ANTICANCER RESEARCH *24*: 2471-2476 (2004)

Association of K-ras Mutations with Liver Metastases from Colorectal Carcinoma

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Abstract. *Background: Colorectal carcinomas were studied regarding early proof of liver metastases through determination of K-ras mutations. Patients and Methods: Seventy-seven colorectal carcinomas were investigated for the presence of point mutations in codon 12 and 13 of the K-ras gene, using single-strand conformation polymorphism (SSCP) analysis and direct sequencing. Results: Twenty-six carcinomas were positive for K-ras mutations, of which 21 had codon 12 and 5 had codon 13 mutations. Twenty patients with K-ras-positive tumor (20 out of 26: 77%) developed liver metastases, of which 13 had simultaneous metastases and 7 had metachronous metastases. There was a significant association between K-ras mutations and liver metastases ($p=0.03$). A multivariate logistic regression model demonstrated that the involvement of lymph node ($p<0.01$) and K-ras mutations ($p=0.02$) were predictive factors for liver metastases from colorectal carcinoma. Sequencing in carcinomas without liver metastases showed the base change in the first position of codon 12, whereas with liver metastases it showed significantly frequent base change in the second position of codon 12 ($p<0.01$). Conclusion: It is suggested that the presence of K-ras mutation, especially base change in the second position of codon 12, may predict liver metastases from colorectal carcinoma.*

Prognosis of patients with colorectal carcinoma is influenced by liver metastases. Natural history studies of patients with untreated liver metastases suggested that survival after diagnosis of hepatic involvement ranges from 3 to 24 months, with few patients surviving more than 5 years (1-3). Thus, about 70% of all patients dying of colorectal carcinoma showed liver metastases at autopsy (4).

Colorectal carcinogenesis is regarded as a multi-step process involving several genetic alterations. Mutations within the ras-gene family, especially in codons 12 and 13 of K-ras, are among the first abnormal genes to appear in sporadic colorectal adenocarcinoma (5-7). The incidence of K-ras codon 12 and 13 mutations is reported in nearly half of the carcinomas which mutated (5-8). A series of investigations on the occurrence of K-ras mutations had been undertaken on colorectal carcinomas, resulting in the belief that the precise K-ras nucleotide change might have predictive value for patients at high risk of recurrence and poor prognosis (9-11).

Several studies have focused on K-ras mutations and liver metastases from colorectal carcinomas (12-14). However, there is little knowledge about the relationship between K-ras mutations and metachronous liver metastases from colorectal carcinoma. In the present study, we evaluated clinical data for primary colorectal carcinoma aiming at the detection of K-ras mutations and investigated whether the base substitution in the K-ras gene might have predictive value for patients at high risk of liver metastases.

Patients and Methods

Patients and tumor specimens. Tumor samples were obtained from resection colon specimens from 77 patients undergoing elective surgery for primary carcinoma. No patients received neoadjuvant chemotherapy or radiation treatment. All the patients had given their written informed consent for the detection and identification of K-ras mutations in codon 12 and 13 by single-strand conformation polymorphism (SSCP) and direct sequence analysis.

The median age of the patients was 64 years (range, 37 to 84), and there were 46 men and 31 women. The primary tumors were located as 42 in the colon and 35 in the rectum. Regarding the tumor differentiation, 37 tumors were diagnosed as well-differentiated, 36 as moderately-differentiated, 1 as poorly-differentiated and 3 with mucinous carcinomas. The depth of invasion was 36 in between the submucosa and subserosa and 41 in

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Key Words: Colorectal carcinoma, K-ras mutation, liver metastases.

Table I. *Relation of clinicohistopathological and K-ras parameters to liver metastases from colorectal carcinoma.*

	Tumor without liver metastases	Tumor with liver metastases	*P* value
No. of tumors	32	45	
Age (median 64)			
<64	14	22	NS
≥64	18	23	
Sex			
male	18	18	NS
female	14	17	
Location			
colon	18	24	NS
rectum	14	21	
Differentiation			
well	15	22	NS
mod, poor, muc	17	23	
Depth of invasion			
sm; mp; ss, si	20	16	0.03
se, a2; si, ai	12	29	
Involvement of lymph node			
negative	22	10	<0.01
positive	10	35	
K-ras gene			
no mutation	26	25	0.03
mutation	6	20	

Analysis by Chi-square test.

the subserosa or exceeding serosa, whereas the involvement of lymph nodes was 32 with negative nodes and 45 with positive nodes. According to Dukes' stage at initial presentation, there were 3 in stage A, 23 in stage B, 19 in stage C and 32 in stage D with liver metastases and without other recurrence. We selected two groups of patients with colorectal carcinomas. The first group consisted of 32 patients who remained free of recurrent disease for at least 3 years after the initial operation. The second group was formed by 45 patients, who were detected with liver metastases peri-operatively and within 3 years after the initial operation by CT-scan or ultrasonography.

The tumor tissue had been taken from the center of the tumor, each tissue sample was placed in liquid nitrogen immediately and stored at -80˚C until further processing.

DNA extraction. DNA was extracted from each tumor and the corresponding normal tissue as described previously (15) using a DNA extraction kit (Sepa Gene; Sanko Junyaku Co. Ltd., Tokyo, Japan). The DNA was dissolved in TE buffer (10 mM Tris, 1 mM ethylenediamine tetraacetic acid) and weighed.

Table II. *Univariate logistic regression model with regard to liver metastases from colorectal carcinoma.*

Covariate	OR	95% CI	*P* value
Age	0.936	0.291–3.013	0.91
Sex	0.752	0.244–2.323	0.62
Location	0.759	0.235–2.450	0.64
Differentiation	0.585	0.184–1.886	0.37
Depth of invasion	1.396	0.439–4.434	0.57
Involvement of lymph node	9.445	2.735–32.618	<0.01
K-ras mutations	4.100	1.170–14.376	0.02

OR: odds ratio ; 95% CI: 95% confidence interval

Table III. *Multivariate logistic regression model with regard to liver metastases from colorectal carcinoma.*

Covariate	OR	95% CI	*P* value
Involvement of lymph node	8.949	2.947–27.169	0.0001
K-ras mutations	4.422	1.289–15.171	0.0181

OR: odds ratio ; 95% CI: 95% confidence interval

Mutation analysis. DNA samples were amplified using polymerase chain reaction (PCR) and analyzed by the single-strand conformation polymorphism (SSCP) method. The primers for the K-ras mutation were the ras Gene Primer set (Takara Biochemicals, Kyoto, Japan). Conditions for PCR were the same as those described previously (16). When abnormal bands were detected in the SSCP analysis, single-stranded DNA fragments were extracted, amplified by asymmetrical PCR and then subjected to direct sequencing by a dideoxy chain-termination reaction (16).

PCR-SSCP analysis. DNA samples were amplified for SSCP analysis of the K-ras gene using PCR (5 min at 97˚C, once: 1 min at 95˚C, 1 min at 58˚C, and 1 min at 72˚C, for 35 cycles; 10 min at 72˚C, once). The reaction mixture (5 μl) contained 200 ng of genomic DNA, the proper pair of each 0.2 μM primer, 25 μM each four deoxynucleoside triphosphate, 1xPCR buffer, Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), and [α-^{32}P]dCTP. Oligodeoxynucleotide primers for the K-ras mutation were the ras Gene Primer Set (Takara Biochemicals, Kyoto, Japan). PCR products were diluted 10-fold with formamide-dye solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and a 2 μl sample of the diluted reaction mixture was heated for 5 min at 80˚C, followed by electrophoresis in 5% polyacrylamide gel containing 5% glycerol, as described previously (17). After electrophoresis at 20-25˚C, the gel was exposed to X-ray film at -70˚C. When abnormal bands were detected in tumor DNAs in the PCR-SSCP analysis, the DNAs from the corresponding normal tissues were also analyzed under the same conditions. The abnormal bands that were detected in both tumors and the corresponding normal tissues were classified as being due to germ line mutation. The abnormal bands that were present in DNAs from tumors but were absent in DNAs from corresponding normal tissues were classified as mutant bands due to

Table IV. *Incidence of K-ras mutations according to liver metastases status.*

Tumor without liver metastases	Tumor with liver metastases	
	synchronous	metachronous
6/ 32 (19%)*	13/ 32 (44%)	7/ 13 (54%)

* $p=0.0555$ *vs.* synchronous, $p=0.0186$ *vs.* metachronous

Table V. *Incidence of K-ras mutations in relation to the Dukes' stage.*

Stage	Without liver metastases [no. (%)]	With metachronous liver metastases [no. (%)]	*P* value
Dukes' A	0/3 (0%)	0/0 (0%)	–
B	4/19 (21%)	3/4 (75%)	0.06[a]
C	2/10 (20%)	4/9 (44%)	0.34[a]

Analysis by a Fisher's exact test

somatic mutations in the tumors. DNA samples exhibiting germ line and/or somatic mutation were subjected to PCR-SSCP at least twice and only the reproducible cases were used.

Sequencing of the mutated strand. Abnormal single-stranded DNA fragments were extracted with distilled water from the corresponding bands on PCR-SSCP gels as described previously (18, 19). The DNA fragments were amplified through the asymmetrical PCR in a 100 μl mixture under the same conditions as those for PCR-SSCP analysis, with the exception that the ratio of primers was 100/1 or 1/100 for sense and antisense primers, respectively. The amplified DNA samples were purified using a QIAGEN spin 20 column (QIAGEN Inc., Chatsworth, CA, USA), and they were sequenced with the dideoxy chain-termination reaction using Sequenase version 2.0 (United State Biochemical Co., Cleveland, OH, USA). Primers used for sequencing were the same as those in the PCR-SSCP. Sequencing was performed more than twice for each DNA fragment.

Statistical analysis. Frequency distributions (patient characteristics and the base change in codon 12 and 13 of K-ras gene) were analyzed using the Chi-square test. Univariate and multivariate logistic regression models were calculated to identify independent prognostic factors by Stat View 5.0 software. The incidence of K-ras mutation in relation to the Dukes' stage was analyzed using the Chi-square test and Fisher's exact test. Results were considered significant at $p<0.05$.

Results

Twenty-six mutations were found in 77 colorectal carcinomas (34%), while no mutation was noticed in normal tissues. The relationship of K-ras mutation and clinicopathological characteristics to the liver metastases from colorectal carcinoma is shown in Table I. There was no relationship between the liver metastases from colorectal carcinoma and patient's age, sex, location of tumors, or differentiation of tumors, whereas the significant association of liver metastases with depth of invasion ($p=0.03$), involvement of lymph nodes ($p<0.01$), or K-ras mutations ($p=0.03$) was found. A univariate logistic regression model demonstrated that involvement of lymph node ($p<0.01$) and K-ras mutations ($p=0.02$) were predictive factors for liver metastases from colorectal carcinoma. The patient's age, sex, location of tumors, differentiation of tumors and depth of invasion were not associated with the risk of liver metastases (Table II). A multivariate logistic regression model included involvement of lymph nodes and K-ras mutations. In this model, both of them remained the statistically significant prognostic factor for liver metastases (Table III).

Table VI. *Incidence of K-ras mutation and base substitutions in codon 12 and 13.*

		Tumor without liver metastases	Tumor with liver metastases	*P* value*
Codon				
Codon 12		4	17	NS
Codon 13		2	3	
Base substitution				
Codon 12	first base	4	3	<0.01
	second base	0	14	
Codon 13	first base	0	0	NS
	second base	2	3	

*Analysis by Fisher's exact test

Table VII. *Base substitutions in K-ras mutations related to liver metastases status from colorectal carcinoma.*

Base substitution and amino acid		Simultaneous liver metastases	Metachronous liver metastases
Codon 12	(GGT glycine)		
first base	AGT serine	1	0
	TGT cysteine	0	0
	CGT arginine	2	0
second base	GAT aspartic acid	4	3
	GTT valine	4	1
	GCT alanine	1	1
Codon 13	(GGC glycine)		
second base	GAC aspartic acid	1	2

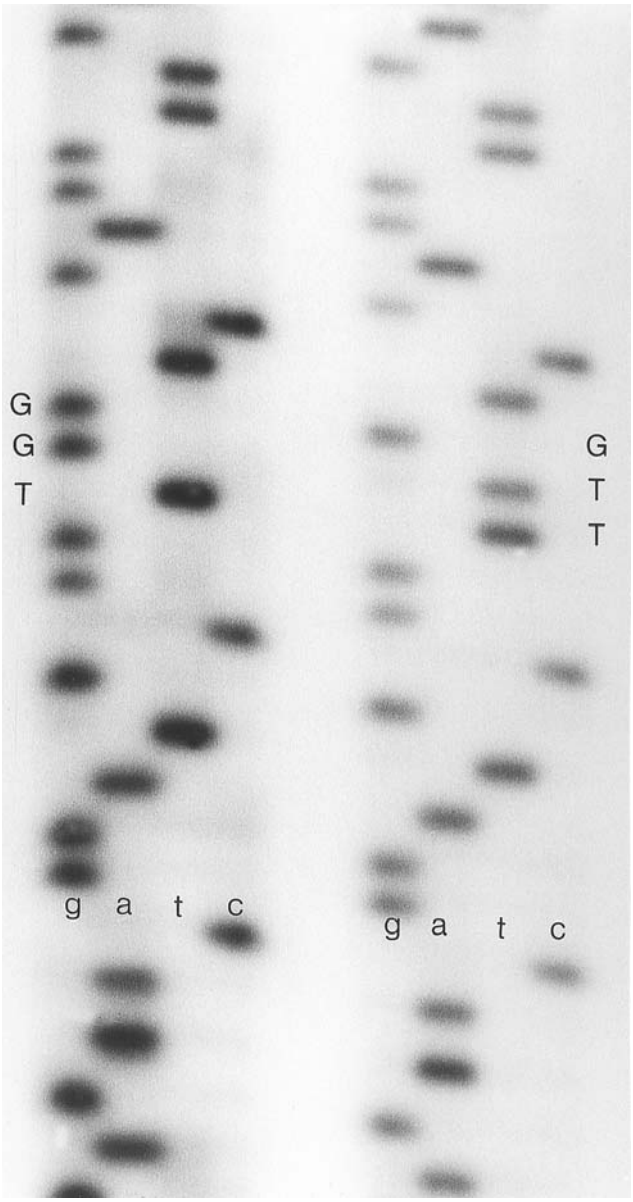

Figure 1. *Representative integrated morphologic-genetic analysis: DNA sequencing of K-ras PCR product. The first coding exon of the K-ras gene has been amplified and sequenced from mixtures of normal DNA (left lane) and mutated DNA (right lane).*

The correlation of K-ras mutations with liver metastases status was examined (Table IV). The incidence of K-ras mutations in patients with metachronous liver metastases was significantly higher than those without liver metastases ($p=0.0186$). The incidence in patients with synchronous liver metastases was also high, but did not reach significance ($p=0.0555$).

Table V shows the correlation between K-ras mutation and Dukes' stage of colorectal carcinomas at initial presentation. The relationship of metachronous liver metastases and K- ras mutation in Dukes' B tumors was high, but this did not reach significance due to the small subgroup numbers ($p=0.06$). In Dukes' C tumors, the relationship was not observed ($p=0.34$). The incidence of K-ras mutation and base substitutions in codon 12 and codon 13 related to liver metastases are shown in Table VI.

The incidence of the mutation in codon 12 (21/26) was higher than that in codon 13 (5/26), but there was no significant difference between the incidence of the mutation in codon 12 or codon 13, and the presence of liver metastases. For the carcinomas without liver metastases, the base substitution was present at the first position of codon 12, whereas the mutation at the second position of codon 12 was markedly predominant in the carcinomas with liver metastases ($p<0.01$).

In all cases in which the mutation of the K-ras gene was detected, direct sequence analysis was performed confirming and identifying the K-ras mutational change (Figure 1). Base substitutions in K- ras mutations related to liver metastases status are shown in Table VII. In codon 12, the predominant mutation was a G to A transversion in the second base pair (7/21), encoding aspartic acid; and then a G to T transversion in the second base pair, encoding valine, was observed in 5 tumors. Especially in those with the metachronous liver metastases, the base substitutions were only at the second position of codon 12. In codon 13, the mutation was G to A transition at the base pair, encoding aspartic acid.

Discussion

The main finding in the present study was that K-ras mutations in codon 12 and 13 in colorectal carcinoma were the independent risk factor for liver metastases and the striking difference was proved between the position of mutations at codon 12 of the K-ras gene and the presence of liver metastases.

The incidence of K-ras codon 12 and 13 mutations in tumor tissue was 34% in the present study, which is slightly less frequent than previous studies, reporting an incidence of 40- 50% (5, 6, 20).

Concerning the prognostic significance of K-ras mutation in colorectal cancer, conflicting results have been reported in previous studies, some reporting a poor survival in patients with K-ras mutated tumors (9, 10, 21) and others no correlation at all (22, 23). While, in clinical practice, the prediction of metachronous liver metastases following potentially curative resection for patients with colorectal cancer is expected. Current clinical diagnostic procedures, such as computed tomography, detect metastases with a sensitivity of 94% when they exceed 1 cm in diameter (24) and only 31% when the metastases are below this size (25), but are unable to

detect micrometastases. Schimanski *et al.* (14) detected K-ras mutation in the liver tissue at the time of surgery, which may allow early proof of metachronous metastases. In the present study, the significant predictive value of overall K-ras mutation on liver micrometastases was shown in patients with potentially curative resection for cancer.

The predictive value of overall K-ras mutation was shown when the cases were stratified according to Dukes' stage in the present study. On the association with Dukes' classification, the frequency of K-ras mutated tumors in Dukes' B suffering from liver metastases was 75% and a good correlation was expected between liver metastases and the presence or absence of K-ras mutations in Dukes' B.

However, predictive value was not shown in the Dukes' C group. It could be explained by the fact that the other prognostic parameter, involvement of lymph nodes, masked the predictive power of the K-ras mutation. When the tumor has spread through the lymphatics, the risk of liver metastases for Dukes' C tumors no longer depends only on its genotype; it also depends on the fact that it has spread beyond the organ of origin. Our result is in disagreement with those of Bennett *et al.* (22) or Bouzourene *et al.* (23), who found that negative results were obtained for K-ras mutations when the study was restricted to Dukes' B. On the other hand, in the report of Benhattar *et al.* (10), the relationship of local or distant recurrence and K-ras mutation in Dukes' B tumors was highly significant, Also, Ahnen *et al.* (21) found K-ras mutation to correlate with poor prognosis in Dukes' B not in Dukes' C stage. The risk of recurrence including liver metastases in Dukes' B carcinoma may be given by K-ras mutations, which appeared to be a genetic marker of tumor aggressiveness.

Several studies have shown that carcinomas contain a homogenous K-ras profile in different parts of the tumor (5, 11, 26) and that secondary deposits manifest the same mutation as the primary tumor (9, 11, 12, 14). Most authors also reported that the G-A alteration of codon 13 was a indolent mutation for the metastases and the mutations in the second position of codon 12 might characterize the most aggressive tumor behavior. However, the reports were conflicting with regard to the base substitutions of codon 12. Benhattar *et al.* (10) reported that the most frequent mutation was GGT to GA T (Asp) in codon 12 in recurring tumor. Similarly, Finkelstein *et al.* (9) showed that the G-A mutation of K-ras codon 12 was most prevalent in liver metastases, but there was no G-T mutation in the second position of codon 12 in liver metastases. On the other hand, Moerkerk *et al.* (27) concluded that G-T and G-C transversions were associated with metastatic behavior, whereas G-A transversions were not. Andersen *et al.* (11) reported that all kinds of base substitution showed metastatic behavior. In the present study, the mutation in the second position of codon 12 found in colorectal carcinomas from a group of patients with liver metastases compared with that seen in a non-liver metastases group revealed a statistically significant difference G-A and G-T transversion occurred almost exclusively in tumors with liver metastases. Furthermore, our study might suggest that the mutation in the second position of codon 12 was strongly associated with the metachronous liver metastases resulting from liver micrometastases. It was suggested that the mutation in the first position of codon 12 had a rather low risk of metastatic spread, whereas that in the second position bore a measurable risk of liver metastases.

Liver metastases are delineating factors of postoperative survival in patients suffering from colorectal carcinoma. If we could determine the presence of liver micrometastases at the time of primary tumor resection, the adjuvant chemotherapy including the intrahepatic infusion of anti-cancer agents might be commenced early postoperatively. In this respect, it was suggested that the detection of K-ras codon 12 mutation of primary tumor could predict liver metastases from colorectal carcinoma, and a further prospective study will be needed to find the effect of preventive chemotherapy to liver micrometastases based on the presence of K-ras mutations.

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Received February 18, 2004
Accepted May 6, 2004