Deletion of Chromosome 1p Loci and Microsatellite Instability in Neuroblastomas Analyzed with Short-Tandem Repeat Polymorphisms

Tommy Martinsson, Rose-Marie Sjöberg, Fredrik Hedborg, and Per Kogner

Department of Clinical Genetics, University of Göteborg, East Hospital, S-416 85 Gothenburg, IT, M. S.; Department of Pathology, University of Uppsala, University Hospital, S-751 85 Uppsala, F. H.; and Department of Pediatrics, Karolinska Institute, Karolinska Hospital, S-171 76 Stockholm, P. K., Sweden.

ABSTRACT

We have analyzed DNA from 46 neuroblastoma tumors of all clinical stages and five ganglioneuroma tumors together with corresponding control DNA for loss of heterozygosity (LOH) on the distal 1p chromosomal region (1p-LOH). The markers used for the analyses were genetically mapped DNA polymorphisms detectable with PCR analysis. In general, there was concordance among aggressive tumor stage, 1p deletion, and N-myc amplification, although exceptions were found. Twelve (26%) of the 46 neuroblastoma tumors displayed 1p-LOH, 11 being stage 4 and 1 stage 2 (which progressed subsequently to stage 4), whereas 10 stage 4 tumors showed no 1p-LOH. Of 12 neuroblastomas shown to have \textit{N-myc} amplification, 10 had 1p-LOH. In 8 cases it was possible to test for parental origin of the chromosome involved in 1p-LOH. No significant correlation between 1p-LOH and maternal or paternal allele was found. Commonly deleted loci in the distal 1p region in the neuroblastoma tumors indicated that the region for a tentative neuroblastoma tumor suppressor gene is defined proximally by marker D1S244 and distally by marker D1S80. One striking feature of three stage 2 neuroblastomas and one of the stage 3 tumors was the presence in the tumor DNA of alleles not present in the constitutional DNA of the patients, \textit{i.e.}, microsatellite instability. The significance of this phenomenon in localized neuroblastoma tumors remains to be clarified.

Aggressive neuroblastoma in young children (younger than 2 years of age) seems to be a homogenous disorder consistently showing concomitant 1p-LOH and \textit{N-myc} amplification. In the majority of unfavorable neuroblastoma in older children, however, neither 1p-LOH nor \textit{N-myc} amplification could be detected. This indicates that neuroblastoma in older children is a biologically more heterogenous disorder in which genetic alterations other than deletions of chromosome 1p and amplification of \textit{N-myc} also may contribute to tumorigenesis.

INTRODUCTION

Neuroblastoma is a neural crest-derived malignancy, usually occurring during infancy and early childhood. It is the most common extracranial solid tumor of early childhood, with an annual incidence of 27.75/10^6 (about 1/6000) for children younger than 5 years of age (1). Neuroblastoma tumors show marked differences in clinical and biological behavior, ranging from total spontaneous regression to malignant progression, giving tumors resistant to therapy. Cytogenetic analyses of neuroblastoma tumors and cell lines have revealed several specific alterations. One of these is the presence of cytogenetic signs of gene amplification, \textit{i.e.}, double minutes or homogeneously staining regions. These have been shown to contain amplified copies of the oncogene \textit{N-myc} (2, 3).

Another alteration present in about 30% of neuroblastomas is a deletion or unbalanced translocation resulting in a loss of genetic material from the distal short arm of chromosome 1 (1p). These deletions have been detected by cytogenetic analysis (4–6), by an \textit{in situ} hybridization technique (7, 8), and by detection of LOH\textsuperscript{3} (9–11). The deletion of 1p often involves a large proportion of the distal region of the short arm, but some tumors and cell lines display smaller deletions, involving only region 1p36.1–2 (9, 10, 12–15). This region, therefore, is a candidate position for one or several NBS genes(s). Further support for a NBS gene in 1p36 is given by two cases in which patients with neuroblastoma carried constitutional aberrations in the 1p36 region (16, 17). Furthermore, in a recent series of articles (13–15, 18), several groups presented data suggesting two or more neuroblastoma tumor suppressor genes in the 1p region.

We report that in our patient material, molecular data for 1p deletions, microsatellite instability, and \textit{N-myc} amplification correlate with clinical features such as age and stage at presentation and outcome. Furthermore, a subgroup of neuroblastomas was found to carry very distal 1p deletions. The 1p break points in these tumors were fine mapped using the PCR-based markers, and a proximal and distal border for a NBS gene could be determined subsequently.

MATERIALS AND METHODS

Patient Material, DNA Extraction, and Southern Analysis. Tumor specimens and corresponding normal tissues (fibroblast biopsy or blood sample) were obtained from 51 children, 46 with neuroblastoma of all different stages and 5 with ganglioneuroma (Table 1). The clinical material is part of an ongoing, population-based Swedish neuroblastoma study. Thus, some of the patients have been described earlier, and the numbering is in accordance with that of the earlier report (19). The children were diagnosed, staged, and evaluated for clinical outcome according to the International Neuroblastoma Staging System criteria (20, 21). The 25 children currently surviving have been followed for 3–92 months (median, 32 months), and the remaining 21 died after 0–96 months (median, 10 months; Table 1). Genomic DNA was extracted from blood, fibroblasts, and fresh frozen (−70°C) tissue samples using standard procedures. Tumor cell content of the samples was assessed histologically in tumor tissue adjacent to that used for DNA extraction. DNA was also extracted from blood samples from parents of eight patients shown to carry tumor-specific deletions in the 1p region. The \textit{N-myc} gene copy number was analyzed using a Southern blot as reported previously (19). \textit{N-myc} amplification was scored when the gene copy number per haploid genome was 3 or more. Results of \textit{N-myc} analyses have been reported previously (19) for some of the tumors included in the current study.

Detection of 1p Deletions Using PCR-based DNA Polymorphisms. First, PCR-based polymorphisms located on the distal 1p chromosome region were analyzed using primer pairs D1S80 (22), D1S243, D1S200 (23, 24), D1S170, D1S160 (25), and GGAT2A07 (CHLC, University of Iowa, Iowa City, IA; Ref. 26). Genetic markers were derived from the CHLC framework map (chromosome 1 framework map, sex-averaged, version 1; via anonymous ftp to ftp.chlc.org) and for markers D1S80, D1S170, GGAT2A07, and MYCL from the CHLC likely location map (Likely-loc, version 1). Some neuroblastomas found with the first set of markers to carry 1p deletions with distal break points were subjected to further study with a more dense marker set derived from parents of eight patients shown to carry tumor-specific deletions in the 1p region. The \textit{N-myc} gene copy number was analyzed using a Southern blot as reported previously (19). \textit{N-myc} amplification was scored when the gene copy number per haploid genome was 3 or more. Results of \textit{N-myc} analyses have been reported previously (19) for some of the tumors included in the current study.

Received 5/30/95; accepted 9/28/95.

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1 Supported by the Swedish Cancer Society, the King Gustav V Jubilee Clinic Cancer Research Foundation, the Swedish Child Cancer Research Fund, and the Assar Gabrielson Foundation.

2 To whom requests for reprints should be addressed.

\textsuperscript{3}The abbreviations used are: LOH, loss of heterozygosity; NBS, neuroblastoma tumor suppressor; CHLC, Cooperative Human Linkage Center; ftp, file transfer protocol.
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Detection of LOH and Mapping of ip Deletions. Patients with neuroblastoma (n = 46) and ganglioneuroma (n = 5) were subjected to analysis with seven PCR-based microsatellite short tandem repeat polymorphisms (D1S243, D1S160, D1S228, D1S170, GATZ2A07, and D1S200) and one PCR-based variable number of tandem repeats polymorphism (D1S80).

Constitutional DNA and DNA extracted from the tumor of the patient were run in adjacent lanes, and the pattern was compared. Representative analyses are shown in Fig. 1. Twelve of the tested neuroblastosomas (26%) but none of five ganglioneuromas (P = 0.25) displayed LOH in at least one loci (Table 1).

Two tumors (tumors 121 and 189) displayed LOH for distal markers D1S243 and D1S160 but not for more proximal markers D1S244, GATZ2A07, and D1S200. Case 52 however, had both alleles retained for the most distal marker D1S80 but LOH for more proximal markers D1S243, D1S244, and D1S170. These tumors were subjected to analyses with more markers in the region to characterize their ip-deletion break points further (Figs. 2 and 3). It could be shown that for cases 121 and 189, D1S808 displayed LOH, whereas for both tumors, the two constitutional alleles of marker D1S244 were retained (Figs. 2 and 3).

Parental Origin of Deleted Alleles. In eight of the patients with 1p-LOH, the parents could be included in the study for testing of which chromosome, the maternal or the paternal, was involved in the LOH process. Subsequently, polymorphisms that were informative and showed LOH for the children were tested for the family. In four of the cases (cases 55, 95, 121, and 184), the deleted chromosome regions were derived from the maternal chromosomes, whereas in the four other cases (cases 155, 163, 169, and 174), the paternaly derived alleles were deleted in the tumor DNA (Fig. 4).

Presence of Microsatellite Instability in Localized Neuroblastoma. Microsatellite instability was detected in 4 of the 46 tested neuroblastoma tumors (Fig. 1 and Table 1) and, notably, in only nonmetastasizing stages (stages 2 and 3; P = 0.077). Especially for the stage 2 tumors, 3 of the 11 tumors displayed microsatellite instability for at least one loci. Case 69, with a stage 3 tumor, displayed microsatellite instability for five of the 1p loci tested. D1S200, the most proximal of the tested loci, was informative for the displayed microsatellite instability for five of the ip loci tested.

Table 1. Summary of clinical parameters and experimental data from patients with neuroblastoma and ganglioneuroma.

Patient Stage Age (yrs) ip analysis Deletion Parental origin Microsatellite instability

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RESULTS

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Fig. 1. PCR analysis of tumor sample DNA (T) and corresponding normal DNA (N) of the neuroblastoma cases, indicated by numbers. The marker used is indicated with bold letters above each photograph. The result of each comparison is detailed under the photograph. n.i., noninformative; m.i., microsatellite instability. The presence of extra bands in the microsatellite cases is indicated by arrows.

pattern, case 69 was tested with several microsatellite loci on different locations on chromosome 17. Several of these markers were informative for the patient, but no LOH or microsatellite instability could be detected.

**1p-LOH Correlation with N-myc Amplification and Clinical Features.** LOH for 1p was detected in 10 of 12 neuroblastomas with N-myc amplification but in only 2 of the remaining 31 tumors without N-myc amplification (P < 0.0001). Two cases with N-myc amplification (cases 105 and 126) had no 1p-LOH, whereas two cases (121 and 155) had 1p-LOH without having N-myc amplification.

LOH for 1p correlated significantly with stage, because 11 of 12 tumors showing 1p-LOH were stage 4 tumors (P = 0.0002). The only child with a favorable clinical stage at diagnosis and poor outcome due to progressive disease (case 121; stage 2A) had 1p-LOH. Survival probability for the whole neuroblastoma material was 48.7 ± 8.3% at 36 and 60 months’ follow-up. Children with tumors showing 1p-LOH had inferior prognoses (survival probability, 18.2 ± 11.6% at 36 months and 0% at 96 months) compared with those without 1p-LOH (59.7 ± 9.7% at 36 months; P = 0.001; Fig. 5).

**DISCUSSION**

Childhood neuroblastoma is an extremely heterogeneous disease in terms of clinical behavior, in which biological studies may clarify underlying mechanisms for tumor development and progression. In the present study, we have used PCR-based microsatellites to study 1p...
and found a correlation between 1p deletions and N-myc amplification, metastatic phenotype, and an unfavorable clinical outcome. Furthermore, in a limited number of localized tumors, microsatellite instability could be detected. From the present investigation, we may suggest specific clinical subsets with characteristic molecular aberrations.

Using several markers in the distal 1p region (Fig. 1), we screened a group of 51 tumors (46 neuroblastomas and 5 ganglioneuromas) for 1p-LOH (Table 1 and Fig. 1). LOH for distal 1p could be demonstrated in a subset of neuroblastomas (12/46; 26%) but not in any of the benign ganglioneuromas. Two cases with distal 1p deletions had their deletion break points very distal in 1p, i.e., between markers D1S80 and D1S244 (cases 121 and 189; Figs. 2 and 3). The combined information of the 12 cases with 1p deletions, therefore, gave a consensus deletion region for a tentative NBS gene ranging from the telomere to at least 1p35, and these deletions were of random parental origin (18 of 30 had maternal LOH; 2, LOH; —, uninformative. The shortest region of overlap of deletions based on the three cases is indicated to the left as bar d. The shortest region of overlap is compared with earlier published data of deletions in neuroblastoma tumors and rearrangements in cell lines from White et al. (28; bar a), Caron et al. (18; bar b), and Amler et al. (29; bar c). The NBS consensus region of the previous studies and of our present study, defined distally by marker D1S214 and proximally by marker D1S244, is indicated by the gray area.

In eight cases, it was possible to determine the parental origin of the 1p-LOH chromosome. In four of the cases, the maternal copy was lost in the tumor (Fig. 3), whereas in four tumors, the paternal allele was lost. Caron et al. (11) showed that, in their patient material, the maternal allele was preferentially lost (in 13 of 15 cases), suggesting that genomic imprinting could be involved in gene regulation in the 1p region. Cheng et al. (30) came to another conclusion. In their study, 6 of 10 N-myc-amplified cases involved a deletion of the paternally derived 1p alleles, consistent with a random distribution in their material. The differences in selection criteria of patients in the two studies can account possibly for the discrepancy of results. Recently, Caron et al. (18) addressed the question of imprinting in an extended study of neuroblastoma tumors. Of the 47 tumors with 1p-LOH studied, those with N-myc in single copy had preferentially lost the 1p36 allele of maternal origin (16 of 17 cases). These tumors also had a very distal commonly deleted region (i.e., mapping to 1p36.3-p36.2). In contrast, all N-myc-amplified neuroblastomas had larger 1p deletions, extending from the telomere to at least 1p35, and these deletions were of random parental origin (18 of 30 had maternal LOH). Therefore, Caron et al. (18) suggested that at least two different tumor suppressor genes on 1p can be inactivated in different neuroblastoma subtypes. Also, other authors have presented data indicating more than one suppressor gene in 1p (13–15). The data of our material, although small in number, did contradict at least partly those of Caron et al. (18). First, case 155 had a 1p deletion without N-myc amplification, and the deleted copy was of paternal origin. Second, case 189 had the smallest 1p deletion in the material but displayed N-myc amplification.

One surprising feature of our data was the presence of microsatellite instability in nonmetastasizing neuroblastomas but not in any of
the stage 4 tumors. Microsatellite instability (for recent review see Ref. 31) was observed first in colon cancer (32, 33), and it was suggested further that it was present preferentially in carcinomas and not in adenomas (34). In hereditary nonpolyposis colon cancer (Lynch syndrome) the microsatellite instability is caused apparently by mutations in the MSH2 (35) and MLH1 (36) genes. In one study, Gonzales-Zulueta et al. (37) found microsatellite instability in six patients among 200 with bladder cancer. The six patients were among those with lower-stage tumors, whereas no microsatellite instability could be detected in higher-stage tumors. This may suggest that microsatellite instability is an early event in bladder cancer and may contribute to the progression of tumorigenicity in this tumor type. In the neuroblastoma tumors studied by us, microsatellite instability was found only in localized tumors, especially in stage 2A and 2B tumors (Table 1). In two other recent reports, microsatellite instability was investigated and detected in only a limited number of neuroblastoma tumors without any obvious correlation with clinical features (13, 38). The significance of this phenomenon and whether genetic instability may contribute to the neuroblastoma phenotype in a subset of tumors remains to be clarified.

We could demonstrate significant correlations among 1p-LOH, the presence of N-myc amplification, and an unfavorable metastatic phenotype. There were, however, cases in our clinical material with 1p-LOH without N-myc amplification, as well as the reverse. Previous studies have given conflicting results concerning a correlation between 1p-LOH and N-myc amplification. One study by Caron et al. (11) reported N-myc amplification only in tumors with 1p-LOH, whereas other studies, including this one, have found tumors with N-myc amplification without concomitant detection of 1p-LOH (13, 14, 39). Interestingly, it was reported recently that localized tumors with N-myc amplification did not necessarily have unfavorable outcomes, especially when a 1p deletion was not present (39). A similar favorable outcome of a stage 4 patient with N-myc amplification but without 1p-LOH was noted in this material (Table 1, patient 105). Whether the poor outcome for children with tumors showing 1p-LOH is due to this aberration alone, or whether the concomitant amplification of N-myc in most cases may be the genetic reason for an aggressive phenotype and poor prognosis, remains to be addressed in larger studies.

When analyzing our data further, the association of 1p-LOH and N-myc amplification was most significant in the children diagnosed with neuroblastoma younger than the age of 2 years. Six of these had 1p deletions and N-myc amplification, and the other 16 had neither 1p-LOH nor N-myc amplification (P < 0.0001). Among children older than 2 years of age at diagnosis, 6 showed 1p-LOH (four N-myc amplified), and of the remaining 15, 2 had N-myc amplification (P = 0.03). All children younger than 2 years of age at diagnosis who died of tumor progression (5 of 25) had both 1p-LOH and N-myc amplification. Among children older than 2 years who died of tumor progression (15 of 21), 4 had 1p-LOH with N-myc amplification, 1 had only 1p-LOH, 1 had only N-myc amplification, and 9 had neither 1p-LOH nor N-myc amplification. These data indicate clearly that unfavorable neuroblastoma in older children is a heterogeneous disease by molecular means, whereas in younger children, a deletion of chromosome 1p and a concomitant amplification of N-myc were constant findings.

LOH of 1p could be detected in a subset of childhood neuroblastoma tumors in the present material (12 of 46; 26%) almost exclusively in tumors showing unfavorable metastatic stages and poor clinical outcomes. The loss of genetic material confined to this chromosomal region in aggressive tumors indicates the position of one or several neuroblastoma tumor suppressor gene(s). Using a panel of genetically mapped DNA polymorphisms detectable by PCR, the region showing LOH in tumor DNA was defined proximally by the marker D1S244 and distally by the marker D1S80. Microsatellite instability could be detected in only nonmetastasizing neuroblastoma tumors. The significance of this phenomenon and whether genetic instability may be of significance in a subset of neuroblastoma tumors remains to be further investigated. Finally, our data indicated an age-dependent heterogeneity among aggressive neuroblastomas. In the present material, a consistent finding was concomitant 1p-LOH and N-myc amplification in all unfavorable progressive tumors in children younger than 2 years of age. On the other hand, aggressive
neuroblastoma in older children seems to be a heterogeneous disease with all possible combinations of the presence and absence of 1p-LOH and N-myc amplification. These data indicate the significant contribution of several molecular aberrations to tumorigenesis in different subsets of neuroblastoma tumors in children older than the age of 2 years.

ACKNOWLEDGMENTS

We acknowledge the clinical and scientific support of Drs. Idilko Marky, Sven Påhlman, and Magnus Nordenskjöld.

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