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

Dian-Chun Fang, Jeremy R Jass, Dong-Xu Wang

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. 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. The Deleted in Colorectal Cancer (DCC) gene was cloned following the demonstration of frequent deletions affecting the 18q21 region in colon cancer. Subsequently, loss of heterozygosity or loss of expression of DCC has been reported in several additional tumour types including malignancies of the breast, pancreas, prostate, and testis, glioblastoma, neuroblastoma, and haematological malignancies. In a previous study of human gastric cancer, loss of heterozygosity of 18q was detected in more than 60% of cases. 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 method and high molecular weight DNA was extracted by proteinase K digestion and phenol–chloroform–isoamylalcohol extraction as previously described.

RT-PCR Assay of DCC Gene Expression

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described previously with some modifications. 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. 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'-TTCGCCCATGGTTTTTATA-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 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

Department of Gastroenterology, 3rd Military Medical University, Southwest Hospital, Chongqing, People’s Republic of China

D-C Fang

D-X Wang

Department of Pathology, The University of Queensland Medical School, Herston, Queensland, Australia

J R Jass

Correspondence to: Professor J R Jass, Department of Pathology, The University of Queensland Medical School, Herston Road, Herston, Queensland 4006, Australia; email: j.jass@mailbox.uq.edu.au

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MgCl₂, 0.1 µg/µl bovine serum albumin, and 1 µM concentrations of sense and antisense primers. Then 2.5 units of AmpliTaq 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, 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’-TGCACCATGCTGAGTTGT-3’ (M2 sense); 5’-AGTACACACAAGG1ATGTG-3’ (M2 antisense); 5’-CGACTCGATCCTACAAAAT-3’ (M3 sense); 5’-TCTACCCAGGTCCTCAGAG-3’ (M3 antisense); 5’-GATGACATTTTTCCTCTTAG-3’ (VNTR sense); and 5’-GTGGTTATTCCTTGAAAAG-3’ (VNTR antisense). Negative controls without genomic 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 χ² 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 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 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 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 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 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 of gastric cancer.

Correlations between loss of heterozygosity and loss of expression of DCC and clinico-pathological data of gastric cancer are illustrated in table 1. A χ² 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).

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.

Figure 2  Expression of DCC gene in gastric carcinoma. Case 2 showed almost total loss of expression of DCC. N, normal; T, tumour.
The DCC gene encodes a molecule which shares high homology with the neural cell adhesion molecule. 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. 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. They showed that the inactivation of the DCC gene was associated with the progression of early stage carcinoma to advanced stage. Itoh et al found that the expression level of DCC mRNA was lower in liver metastasis than in primary carcinoma. 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.

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. 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, 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. The possibility that 18q carries a colonic tumour suppressor gene is supported by

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

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

*p < 0.05 vs stages I–II.
chromosomal transfer experiments showing that one normal copy of chromosome 18 is sufficient to suppress the tumorigenicity of human colon cancer cell lines. However, the accumulated evidence has failed to implicate DCC conclusively as the colorectal tumour suppressor gene on chromosome 18q. Recent in vitro studies in rodent cell lines 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 growth, defining the DCC gene as an important regulator of axon guidance. In addition, a growth inhibitor encoded by the mouse DCC gene caused defects in axonal guidance, and the murine DCC gene caused defects in axonal growth in vitro. Inactivation of the human DCC gene on chromosome 18q has been shown in colorectal cancer cell lines. Recent in vitro studies supporting the role of DCC as a tumour suppressor gene have been supported by the observation that DCC cDNA will be required to understand the role of DCC in tumour progression.

Somatic inactivation of the DCC gene has been observed in colorectal cancer cell lines. The loss of DCC expression in colorectal cancer cell lines has been shown to be associated with loss of heterozygosity on chromosome 18q in these cell lines. Inactivation of the DCC gene on chromosome 18q has been shown to be associated with loss of heterozygosity and K-ras gene mutation in gastric cancer. The loss of DCC expression in colorectal cancer cell lines has been shown to be associated with loss of heterozygosity on chromosome 18q. The loss of DCC expression in colorectal cancer cell lines has been shown to be associated with loss of heterozygosity on chromosome 18q.

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 the DCC functions as a 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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