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Infrequent Inactivation of *DCC* Gene in Replication Error-Positive Colorectal Cancers

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Colorectal cancers with and without the replication error (RER) exhibit fundamental differences in genotype and phenotype. While alterations in *APC, p53,* and *K-ras* genes have been characterized between RER+ and RER− colorectal cancers, the status of *deleted in colorectal carcinomas (DCC)* gene has not been yet. Alterations of *DCC* gene were analyzed in stage-matched two panels of 30 RER+ and 30 RER− colorectal cancers using semiquantitative reverse transcription-PCR and PCR-LOH analyses. Loss or reduction of DCC mRNA expression and allelic loss at the *DCC* locus were significantly less frequent in RER+ cancers than in RER− cancers. Interestingly, reduced DCC mRNA expression was observed in all 5 RER− cancers with liver metastasis. Our results support the concept that RER+ and RER− colorectal cancers represent different pathways of carcinogenesis and may give a hint for clarifying the specific mechanism of DCC inactivation in RER− colorectal cancers. © 1998 Academic Press

A subset of colorectal cancers has a novel type of genetic instability characterized by alterations within simple repeated sequences termed replication error (RER) (1-3). This phenotype is also known as microsatellite instability (MI) or microsatellite mutator phenotype (MMP). The majority of hereditary non-polyposis colorectal cancer and about 15% of sporadic colorectal cancers belong to the RER pathway of carcinogenesis (4). The clinicopathological characteristics of sporadic and hereditary RER-positive (RER+) colorectal cancers are similar, although not identical (5). RER+ and RER-negative (RER−) colorectal cancers exhibit fundamental differences in the clinical, pathological and molecular characteristics (1-10). RER+ colorectal cancers exhibit a low incidence of somatic mutations in the *p53* tumor suppressor gene (3, 5, 9) and the *c-K-ras* protooncogene (3, 9) and low frequency of chromosome loss and loss of heterozigosity (LOH) (2, 9, 10). The status of *APC* gene in RER+ colorectal cancers is still a matter of argument (9, 11, 12). For the better understanding of the clinicopathological and molecular characteristics of RER+ colorectal cancers, the uniform use of a definition of the RER+ phenotype has been proposed (12, 13). On the other hand, RER+ colorectal cancers have a high rate of slippage-induced frameshift mutations in genes such as *TGFβtypeII receptor* (14), *β2 microglobulin* (15), *hMSH3* (16), *hMSH6* (16), and *BAX* (17) and a high frequency of aberrant DNA methylation (18).

While alterations in *APC, p53,* and *K-ras* genes have been characterized between RER+ and RER− colorectal cancers (3, 5, 9, 11, 12), *deleted in colorectal carcinomas (DCC)* gene, which was identified as a candidate colorectal tumor suppressor gene (19, 20), has not been yet. Marked reduction or loss of DCC mRNA expression has been frequently observed in primary colorectal cancers, especially in cancers with liver metastasis and in liver metastatic foci (21-23). A correlation between the reduced DCC mRNA expression and nodal metastasis in colorectal cancer has been reported as well (24). Allelic loss at the *DCC* locus was found in nearly 100% of colorectal cancers with liver metastasis and of liver metastatic foci (25, 26). Recent study has provided evidence that loss of DCC expression may be associated with poor prognosis of stage II and III colorectal cancers (27). On the contrary, it has been reported that inactivation of the murine *DCC* gene did not affect growth, differentiation, morphogenesis or tumorigenesis in mouse intestine (28). This study suggests that

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Abbreviations used: RER, replication error; LOH, loss of heterozygosity; DCC, deleted in colorectal carcinomas.

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DCC plays little if any part in the physiology of the intestinal epithelium in the mouse, although this does not deny definitively its role in human colorectal carcinogenesis (28). As is the case in pancreatic cancer (29), it is also suggested that the loss of DCC expression may be a consequence of events affecting a linked gene (28). Thus, it is still a matter of argument whether *DCC* gene is a colorectal tumor suppressor or not.

In this regard, comparison of the status of *DCC* gene between RER+ and RER− colorectal cancers may helpful not only for the understanding of these two pathways of colorectal cancers but also for arguing colorectal tumor suppressor role of DCC. In the current study, we analyzed alterations of *DCC* in stage-matched panels of 30 RER+ and 30 RER− primary colorectal cancers using reverse transcription-PCR and PCR-LOH analyses.

MATERIALS AND METHODS

Tumor samples and analysis of RER status. Surgical specimen pairs of primary colorectal cancer and adjacent noncancerous tissue were obtained from patients receiving surgical treatment. Informed consent was obtained from each subject. All tissues were frozen in liquid nitrogen and used for DNA and RNA extraction. Genomic DNA was extracted with phenol/chloroform. Somatic microsatellite alterations were analyzed by PCR using the mononucleotide (BAT25, BAT26, and BAT40) and dinucleotide markers (D2S123, Mfd15, D5S346, D10S197, D18S58, D18S69, and MYCL1). These markers were chosen based on the recommendation in the recent relevant publication (13). The RER was defined as any length change (insertions or deletions) identified within a microsatellite in a given tumor. Finally, tumors exhibiting RER in more than 40% of loci were defined as the RER+ phenotype (13).

Analysis of DCC mRNA expression by RT-PCR. Total RNA was extracted from specimens by the acid guanidinium thiocyanate-phenol-chloroform extraction method and treated with DNaseI. The cDNA was synthesized from 1 μg of total RNA by MMLV reverse transcriptase (Perkin-Elmer/Cetus, Norwalk, CT) with random hexamers (30). The cDNA was amplified by PCR using DCC specific oligonucleotides (19) (5′-TTCCGCCATGGTTTTTAAATCA-3′ and 5′-AGCCTCATTTTCAGCCACACA-3′) or β-actin specific oligonucleotides (31) (5′-CTGTCTGGCGGCACCACCAT-3′ and 5′-GCAACTAAGTCATAGTCCGC-3′). β-actin served as an internal control of the reaction. PCR amplification was carried out in a thermal cycler (Perkin-Elmer/Cetus) under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min; 30 cycles for DCC; 20 cycles for β-actin. Control reactions without reverse transcriptase were performed for each set of PCR reaction.

Southern blotting of PCR products. The PCR products were electrophoresed in a 3% Nusieve agarose gel (FMC Bioproducts, Rockland, ME) and transferred to Hybond N plus membrane (Amersham, Arlington Heights, IL) in 0.4N NaOH. The membranes were hybridized with cDNA probes labeled by the random primer method in 50% formamide/5 × Denhardt's solution/3 × SSC/100 μg/ml salmon sperm DNA/1% SDS at 42°C. Probes were partial cDNAs obtained by RT-PCR, each of which was respectively revealed to be identical to the original DCC and β-actin cDNA with DNA sequencing. After overnight hybridization, followed by washing, the membranes were exposed to X-ray films. Hybridization signals on the films were quantified with a densitometer (Bio-Rad, Richmond, CA).

Preparation of standard curves. Standard curves for PCR were prepared as previously described (22). Plasmids (1 μg) containing amplified regions of DCC or β-actin were diluted serially and logarithmically. Diluted samples were amplified with PCR by the same intervals and cycles as those used for the clinical samples. Southern blotting of amplified products was done with [^{32}P] radiolabeled DCC or β-actin cDNA probe. The resulting band intensities of autoradiograms were measured with the densitometer. The band intensity of each sample was expressed as the peak height (mm) of the curve drawn with the densitometer. Correlation between the quantity of cDNA before PCR and band intensities of DCC and β-actin was analyzed by a hemilogarithmic scale.

Immunohistochemical analysis. Tissue sections were deparaffinized and subjected to heat-induced antigen retrieval in a target unmasking fluid (PharMingen, San Diego, CA). Immunohistochemical staining was performed with the Vectastain Elite ABC reagent kit (Vector Laboratories, Burlingame, CA) according to the manufacture's instructions. Mouse monoclonal antibody to DCC (clone G97-449, PharMingen, CA) was used at a dilution of 1:500. Sections were counterstainned with hematoxylin, dehydrated, cleared, and coverslipped.

PCR-LOH analysis. PCR-LOH analysis using VNTR, M2, and M3 polymorphism was performed as previously described (32). For VNTR polymorphism, PCR products were directly electrophoresed on 2.5% gels. For M2 and M3 polymorphism, PCR products were digested with MspI and separated on 2.5% agarose gels.

Statistical analysis. Fisher's exact test was used to determine the statistical significance. Results were judged as being significant at $P < 0.05$.

RESULTS

Analysis of RER Status

Forty colorectal cancers were classified as RER+. Among them, 10 cancers were excluded from the current study since good quality RNA suitable for semiquantitative RT-PCR was not available. Finally, 30 RER+ colorectal cancers were used for further analyses. TNM stages were as follows: 4 stage I; 14 stage II, 12 stage III. For statistical analysis, stage-adjusted panel of 30 RER− colorectal cancers was chosen and used for further analyses. In addition to these two panels of colorectal cancers, 5 stage IV RER− cancers were also analyzed. Except for the predominance of RER+ cancers in right colon ($P < 0.01$), clinicopathological variables such as age, gender, histological grade were not statistically different between these two panels of colorectal cancers (data not shown). Comparison of these variables between 40 RER+ colorectal cancers and the all remaining RER− cancers revealed the significant association between RER+ and right sided location, poor differentiation, and less prevalent lymphnode and distant metastasis. The more detailed results of the RER status in our panel of all colorectal cancers and its relationship with clinicopathological variables will be described elsewhere.

Preparation of Standard Curves

When RNA samples had been incubated in reverse transcription reactions without reverse transcriptase,

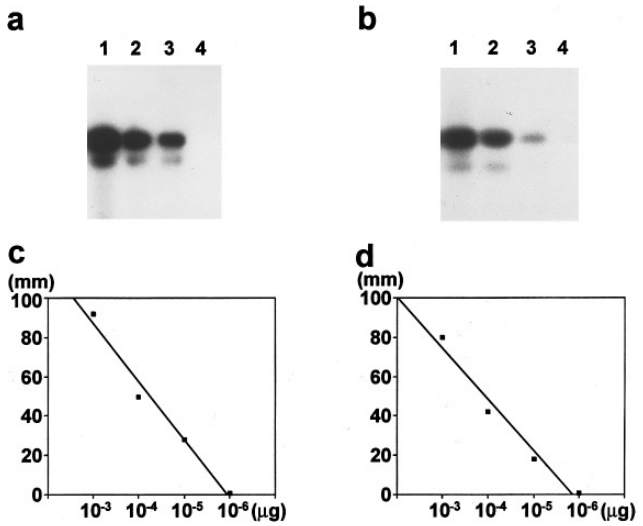

FIG. 1. Correlation between band intensities and amounts of serially diluted cDNAs. *a*, autoradiogram of hybridization after PCR of DCC cDNA. Lanes 1-4 correspond to 10^{-3}-10^{-6} μg of plasmids containing the amplified region of DCC. *b*, autoradiogram of β-actin cDNA. Lanes 1-4 correspond to 10^{-3} – 10^{-6} μg of plasmids. *c*, standard curve for DCC cDNA. Correlation coefficients (r) = −0.980, $P < 0.05$; paired *t*-test. *Vertical and horizontal axes*, band intensity (mm) and the amount of serially diluted cDNA (μg), respectively. *d*, standard curve for β-actin cDNA. $r = -0.968$, $P < 0.05$; paired *t*-test.

no gene products of interest were amplified by PCR (data not shown). This indicates that a trace amount of residual genomic DNA did not show any band even after hybridization with the DCC cDNA probe. For semiquantitative detection using PCR, standard curves were drawn as shown in Figure 1. The correlation between the quantity of cDNA before PCR (Q) and the band intensities (I) of DCC and β-actin was analyzed using a hemilogarithmic scale. The linear relationships, determined by the leastsquares approximation, were: I (DCC) = −29.5 Log Q + 116.5; I (β-actin) = −26.1 Log Q + 100.5. The relations between band intensities and plasmid concentrations were almost linear within the ranges corresponding to 10^{-3}-10^{-6} (μg) of plasmids containing DCC (correlation coefficients; $r = -0.980$, $P < 0.05$; paired *t*-test) and 10^{-3}-10^{-6} (μg) of β-actin plasmids ($r = -0.968$, $P < 0.05$; paired *t*-test). These results indicate that the expression of DCC and β-actin mRNA could be evaluated semiquantitatively within these ranges.

Expression of DCC mRNA in RER+ and RER− Colorectal Cancers

We examined DCC mRNA expression using semiquantitative RT-PCR in stage-matched two panels of 30 RER− and 30 RER+ colorectal cancers with corresponding normal tissues. Figure 2 shows the representative results of 10 RER+ (upper panel) and 10 RER− (bottom panel) cancers. The tumor-normal ratio of DCC mRNA expression was corrected for that of β-actin expression. The ratio of less than one-tenth was considered to be a reduction of DCC mRNA in cancer tissue. Loss or reduction of DCC mRNA expression was significantly less frequent in RER+ cancers than in RER− cancers (5/30, 17% *versus* 16/30, 53%; $P = 0.003$). Interestingly, all 5 stage IV RER− cancers with liver metastasis had reduced DCC mRNA expression (data not shown).

Using the mouse monoclonal antibody to DCC, we could perform the immunohistochemical analysis as well as RT-PCR analysis in 8 cases. Six of 8 cases with normal DCC mRNA expression were DCC-positive, while the remaining 2 cases with markedly reduced DCC mRNA expression were DCC-negative (data not shown).

PCR-LOH Analysis

Allelic loss at the *DCC* locus was determined by PCR-LOH analysis. Three polymorphic sites within *DCC* gene, VNTR, M2 and M3 (MspI RFLP) were used in this study. A positive allelic loss was judged by LOH at least one of the three sites. In 30 RER− cancers, allelic loss was detected in 9/18 (50%), 4/11 (36%), and 5/11 (45%) of informative cases at the VNTR, M2, and M3 sites, respectively. Representative positive cases of LOH are shown in Figure 3. In 30 RER+ cancers, 11 (37%), 10 (33%), and 14 (47%) were informative at VNTR, M2 and M3 sites, respectively, but allelic loss was detected in only 2 cases at M3 site. Overall, allelic loss at the *DCC* locus was less frequently observed in RER+ cancers than in RER− cancers (2/22, 9% *versus* 12/21, 57%; $P = 0.0009$). In 5 RER− cancers with liver metastasis, the frequency of allelic loss at the *DCC* locus was 100% (4/4 of informative cases).

In RER− cancers, 7 of 12 cases with LOH at the *DCC* locus showed reduced DCC mRNA expression, but the remaining 5 did not. On the other hand, 5 of 9 cases without LOH showed reduced DCC mRNA expression. In RER+ cancers, DCC mRNA expression was reduced in 1 of 2 cases with LOH at the *DCC* locus and in 2 of 20 cases without. Thus, reduced DCC mRNA expression in cases without LOH was also less frequent in RER+ cancers than in RER− cancers (2/20, 10% *versus* 5/9, 56%; $P = 0.016$).

DISCUSSION

RER+ and RER− colorectal cancers exhibit fundamental differences in the clinical, pathological and molecular characteristics (1-10). In this kind of study, the definition of the RER+ phenotype is the most im-

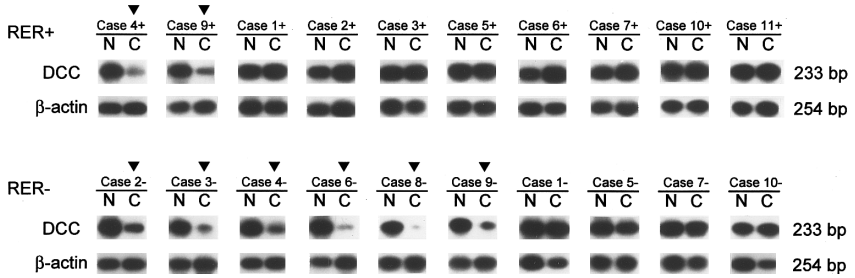

FIG. 2. RT-PCR analysis of DCC mRNA expression in RER+ and RER− colorectal cancers. Representative cases are shown. The frequency of reduced DCC expression in 10 RER+ (20%) and 10 RER− (60%) cancers shown roughly corresponds to that in 30 RER+ (17%) and 30 RER− (53%) cancers examined in this study. β-actin served as an internal control. N and C, matched RNA samples isolated from normal and cancer tissue, respectively. Triangles pointing down indicate the reduction of DCC mRNA expression.

portant issue (12, 13). In support of this, it has been reported that colon cancers with a single replication error positive loci and cancers without positive loci share clinicopathological features that differ from those of cancers with multiple positive loci (6). Recently, the uniform use of a panel of 10 microsatellites and a definition of the RER+ phenotype has been proposed (13). To make the results more comparable between studies, we followed the recommendation (13), and 40 cancers were classified as the RER+ phenotype.

In this study, we showed that loss or reduction of DCC mRNA expression was significantly less frequent in RER+ colorectal cancers than in RER− cancers. As one of the possible mechanisms underlying the low incidence of reduced DCC mRNA expression in RER+ colorectal cancers, allelic loss at the *DCC* locus was then examined. LOH of 18q, where *DCC* gene locates, has been reported to be less frequent in RER+ cancers compared with RER− cancers (2, 33). Consistent with these reports, LOH at the *DCC* locus was less frequently observed in RER+ cancers than in RER− cancers. However, reduced DCC mRNA expression was not always observed in association with LOH at the *DCC* locus, and reduced DCC mRNA expression in cases without LOH was also less frequent in RER+ cancers than in RER− cancers. These results are consistent with the previous studies showing that LOH is not always necessary for inactivating *DCC* gene (21,34). Therefore, infrequent LOH at the *DCC* locus seems not to be sufficient to account for the low incidence of reduced DCC mRNA expression in RER+ colorectal cancers.

Other mechanisms such as mutations in the coding region of *DCC* gene, aberrant splicing of DCC transcripts, allele-specific loss of transcripts, intronic deletions, mutations at initiator or promoter sites of *DCC* gene have been considered to be responsible for the inactivation of DCC expression, but the specific mechanism remains to be determined (35). Aberrant DNA methylation is also considered to be an important mechanism by which tumor suppressor genes are inactivated in human cancers (36). Recently, a link between the RER phenotype and aberrant DNA methylation was shown in colorectal cancers (18). However, no differences in the methylation sequences of flanking *DCC* exon1 were found between tumors with and without DCC expression (35).

The colorectal tumor suppressor role of DCC is still a matter of controversy. One possible interpretation of our results is that DCC may be a target tumor suppres-

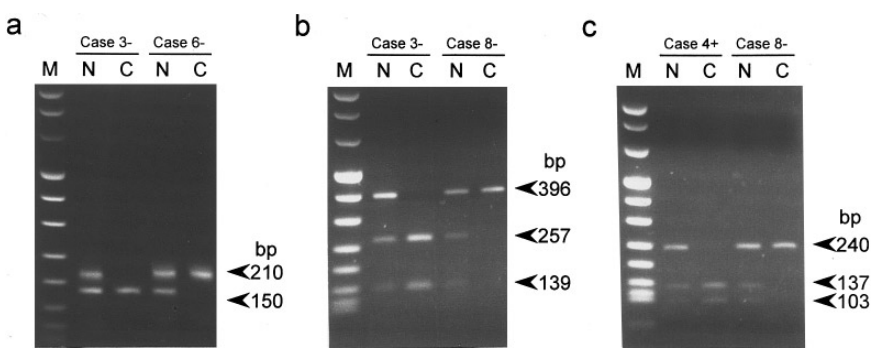

FIG. 3. PCR-LOH analysis at the *DCC* locus in colorectal cancers. Representative cases of LOH at VNTR (*a*), M2 (*b*), and M3 (*c*) are shown. N and C, matched DNA samples isolated from normal and cancer tissue, respectively.

sor gene in RER− colorectal cancers. In support of this, all 5 RER− cancers with liver metastasis showed reduced DCC mRNA expression and all 4 informative cases had allelic loss at the *DCC* locus. These results are consistent with the previous studies suggesting the association of DCC inactivation with metastatic potential of colorectal cancers (21-26). If this interpretation is true, we can also suggest that the infrequent inactivation of DCC expression may contribute to the lower tendency of metastasis in RER+ colorectal cancers compared with RER− cancers (3). Recent study has demonstrated an association between loss of DCC expression in stage II and III colorectal cancers and poor prognosis (27). RER+ colorectal cancers have been shown to have a better prognosis compared with RER− cancers (2, 6). Diploid predominance in RER+ colorectal cancers could be one of the possible explanations accounting for their better prognosis (6, 37). It is also possible to speculate that infrequent DCC inactivation in RER+ colorectal cancers may contribute to their better prognosis. Alternatively, it is also possible to interpret that although DCC may not be a target tumor suppressor gene, its expression is frequently reduced in RER− colorectal cancers (28, 29).

In either case, the specific mechanism accounting for the reduction of DCC expression in colorectal cancers is an important issue needs to be clarified. Although the mechanism is still incompletely defined, the low incidence of reduced DCC expression should be dependent on the nature of cancers in the RER pathway of colorectal carcinogenesis. In this regard, our results may give a hint for addressing the issue, which is very important to clarify whether DCC inactivation is relevant or not in colorectal carcinogenesis.

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