).

Conclusion. Although *BRAF* mutation has been reported in a variety of other human cancers, it is a rare event in the carcinogenesis and progression/development of gastric cancer.

Key words Gastric cancer · *BRAF* · *K-ras* · Microsatellite instability (MSI)

Introduction

Ras genes are the most frequently mutated oncogenes in human cancer [1,2]. The vast majority of *Ras* muta-

tions found in human disease occur in *K-ras*, with mutations in *H-ras* being quite rare [3]. Activating point mutations usually occur in codons 12 and 13 [2], and a high frequency of codon 61 *K-ras* point mutations in lung and Harderian gland neoplasms of B6C3F1 mice exposed to chloroprene was also reported [4]. Recently, mutations in *BRAF*, a member of the *RAF* family of genes, were reported in human malignancies such as colon cancer and melanoma cells [5,6]. Almost all *BRAF* mutations have been reported within two kinase domains (G-loop domain and kinase domain), and the most common mutation is a single substitution, V599E [5–8]. *BRAF* protein plays a central role in the classical RAS/RAF/MEK/ERK pathway, acting to relay signals from activated RAS proteins [9,10]. It has been reported that dysfunction of RAS protein is not required for the growth of cancer cells with *BRAF* mutation [5].

Hypermethylation of cytosine guanine dinucleotides (CpG) islands is a common change in human cancers, and is associated with the silencing of various tumor suppressor genes in their promoters [11–13]. Wang et al. [14] reported that, in colorectal cancer, *BRAF* mutation was always found in tumors with microsatellite instability (MSI), and these tumors were found most often with an abnormality of *hMLH1* and with hypermethylation of the *hMLH1* promoter. Koinuma et al. [15] suggested that *BRAF* activation may participate in the carcinogenesis of sporadic colorectal cancers with *hMLH1* hypermethylation in the proximal colon, independently of *K-ras* activation. However, *BRAF* mutation in gastric cancer is rare [16–18], and Zhao et al. [19] suggested that was not associated with MSI. *O*⁶-Methylguanine DNA methyltransferase (*MGMT*) is a DNA repair protein that removes mutagenic and cytotoxic adducts from the *O*⁶-position of guanine. *O*⁶-Methylguanine is broken down to adenine by DNA polymerases, thus leading to the frequent generation of G-to-A transitions in *K-ras* [20].

In the present study, to discover whether alterations in *BRAF* and *K-ras* might play a role in stomach carcinogenesis, we analyzed the occurrence of *BRAF* and *K-ras* mutations in gastric cancer cell lines and tissues. We also studied the microsatellite status and hypermethylation of promoter regions in *hMLH1* and *MGMT* to find whether these factors were related to mutations in the *BRAF* and *K-ras* genes.

Subjects, materials, and methods

Samples and DNA extraction

Sixteen gastric cancer cell lines were used in this study. Ten gastric cell lines of the HSC series (HSC-39, -41, -42, -44PE, -58, -60, -57, -59, -64, and SH101-P4) were provided by Dr. K. Yanagihara (National Cancer Center, Tokyo, Japan) [21,22], and 5 human gastric carcinoma cell lines of the MKN series (MKN-1, -7, -28, -45, and -74) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima, Japan) [23]. The TMK-1 gastric carcinoma cell line was established from poorly differentiated adenocarcinoma of the stomach by Ochiai et al. [24]. The original histological type of HSC-39, -44PE, -57, -58, -59, -60, and -64; MKN-45 and -74; and TMK-1 is diffuse type of gastric cancer, and that of HSC-41 and -42, and SH101-P4, as well as MKN-1, -7, and -28, is intestinal type of gastric cancer. DNA was extracted with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Sixty-two primary gastric cancer samples were obtained from patients undergoing surgery at Hiroshima University Hospital (Hiroshima, Japan). Tumor tissues and normal control tissues were immediately frozen in liquid nitrogen after operation, and stored at -80°C until use. Informed consent was obtained from all patients, with the approval of the local ethics committee (approval number 23). Genomic DNAs in this tumor series were extracted as described previously [25]. We confirmed, microscopically, that the tumor specimens consisted mainly (>80%) of carcinoma tissue. Clinicopathological information was obtained from medical charts, and histopathological examination was performed according to the *Japanese classification of gastric carcinoma* [26].

Direct sequencing analyses

BRAF exons 11 and 15 and *K-ras* exons 2 and 3, were amplified by polymerase chain reaction (PCR), using the following primer sets: *BRAF*-exon 11, 5'-TCCCTC TCAGGCATAAGGTAA-3' (sense)/5'-CGAACAG TGAATATTTCTTTGAT-3' (antisense); *BRAF*-exon 15, 5'-TCATAATGC TTGCTCTGATAGGA-3'

(sense)/5'-GGCCAAAAATTTAATCAGTGGA-3' (antisense); *K-ras*-exon 2, 5'-GTGTGACATGTTC TAATATAGTCA-3' (sense)/5'-GAATGGTCCTGC ACCAGTAA-3' (antisense); and *K-ras*-exon 3, 5'-TCAAGTCCTTTGCCCATTTT-3' (sense)/5'-TGCA TGGCATTAGCAAAGAC-3' (antisense). Polymerase chain reaction (PCR) was performed in total 15- μl reaction volumes containing 1 μl template DNA; 0.56 μM of each primer; 74.7 μM each of dATP, dGTP, dCTP, and dTTP; 4.5 mM of MgCl_2 ; and 0.075 units of *AmpliTaq Gold* (Applied Biosystems, Foster City, CA, USA). The PCR amplification was used with "hot-start PCR" and consisted of 35 cycles (94°C for 30s, at 58°C [*BRAF* exons 11 and 15 and *K-ras* exon 2] or 64°C [*K-ras* exon 3] for 30s, 72°C for 30s) after the initial *Taq* DNA polymerase activation step (95°C for 12min), followed by a final extension for 3 min at 72°C .

PCR products were sequenced using each sense primer and the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready Reaction (Applied Biosystems) and an automated DNA sequencer. Mutations in these genes were confirmed by sequencing reaction using each antisense primer. All experiments were duplicated precisely.

Microsatellite analyses

Five microsatellite markers (D1S191, D5S346, D17S250, BAT-25, and BAT-26) were analyzed [27]. The forward primers were fluorescein-labeled with [6-FAM] (D1S191, D17S250, and BAT-26); [VIC] (D5S346); and [TAMRA] (BAT-25). PCR was performed as described above. The PCR amplification consisted of 35 cycles (94°C for 30s, 55°C for 30s, 72°C for 30s) after the initial *Taq* DNA polymerase activation step (95°C for 10min), followed by a final extension for 10min at 72°C . PCR products were electrophoresed in an ABI PRISM 310 Genetic Analyzer, along with the GeneScan-500 [ROX] molecular weight standard (Applied Biosystems). The size of the PCR product was analyzed using GeneScan software (Applied Biosystems). We classified the MSI status in each tumor according to the criteria of Boland et al. [27]: microsatellite stable (MSS), MSI was not observed in any microsatellite locus examined; low frequency of MSI (MSI-L), one of five loci revealed MSI; high frequency of MSI (MSI-H), two or more microsatellite loci showed MSI.

Bisulfite PCR and methylation-specific PCR (MSP)

To examine the DNA methylation patterns, we treated genomic DNA with sodium bisulfite, as described by Herman et al. [28] and Esteller et al. [29]. For the analysis of DNA methylation of *MGMT*, we performed MSP.

Table 1. Mutations of *BRAF* and *K-ras* in gastric cancer cell lines

Cell line	<i>BRAF</i> point mutation		<i>K-ras</i> point mutation	
	Exon 11	Exon 15	Exon 2	Exon 3
HSC-39	—	—	—	—
HSC-41	—	—	G216A /G12D	—
HSC-42	—	—	G216A /G12D	—
HSC-44PE	—	—	G216A /G12D	—
HSC-57	—	—	G216A /G12D	—
HSC-58	—	—	—	—
HSC-59	—	—	—	—
HSC-60	—	—	—	—
HSC-64	—	—	—	—
SH101-P4	—	—	G216A /G12D	—
MKN-1	—	—	—	—
MKN-7	—	—	—	—
MKN-28	—	—	—	—
MKN-45	—	—	—	—
MKN-74	—	—	—	—
TMK-1	—	—	—	—

For the analysis of DNA methylation of *hMLH1*, we performed bisulfite-PCR, followed by restriction digestion, as previously described elsewhere [25,30]. The PCR products were loaded onto 8% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light.

Statistical analyses

Associations between the MSI and the methylation status of the *hMLH1* and *MGMT* gene promoter regions, and the relationship between the MSI status and clinicopathological factors, were assessed by the χ^2 test. A *P* value of less than 0.05 was regarded as statistically significant.

Results

Point mutation of *BRAF* and *K-ras*

We first examined the gastric cancer cell lines and tissue samples for the presence of point mutations in *BRAF* exons 11 and 15 and *K-ras* exons 2 and 3 by direct sequencing. No *BRAF* point mutation was found in any of the gastric cancer cell lines examined (Table 1). One (1.6%) gastric cancer tissue sample (A771: por2, T3, N2, stage III) showed *BRAF* point mutation (GTG → GAG at codon 599, Val → Glu) (Fig. 1A). The frequency of *BRAF* mutations in the gastric cancer cell lines and tissues were 0% and 1.6%, respectively. On the other hand, HSC-41, -42, -44PE, -57, and SH101-P4 (Table 1) and one (1.6%) gastric cancer tissue sample (H205: tub1, T2, N0, stage I) (Fig. 1B) had a *K-ras* point muta-

tion (GGT → GAT at codon 12, Gly → Asp). Two of the ten (20%) cell lines originating from diffuse-type gastric cancer and three of the six (50%) cell lines originating from intestinal-type gastric cancer had a *K-ras* mutation.

Microsatellite analyses in gastric cancer tissue samples

We performed microsatellite assays in the gastric cancer tissue samples, using five microsatellite markers. Results are shown in Table 2. Overall, MSI was detected in 23 (37%) of the 62 samples. MSI-H in 14 (23%), and MSI-L in 9 (14%). Of note, MSI was frequently observed at the following loci: D1S191 (18 samples; 29%); BAT 25 (11 samples; 18%); and D5S346 (10 samples; 16%). The sample with the *BRAF* point mutation (A771) showed MSS, while the sample with the *K-ras* point mutation (H205) demonstrated MSI-H.

When we analyzed the relationship between MSI status and clinicopathological factors (Table 3), gastric cancers with MSS showed frequent lymphatic invasion (*P* = 0.050).

Methylation status of *hMLH1* and *MGMT* in gastric cancers

In the 62 gastric cancer tissue samples, DNA hypermethylation was detected at the following frequencies: 6 (10%) for *hMLH1* and 13 (21%) for *MGMT* (Table 2). Of the 6 samples with *hMLH1* hypermethylation, 3 were associated with MSI (MSI-H, 2 samples; MSI-L, 1 sample). The sample with *BRAF* mutation (A771) demonstrated MSS, and did not have

Fig. 1A,B. *BRAF* and *K-ras* mutations in gastric carcinomas. **A** The gastric cancer tissue sample with *BRAF* mutation (A771). A point mutation at codon 599 was detected in the cancer lesion (GTG → GAG). **B** Gastric cancer tissue with *K-ras* mutation and microsatellite instability (MSI; H205). A point mutation at codon 12 was detected in the cancer lesion (GGT → GAT)

hypermethylation in the promoter region of *hMLH1* or *MGMT*. Although MSI-H was detected in the sample with *K-ras* mutation (H205), no hypermethylation was detected in the promoter regions of *hMLH1* or *MGMT*.

No significant correlation was observed between clinicopathological factors and hypermethylation in the promoter regions of *hMLH1* or *MGMT* in the present series of gastric carcinomas.

Discussion

Recently, *BRAF* mutations have been found in a wide range of human cancers [5], especially in melanoma cell lines (59%) and tissues (56%–68%) [5,31,32], thyroid cancer cell lines (80%) and tissues (10%–45%) [33,34], and colorectal cancer cell lines (7%) and tissues (5.1%–22%) [14,15,18,35]. On the other hand, *BRAF* mutation was infrequent (1.6%) in the gastric cancer tissues in our study; a finding that is compatible with those reported previously (0–2.2%) [16–19]. No information has been available up to now regarding *BRAF* mutation in gastric cancer cell lines. We could not detect any *BRAF* mutations, in exons 11 and 15, in the 16 gastric cancer cell lines studied.

The frequency of *K-ras* mutation was reported previously to be 70%–100%, 7%–80%, and 10%–48% in pancreatic, colorectal, and lung cancer tissues, respectively [35–38]. In gastric cancer, the frequency of *K-ras* mutation was reported to be 2.8%–20% [17,19,39,40]. In the present study, *K-ras* mutation was also infrequent

(1/62; 1.6%) in gastric cancer tissues. In contrast, the frequency of *K-ras* mutation in gastric cancer cell lines was higher (31%) in this study than in those reported previously (19%) [16]. Miki et al. [41] reported that the intestinal type of gastric cancer had a higher frequency of *K-ras* mutations than diffuse-type tumors. We considered that the frequency of *K-ras* mutation was also higher in cell lines originating from intestinal-type gastric cancer than in originating from the diffuse type.

Ten percent of the gastric cancer tissues in this study showed hypermethylation in the promoter region of *hMLH1*, which was slightly lower than the frequencies reported previously (14%–37%) [25,42–44]. The frequency of hypermethylation in the promoter of *MGMT* in the present study (21%) matched findings reported lately (16%–61%) [25,42,44–46]. In the present study, only 14% and 21% of MSI-H gastric cancers were associated with hypermethylation in the promoter regions of *hMLH1* and *MGMT*, respectively, findings that were obviously lower than those reported previously (37%–87.5%, and 61%, respectively) [42,43,47]. MSS tumors showed frequent lymphatic invasion, which may be related to a previous observation that MSS tumors tended to have a poorer prognosis than MSI ones [48,49].

Although mismatch repair (MMR)-deficient colorectal cancers have been reported to show a higher incidence of *BRAF* mutations and a lower incidence of *K-ras* mutations compared with MMR-containing colorectal cancers [6], we could not find any relationship between MMR deficiency and mutations of *BRAF* and

Table 2. Status of MSI and methylation in promoter regions of *hMLH1* and *MGMT* in gastric cancer tissue samples

Sample identification	<i>BRAF</i> mutation	<i>K-ras</i> mutation	Microsatellite status	<i>hMLH1</i> hypermethylation status	<i>MGMT</i> hypermethylation status
A517	—	—	MSI-H	—	+
A803	—	—	MSI-H	—	—
G102	—	—	MSI-H	—	—
G109	—	—	MSI-H	+	—
G209	—	—	MSI-H	—	+
H204	—	—	MSI-H	—	—
H205	—	G12D	MSI-H	—	—
H206	—	—	MSI-H	—	+
H208	—	—	MSI-H	—	—
K108	—	—	MSI-H	—	—
K111	—	—	MSI-H	—	—
K116	—	—	MSI-H	+	—
K202	—	—	MSI-H	—	—
R102	—	—	MSI-H	—	—
A769	—	—	MSI-L	—	—
G105	—	—	MSI-L	—	+
G106	—	—	MSI-L	—	—
G202	—	—	MSI-L	—	—
G210	—	—	MSI-L	+	—
H203	—	—	MSI-L	—	—
H207	—	—	MSI-L	—	—
H210	—	—	MSI-L	—	—
K101	—	—	MSI-L	—	—
A518	—	—	MSS	—	—
A519	—	—	MSS	—	—
A529	—	—	MSS	—	—
A531	—	—	MSS	—	—
A734	—	—	MSS	—	+
A741	—	—	MSS	—	—
A768	—	—	MSS	—	+
A771	V599E	—	MSS	—	—
A774	—	—	MSS	—	+
A776	—	—	MSS	—	—
A777	—	—	MSS	—	—
A778	—	—	MSS	—	—
G103	—	—	MSS	—	—
G104	—	—	MSS	—	+
G107	—	—	MSS	—	+
G108	—	—	MSS	—	—
G110	—	—	MSS	—	—
G112	—	—	MSS	—	—
G114	—	—	MSS	—	—
G201	—	—	MSS	—	—
G212	—	—	MSS	—	+
H201	—	—	MSS	+	—
H202	—	—	MSS	—	—
H209	—	—	MSS	—	—
H211	—	—	MSS	—	—
H212	—	—	MSS	—	+
J201	—	—	MSS	—	+
K113	—	—	MSS	—	—
K118	—	—	MSS	—	+
K201	—	—	MSS	+	—
K206	—	—	MSS	—	—
K208	—	—	MSS	—	—
R101	—	—	MSS	+	—
R103	—	—	MSS	—	—
R104	—	—	MSS	—	—
S202	—	—	MSS	—	—
S203	—	—	MSS	—	—
S204	—	—	MSS	—	—
A8086	—	—	MSS	—	—

Table 3. Relationship of clinicopathological parameters of adenocarcinoma of the stomach to MSI and MSS

	MSI status						<i>P</i> value ^b
	Total		MSI		MSS		
	<i>n</i>	(%)	<i>n</i>	(%)	<i>n</i>	(%)	
Total	62	(100.0)	23	(37.0)	39	(63.0)	
Histological type							<i>P</i> = 0.320
tub1	9	(14.5)	2	(8.7)	7	(17.9)	
tub2	17	(27.4)	8	(34.8)	9	(23.1)	
pap	7	(11.3)	4	(17.4)	3	(7.7)	
por1	9	(14.5)	5	(21.7)	4	(10.3)	
por2	16	(25.8)	3	(13.0)	13	(33.3)	
sig	3	(4.8)	1	(4.3)	2	(5.1)	
und	1	(1.6)	0	(0.0)	1	(2.6)	
Depth of invasion ^a							<i>P</i> = 0.462
T1 (M, SM)	3	(4.8)	1	(4.3)	2	(5.1)	
T2 (MP, SS)	27	(43.5)	13	(56.5)	14	(35.9)	
T3 (SE)	29	(46.8)	8	(34.8)	21	(53.8)	
T4 (SI)	3	(4.8)	1	(4.3)	2	(5.1)	
Lymphatic invasion							<i>P</i> = 0.050
ly (−)	7	(11.3)	5	(21.7)	2	(5.1)	
ly (+)	55	(88.7)	18	(78.3)	37	(94.9)	
Lymph node metastasis ^a							<i>P</i> = 0.780
N0	19	(30.6)	8	(34.8)	11	(28.2)	
N1	14	(22.6)	5	(21.7)	9	(23.1)	
N2	17	(27.4)	7	(30.4)	10	(25.6)	
N3	12	(19.4)	3	(13.0)	9	(23.1)	
Venous invasion							<i>P</i> = 0.356
v (−)	10	(16.1)	5	(21.7)	5	(12.8)	
v (+)	52	(83.9)	18	(78.3)	34	(87.2)	
Liver metastasis							<i>P</i> = 0.605
H0	58	(93.5)	22	(95.7)	36	(92.3)	
H1	4	(6.5)	1	(4.3)	3	(7.7)	
Peritoneal metastasis							<i>P</i> = 0.841
P0	56	(90.3)	21	(91.3)	35	(89.7)	
P1	6	(9.7)	2	(8.7)	4	(10.3)	
Other distant metastases							<i>P</i> = 0.409
M0	57	(91.9)	22	(95.7)	35	(89.7)	
M1	5	(8.1)	1	(4.3)	4	(10.3)	
Tumor stage ^a							<i>P</i> = 0.922
I	16	(25.8)	6	(26.1)	10	(25.6)	
II	7	(11.3)	3	(13.0)	4	(10.3)	
III	22	(35.5)	7	(30.4)	15	(38.5)	
IV	17	(27.4)	7	(30.4)	10	(25.6)	

^aDepth of invasion, lymph node metastasis, and tumor stage were evaluated according to the criteria of the *Japanese classification of gastric carcinoma* [26]

^bStatistical analysis was performed by the χ^2 test. A *P* value of less than 0.05 was regarded as significant

K-ras in gastric carcinomas. Moreover, no connection was shown between *K-ras* mutation and hypermethylation of the promoter region in *MGMT* in gastric cancer. These findings indicate that there may be differences between gastric and colorectal carcinomas in terms of their tumorigenesis. Further investigation will be required to clarify this point.

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References

- Barbacid M. *ras* genes. *Annu Rev Biochem* 1987;56:779–827.
- Bos JL. *ras* oncogenes in human cancer: a review. *Cancer Res* 1989;49:4682–9.
- Ellis CA, Clark G. The importance of being K-*Ras*. *Cell Signal* 2000;12:425–34.
- Sills RC, Hong HL, Melnick RL, Boorman GA, Devereux TR. High frequency of codon 61 K-*ras* A→T transversions in lung and Harderian gland neoplasms of B6C3F1 mice exposed to chloroprene (2-chloro-1,3-butadiene) for 2 years, and comparisons with the structurally related chemicals isoprene and 1,3-butadiene. *Carcinogenesis* 1999;20:657–62.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the *BRAF* gene in human cancer. *Nature* 2002;417:949–54.
- Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: *RAF/RAS* oncogenes and mismatch-repair status. *Nature* 2002;418:934.
- Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Guerrero R, et al. *BRAF* and *RAS* mutations in human lung cancer and melanoma. *Cancer Res* 2002;62:6997–7000.
- Naoki K, Chen TH, Richards WG, Sugarbaker DJ, Meyerson M. Missense mutations of the *BRAF* gene in human lung adenocarcinoma. *Cancer Res* 2002;62:7001–3.
- Marais R, Marshall CJ. Control of the ERK MAP kinase cascade by Ras and Raf. *Cancer Surv* 1996;27:101–25.
- Marshall CJ. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet Dev* 1994;4:82–9.
- Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? *Trends Genet* 1997;13:444–9.
- Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16/CDKN2/MTS1* in human cancers. *Nat Med* 1995;1:686–92.
- Razin A, Cedar H. DNA methylation and gene expression. *Microbiol Rev* 1991;55:451–8.
- Wang L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, et al. *BRAF* mutations in colon cancer are not likely attributable to defective DNA mismatch repair. *Cancer Res* 2003;63:5209–12.
- Koinuma K, Shitoh K, Miyakura Y, Furukawa T, Yamashita Y, Ota J, et al. Mutations of *BRAF* are associated with extensive *hMLH1* promoter methylation in sporadic colorectal carcinomas. *Int J Cancer* 2004;108:237–42.
- Kim IJ, Park JH, Kang HC, Shin Y, Park HW, Park HR, et al. Mutational analysis of *BRAF* and *K-ras* in gastric cancers: absence of *BRAF* mutations in gastric cancers. *Hum Genet* 2003; 114:118–20.
- Lee SH, Lee JW, Soung YH, Kim HS, Park WS, Kim SY, et al. *BRAF* and *KRAS* mutations in stomach cancer. *Oncogene* 2003; 22:6942–5.
- Oliveira C, Pinto M, Duval A, Brennetot C, Domingo E, Espin E, et al. *BRAF* mutations characterize colon but not gastric cancer with mismatch repair deficiency. *Oncogene* 2003;22:9192–6.
- Zhao W, Chan TL, Chu KM, Chan AS, Stratton MR, Yuen ST, et al. Mutations of *BRAF* and *KRAS* in gastric cancer and their association with microsatellite instability. *Int J Cancer* 2004;108: 167–9.
- Mitra G, Pauly GT, Kumar R, Pei GK, Hughes SH, Moschel RC, et al. Molecular analysis of *O6*-substituted guanine-induced mutagenesis of *ras* oncogenes. *Proc Natl Acad Sci U S A* 1989;86: 8650–4.
- Yanagihara K, Seyama T, Tsumuraya M, Kamada N, Yokoro K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the *c-myc* oncogene. *Cancer Res* 1991;51:381–6.
- Yanagihara K, Ito A, Toge T, Numoto M. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res* 1993;53:5815–21.
- Hojo H. Establishment of cultured cell lines of human stomach cancer—origin and their morphological characteristics. *Niigata Igaku Zasshi (Niigata Med J)* 1977;91:737–52.
- Ochiai A, Yasui W, Tahara E. Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. *Jpn J Cancer Res* 1985;76:1064–71.
- Oue N, Oshimo Y, Nakayama H, Ito R, Yoshida K, Matsusaki K, et al. DNA methylation of multiple genes in gastric carcinoma: association with histological type and CpG island methylator phenotype. *Cancer Sci* 2003;94:901–5.
- Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma. 2nd English ed. *Gastric Cancer* 1998;1:10–24.
- Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 1998;58:5248–57.
- Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996;93:9821–6.
- Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene *O⁶-methylguanine-DNA methyltransferase* by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999;59:793–7.
- Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, et al. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999;59:5438–42.
- Kumar R, Angelini S, Czene K, Sauroja I, Hahka-Kemppinen M, Pyrhonen S, et al. *BRAF* mutations in metastatic melanoma: a possible association with clinical outcome. *Clin Cancer Res* 2003; 9:3362–8.
- Uribe P, Wistuba II, Gonzalez S. *BRAF* mutation: a frequent event in benign, atypical, and malignant melanocytic lesions of the skin. *Am J Dermatopathol* 2003;25:365–70.
- Xu X, Quiros RM, Gattuso P, Ain KB, Prinz RA. High prevalence of *BRAF* gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. *Cancer Res* 2003;63:4561–7.
- Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, et al. *BRAF* mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. *J Clin Endocrinol Metab* 2003;88:5399–404.
- Yuen ST, Davies H, Chan TL, Ho JW, Bignell GR, Cox C, et al. Similarity of the phenotypic patterns associated with *BRAF* and *KRAS* mutations in colorectal neoplasia. *Cancer Res* 2002;62: 6451–5.
- Lohr M, Maisonneuve P, Lowenfels AB. *K-Ras* mutations and benign pancreatic disease. *Int J Pancreatol* 2000;27:93–103.
- Fong KM, Zimmerman PV, Smith PJ. *KRAS* codon 12 mutations in Australian non-small cell lung cancer. *Aust N Z J Med* 1998;28:184–9.
- Minamoto T, Mai M, Ronai Z. *K-ras* mutation: early detection in molecular diagnosis and risk assessment of colorectal, pancreas, and lung cancers—a review. *Cancer Detect Prev* 2000;24:1–12.
- Hongyo T, Buzard GS, Palli D, Weghorst CM, Amorosi A, Galli M, et al. Mutations of the *K-ras* and *p53* genes in gastric adenocarcinomas from a high-incidence region around Florence, Italy. *Cancer Res* 1995;55:2665–72.
- Lee KH, Lee JS, Suh C, Kim SW, Kim SB, Lee JH, et al. Clinicopathologic significance of the *K-ras* gene codon 12 point mutation in stomach cancer. An analysis of 140 cases. *Cancer* 1995;75:2794–801.

41. Miki H, Ohmori M, Perantoni AO, Enomoto T. *K-ras* activation in gastric epithelial tumors in Japanese. *Cancer Lett* 1991;58:107–13.
42. Carvalho B, Pinto M, Cirnes L, Oliveira C, Machado JC, Suriano G, et al. Concurrent hypermethylation of gene promoters is associated with a MSI-H phenotype and diploidy in gastric carcinomas. *Eur J Cancer* 2003;39:1222–7.
43. Fang DC, Wang RQ, Yang SM, Yang JM, Liu HF, Peng GY, et al. Mutation and methylation of *hMLH1* in gastric carcinomas with microsatellite instability. *World J Gastroenterol* 2003;9:655–9.
44. Oue N, Sentani K, Yokozaki H, Kitadai Y, Ito R, Yasui W. Promoter methylation status of the DNA repair genes *hMLH1* and *MGMT* in gastric carcinoma and metaplastic mucosa. *Pathobiology* 2001;69:143–9.
45. Oue N, Shigeishi H, Kuniyasu H, Yokozaki H, Kuraoka K, Ito R, et al. Promoter hypermethylation of *MGMT* is associated with protein loss in gastric carcinoma. *Int J Cancer* 2001;93:805–9.
46. Park TJ, Han SU, Cho YK, Paik WK, Kim YB, Lim IK. Methylation of *O(6)*-methylguanine-DNA methyltransferase gene is associated significantly with *K-ras* mutation, lymph node invasion, tumor staging, and disease free survival in patients with gastric carcinoma. *Cancer* 2001;92:2760–8.
47. Pinto M, Oliveira C, Machado JC, Cirnes L, Tavares J, Carneiro F, et al. MSI-L gastric carcinomas share the *hMLH1* methylation status of MSI-H carcinomas but not their clinicopathological profile. *Lab Invest* 2000;80:1915–23.
48. Samowitz WS, Curtin K, Ma KN, Schaffer D, Coleman LW, Leppert M, et al. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. *Cancer Epidemiol Biomarkers Prev* 2001;10:917–23.
49. Guidoboni M, Gafa R, Viel A, Doglioni C, Russo A, Santini A, et al. Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis. *Am J Pathol* 2001;159:297–304.