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Original Paper

Alterations of the Tumour Suppressor Gene *DCC* in Neuroblastoma

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The deleted in colorectal carcinoma (*DCC*) gene, a candidate tumour suppressor, might be inactivated in a number of human cancers. In order to evaluate the possible role of *DCC* alterations in the pathogenesis of neuroblastoma, we examined 25 neuroblastoma cell lines and 16 primary tumours, including 6 samples with loss of heterozygosity (LOH) at the *DCC* locus for *DCC* mRNA expression, by using the reverse transcriptase–polymerase chain reaction (RT–PCR) technique. The level of *DCC* expression was significantly reduced or undetectable in 12 of 25 (48%) cell lines and 7 of 16 (44%) primary tumours, suggesting that inactivation of the *DCC* gene is involved in the development of neuroblastoma. Three of the 6 tumours with LOH at the *DCC* locus revealed reduced *DCC* mRNA expression, indicating that LOH at the *DCC* locus might have affected the levels of *DCC* mRNA. We also screened for mutations in 4 exons of the *DCC* gene in 12 cell lines by using PCR–single strand conformation polymorphism (PCR–SSCP) analysis. Point mutations were not found except a polymorphic change at codon 201. The mechanism for inactivation of the *DCC* gene will be further investigated. © **1997 Elsevier Science Ltd.**

Key words: tumour suppressor gene, *DCC* gene, neuroblastoma, reverse transcriptase–polymerase chain reaction (RT–PCR), PCR–single strand conformation polymorphism (PCR–SSCP)
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INTRODUCTION

INACTIVATION OF tumour suppressor genes has been shown to play an important role in the development of a variety of human cancers [1]. Recently, several major tumour suppressor genes such as retinoblastoma (*RB*), *p53*, Wilms' tumour (*WT*), neurofibromatosis type I (*NF1*), mutated in colorectal cancer (*MCC*), adenomatous polyposis coli (*APC*), *p16*/*MTS1* and the deleted in colorectal carcinoma (*DCC*) genes have been cloned and characterized [1–3].

The *DCC* gene, located on human chromosome band 18q21, was identified as a potential tumour suppressor gene in colorectal cancers by Fearon and associates in 1990 [3]. The 29 exons of the *DCC* gene span nearly 1.4 megabases and the open reading frame encompasses approximately 4.35 kb [3, 4]. The cDNA of the *DCC* gene predicts a 1447 amino acid transmembrane protein with four immunoglobulin-like and six fibronectin type III-like extracellular domains which show a high similarity to the neural cell adhesion molecule (NCAM) family of cell surface proteins [3, 5]. *DCC* transcripts and proteins are expressed highly in central and peripheral nervous system axons and most abundantly in brain tissues [3, 5, 6]. Two forms of DCC proteins with an M_r of approximately 170 000 and 180 000 have been demonstrated recently [5]. One form is mainly expressed in the normal colon, while the other is expressed mainly in the reticuloendothelial system [7].

The function of the *DCC* gene is still poorly understood, although some data suggest that *DCC* plays a role in regulating cell growth and differentiation, particularly in the nervous system [8, 9]. A recent study has shown that the full-length wild type, but not truncated *DCC*, inhibits tumorigenicity in

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human epithelial cells that have allelic loss and reduced expression of *DCC* [10]. This directly demonstrated that the *DCC* gene functions as a tumour suppressor [10]. Loss of heterozygosity (LOH) for 18q and/or alterations in *DCC* expression have been detected in colorectal carcinomas, brain tumours, cancers of the prostate, pancreas, lung, oesophagus, stomach, germ cells, female reproductive tract and bladder, lymphoma and leukaemia. The results indicate that *DCC* inactivation might contribute to the development of a variety of human cancers [3, 11–13].

Neuroblastoma is one of the most common childhood tumours with an incidence of approximately 1 in 6000 children under 5 years of age [14]. It originates from neural crest cells of the sympathetic nervous system. It is well known that *MYC*N amplification and abnormalities of the short arm of chromosome 1 are strongly correlated with advanced stages of neuroblastoma and poor prognosis [15, 16]. However, since low-stage tumours and more than half advanced stage cases do not have *MYC*N amplification and/or 1p abnormalities, other tumour related genes might be associated with the development and progression of neuroblastoma. Recently, we found that 18q LOH occurred in 31% of neuroblastomas [17]. This relatively high incidence suggests that *DCC* alterations may be involved in the development of neuroblastoma. Based on the hypothesis that inactivation of this gene may play an important role in the pathogenesis of neuroblastoma, we examined neuroblastoma cell lines and primary tumours for *DCC* mRNA expression by using a reverse transcriptase–polymerase chain reaction (RT–PCR) method, and screened for potential mutations in 4 exons of the *DCC* gene by PCR–single-strand conformation polymorphism (PCR–SSCP) analysis followed by sequencing.

MATERIALS AND METHODS

Cell lines and primary tumours

Twenty-five neuroblastoma cell lines were investigated: SCMC-N2 ~ SCMC-N6, NH12, TGW, NB-1, GoTo, TNB-9, IMR-32, NB-19, LAN5, SK-N-SH, CHP134, NB-16, NB-69, LAN-1, LAN-2 and SJNB-1 ~ SJNB-5, SJNB 8. All the cell lines were cultured in RPMI-1640 medium supplemented with 9% fetal bovine serum.

Primary tumour specimens were obtained from 16 patients with neuroblastoma. The majority of patients underwent initial surgical resection either at Saitama Children's Medical Centre or at The University of Tokyo Hospital. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until processed. Non-cancerous thymus gland and skeletal muscle tissues obtained from 2 patients and peripheral blood obtained from volunteers were used as normal controls. Of the 16 patients, 9 were classified as stage I, 4 as stage II, 2 as III and 1 as IV.

Total RNA isolation and DNA extraction

Total RNAs were prepared from above cell lines and tissues by the acid guanidine thiocyanate–phenol chloroform (AGTC) method [18]. Tissues were ground to powder in liquid nitrogen before RNA isolation. DNA from cell lines and tissues was extracted as previously described [19].

RT–PCR

Randomly primed cDNAs were reverse transcribed from total RNAs with a cDNA synthesis kit (Boehringer Mannheim, Corp., Germany) as previously described [18]. The cDNAs were amplified by PCR using *DCC*-specific oligonucleotide primers, DCC2 and DCC3, synthesised on the basis of the nucleotide sequence used by Fearon and associates [3]: DCC2, 5′-TTCCGCCATGGTTTTTAAATCA-3′ as a sense primer and DCC3, 5′-AGCCTCATTTTCAGCCACACA-3′ as an antisense primer. The β-actin specific oligonucleotides (5′-CTGTCTGGCGGCACCACCAT-3′ as a sense primer, 5′-GCAACTAAGTCATCATAGTCCGC-3′ as an antisense primer) provided quantitative control of the reaction. PCR for *DCC* amplification was performed on a DNA thermal cycler (Perkin-Elmer Cetus) by 40 cycles of amplification: denaturation at 94°C, annealing at 58°C, extension at 72°C for 1, 1 and 2 min of each cycle, respectively. PCR for β-actin amplification were run at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 30 cycles. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide and photographed under UV light. Then the products on the gel were transferred to a nylon membrane, followed by hybridisation with a ^{32}P-labelled oligonucleotide probe from cDNA of the *DCC* gene, and exposed to X-ray film for 1–2 h.

PCR–SSCP and direct sequencing

Four pairs of oligonucleotide primers were synthesised based on previous report [20] and used to amplify four exons of the *DCC* gene (exons 3, 6, 7 and 8). SSCP was performed as described previously with a little modification [19]. Briefly, PCR for genomic DNA amplification was carried out by 35 cycles of 1 min at 94°C for denaturation, 1–2 min at an appropriate temperature for annealing, and 1 min at 72°C for extension on the DNA thermal cycler. Following amplification, the PCR mixture was heated for 5 min at 80°C with 45 μl of formamide denaturing dye mixture (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), and then electrophoresed in a 5% non-denaturing polyacrylamide gel with and without 10% glycerol at 40 W for 2–3 h, with the temperature of the gels being kept at 26°C or 4°C. The gels were dried on a filter paper and exposed to X-ray film at –80°C overnight.

Direct sequencing was carried out by the dideoxy chain termination method [19]. Electrophoresis was performed on a polyacrylamide gel containing 7 mol/l of urea.

RESULTS

Expression of *DCC* mRNA was examined in 25 neuroblastoma cell lines and 16 primary tumours by RT-PCR analysis. Of the 16 primary tumours, 13 have been proved to be informative cases at the *DCC* locus in a previous study [17], and 6 of the 13 cases had LOH at the *DCC* locus. RT–PCR analysis showed that the expected *DCC* mRNAs of 233 bp were observed in all of the 8 normal peripheral blood, one thymus and one muscle control samples with little variability in intensity. Southern blotting analysis of RT–PCR products using an oligonucleotide probe between *DCC2* and *DCC3* demonstrated that the fragment of 233 bp was the only *DCC*-specific fragment. Of the 25 neuroblastoma cell lines, *DCC* transcripts were absent or significantly reduced in 12 (48%) cell lines (Figure 1). Of the 16 primary tumours, 7 (44%) showed reduced *DCC* expression (Table 1). Of the 6 tumours with LOH at the *DCC* locus, 3 had reduced expression of the *DCC* mRNA, but the other 3 showed normal expression. Four of 13 (31%) stage I and II tumours and 3 of 3 (100%)

Table 1. *Correlation of DCC mRNA expression with biological and clinical findings in primary neuroblastomas*

Findings	Total*	Stage†		*MYCN*‡		Survival§		LOH¶	
		I, II, IVs	III,IV	+	–	+	–	+	–
Reduced *DCC* expression	7/16 (44%)	4/13	3/3	1/1	6/15	6/15	1/1	3/6	1/7

*All 16 children were less than 1 year of age. †Stage of neuroblastoma. ‡+, *MYC*N was amplified; –, *MYC*N was not amplified. §+, alive; –, dead. ¶LOH, loss of heterozygosity.

stage III and IV tumours showed reduced expression of DCC mRNA, but this was not significantly different because of the small number of cases examined in this study (P=0.063, by Fisher's exact test). All patients were alive except 1 who died 8 months after diagnosis.

Of the 25 cell lines, 12 (4 with reduced *DCC* expression) were investigated for mutations in 4 exons of the *DCC* gene using PCR–SSCP analysis. Mobility shifts in exon 3 were found in five cell lines. Sequence analysis showed a CGA (Arg) to GGA (Gly) transition at codon 201 in exon 3 (Figure 2). There was no mobility shift in the other 3 exons.

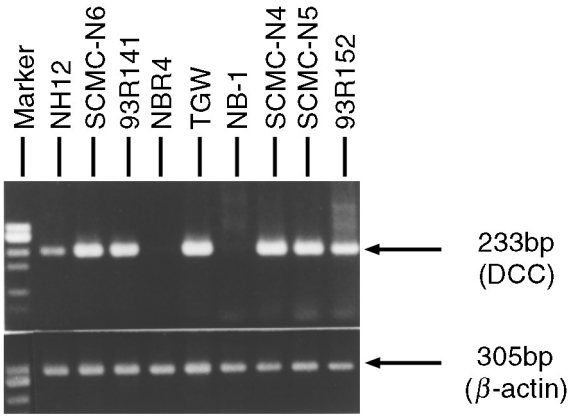

Figure 1. *DCC* mRNA expression in neuroblastoma cell lines and primary tumours. Marker, φX174 HaeIII; NH-12, SCMC-N6, TGW, NB-1, SCMC-N4 and SCMC-N5 were cell lines; 93R141, NBR4 and 93R152 were primary tumours. *DCC* transcripts were markedly reduced in NH12 and almost undetectable in NBR4 and NB1. The β-actin fragments were used as a quantitative control.

DISCUSSION

Recent studies have shown that the *DCC* tumour suppressor gene is involved in many human cancers by the evidence of frequent LOH, loss of expression or somatic mutations of the *DCC* gene [12]. The present study showed that loss or significantly reduced *DCC* expression was relatively frequent in neuroblastoma cell lines and primary tumours, suggesting that inactivation of the *DCC* gene may play an important role in the development of neuroblastoma. Although the frequency of reduced expression in stages III and IV (100%) was much higher than that in stage I and II (30%) patients, there was no statistical difference between them since the number of cases examined was small. Therefore, we cannot conclude that *DCC* inactivation is associated with progression of neuroblastoma and further investigation with more cases will be necessary to clarify this issue.

Inactivation of a tumour suppressor gene may occasionally be accomplished through protein–protein interactions, but suppressor genes in most human tumours usually appear to be inactivated by a point mutation of one allele and loss of the remaining allele. In an effort to determine if the LOH at the *DCC* locus is the mechanism of *DCC* inactivation in neuroblastoma, we examined 6 primary neuroblastomas with LOH and 7 without LOH for *DCC* mRNA expression. Reduced expression was found in 3 of the 6 tumours with LOH, which suggested that LOH at the *DCC* locus might have affected the levels of *DCC* mRNA. The other 3 samples with LOH did not show abnormal expression, which may result from contamination of non-cancerous cells in tumour specimens and some other factors [11]. Only reduction but no complete absence of *DCC* expression in the primary tumours may be caused by the contamination of adjacent non-cancerous cells in tumour specimens. One of the 7 samples without LOH

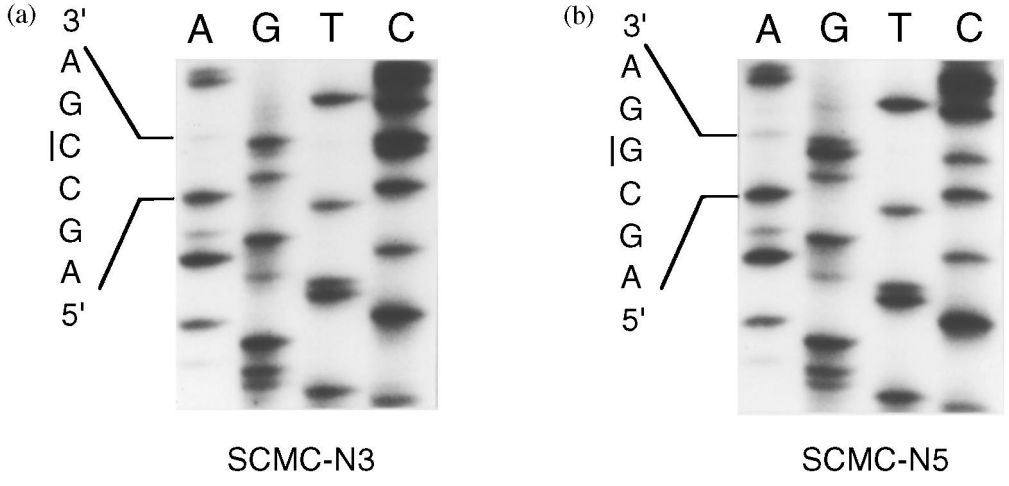

Figure 2. Genetic polymorphism in exon 3 of the *DCC* gene in neuroblastoma cell lines. DNA amplified products of cell lines SCMC-N3 and SCMC-N5 were sequenced; a transition from CGA (a) to GGA (b) was noted at codon 201.

was also found to have reduced *DCC* mRNA expression. It could be speculated that a point mutation, or an alteration in the initiator or promoter area of the *DCC* gene, may be responsible for the reduced *DCC* expression.

In order to clarify the assumption that nonsense, missense or frame shift mutations in the coding region of the *DCC* gene may contribute to altered expression of the *DCC* gene in neuroblastoma, we examined 4 exons for possible mutations. Mutation at codon 201 was observed in 5 of 12 cell lines. This mutation was considered to be a polymorphic change since it was also noted in normal mucosal DNA by Miyake and associates [20]. Of the 12 cell lines, 4 showed reduced or undetectable levels of *DCC* mRNA by RT–PCR analysis. Since we did not examine all the 29 exons of the *DCC* gene, we could not exclude that the altered expression in the 4 cell lines was caused by nonsense, missense or frameshift mutations. Mutations may be detected if the other 25 exons are investigated.

Recently, aberrant DNA methylation of the *CDKN2*/*p16*/*MTS1* tumour suppressor gene was noted to be associated with loss of transcription in various primary neoplasms, and this mode was considered to be the alternative mechanism of inactivation of the tumour suppressor gene in human cancers [21]. However, no apparent differences in the methylation of sequences of flanking *DCC* exon 1 between those brain tumours with abundant *DCC* expression and those with undetectable expression indicates that this is not the frequent pathway of *DCC* inactivation [11]. Abnormal splicing of *DCC* transcripts and allele-specific loss of transcripts might be one alternative important mechanism for altered *DCC* expression in tumours since transcripts from the aberrant splicing will result in a frame shift and premature truncation upon translation, producing a truncated protein [11]. It seemed that altered *DCC* expression in tumours results from multiple mechanisms in addition to LOH and mutations.

In conclusion, we have shown that altered *DCC* expression is relatively frequent in neuroblastoma, which suggests that inactivation of the *DCC* gene may play an important role in the pathogenesis of neuroblastoma. The mechanisms for *DCC* inactivation will be further investigated.

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