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Advances in Brief

Loss of Expression of the *DCC* Gene during Progression of Colorectal Carcinomas in Familial Adenomatous Polyposis and Non-Familial Adenomatous Polyposis Patients[1]

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Abstract

We have previously observed that the frequency of loss of heterozygosity (LOH) on chromosome 18q was low in adenomas and intramucosal carcinomas, whereas invasive carcinomas exhibited a high frequency in familial adenomatous polyposis patients (M. Miyaki *et al.*, Cancer Res., *50:* 7166-7173, 1990). In the present study, LOH at the *DCC* locus on chromosome 18q and the expression of *DCC* gene into mRNA were analyzed in colorectal tumors with distinct histopathological types. The carcinomas that showed 18q LOH also lost the *DCC* locus. The expression of *DCC* gene into mRNA was examined at the level of 233-base pair fragments of nucleotide 986-1218 in *DCC* complementary DNA. In a moderate-to-severe adenoma, 5 carcinoma-in-adenomas, and 4 intramucosal carcinomas, the level of expression was as high as in normal colorectal mucosa, whereas it was greatly reduced or not detectable in most (13 of 16) invasive carcinomas. Among these invasive carcinomas, 7 of 11 showed 18q LOH, but 4 showed no LOH. These results suggest that the *DCC* gene is included in the allelic deletion on chromosome 18q, and that the progression of colorectal carcinoma from early stage to advanced stage accompanies the inactivation of the *DCC* gene through LOH and other mechanisms.

Introduction

Colorectal carcinoma has been regarded as generating mainly from polyps through multiple transition stages both in FAP,[3] an autosomal dominant disease with a high risk of cancer, and in non-FAP. The progression is presumed to be as follows: from adenoma with moderate dysplasia, through adenoma with severe dysplasia, and intramucosal carcinoma, to invasive carcinoma. In this course, multiple genetic changes occur, including inactivation of tumor suppressor genes. In a previous study on the mechanism of colorectal carcinogenesis in FAP, we demonstrated that the LOH on chromosome 18q was frequently detected in invasive carcinoma but rarely in the earlier stage, such as adenoma and intramucosal carcinoma (1). LOH on chromosome 18 in sporadic colorectal carcinoma has also been observed in other reports (2–7). Therefore, LOH on chromosome 18q seemed to be included in the progression of carcinoma from an early stage to an advanced stage. Fearon *et al.* (8) identified a candidate tumor suppressor gene on chromosome 18q, the *DCC* gene (deleted in colorectal carcinoma). There is a possibility that a target of LOH on chromosome 18q may be the *DCC* gene. In the present study, to clarify the nature of LOH on chromosome 18q, we investigated LOH at the *DCC* locus and the expression of *DCC* gene into mRNA in colorectal tumors of distinct histopathological stages. We confirmed that the *DCC* gene was included in the deleted region and that its expression into mRNA was much reduced in invasive carcinoma.

Materials and Methods

Tumor Specimens. In the present study, 23 tumors from 14 FAP patients and 4 tumors from 4 non-FAP patients were analyzed. A part of each specimen was fixed with formalin for diagnosis by histopathological staining. The remaining part was used for analyses of DNA and poly(A) RNA.

Histopathological diagnosis was performed as previously described (1), according to the General Rules of the Japanese Research Society for Cancer of Colon and Rectum (9). The tumors included "adenoma with moderate dysplasia," "carcinoma-in-adenoma," "intramucosal carcinoma," and "invasive carcinoma."

Analysis of LOH. Genomic DNA was extracted from each tumor and from normal colorectal mucosa corresponding to it, and LOH on chromosome 18q was analyzed as previously described (1). The following enzymes and probes were used in the present study: *Pst*I and *Os4* (10); *Msp*I and p15-65 (8); *Eco*RI and 1.65 DCC-cDNA (8).

Analysis of Poly(A) RNA. Poly(A) RNA was prepared from each frozen tissue using guanidium thiocyanate and oligodeoxythymidylate cellulose. The expression of *DCC* gene into mRNA was analyzed as described by Fearon *et al.* (8). First strand cDNA was synthesized from poly(A) RNA by using the antisense primer: 5′-ATGCGAATT-CAGCCTCATTTTCAGCCACACA-3′. The 233-base pair fragment of *DCC* cDNA (nucleotide 986–1218) was then amplified by polymerase chain reaction by using the sense primer, 5′-ATGCGA-ATTCTTCCGCCATGGTTTTTAAATCA-3′ and the antisense primer. The 233-base pair fragment was separated in agarose gel, transferred to a nitrocellulose membrane, and hybridized with ^{32}P-labeled 57 nucleotide oligomer coding 1141–1197 in *DCC* cDNA, as described previously (11).

Results

We have previously detected LOH at high frequency in invasive colorectal carcinomas from FAP patients (63%) using *Os4* (18q21.3-qter) (1). In the present investigation, we analyzed LOH at *DCC* locus in invasive carcinoma that showed LOH at the *Os4* locus (Fig. 1). For example, hybridization of *Os4* to *Pst*I-fragment of DNA from PLK89N detected 7.5 kilobases and 4.8-kilobase bands, and the former was lost in the carcinoma in PLK89Ca. Hybridization of p15-65 to *Msp*I-fragment of DNA from the same patient detected 7.8 kilobases and 10.5-kilobase bands, the latter of which was deleted in carcinoma PLK89Ca. The combination of *Eco*RI and 1.65 *DCC* cDNA gave 20 kilobases and 16-kilobase bands, which were confirmed to be polymorphic. In PLK89Ca, the 16-kilobase band was kept, while the 20-kilobase band was lost on this patient. These results indicate that loss at *Os4* corresponds to those at p15-65

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; LOH, loss of heterozygosity; cDNA, complementary DNA; poly(A) RNA, polyadenylated RNA.

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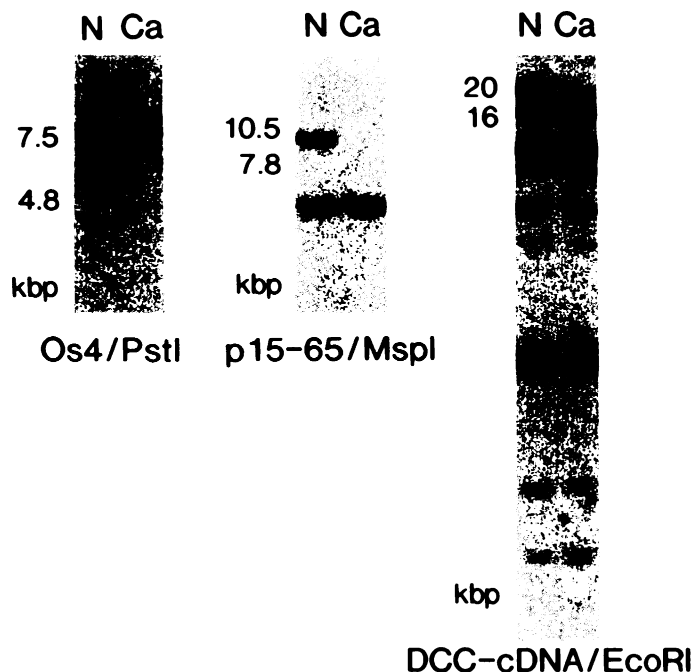

Fig. 1. LOH on chromosome 18q in normal colorectal mucosa and carcinoma from patient PLK89. *N*, normal tissue; *Ca*, carcinoma.

and 1.65 DCC-cDNA. There were many other carcinomas that showed similar LOH patterns. Therefore, it is suggested that the *DCC* gene is the target of LOH on chromosome 18q in colorectal carcinomas from FAP and non-FAP patients.

The *DCC* gene has been demonstrated to be inactivated in sporadic colorectal carcinomas by experiments showing loss of expression of this gene into mRNA (8). To clarify whether the inactivation of *DCC* gene occurs in the early or late stage of carcinogenesis in FAP patients, we examined the expression of the *DCC* gene into mRNA in colorectal tumors with distinct histopathological types, including an adenoma with moderate dysplasia, intramucosal carcinomas, and invasive carcinomas. The expression of *DCC* mRNA was determined by intensity of the 233-base pair band of nucleotide 986–1218 in DCC-cDNA, which was hybridized with synthetic 57-mer oligonucleotide (encoding 1141–1197 in *DCC* cDNA). This hybridization also detected the 180-base pair band, which may be an alternatively spliced transcript. As shown in Fig. 2, *DCC* mRNA was detected in adenoma with moderate dysplasia, PLK121Am, at the same degree as in normal mucosa from FAP (PLK121N) and non-FAP (*N muc*). *DCC* mRNA was also detectable in carcinoma-in-adenomas (PLK121A1, -A2, -A3, and A6, and PLK122A1) and intramucosal carcinomas (PLK121A4, -A5, and PLK122A2 and -A3), although its level was not as high as in normal mucosa and moderate adenoma. The expression of *DCC* mRNA was absent in most invasive carcinomas, except PLK36Ca, PLK58Ca, and PLK60Ca. *DCC* mRNA in even these three carcinomas was considerably reduced when compared with that in normal mucosa.

Table 1 is a list of the individual tumors examined for 18q LOH and the expression of the *DCC* gene into mRNA in the present study. Moderate adenoma, almost all carcinoma-in-adenomas, and intramucosal carcinomas expressed *DCC* mRNA and retained both alleles of the *DCC* gene. A carcinoma-in-adenoma, PLK121A1, and an intramucosal carcinoma, PLK121A4, showed LOH on 18q; however, it still expressed *DCC* mRNA. On the contrary, there were many invasive carcinomas without expression of the *DCC* gene, 64% of which (7 of 11) also showed LOH on 18q, but 36% of which (4 of 11) did not exhibit 18q LOH for the probes including *Os4*, p15-65, and *DCC* cDNA.

Discussion

In most FAP cases, it appears that moderate colorectal adenoma develops into severe adenoma, converts into early-stage carcinoma, and advances to invasive carcinoma, with an accumulation of genetic changes in tumor suppressor genes. With respect to the LOH on 18q, the frequency was very low in moderate and severe adenomas and in intramucosal carcinomas, but it was high in invasive carcinomas (1). These data strongly suggested that inactivation of a tumor suppressor gene on chromosome 18q was associated with the progression of early-stage carcinoma into advanced-stage carcinoma.

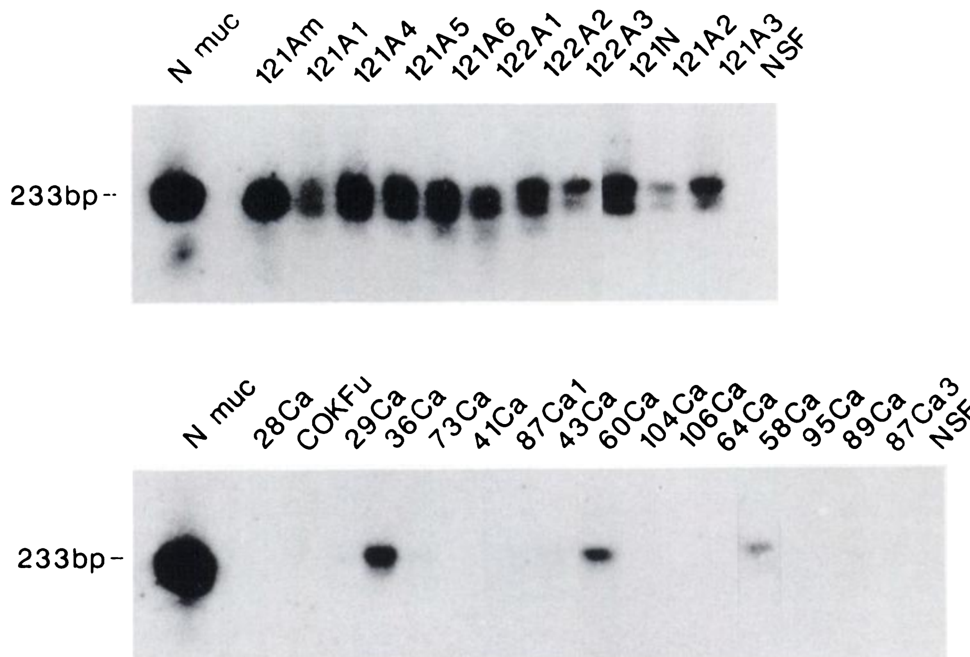

Fig. 2. Expression of *DCC* gene in colorectal tumors. The 233-base pair fragment of *DCC* cDNA was synthesized from mRNA, amplified by polymerase chain reaction, and detected by 57 synthetic nucleotides encoding 1141–1197 in *DCC* cDNA. The name of samples are the same as those in Table 1. *N muc*, and *N*, normal colorectal mucosa; *Am*, adenoma with moderate dysplasia; *A*, carcinoma-in-adenoma or intramucosal carcinoma; *Ca* invasive carcinoma; *NSF*, normal skir fibroblast.

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Although the highest frequency of LOH on chromosome 18q was observed on the Os4 locus (18q21.3-qter), we could not define the specific region for the target of LOH, since no specimen exhibited partial deletion on chromosome 18q. Fearon *et al.* (8) isolated a candidate tumor suppressor gene on chromosome 18q that is altered in sporadic colorectal carcinoma, termed "*DCC gene*." Then, we examined the possibility that a target for this LOH is the *DCC* gene. We detected LOH on p15-65, which is located within an intron of the *DCC* gene, in colorectal carcinomas that showed LOH on the *Os4* locus (Fig. 1). LOH was also observed when 1.65 DCC-cDNA was used as a probe. These data indicate that the *DCC* gene is included in the allelic deleted region on chromosome 18q.

We could also observe a correlation between the malignancy of carcinoma and the level of *DCC* mRNA. The amount of *DCC* mRNA was determined after synthesis and amplification of cDNA from poly(A) RNA, since *DCC* mRNA has been described to be undetectable in Northern blot analysis (8). The expression of the *DCC* gene into mRNA was absent or greatly reduced in almost all invasive carcinomas, but it was high in normal mucosa, adenomas, intramucosal carcinomas, and early-stage carcinomas (Fig. 2). These results were reproducible, although this method seems to be semiquantitative. The suppression of tumorigenicity has been demonstrated by the appearance of *DCC* mRNA after introduction of normal human chromosome 18 into advanced carcinoma COKFu (11). Therefore, it is probable that the inactivation of *DCC* gene is involved in advancement of early carcinoma into invasive carcinoma.

Table 1 *Expression of DCC* gene and LOH on chromosome 18q in colorectal tumors

Tumor	Status[a]	Histopathological type[b]	*DCC* mRNA[c]	LOH on 18q[d]
Mucosa	S	Normal	+++	12
PLK121N	F	Normal	++	12
PLK121Am	F	Moderate Ad	+++	12
PLK121A1	F	Ca-in-Ad	+	Loss
PLK121A2	F	Ca-in-Ad	+	12
PLK121A3	F	Ca-in-Ad	++	12
PLK121A6	F	Ca-in-Ad	++	12
PLK122A1	F	Ca-in-Ad	++	12
PLK121A4	F	Intramuc Ca	++	Loss
PLK121A5	F	Intramuc Ca	++	12
PLK122A2	F	Intramuc Ca	++	12
PLK122A3	F	Intramuc Ca	++	12
PLK41Ca	F	Inv Ca (mucinous)	−	Loss
PLK60Ca	F	Inv Ca (mucinous)	+	12
PLK104Ca	F	Inv Ca (mucinous)	−	—
PLK43Ca	F	Inv Ca (well-diff)	−	Loss
PLK87Ca1	F	Inv Ca (well-diff)	−	12
PLK28Ca	F	Inv Ca (mod-diff)	−	12
PLK36Ca	F	Inv Ca (mod-diff)	+	Loss
PLK58Ca	F	Inv Ca (mod-diff)	+	Loss
PLK64Ca	F	Inv Ca (mod-diff)	−	Loss
PLK73Ca	F	Inv Ca (mod-diff)	−	12
PLK87Ca3	F	Inv Ca (mod-diff)	−	Loss
PLK89Ca	F	Inv Ca (mod-diff)	−	Loss
COK29Ca	S	Inv Ca (mod-diff)	−	Loss
COK95Ca	S	Inv Ca (mod-diff)	−	12
COK106Ca	S	Inv Ca (mod-diff)	−	—
COKFu	S	Inv Ca (poor-diff)	−	Loss

[a] F, FAP; S, non-FAP.
[b] Moderate Ad, adenoma with moderate dysplasia; Ca-in-Ad, carcinoma-in-adenoma; Intramuc Ca, intramucosal carcinoma; Inv Ca, invasive carcinoma; well-diff, well differentiated; mod-diff, moderately differentiated; poor-diff, poorly differentiated.
[c] The level of *DCC* mRNA was determined as that of 233 base pair (1141–1197) fragment of *DCC* cDNA.
[d] Loss, with LOH; 12, without LOH; —, not informative.

The majority of the cases seem to progress through this mechanism, although there may be some exceptions such as the case in which the 18q LOH precedes the 17p LOH (12).

The mechanism of loss or reduction of expression of *DCC* gene into mRNA remains to be resolved. As seen in Table 1, among 13 invasive carcinomas without *DCC* expression, 7 showed LOH, but 4 retained both alleles. Among the other 3 (PLK36Ca, PLK58Ca, and PLK60Ca) expressing *DCC* mRNA at a very low level, 2 had 18q LOH and 1 had no LOH. There were two carcinomas having both *DCC* mRNA and LOH: a carcinoma-in-adenoma PLK121A1 and an intramucosal carcinoma PLK121A4 still expressed *DCC* mRNA at a high level despite showing LOH. Accordingly, LOH does not seem to be necessary for loss of expression of *DCC* mRNA. There seem to be other causes, such as alterations in sequences controlling transcriptional regulation, point mutations or insertions within the DCC gene, or alterations in other genes controlling *DCC* gene expression. It will be of interest to examine these possibilities in future studies.

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