

Not Infrequent *K-ras* Mutations in Depressed-type Early Colorectal Carcinomas Larger than 10 mm

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The aim of this study was to elucidate whether *K-ras* (codons 12 and 13) mutations occur in depressed-type early colorectal carcinomas (DECas) larger than 10 mm in size. Thirty-four cases of DECas including 27 larger than 10 mm were examined for *K-ras* mutations by means of microdissection, PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism), and direct sequencing. Although *K-ras* mutation was infrequent (1/7, 14%) in small (less than 10 mm) DECas, 16/27 (59%) and 17/27 (63%) of DECas larger than 10 mm revealed codon 12, or either codon 12 or 13 mutations, respectively. None of the evaluated pathological factors except size showed a correlation with *K-ras* mutation. These data indicate that although *K-ras* mutation could not be involved in the early stage of the progression of DECas, it might play a role at a later stage when the tumor size is over 10 mm.

Key words: Early colorectal carcinoma — Depressed type — *K-ras* gene — Heterogeneity

It has been believed that colorectal carcinomas mainly arise from pre-existing polypoid adenomas and develop into ulcerative circumscribed carcinomas. This concept has been generally accepted as the adenoma-carcinoma sequence theory.¹⁾ Molecular studies to elucidate the multi-step genetic alterations of colorectal carcinogenesis have been based on this pathological concept,²⁾ and *K-ras* gene mutations, predominantly codon 12,²⁻⁴⁾ are considered to be associated with growth and progression of adenomas.

On the other hand, in Japan, sporadic non-polypoid colorectal adenomas and early carcinomas including flat or depressed type have been detected by advanced routine colonoscopy.⁵⁾ The depressed-type early carcinomas (DECas) are thought to arise *de novo*,^{6,7)} to show rapid growth and aggressive behavior,^{8,9)} and to play an important role in the developmental process of colorectal carcinoma,^{10,11)} rather than the polypoid adenoma-carcinoma sequence.

From the genetic point of view, the frequency of *K-ras* mutations in flat or depressed neoplasms has been reported to be low.¹²⁻¹⁸⁾ Consequently, it has been suggested that different genetic pathways are involved in progression of these lesions compared with their polypoid counterparts. However, earlier studies reporting infrequent *K-ras* mutation dealt with relatively small numbers of samples mainly consisting of small-sized lesions (not more than 10 mm), or did not take into consideration the size of tumors.^{12,18)} It remains to be seen whether *K-ras* mutation might be involved in flat or depressed-type tumors at a later stage of their development.

In this study, we have analyzed the frequency of *K-ras* mutations in DECas, focusing especially on those more than 10 mm in size. We found that *K-ras* mutation in these tumors was not infrequent when they became larger, regardless of their histogenesis, suggesting that this mutation could be involved in the progression of these tumors.

MATERIALS AND METHODS

Samples Thirty-four surgically resected DECas were selected from the archival pathology files of the First Department of Pathology at Niigata University, School of Medicine from 1989 to 1999. During the period, 803 early colorectal carcinomas were entered into the files and DECas amounted to 80 cases (10.0%). Samples comprised 18 intramucosal and 16 submucosal carcinomas. Eight DECas were associated with tubular adenoma located at the periphery of the tumor. Twenty-seven out of 34 samples were larger than 10 mm in size and the average size of the tumors was 15.0 ± 6.7 mm. The size of tumors was defined as their maximal diameter. Cases with a family history or clinical features suggestive of hereditary colorectal cancers and cancers complicating inflammatory bowel disease were excluded. All samples were fixed in 10% formalin and the entire lesion was cut into stepwise sections, embedded in paraffin, and stained with hematoxylin and eosin (H&E). All sections were histologically examined.

Morphological examinations Depressed-type tumor was defined as tumor either showing a pure depression or one with marginal elevation in which the mucosal thickness

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was less than twice that of the surrounding normal mucosa. The size of the cancer associated with adenoma was measured including the adenomatous component. Well-differentiated adenocarcinoma was subclassified as high-grade atypia (CAH) or low-grade atypia (CAL) according to the cytological degree of atypia, as described previously.¹⁷⁾

DNA extraction For each tumor, representative paraffin specimens were selected and DNA was extracted from five 10 μm -thick serial sections. Target foci, consisting of 2–27 neoplastic tubules per serial section, were microdissected and DNA was extracted according to the methods described elsewhere.¹⁹⁾ Areas with more than one histological diagnosis (adenoma, CAL, CAH) were microdissected separately.

Furthermore, considering the possibility of intratumoral heterogeneity of K-ras mutational status,²⁰⁾ several foci with identical histological diagnosis (adenoma, CAL and CAH, respectively) were selected for DNA extraction. Altogether 241 foci were microdissected separately from 34 samples (mean 7.1 ± 4.1 per sample, range: 2–19). DNA from normal colorectal mucosa was also extracted from each specimen as a negative control.

Analysis of K-ras mutation Mutations of codon 12 and 13 were analyzed by nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For codon 12, PCR amplification was performed using the primers and conditions described by Ohshima *et al.*,²¹⁾ except that annealing was carried out for 1.5 min. PCR products encoding the wild-type and mutant sequences were distinguished as 86 base pair (bp) and 106 bp fragments, respectively, by digestion with the restriction enzyme *Mva*I (TaKaRa, Kyoto). For codon 13, K-ras (13)-5' primer described by Nollau *et al.*²²⁾ was used instead of primer 3 described by Ohshima *et al.*²¹⁾ The wild type was cleaved into 83 and 32 bp fragments by the restriction enzyme *Bgl*II (TaKaRa), whereas the PCR product having a mutant sequence could not be digested by this enzyme. In each PCR run, three control reactions were included: a) negative control (containing no template DNA), b) normal control (normal human placental DNA), and c) positive control (for codon 12, Asp1 pancreas cancer cell line DNA with GTT to GAT mutation, and for codon 13, HCT116 colon cancer cell line DNA with GGC to GAC mutation). PCR, enzyme digestion and electrophoresis were performed at least twice to confirm the reproducibility of the result.

The sensitivity for detecting mutant K-ras gene was determined preliminarily, by testing samples with several ratios of mutant (Asp1 or HCT116) and wild-type (human placental) DNA. Mutations in K-ras were detectable when mutant DNA comprised more than 10% of genomic DNA (Fig. 1).

Direct DNA sequencing For amplified DNA samples with codon 12 mutation, mutant DNA fragments were

removed from the gel and purified and direct DNA sequencing was carried out using an Auto Loat Solid-Phase Sequence Kit (Pharmacia Biotech, Uppsala, Sweden) and an automated DNA sequencer (ALF-II, Pharmacia) equipped with an ALF manager (version 2.5).

Statistical analysis The χ^2 test was used for statistical analysis and a *P* value of less than 0.05 was considered significant. Fisher's exact test was applied whenever appropriate.

RESULTS

The K-ras mutation in DECAs was detected in 17/34 (50%) and 9/34 (26%) for codons 12 and 13, respectively (Table I). Statistical significance was observed for the relation of tumor size to mutation at codon 12 ($P=0.042$) and at codon 12 or 13 ($P=0.029$). Although only one of 7 tumors (14%) less than 10 mm revealed mutation at codon 12, 16/27 (59%) and 9/27 (33%) of tumors of 10 mm or more showed codon 12 and 13 mutation, respectively. No statistical significance was observed for other pathological factors.

K-ras mutation was detected in the adenomatous component in 2 out of the 8 DECAs associated with adenoma (at the periphery next to the carcinoma). In one case, mutational status was identical between the adenomatous and carcinomatous component (mutation in codon 12 GGT→GAT) (Fig. 2) and in the other, it was different (mutation in codon 13 in adenoma, and in codon 12 in carcinoma).

Table II shows K-ras codon 12 mutational status and geometrical distribution of the K-ras mutation-positive portion in 17 mutation-positive tumors. In only two of 17 tumors was single identical mutation detected in all microdissected foci. In the remaining 15 tumors, the mutational

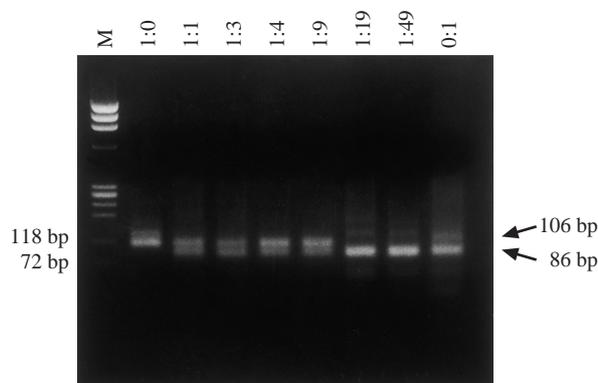


Fig. 1. K-ras codon 12 titration assay. DNA mixtures with the indicated ratio of mutant (Asp1, 50 $\mu\text{g}/\text{ml}$) to normal (human placenta, 50 $\mu\text{g}/\text{ml}$) DNA were examined by nested PCR-RFLP analysis. The mutant band of 106 bp was detected until 1:9 dilution.

Table I. *K-ras* Mutations in Depressed-type Early Colorectal Carcinomas by Various Pathological Factors

	Frequency of mutation		
	Codon 12 (%)	Codon 13 (%)	Codon 12 or 13 (%)
Depth of invasion			
Mucosal	8/18 (44%)	5/18 (28%)	14/18 (78%)
Submucosal	9/16 (56%)	4/16 (25%)	9/16 (56%)
Accompanying adenoma			
Present	4/8 (50%)	3/8 (38%)	4/8 (50%)
Absent	13/26 (50%)	6/26 (23%)	14/26 (54%)
Histology			
Adenoma	1/8 (13%)	1/8 (13%)	2/8 (26%)
CAL	14/23 (61%)	7/23 (30%)	14/23 (61%)
CAH	3/11 (27%)	2/11 (18%)	4/11 (36%)
Tumor size (mm)			
X<10	1/7 (14%)	0/7 (0%)	1/7 (14%)
X≥10	16/27 (59%)	9/27 (33%)	17/27 (63%)
Total	17/34 (50%)	9/34 (26%)	18/34 (53%)

CAL: well-differentiated adenocarcinoma, low-grade atypia. CAH: well-differentiated adenocarcinoma, high-grade atypia.

* $P=0.042$ (1/7 vs. 16/27); ** $P=0.029$ (1/7 vs. 17/27), by Fisher's exact test.

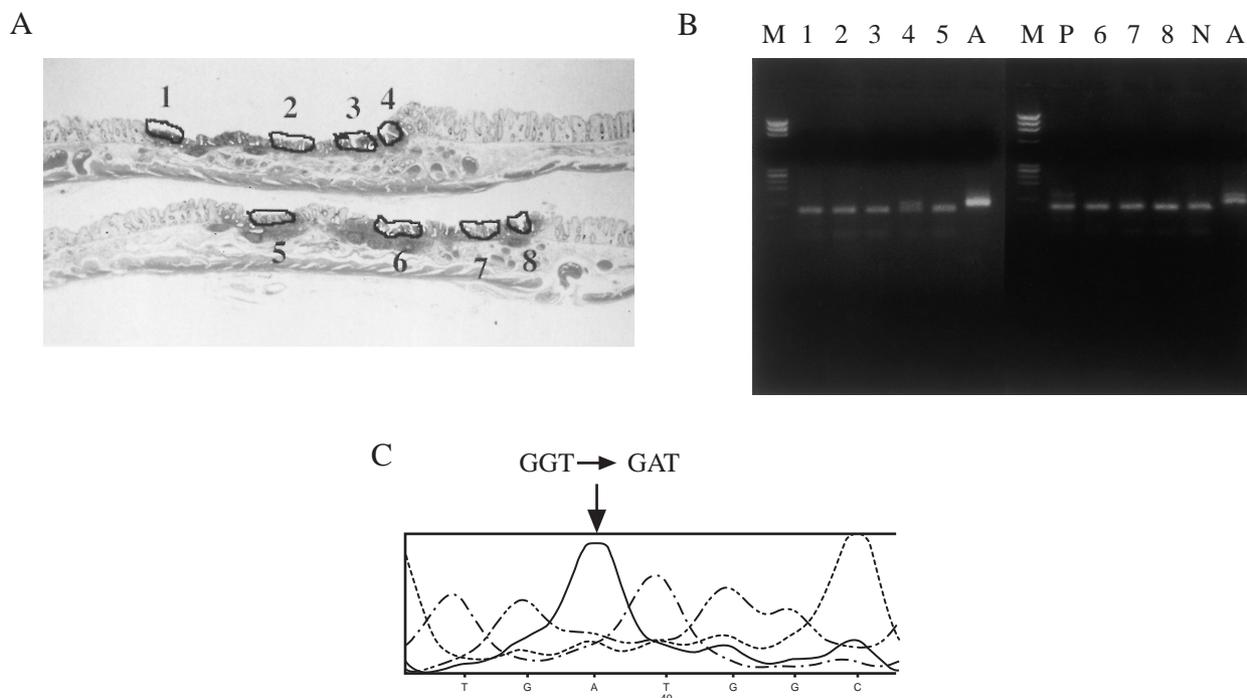


Fig. 2. A: DECa 15 mm in size. Microdissection was carried out from the site outlined by the square (HE stain; original magnification $\times 2.5$). B: Result of PCR-RFLP analysis of *K-ras* codon 12 mutation in this case. M, ϕ X174/*Hae*III DNA size marker; lane P, normal control (human placental DNA) showing cleaved wild band (86 bp) only; lanes 1 to 5; DNA samples microdissected from the lesion showing both mutant (106 bp) and wild-type bands (heterozygous); lane N, DNA sample from normal colorectal mucosa showing cleaved wild band only; lane A, positive mutant control (Aspc 1) showing uncleaved mutant band only. C: DNA direct sequence analysis. All samples presented an identical mutation (codon 12, GGT \rightarrow GAT).

Table II. Intratumoral Heterogeneity of K-ras Codon 12 Mutations in Depressed-type Early Colorectal Carcinomas

Mutational status	Total (%)	GEM			Base change (%)	
		P	C	C+P		
Single mutation alone	2/17 (11.8%)	—	—	—	GGT→GAT	1/17 (5.9%)
Wild+single mutation	13/17 (76.5%)	2	4	7	GGT→GTT	1/17 (5.9%)
					GGT→GAT	12/17 (70.6%)
Wild+double mutations	2/17 (11.8%)	0	0	2	GGT→GTT	1/17 (5.9%)
					GGT→GAT/GTT	

GEM: geometrical distribution of mutation in the tumor. P, peripheral portion; C, central portion; C+P, central and peripheral portion.

status was heterogeneous. Thirteen tumors consisted of two populations of subclones, each of which carried a single identical mutation or wild-type gene, and 2 tumors consisted of wild-type and identical double mutations. For codon 12 mutation-positive tumors, the rate of mutation-positive foci in each tumor (number of mutation-positive foci/total number of microdissected foci) was calculated (Table III). The mean rate was $35.1 \pm 30.0\%$ in tumors larger than 10 mm, and in 9 out of 16 (56.3%), they contained mutation-positive foci at a rate of less than 25%.

DISCUSSION

Earlier studies of K-ras mutation in depressed-type colorectal tumors have suggested that K-ras mutation is not involved in the progression of these types of tumors.^{12–18} Most, if not at all, of these earlier studies were based on the examination of small depressed-type tumors (less than 10 mm), presumably because depressed-type tumors in the colorectum are usually rather small compared to the polypoid type. In large-scale studies, 96% of depressed-type adenomas were less than 3 mm in size,²³ and 87% of depressed-type early carcinomas were less than 10 mm.⁵ However, K-ras mutation, predominantly in codon 12, is known to up-regulate mitogenic signal transduction pathways²⁴ and is considered to result in subclonal evolution of colorectal adenomas, represented morphologically as an increase in size and grade of atypia.²⁵ Considering these *in vivo* effects of K-ras mutation in colorectal tumors, larger size depressed-type tumors must be investigated to conclude whether or not K-ras mutation plays a role in the progression of these tumors.

In the present study, K-ras mutation was infrequent (1/7, 14%) in small (less than 10 mm) DECAs, consistent with earlier studies.^{14–18} However, 16/27 (59%) and 17/27 (63%) of DECAs whose size was larger than 10 mm showed K-ras codon 12 mutation, or either codon 12 or 13 mutation, respectively, which were significantly more frequent than in tumors of less than 10 mm. The present results seem to indicate that although K-ras mutation

Table III. Rate of Mutation-positive Foci in K-ras (Codon 12) Mutation-positive Tumors

Mutation-positive rate	Size of tumors (mm)	
	X<10	X≥10
100%	0/1 (0%)	2/16 (12.5%)
≥50%	0/1 (0%)	5/16 (31.3%)
≥25%	1/1 (100%)	9/16 (56.3%)
Average	25.0%	35.1±30.0%

Mutation-positive rate: (mutation-positive foci/all microdissected foci) × 100.

could not be involved in the early stage, it might play a role in a later stage of progression of DECAs.

A few^{15, 16} investigations of K-ras mutations in DECAs larger than 10 mm have been done, and these earlier studies showed infrequent K-ras mutation (0–24%) in contrast to our present results. This discrepancy may be explained, at least partly, by intratumor heterogeneity of K-ras mutational status and the absence in the earlier studies of microdissection of several foci from one tumor. In 17 samples larger than 10 mm in the present study, only 2/17 (12%) showed K-ras (codon 12) mutation in all microdissected foci (mean of 7.1 foci per sample) and 15/17 (88%) showed heterogeneity in mutational status, either wild-type and single mutation, or wild-type and double mutation (Table II). The rate of mutation-positive foci was 35.1% on the average (Table III). These data imply that K-ras mutation in large DECAs (not small ones less than 10 mm in size) may not appear positive when the DECAs consist of mixed mutation-positive and negative areas, unless microdissection is used to examine several foci and mutation-positive areas.

In colorectal adenomas and carcinomas, codons 12, 13, and 61 are known to be hot spots of K-ras point mutation.²⁴ As to codon 13, the mutation rate in sporadic adenomas is reported to be 9–19%^{26, 27} and the 'RASCAL' study summed up the mutation rate of advanced colorectal cancers as 6.6%.²⁸ In the present study, we have examined, for the first time, the frequency of codon 13 mutation

in DECAs and detected it in 9/27 cases (33%) of those larger than 10 mm (no mutation was detected in those less than 10 mm).

The role of *K-ras* mutation in DECAs remains to be elucidated. A possible role could be an increase in size of the tumor through clonal expansion of mutation-positive cells which have gained a growth advantage, since none of the various pathological factors evaluated (histogenesis, histological atypia, depth of cancer invasion), except for the size, showed any correlation with *K-ras* mutation (codons 12 and 13) (Table I). However, it seems unjustified to speculate about a connection between *K-ras* mutation in DECAs and increase of their size. If enlargement of the tumor is lead by *K-ras* mutation, the mutational status is expected to be homogeneous due to clonal expansion, but it was heterogeneous in our study. Our present data indicate only that multiple subclones with *K-ras* mutation

could occur when DECAs reached a size over 10 mm. To elucidate the cryptic role of *K-ras* mutation in DECAs, further investigation is necessary, including studies of cell proliferation activity and/or angiogenesis,^{17, 29, 30} which are known to be related to *K-ras* mutation, correlation with other genetic alterations such as in the *APC* or *p53* gene, and investigation of advanced colorectal cancers, which are believed to develop from early DECAs.

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REFERENCES

- 1) Muto, T., Bussey, H. J. and Morson, B. C. The evolution of cancer of the colon and rectum. *Cancer*, **36**, 2251–2271 (1975).
- 2) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smiths, M. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1988).
- 3) Bos, J. L., Fearon, E. R., Hamilton, S. R., Vries, M. V., Boom, J. H., Eb, A. J. and Vogelstein, B. Prevalence of ras gene mutations in human colorectal cancers. *Nature*, **327**, 293–297 (1987).
- 4) Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. and Perucho, M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature*, **327**, 298–303 (1987).
- 5) Kudo, S. Endoscopic mucosal resection of flat and depressed types of early colorectal cancer. *Endoscopy*, **25**, 455–461 (1993).
- 6) Ishii, H., Tatsuta, M., Tsutsui, S., Imanishi, K., Otani, T., Okuda, S., Ishiguro, S. and Taniguchi, H. Early depressed adenocarcinomas of the large intestine. *Cancer*, **68**, 2406–2410 (1992).
- 7) Kuramoto, S. and Oohara, T. How do colorectal cancers develop? *Cancer*, **75**, 1534–1538 (1995).
- 8) Minamoto, T., Sawaguchi, K., Ohta, T., Itoh, T. and Mai, M. Superficial type adenomas and adenocarcinomas of the colon and rectum: a comparative morphological study. *Gastroenterology*, **106**, 1436–1443 (1994).
- 9) Kudo, S., Tamura, S., Nakajima, T., Hirota, T., Asano, M., Ito, O. and Kusaka, H. Depressed type of colorectal cancer. *Endoscopy*, **27**, 54–57 (1995).
- 10) Kudo, S., Tamura, S., Hirota, S., Sano, Y., Yamano, H., Serizawa, M., Fukuoka, T., Mitsuoka, H., Nakajima, T. and Kusaka, H. The problem of *de novo* colorectal carcinoma. *Eur. J. Cancer*, **31A**, 1118–1120 (1995).
- 11) Shimoda, T., Ikegami, M., Fujisaki, J., Matsui, T., Aizawa, S. and Ishikawa, E. Early colorectal carcinoma with special reference to its development *de novo*. *Cancer*, **64**, 1138–1146 (1989).
- 12) Soh, K., Yanagisawa, A., Hiratsuka, H., Sugano, H. and Kato, Y. Variation in *K-ras* codon 12 point mutation rate with histological atypia within individual colorectal tumors. *Jpn. J. Cancer Res.*, **84**, 388–393 (1993).
- 13) Yamagata, S., Muto, T., Uchida, Y., Masaki, T., Sawada, T., Tsuno, N. and Hirooka, T. Lower incidence of *K-ras* codon 12 mutation in flat colorectal adenomas than in polypoid adenomas. *Jpn. J. Cancer Res.*, **85**, 147–151 (1994).
- 14) Fujimori, T., Satonaka, K., Yamamura-Idei, Y., Nagasaki, K. and Maeda, S. Non-involvement of ras mutations in flat colorectal adenomas and carcinomas. *Int. J. Cancer*, **57**, 51–55 (1994).
- 15) Minamoto, T., Sawaguchi, K., Mai, M., Yamashita, N., Sugimura, T. and Esumi, H. Infrequent *K-ras* activation in superficial-type (flat) colorectal adenomas and adenocarcinomas. *Cancer Res.*, **54**, 2841–2844 (1994).
- 16) Hasegawa, H., Ueda, M., Watanabe, M., Teramoto, T., Mukai, M. and Kitajima, M. *K-ras* gene mutations in early colorectal cancer—flat elevated vs. polyp-forming cancer. *Oncogene*, **10**, 1413–1416 (1995).
- 17) Kobayashi, M., Watanabe, H., Ajioka, Y., Honma, T. and Asakura, H. Effect of *K-ras* mutation on morphogenesis of colorectal adenomas and early cancers: relationship to distribution of proliferating cells. *Hum. Pathol.*, **27**, 1042–1049 (1996).
- 18) Yagi, O. K., Akiyama, Y., Ohkura, Y., Ban, S., Endo, M., Saitoh, K. and Yuasa, Y. Analyses of the *APC* and *TGF- β type II receptor* genes, and microsatellite instability in mucosal colorectal carcinomas. *Jpn. J. Cancer Res.*, **88**, 718–724 (1997).

- 19) Moskaluk, C. A. and Kern, S. E. Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. *Am. J. Pathol.*, **150**, 1547–1552 (1997).
- 20) Saraga, E., Bautista, D., Dorta, G., Chaubert, P., Martin, P., Sordat, B., Protiva, P., Blum, A., Bosman, F. and Benhattar, J. Genetic heterogeneity in sporadic colorectal adenomas. *J. Pathol.*, **181**, 281–286 (1997).
- 21) Ohshima, S., Shimizu, Y. and Takahama, M. Detection of c-Ki-ras gene mutation in paraffin sections of adenocarcinoma and atypical bronchioloalveolar cell hyperplasia of human lung. *Virchows Arch.*, **424**, 129–134 (1994).
- 22) Nallau, P., Moser, C., Weinland, G. and Wagner, C. Detection of K-ras mutations in stools of patients with colorectal cancer by mutant-enriched PCR. *Int. J. Cancer*, **66**, 332–336 (1996).
- 23) Kubota, O., Kino, I., Kimura, T. and Harada, Y. Nonpoly-ploid adenomas and adenocarcinomas found in background mucosa of surgically resected colons. *Cancer*, **77**, 621–626 (1996).
- 24) Boss, J. L. The ras gene family and human carcinogenesis. *Mutat. Res.*, **195**, 255–271 (1988).
- 25) Ilyas, M. and Tomlinson, I. P. M. Genetic pathways in colorectal cancer. *Histopathology*, **28**, 389–399 (1996).
- 26) McLellan, E. A., Owen, R. A., Stepniewska, K. A. and Lemoine, N. R. High frequency of K-ras mutations in sporadic colorectal adenomas. *Gut*, **34**, 392–396 (1993).
- 27) Ohnishi, T., Tomita, N., Monden, T., Ohue, M., Yana, I., Takami, K., Yamamoto, H., Yagyu, T., Kikkawa, N., Shimano, T. and Monden, M. A detailed analysis of the role of K-ras gene mutation in the progression of colorectal adenoma. *Br. J. Cancer*, **75**, 341–347 (1997).
- 28) Andreyev, H. J., Norman, A. R., Cunningham, D., Oates, J. R. and Clarke, P. A. Kirsten ras mutations in patients with colorectal cancer: the multicenter “RASCAL” study. *J. Natl. Cancer Inst.*, **90**, 675–684 (1998).
- 29) Rak, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Shirasawa, S., Sasazuki, T. and Kerbel, R. S. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res.*, **55**, 4575–4580 (1995).
- 30) Konishi, T., Huang, C. L., Adachi, M., Taki, T., Inufusa, H., Kodama, K., Kohno, N. and Miyake, M. The K-ras gene regulates vascular endothelial growth factor gene expression in non-small cell lung cancers. *Int. J. Oncol.*, **16**, 501–511 (2000).