Point Mutations and Allelic Deletion of Tumor Suppressor Gene DCC in Human Esophageal Squamous Cell Carcinomas and Their Relation to Metastasis

Satoshi Miyake, Kagami Nagai, Kunihide Yoshino, Michiei Oto, Mitsuo Endo, and Yasuhito Yuasa

ABSTRACT

Since tumor suppressor gene DCC exhibits amino acid sequence homology to the neural cell adhesion molecule, there is a possibility that DCC might be related to tumor metastasis. In the present study, we examined 51 cases of primary esophageal carcinomas with regard to point mutations and loss of the DCC gene. We detected point mutations in two cases by screening using polymerase chain reaction-single strand conformation polymorphism analysis. When we determined the sequences, one case with lymph node metastasis showed an ATG (Met) to ACG (Thr) missense mutation in codon 168. Another case showed a CGA (Arg) to GGA (Gly) mutation in codon 201, which might be a polymorphic change, and two other mutations resulting in no amino acid change. We also examined loss of heterozygosity of the DCC gene. Forty-four of the 51 cases (86%) were informative, and among them 10 cases (23%) showed allelic deletion. The further away the lymph node metastasis was from the primary tumor, the higher the frequency of allelic deletions became. We also found allelic deletions in moderately and poorly differentiated squamous cell carcinomas but not in well differentiated ones. These results indicate that alterations of the DCC gene are related to the degree of lymph node metastasis and the degree of differentiation.

INTRODUCTION

Esophageal carcinomas are the sixth most frequent cause of cancer found in males throughout the world (1). Their prognosis is still poor because of its high metastatic and invasive properties. Previous studies revealed genetic changes in esophageal carcinomas. These include LOH of p53 (2–4), Rb (5), APC/MCC (6) and DCC (7), point mutations of p53 (3, 8, 9), and amplification of int-2/hst-1 (10, 11), cyclin D (12), EGFR (13, 14), c-erbB-2 (14) and c-myc (15). How other mutations resulting in no amino acid change. We also examined loss of heterozygosity of the DCC gene. Forty-four of the 51 cases (86%) were informative, and among them 10 cases (23%) showed allelic deletion. The further away the lymph node metastasis was from the primary tumor, the higher the frequency of allelic deletions became. We also found allelic deletions in moderately and poorly differentiated squamous cell carcinomas but not in well differentiated ones. These results indicate that alterations of the DCC gene are related to the degree of lymph node metastasis and the degree of differentiation.

Materials and methods

Tissue Preparation and DNA Extraction. Tissue samples were obtained from 51 patients receiving surgical treatment in our department from 1990 to 1992. None had undergone either chemotherapy or irradiation prior to surgery, which may cause genetic changes. Primary tumors, normal esophageal mucosa, and, if available, metastatic lymph nodes were collected from operative specimens. In order to prevent the contamination by normal cells, we matched the frozen specimens with hematolx-in eosin staining sections. The contamination by normal cells was less than 20% in primary tumors and almost undetectable in metastatic lymph nodes. Metastatic lymph node specimens could be obtained from 27 of the 43 cases with lymph node metastasis. Germline DNA was obtained from peripheral lymphocytes. These materials were immediately frozen in liquid nitrogen and stored at −80°C until DNA extraction. High molecular weight DNA was prepared by proteinase K digestion and phenol-chloroform extraction (34).

SSCP Analysis. PCR was carried out as described previously (35) with minor modifications. Typical reaction conditions were as follows. The initial denaturing step was for 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at an annealing temperature, 1 min at 72°C, and 10 min at 72°C for the final extension step. The annealing temperature was optimized for each primer set. The primer sequences were: DCC B-U, 5'-ATCTGGAACTTTCCTTCTCTC-3'; DCC B-D, 5'-CTGTAAGGCAAACAAAGAGA-3'; DCC C-U, 5'-TTCTCCTTTGCGACCTGGCC-3'; DCC C-D, 5'-GAAAACAAATTACACTC-3'; DCC D-U, 5'-ATCACCTGCTTTCCTTCTC-3'; DCC D-D, 5'-CAACACAAAGGTCCTTCCCAT-3'; DCC E-U, 5'-TCTGTAGATCCTCTTCTTCTC-3'; and DCC E-D, 5'-TATATGATGATCCTACCACTAC-3'.

DCC B, C, D, and E primer sets flanked codons 138 to 232, 358 to 380, 381 to 420, and 421 to 473, respectively (36). After PCR amplification, the products were checked by 2% agarose gel electrophoresis, and then SSCP analyses were carried out as described previously. (35) A PCR aliquot (4 μl) was diluted with 11 μl of loading buffer (0.1% xylene cyanol-0.1% bromophenol blue in formamide), of which 3 μl was applied to a polyacrylamide gel.

Downloaded from cancerres.aacrjournals.org on April 9, 2017. © 1994 American Association for Cancer Research.
The running conditions were 450V for 2 h at 15°C. After electrophoresis, the gel was stained with silver (2D-Silver Stain II; Daiichi Pure Chemicals, Tokyo, Japan).

**Subcloning and Sequencing.** Subcloning was carried out with the PCR products using two primers containing XbaI and XhoI sites at the 5' end, respectively. These products were digested with XbaI and XhoI and then ligated to the pBluescript II plasmid digested with XbaI and XhoI. The nucleotide sequences of both strands were determined by the dideoxy chain termination method (37).

**LOH Analysis.** LOH was analyzed by three different methods, i.e., PCR-RFLP, PCR-LOH, and PCR-SSCP analyses. The primer sequences were: DCC 1-U, 5'-GATGACATTTCCTCTAG-3'; DCC 1-D, 5'-GTGGTATTGCACTGAAAG-3'; DCC 2-U, 5'-TGACACCCTGCTGAGATTG-3'; and DCC 2-D, 5'-AGTACAACACAGGTATG-3' (7). After amplification, the PCR products were electrophoresed on polyacrylamide gels after digestion with MspI (primer set DCC-1) or directly [primer set DCC-2 (7)] and detected by ethidium bromide staining.

**RESULTS**

**Point Mutations.** We investigated four exons (B, C, D, and E) of the DCC gene (33) by PCR-SSCP analysis in 51 primary tumors and 27 metastatic lymph nodes. There was no mobility shift within exon C, D, or E. We found a mobility shift in 2 of the 51 cases (4%) in the case of exon B (Fig. 1A). In case E9107, a mobility shift was observed only in the metastatic lymph node, i.e., not in the primary tumor. The PCR product of DCC exon B of the metastatic lymph node of case E9107 was subcloned. Six of the 10 subclones gave the same mutant allele, and the remaining four subclones gave the wild-type allele on PCR-SSCP analysis. Sequence analysis of the mutant clones showed an ATG (Met) to ACG (Thr) transition in codon 168 (Fig. 1B). Subclones showing the wild-type pattern showed the same sequence as described before (data not shown).

In case E9008, 9 of the 10 subclones gave the mutant pattern on PCR-SSCP analysis, and the other one gave the wild-type pattern. The mutant subclones had three mutations, GTG (Val) to GTT (Val) in codon 149, TTG (Leu) to TTA (Leu) in codon 197, and CGA (Arg) to GGA (Gly) in codon 201. When we sequenced the subclones from normal mucosal DNA of case E9008, we found the same sequence GGA in codon 201 as that of the carcinoma, GTG in codon 149 and TTG in 197, which were wild-type sequences (data not shown).

**LOH.** Fifty-one primary tumors and their 27 metastatic lymph nodes were analyzed using three primer sets. The PCR-SSCP pattern of normal cell DNA showed heterozygosity in the case of exon B of the DCC gene. Therefore, LOH analysis was carried out in this exon. Heterozygosity was observed in 20 of the 51 (39%) cases, and LOH of this locus was detected in 2 of the 20 (10%) informative cases (Fig. 2A). As for primer set DCC-1, which showed a variable number of tandem repeats (Fig. 2B), 33 of the 51 (65%) cases were informative, and LOH was observed in 8 of the 33 (24%) informative cases. MspI-digested PCR products (primer set 2; Fig. 2C) were informative in 13 of the 51 (25%) cases, and LOH was detected in 2 of the 13 (15%) informative cases. In total, 44 of the 51 (86%) cases were informative for at least one marker, and the DCC gene exhibited allelic deletion in 10 of the 44 (23%) informative cases. The two cases which had point mutations in the DCC gene also showed allelic loss.

The correlation of allelic loss of the DCC gene and the clinicopathological data is summarized in Table 1. LOH of DCC was detected only in moderately and poorly differentiated squamous cell carcinomas, i.e., not in well-differentiated ones, while LOH negativity was seen in well-differentiated ones as well as in moderately and poorly differentiated ones (P < 0.05, x² test). As for correlation of LOH of DCC and lymph node metastasis, the further away the lymph node metastasis was from the primary tumor, the higher the frequency of DCC deletion became (P < 0.05, Mann-Whitney U test). Similarly, a higher frequency of LOH of DCC was seen in late stages of esophageal carcinomas.

**DISCUSSION**

Here we reported point mutations within the coding region of the DCC gene in 2 of the 51 cases (4%), which is the first report to our knowledge. In E9107, a missense mutation from ATG (Met) to ACG (Thr) in codon 168 was detected, which was observed only in the metastatic lymph node, i.e., not in the primary tumor. Codon 168 is located in the second domain of four domains, which exhibit homology to the amino acid sequence of NCAM (16). The methionine coded by codon 168 is located between two prolines, which are highly conserved among homologous domains of DCC and NCAM of chicken and mouse. These prolines usually affect the structure of a protein molecule due to their ability to bend the primary structure of the protein. We also found the allelic loss of DCC in this metastatic lymph node. These results indicate the "two hit" inactivation of the DCC gene in this tumor, similar to that of other tumor suppressor genes such as p53, Rb, and APC reported previously (38-40).

In the second case, we detected three mutations. Two of them did not change the coding amino acid, whereas the remaining one caused an amino acid change from arginine to glycine in codon 201. This change was also found in the normal mucosa of the same patient. The
ALTERATIONS OF DCC IN ESOPHAGEAL CANCERS AND METASTASIS

A. DCC exon B (354bp, SSCP)

![Image of DCC exon B electrophoresis](image)

B. DCC-1 (150-210bp, VNTR)

![Image of DCC-1 electrophoresis](image)

C. DCC-2 (396bp, Msp I)

![Image of DCC-2 electrophoresis](image)

Fig. 2. A, LOH analysis of esophageal carcinomas. The denatured PCR products of DCC exon B were electrophoresed on a 10% polyacrylamide gel. Normal mucosa shows two different alleles. In case E9009, allelic deletions can be seen in both the primary tumor and the metastatic lymph node. B, PCR-LOH analysis using primer set 1. After PCR amplification, the products were electrophoresed on a 10% polyacrylamide gel and then stained with ethidium bromide. A spectrum of alleles ranging in size from 150 to 210 base pairs was generated. A decrease in the number of signals can be seen in case E9002, although LOH cannot be detected in case E9001. C, a 396-base pair product was digested with MspI and then electrophoresed. RFLP can be seen in case E9107 and LOH only in the metastatic lymph node (LN).

same alteration was detected previously. They reported polymorphisms in codon 201, i.e., CGA (Arg) and GGA (Gly). This change is also located in the second domain, but the sequence neighboring codon 201 is not so highly conserved. We could not determine the significance of this alteration from our results. In the present study, we only examined four exons of the DCC gene. Therefore, the frequency of point mutations may increase if other portions are investigated.

We detected LOH of DCC in 23% of the informative cases of esophageal squamous cell carcinomas. A previous paper reported that 24% of esophageal carcinomas (both squamous and adenocarcinomas) showed LOH of DCC. The authors did not find any correlation between the loss and clinicopathological parameters (7). In the present study, we used three markers to detect LOH. We found inconsistency in the allelic loss among the three markers. Since the precise location of DCC 1 and DCC 2 has not been published, we cannot draw a conclusion about the importance of the discrepancies.

There was no significant correlation between LOH of DCC and the clinicopathological backgrounds of the patients such as age, sex, and alcohol and tobacco use, except for the degree of differentiation and the degree of lymph node metastasis. Allelic deletion was only seen in the cases of moderately and poorly differentiated squamous cell carcinomas, i.e., not in those of well-differentiated ones. These results may suggest the direct involvement of LOH of DCC as to the degree of differentiation. There is a possibility that loss of DCC function accelerates the scattering of cancer cells and dedifferentiation. Further investigations involving in situ hybridization, immunohistochemistry, and LOH analysis of paraffin-embedded blocks are necessary to clarify the relation between DCC and the degree of differentiation.

Recently, a model of multistep carcinogenesis, especially in colorectal carcinomas, was proposed (31). Some reports showed that LOH of DCC is a relatively late event in colorectal and lung carcinomas (32, 33). As for esophageal carcinomas, the order of the multiple genetic changes has not been reported yet. Since we did not examine esophageal carcinomas at relatively early stages, we could not determine when the DCC change occurs. However, our results indicate that DCC alteration is associated with the metastatic potential and therefore may be a relatively late event in esophageal carcinogenesis.

Of 10 cases showing LOH of DCC, 9 had lymph node metastasis. We detected LOH only in the metastatic lymph node, i.e., not in the primary tumor, in 2 of the 9 cases which had allelic deletion in the metastatic lymph node. In addition, the degree of the metastasis was correlated with the frequency of the allelic deletion. Two of the 51 cases had distant organ metastasis at the time of operation and showed allelic loss of DCC. It was reported that allelic loss of DCC was related to the distant organ metastasis in colorectal and lung cancers (32, 33). Other tumor suppressor genes have not been reported to be associated with metastases in esophageal carcinomas (4–8). Therefore, our results suggest that inactivation of DCC due to allelic

---

Table 1 Correlation of allelic loss of DCC with clinicopathological data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LOH(+)</th>
<th>LOH(—)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>0 0 1</td>
<td>N0 N1 N2 N3 N4</td>
</tr>
<tr>
<td>Lymph node</td>
<td>N0 1 8</td>
<td>N1 0 2 N2 2 13 N3 2 8 N4 5 3</td>
</tr>
<tr>
<td>Histology</td>
<td>Well 11</td>
<td>Mod 10 Por 13</td>
</tr>
</tbody>
</table>

---

*According to the classification of the Japanese Society for Esophageal Diseases (41).

**Well, well differentiated squamous cell carcinoma; Mod, moderately differentiated squamous cell carcinoma; Por, poorly differentiated squamous cell carcinoma.

---

ALTERATIONS OF DCC IN ESOPHAGEAL CANCERS AND METASTASIS

deletion and/or point mutations may cause both lymphatic and hematogenous metastasis of esophageal squamous cell carcinomas.

ACKNOWLEDGMENTS

We thank Dr. K. Maruyama for technical support and the critical review of the manuscript.

REFERENCES

Point Mutations and Allelic Deletion of Tumor Suppressor Gene DCC in Human Esophageal Squamous Cell Carcinomas and Their Relation to Metastasis


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/11/3007

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.