Reduced expression of deleted colorectal carcinoma (DCC) protein in established colon cancers

T Goi1, A Yamaguchi1, G Nakagawara1, T Urano2, H Shiku3 and K Furukawa4

1The First Department of Surgery, Fukui Medical School, Fukui, Japan; 2Department of Biochemistry, Tufts University School of Medicine, Boston, USA; 3Second Department of Internal Medicine, Mie University School of Medicine, Tsu, Japan; 4Department of Oncology, Nagasaki University School of Medicine, Nagasaki, Japan

Summary Using a bacterial fusion protein, a deleted colorectal carcinoma (DCC)-specific monoclonal antibody (MAb) 127–22 was established. Although MAb 127–22 reacted with almost all normal tissues, it did not react or only weakly reacted with many cancer cell lines, including colon cancer lines, in flow cytometry. In Western immunoblots, the MAb reacted with a single 190-kDa molecule in a myeloma line ARA-10 extract. This component was scarcely detected in colon cancer cell lines. Immunoblots of samples from 25 pairs of colon cancers and adjacent normal tissues and from five adenoma tissues revealed that normal colon and adenoma tissues significantly expressed the DCC protein, whereas colon cancer tissues showed poor expression. These results indicate not only deletion of and lowered mRNA expression of the DCC gene, but also marked reduction of DCC protein occurred in colon cancer tissues. In addition, colon cancer patients with liver metastasis expressed significantly lower levels of DCC than those without, suggesting the prognostic value of DCC expression.

Keywords: tumour-suppressor gene; deleted colorectal carcinoma; colon cancer; metastasis

The development of human cancer has been proposed to be a multistep process (Nowell, 1986). Vogelstein et al (1988) showed that colonic tumorigenesis provides the systematic course to the multistep hypothesis at the molecular level. Several genes have been identified that alter during tumour progression. Frequent and consistent loss of heterozygosity (LOH) of specific chromosomes in human cancers has been associated with the presence of tumour-suppressor genes (Friend et al, 1986; Baker et al, 1989). In particular, the long arm of chromosome 18 has been shown to be lost in about 75% of colon cancers (Vogelstein et al, 1988).

The tumour-suppressor gene DCC (deleted in colorectal carcinoma), located on the long arm of chromosome 18, encodes a cell-surface protein containing homology with N-CAM (Fearon et al, 1990). There have been many reports on the loss of heterozygosity at the DCC gene locus in human colon cancers (Kern et al, 1989; Kikuchi-Yanoshita et al, 1992; Itoh et al, 1993; Turley et al, 1995; Thiagalingam et al, 1996), suggesting that DCC might be a tumour-suppressor gene. Some reports also claim marked reduction of the gene expression in colon cancers based on the results of reverse transcription polymerase chain reaction (RT-PCR).

However, only a few studies have been performed on the alteration of DCC protein in colon cancer cells compared with that in normal cells (Hedrick et al, 1992, 1994; Shibata et al, 1996).

In the present study, we generated a DCC-specific mouse monoclonal antibody (MAb) and analysed the expression levels of DCC proteins in various normal tissues, cancer cell lines and benign and malignant colon tumours. Here, we demonstrate dramatic decreases of DCC protein as well as its mRNA in colon cancer tissues. Furthermore, we also investigated the clinical significance of the reduction of DCC expression in the diagnosis and treatment of colorectal cancer patients.

MATERIALS AND METHODS

RT-PCR

Total RNA was extracted from ARA 10 (myeloma) using guanidinium thiocyanate (Chomczynski and Sacchi, 1987). Single-strand cDNA prepared from 3 μg of total RNA using Moloney murine leukaemia virus reverse transcriptase (GIBCO-BRL, Bethesda, MD, USA) with an oligo(dT)14 primer was used as the template for the polymerase chain reaction (PCR). The primers for PCR to amplify the DCC gene-coding region were as follows: the 5\′ primer DCC-AX encompassed positions 208–224 of the published human DCC sequence (Fearon et al, 1990), 5\′-GGGATCC-CTCAATTGCTACCAAAGGAAATCGG-3\′ (contained a BamHI site); the 3\′ primer DCC-BX encompassed positions 416–432, 5\′-GGGATCCCTCAATTGCTACCAAAGGAAATCGG-3\′ (contained an EcoRI site) (Fearon et al, 1990). These primers and an oligo(dT)14 primer were constructed using a 380B DNA synthesizer (Applied Biosystems, Tokyo, Japan). Thirty cycles of denaturation (94°C, 1 min), annealing (50°C, 1.5 min) and extension (72°C, 1 min) were carried out in a thermal cycler (Program Temp Control System PC-700, Astec, Fukuoka, Japan). Ten microlitres of the PCR products were resolved by electrophoresis in polyacrylamide (12%) gels.

Construction of plasmid pBSK-DCC and DNA sequencing

The PCR products were digested with BamHI and EcoRI, separated by polyacrylamide gel, purified by electrophoresis, and cloned into the BamHI and EcoRI sites of pBluescript II SK+ (Stratagene).
DNA was sequenced using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, USA) with [α-32P]dCTP.

**Construction of plasmid pGEX-DCC used to express DCC in E. coli**

Plasmid pGEX-DCC was constructed to express DCC proteins fused with a 26-kDa glutathione S-transferase (GST) in *E. coli*, using the BamHI–EcoRI fragments (225 base pairs) of pBSK-DCC. After digestion, the fragment was subcloned into the BamHI and EcoRI sites of pGEX-2T (Pharmacia, Uppsala, Sweden). DH5α *E. coli* transformed with pGEX-2T vector was used as control.

**Preparation and affinity purification of bacterial extracts**

This is performed principally as described by Smith and Johnson (1988). In brief, overnight bacterial cultures were diluted 1:10 (to 400 ml) in fresh medium and incubated for 2 h. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM and incubated for a further 4 h. The cells were then pelleted, resuspended in 10 ml of MTPBS (150 mM sodium chloride, 16 mM disodium hydrogen phosphate, 4 mM sodium dihydrogen phosphate, pH 7.3) containing 1% Triton X-100. The cells were lysed on ice by mild sonication, then centrifuged at 10 000 g for 5 min at 4°C. The supernatants were loaded onto a column containing glutathione sepharose 4B (Pharmacia). After washing the column twice with five bed volumes of MTPBS, the bound fractions were eluted with about four bed volumes of elution buffer containing 5 mM reduced glutathione (KOJIN, Tokyo, Japan) in 50 mM Tris-HCl, pH 8.0. The purity of the proteins was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by staining with Coomassie blue. The protein concentration was estimated from the absorbance at 280 nm (1 A280 = 0.5 mg ml⁻¹).

**Monoclonal antibody**

A mouse was immunized s.c. three times with DCC fusion protein at 2-week intervals: the first time with 50 μg of protein with complete Freund’s adjuvant, the second with 100 μg of protein with incomplete Freund’s adjuvant and the third with 100 μg of protein alone. Spleen cells were obtained from the mouse and fused with the murine myeloma cell line NS-1. The hybridoma culture supernatants were assayed for reactivity with the DCC protein using an enzyme-linked immunosorbent assay and immunoblotting. Positive cultures were cloned by limiting dilution three times to obtain the MAb DCC127–22, which is specifically reactive with the DCC protein.

**Cells**

The cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum (leukaemia lines) or in Dulbecco’s modified Eagle medium containing 7.5% fetal bovine serum (monolayer cells) and cultured in a carbon dioxide incubator at 37°C. The derivation of the cell lines was as follows – human colorectal cell lines: CCK-81, CoCM-1, RCM-1, WiDr and VMRC-MELG (melanoma in the colon); stomach cell lines: AZ-521, MKN-1 and SCH; and B-cell lines: BALL-1, CCRF-SB, HS-Sultan, IM9 and Ramos (obtained from the Japanese Cancer Research Resources Bank). Other cell lines were obtained as described in Yamashiro et al (1993).

**Flow cytometry**

Flow cytometry was performed as described previously (Yamashiro et al, 1993). Briefly, the cells were incubated with appropriately diluted MAb for 45 min on ice. After two washes with phosphate-buffered saline (PBS), the cells were incubated with 100 μl of 100-fold-diluted fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Cappel, West Chester, PA, USA) for 30 min on ice. After two washes, the cells were examined using a FACScan (Becton-Dickinson, Mountain View, CA, USA).

**Western blot**

Cells were lysed in 0.01 M Tris buffer, pH 7.3, containing 0.15 M sodium chloride, 0.01 M magnesium chloride, 0.5% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma, St Louis, MO, USA) and 20 μl⁻¹ of aprotonin (Bayer, Leverkusen, Germany). Usually, 100 μg of protein determined using the Bradford method (Bradford, 1976) was resolved by SDS-PAGE according to Laemmli (1970) and transferred to a PVDF membrane (Immobilon, 0.22-μm pore size) (Nihon Millipore Kogyo KK, Tokyo, Japan) for 4.5 h at 70 V in blotting buffer consisting of 0.025 M Tris, 0.192 M glycine and 20% methanol. The protein blots were incubated in PBS with 5% non-fat dry milk (Yukijirushi, Sapporo, Japan) and 0.02% sodium azide at 4°C overnight. The membranes were incubated with MAb DCC127–22 at room temperature for 1 h and washed with T-PBS (PBS containing 0.05% Tween-20) three times. Proteins were immunodetected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. The proteins were visualized using a Konica immunostaining HRP kit (Konica, Tokyo, Japan). The intensity of the bands was measured by a densitometer (Yamashiro et al, 1995), and the ratio of the DCC bands in cancer tissues to those of the adjacent normal mucosa was calculated. Data were presented as the mean ± standard deviation. Statistical analysis was performed by the chi-square test. Differences were taken as being significant when the P-value was less than 0.05.

**Tumour specimens**

Primary tumour samples and normal tissues were obtained from surgical resection specimens from patients with colon cancer or endoscopic polypectomy specimens of adenoma at the First Department of Surgery, Fukui Medical School, Fukui, Japan. All samples had been snap frozen in liquid nitrogen immediately after surgical excision and stored at −80°C until use. Of the primary carcinomas, eight were well differentiated, 13 were moderately differentiated, two were mucinous and one case was a poorly differentiated adenocarcinoma. These studies have been carried out with approval of the ethical committee of Fukui Medical School.

**Immunohistochemical staining of tissues**

Paraffin-embedded blocks of acetone, methanol and xylene-fixed (Amex method; Sato et al, 1986) tissues were sliced into 4-μm sections, deparaffinized and immersed in methanol containing 1% hydrogen peroxide for 20 min to eliminate endogenous peroxidase activity. After preincubation with normal goat serum for 20 min at room temperature, the sections were incubated with MAb DCC127–22 (1 μg ml⁻¹) for 4 h at room temperature. They were
QUESTION: What is the purpose of using Coomassie blue staining in this context?  

ANSWER: Coomassie blue staining is used to visualize the soluble fusion protein purified from E. coli. The purpose of the staining is to identify the protein band corresponding to DCC fusion protein. The figure (Figure 1A) shows the Western blot of the purified DCC fusion protein (lane 1) and GST protein (lane 2) as compared to the Coomassie blue stained protein bands. This method allows for the identification of the protein of interest among other proteins present in the sample. The entire DCC gene-coding region was translated as a fusion protein with a 26-kDa GST. The partly soluble fusion proteins were purified by affinity chromatography using glutathione sepharose 4B and migrated as a few bands at 40 kDa in SDS-PAGE (Figure 1A). Using these fusion proteins, a MAb DCC127–22 was generated that specifically reacted with DCC proteins but not with GST as shown in Figure 1B. The Ig subclass of MAb DCC127–22 was IgG1.

**Cell-surface expression of DCC recognized by MAb DCC127–22**

We examined whether MAb DCC127–22 was reactive with cell-surface molecules on various human cell lines by means of flow cytometry. As shown in Figure 2, MAb DCC127–22 was reactive with ARA10 and RPMI8226. It was not reactive with HT29 and DLD-1. A summary of the flow cytometric analysis of cell lines is shown in Table 1. Many haematopoietic and non-haematopoietic cell lines examined were not reactive with MAb DCC127–22, except some leukaemia lines, suggesting that the DCC epitope is rarely expressed in a wide range of cancer cells.
Western blots with various cell lines

DCC expression in cancer cell lines was analysed by immunoblotting using MAb 127–22. A representative example is shown in Figure 3. A specific band of 190 kDa was observed in ARA10 and RPMI-8226. Colorectal and stomach cancer lines were negative.

mRNA expression of DCC in haematopoietic and colon cancer cell lines

The expression of DCC mRNA was analysed by RT-PCR followed by ethidium bromide staining. There was a major amplified band at 225 bp in ARA10 and IMR32, but not in human colonic cancer cell lines (data not shown). A summary of the flow cytometric analysis and RT-PCR is shown in Table 1. Consequently, the expression of a 190-kDa band detected in immunoblot corresponded well with mRNA expression of DCC gene as measured by RT-PCR.

Immunohistochemistry of normal human tissues and colonic cancers using MAb 127–22

The expression of DCC in normal human tissues was analysed using immunohistostaining. All tissues expressed some levels of DCC, although there were some differences in the intensities of the staining. Colonic cancer tissue and corresponding normal tissue were then examined using immunohistochemistry. For normal tissue, the staining was intense, mainly on the apical aspect of the cells, while colonic cancer tissue was not stained (Figure 4A). Figure 4B shows a high magnification of the border between colon cancer tissue and normal tissue and demonstrates a distinct staining pattern.
Decreased expression of protein in colonic cancer

The expression of DCC protein in colonic cancers, adjacent normal mucosa and adenomas was analysed by immunoblotting using MAb 127–22. Representative examples are shown in Figure 5. Prominent 190-kDa bands of DCC were observed in the extracts from normal colonic mucosa and adenomas, whereas bands were undetectable in the extracts from corresponding colonic cancers. In all samples examined, DCC protein was more prominently expressed in the adjacent normal colonic mucosa and adenoma tissues than in the colonic cancer tissues. Figure 6 is a summary of Western immunoblots showing the ratio of DCC bands (tumour/normal) in adenoma and colon cancer samples. Thus, development of cancers appears to result in the marked reduction of DCC protein, as benign adenomas contained levels equivalent to those in normal tissues.

Liver metastasis and DCC expression

Among 25 colon cancer tissues, 15 samples with no liver metastasis showed significantly higher levels of DCC than ten samples with liver metastasis as shown in Figure 7. These results indicate the possible use of DCC as a prognostic factor in colon cancer patients.

DISCUSSION

Since DCC was discovered by Vogelstein et al in 1990 (Fearon et al, 1990), there have been many reports describing the high frequency of LOH at the DCC locus in the colon cancer tissues (Fearon et al, 1994; Iacobetta et al, 1994; Iino et al, 1994), and in many other cancers (Hohne et al, 1992; Miyake et al, 1994; Munty et al, 1994; Kashiwaba et al, 1995; Cho et al, 1996). DCC has been evaluated as a tumour-suppressor gene candidate on chromosome 18 in colorectal cancers (Thiagalingam et al, 1996). RT-PCR approaches have also been used to show lower expression of the DCC gene in colon cancer tissues compared with adjacent normal colonic mucosae (Kikuchi-Yanoshita et al, 1992; Itoh et al, 1993; Iino et al, 1994). However, only a few reports on the DCC protein levels in colon cancer and normal colonic tissues in individual patients have been published. Protein analysis has been reported in normal tissues (Turley et al, 1995), for leukaemia and MDS (Inokuchi et al, 1996) and for brain tumours (Ekstrand et al, 1995). In the present study, we have established a DCC-specific MAb by using a GST-DCC fusion protein for immunization. This MAb proved to be very useful in Western immunoblot, flow cytometry and immunohistochemical staining. As expected, DCC protein was scarcely detectable in human cancer cell lines, except for some haematopoietic lines. Colon cancer tissues from patients were also negative or they expressed at a very low level. On the other hand, normal colonic tissues and adenoma tissues showed almost equivalent intensities of DCC bands in immunoblots. These results are very similar to the immunohistochemical studies reported recently by Shibata et al (1996). The fact that five adenomas showed equivalent expression levels to normal tissues suggested that the reduction observed in carcinomas is a relatively late phenotypic event. These results indicate that DCC may play an important role as a tumour-suppressor gene, and down-regulation or defect of the gene may trigger progression of colon cancers from adenomas.

The immunoblot results of DCC showed fairly broad variations in the cancer/normal expression ratio between samples as shown in Figures 6 and 7. This may be partly due to varying levels of contamination of non-tumour tissue present in each case. The data for cancer cell lines in which DCC bands could be scarcely seen also suggest this possibility.

Furthermore, it has been demonstrated that the inactivation of the DCC gene or reduction of DCC gene expression closely correlates with cancer metastasis. Recent studies have indicated the importance of DCC alterations in liver metastasis (Itoh et al, 1993; Ookawa et al, 1993; Iino et al, 1994; Kato et al, 1996) or nodal metastasis (Katoaka et al, 1995) of colorectal cancers. Our quantitative data of DCC protein also indicate a reverse correlation between DCC levels and frequency of liver metastasis in colon cancers, suggesting its value as a prognostic marker. As the observation time after operation was not long enough, the prognosis of each patient has not been examined. However, the results shown in Figure 7 may be useful in identifying primary tumours likely to have liver metastases. The study of a larger number of tumours is also needed.

As for the biological function of DCC, no definite roles of the molecule have been elucidated so far. As DCC has high homology with N-CAM, one of the adhesion molecules abundantly expressed in nervous tissues, it might play an important role in cell–cell or cell–extracellular matrix interaction during development or differentiation (Fearon et al, 1994). Some reports strongly indicate that DCC is important for cell differentiation (Hedrick et al, 1994), particularly in the differentiation of neuronal cells (Laelor and Narayanan, 1992). Recently, Goyette has shown that chromosome 18 restores TGF-β responsiveness and reduces tumorigenicity in the human colon cancer cell line SW480. It may be important to determine whether the DCC gene is sufficient to restore responsiveness to TGF-β and suppress tumorigenicity. In fact, transformed epithelial cells have been reversed from the malignant phenotype by the introduction of the DCC gene (Klingelhutz et al, 1995). Further understanding of the biological
functions of DCC will provide more effective methods of its application for diagnostic and therapeutic purposes.

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