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# Higher Frequency of Point Mutations in the c-K-*ras* 2 Gene in Human Colorectal Adenomas with Severe Atypia than in Carcinomas

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**Human colorectal carcinomas may be induced from adenomas or they may occur *de novo*. To examine which is the main pathway, we analyzed point mutations at codon 12 in the c-K-*ras* 2 gene in 73 colorectal carcinomas, 13 metastatic tumors, 72 adenomas and 30 normal tissues. The c-K-*ras* 2 codon 12 mutation frequency was 0/30 in normal tissues, 0/17 in adenomas with mild atypia, 3/37 (8.1%) in adenomas with moderate atypia, 15/18 (83.3%) in adenomas with severe atypia, 19/73 (26.0%) in primary carcinomas and 3/13 (23.1%) in metastatic tumors. The mutation frequency in adenomas with severe atypia was much higher than that in carcinomas. These results indicate that many colorectal carcinomas may not be induced through adenomas with severe atypia.**

Key words: Colorectal carcinoma — Adenoma — Familial adenomatous polyposis — c-K-*ras* 2 gene — Point mutation

Several oncogenes and tumor suppressor genes, which might be associated with human colorectal tumorigenesis, have been identified recently. For example, there are point mutations in the c-K-*ras* 2 gene,[1-3] and alterations of the DCC[4] and p53 genes.[5] Analysis of these markers has supported the hypothesis that most colorectal carcinomas arise through adenomas.[6] An adenoma-carcinoma sequence had already been proposed on the basis of clinical and pathological considerations.[7]

Other investigators have presented a different hypothesis. It was suggested, on the basis of the results of morphometry, that the frequency of the adenoma-carcinoma sequence is 20 to 30% and that other colorectal carcinomas arise from normal mucosae directly (*de novo*).[8,9] Indeed, there has been an increasing number of reports of the detection of early carcinomas of a flat or depressed type.[10] This issue is very important not only for the clarification of colorectal carcinogenesis but also for the clinical treatment of adenomas. We report here determination of the mutation frequency of codon 12 in the c-K-*ras* 2 gene in colorectal tumors in order to analyze the origin of carcinomas.

Most point mutations in the c-K-*ras* 2 gene occur at codon 12 in colorectal tumors. Therefore, we used the polymerase chain reaction (PCR) and *Msp*I digestion to detect the point mutations in codon 12.[11] As shown in Fig. 1A, two oligonucleotides, a 22mer and a 20mer, were used as primers, as described previously. Substitution of C for T at the 3′ end of the 22mer introduces an artificial *Msp*I site (CCGG) and creates a new restriction fragment length polymorphism (RFLP) indicative of the point mutation in codon 12. A single base change at the first or second base of codon 12 changes the 12th amino acid, altering the *Msp*I recognition site at that location. Thus, 78- and 21-base pair (bp) fragments should be generated in the normal c-K-*ras* 2 gene. In contrast, *Msp*I should generate a 99-bp fragment in the mutated c-K-*ras* 2 gene. Since alterations at the third base of the 12th codon do not alter the amino acid, glycine, this method can detect all of the point mutations and amino acid changes in the 12th codon.

Although there are two and one base differences in the 5′ and 3′ primers, respectively, between the c-K-*ras* 2 gene and c-K-*ras* 1,[12] a pseudogene of c-K-*ras* 2, the 99-bp fragment might be amplified from c-K-*ras* 1, too. Since the sequences of codons 11 and 12 of both genes are the same,[12] the *Msp*I digestion should also generate the 78- and 21-bp fragments in c-K-*ras* 1.

This method is not only rapid but also very sensitive. We can detect a mutation if it is present in only 2.5 to 10% of the studied cells. To test the effect of the sampling site, three independently located samples from each of the 14 carcinomas were taken and examined for mutations. The DNAs in all sets of three samples gave the same results, indicating that there is no difference due to the location. To avoid false-positives with the PCR the experiments were carried out according to recommendations reported previously.[13]

DNAs were extracted from 8 colorectal carcinomas, 59 adenomas, 18 normal colonic mucosae and 8

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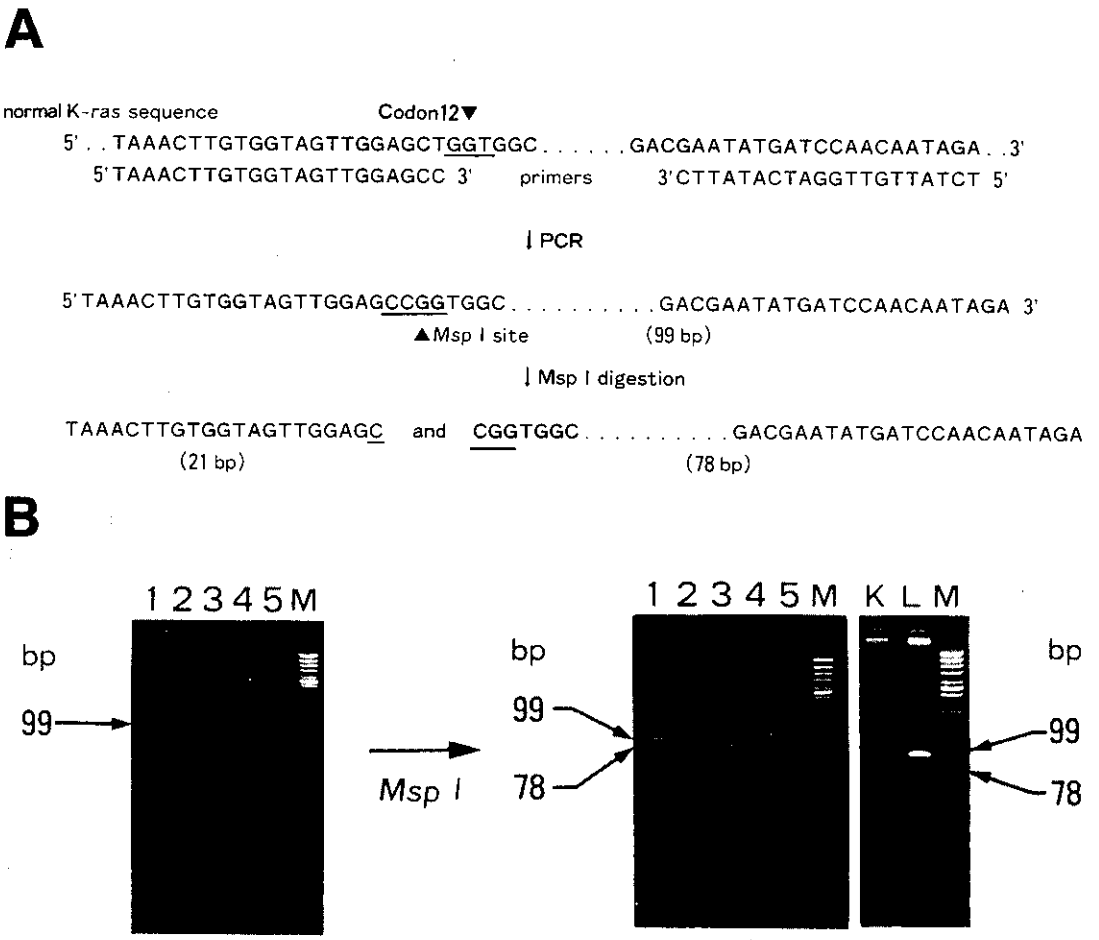


Fig. 1. Detection of point mutations in codon 12 using PCR and *Msp*I digestion. (A) PCR with modified primers creating artificial RFLP. Two oligonucleotides, a 22mer (5′ TAAACTTGTGGTAGTTGGAGCC 3′) and a 20mer (5′ TCTATTGTTGGATCATATTC 3′), were prepared. The 22mer was modified to contain GCC instead of GCT (codon 11) to introduce the *Msp*I restriction site if codon 12 is normal. (B) Electrophoretic analysis of PCR products (left panel) and the RFLP introduced by *Msp*I degestion (right panel) on 4% Nusieve agarose gels. Genomic DNA (1 $\mu$g) was amplified by PCR (40 cycles) using the primers described in Fig. 1A on automated apparatus (Takashow Trio Thermojupiter).[11] Each cycle includes three steps: denaturation of DNA at 94°C for 78 s, annealing of the primers at 50°C for 4 min and enzymatic extension at 74°C for 102 s. Amplified DNA (about 1 $\mu$g) was digested with *Msp*I (24 units). Representative results are shown: an advanced carcinoma (lane 1), an adenoma with mild atypia (lane 2), an adenoma with moderate atypia (lane 3), an adenoma with severe atypia (lane 4) and a normal mucosa (lane 5). Lanes K, KMS-4,[1] human colon carcinoma cells; L, Lu-65,[14] human lung cancer cells. Both cells contain TGT as codon 12. Coelectrophoresed DNA fragments of *Hinc*II-digested $\phi$X174 DNA (lane M) served as standards. The point mutations were detected in lanes 1, 4, K and L (right panel).

hyperplastic mucosae from 22 familial adenomatous polyposis (FAP) patients. DNAs were also extracted from 65 primary advanced carcinomas, 13 metastatic tumors, 3 elevated mucosal carcinomas, 13 adenomas and 4 normal colonic mucosae from 71 nonFAP patients. All samples were freshly taken and kept frozen. Neighboring tissues were fixed with formalin and embedded in paraffin. Histological sections were made, and stained with hematoxylin and eosin. Pathological diagnosis was performed by a single pathologist without any information as to the results of DNA analyses. Atypia of adenomas was classified histologically into three categories: mild, moderate and severe.

Fig. 1B shows representative electrophoretic analysis data of PCR products and *Msp*I digestion. To avoid partial digestion with *Msp*I, the PCR product of normal colonic mucosae was included as a control in each experiment. Moreover, when the result was unclear, for exam-

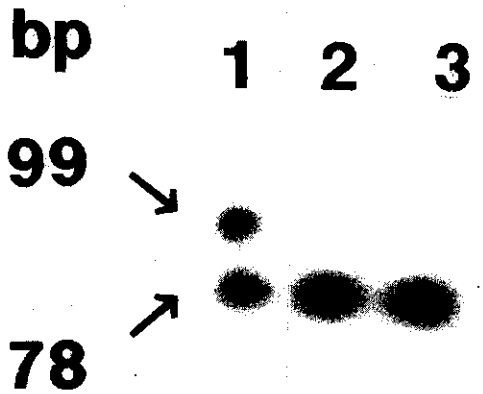


Fig. 2. Hybridization of *Msp*I-digested samples with an internal oligonucleotide probe. After the electrophoresis, the fragments were transferred onto nylon membrane (Hybond N+, Amersham) as described before.[15] The membrane was hybridized to the $^{32}$P-labeled oligonucleotide probe according to the supplier's instruction manual. The probe was 5′-GCTAATTCAGAATCATTTTG-3′, which is located between the two primers. Each lane corresponds to the sample shown in Fig. 1B.

Table I. Frequency of Mutations in Codon 12 of K-*ras* Genes in Colorectal Tissues

| Tissue | FAP | nonFAP | Total (%) |
|---|---|---|---|
| Normal mucosae[a] | 0/18 | 0/4 | 0/22 (0) |
| Hyperplastic mucosae | 0/8 | | 0/8 (0) |
| Adenomas with | 16/59 | 2/13 | 18/72 (25) |
| mild atypia | 0/14 | 0/3 | 0/17 (0) |
| moderate atypia | 3/30 | 0/7 | 3/37 (8.1) |
| severe atypia | 13/15 | 2/3 | 15/18 (83.3) |
| Mucosal carcinomas | | 2/3 | 2/3 |
| Advanced carcinomas | 3/8 | 16/65 | 19/73 (26.0) |
| Metastatic tumors[b] | | 3/13 | 3/13 (23.1) |

*a*) Normal mucosae were collected from flat mucosae of surgically resected specimens from locations more than 10 cm away from cancerous lesions. Normal mucosae mean not only flat mucosae but also ones proved histologically to be normal. *b*) Of 13 metastatic tumors, 1/5 in lymph nodes, 1/6 in liver, 0/1 in skin and 1/1 in peritoneal metastases had mutations in K-*ras* codon 12.

ple, weak visualization of the 99-bp fragment, the sample was re-examined using twice as much *Msp*I (48 units). As positive controls, two human cancer cells, KMS-4 and Lu-65, containing point mutations at the 12th codon of the c-K-*ras* 2 gene,[1, 14] were examined and were shown to contain the 99-bp fragment (Fig. 1B, right panel).

Hybridization of the *Msp*I-digested fragments with an internal radiolabeled oligonucleotide probe was carried out (Fig. 2). Both the 99-bp and 78-bp fragments were hybridized, indicating that the PCR products were derived from the expected K-*ras* gene.

Mutations at codon 12 of the c-K-*ras* 2 gene were not detected in the normal tissues or adenomas with mild atypia of the FAP patients (Table I). Ten percent of 30 FAP adenomas with moderate atypia contained c-K-*ras* 2 gene mutations and 86.7% of 15 FAP adenomas with severe atypia had mutations (Table I). However, only 37.5% of 8 FAP carcinomas contained such mutations. The mutation frequency in FAP carcinomas was significantly lower than that in adenomas with severe atypia ($P<0.05$ by $\chi^2$ test). NonFAP samples showed a similar mutation frequency to the FAP samples (Table I). Overall, the c-K-*ras* 2 codon 12 mutation fraquency was 0/30 in normal tissues, 0/17 in adenomas with mild atypia, 3/37 (8.1%) in adenomas with moderate atypia, 15/18 (83.3%) in adenomas with severe atypia, 19/73 (26.0%) in primary advanced carcinomas and 3/13 (23.1%) in metastatic tumors. These results indicate that the mutation frequency in adenomas increased as atypia progressed. However, the mutation frequency in advanced carcinomas was significantly lower than that in adenomas with severe atypia ($P<0.0001$ by $\chi^2$ test). The mutation frequency was also low in metastatic tumors.

There are two possible mechanisms for colorectal tumorigenesis. Adenomas may progress to an increasingly malignant phenotype and finally become carcinomas. Alternatively, colorectal carcinomas may arise from colorectal mucosae directly. It is generally thought that most colorectal carcinomas develop from adenomas and that only a small portion of them occur *de novo*. Recently, the results of molecular genetic analyses supported the proposition that most carcinomas arise from adenomas.[6]

In contrast, there have been several reports opposing the adenoma-carcinoma sequence. Firstly, the distribution of adenomas and carcinomas was found to be different.[16] Secondly, the numbers of adenomas and carcinomas were determined in FAP and nonFAP patients, and the rates of appearance of carcinomas per adenoma were compared between the two groups. The rate was found to be lower in FAP patients than in nonFAP patients. If most carcinomas arise from adenomas, there should be a greater number of carcinomas in FAP patients.[17] Thirdly, most advanced colorectal carcinomas are of Bormann type II, having a depressed shape. If most carcinomas are derived from polypoid adenomas, there should be intermediate forms. However, such intermediate lesions are not often seen endoscopically. Nakamura has proposed, on the basis of pathological and statistic analyses using the nucleus-gland ratio and structural atypia as indices, that 20 to 30% of colorectal carcinomas may arise from adenomas, but that other carcinomas develop *de novo*.[8, 9]

Our results showed that the c-K-*ras* 2 mutation frequency was similar in the FAP and nonFAP tumors, as described previously.[18] The mutation frequency increased as adenomas progressed. However, the mutations were much less common in carcinomas than in adenomas with severe atypia. If most carcinomas arise from adenomas with severe atypia, the c-K-*ras* 2 gene mutation frequency in carcinomas should be the same as or higher than that in these adenomas. Therefore, our results suggested that many colorectal carcinomas do not arise from adenomas with severe atypia, but from normal mucosae or other lesions. However, the adenoma-carcinoma sequence may occur sometimes, since 26.0% of carcinomas contained c-K-*ras* 2 gene mutations.

There have been several reports on the mutation frequency in colorectal tumors. Forrester *et al.* reported that all of 8 adenomas and 39.4% of 66 carcinomas contained c-K-*ras* 2 gene mutations.[3] Vogelstein *et al.* reported that 57% of class III (most advanced) adenomas and 47% of carcinomas had c-K-*ras* 2 or N-*ras* gene mutations.[6] Although the c-K-*ras* 2 gene mutation frequency was lower in our carcinoma samples, every report showed a higher mutation frequency in adenomas than in carcinomas. It is not clear why the mutation frequency in our carcinoma samples was lower than that in carcinomas in other reports. However, we only examined codon 12 in the c-K-*ras* 2 gene. If we look for mutations in codons 13 and 61 in the c-K-*ras* 2 gene, and mutations in the N-*ras* gene, we would expect to find more mutations.

What is the role of the mutated c-K-*ras* 2 gene in colorectal tumors? In the case of adenomas, the mutated c-K-*ras* 2 gene must offer some growth advantage, since the mutation frequency increases as adenomas advance. It is unclear what role the mutated *ras* gene plays in colorectal carcinogenesis.

The origin of colorectal carcinomas is a very important issue from a clinical viewpoint, too. More colorectal tumors should be examined for *ras* gene mutations. Other genetic markers such as the DCC and p53 genes should also be analyzed to clarify the origin of colorectal carcinomas.

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