

Loss of heterozygosity and loss of expression of the DCC gene in gastric cancer

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Abstract

Aim—To investigate the role of DCC gene inactivation in the development and progression of gastric cancer.

Methods—Loss of heterozygosity and loss of expression of the DCC gene was studied in 51 surgical specimens of gastric cancer using detection based on polymerase chain reaction.

Results—Loss of heterozygosity was found in 35.3% (18 of 51) of specimens and was detected more often in stage III and IV (50%) than in stage I and II cancers (14.3%) ($p < 0.05$). Occurrence of loss of heterozygosity was not correlated with histological type, tumour size, depth of invasion, or lymph node metastasis. Loss of expression was found in 49% of cases (25 of 51). Loss of expression was not significantly correlated with any clinicopathological variable.

Conclusions—Loss of heterozygosity and loss of expression of the DCC gene are often encountered in gastric cancer. Loss of heterozygosity of the DCC gene is a late event and associated with malignant progression.

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Keywords: gastric cancer; DCC gene; heterozygosity; mRNA expression

Tumour suppressor genes play an important role in regulating normal cellular proliferation.^{1,2} Conversely, inactivation of tumour suppressor genes at both alleles may allow a cell to escape normal growth control and so acquire a malignant phenotype. This inactivation may occur by a variety of mechanisms including deletion, rearrangement, point mutation, and binding of suppressor gene products with viral or cellular inactivating proteins.^{1,3} The Deleted in Colorectal Cancer (DCC) gene was cloned following the demonstration of frequent deletions affecting the 18q21 region in colon cancer.^{4,5} Subsequently, loss of heterozygosity or loss of expression of DCC has been reported in several additional tumour types including malignancies of the breast,^{6,7} pancreas,⁸ prostate,⁹ and testis,¹⁰ glioblastoma,^{11,12} neuroblastoma,¹³ and haematological malignancies.^{14,15} In a previous study of human gastric cancer, loss of heterozygosity of 18q was detected in more than 60% of cases.¹⁶ However, little is known of the role of loss of expression of the DCC gene in gastric cancer. To investigate the effects of alteration of the DCC gene on the development and progression of gastric cancer, we studied loss of heterozygosity and loss of expression of this gene using detection methods based on polymerase chain reaction.

Methods

TISSUE SPECIMENS

Tumour and corresponding non-cancerous tissues were obtained from 51 patients who underwent surgical resection for gastric carcinoma between January 1995 and October 1997 at the Southwest Hospital, Chongqing. No radiotherapy or chemotherapy preceded tumour excision. Each pair of tumour and corresponding non-tumour tissue was taken immediately after the resection and stored at -80°C for later use. A 6 μm section was cut from each specimen and stained with haematoxylin and eosin for pathological diagnosis. After diagnostic confirmation, a visual assessment was made of the approximate proportion of tumour cells versus normal cells in tumour samples. Only those specimens in which tumour cells represented 50% of the tumour tissue were accepted for loss of heterozygosity and loss of expression analysis.

TOTAL RNA ISOLATION AND DNA EXTRACTION

Total RNA was prepared from tumour and non-cancerous tissue using the acid guanidinium thiocyanate method¹⁷ and high molecular weight DNA was extracted by proteinase K digestion and phenol-chloroform-isoamylalcohol extraction as previously described.¹⁸

RT-PCR ASSAY OF DCC GENE EXPRESSION

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described previously with some modifications.¹⁹ DCC complementary DNA was amplified at 94°C for 40 seconds, 49°C for 40 seconds, and 72°C for one minute for 35 cycles. DCC primers are located on exons 0 and P and amplify a 233 base pair fragment from human mRNA.²⁰ A fragment of this size cannot be amplified from genomic DNA, since the primers were designed to frame sequences that cross an intron on the DCC gene. RT-PCR without RNA or without reverse transcriptase was included in each experiment as negative controls; primers used were 5'-TTCCGCCATGGTTTTTAAATCA-3' (DCC sense), which corresponds to nucleotides 986-1007 in the cDNA sequence, and 5'-AGCCTCATTTTCAGCCACACA-3' (DCC antisense), which corresponds to nucleotides 1218-1198. Amplifiable cDNA was demonstrated in lanes showing DCC loss of expression using β_2 microglobulin primers.

PCR LOSS OF HETEROZYGOSITY ANALYSIS

Genomic DNA (50 to 500 ng) was held at 95°C for five minutes in 20 μl buffer containing 10 mM Tris (pH 8.3), 5 mM KCl, 2.5 mM

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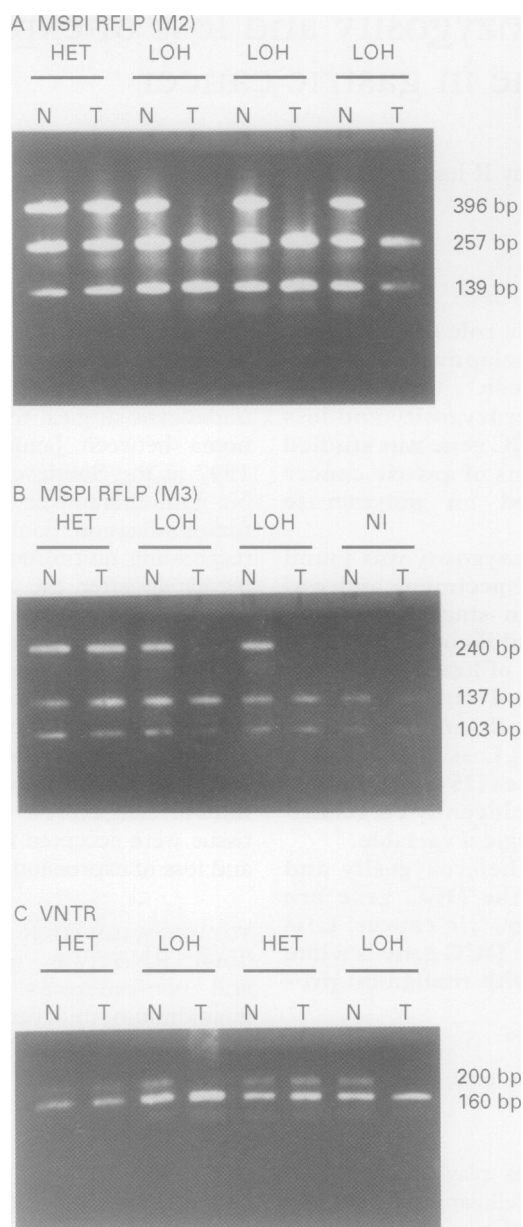


Figure 1 Loss of heterozygosity (LOH) assay of DCC gene in gastric carcinoma showing loss of heterozygosity in representative cases at M2 (A), M3 (B), and VNTR (C). HET, retained heterozygosity; N, normal DNA; NI, not informative; T, tumour DNA.

MgCl₂, 0.1 µg/µl bovine serum albumin, and 1 µM concentrations of sense and antisense primers. Then 2.5 units of Ampli Taq DNA polymerase were added and PCR was run at 94°C for 40 seconds, 56°C for 40 seconds, and 72°C for one minute for 35 cycles. For M2 and M3 polymorphism,²¹ PCR products were digested with MspI and analysed on 2.5% agarose gels. For VNTR polymorphism,^{22, 23} PCR products were directly separated on 2.5% gels. The gel was then stained with ethidium bromide and photographed under ultraviolet light. The primers were: 5'-TGCACCATGC-TGAAGATTGT-3' (M2 sense); 5'-AGTCAACACAAGGTATGTG-3' (M2 antisense); 5'-CGACTCGATCCTACAAAAT-3' (M3 sense); 5'-TCTACCCAGGTCTCAGAG-3' (M3 antisense); 5'-GATGACATTTTC-CCTCTAG-3' (VNTR sense); and 5'-GTGGTTATTGCCTTGAAAAG-3' (VNTR antisense). Negative controls without genomic



Figure 2 Expression of DCC gene in gastric carcinoma. Case 2 showed almost total loss of expression of DCC. N, normal; T, tumour.

DNA were performed for each set of PCR reactions.

DATA ANALYSIS

Photographs of ethidium stained gels were read by two independent observers. Loss of heterozygosity and loss of expression were defined by a visible change in that allele to allele ratio in tumour compared with matching normal tissue. A reduction of allelic intensity in tumour of more than 50% compared with matching tissue was taken to indicate loss of heterozygosity or loss of expression.

Associations between variables were tested with the χ^2 test. A probability (p) value of less than 0.05 was considered to be statistically significant.

Results

Loss of heterozygosity of the DCC gene was determined by PCR in 51 specimens of gastric cancer. To increase assay sensitivity, three different sites—M2, M3, and VNTR—were used in the study. Loss of heterozygosity of DCC was observed in nine of 47 (19.0%) at M2, seven of 50 (14%) at M3, and three of 26 (11.5%) at VNTR sites of informative cases, respectively (fig 1). If a positive allelic deletion of DCC was judged by loss of heterozygosity at one or any combination of these three sites, the incidence of loss of heterozygosity at the DCC locus was 35.3% (18/51). Loss of heterozygosity was detected in 38.5% (10/26) of intestinal type of gastric cancer and 34.8% (8/23) of diffuse type of gastric cancer. Loss of expression of the DCC gene was observed in 49% of cases (25/51) (fig 2). The incidence of loss of expression was 61.5% (16/26) in intestinal type and 39.1% (9/23) in diffuse type. A χ^2 test showed that there was no significant difference in the distribution of loss of heterozygosity and loss of expression between the two types of gastric cancer ($p > 0.05$).

Correlations between loss of heterozygosity and loss of expression of DCC and clinicopathological data of gastric cancer are illustrated in table 1. A χ^2 test showed that loss of heterozygosity of DCC was significantly greater in the gastric cancer of stage III–IV than that in stage I–II ($p < 0.05$). The association of loss of heterozygosity and loss of expression was such that most cases of loss of heterozygosity (17/18) were accompanied by loss of expression, although the frequency of loss of expression was greater than of loss of heterozygosity.

Table 1 Relation of loss of heterozygosity (LOH) and loss of expression (LOE) of DCC with clinicopathological variables

Clinicopathological variable	No	LOH (%)	LOE (%)
<i>Grade (differentiation)</i>			
Well/moderately differentiated	24	9 (37.5)	13 (54.2)
Poorly differentiated	16	7 (43.8)	8 (50.0)
Mucinous carcinoma	11	2 (18.2)	4 (36.4)
<i>Tumour size</i>			
≤ 5 cm	20	5 (25.0)	11 (55.0)
< 5 cm	31	13 (41.9)	14 (45.2)
<i>Serosal invasion</i>			
Absent	18	5 (27.8)	6 (33.3)
Present	33	13 (39.4)	19 (57.5)
<i>Lymph node metastasis</i>			
Absent	24	6 (25.0)	9 (37.5)
Present	27	12 (44.4)	16 (59.2)
<i>Clinical staging</i>			
Stages I–II	21	3 (14.3)	7 (33.3)
Stages III–IV	30	15 (50.0)*	18 (60.0)

*p < 0.05 v stages I–II.

Discussion

The DCC gene is located on human chromosome 18q21.3. Inactivation of this gene is closely related to the pathogenesis of colorectal, oesophageal, and pancreatic carcinoma. DCC is regarded as a susceptibility gene in the stepwise evolution of gastrointestinal neoplasms. Uchino *et al* and Ranzani *et al* reported rates of loss of heterozygosity of DCC in gastric cancer of 61% and 42.9% respectively.^{16, 23} The rate was 35.3% in our study. These findings suggest that involvement of the DCC gene in the pathogenesis of gastric cancer occurs through loss of heterozygosity.

Although there are many reports on loss of heterozygosity of chromosome 18q, there are few reports on DCC mRNA levels in clinical cases.²⁴ The RT-PCR method has been used to detect DCC mRNA in most investigations because the amount is too small to be detected by northern hybridisation. In this study we have confirmed that there is loss of expression of DCC mRNA in gastric cancers using RT-PCR. This suggests that the inactivation of DCC plays an important role in the pathogenesis of gastric cancer.

The histological progression associated with intestinal type gastric cancer has been well documented, with apparent evolution through a sequence of chronic active gastritis, intestinal metaplasia, and dysplasia.^{25, 26} Lesions indicating an adenoma–carcinoma sequence similar to that in the colorectum is also observed in the stomach.²⁷ In the present study we found that the frequency of loss of expression of DCC was 61.5% in the intestinal type of gastric cancer but only 39.1% in diffuse cancers. Although the rate of loss of expression showed no statistical difference between the two types of gastric cancer, similarities in genetic alterations between colorectal carcinoma and the intestinal type of carcinoma may reflect a common pathogenic pathway. On the other hand, the rate of loss of heterozygosity at the DCC locus was similar between the two types of gastric cancer. Whether the mechanisms underlying loss of heterozygosity and loss of expression of the DCC gene are different in the two types of gastric cancer needs further investigation.

The DCC gene encodes a molecule which shares high homology with the neural cell adhesion molecule.²⁰ Cell adhesion molecules are cell surface receptors which play critical roles during processes such as embryogenesis, thrombosis, wound healing, cell homing, immunoreaction, and tumour progression and metastasis. The inactivation of the DCC gene may result in malignant transformation of the cells and invasion and metastasis by a tumour. Kato *et al* found that the incidence of loss of heterozygosity at the DCC locus in colorectal carcinoma was significantly greater for patients with liver metastases than those without.²⁸ Kikuchi-Yanoshita *et al* and Iino *et al* observed that the expression of DCC mRNA was greatly reduced or not detectable in invasive colorectal carcinoma in comparison with malignant adenoma and intramucosal carcinoma.^{17, 29} They showed that the inactivation of the DCC gene was associated with the progression of early stage carcinoma to advanced stage.²⁹ Itoh *et al* found that the expression level of DCC mRNA was lower in liver metastasis than in primary carcinoma.³⁰ These findings imply that the inactivation of the DCC gene occurs at a late stage in the evolution of colorectal carcinoma and may be of prognostic significance. Similar conclusions were expressed in studies of oesophageal and pancreatic carcinomas.^{8, 31}

In our study the rate of loss of heterozygosity of DCC increased in parallel with increase in tumour size, depth of invasion, and metastasis to lymph nodes, and loss of expression of DCC often occurred in gastric cancer of stages III–IV with lymph node metastasis. Although these findings did not show statistical significance, they suggest that DCC may play a role in the proliferation, invasion, and metastasis of gastric cancer. The frequency of loss of heterozygosity was significantly higher in stage III–IV than that in stage I–II, indicating a relation to advancing malignancy. Ranzani *et al* described similar findings.²³ Thus it is possible that loss of heterozygosity and loss of expression of DCC may serve as prognostic factors for gastric cancer. However, more cases need to be examined to show the correlations between loss of heterozygosity and loss of expression with other variables and their independent prognostic significance.

In a preliminary study of the relation between loss of heterozygosity and loss of expression we have shown, as have others,³² that loss of heterozygosity does not seem to be necessary for loss of expression of DCC mRNA. Presumably there are several explanations for loss of expression such as alterations in sequences controlling transcriptional regulation, point mutation or insertions within the DCC gene, or alterations in other genes controlling DCC gene expression. Further studies are required to examine these alternative mechanisms.

The DCC gene was originally identified as a candidate tumour suppressor gene in colon carcinogenesis on the basis of allelic losses in chromosome 18q21 in more than 60% of colon cancers.^{16, 20, 33} The possibility that 18q carries a colonic tumour suppressor gene is supported by

chromosomal transfer experiments showing that one normal copy of chromosome 18 is sufficient to suppress the tumorigenicity of human colon carcinoma cell lines.^{34,35} However, the accumulated evidence has failed to implicate DCC conclusively as the colorectal tumour suppressor gene on chromosome 18q. Recent *in vitro* studies in rodents have provided evidence that DCC might function as a receptor for the axonal chemoattractant netrin-1. Inactivation of the murine DCC gene caused defects in axonal projection that are similar to those observed in netrin-1 deficient mice but did not affect growth, differentiation, morphogenesis, or tumorigenesis in mouse intestine.^{36,37} In addition, a study of the pattern of DCC mRNA and protein expression in surgically resected colonic cancer tissues has found that DCC protein was detected consistently in colorectal carcinoma and colon liver metastases by western blot analysis, and was not completely lost in any of the tumour specimens examined, even within metastatic cancers.³⁸ These data may suggest that the DCC gene is not the main tumour suppressor gene in the region of chromosome 18q21. Other tumour suppressor genes present in the region of homozygous deletion at chromosome 18q21 may contribute to the development of colorectal carcinoma, such as the candidate tumour suppressor DPC4 gene in pancreatic cancer.³⁹ In our opinion this is unlikely, since there is functional evidence supporting the role of DCC as a tumour suppressor.⁴⁰ Moreover, recent studies have shown that the frequency of alterations of DPC4 was relatively low, at 16.7% (five of 31 colorectal cancers) compared with 60% cases reported to have allelic loss at 18q21.⁴¹

Our data suggest that loss of heterozygosity and loss of expression of DCC appear to play an important role in the progression of gastric cancer, supporting the view that DCC functions as suppressor gene. However, additional functional studies in model systems using the full length DCC cDNA will be required to understand the role of DCC in tumour progression.

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