Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 1949–1952, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00286-4

Original Paper

Restriction Fragment Length Polymorphism Analysis Reveals Different Allele Frequency and a Linkage Disequilibrium at Locus *D1S94* in Neuroblastoma Patients

P. Perri,[1] A. Pession,[1] K. Mazzocco,[2] P. Scaruffi,[2] P. Strigini,[3] A. Iolascon,[4] M.P. Albergoni,[1] G. Basso[5] and G.P. Tonini[2]

[1]Department of Experimental Pathology, University of Bologna; [2]Unit of Solid Tumor Biology, Laboratory of Oncology, G. Gaslini Institute/Advanced Biotechnology Centre, Largo R. Benzi 10, 16136 Genoa; [3]Session of Genetic Epidemiology, National Institute for Cancer Research, Genoa; [4]Department of Biomedicine of the Evolution Age, University of Bari; and [5]Department of Paediatrics, University of Turin, Turin, Italy
For the Italian Neuroblastoma Research Programme

Deletion of chromosome 1p and *MYCN* amplification have been reported as frequent abnormalities in human neuroblastoma. We studied loss of heterozygosity (LOH) in 50 (48 informative) Italian neuroblastoma patients by restriction fragment length polymorphisms (RFLPs) analysis using anonymous and hypervariable region (HVR) sequences. Twelve cases (25%) showed LOH at one or more loci. Locus *D1S94* was the most frequently involved in LOH events (8/12) of deleted cases (66.6%). *MYCN* amplification was observed in 20% of patients which showed a significantly lower event-free survival probability (EFSp) ($P = 0.004$). We also studied the allelic distribution in the constitutional DNA of neuroblastoma patients ($n = 44$) and a matched group of healthy Italian subjects ($n = 79$) for loci *D1S112* and *D1S94*. A significantly ($P = 0.01$) different allele frequency was detected for the two groups at locus *D1S94*, but not at *D1S112*. Moreover, the neuroblastoma population did not confirm the Hardy–Weinberg expectations at the former locus. This observation suggests the existence of an allelotype associated with neuroblastoma susceptibility. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, loss-of-heterozygosity, *MYCN* amplification, chromosome 1, allelic frequency, linkage disequilibrium, *D1S112*, *D1S94*, Hardy–Weinberg
Eur J Cancer, Vol. 33, No. 12, pp. 1949–1952, 1997

INTRODUCTION

RECENT ADVANCES in understanding the molecular genetics of tumours, especially solid tumours, indicate that cancer develops as a result of an accumulation of genetic changes including activation of oncogenes and inactivation of tumour suppressor genes. Neuroblastoma, the most common extracranial solid tumour in childhood, has been genetically characterised by *MYCN* gene amplification and by deletion of the short arm of chromosome 1. Both alterations are associated predominantly with advanced stages of disease and poor prognosis [1, 2].

While amplification of *MYCN* is seen only in a fraction of neuroblastomas (20–25%), alterations of chromosome 1p are much more frequent (60% of advanced stages). Cytogenetic and molecular studies of 1p indicate the presence of distinct loci for tumour suppressor genes. Chromosome 1p deletions have been confirmed as loss of heterozygosity (LOH) in several studies and show an incidence ranging from 25% to 89% [3–8]. These differences are probably due to the variety and location of the probes used and to the stages of analysed tumours. The most frequently deleted regions are 1p36.2–1p36.1 [3, 4, 9, 10], 1p36.2–3 and 1p35–36.1 [2] and 1pter–1p32 [5, 9]. Some authors have suggested a significant correlation between *MYCN* amplification and loss of DNA sequence for 1p [4], while others have failed to support this association [3]. Above all, limited data are currently available concerning the allelic distribution of the loci involved in the deletion of 1p36, for both neuroblastoma patients and the healthy population.

Correspondence to G. Paolo Tonini.

We used restriction fragment length polymorphism (RFLP) analysis to determine LOH in patients with neuroblastoma at different stages of the disease and to establish whether a recurrent pattern of putative gene inactivation exists for the 1p36 region. We also analysed RFLP in a group of healthy subjects to define the genotype of this population for comparison with the neuroblastoma patients. Finally, we also evaluated a possible correlation between 1p LOH and *MYC*N amplification.

MATERIALS AND METHODS

Tumour and blood specimens

Primary tumour and skin tissues (50 samples) were obtained from untreated patients affected with neuroblastoma belonging to the Italian Tissue Bank [11]. Moreover, blood samples were obtained from 79 unrelated healthy subjects.

Nucleic acid isolation

Genomic DNA was isolated from tumour and skin surgically resected. Material was snapped frozen in liquid nitrogen and stored at −80°C until use. DNA was also purified from peripheral blood mononuclear cells of controls. The tissue samples were mechanically disrupted, treated with proteinase K, extracted in phenol–chloroform–isoamilic alcohol (25:24:1) and precipitated in ethanol [12].

Evaluation of MYCN amplification

For analysis of *MYC*N amplification, the purified DNA was digested with Eco RI, electrophoresed in a 0.8% agarose gel in a saline buffer (TBE 1X: 0.089 M Tris-borato, 0.2 mM EDTA, pH 8.0) and blotted on to nylon membrane positive charged (Hybond-N+, Amersham, U.K.) according to Southern blot analysis [13]. For evaluation of gene amplification, human lymphocyte DNA as a control of single-copy gene and DNA of the neuroblastoma cell line IMR 32 as *MYC*N amplified control were used. Filters were hybridised with probe NB 19-21 (kindly provided by Dr F. Alt, Columbia University, New York, U.S.A.). Probe labelling, Southern hybridisation and autoradiography were performed as described below. The signal intensity of autoradiography film was evaluated by densitometric analysis. Samples were considered amplified if more than three copies were present. Filters were then hybridised with a ^{32}P-labelled β-actin gene probe as internal control.

RFLP analysis

LOH was detected in tumour DNA by hypervariable region (HVR) polymorphic minisatellite MS1 sequence for the *D1S*7 locus (1p35–36.1); p1–31 and p1–24 probes, derived from microdissection and microcloning of short arm of chromosome 1 (kindly provided by Manfred Schwab, Institute for Experimental Pathology, Heidelberg), were used for the loci *D1S112* (1p36.11) and *D1S94* (1p36.33), respectively [14]. The following probe–enzyme combinations were used: MS1–Hinf I, p1–31–Ssp I and p1–24–Sph I. The fragments were separated by 0.7–1.0% agarose gel electrophoresis; constitutional and tumour DNAs from each patient were loaded in parallel. RFLP analysis was also carried out on 79 control DNAs to define the genotype of an Italian healthy population.

Hybridisation conditions

Probes were labelled with [α^{32}P]dCTP (3000 Ci/mmol, Amersham, U.K.) by a nick translation method (Promega, U.S.A). Prehybridisation and hybridisation were performed in 1 mM EDTA, 7% SDS, 0.25 M Na_2HPO_4, 5% dextran sulphate and carried out at 65°C for 1 h and 20 h, respectively. Probes were used at a concentration of 20 ng/ml hybridisation solution. After washing, the filters were exposed to sensitive X-ray film at −80°C.

Statistical analysis

Fisher's exact test was used for statistical analysis of the data [15] and event-free survival probability (EFSp) was estimated by the Kaplan–Meier method [16] on all patients, and updated to March 1995 (median follow-up was 17 months). Terminal events were relapse and death due to any cause, whichever came first. The log-rank test [17] was adopted to assess differences in univariate analysis. Univariate analysis, with EFSp as a dependent variable, was conducted for 1p LOH and *MYC*N amplification to verify their prognostic value. Fisher's exact test and χ^2 goodness of fit were also used for allelic frequency comparison and to verify the Hardy–Weinberg equilibrium.

RESULTS

We employed RFLP and HVR probes for 1p loci to detect allelic loss in 50 neuroblastomas. Only patients with constitutional heterozygosity were considered informative at one or more loci and studied for LOH. Forty-eight analysed patients were informative for one or more loci. LOH was found in 12/48 (25%), close to the result obtained by Takeda [9]. In informative cases, the intensities of the hybridisation signals from tumour and constitutional DNAs were compared by densitometrical scanning of autoradiographs. Some tumours showed changes in the ratio of one allele to the other when compared with the corresponding normal tissues: a decrease of at least 60–70% in the hybridisation signal corresponding to a given allele was considered an allelic loss (Figure 1). This faint band may be ascribed to a contamination of non-neoplastic tissue in the tumour or to the heterogeneity of tumour cells with respect to the genetic alteration.

Constitutional heterozygosity was present in 95.6% of our cases for the *D1S7* locus, and 54.5% and 65.9% for the *D1S112* and the *D1S94* loci, respectively. Analysis of locus *D1S7* by probe MS1 was performed on 45/50 samples and 43 of them were informative; 5/43 samples (11.6%) showed LOH and relative LOH was 41.6% (5/12 of deleted cases). Analysis of the locus *D1S112* was carried out on 44 samples. The p1–31 probe recognised two bands in informative cases: one of 15 kb and another of 10 kb. LOH was detected in 5/24 informative samples (20.8%) and relative LOH was 41.6% (5/12 of deleted cases). At locus *D1S94*, the p1–24 probe identified two bands of 16 kb and 8.5 kb, respectively. Eight

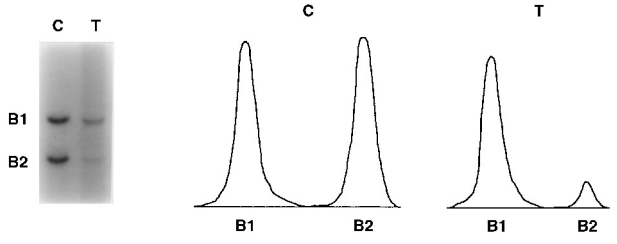

Figure 1. Representative scanning analysis of RFLP for locus *D1S94*. C, constitutional DNA; T, tumour DNA; B_1, 16 kb allele; B_2, 8.5 kb allele.

of 29 informative samples (27.6%) showed LOH with a relative LOH of 66.6% (8/12 of deleted cases). We also observed a new 5.4 kb allele at this locus in the constitutional DNA of a stage III neuroblastoma patient. This allele was lost in tumour DNA.

Our results show that the most frequently involved locus was the *D1S94* (relative LOH = 66.6%). Four cases showed a larger deletion encompassing the 1p35–36 region. It is worth noting that LOH occurred in stages III and IV (9/30 = 30%), as well as in stages I and II (3/16 = 18.7%). None of the analysed 4S stage tumours showed 1p LOH.

*MYC*N amplification was detected in 10/50 (20%) cases, confirming the frequency of the previous study [18]. The presence of extra copies of *MYC*N was associated with a significantly lower EFSp ($P = 0.004$). No correlation between LOH and EFSp was indicated.

Allele frequencies at loci *D1S112* and *D1S94* for constitutional DNA were calculated for neuroblastoma patients and compared with those found in control subjects. Forty-four out of 48 informative cases (4 DNA samples were not available) represented the neuroblastoma population, whereas the control group of 79 was recruited from healthy Italian individuals aged over 20 years, an age above which neuroblastoma rarely occurs [19]. Since no significant difference in the allele frequencies or in heterozygous fraction was associated with regional origin among controls ($\chi^2 = 0.97$; $P = 0.8$), the entire control and neuroblastoma groups were compared for locus *D1S94*. The results are summarised in Table 1. Allele frequencies at *D1S94* were calculated for the neuroblastoma population and compared with those found in the control group. Allele frequencies at locus *D1S112* in the two groups did not show any significant difference ($P = 0.6$) and no significant departure from the Hardy–Weinberg expectation was detected in either population. A rare allele (B_3) was observed at locus *D1S94* only in a stage III tumour. Disregarding that single patient, a significant difference ($\chi^2 = 6.13$; $P = 0.01$) of allele frequencies was found at *D1S94* locus: the B_1 allele being more frequent in neuroblastoma than in the control group (0.349 versus 0.196). Genotype distribution among controls at locus *D1S94* was in good agreement with the Hardy–Weinberg law. In contrast, a significant deviation ($\chi^2 = 8.07$; $P = 0.005$) from the expected Hardy–Weinberg equilibrium was detected at *D1S94* locus in the neuroblastoma group, mostly due to an excess of B_1/B_2 heterozygotes. The presence of the B_1 allele seemed to be more common in advanced cases (20/25 in III and IV versus 9/19 in I, II and IVS): larger case series should be analysed to assess the significance of such an observation.

Table 1. *Genotype and allele frequency comparison at locus* D1S94 *in neuroblastoma patients and healthy subjects*

Locus *D1S94*	Neuroblastoma	Controls
Observed genotypes*		
B_1/B_1	1	3
B_1/B_2	28	25
B_2/B_2	14	51
B_2/B_3	1	0
Allele frequency		
$p(B_1)$	30/86†	31/158
χ^2_c	6.13 ($P = 0.01$)	
H–W equilibrium		
χ^2 goodness of fit	8.07 ($P = 0.005$)	

*Alleles detected where $B_1 = 16$ kb, $B_2 = 8.5$ kb, $B_3 = 5.4$ kb at locus *D1S94*. B_3, rare allele at locus *D1S94*, was disregarded for both allele frequency and Hardy–Weinberg equilibrium comparison. †Disregarding the rare B_2/B_3 genotype. χ^2_c, Chi-square with Yates correction.

DISCUSSION

Recent studies indicate that the short arm of chromosome 1 is deeply involved in the genesis of neuroblastoma [2, 6, 9]. Here we report the first analysis of LOH for 1p in Italian neuroblastoma. We found an incidence of LOH of 25%. Previous studies have shown a great variability of LOH incidence (ranging from 25% to 89%) which could be ascribed to differences in the techniques, the probes employed and the stage distribution among observed subjects. In our work, the distribution of advanced (stage III and IV) and non-advanced cases (stages, I, II and IVS) was comparable with that found in a larger cohort by the Italian Neuroblastoma Cooperative Study Group [18, 20]. Therefore, no selection bias towards clinical stage was detected in the study. LOH was often (66.6%) found at the distal *D1S94* locus, indicating that deletion frequently involved the putative neuroblastoma suppressor assigned to the 1p36.33 region. This finding is in keeping with those reported by Takayama [7].

The 20% (10/50) of patients studied showed *MYC*N amplification (*MYC*N copy number ≥ 3), close to that found in a previous study on a larger population [20]. This frequency can be considered to be a characteristic of Italian neuroblastoma.

Patients whose tumour showed extra copy numbers of *MYC*N gene had a significantly lower ($P = 0.004$) EFSp [11]. After dividing patients in two groups, with or without 1p LOH, we did not find any significant difference in EFSp at 5 years, even if we considered only cases in advanced stages (III and IV). Moreover, no additional effect of LOH on the *MYC*N prognostic value was detectable in all *MYC*N amplified cases (3 cases with LOH out of 10 amplified cases), even considering only advanced neuroblastoma. The contrasting findings by Caron [2] might be a reflection of a larger study population. EFSp evaluation showed the effect of the poor sample dimension and the low number of events. Moreover, the median follow-up time was only 17 months. Indeed, considering only localised tumours with 1p LOH in a larger cohort of patients we are studying, allelic loss assumed a significantly poor prognostic value (data not shown).

While allele frequency and genotype distribution at the *D1S94* locus in the control group were in good agreement with Hardy–Weinberg expectation, a significantly different allele frequency was evident between neuroblastoma patient and controls. The higher B_1 allele frequency, due to an excess of heterozygous individuals in the patient group, suggests a non-random allelic combination, associated with the susceptibility to neuroblastoma. Furthermore, neuroblastoma patients represent a non-panmictic population. These data indicate that the *D1S94* locus might be in a linkage disequilibrium with a gene or a sequence involved in the genesis of the neoplasia. Since a normal allele frequency and genotype distribution were found at nearby locus *D1S112* among neuroblastoma patients, selection bias of cases or controls may be ruled out. Linkage disequilibrium appears to be widely distributed in anonymous regions of the genome [21]. The study of linkage disequilibrium is useful in mapping disease genes and our result may give additional information

for better defining the position of the neuroblastoma suppressor gene.

1. Schwab M. Molecular cytogenetics of human neuroblastoma. *Biochim Biophys Acta* 1992, **1114**, 43–50.
2. Caron H, Peter M, van Sluis P, *et al.* Evidence for two tumor suppressor loci on chromosomal bands 1p35-36 involved in neuroblastoma: one probably imprinted, another associated with N-myc amplification. *Hum Mol Genet* 1995, **4**, 535–539.
3. Weith A, Martinsson T, Cziepluch C, *et al.* Neuroblastoma consensus deletion maps to 1p36.1-2. *Genes Chrom Cancer* 1989, **1**, 159–166.
4. Fong CT, Dracopoli NC, White PS, *et al.* Loss of heterozygosity for short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci USA* 1989, **86**, 3753–3757.
5. Hunt JD, Tereba A. Molecular evaluation of abnormalities of short arm of chromosome 1 in neuroblastoma. *Genes Chrom Cancer* 1990, **2**, 137–146.
6. Peter M, Michon J, Vielh P, *et al.* PCR assay for chromosome 1p deletion in small neuroblastoma samples. *Int J Cancer* 1992, **52**, 544–548.
7. Takayama H, Suzuki T, Mugishima H, *et al.* Deletion mapping of chromosome 1p deletion in small neuroblastoma samples. *Oncogene* 1992, **7**, 1185–1189.
8. Caron H. Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavorable prognostic markers in neuroblastoma. *Med Pediatr Oncol* 1995, **24**, 215–221.
9. Takeda O, Homma C, Maseki N, *et al.* There may be two tumor suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chrom Cancer* 1994, **10**, 30–39.
10. White PS, Maris JM, Beltinger C, *et al.* A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2–36.3. *Proc Natl Acad Sci USA* 1995, **92**, 5520–5524.
11. Tonini GP. Report of the Italian Pediatric Cancer Research Group on the Neuroblastoma Research Programme. *Eur J Cancer* 1993, **28A**, 1000–1001.
12. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York, 1989.
13. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975, **98**, 503–517.
14. Martinsson T, Weith A, Cziepluch C, Schwab M. Chromosome 1 deletions in human neuroblastoma: generation and fine mapping of microclones from distal 1p region. *Genes Chrom Cancer* 1989, **1**, 67–78.
15. Pearson ES, Hartley HO. *Biometrika Tables for Statisticians*, vol. 1, 3rd edn. Cambridge University Press, Cambridge, 1966.
16. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958, **53**, 457–481.
17. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959, **22**, 719–748.
18. Sansone R, Strigini P, Badiali M, *et al.* for the Italian Neuroblastoma Research Program. Age-dependent prognostic significance of N-myc amplification in neuroblastoma: the Italian experience. *Cancer Genet Cytogenet* 1991, **54**, 253–257.
19. Lopez R, Karakousis C, Rao U. Treatment of adult neuroblastoma. *Cancer* 1980, **45**, 840–844.
20. Tonini GP, Boni L, Pession A, *et al.* MYCN oncogene amplification in neuroblastoma is associated with worse prognosis, except in stage 4s. The Italian experience with 295 children. *J Clin Oncol* 1997, **15**(1), 85–93.
21. Peterson AC, Di Rienzo A, Lehesjoki AE, *et al.* The distribution of linkage disequilibrium over anonymous genome regions. *Human Mol Genet* 1995, **4**, 887–894.

Acknowledgements—This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), by Italian Neuroblastoma Association, by Projects CNR-ACRO N. 94.01259/PF39 for G.P.T. and by grants from Ministero Pubblica Istruzione 60% for A.P. The authors thank Miss Alessandra Zanotta for her assistance in the editing of the manuscript and Dr Christine M. Betts for the language revision. This work was carried out thanks to the collaboration of the following members in the National Tissue Bank-AIEOP: Prof. G. Paolucci, Prof. A. Pession (Bologna), Prof. M. Carli, Prof. G. Basso, Dr A. Rosolen (Padua), Prof. A. Iolascon (Bari), Dr A. Pagani (Turin), Dr R. Luksch (Milan), Dr A. Cavazzana, Dr C. Dominici, Prof. R. Mastrangelo, Prof. M. Castello, Prof. A. Donfrancesco (Rome), Dr A. Garaventa, Prof. L. Massimo (Genoa), Prof. M. Lo Curto (Palermo), Prof. S. Cutillo (Naples).