

Detailed deletion mapping of chromosome band 14q32 in human neuroblastoma defines a 1.1-Mb region of common allelic loss

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Summary Neuroblastoma (NB) is a well-known malignant disease in infants, but its molecular mechanisms have not yet been fully elucidated. To investigate the genetic contribution of abnormalities on the long arm of chromosome 14 (14q) in NB, we analysed loss of heterozygosity (LOH) in 54 primary NB samples using 12 microsatellite markers on 14q32. Seventeen (31%) of 54 tumours showed LOH at one or more of the markers analysed, and the smallest common region of allelic loss was identified between D14S62 and D14S987. This region was estimated to be 1-cM long from the linkage map. Fluorescence in situ hybridization also confirmed the loss. There was no statistical correlation between LOH and any clinicopathologic features, including age, stage, amplification of *MYCN* and ploidy. We further constructed a contig spanning the lost region using bacterial artificial chromosome and estimated this region to be approximately 1.1-Mb by pulsed-field gel electrophoresis. Our results will contribute to cloning and characterizing the putative tumour-associated gene(s) in 14q32 in NB. © 2000 Cancer Research Campaign

Neuroblastoma (NB) is one of the most common malignant diseases of childhood. The most frequent genetic abnormality reported to date is the deletion of the short arm of chromosome 1 (Brodeur et al, 1981; Gilbert et al, 1984). Recent investigations have reported that the frequency of loss of heterozygosity (LOH) in 1p ranged from 20 to 89% (Fong et al, 1989, 1992; Suzuki et al, 1989; Weith et al, 1989; Takayama et al, 1992; Caron et al, 1993, 1996; Schleiermacher et al, 1994; Takeda et al, 1994; Martinsson et al, 1995), and two or more tumour suppressor genes have been thought to be located in chromosome 1p (Schleiermacher et al, 1994; Takeda et al, 1994; Caron et al, 1995; Cheng et al, 1995). However, the genes on chromosome 1 responsible for NB have not yet been reported. On the other hand, allelic loss in the long arm of chromosome 14 (Suzuki et al, 1989; Fong et al, 1992; Takayama et al, 1992; Srivatsan et al, 1993; Caron et al, 1996) has also been frequently reported, and these investigations have suggested that a tumour suppressor gene(s) might be located in chromosome bands 14q32-qter (Suzuki et al, 1989; Fong et al, 1992; Takayama et al, 1992). Furthermore, in chromosome arm 14q, commonly deleted regions have also been reported in several other malignancies, including renal cell carcinoma (Kovacs and Frisch, 1989), colorectal carcinoma (Sasaki et al, 1989; Young et al, 1993) and ovarian carcinoma (Bandera et al, 1997). These deleted regions are likely to overlap with that in NB; there may be some common pathways or genes associated with the tumorigenesis of these tumours. As the first step towards the isolation and characterization of the

tumour-associated gene responsible for initiation and/or progression of NB, we analysed 54 paired samples of tumour and normal tissues from patients with NB for LOH analyses at 12 microsatellite loci. Herein we report the localization of a putative tumour suppressor gene to a 1.1-Mb region on 14q32.

MATERIALS AND METHODS

Patients with NB analysed

The Ethical Committee of Tohoku University School of Medicine approved all protocols and samples used in the present study. A total of 54 patients with NB were analysed. Mass screening was performed for 52 of 54 patients, and 36 (69%) were positive. Clinical characteristics of the tumours are summarized in Table 1. The ages of the patients at the time of operation ranged from 6 months to 8 years. They were staged according to Evans' classification (Evans et al, 1971). Primary sites of NB were as follows: 37 from adrenal gland, 12 from retro-peritoneum and five from mediastinum. Six patients had been treated with chemotherapy before operation. None had been treated with radiotherapy.

Tissue samples

Tumour and corresponding normal tissues were obtained from NB patients treated at the Tohoku University Hospital (Sendai, Japan), Saitama Cancer Center Hospital (Saitama, Japan), and their related institutions within the period from September 1988 to June 1995. Tumour samples were frozen in liquid nitrogen immediately after resection and stored at -80°C until use. In each case, corresponding normal tissue was obtained from one of the following: peripheral

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Table 1 Summary of samples analysed

Stage	Number of patients ^a			MYCN		Chromosome ploidy		
	Younger ^f	Older ^g	Total	-	+	^b AP	^c DP	^d ND
I	9 (2)	1 (0)	10 (2)	9	0	8	1	1
II	14 (4)	4 (4)	18 (8)	18	0	12	2	4
III	5 (1)	4 (0)	9 (1)	9	0	6	0	3
IV	2 (0)	13 (5)	15 (5)	7	6	5	3	7
IVs	2 (1)	0 (0)	2 (1)	2	0	2	0	0
Total	32 (8)	22 (9)	54 (17)	45	6	33	6	15

^aNumber of patients who showed loss of heterozygosity on chromosome arm 14q are indicated in brackets. ^bAP: aneuploid, ^cDP: diploid, ^dND: unable to examine. ^eAmplification of MYCN was not determined in three patients. ^fYounger: younger than 12 months of age at operation.

^gOlder: older than 12 months of age at operation.

Table 2 Nucleotide sequences of the primers for each microsatellite marker

Locus symbol	Annealing temperature	Nucleotide sequence	
		CA strand	GT strand
D14S990	55°C	AGCCACATGCATCCTCTGTC	GAATAAAGTTGCACTGTGACTG
D14S63	55°C	CTCTAACACTCACCATGTTTCAT	CTGCCAGAGAGCCACACTG
D14S81	55°C	ACTTATCCTAAAATGAACTNCA	CAGAGCAGGACCCTTTCTCA
D14S265	55°C	GTTTTTGGCTATTATGAATATGG	CATACATATGCATGCACTCTG
D14S62	55°C	GAGGCTTCAGCCTTGCTGT	TCTGTGTGTGATGTATCTGCT
D14S987	55°C	CTTGAGGCCAGGAGTTTGAG	AGCTGAATAATTTAATTCAATTGT
D14S979	55°C	TGTACCACCACCTCCTTATAC	GATGCTCAATGAACAGCCTGA
D14S65	55°C	CCCTAAAGATCCCTGCCATC	CTGTTCAAATCAGGACCA
D14S998	55°C	CATCCAAGGGAAAATGAGAG	TTCTCAGATAAATGACAGCGT
D14S267	55°C	GTTCTTTAAGAGCCAAACATAC	GGATTATTTTNCAGGTTTCGTA
D14S250	55°C	TGATGCAGCAATCACTGGAC	ACCCCTGCATTGTTTGAG
D14S260	55°C	TTTAAAAATGTAAAAGTGTATTCC	AAAACGGTCACTATGTTTTCCA

Markers are ordered from the centromeric end to the telomeric one on chromosome band 14q32.

white blood cells, non-cancerous tissue obtained mainly by biopsy of the liver, or non-metastatic lymph node. Some of the samples were obtained from formalin-fixed and paraffin-embedded tissues.

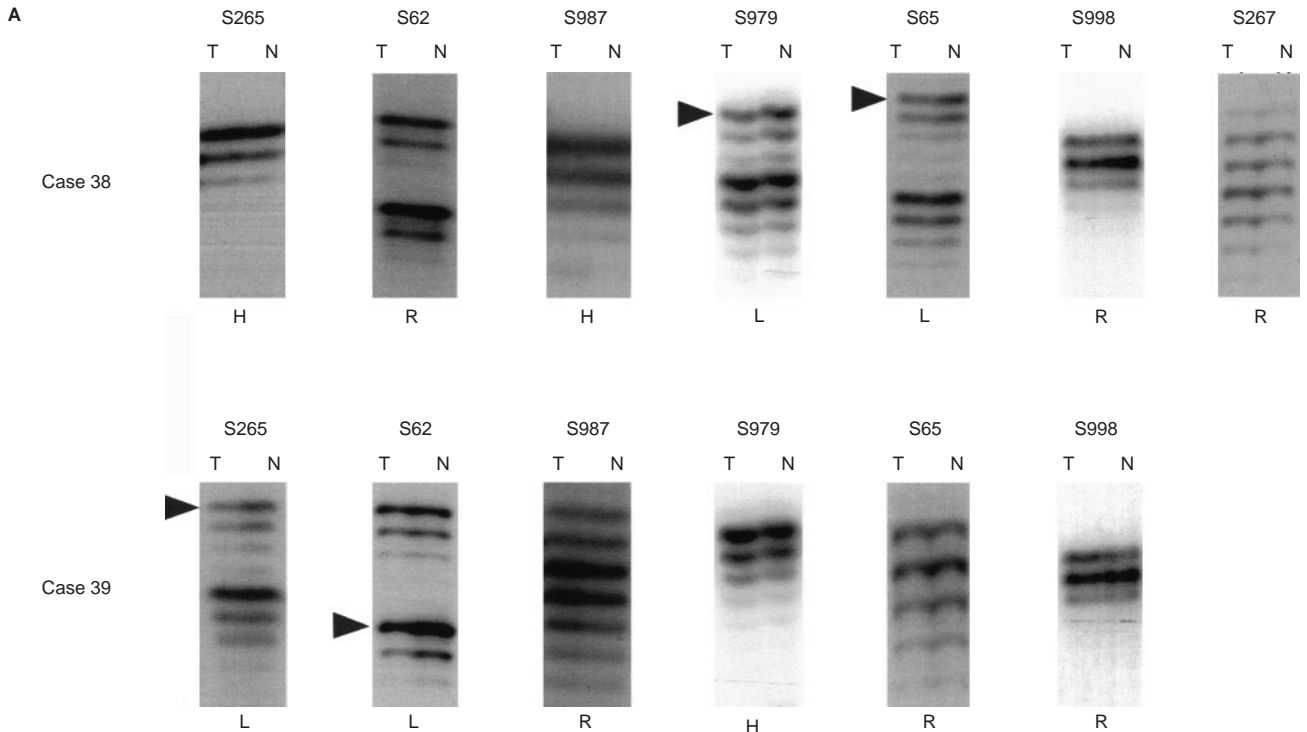
Analysis of LOH

To obtain the genomic DNAs for microsatellite analysis, we performed micro dissection; tissue samples embedded in paraffin blocks were cut into 50-µm-thick slices and deparaffinized with xylene. The genomic DNAs were extracted as previously described (Kimura et al, 1996). DNAs from fresh frozen tumour tissues and peripheral blood cells were also extracted as previously described (Sambrook et al, 1989; Sato et al, 1990). Based on the Génethon human linkage map (Dib et al, 1996), nucleotide sequences of primer sets for microsatellite markers on chromosome band 14q32 were designed to amplify less than or around 100-bp so that we could perform polymerase chain reaction (PCR) amplification from the DNA prepared from paraffin sections. Nucleotide sequences and annealing temperatures of these primers are summarized in Table 2. In each primer set, the primer corresponding to the DNA strand harbouring the CA dinucleotide repeat was labelled with [γ -³²P] ATP, and PCR amplification was performed as described previously (Kimura et al, 1996). We performed electrophoreses of the PCR products in 6% polyacrylamide/8 M urea/32% formamide denatured gels, obtained autoradiograms, and analysed them densitometrically. When a sample

exhibited some heterozygosity, allelic imbalance, probably loss, was assigned if the intensity of one of the bands in the tumour sample showed more than a 50% reduction from that of the corresponding normal tissue. At least two independent experiments as well as experiments with the labelled antisense primer (corresponding to the strand for the GT dinucleotide repeat) were performed to confirm our results. Fluorescence in situ hybridization (FISH) analysis was also performed in the critical cases to confirm our microsatellite analysis (see below).

Isolation of the genomic clones harbouring the commonly deleted region

A yeast artificial chromosome (YAC) clone y964D10 harbouring microsatellite marker D14S62 that had been isolated by PCR-based screening was purchased from Research Genetics (Huntsville, AL, USA). A cosmid library was constructed using the total yeast DNA of this YAC clone by methods described previously (Ariyama et al, 1995), and a clone named c14-16 harbouring D14S62 was isolated by colony hybridization. This clone was used for FISH analysis (see below). BAC clones were obtained by screening two total human BAC libraries; one was constructed by Asakawa et al (1997), and the other was purchased from Research Genetics (Huntsville, AL, USA). From the latter, we prepared a PCR-based BAC screening system and isolated BAC clones. The BAC DNAs were extracted by automated DNA



B

Case No.	3	49	5	6	9	10	12	14	26	69	17	20	22	38	10	54	35	LOH
	Stage I			II				III		IV				IVs				
11.1	D14S990	● ●	○ H	● H	● H	○ ○	-	○ ●	○ ●	H	●	●	●	●	H	●	●	7/34 (21%)
11.2	D14S63	H H	H H	● ●	● ●	● ●	H H	H	○ ●	○ ●	-	H	●	○	H	●	●	6/27 (22%)
12	D14S81	● ●	● ●	● ●	● ●	● ●	● ●	● ●	H	● ●	○ ●	● ●	○ ●	H	●	●	●	14/45 (31%)
13	D14S265	● H	● H	H	● ●	● ●	H ●	● ●	● ●	● ●	● ●	● ●	H ●	● ●	● ●	● ●	● ●	12/28 (42%)
21	D14S62	H ●	● H	H	● ●	H ●	● ●	● ●	● ●	● ●	● ●	H ●	○ ●	H ●	● ●	● ●	H ●	8/31 (26%)
22	D14S987	● ●	● ●	● ●	H ●	H ●	● ●	● ●	○ ●	H ●	● ●	● ●	H ●	- ●	● ●	● ●	● ●	8/27 (30%)
23	D14S979	● H	● ●	● ●	● ●	● ●	H ●	● ●	● ●	● ●	● ●	● ●	H ●	● ●	● ●	● ●	● ●	12/36 (33%)
24	D14S65	● H	● ●	● ●	H ●	● ●	● ●	○ ●	H ●	● ●	● ●	● ●	● ●	● ●	● ●	● ●	● ●	12/44 (27%)
31	D14S998	● ●	● ●	● ●	H ●	H ●	H ●	● ●	○ ●	● ●	● ●	H ●	H ●	○ ●	- ●	● ●	● ●	9/28 (32%)
32	D14S267	H H	H H	H H	H H	● ●	● ●	H ●	- ●	H ●	● ●	H ●	H ●	○ ●	● ●	● ●	● ●	5/23 (22%)
	D14S250	● ●	● ●	● ●	H ●	● ●	H ●	● ●	○ ●	● ●	● ●	● ●	○ ●	● ●	● ●	● ●	H ●	11/38 (29%)
	D14S260	● H	● ●	● ●	● ●	● ●	● ●	● ●	○ ●	H ●	● ●	● ●	H ●	○ ●	● ●	● ●	● ●	12/42 (29%)
	Origin of tumour	A	A	R	M	A	R	A	A	A	R	A	A	A	R	R	A	

Figure 1 (A) LOH study employing microsatellite markers on chromosome band 14q32. Intensities of bands of DNA from tumours indicated by arrows are significantly reduced when compared with those of corresponding normal tissues. Cut-off value of allelic loss applied in this study was more than 50% reduction. In case 38, the intensities of the upper bands indicated by arrows were reduced by 65% and 60% at D14S979 and D14S65 respectively. In case 69, the intensities of the bands indicated by arrows were reduced by 58% and 56% from the corresponding normal bands at D14S265 and D14S62 respectively. T, tumour samples; N, normal tissue samples; H, homozygous; L, loss of heterozygosity; R, retention. (B) Summary of 17 neuroblastoma patients who showed allelic losses on chromosome band 14q32. A closed bar on the right side indicates the commonly deleted region. Open circle, retained heterozygosity; closed circle, loss of heterozygosity; H, constitutionally homozygous; -, not determined. Origins of the tumour are indicated at the bottom; A, adrenal gland; R, retroperitoneum; M, mediastinum

Table 3 Summary of cases showing 14q LOH

Case no.	Age	Stage	N-myc gene amplification	Ploidy	Mass screening
3	10 months	I	ND	AP	MS
5	8 months	II	-	AP	MS
6	1 year 1 month	II	-	AP	MS
9	1 year 10 months	II	-	AP	MS
10	2 years	II	-	AP	FL
12	8 months	II	-	AP	MS
14	6 months	II	-	ND	MS
17	1 year	III	-	AP	FL
20	4 year	IVA	ND	ND	FL
22	1 year 1 month	IVA	ND	AP	FL
26	1 year 11 months	II	-	ND	MS
35	6 months	IVs	-	AP	MS
38	3 years	IVA	-	DP	FL
40	6 years	IVA	+	ND	FL
49	8 months	I	-	AP	MS
54	1 year 5 months	IVB	-	AP	FL
69	8 months	II	-	AP	MS

AP, aneuploid; DP, diploid; ND, not examined. MS, picked up by mass screening; FL, failed to pick up by mass screening.

33 (85%) of 39 informative cases were aneuploid. These data are summarized in Table 1. We could not detect any significant correlations between age of onset, chromosomal ploidy and amplification of *MYCN*. There were significant correlations between the stage classification (early stages: stage I, II, and IVs; late stages:

stage III and IV except for IVs) and age of onset or chromosomal ploidy or amplification of *MYCN* ($P = 0.00008$, $P = 0.04$ and $P = 0.00006$ respectively). Since 69% of our cases were selected by mass screening, the above mentioned correlation may have been influenced.

We then analysed allelic imbalances of chromosome band 14q32 in the 54 cases. We first analysed seven microsatellite loci (D14S81, D14S265, D14S62, D14S65, D14S267, D14S250 and D14S260) by a microsatellite-based PCR-LOH method and then added five more microsatellite loci (D14S990, D14S63, D14S987, D14S979 and D14S998) for a total of 12 loci on chromosome arm 14q. Seventeen (31%) of 54 patients showed allelic imbalances, probably LOHs, at one or more loci on 14q. None of the six patients who had been treated with chemotherapy before their operations showed allelic imbalances. In 15 (88%) of the 17 patients that showed allelic imbalances, such imbalances, predicted to be LOHs, were observed at almost all informative loci; deletion of large segment of chromosome arm 14q including 14q32 was suggested in these patients. On the other hand, cases 38 and 69 had more specific patterns of loss. In case 38, the LOHs were observed at the D14S979 and D14S65 loci; the intensities of the upper bands were reduced by 65% and 60% respectively, from those of the corresponding normal bands. In this patient, heterozygosity was retained at two flanking markers D14S62 and D14S998. In case 69, the intensity of the bands indicated by arrows was reduced by 58% and 56% from the corresponding normal bands at D14S265 and D14S62 loci respectively. Heterozygosity was retained at the D14S987 and D14S65 loci in

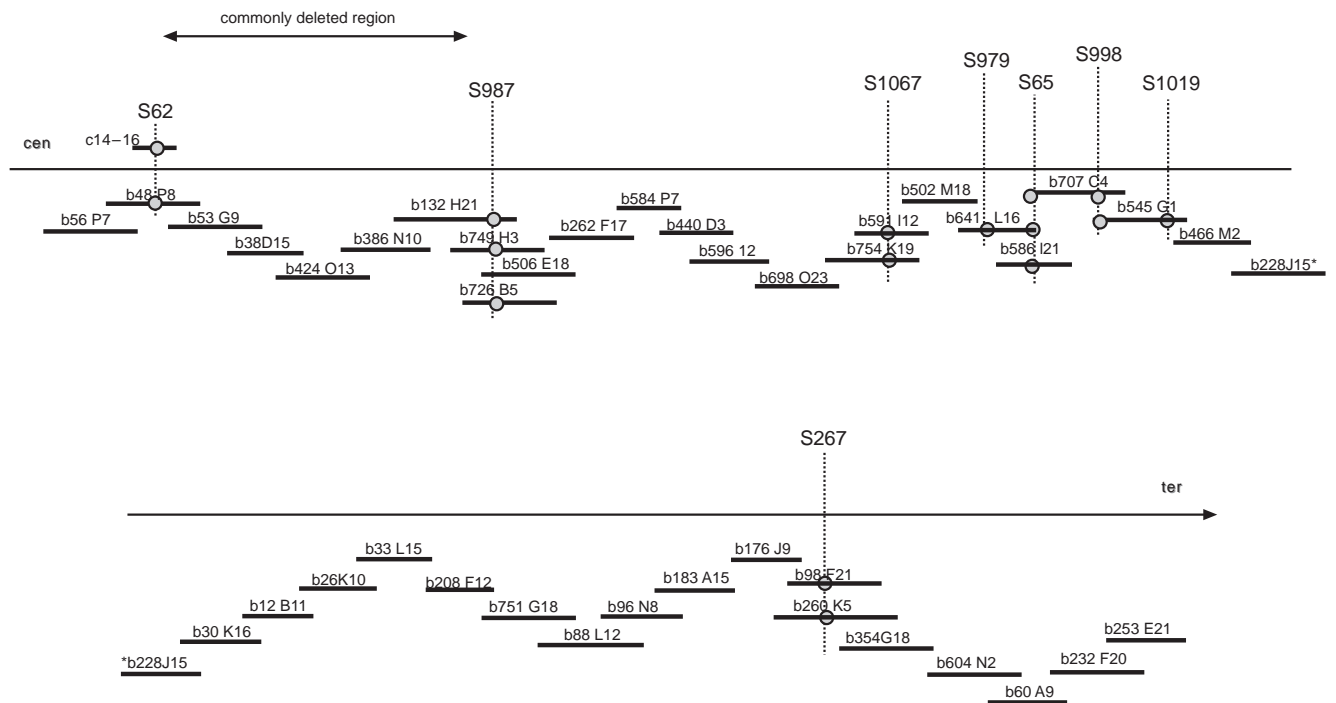


Figure 3 A sequence-ready BAC contig covering the deleted region. Based on this contig, the commonly deleted region was covered with six overlapping BAC clones, and the physical distance was approximately 1.1 Mb. The centromeric portion is on the upper layer, and telomeric portion is on the bottom layer. The clone b228J15, indicated by an asterisk, bridges the two clones, b466M2 (in the upper layer) and b30K16 (in the bottom layer). The cosmid clone c14-16 that was isolated initially is also shown. Sizes of the BAC clones covering the commonly deleted region are also provided in the parenthesis

this tumour. Thus these two cases showed interstitial deletions on chromosome band 14q32; according to the results, the smallest region of common allelic loss was defined between D14S62 and D14S987. The results of the microsatellite analysis are indicated in Figure 1. None of the tumours showed microsatellite instability (MSI) at any of the tested microsatellite loci.

To confirm the microsatellite analysis on chromosome band 14q32, dual-colour FISH analysis was performed in case 69. We could not sufficiently analyse case 38, because the tumour specimen was not for FISH analysis. Typical results are shown in Figure 2. A majority of the nuclei showed three signals for b726B5, a BAC clone harbouring microsatellite marker D14S987 (rhodamine, red), but only two signals for c14-16, a cosmid clone harbouring D14S62 (FITC, green). Signal numbers that were counted in more than 50 nuclei are shown by dots on the right side of Figure 2. This result indicates a decrease in copy number at D14S62 from that at D14S987. These results were consistent with those of microsatellite analyses in case 69. We further performed FISH analyses with marker sets, D14S65 and D14S998, D14S990 and D14S62, and D14S990 and D14S998; the results showed signal count reductions at D14S65 and D14S62 with no reduction but rather triploidy at both D14S990 and D14S998 respectively (data not shown). These results supported our results of PCR-LOH analysis; case 69 appeared to harbour an interstitial deletion between D14S81 and D14S62. Tumours with LOHs on 14q are summarized in Table 3; case 38 was diploid, and case 69 was aneuploid.

To investigate further, we constructed a BAC contig spanning an 8-cM region including the commonly deleted region, as shown in Figure 3. The relative order of the microsatellite markers that were not determined precisely in the linkage map was found to be as follows: 14cen-D14S62-D14S987-D14S1067-D14S979-D14S65-D14S998-D14S1019-D14S267-14qter. The region between D14S62 and D14S267, approximately 8-cM according to the linkage map, was covered with 32 clones of minimum tiling path, and the commonly deleted region was covered with a minimum tiling path consisting of six overlapping BAC clones. PFGE with *NotI* digested BAC DNA was performed to measure the physical distance of the commonly deleted region; it was estimated to be approximately 1.1-Mb.

DISCUSSION

In the present study, 31% allelic loss was observed on chromosome arm 14q, in good agreement with other reports on the LOH analysis of 14q (Suzuki et al, 1989; Fong et al, 1992; Takayama et al, 1992). The centromeric and telomeric borders of the commonly deleted region were D14S62 and D14S987; the interval was 1-cM according to the Génethon human linkage map (Dib et al, 1996). We established that the physical size of this region is approximately 1.1-Mb by PFGE. This region overlaps that of the previous work (14q32-qter) by Takayama et al (1992), which was done using restriction fragment length polymorphism (RFLP) markers. Another report on NB (Theobald et al, 1999) suggests the presence of two or more loci for putative tumour suppressor genes on chromosome 14 by RFLP and microsatellite analysis; one of them is between markers D14S1 and D14S16 and the other one is between markers D14S17 and D14S23 in 14q32. However, those areas are large; our study may be a first report of fine deletion mapping of 14q32 in NB.

Recently, disruption of the DNA mismatch repair system has been found to important associations with carcinogenesis of various human cancers and to involve microsatellite instability (MSI). No

MSI was observed in this study, in good agreement with those in previous reports (Schleiermacher et al, 1994; White et al, 1995). MSI is thus not generally involved in the tumorigenesis in NB.

In NB, 1p deletion and 17q amplification are associated with the age of onset and/or the clinical stage of NB, or with chromosomal ploidy (Fong et al, 1989; Takeda et al, 1994; Bown et al, 1999). Amplification of *MYCN* and 17q was associated with poor prognosis (Brodeur et al, 1992; Bown et al, 1999) as was a relatively large deletion of chromosome 1p (Schleiermacher et al, 1994; Takeda et al, 1994; Caron et al, 1995). Thus genetic alterations in NB appear to be associated with some clinical features. In this study, however, there was no correlation between LOHs on 14q32 and any clinical feature, including stage, chromosomal ploidy, *MYCN* amplification and age of onset ($P = 0.38$, $P = 0.31$, $P = 0.45$ and $P = 0.19$ respectively, by Fisher's exact test). In the present study, 36 cases were picked up by mass screening of 52 patients; the efficiency was 69% (36/52). More than 80% of the patients examined showed aneuploidy. Although we cannot exclude the possibility of a sampling bias, there is also no correlation between the mass screening and 14q32 LOH ($P = 0.83$). It is interesting that the LOH on 14q in this study showed the same frequency as reported previously despite the possible sampling bias.

In this study, we further performed FISH analysis to confirm the microsatellite analysis. By FISH analysis, we could detect an interstitial allelic deletion in case 69 that was consistent with the microsatellite analysis. In the LOH analysis of this study, we cannot exclude the possibility of 'pseudo' allelic loss derived from aneuploidy, but the FISH analysis clearly demonstrated an allelic imbalance, so we could confirm that the LOHs by microsatellite analysis were caused by true allelic losses in 14q32.

In this study, we further constructed a sequence-ready BAC contig in the region spanning D14S62 through D14S267. A minimum tiling path consisting of 32 overlapping BAC clones covered the entire region. The smallest region of common allelic loss in NB identified in the present study was approximately 1.1-Mb. Further investigations are necessary to understand and characterize the role of a putative tumour suppressor gene in 14q32 in the pathogenesis of NB. Moreover, the contig presented in this study will be a great help in cloning and characterizing the gene(s) that is closely associated with pathogenesis of tumours of the colorectum, ovary, and kidney as well as NB.

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