Loss of expression and loss of heterozygosity in the DCC gene in neoplasms of the human female reproductive tract

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Summary In order to identify the possible role of the DCC gene in neoplasms of the human female reproductive tract, messenger RNA expression of the DCC gene was examined by reverse transcriptase-polymerase chain reaction, and expression of the DCC gene product was detected immunohistochemically. While histologically normal endometrium, cervical epithelium and ovary expressed detectable mRNA of the DCC gene, three of eight (37.5%) endometrial carcinomas, one of two (50%) cervical carcinomas and 9 of 22 (41%) ovarian malignant tumours had significantly reduced or negligible DCC expression, and another endometrial carcinoma and two other ovarian tumours underexpressed DCC when compared with histologically normal endometrial or ovarian tissues. Impaired DCC mRNA expression was detected more frequently in grade 3 ovarian epithelial tumours than in grade 1 tumours (P=0.002). Loss of expression of the DCC gene product detected by immunohistochemistry significantly correlated with the loss of mRNA expression in ovarian carcinomas (P=0.01 by chi-square test) or in both endometrial and ovarian carcinomas combined (P=0.001). Loss of heterozygosity of the DCC gene was also evaluated by restriction fragment polymorphism analysis of the polymerase chain reaction-amplified DNA fragment. Loss of heterozygosity of the DCC gene was detected in one of seven (14%) informative informative cases of cervical carcinomas and two of six (33%) informative cases of ovarian tumours. These results demonstrate that inactivation of the DCC gene, especially by the loss of expression, plays a significant role in the aetiology of neoplasms of the human reproductive tract.

Keywords: DCC, ovarian carcinoma; endometrial carcinoma; cervical carcinoma; LOH; immunohistochemistry

Materials and methods

Tissue preparations of DNA and RNA

Samples used in this study were from patients who had been admitted to the Department of Obstetrics and Gynecology at the Osaka University Hospital in Osaka, Japan. No initial
chemotherapy or radiation therapy was performed prior to tumour excision. Surgically removed tissues were sampled for histopathological diagnosis and the remainder were quickly frozen for extraction of RNA or DNA or for immunohistochemical analysis. Histological classification of tumours was in accordance with the WHO international system. Surgical staging was established according to the International Federation of Gynecology and Obstetrics. A total of 32 neoplasms, including eight endometrial adenocarcinomas, 22 ovarian malignant and low-grade malignant tumours (three endometrioid carcinomas, five mucinous adenocarcinomas, one mucinous carcinoma of low malignant potential, ten serous adenocarcinomas, one clear cell carcinoma, one immature teratoma and one dysgerminoma) and two cervical carcinomas, were studied for the mRNA expression of the DCC gene. RNA was extracted with guanidinium isothiocyanate followed by centrifugation in a caesium chloride solution. RNA was also extracted from histologically normal ovary, endometrium, cervix and colon mucosa. Expression of the DCC gene product was detected immunohistochemically in a total of 18 neoplasms, including five endometrial adenocarcinomas and 13 malignant ovarian tumours (eight serous adenocarcinomas). Immunohistochemical amplification of the endometrioid adenocarcinomas. Loss of heterozygosity of the DCC gene was also analysed in a total of 95 neoplasms, including 29 cervical squamous cell carcinomas, 34 ovarian tumours, and 32 endometrial adenocarcinomas and seven endometrial atypical hyperplasias. High molecular weight DNA was extracted following the procedures previously described (Enomoto et al., 1990). DNA was also extracted from white blood cells or histologically normal myometrium from the patients.

Reverse transcription–polymerase chain reaction analysis
For complementary DNA synthesis, 1 μg of total cellular RNA was annealed with random hexamers (pd(N)₆) at 26°C for 10 min and transcribed with 10 units of AMV reverse transcriptase (Gibco-BRL) in the presence of the ribonuclease inhibitor RNasin (1 μl; Promega, Madison, WI, USA) (Enomoto et al., 1993a). A cDNA aliquot, corresponding to 200–500 ng of RNA, was used as the template of PCR amplification. Primers used were 5'-AGCCCTATTCTTGACCATGCC-3' for antisense primer, which corresponds to nucleotides 1218–1198 in the cDNA sequences, and 5'-TTCCGGCATGTTTTAAATACA-3' for sense primer, which corresponds to nucleotides 986–1007 (Fearon et al., 1990). For an internal control of RT–PCR, a 319 bp fragment of β-actin was simultaneously amplified using primers 5'-ATCA- TGTTTGGACAGCTTCCA-3' and 5'-CATCTCTTGGCTGAGTCCA-3' (Fuqua et al., 1990). The PCR mixture (total 50 μl) contained cDNA (200–500 ng), 0.5 μM of each primer for DCC, 0.05 μM of each primer for β-actin, 100 μM of each deoxynucleotide triphosphate, 0.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM Tris–HCl (pH 8.3) and 0.01% gelatin. One cycle consisted of 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (elongation) and a total of 28 cycles of PCR amplification was performed. The PCR products were fractionated in 12% polyacrylamide gels, and DNA fragments were visualised by staining with ethidium bromide. Quantitation was done by densitometric analysis using the software NIH Image version 1.44.

Immunohistochemistry
The tissue localisation of DCC protein was determined immunohistochemically by the avidin–biotin–peroxidase complex method on frozen sections. Sections (4 μm) were cut with a cryostat, placed on glass slides coated with 0.02% polylysine and fixed in 1% glutaraldehyde and 2% acetone for 10 min at room temperature. The sections were immersed in 0.3% hydrogen peroxide to block endogenous peroxidase activity, and the staining procedures were then carried out according to the manufacturer’s recommendation (Vector Laboratories, Burlingham, CA, USA). A monoclonal antibody to the DCC gene product (Ab-1) (OncoGene Science, Uniondale, NY, USA) was used as primary antibody for the present immunohistochemistry. Sections from normal proliferative endometrium and normal ovary were used as positive controls for the expression of DCC. Sections on which normal mouse serum were incubated instead of the corresponding primary antibody were used as negative controls. The relative number of immunoreactive cells was graded from negative to grade 3 (+++) as follows. (−), no staining was observed in any tumour cell; +, less than 10% of the tumour cells were stained positively; + +, between 10% and 50% of the tumour cells were stained positively; + + +, more than 50% of the tumour cells were stained positively.)

Detection of loss of heterozygosity by PCR–RFLP analysis
PCR amplification was performed to generate 367 bp DNA fragments around the polymorphic MspI sites of the DCC gene. The PCR mixture was prepared from a GeneAmp PCR amplification reagent kit (Perkin-Elmer Cetus) following the manufacturer’s instructions. Primers used were 5'-TTGACCATGCTGAAGATTCTT-3' (upstream) and 3'-ATTTCAGTAGTCCCCCTCCCA-5' (downstream) (Parry et al., 1991). DNA (300 ng) was added to the 50 μl reaction mixture and incubated initially for 5 min at 100°C. Then 0.25 units of Taq polymerase was added. One cycle consisted of 1 min at 95°C (denaturation), 1 min at 59°C (annealing) and 1 min at 72°C (elongation) of PCR amplification. The amplified 367 bp fragment was cut into 227 bp and 140 bp fragments when the restriction site was present. Cases in which DNA derived from white blood cell (WBCs) yielded three fragments (367 bp, 227 bp and 140 bp) were considered informative, and DNA derived from the tumours was further analysed for loss of heterozygosity.

Results
Loss of expression of the DCC gene
Expression of mRNA from the DCC gene was examined in a total of 32 neoplasms of the human female reproductive tract by RT–PCR analysis, as well as in histologically normal ovary, uterine endometrium, cervix and colon mucosa as positive controls. PCR amplification was performed from the complementary DNA to generate the 233 bp fragment of the DCC gene and the 319 bp fragment of β-actin simultaneously. Both fragments were successfully amplified in the histologically normal tissues, suggesting that the DCC gene is expressed in the normal tissues of the ovary, uterine endometrium, cervix, and colon mucosa (Figure 1). On the other hand, the 233 bp fragment of the DCC gene was amplified little or not at all, while the 319 bp fragment of the β-actin gene was successfully generated in three of eight (37%) endometrial adenocarcinomas, one of two (50%) cervical squamous cell carcinomas and 9 of 22 (41%) ovarian tumours [one of three endometrioid carcinomas (33%), one of six mucinous carcinomas (17%), five of ten serous carcinomas (50%), one clear cell carcinoma and one of two germ-line tumours], suggesting that DCC mRNA expression was significantly reduced or absent in these tumours (Table I). Moreover, in one endometrial carcinoma (case 6) and two serous adenocarcinomas of the ovary (cases 3 and 7), the intensity of the 233 bp fragment of the DCC gene was only about one-third of the intensity of those derived from the histologically normal endometrial or ovarian tissues. In order to test the validity of the experiment regarding the semiquan-
Figure 1 Demonstration of loss of expression in the DCC gene by RT-PCR analysis. PCR amplification was performed on complementary DNA to generate a 233 bp fragment of the DCC gene and a 319 bp fragment of the β-actin gene simultaneously. The PCR products were fractionated in a 12% polyacrylamide gel and visualised by staining with ethidium bromide. Note that the 319 bp fragment of β-actin was successfully generated in all the samples analysed, whereas fragments of the DCC were not amplified in lanes 3, 7, 10, 13, 14 and 15, suggesting the loss of expression in these tumours. In lane 4, intensity of the 233 bp fragment of DCC was weak, suggesting decreased DCC expression. Lanes 1–5, endometrial carcinomas, lanes 6 and 7, cervical carcinomas; lanes 8–15, ovarian carcinomas; lanes 16–18, normal uterine endometrium, normal ovary, and normal colon mucosa.

Table 1 Expression of the DCC gene in the neoplasms of the human reproductive tract

| Case Histology | Age | Grade | Stage | mRNA expression | Immunohistochemistry |
|----------------|-----|-------|-------|-----------------|---------------------|
| **Endometrial carcinoma** |     |       |       |                 |                     |
| 1 Endometrioid | 50  | 1     | 1     | +               | NT                  |
| 2 Endometrioid | 67  | 1     | 1     | +               | NT                  |
| 3 Endometrioid | 44  | 2     | 3     | +               | +                   |
| 4 Endometrioid | 28  | 2     | 1     | –               | –                   |
| 5 Endometrioid | 72  | 3     | 3     | +               | –                   |
| 6 Endometrioid | 49  | 3     | 3     | ±               | NT                  |
| 7 Endometrioid | 68  | 3     | 2     | –               | –                   |
| 8 Endometrioid | 66  | 3     | 3     | –               | –                   |
| **Ovarian tumour** |     |       |       |                 |                     |
| 1 Serous       | 39  | 1     | 1     | +               | +                   |
| 2 Serous       | 50  | 1     | 3     | –               | –                   |
| 3 Serous       | 62  | 2     | 3     | ±               | +                   |
| 4 Serous       | 52  | 2     | 4     | +               | +                   |
| 5 Serous       | 55  | 2     | 3     | +               | +                   |
| 6 Serous       | 31  | 2     | 3     | –               | –                   |
| 7 Serous       | 61  | 3     | 4     | ±               | +                   |
| 8 Serous       | 53  | 3     | 3     | –               | NT                  |
| 9 Serous       | 38  | 3     | 3     | –               | –                   |
| 10 Serous      | 46  | 3     | 3     | –               | –                   |
| 11 Mucinous    | 29  | 1     | 1     | +               | NT                  |
| 12 Mucinous    | 54  | 1     | 2     | +               | –                   |
| 13 Mucinous    | 55  | 1     | 2     | +               | NT                  |
| 14 Mucinous    | 38  | 1     | 3     | +               | +                   |
| 15 Mucinous    | 47  | 1     | 3     | –               | NT                  |
| 16 Mucinous (LPM) | 63 | NA | 1     | +               | NT                  |
| 17 Endometrioid| 55  | 2     | 2     | +               | +                   |
| 18 Endometrioid| 47  | 2     | 3     | +               | +                   |
| 19 Endometrioid| 39  | 3     | 3     | –               | –                   |
| 20 Clear cell  | 49  | 3     | 1     | –               | NT                  |
| 21 Dysgerminoma| 19  | NA | 1     | –               | NT                  |
| 22 Immature teratoma | 21 | 2 | 1     | +               | NT                  |
| **Cervical carcinoma** |     |       |       |                 |                     |
| 1 Squamous cell | 56  | 3     | 1     | –               | NT                  |
| 2 Squamous cell | 62  | 1     | 1     | +               | NT                  |

*+*, Intensity equal to that observed in normal tissue; ±, approximately one-half of normal; ‒, no detectable expression or much less than half of normal; ‒+ + ‒+, more than 50% of the tumour cells were stained positively; + ‒+ ‒+, between 10% and 50% of the tumour cells were stained positively; ‒+, less than 10% of the tumour cells were stained positively; ‒, no staining was observed in any tumour cell. Abbreviations: NT, not tested; LPM, low potential malignancy; NA, not applicable.

Fractional assay, the following control experiments were performed. Complementary DNA derived from normal endometrium which expressed DCC was diluted 2-fold serially with the cDNA derived from the endometrial carcinoma (case 4), which did not express DCC, and 20, 28, and 35 cycles of PCR amplification were performed. The PCR products were fractionated in 12% polyacrylamide gels, and DNA fragments were visualised by staining with ethidium bromide. Quantitation was done by densitometric analysis. The amount of PCR product of the DCC gene correlated well with the proportion of the cDNA derived from normal endometrium after 28 cycles of PCR amplification (Figure 2) but did not correlate well after 35 cycles, probably because the reaction plateaued. PCR for 20 cycles did not yield sufficient product to be detected (data not shown). Densitometric analysis showed that the ratio of DCC/β-actin was 1.10, 0.79, 0.63, 0.33, 0.16 for 1-, 2-, 4-, 8- and 16-fold dilution respectively. From these observations we conclude that DCC expression was reduced in cases 6 (endometrial carcinoma) 3 and 7 (ovarian carcinoma), in DCC β-actin ratios were 0.29, 0.38 and 0.16 respectively.

Association of impaired DCC mRNA expression with histological grade or clinical stage was evaluated. Impaired expression was observed in one of three (33%), one of one (100%) and two of five (50%) endometrial carcinomas of stage 1, 2 and 3, respectively, and two of four (50%), none of three (0%), seven of ten (70%) and both (100%) ovarian epithelial tumours of stage 1, 2, 3 and 4 respectively. Thus, there was no clear association of impaired expression with
clinical stage. Conversely, impaired expression was observed in none of two (0%), one of two (50%) and three of four (75%) endometrial carcinomas of grade 1, 2 and 3, respectively, and one of seven (14%), two of six (33%) and all seven (100%) ovarian epithelial tumours of grade 1, 2 and 3 respectively (Table II). The prevalence of impaired DCC expression in grade 3 ovarian epithelial tumours was significantly higher than that in grade 1 tumours \( (P = 0.002 \) by Fisher’s exact test). Therefore reduced or absent expression of the DCC gene tended to occur in tumours of higher histological grade. In the ovary, decreased or loss of expression was found more frequently in serous tumours (7/10, 70%) than in mucinous tumours (1/6, 17%).

Immunohistochemical detection of the DCC gene product

The DCC gene product was examined immunohistochemically in five endometrial adenocarcinomas and 13 ovarian adenocarcinomas. While the normal endometrium and the surface epithelium of the normal ovary showed strong positive staining, many tumours showed either no staining or weak staining to a variable extent (Figure 3). Even in a single tumour, staining intensity varied from area to area, although there was a tendency that it was less evident in areas of histologically more aggressive lesion. Four of five endometrial carcinomas and 5 of 13 ovarian adenocarcinomas showed negative staining (Table I). Three endometrial adenocarcinomas (cases 4, 7 and 8) and four ovarian adenocarcinomas (cases 2, 6, 9 and 19) which showed negative staining did not express DCC mRNA. Two ovarian adenocarcinomas which showed weak positive staining (cases 3 and 7) revealed reduced DCC mRNA expression. The intensity of the immunohistochemical staining correlated significantly with the level of mRNA expression in ovarian tumours \( (P = 0.01 \) by chi-square test) or in both endometrial carcinomas and ovarian tumours combined \( (P = 0.001 \) by chi-square test). Although a single case of endometrial carcinoma (case 5) and a single case of ovarian adenocarcinoma (case 12) showed no staining in spite of positive DCC mRNA expression, there was, thus, a significant correlation between the mRNA expression and immunohistochemistry.

Detection of loss of heterozygosity in the DCC gene

Loss of heterozygosity in the DCC gene was detected by PCR–(RFLP) analysis (Figure 4). Of a total of 102 samples analysed, DNA derived from WBCs or the histologically normal tissue from 7 of 32 (22%) endometrial adenocarcinomas, one of seven (14%) endometrial atypical hyperplasias, 11 of 29 (38%) cervical carcinomas and 6 of 34 (18%) ovarian tumours showed heterozygosity and were therefore informative for the LOH analysis. DNA derived

![Figure 3](image-url)  
Immunohistochemical analysis of the DCC gene product in normal and malignant tissues of the female genital tract. (a) Glandular cells of proliferative endometrium showed a granular staining at the cell boundaries, with weak staining in cytoplasm. (b) The foci of well-differentiated adenocarcinoma of the uterine endometrium showed intense staining, whereas histologically more aggressive lesion showed faint staining (arrows). (c) Poorly differentiated adenocarcinoma of the uterine endometrium showed no staining. (d) Surface epithelium of the normal ovary showed positive staining while stromal cells showed negative staining.
from the tumours of those informative cases was further analysed. Of those one of seven (14%) endometrial carcinomas, 1 of 11 (9%) cervical squamous cell carcinomas and two of six (33%) ovarian adenocarcinomas showed loss of heterozygosity at the DCC locus (Table III). In these four cases of LOH, either the 367 bp fragment or the 227 bp and 140 bp fragments were lost in DNA derived from the tumours.

Discussion
In this study, we analysed the possible involvement of the DCC gene in neoplasms of the human female reproductive tract. We show, for the first time, that the DCC gene is frequently decreased in expression or undetectable in these neoplasms. Significantly reduced or absent mRNA expression was observed in about 40% of the ovarian malignant tumours and endometrial carcinomas. Three other tumours (one endometrial carcinoma and two serious adenocarcinomas of the ovary) expressed DCC mRNA levels that were much less than one-half of that of the normal tissues. Therefore, impaired expression of the DCC gene was observed in four of eight (50%) endometrial carcinomas and 12 of 22 (55%) ovarian tumours. Impaired expression of mRNA was more frequently detected in the tumours of higher histological grade, corresponding well to the immunohistochemical analysis. The high incidence of impaired expression of the DCC gene is somewhat similar to the frequencies reported for other human malignancies such as colorectal (57%), pancreatic (50%) and prostatic carcinomas (86%) (Hohme et al., 1992; Itoh et al., 1993; Gao et al., 1993). We also showed that loss of heterozygosity of the DCC gene was detected in 14% of endometrial carcinoma, 9% of cervical carcinomas and 33% of ovarian tumours, which is not as frequent as reported for colorectal or gastric carcinoma. Loss of heterozygosity of the DCC gene in endometrial carcinoma was previously examined by Okamoto et al. (1991a) and Imamura et al. (1992) using the probe OLVIE10, and loss of heterozygosity was found in one of eight (13%) and 4 of 13 (31%) endometrial carcinomas respectively, similar to the present data. Chenex-Trench et al. (1992) reported loss of heterozygosity in about 40% of adenocarcinomas of the ovary, which is also similar to the present data. However, intensive analysis using several chromosomal markers which localise to 18q showed that the smallest region of overlap appears to exclude the DCC gene (18q21.3) and to be between D18S55 (18q21.3–qter) and D18S11 (18q23) loci, suggesting that another locus, in addition to DCC, may be involved in ovarian carcinogenesis. Nevertheless, the DCC gene is implicated in tumorigenesis of endometrium and ovarian epithelium since impaired expression has frequently been observed in such tumours. In colorectal carcinoma, DCC expression was reduced during tumour progression from intramucosal to invasive carcinoma (Kikuchi-Yanoshita et al., 1992), suggesting that the DCC gene may act as a metastatic suppressor. Similarly, impaired DCC expression was detected in 100% of colorectal tumours with liver metastasis (Itoh et al., 1993). Loss of heterozygosity of the DCC gene was observed more frequently in metastatic liver tumours from colon than in primary colorectal carcinomas (Okawa et al., 1993). In endometrial carcinomas and ovarian epithelial tumours, we found that impaired DCC expression occurs more frequently in tumours of higher histological grade, suggesting that alteration in the DCC gene is associated with the aggressiveness of these tumours.

Among the tumour-suppressor genes, alterations of the p53 gene are observed in a wide variety of human cancers and are currently the most commonly found alterations associated with human cancer (Nigro et al., 1989; Levine et al., 1991). Mutations of both p53 alleles, one through deletion and the other through a base substitution, occur in many human cancers including endometrial carcinoma (Okamoto et al., 1991a; Enomoto et al., 1993) and ovarian carcinoma (Mazars et al., 1991; Okamoto et al., 1991b). The mechanism of inactivation of the RB tumour-suppressor gene seems to be different from that of the p53 gene. There is no correlation between the loss of expression of the RB protein and loss of heterozygosity of the RB gene in bladder carcinoma (Ishikawa et al., 1991) and breast cancer (Borg et al., 1992). We previously reported that loss of heterozygosity does not accompany loss of mRNA expression in an endometrial carcinoma (Enomoto et al., 1993a). Inactivation of the RB gene seems to occur as a result of mutations in both alleles rather than a loss of one allele and mutation in another allele, as observed for p53. The association of loss of expression with the loss of heterozygosity in the present study was therefore evaluated. Of seven cases of endometrial carcinomas which were informative for loss of heterozygosity analysis, a single case (case 7) was also analysed for DCC expression. Loss of mRNA expression was observed in this case, although there was no concomitant loss of heterozygosity of nine cases of ovarian carcinomas which were informative for loss of heterozygosity analysis, four cases (cases 4, 5, 7 and 22) were also analysed for DCC expression. Loss of heterozygosity was detected in two cases (cases 7 and 22). Decreased mRNA expression accompanied LOH in one case (case 7), but DCC expression was not altered in another case of immature teratoma (case 22). In the latter tumour, another locus which is located very close to the DCC gene may function in carcinogenesis as proposed by Chenex-Trench et al. (1992). Among ovarian tumours in which loss
of heterozygosity was not observed (cases 4 and 5), decreased mRNA expression was observed in only a single case (case 4). These findings suggest that inactivation of the DCC tumour-suppressor gene does not necessarily occur through deletion in one allele and mutation in the other allele, as observed for the p53 gene. Since the expressions of the DCC locus are scattered over approximately 370 kb, and the locus we analysed in the present study, D18S58, is in the middle of this segment, rearrangements or partial deletions of DCC may occur upstream or downstream of this locus. The present findings suggest that inactivation of the DCC gene can occur through mutations in both alleles, which could lead to loss of expression of the gene. The observation that the prevalence of loss of expression is higher than loss of heterozygosity seems to support this hypothesis.

In conclusion, inactivation of the DCC gene by loss of expression or by allelic loss occurs rather frequently in neoplasms of the human female reproductive tract, and therefore may play an important role as one of the alterations in multistep carcinogenesis. Other types of DCC alterations, such as insertions, deletions or point mutations, warrant further study.

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