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Allelic imbalance on chromosome 18 in neuroblastoma

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Abstract

We previously demonstrated that chromosome 18 is frequently deleted in neuroblastoma. To further elucidate the role of chromosome 18 deletions in the development of neuroblastomas we examined 82 cases of neuroblastomas for allelic imbalance (AI) at 17 loci on chromosome 18 to define the common region of AI in neuroblastoma. AI at one or more loci on chromosome 18 was detected in 18/82 (22%) cases. AI on 18q was detected in 17/82 (21%) cases, whereas AI on 18p was detected in 4/82 (5%) cases. There was a distinct common region of AI at 18q21.1 between the *D18S363* and *D18S858* loci. In addition, cases 16 and 53, which did not show AI at 18q21.1, showed AI at 18pter-q12.3 between the *D18S52* and *D18S36* loci, indicating that another common region of AI may exist on chromosome 18. AI on chromosome 18 did not significantly correlate with any clinicopathological findings of patients with neuroblastoma. The common region of AI at 18q21.1 includes the *DCC* gene but not the *Smad2* and *Smad4* genes. However, our previous studies together with the present study indicated that the incidence of *DCC* mutation is much less than that of AI at 18q21.1 in neuroblastoma. These results indicate that novel tumour suppressor genes involved in the development of neuroblastoma are present at 18q21.1, and possibly at 18pter-q12.3.

Keywords: Allelic imbalance; Chromosome 18; Neuroblastoma; *DCC*

1. Introduction

Chromosomal deletion is a common genetic event in human cancer [1], and is generally accepted to indicate the existence of tumour suppressor genes within the deleted regions. High incidence of loss of heterozygosity (LOH) on chromosome 18 has been observed in a variety of human cancers, including colorectal cancer [2,3], lung cancer [4,5], head and neck squamous cell carcinoma [6], pancreatic cancer [7] and prostate cancer [8,9]. To date, three candidate tumour suppressor genes, *Smad2*, *Smad4* and *DCC*, have been identified from the regions of LOH on chromosome 18 [2,10,11]. The *Smad2* and *Smad4* genes share a similarity to the *Drosophila melanogaster* gene, *MAD*, which is known to reside in a pathway of transforming growth factor β signalling. *Smad4* alterations were observed in a significant portion of pancreatic cancers and in a subset of other types of cancers [12], whereas *Smad2* alterations were detected in only a few cases of colorectal and lung cancers [13,14]. The *DCC* gene encodes a transmembrane protein with significant homology to proteins of the neural cell adhesion molecule (N-CAM) family, which have four immunoglobulin-like and six fibronectin type III-like domains [15]. Recently, it was reported that DCC might function as a receptor for the axonal chemoattractant netrin-1 [15]. Furthermore, functional studies indicated that the *DCC* gene may play an important role in mediating cell differentiation in the nervous system [16,17] and in apoptotic processes [18]. However, mutations of the *DCC* gene occur very rarely in human cancers [19–21]. Regions of deletions in colorectal cancer and head and neck squamous cell carcinoma include the *DCC*, *Smad2* and *Smad4* genes, whilst those in pancreatic and prostate cancers include the *Smad4* gene but not the *Smad2* and *DCC* genes. In

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contrast, all three genes are excluded from the commonly deleted region in lung cancer [22]. Therefore, it is possible that a novel tumour suppressor gene inactivated in several types of human cancers is present on chromosome 18.

Previously, we reported that LOH on chromosome 18 frequently occurs in neuroblastoma [23]. Furthermore, we and others also reported that reduced DCC expression was significantly associated with advanced stage of the disease and metastatic spread [21,24,25]. However, genetic alterations of the *DCC* gene were detected in only a limited fraction of neuroblastomas [21]. Furthermore, no mutations or homozygous deletions of either the *Smad2* gene or the *Smad4* gene were detected in neuroblastomas [21]. Thus, it is possible that another tumour suppressor gene involved in the genesis and/or progression of neuroblastoma is present on chromosome 18. Therefore, to elucidate the role of chromosome 18 aberrations in neuroblastoma more critically, we determined common regions of allelic imbalance (AI) including LOH on chromosome 18 in 82 cases of neuroblastoma using 17 microsatellite markers and two polymorphic DNA markers.

2. Patients and methods

2.1. Primary tumours

Tumours were randomly obtained at surgery or autopsy from 82 patients with neuroblastoma who had been admitted to various institutions between May 1987 and July 1993 [26]. Corresponding normal tissues were available in all cases. The patients were staged according to the classification of staging in neuroblastoma [27]. Of the 82 cases, 21 were classified as being stage I, 27 as II, 9 as III, 17 as IV and 8 as IV-S. Patients with stage I, II or IV were treated with either surgery alone or surgery plus chemotherapy consisting of vincristine and cyclophosphamide with or without radiotherapy. Patients with stage III or IV were treated with multidrug chemotherapy consisting of cyclophosphamide, doxorubicin, cisplatin and etoposide with or without surgery and radiotherapy.

2.2. DNA isolation and southern blot analysis

High molecular weight DNA was isolated from tumours and normal tissues by proteinase K digestion and phenol/chloroform isoamylalcohol (24:1) extraction as previously described [28]. Approximately 10 μg of DNA were digested to completion with appropriate restriction enzymes, subjected to electrophoresis on 0.8% agarose gel for 12–24 h, transferred to nylon filters, and fixed by ultraviolet (UV) irradiation. The filters were hybridised under stringent conditions of 5×SSC (1×SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) and 50% formamide at 42°C with ^{32}P-labelled probes. After hybridisation, filters were washed twice in 0.1×SSC at 65°C and exposed to Kodak XAR-5 films at −70°C. The filters were hybridised repeatedly with two probes for the *DCC* locus, p16-65 and SMA1.1 [23].

2.3. PCR-AI analysis

Seventeen microsatellite markers used for PCR-AI analysis are listed in Table 1. Total reaction volumes were 10 μl containing 50–100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM of each deoxynucleotide triphosphate, 0.01% gelatine, 125 ng of each primer, 1.14 μCi of [α-^{32}P] dCTP and 1 unit of Taq DNA polymerase (Pharmacia, Tokyo, Japan). PCR was performed for 35 cycles consisting of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 90 s in a Gene Amp PCR system 9600 (Perkin-Elmer Cetus, NJ, USA) as previously described [26]. One-tenth of the PCR product was loaded onto 6% urea-formamide-polyacrylamide gels and fragments separated by electrophoresis. The gels were dried and exposed to Kodak XAR film for 12–48 h at −80°C.

2.4. Definition of allelic imbalance

The signal intensity of the polymorphic alleles was quantified and calculated by the scanning densitometer and data analysis system (The Discovery Series™, Quantity One, *pdi*, New York, USA). AI was considered to be present if the rates of the signal intensities

Table 1
Incidence of allelic imbalance at loci on chromosome 18 in neuroblastoma

Marker	Chromosomal location	Allelic imbalance/ informative cases (%)[a]
D18S52	18pter-p11.2	1/41 (2)
D18S62	18pter-p11.2	1/37 (3)
D18S542	18pter-qter	1/39 (3)
D18S45	18q11.2-q11.1	1/43 (2)
D18S877	18q12.1-q12.3	2/43 (5)
D18S36	18q12.2-q12.3	2/39 (5)
D18S535	18q12.2-q12.3	3/46 (7)
D18S454	18q12.2-q21	3/41 (7)
D18S474	18q12.2-q21.3	3/37 (8)
D18S46	18q12.2-q21.3	3/39 (8)
D18S363	18q12-q21	2/37 (5)
DCC	18q21.1-q21.2	12/57 (21)
D18S858	18q21.2	7/44 (16)
D18S38	18q21.1-q21.31	1/47 (2)
D18S64	18q21.1-q21.32	1/41 (2)
D18S42	18q22.1	1/34 (3)
D18S58	18q22.3-q23	1/39 (3)

[a] Some cases show allelic imbalance at more than one chromosomal location.

in the tumours were reduced by more than 40% as previously described [26].

2.5. Statistical analysis

Significance of the differences in various biological and clinical features of the disease amongst the patient group was examined by Fisher's exact test. The vital status of the patients was observed until 31 December 1997. All patients were followed up for at least 10 months and up to 112 months. The survival curves for each group of the patients were estimated by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test.

3. Results

3.1. Frequency and common regions of AI on chromosome 18 in neuroblastoma

Eighty-two cases of neuroblastoma were examined for AI on chromosome 18 using 17 microsatellite polymorphic markers and two polymorphic DNA markers. The incidence of AI at each locus is summarised in Table 1. All cases showed heterozygous genotypes in their normal tissues at one or more loci on chromosome 18, and AI at one or more loci was detected in 18 of 82 cases (22%). AI on chromosome 18q was detected in 17 of 82 cases (21%), whilst AI on chromosome 18p was detected in 4 of 82 cases (5%). AI on chromosome 18 was schematically summarised in Fig. 1. In cases 15, 17, 26, 29 and 61, AI was detected at the *DCC* locus, whilst heterozygosity was retained at the *D18S363* locus. In cases 3, 15 and 26, AI was detected at the *DCC* locus, but heterozygosity was retained at the *D18S858* locus. The result from these six cases indicates the presence of a distinct common region of AI between the *D18S363* and *D18S858* loci on chromosome 18q21.1. Case 16 showed AI at the *D18S877* and *D18S36* loci and retention of heterozygosity at *D18S52* and all informative loci distal to the *D18S454* locus. Case 53 showed AI at the *D18S52* and *D18S542* loci and retention of heterozygosity at all informative loci distal to the *D18S36* locus. Therefore, it is possible that there is another common region of AI on 18pter-q12.3 between the *D18S52* and *D18S36* loci. Representative autoradiograms of cases 15, 16, 26 and 53 are shown in Fig. 2.

Cytogenetic data was obtained in 38 cases (46%). Thus, a preliminary review of the relationship between the modal chromosomal number and AI on chromosome 18 was carried out for these cases. Amongst 14 early stage patients with AI on chromosome 18, cytogenetic data were available for 10 patients, and 3 of them were scored as having trisomy of chromosome 18. Furthermore, only 1 of 4 advanced stage patients was scored as having both AI and trisomy of chromosome 18. Thus, AI observed in the tumours was not linked to trisomy of chromosome 18 (data not shown).

3.2. Relationship between AI on chromosome 18 and clinicopathological findings in neuroblastoma

Since age, stage, and *N-myc* amplification are known to be associated with the prognosis of patients with neuroblastoma, the relationship between AI on chromosome

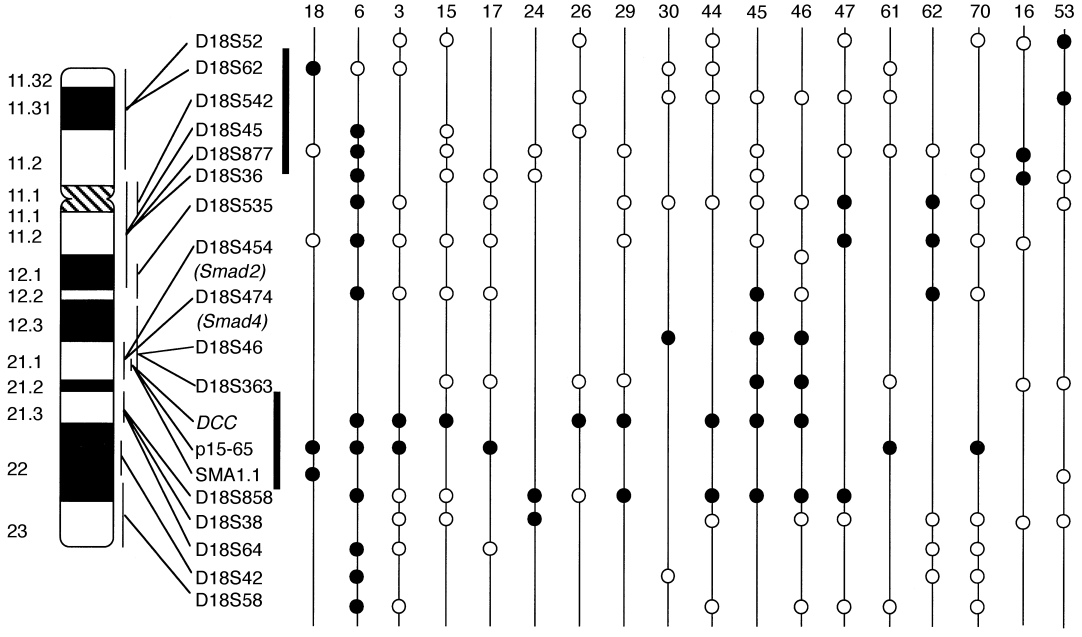

Fig. 1. Schematic representation of allelic imbalance (AI) on chromosome 18 in neuroblastoma. The approximate locations of markers used are shown on the left and tumour numbers are shown above. ●, AI; ○, heterozygosity retained; and, no symbol, not informative.

18 and these clinicopathological findings was examined. In our 82 neuroblastoma patients, 74 patients were infants under 1-year of age at diagnosis and 8 patients were over 1-year old. There were 56 patients with stage I + II + IV-S, 9 patients with stage III, and 17 patients with stage IV. N-myc amplification was detected in 11 of the 82 cases. Since 62 of the 82 cases (76%) were found by a mass screening programme, the ratio of infantile and early stage patients in this study was higher than those in previous studies [29–32]. Therefore, the population of our cases is extremely biased for low stage disease. However, age, stage and genotype of N-myc were significantly associated with survival of these patients ($P < 0.001$). Thus, the clinical outcome of patients in this study was considered to be similar to that of patients in previous studies. There was no statistically significant relationship between AI on chromosome 18 nor AI on chromosome 18q21.1 and any of these parameters. AI on chromosome 18 was also not associated with survival of these patients (Table 2).

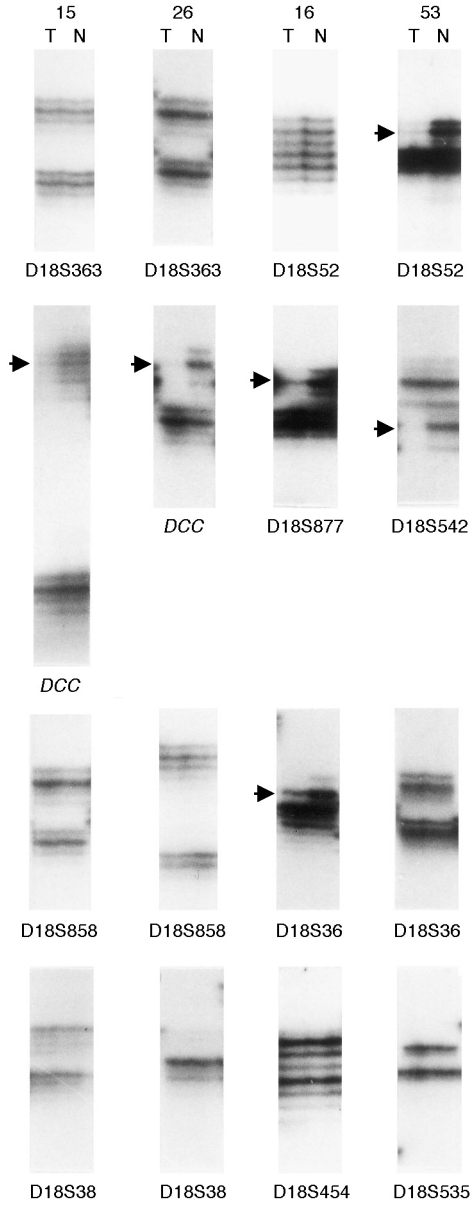

Fig. 2. Representative results of allelic imbalance (AI) on chromosome 18 in neuroblastoma determined by PCR of microsatellite loci. DNA was isolated from tumours (T) and corresponding normal tissues (N) of patients 15 (lane 1), 26 (lane 2), 16 (lane 3) and 53 (lane 4). Allelic fragments that showed AI are indicated by arrowheads.

3.3. Correlation of AI on chromosome 18q21.1 with the expression and mutation of the DCC gene in neuroblastoma

We previously reported that *DCC* expression was reduced in 14 of 32 (44%) primary neuroblastomas [21]. Of the 82 cases, 13 (16%) were analysed for *DCC* expression in the previous study. Therefore, to address whether AI is a mechanism for inactivation of the *DCC* gene in neuroblastoma, we analysed the relationship between AI at chromosome 18q21.1 and expression of the *DCC* gene in these 13 cases. Amongst 6 cases with AI at 18q21.1, mRNA expression of the *DCC* gene was reduced in 3 cases and not reduced in the remaining 3 cases. In contrast, only 1 out of 7 patients without AI at 18q21.1 had reduced *DCC* expression. However, the correlation between AI at 18q21.1 and *DCC* expression was not statistically significant. Since it was considered that this contradiction might be due to the small number of patients analysed, we further examined for DCC expression in 41 of the 82 cases, including 11 cases with

Table 2
Correlation of allelic imbalance on chromosome 18 with biological and clinical variables in neuroblastoma

Variable	Allelic imbalance	
	18	18q21.1
Age		
< 1 y	15/74 (20%)	13/74 (18%)
⩾ 1 y	3/8 (38%) (0.451)[a]	3/8 (38%) (0.521)
Stage[a]		
I,II,IVS	14/56 (25%)	12/56 (21%)
III	0/9	0/9
IV	4/17 (24%) (0.894)	4/17 (24%) (0.689)
Result of screening		
+	16/62 (26%)	14/62 (23%)
–	2/20 (10%) (0.195)	2/20 (10%) (0.201)
N-myc amplification		
+	2/11 (18%)	1/11 (9%)
–	16/71 (23%) (0.341)	15/71 (21%) (0.338)
Survival[b]	0.685	0.551

[a] *P*-value, Fisher's exact test.
[b] *P*-value, log rank test.
$P < 0.05$ was considered significant.

AI and 30 cases without AI at 18q21.1, by immunohistochemical analysis. However, there was no significant correlation between DCC expression and AI at chromosome 18q21.1 (data not shown). Although 18 of 82 cases (22%), including three cases with AI at 18q21.1, were analysed for *DCC* mutations in our previous study, no mutations were detected in these 18 cases.

4. Discussion

We demonstrated here the presence of two common regions of AI on chromosome 18 in neuroblastoma. A distinct common region of AI was detected at 18q21.1 between the *D18S363* and *D18S858* loci. In addition, it was suggested that there might be another common region of AI at 18pter-q12.3 between the *D18S52* and *D18S36* loci. The *DCC* gene has been mapped to chromosome 18q21.1 between the *D18S363* and *D18S858* loci, whereas the *Smad2* and *Smad4* genes have been mapped proximal to the *D18S363* locus. Thus, the *DCC* gene is located inside the common region of AI, whereas both the *Smad2* and *Smad4* genes were excluded from the common region of AI. We previously reported that reduced or absent mRNA expression of the *DCC* gene was frequent and that of the *Smad2* and *Smad4* genes was infrequent in neuroblastoma [21]. It was also shown that the status of DCC expression in primary neuroblastoma, assessed by immunohistochemical analysis, was associated with neuroblastoma dissemination [24,25]. These results suggested that the *DCC* gene, but not the *Smad2* and *Smad4* genes, is inactivated during the progression of neuroblastoma. However, in this study, there was no significant correlation between AI at chromosome 18q21.1 and DCC expression in neuroblastoma. Moreover, we also reported that mutations of the *DCC* gene occur infrequently in neuroblastoma [21]. Thus, although reduced DCC expression occurs in a subset of neuroblastoma, it is unlikely that the *DCC* gene is a target tumour suppressor gene inactivated by mutation and/or deletion at 18q21.1 in neuroblastoma. It is highly possible that another tumour suppressor gene involved in the development of neuroblastoma is present at 18q21.1. More detailed analysis will be required to identify a target tumour suppressor gene on chromosome 18 in neuroblastoma.

The present results also suggests that there is another common region of AI on chromosome 18pter-q12.3. Previously, it was reported that chromosome 18q11.1-q12.3 is deleted in a subset of head and neck squamous cell carcinomas without LOH on 18q21.1 [6]. In addition, frequent LOH on chromosome 18p was observed in ovarian adenocarcinomas without deletion of 18q21 region [33]. Taken together, it is possible that there is a tumour suppressor gene on 18pter-q12.3, which is inactivated in several types of human cancers.

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