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

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Running tile: *BRAF* mutation in gastric cancer

Key words: gastric cancer, *BRAF*, K-*ras*, microsatellite instability (MSI)

Mini-abstract. *BRAF* mutation is rare in the carcinogenesis and progression/ development of the gastric cancer. *BRAF* and K-*ras* mutation did not correlate with either MSI or hypermethylation of *hMLH1* and *MGMT* promoters.

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Abstract

Background. *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 the relationship with microsatellite instability (MSI) and 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 with direct sequencing. We also performed microsatellite assay and investigated methylation status in promoter regions of *hMLH1* and *MGMT*.

Results. *BRAF* mutation was not found in cancer cell lines examined. One (1.6%) cancer tissue sample showed point mutation in *BRAF* gene (GTG-->GAG, V599E). K-*ras* mutation (GGT-->GAT, G12D) was detected in 5 (31%) gastric cancer cell lines and one (1.6%) gastric cancer tissue sample. In gastric cancer tissues examined, MSI was detected in 23 (37%) cases. Hypermethylated promoter regions were detected in 6 (10%) cases and 13 (21%) cases in *hMLH1* and *MGMT*, respectively. MSS (microsatellite stable) tumors showed frequent lymphatic invasion (P = 0.050).

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

Introduction

Ras genes are the most frequently mutated oncogenes in human cancer [1,2]. The vast majority of *Ras* mutations found in human disease occur in K-*ras*, with mutations in H-*ras* being quite rare [3]. Activating point mutations usually occur at codon 12 and 13 [2], and high frequency of codon 61 K-*ras* point mutation in lung and Harderian gland neoplasm of B6C3F1 mice exposed to chloroprene was also reported [4]. Recently, mutations in *BRAF*, a member of *RAF* family gene, 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 dyfunction of RAS protein is not required for the growth of cancer cells with *BRAF* mutation [5].

Hypermethylation of CpG islands is common changes in human cancers, and associated with silencing of various tumor suppressor genes in their promoters [11-13]. Wang et al. [14] reported that *BRAF* mutation was always found in tumors with microsatellite instability (MSI) in colorectal cancer and these tumors were found most often with an abnormality of hMLH1 and with hypermethylation of *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 it did not associate 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 considered as 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 the possibility that the alterations in *BRAF* and K-*ras* might play a role in stomach carcinogenesis, we analysed the occurrence of *BRAF* and K-*ras* mutations in gastric cancer cell lines and tissues. We also studied microsatellite status and hypermethylation of promoter regions in *hMLH1* and *MGMT* to know the correlation with *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]. TMK-1 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, -64, MKN-45, -74 and TMK-1 is diffuse type of gastric cancer and HSC-41, -42, SH101-P4, MKN-1, -7 and -28 is intestinal type of gastric cancer. DNA was extracted with Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Sixty-two primary gastric cancer samples were obtained from patients undergoing surgery in Hiroshima University Hospital (Hiroshima, Japan). Tumor tissues and normal control tissues were frozen immediately in liquid nitrogen after operation, and stored at --80°C until use. Informed consent was obtained from all patients under approval by local ethical committee (approval number 23). Genomic DNAs in this series of tumor 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 exon 11 and 15, K-*ras* exon 2 and 3, were amplified by PCR using the following primer sets: *BRAF* exon 11, 5'-TCCCTCTCAGGCATAAGGTAA-3' (sense)/

5'-CGAACAGTGAATATTTCCTTTGAT-3' (antisense), *BRAF*-exon 15, 5'-TCATAATGC TTGCTCTGATAGGA-3' (sense)/5'-GGCCAAAAATTTAATCAGTGGA-3' (antisense), K-*ras*-exon 2, 5'-GTGTGACATGTTCTAATATAGTCA-3' (sense)/5'- GAATGGTCCTGCA CCAGTAA-3' (antisense), K-*ras*-exon 3, 5'-TCAAGTCCTTTGCCCATTTT-3' (sense)/5'-TGCATGGCATTAGCAAAGAC-3' (antisense). Polymerase chain reaction (PCR) was performed in total 15-μl reaction volumes containing 1-μl template DNA, 0.56 μ*M* of each primer, 74.7 μ*M* each of dATP, dGTP, dCTP, dTTP, 4.5 m*M* of $MgCl_2$, 0.075 unit of Ampli*Taq* Gold (Applied Biosystems, Foster City, CA, USA). The PCR amplification was used with "hot-start PCR" and consisted of 35 cycles (94˚C for 30 s, 58˚C [*BRAF* exon 11, 15 and K-*ras* exon 2] or 64˚C [K-*ras* exon 3] for 30 s, 72˚C for 30 s) after the initial *Taq* DNA polymerase activation step (95˚C for 12 min), 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 30 s, 55˚C for 30 s, 72˚C for 30 s) after the initial *Taq* DNA polymerase activation step (95˚C for 10 min), followed by a final extension for 10 min at 72˚C. PCR products were electrophoresed in ABI PRISM 310 Genetic Analyzer along with GeneScan-500 [ROX] molecular weight standard (Applied Biosystems). The size of the PCR product was analysed using GeneScan software (Applied Biosystems). We classified the status of MSI in each tumor according to the criterion by Boland et al. [27]: microsatellite stable (MSS), MSI was not observed in any microsatellite locus examined; low frequency of MSI (MSI-L), one out of 5 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. [28,29]. For analysis of DNA methylation of *MGMT*, we performed MSP. For 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 methylation status of *hMLH1* and *MGMT* gene promoter regions, and relationship between MSI status and clinicopathological factors were assessed by the χ^2 test. A P value 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 presence of point mutations in *BRAF* exon 11, 15 and K-*ras* exon 2, 3 by direct sequencing. No *BRAF* point mutation was found in gastric cancer cell lines examined (Table 1). One (1.6%) gastric cancer tissue (A771: por2, T3, N2, stage III) showed *BRAF* point mutation (GTG-->GAG at codon 599, Val-->Glu) (Fig. 1A). Frequency of *BRAF* mutation in 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 (H205: tub1, T2,

N0, stage I) (Fig. 1B) had K-*ras* point mutation (GGT-->GAT at codon 12, Gly-->Asp). Two out of 10 (20%) cell lines originated from diffuse type gastric cancer and 3 of 6 (50%) cell lines from intestinal type gastric cancer had K-*ras* mutation.

Microsatellite analyses in gastric cancers

We performed microsatellite assay in these gastric cancer samples with 5 microsatellite markers. Results are shown in Table 2. Overall, MSI was detected in 23 (37%) of 62 cases: MSI-H (14 cases, 23%), MSI-L (9 cases, 14%). Especially, MSI was frequently observed at loci of D1S191 (18 cases, 29%), BAT 25 (11 cases, 18%) and D5S346 (10 cases, 16%). The case with *BRAF* point mutation (A771) showed MSS. While the case with 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.

Among these 62 gastric cancer tissues, DNA hypermethylation was detected in the following frequencies: 6 (10%) for *hMLH1*, 13 (21%) for *MGMT* (Table 2). Of 6 cases with *hMLH1* hypermethylation, three were accompanied with MSI (MSI-H, 2 cases;

MSI-L, 1 case). One case with *BRAF* mutation (A771) demonstrated stable microsatellite and did not have hypermethylation in the promoter of *hMLH1* or *MGMT*. Although MSI-H was detected in a case with K-*ras* mutation (H205), hypermethylation of *hMLH1* or *MGMT* was not detected in their promoter regions.

Significant correlation was not observed between clinicopathological factors and promoter hypermethylation of *hMLH1* or *MGMT* in the present series of gastric carcinomas.

Discussion

Recently, *BRAF* mutation was found in 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], 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 gastric cancer tissues in our study; that is conformable to those reported previously (0--2.2%) [16-19]. According to the *BRAF* mutation in gastric cancer cell lines, no information has been available up to now. We could not detect any *BRAF* mutation, exon 11 and 15, in 16 gastric cancer cell lines studied.

K-*ras* mutation frequency 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 those reported previously (19%) [16]. Miki et al. [41] reported the intestinal type of gastric cancer had higher frequency of K-*ras* mutation than those of the diffuse type tumors. We considered that the frequency of K-*ras* mutation was also higher in cell lines from intestinal type gastric cancer than in those from diffuse type.

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

Although MMR-deficient colorectal cancers have been reported to show high incidence of *BRAF* mutations and lower incidence of K-*ras* mutations compared with MMR-proficient colorectal cancers [6], we could not find any relationship between MMR-deficiency and mutation of *BRAF* and 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 may indicate the difference between gastric and colorectal carcinomas on their tumorigenesis. Further investigation will be required to clarify that point.

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References

1. Barbacid M. *ras* genes. Annu Rev Biochem 1987;56:779--827.
2. Bos JL. *ras* oncogenes in human cancer: a review. Cancer Res 1989;49:4682--4689.
3. Ellis CA, Clark G. The importance of being K-Ras. Cell Signal 2000;12:425--434.
4. 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--662.
5. 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--954.
6. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: *RAF/RAS* oncogenes and mismatch-repair status. Nature 2002;418:934.
7. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, et al. *BRAF* and *RAS* mutations in human lung cancer and melanoma. Cancer Res 2002;62:6997--7000.
8. 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--7003.

9. Marais R, Marshall CJ. Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv 1996;27:101--125.

10. Marshall CJ. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev 1994;4:82--89.

11. Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? Trends Genet 1997;13:444--449.

12. 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--692.

13. Razin A, Cedar H. DNA methylation and gene expression. Microbiol Rev 1991;55:451--458.

14. 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--5212.

15. 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--242.

16. 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--120.

17. Lee SH, Lee JW, Soung YH, Kim HS, Park WS, Kim SY, et al. *BRAF* and K*RAS* mutations in stomach cancer. Oncogene 2003;22:6942--6945.

18. 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--9196.

19. Zhao W, Chan TL, Chu KM, Chan AS, Stratton MR, Yuen ST, et al. Mutations of *BRAF* and K*RAS* in gastric cancer and their association with microsatellite instability. Int J Cancer 2004;108:167--169.

20. 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--8654.

21. 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--386.

22. 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--5821.

23. Hojo H. Establishment of cultured cell lines of human stomach cancer - origin and their mophological characteristics. Niigata Igaku Zasshi 1977;91:737--752.

24. 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--1071.

25. 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--905.

26 Japanese Gastric Cancer Association. Japanese Classification of Gastric Carcinoma - 2nd English Edition -. Gastric Cancer 1998;1:10-24.

27. 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--5257.

28. 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--9826.

29. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene *O*^{6}-methylguanine-DNA methyltransferase by promoter

hypermethylation is a common event in primary human neoplasia. Cancer Res 1999;59:793--797.

30. 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--5442.

31 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--3368.

32 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--370.

33 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--4567.

34 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--5404.

35 Yuen ST, Davies H, Chan TL, Ho JW, Bignell GR, Cox C, et al. Similarity of the

phenotypic patterns associated with *BRAF* and K*RAS* mutations in colorectal neoplasia. Cancer Res 2002;62:6451--6455.

36 Lohr M, Maisonneuve P, Lowenfels AB. K-*Ras* mutations and benign pancreatic disease. Int J Pancreatol 2000;27:93--103.

37 Fong KM, Zimmerman PV, Smith PJ. K*RAS* codon 12 mutations in Australian non-small cell lung cancer. Aust N Z J Med 1998;28:184--189.

38 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.

39 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--2672.

40 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--2801.

41 Miki H, Ohmori M, Perantoni AO, Enomoto T. K-ras activation in gastric epithelial tumors in Japanese. Cancer Lett 1991;58:107--113.

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--1227.

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--659.

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--149.

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--809.

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--2768.

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--1923.

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--923.

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.

Figure legends

Figure 1 *BRAF* and K-*ras* mutations in gastric carcinomas. A, the case with *BRAF* mutation (A771). Point mutation at codon 599 was detected in the cancer lesion (GTG-->GAG). B, the case with K-*ras* mutation and MSI (H205). Point mutation at codon 12 was detected in the cancer lesion (GGT-->GAT).

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

Cell line	*BRAF* point mutation		K-*ras* point mutation	
	Exon 11	Exon 15	Exon 2	Exon 3
HSC-39	--	--	--	--
HSC-41	--	--	G216A /G13D	--
HSC-42	--	--	G216A /G14D	--
HSC-44PE	--	--	G216A /G12D	--
HSC-57	--	--	G216A /G15D	--
HSC-58	--	--	--	--
HSC-59	--	--	--	--
HSC-60	--	--	--	--
HSC-64	--	--	--	--
SH101-P4	--	--	G216A /G15D	--
MKN-1	--	--	--	--
MKN-7	--	--	--	--
MKN-28	--	--	--	--
MKN-45	--	--	--	--
MKN-74	--	--	--	--
TMK-1	--	--	--	--

Table 2. Status of MSI and methylation in promoter regions of *hMLH1* and *MGMT* in gastric cancers

Case ID	*BRAF* mutation	K-*ras* mutation	Microsatellite status	*hMLH1* methylation status	*MGMT* methylation 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. Comparison of clinicopathological parameters of adenocarcinomas of the stomach with MSI and MSS

	Total		MSI status				P value[2]
			MSI		MSS		
	n	(%)	n	(%)	n	(%)	
Total	62	(100.0)	23	(22.0)	39	(64.0)	
Histological type							
tub1	9	(14.5)	2	(8.7)	7	(17.9)	P = 0.320
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[1]							
T1 (M, SM)	3	(4.8)	1	(4.3)	2	(5.1)	P = 0.462
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							
ly (-)	7	(11.5)	5	(21.7)	2	(5.3)	P = 0.050
ly (+)	54	(88.5)	18	(78.3)	36	(94.7)	
Lymph node metastasis[1]							
N0	19	(30.6)	8	(34.8)	11	(28.2)	P = 0.780
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							
v (-)	10	(16.1)	5	(21.7)	5	(12.8)	P = 0.356
v (+)	52	(83.9)	18	(78.3)	34	(87.2)	
Liver metastasis							
H0	58	(93.5)	22	(95.7)	36	(92.3)	P =0.605
H1	4	(6.5)	1	(4.3)	3	(7.7)	
Peritoneal metastasis							
P0	56	(90.3)	21	(91.3)	35	(89.7)	P = 0.841
P1	6	(9.7)	2	(8.7)	4	(10.3)	
Other distant metastases							
M0	57	(91.9)	22	(95.7)	35	(89.7)	P = 0.409
M1	5	(8.1)	1	(4.3)	4	(10.3)	
Tumor stage[1]							
I	16	(25.8)	6	(26.1)	10	(25.6)	P = 0.922
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)	

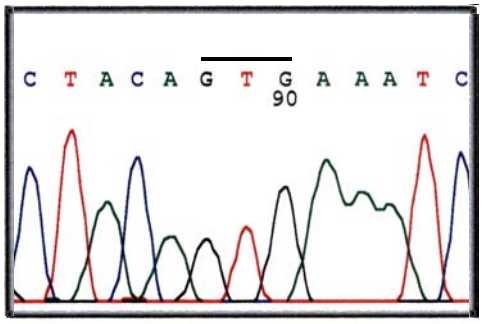
[1] Depth of invasion, lymph node metastasis and tumor stage was evaluated according to

the criteria of the Japanese Classification of Gastric Carcinoma [26].

[2] Statistical analysis was performed by χ^2 test. A P value less than 0.05 were regarded to be significant.

A

BRAF



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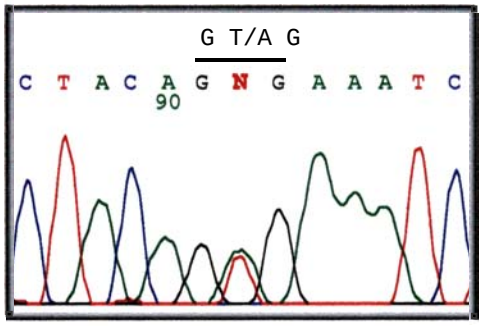
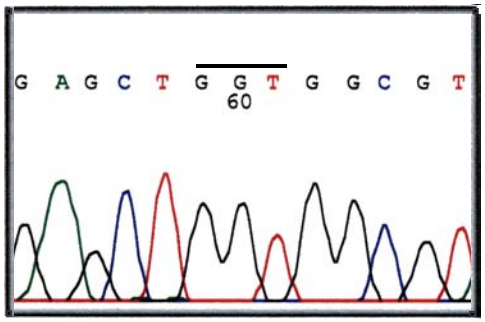


Tumor

B

K-ras



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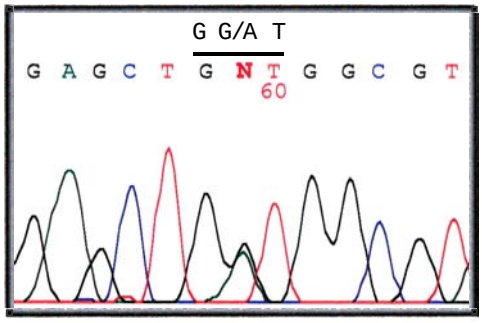

Tumor