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Frequent loss of heterozygosity at the deleted in colorectal carcinoma gene locus and its association with histologic phenotypes in breast carcinoma

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Abstract Loss of heterozygosity (LOH) at the deleted in colorectal carcinoma gene (DCC), a tumour suppressor gene that encodes a protein with high homology to the neural cell adhesion molecule, was investigated in 42 surgical specimens of primary breast carcinoma. LOH was analysed in breast carcinoma by amplifying the DNA, spanning a variable number of tandem repeats site and a restriction fragment length polymorphism site within DCC, using the polymerase chain reaction (PCR). Cell sorting was used to enrich carcinoma cells. The expression of the DCC gene was also investigated using a reverse transcription-PCR method followed by Southern blot hybridization. LOH at the DCC locus was detected in 15 (51.7%) of 29 informative cases and 10 of 13 cases having DCC-LOH showed distinct reduction or loss of DCC expression. The DCC-LOH was closely associated with certain histological phenotypes; DCC-LOH was more frequent in scirrhous carcinomas than in solid-tubular ones ($P<0.05$), and was also more frequent in carcinomas with infiltration into fat tissue over the mammary gland than in those without infiltration ($P<0.05$). DCC-LOH was detected in invasive lobular carcinomas (2/2), but in none of the noninvasive ductal carcinomas (0/2). These observations suggest that malignant histological phenotypes are associated with DCC-LOH.

Key words DCC gene · Breast carcinoma · Histopathology

Introduction

The inactivation of tumour suppressor genes is usually induced by constitutional allelic deletion followed by mutation of the remaining allele [16]. Allelic deletion on a specific region of chromosome 18q has been observed in more than 70% of colorectal carcinomas [35] and a candidate tumour suppressor gene, deleted in colorectal carcinoma (DCC), has been identified at this region [10]. It has recently been reported by Hedrick et al. [13] that DCC encodes a membrane-bound protein of the immunoglobulin-cellular adhesion molecule (CAM) family which is found in axons of the central and peripheral nervous system and in differentiated cell types of the intestine.

During colorectal tumorigenesis, the incidence of loss of heterozygosity (LOH) at the DCC locus is low in adenomas and intramucosal carcinomas, although it is high in invasive carcinomas [23]. The expression of DCC mRNA is detected in intramucosal carcinomas, while it is absent in most invasive carcinomas [15]. In addition, the incidence of DCC-LOH is higher in hepatic metastases than in primary tumours [24]. LOH of chromosome 18q including DCC has been reported not only in colorectal carcinoma but also in 61% of gastric carcinomas [34], 64% of osteosarcomas [36], 60% of ovarian adenocarcinomas [4], 33% of renal cell carcinomas [1] and 11% of lung carcinomas [37]. In breast carcinoma, the prevalence of LOH of chromosome 18q has been reported to range from 2.5% to 69% [7, 9, 20, 27, 33].

To clarify the significance of LOH of DCC in breast carcinoma we investigated DCC-LOH in 42 primary breast carcinomas utilizing the polymerase chain reaction (PCR) combined with cell sorting to avoid the contamination of DNA from normal cells [33, 31] and we analysed the associations between this LOH and the histological phenotypes of the carcinoma. The expression of the DCC gene was also investigated using reverse transcription (RT)-PCR method followed by Southern blot hybridization.

Materials and methods

Tumour tissue and the surrounding normal breast tissue were obtained by surgical mastectomy in 42 patients with primary breast

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carcinoma at the Department of Surgery, Iwate Medical University School of Medicine between April 1992 and December 1993. The specimens were frozen immediately after mastectomy and stored at −80° C until analysis. DNA was extracted by proteinase K digestion and phenol/chloroform extraction. The tissues were homogenized in a denaturating solution of guanidium thiocyanate, then the total RNA was extracted with phenol/chloroform and recovered with iso-propanol.

Tumours were classified histologically according to the criteria proposed by Page and Anderson [26].

For flow cytometry and cell sorting the frozen tumour tissues were minced in 0.2% Triton-X-100 (Sigma Chemical Company, St. Louis, Ma, USA), treated with 0.1% RNase (Sigma), stained with 50 μg/ml of propidium iodide (Sigma) and filtered through a 40 μm nylon mesh. Aneuploid cells and the cells at $S+G_2M$ phases were selected using a cell sorter (FACStar plus, Becton Dickinson, Mountain View, Calif., USA) from aneuploid and diploid tumours, respectively. DNA was then extracted from 10000–50000 sorted nuclei [31].

In PCR-variable number of tandem repeats (VNTR) analysis the sequences of the primers used in the analysis of LOH, at the VNTR site, were 5′-GATGACATTTTCCCTCTAG-3′ and 5′-GAGGTTATTGCCTTGAAAAG-3′ [11]. Extracted DNA was amplified in 10 μl of buffer (50 mM potassium chloride, 0.01% gelatin and 10 mM TRIS buffer at pH 8.3) containing 10 pmol of each primer, 1 mM magnesium chloride, 0.2 mM of each deoxynucleotide triphosphate and 2.5 units of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn., USA) by PCR for 40 cycles consisting of a denaturation step at 95° C for 30 s, an annealing step at 51° C for 2 min and an extension step at 70° C for 2 min with a thermal cycler (Omnigene, Hybaid Limited, Middlesex UK). The PCR products were electrophoresed directly on a 3% agarose gel and stained with ethidium bromide. LOH was defined as a visible change in the allele: allele ratio in the tumour DNA relative to the ratio in the patient's corresponding DNA from normal cells [12].

For PCR followed by restriction fragment length polymorphism (RFLP) analysis, primers flanking a *Msp* I-RFLP site were synthesized. The sequences of the primers were 5′-TGCACCA TGCTGAAGATTGT-3′ and 5′-AGTACAACACAAGGTATGTG-3′ [11]. Extracted DNA was amplified in the same solution as mentioned previously for the PCR-VNTR analysis by PCR for 45 cycles consisting of denaturation at 94° C for 30 s, annealing at 50° C for 1 min and extension at 70° C for 1 min with a thermal cycler (Omnigene). The PCR products were digested with 40 units of *Msp* I (Toyobo, Osaka, Japan) at 37° C for more than 4 h. The digested products were then electrophoresed on a 3% agarose gel and stained with ethidium bromide. LOH was defined as previously mentioned.

The expression of the DCC gene was analysed by the method described by Fearon et al. [10]. The first strand cDNA was synthesized from 400 μg of extracted RNA using the antisense primer, 5′-ATGCGAATTCAGCCTCATTTTCAGCCACACA-3′, by means of the RT reaction at 42° C for 30 min. Then RT was inactivated by heating at 99° C for 5 min. The 233-base pair fragment of DCC cDNA (nucleotide 986–1218) was amplified by PCR using the sense primer, 5′-ATGCGAATTCTTTCCGCCATGGTTTTTAAA TCA-3′, and the antisense primer for 45 cycles consisted of denaturation at 94° C for 40 s, annealing at 55° C for 40 s and extension at 72° C for 75 s. The amplified fragment was electrophoresed in 3% agarose gel, denatured with a solution of 1.5 M sodium chloride (NaCl) and 0.5 M sodium hydroxide, neutralized with a solution of 3 M NaCl and 0.5 M TRIS hydrochloric acid (HCl) at pH 8.0 and transferred to a nylon membrane (Hybond-N, Amersham, UK) using the Pharmasia LKB VacuGene XL (Pharmasia, Freiburg, Germany), and fixed by an ultraviolet linker (Spectronics, Westbury, N.Y., USA). Southern blot hybridization was performed with a ^{32}P-labelled DCC-specific 57-mer oligonucleotide containing the sequence 1141–1197 in DCC cDNA as previously described [32]. The membrane was then prehybridized in a solution of 50 mM TRIS HCl, 1 M NaCl, 10% dextran, 1% sodium dodexyl sulphate with the sheared salmon sperm DNA at 65° C for 2 h. Hybridization proceeded in the same solution containing the probe labelled at the 5′ end with [γ-^{32}P]ATP using 1 unit of polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany). After incubation for 8–16 h at 65° C, the membrane was washed three times at room temperature for 20 min and twice at 65° C for 20 min in standard saline citrate (0.3 M NaCl and 0.03 M sodium citrate at pH 7.0) containing 0.1% sodium dodecyl sulphate. The membrane was then autoradiographed at −80° C for 12–36 h. We used β-actin RNA as quantitative control. The sequences of the β-actin primers were: sense, 5′-CCTGGCACCCAGCACAATGA-3′; and antisense, 5′-TTGGGAAGGTTGGATGTTCG-3′ [22].

For all comparisons, the Fisher's exact test was applied, and a P value <0.05 was considered statistically significant.

Results

PCR-VNTR and PCR-RFLP analyses

Using DNA extracted from unsorted nuclei in the 42 cases examined, there were 23 (55%) informative cases at the VNTR site and 14 (33%) at the RFLP site. LOH at the DCC locus was detected in one patient at the VNTR (4.3%, 1/23) site using DNA extracted from unsorted nuclei. This patient's DNA was homozygous at the RFLP site. LOH was not detected at the RFLP site in any of the cases (0%, 0/14).

The case with LOH at the VNTR site had scirrhous carcinoma and was at Stage IIIb (tumour size 6.0×5.0 cm, no lymph node metastases, oestrogen receptor positive).

Cell sorting combined with PCR-VNTR or PCR-RFLP

LOH was detected in 13 (56.5%) of the 23 informative cases at the VNTR site (Fig. 1), and in 7 of the 14 (50%)

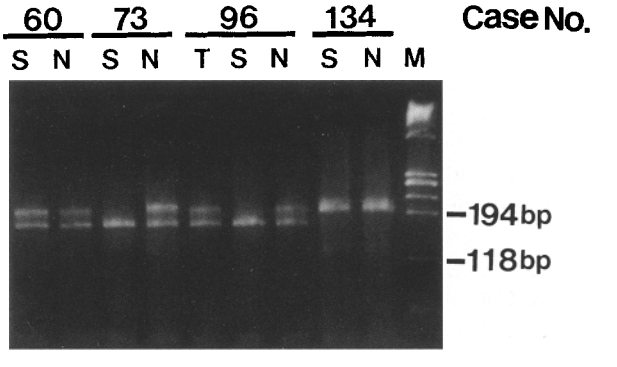

Fig. 1 A representative illustration of the polymerase chain reaction (PCR)-variable number of tandem repeats analysis. Case 60, heterozygous; case 134, homozygous; cases 73 and 96, loss of heterozygosity (LOH). LOH was detectable in case 96 only when DNA extracted from sorted nuclei was used. *S* DNA extracted from sorted nuclei, *N* DNA extracted from normal cells, *T* DNA extracted from unsorted tumour cells, *M* size marker (Hae III-digested ϕX 174 DNA

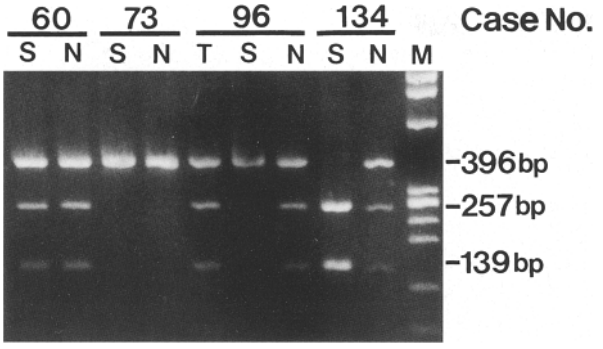

Fig. 2 A representative illustration of the PCR-restriction fragment length polymorphism analysis. LOH is apparent in cases 96 and 134. Case 60, heterozygous; case 73, homozygous

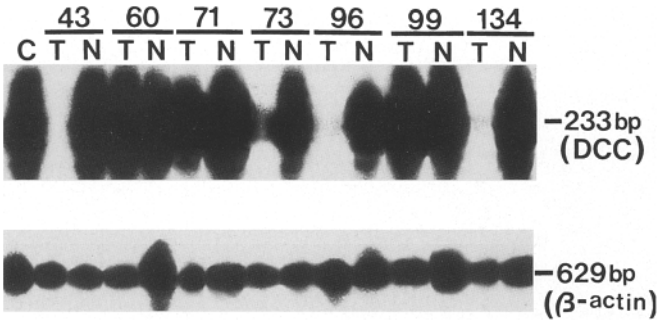

Fig. 3 Analysis of the expression of the deleted in colorectal carcinoma (DCC) gene by Southern blot hybridization after reverse transcription-PCR. β-actin was used as quantitative control. Loss or reduction in DCC expression was confirmed in cases 43, 73, 96 and 134. *C* colonic mucosa as a positive control

at the RFLP site (Fig. 2). From these results using VNTR and RFLP analyses, LOH was judged to be at 51.7% (15/29).

Expression of the DCC gene

We analysed the expression of the DCC gene in paired carcinoma and normal breast tissues in 24 cases. Distinct reduction or loss in expression of DCC was observed in 76.9% (10 cases) of 13 cases having DCC-LOH (Fig. 3).

Association of LOH and clinicopathologic characteristics

The association between DCC-LOH and pathological characteristics is summarized in Table 1. LOH at DCC was closely associated with certain histological types of carcinoma; DCC-LOH was more frequent in scirrhous carcinoma than in solid-tubular carcinoma ($P<0.05$; Fig. 4). It was also found more frequently in carcinomas with infiltration into fatty tissue than in those without ($P<0.05$). DCC-LOH was detected in invasive lobular carcinomas (2/2), but in none of the non-invasive ductal carcinomas (0/2). No significant correlation was found between DCC-LOH and the clinical stage or size of tumour.

Table 1 Loss of heterozygosity (*LOH*) at the deleted in colorectal carcinoma gene (*DCC*) locus and clinicopathologic characteristics in the 29 informative cases (*TNM* tumour, node, metastases; *NS* not significant)

Clinicopathological characteristics	LOH at the DCC locus		
	present	absent[b]	
TNM stage			
I	1	1	
II	8	9	NS
III	5	3	
IV	1	1	
Tumour size			
<2 cm	1	4	
2–5 cm	9	7	NS
>5 cm	5	3	
Histologic type[a]			
papillotubular	4	5	
solid-tubular	0	4	
scirrhous	7	3	$P<0.05$
medullary	1	0	
mucinous	1	0	
invasive lobular	2	0	
non invasive ductal	0	2	
Oestrogen receptor status			
positive	10	5	NS
negative	5	9	
DNA ploidy pattern			
aneuploidy	8	8	NS
diploidy	7	6	
Infiltration into fatty tissue			
positive	14	8	$P<0.05$
negative	1	6	

[a] According to the histological classification of breast tumours (Page and Anderson, 1987 [26])
[b] P value was calculated by Fisher's exact test.

Discussion

Multiple allelic deletions have been reported in human malignancies. In breast carcinoma, frequent allelic deletions have been reported on chromosomes 1p at 47% [2], 1q at 23% [4], 3p at 46%–47% (8, 28], 5q at 28% [33], 11p at 20%–41% [19, 28, 29], 13q at 21%–59% [3, 27, 31], 16q at 45%–55% [27, 29], 17p at 42%–71% [5, 19, 25, 27, 28, 29], 17q at 35%–36% [27], 18q at 2.5%–69% [7, 9, 20, 27, 33] and 22q at 11% [28]. These frequent allelic deletions suggest the existence of tumour suppressor genes on these chromosomes. In fact, almost all tumour suppressor genes exist on some of these chromosomes and other genes of this type are also presumed to exist on these chromosomes.

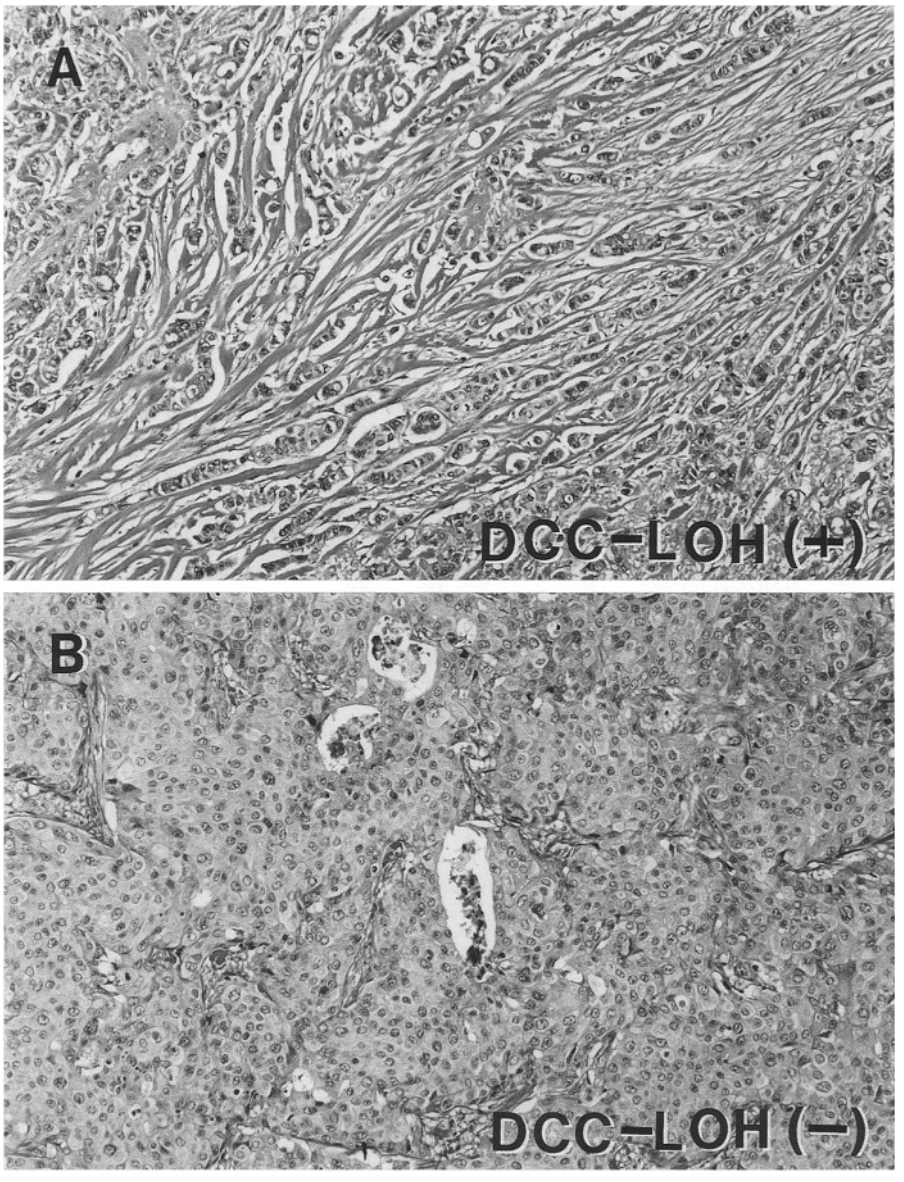

Fig. 4 Microscopic features of scirrhous carcinoma with DCC-LOH (**A**) and solid-tubular carcinoma without DCC-LOH (**B**). Haematoxylin and eosin, ×100

LOH at the DCC locus or deletion of 18q has been reported to range from 2.5% to 69% in breast carcinoma [7, 9, 20, 27, 33]. The differences in the incidence of DCC-LOH according to investigators may be caused by a difference in the methods employed and/or by underestimation of LOH due to contamination with non-cancer cells. We have previously reported that, in breast carcinoma, the incidence of LOH at the retinoblastoma gene locus was significantly higher on sorted cells than on unsorted cells [31].

In this study we found the importance of cell-sorting prior to extraction of DNA. DCC-LOH was detected in 51.7% (15/29) breast carcinomas using sorted cells, whereas it was detectable in only 3.4% (1/29) in a routine manner using unsorted cells.

DCC-LOH may result in loss of cell to cell and/or cell to matrix adhesion because of the inactivation of the DCC product which acts as a cell adhesion molecule [10]. This is supported by the fact that transfection of a vector with an antisense RNA to DCC resulted in cell detachment from the substratum in a fibroblast cell line [23]. A reduced expression of DCC was reported to be an important factor in the development, progression and undifferentiation of pancreatic adenocarcinoma and pancreatic tumour cells [14]. The transfer of normal chromosome 18 by microcell hybridization into a colon carcinoma cell line, which lost an allele on chromosome 18 without DCC expression, exhibited a reduction in the malignant morphological phenotype [32]. In this study we have demonstrated the high frequency of DCC-LOH with its reduction or loss of expression in surgical specimens of primary breast carcinomas, which is concordant with reports in prostatic carcinoma [11]. These results suggest that DCC-LOH is associated with the more ma-

lignant or undifferentiated histological phenotypes and are concordant with our present observations. Furthermore, DCC-LOH is detected more frequently in hepatic metastatic carcinoma than in primary carcinoma [24], and the expression of neural (N)-CAM, which is highly homologous with DCC, is reduced more in pulmonary metastasizing melanoma cell lines than in non-metastasizing melanoma cell lines [17].

In conclusion, DCC-LOH is a frequent event in breast carcinoma and is related to a tendency of invasion. This may be caused by the functional inhibition of normal DCC, because DCC encodes a glycoprotein which functions as a cell to cell and/or a cell to matrix adhesion.

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