Codon 201^{Gly} Polymorphic Type of the *DCC* Gene is Related to Disseminated Neuroblastoma[1]

Xiao-Tang Kong*, Seung Hoon Choi[†], Fumio Bessho*, Miyuki Kobayashi*, Ryoji Hanada[‡], Keiko Yamamoto[‡] and Yasuhide Hayashi*

Department of *Pediatrics; [†]Pediatric Surgery, Graduate School of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; [‡]Division of Hematology/Oncology, Saitama Children's Medical Center, Iwatsuki, Saitama 339-8551, Japan

Abstract

The *deleted in colorectal carcinoma* (*DCC*) gene is a potential tumor-suppressor gene on chromosome 18q21.3. The relatively high frequency of loss of heterozygosity (LOH) and loss of expression of this gene in neuroblastoma, especially in the advanced stages, imply the possibility of involvement of the *DCC* gene in progression of neuroblastoma. However, only few typical mutations have been identified in this gene, indicating that other possible mechanisms for the inactivation of this gene may exist. A polymorphic change (Arg to Gly) at *DCC* codon 201 is related to advanced colorectal carcinoma and increases in the tumors with absent DCC protein expression. In order to understand whether this change is associated with the development or progression of neuroblastoma, we investigated codon 201 polymorphism of the *DCC* gene in 102 primary neuroblastomas by polymerase chain reaction single-strand conformation polymorphism. We found no missense or nonsense mutations, but a polymorphic change from CGA (Arg) to GGA (Gly) at codon 201 resulting in three types of polymorphism: codon 201^{Gly} type, codon 201$^{Arg/Gly}$ type, and codon 201^{Arg} type. The codon 201^{Gly} type occurred more frequently in disseminated (stages IV and IVs) neuroblastomas (72%) than in localized (stages I, II, and III) tumors (48%) (*P*=.035), and normal controls (38%) (*P*=.024). In addition, the codon 201^{Gly} type was significantly more common in tumors found clinically (65%) than in those found by mass screening (35%) (*P*=.002). The results suggested that the codon 201^{Gly} type of the *DCC* gene might be associated with a higher risk of disseminating neuroblastoma. *Neoplasia* (2001) **3**, 267–272.

Introduction

Inactivation of the tumor-suppressor gene has been shown to play an important role in the development of a variety of human cancers [1]. The *deleted in colorectal carcinoma* (*DCC*) gene, initially identified as a candidate tumor-suppressor gene on chromosome 18q21.3 in colorectal carcinoma [2], shows frequent allelic deletion and aberrant expression in many human tumors [3,4]. Alterations of the *DCC* gene are also associated with metastasis of various carcinomas [5–11]. The *DCC* gene encodes a neural cell adhesion molecule (NCAM)-like transmembrane protein. Its extracellular domain has four immunoglobulin (Ig)-like and six fibronectin type III-like domains, which have high homology to frazzled and UNC-40, the two transmembrane proteins of the Ig superfamily that are mainly expressed on motor neurons of the nervous system [12,13]. The cytoplasmic domain of the *DCC* gene shares similarity to neogenin. The expression of neogenin is dynamically regulated in the developing nervous system of chickens [14]. In addition, DCC has been identified as a receptor for netrin-1, a molecule involved in axon guidance and cell migration during development [15]. Thus, the *DCC* gene is a critical gene in regulating the growth and differentiation of the neural-origin cells.

Although there is no increase in the frequency of tumor formation in *DCC* hemizygous mice [16], reestablishment of the expression of *DCC* suppresses tumorigenicity [17,18]. Furthermore, DCC can induce apoptosis in cells outside the region of ligand availability (e.g., metastatic cells or locally invasive cells), suggesting that DCC may function as a tumor-suppressor and play a major role in suppressing invasion or metastasis of tumor cells [19].

Neuroblastoma is a malignant tumor of neural crest origin. It is very common in childhood neoplasm with an incidence of about 1 in 100,000 to 130,000 children under 15 years of age [20]. We have reported the relatively high frequency of loss of heterozygosity (LOH) (31%) at *DCC* locus on chromosome 18q21 in neuroblastoma [21]. Reduced or missing expression of the *DCC* gene at the mRNA and protein levels in advanced stages of neuroblastoma has been observed both by us and

by other groups [22–24], indicating that *DCC* is probably involved in the tumorigenesis of neuroblastoma. However, only a few mutations have been identified in this gene [23]. It is thus possible that mechanisms other than mutations are responsible for the inactivation of this gene. A polymorphic change (Arg to Gly) at *DCC* codon 201 was found to be related to advanced colorectal carcinomas and carcinomas lacking DCC protein expression [25,26]. In our previous study, this polymorphic change was observed in varieties of neuroblastoma cell lines and primary tumors [23]. Thus, we hypothesize that this polymorphic change might be associated with the development or progression of the neuroblastoma in the same way as it is in the colorectal carcinoma. In order to test this hypothesis, we investigated codon 201 polymorphism of the *DCC* gene in 102 primary tumors by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP), followed by sequencing.

Materials and Methods

Primary Tumor Specimens

One hundred and two tumor specimens were obtained from neuroblastoma patients. The samples were taken from the patients either at Saitama Children's Medical Center or at the University of Tokyo Hospital before chemotherapy. Tissue specimens were immediately frozen in liquid nitrogen after the surgical removal and stored at −80°C. Twelve noncancerous tissues including peripheral blood bone marrow were obtained from some of the above patients of stages I and II. The informed consent was obtained from the parents of the patients before the samples were taken. Twenty-one peripheral blood samples as normal controls for PCR-SSCP were obtained from healthy volunteers.

Patient Data

The stages of neuroblastoma were defined according to the classification of Evans et al. [27]. One hundred and two patients, aged from 18 days to 16 years with a median age of 12 months, were examined. These included 68 males and 34 females. Twenty-three were classified as stage I, 27 stage II, 16 stage III, 32 stage IV, and 4 stage IVs. Twenty-six patients including 13 stage I, 12 stage II, and 1 stage III were found by mass screening [28]. The status of the *N-myc* gene was examined in 59 patients including 12 patients in stage IV, five of whom had amplification. Patients in stages I and II were treated by either surgery alone or surgery plus chemotherapy that mainly consisted of vincristine and cyclophosphamide without radiotherapy. Patients in stages III and IV were administered with multidrug chemotherapy consisting of cyclophosphamide, doxorubicin, cisplatinum, and etoposide with or without surgery, radiotherapy, and hematopoietic stem cell transplantation [29].

PCR-SSCP Analysis of DCC Mutations

High-molecular-weight DNA was prepared from tumor tissues using the proteinase-K phenol–chloroform method

and PCR-SSCP was performed as previously described [23]. The oligonucleotide primers for amplifying *DCC* codon 201 span a 353-fragment and were synthesized as described by Miyake et al. [5]. PCR for genomic DNA amplification proceeded in a 5-ml mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl$_2$, 100 mM of each dNTP, 5 pmol of each primer, 2.5 ml of [α-^{32}P] dCTP (3000 Ci/mmol), and 0.25 units of Taq polymerase (Boehringer Mannheim, Mannheim, Germany). The PCR consisted of an initial denaturing step: 2 minutes at 94°C; 35 cycles of amplification: 1 minute at 94°C for denaturation, 1 minute at 56°C for annealing, 1 minute at 72°C for extension, and 7 minutes at 72°C for the final extension step. After amplification, PCR mixtures were heated for 5 minutes 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 resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel containing 45 mM Tris–borate (pH 8.3), 4 mM EDTA, and 1.6% Amp (ammonium peroxodisulfate) with and without 10% glycerol. Electrophoresis proceeded at 40 W for 2 to 3 hours at 26°C or 4°C. Gels were dried on filter paper and exposed to X-ray film at −80°C overnight.

Direct Sequencing of PCR-Amplified Fragments

Sequencing was performed as previously described [23]. Small gel slices containing the shift band detected by SSCP
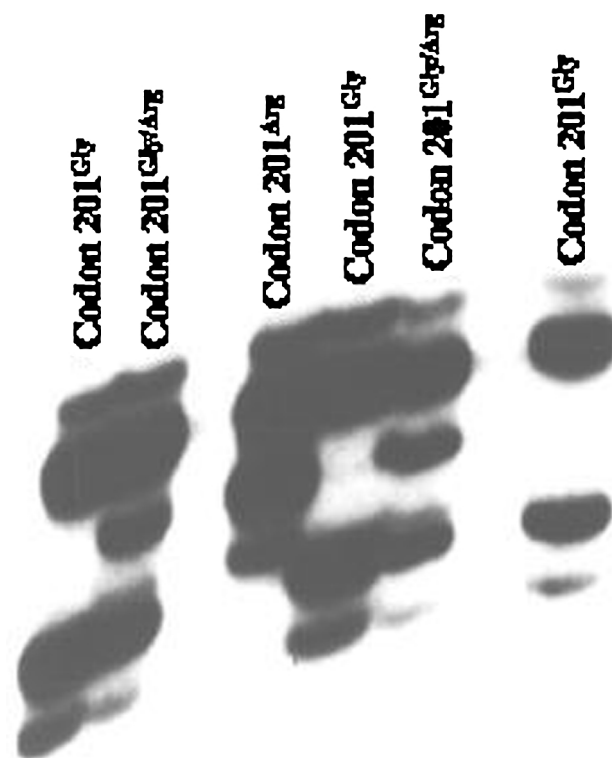

Figure 1. *Three types of codon 201 polymorphism within exon 3 of the DCC gene. Exon 3 was amplified from six primary tumors of neuroblastoma by PCR-SSCP and subjected to polyacrylamide gel electrophoresis. Three patterns of the bandshifts were noted on the electrophoresis gel and they are named as codon 201^{Arg} type, codon 201^{Gly} type, and codon 201$^{Arg/Gly}$ type according to the sequencing result.*
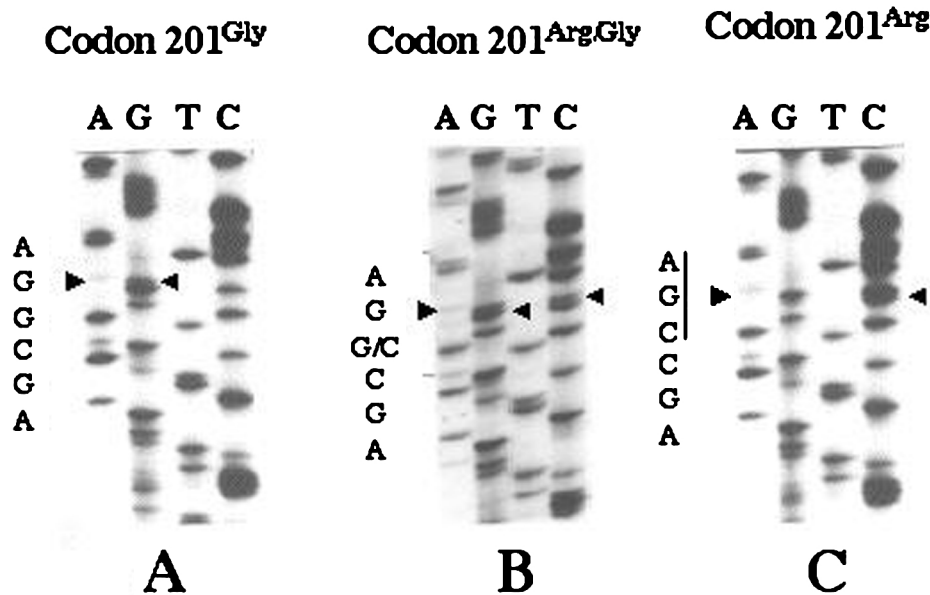
Figure 2. *Position and nature of the codon 201 polymorphism. Small gel slices from the top second bands shown in Figure 1 were excised and applied to sequencing after PCR amplification. Variation of sequences at codon 201 was observed: codon 201^Gly (A), a mutation of CGA (Arg) to GGA (Gly) at codon 201; codon 201^Arg/Gly (B), both GGA (Gly) and CGA (Arg) present at codon 201; codon 201^Arg (C), the wild type CGA (Arg) at codon 201. Arrow heads indicate the polymorphism nucleotides.*

were excised, immersed in 20 μL of water, and placed at room temperature overnight. The extracts were centrifuged and PCR-amplified as described above. PCR products were purified using a Microcon 100 (Amicon, Beverly, MA) and sequenced by the dideoxy chain termination method.

Results

Using PCR-SSCP methodology, we investigated exon 3 of the *DCC* gene for codon 201 polymorphism in the 102 primary tumors, 12 noncancerous tissues, and 21 normal peripheral blood samples. Three patterns of band distribution were noted on the SSCP electrophoresis gels (Figure 1). DNA sequencing revealed that the polymorphism resulted from a CGA (Arg) to GGA (Gly) transversion in codon 201 at one or both alleles of the *DCC* gene (Figure 2). In tumor tissues, the most frequent transversion type was codon 201^Gly (Figure 2A), which had one or two homozygous codon 201^Gly alleles. The less frequent type was codon

201^Arg/Gly type containing heterozygous codon 201^Arg allele and codon 201^Gly allele (Figure 2B). The least observed type was codon 201^Arg type, which consisted of one or two homozygous alleles of wild type codon 201^Arg (Figure 2C). In the 102 primary tumors, the frequencies of the codon 201^Gly, codon 201^Arg/Gly, and codon 201^Arg types were 57% (58/102), 37% (38/102), and 6% (6/102), respectively. The types of codon 201 polymorphism were significantly correlated with the stages of neuroblastoma ($P=.047$, by Kruskal-Wallis test). The frequency of codon 201^Gly type in stage IV+IVs tumors (26/36, 72%) was significantly higher than that in stage I+II+III tumors (32/66, 48%) ($P=.035$, by chi-square analysis) and that in normal controls (8/21, 38%) ($P=.024$, by chi-square analysis) (Table 1). Among the 26 tumors found by mass screening, 9 (35%) were codon 201^Gly type, 12 (46%) were codon 201^Arg/Gly, and 5 (19%) were codon 201^Arg. In the 76 tumors found clinically, the frequencies of the three types were 49 (65%), 26 (34%), and 1 (1%), respectively. Codon 201^Gly type of the *DCC*

Table 1. Correlation of Codon 201 Polymorphic Types of the *DCC* Gene with the Stages of Neuroblastoma.

Stages	Number of total samples	Number (%) of codon 201^Gly	Number (%) of codon 201^Arg/Gly	Number (%) of codon 201^Arg
I*	23	10 (43%)	11 (48%)	2 (9%)
II*	27	13 (48%)	11 (47%)	3 (11%)
III*	16	9 (56%)	6 (44%)	0
IV	32	23 (72%)	8 (25%)	1 (3%)
IVs	4	3 (75%)	1 (25%)	0
Total	102	58 (57%)	38 (37%)	6 (6%)
Normal control**	21	8 (38%)	10 (48%)	3 (14%)

The codon 201 polymorphic types of the *DCC* gene were significantly correlated with the stages of neuroblastoma ($P=.047$, by Kruskal-Wallis test). The frequency of codon 201^Gly type was significantly higher in stages IV+IVs (26/36, 72%) than in stages I, II, and III (32/66, 48%) (*$P=.035$, by chi-square analysis) and in normal control (8/21, 38%) (**$P=.024$, by chi-square analysis).

gene was significantly more frequent in the tumors found clinically (65%) than in those found by mass screening (35%) (P=.002, by chi-square analysis). Of the five tumors with *N-myc* amplification, codon 201^Gly type was detected in two, codon 201^Arg/Gly type in two, and codon 201^Arg type in one tumor, respectively. The corresponding noncancerous tissues from stages I and II patients showed the same codon 201 alleles with the tumor tissues.

Discussion

The *DCC* gene on chromosome 18q21 is considered to be one of the candidate tumor-suppressor genes in neuroblastoma because of the high frequency of LOH at *DCC* locus on chromosome 18 [21], the presence of a functional protein in primary tumors *versus* loss of expression in metastatic deposit, and the frequent reduced or loss of expression in advanced or disseminated tumors [22–24]. However, mechanisms for *DCC* inactivation are still unknown because only few mutations have been detected in neuroblastomas [23].

A polymorphic change from CGA (Arg) to GGA (Gly) at codon 201 of the *DCC* gene has been found to be associated with the progression of the colorectal carcinomas [25,26]. Minami et al. [25] demonstrated that codon 201^Gly type was significantly increased in invasive colorectal carcinomas than adenomas, and 9 of 10 invasive cancer cases with LOH had remained only codon 201^Gly allele. Another study by Schmitt et al. showed that 7 of 15 cases of the malignant colorectal tissues newly changed from codon 201^Arg/Gly to codon 201^Gly/Gly (both were codon 201^Gly alleles) compared with their corresponding normal mucosa. The results from these two studies indicate that change from codon 201^Arg/Gly to codon 201^Gly type (remaining one codon 201^Gly allele due to LOH [25] or two codon 201^Gly alleles due to mutation [26]) is related to the malignant transformation. Previously, we found that the codon 201^Gly type is frequently observed in neuroblastoma [23]; thus, we proposed that the polymorphic change from Arg to Gly at codon 201 of the *DCC* gene might be associated with neuroblastoma progression. Our results from the present study demonstrated that the codon 201^Gly is more prevalent in disseminated neuroblastoma (stages IV and IVs) than in localized tumors (stages I, II, and III). In addition, the frequency of this abnormality is significantly higher in the patients found clinically than in those found by mass screening. This may be explained by the fact that most of the patients found by mass screening have stage I or II tumors. The data from this study suggest that the codon 201^Gly type is likely related to a higher risk of dissemination of tumor cells. However, how codon 201^Gly type becomes more prevalent in disseminated neuroblastoma remains unknown. Twelve noncancerous tissue specimens from stages I and II neuroblastomas (nondisseminated tumors) had the same codon 201 polymorphism types as the tumor tissues in the present study, indicating that the variation at codon 201 of the *DCC* gene is an inherited polymorphism. Thus, the possible explanations for the high incidence of codon 201^Gly type in the disseminated neuroblastomas might be as

follows. One possibility is that the codon 201^Arg allele is preferentially lost in tumor cells due to LOH or mutation during progression of neuroblastoma similar to the scenario that happened in the advanced colorectal carcinoma [25,26]. The other possibility is that individuals with two codon 201^Gly alleles are more susceptible to disseminated neuroblastoma than those without the codon 201^Gly allele. We considered that the first mechanism is more possible in neuroblastoma based on the studies of others [25,26]; however, the second cannot be excluded until we compare the codon 201 polymorphism between tumors and the adjacent normal tissues from the patients with disseminated neuroblastomas in the future.

Not only unique somatic mutations in a gene could change the protein conformation and reduce or abolish the function of the gene, many functional polymorphisms intend to affect the expression of the protein and its activities [30–33]. The polymorphic change from CGA (Arg) to GGA (Gly) at codon 201 was also found to be associated with loss of the DCC protein in colorectal carcinomas [26]. Eight of nine colorectal tumors with both codon 201^Gly alleles had no detectable *DCC* expression. Importantly, seven of them acquired a new codon 201^Gly allele through mutation of Arg to Gly. Although we did not examine the DCC protein expression due to the limited amount of neuroblastoma samples, the polymorphic change from Arg to Gly at codon 201 resulting in codon 201^Gly type documented in our study seems to have a good agreement with the loss of protein expression in neuroblastoma reported by Reale et al. [22]. First, our finding that codon 201^Gly type was more prevalent in the disseminated tumors is consistent with Reale et al.'s result that loss of DCC protein expression was more frequently observed from patients with stage IV or IVs tumors. Second, stage IVs neuroblastomas occur in patients less than 1 year of age, and the majority of tumors spontaneously regress despite dissemination to distant sites [34]. Three of the four tumors from stage IVs patients showed the codon 201^Gly type, implying that this mutation is probably related only to the higher risk of tumor cell dissemination, regardless of their true malignant potential. This result is consistent with the finding that DCC protein expression was undetectable in most stage IVs tumors [22]. Third, our study found that five neuroblastomas with *N-myc* amplification did not show a prediction to any codon 201 polymorphic type, indicating that the polymorphic change at codon 201 might be involved in the dissemination, not through the pathway regulated by *N-myc*. This phenomenon also agrees with the finding that there is no consistent relationship between loss of DCC protein and *N-myc* amplification [22]. However, because only a small number of cases in the second and third scenarios were investigated, further studies involving larger patient populations are required. In any case, it will be very interesting in the future to investigate the relationship between loss of DCC protein expression and codon 201 genotypes in disseminated neuroblastoma.

The association of the polymorphic change from Arg to Gly at codon 201 with the tumor progression or dissem-

ination indicates that codon 201^{Gly} type protein might have a weaker function than codon 201^{Arg} type protein. Codon 201 is in the second of the four Ig-like domains of the *DCC* gene. This domain is in a highly conserved region that shares high similarity with adhesion molecule NCAM [2]. Developing neoplasia and metastasis is often associated with disruption of cell adhesion or cell-to-cell contacts [35,36]. The polymorphic change at codon 201 is from the amino acid Arg to Gly. The Arg is a basic, hydrophilic amino acid and has a big basic side chain, whereas Gly is a nonpolar amino acid with the least side chain volume. More importantly, occurrence of Gly residue would destabilize an α-helix structure in the second Ig-like domain because it has more conformational flexibility than other amino acids [37]. Taking together the changes in the side chain volume, hydropathy, and α-helix stability due to Gly substitution for Arg at codon 201 may alter the conformation of the DCC protein, resulting in the activity of the codon 201^{Gly} type DCC protein much weaker than that of the wild type codon 201^{Arg} DCC protein. This may diminish the tumor-suppressor function of DCC and contribute to the progression or dissemination of the tumors. Because codon 201$^{Arg/Gly}$ type is not shown to be related to the progression of the colorectal carcinomas [25,26] and the dissemination of neuroblastoma in this study, it is likely that the presence of one codon 201^{Arg} allele is enough to produce functional DCC protein. Alternatively, the codon 201^{Arg} allele is selectively transcribed. Loss of *DCC* expression and/or function activity may occur only when both of codon 201^{Arg} alleles are deleted, for example, in codon 201^{Gly} type tumors.

In summary, our study showed that the CGA (Arg) to GGA (Gly) substitution at codon 201 in both alleles is more frequent in disseminated neuroblastomas, suggesting that the polymorphism change from codon 201^{Arg} type to codon 201^{Gly} type might be involved in tumor dissemination. The high frequency of codon 201^{Gly} type of the *DCC* gene in disseminated neuroblastomas also suggests that this variation might be a helpful genetic marker for these tumors.

Acknowledgements

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