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Regression and Progression in Neuroblastoma. Does Genetics Predict Tumour Behaviour?

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Neuroblastoma (NB) is a heterogeneous disease. The clinical course may range from spontaneous regression and maturation to very aggressive behaviour. Stage 4s is a unique subcategory of NB, generally associated with good prognosis, despite skin and/or liver involvement and the frequent presence of tumour cells in the bone marrow. Another type of NB is the locally invasive tumour without bone and bone marrow involvement which can also have a good prognosis, irrespective of lymph node involvement. Unfortunately, there is only limited biological information on such tumours which have not been treated with cytotoxic therapy despite lymph node involvement, residual tumour mass after surgery and/or bone marrow infiltration. In order to find specific genetic changes common to NBs with a benign clinical course, we studied the genetic abnormalities of these tumours and compared them with highly aggressive tumours. We analysed a series of 54 localised and stage 4s tumours by means of *in situ* hybridisation performed on fresh cells or on paraffin embedded tissues. In addition, we performed classical cytogenetics, Southern blotting and PCR analysis on fresh tumour tissue. The majority of patients had been treated with surgery alone, and in a number of patients tumour resection was incomplete. Deletions at 1p36 and amplifications of the *MYCN* oncogene were absent, and diploidy or tetraploidy were not seen in any case, with residual localised tumours possessing a favourable outcome. Unexpectedly, one patient with a tetraploid 4s tumour without any genetic structural changes not receiving any cytotoxic treatment, did well. Interestingly, this genetic spectrum contrasted with that of progressing tumours, in which most had genetic aberrations, the deletion at 1p36 being the most common event. These data, although limited, suggest that an intact 1p36 (recognised by D1Z2), the absence of *MYCN* amplification and near-triploidy (at least in localised tumours), represent prerequisites for spontaneous regression and/or maturation.

Key words: neuroblastoma, genetics, deletion 1p36, *MYCN*, cytotoxic treatment, *in situ* hybridisation, localised tumours, stage 4s, regression, progression

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INTRODUCTION

NEUROBLASTOMAS (NBs) are extremely heterogeneous in terms of their biological and clinical behaviour. The majority of tumours have an unfavourable outcome, very often associated with chromosome 1p deletions [1–6], diploidy [3, 4, 7] and *MYCN* amplification [7–9] (for review see [10]). In contrast, subsets of tumours, either localised [11–13] or even widespread (4s), have the ability to spontaneously regress and/or mature [14–17]. There is a growing number of reports indicating that even patients with residual tumour, not receiving any cytotoxic treatment have an excellent outcome [11–12]. These phenomena can be explained by spontaneous regression and/or maturation processes. The mechanisms leading to differentiation of stroma-rich tumours have begun to be elucidated. In these tumours, the Schwann cell, which is now recognised to be a normal cell [13] seems to play a key role. By "invading" the tumours they probably promote the maturation processes of the neuroblasts in these NBs (Ambros and Ambros, this issue, pp. 429–434). Unfortunately, the mechanisms underlying the regression processes are at best only vaguely understood. In addition, there are only a few reports on the genetics of tumours that are able to undergo regression and/or maturation. The reports on ploidy, 1p deletions, and *MYCN* status are controversial and do not answer the questions of whether these cells show a common genetic make-up. This is mainly because terminally differentiated cells and regressing cells are unavailable for cytogenetic analysis as they do not grow in culture. In addition, the presence of a high proportion of normal cells (e.g. Schwann cells and lymphocytes) within the tumour sample may obscure flow cytometric analysis and molecular studies using PCR or Southern blotting. Generally, triploidy was considered to be associated with this type of tumour [3, 7, 18], but diploidy was also found, especially in ganglioneuromas, presumably due to the high Schwann cell content of the tumours analysed [19].

Based on clinical reports [11, 12], there is encouragement for the avoidance of cytotoxic treatment in localised tumours. Unfortunately, a small number of patients with localised NBs show progressive disease. To minimise the risk for these patients carrying a tumour, with the potential to progress into a stage 4 tumour, a treatment regime adapted to the biology of these tumours would be ideal. One of the aims for the future is the definition of pertinent biological markers which will allow unambiguous discrimination of localised tumours, which will progress from those that will not. To shed some light on the genotypes of these tumours, we have introduced methods which allowed the study of chromosomal aberrations in terminally differentiated cells and in tumours otherwise inaccessible to genetic analysis. In addition, *in situ* hybridisation techniques on paraffin sections [13, 20, 21] allowed an unambiguous consignment of the genetic aberrations to specific individual cells or cell types. Aneusomies, deletions at 1p36 and also amplifications of the *MYCN* oncogene were identified in interphase nuclei by using centromere-specific DNA probes, a repetitive probe identifying a VNTR-region at the sub-telomeric position of the short arm of chromosome 1 [22, 23] and a DNA probe identifiying the *MYCN* gene [8, 24].

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MATERIALS AND METHODS

Patients and material

Tumour material from 54 patients with NB was analysed. 38 patients (age range, 1 month–13 years) had localised tumours and did not receive cytotoxic treatment, irrespective of residual disease (8/38), 5 patients (age range, 11 months–7 years) had local disease that progressed to metastatic disease, despite cytotoxic treatment. 11 patients (age range, 1–11 months) presented with widespread disease that clinically fulfilled the criteria of stage 4s. Of these, 7 showed spontaneous regression of disease without cytotoxic treatment, and 4 had progression of disease, which was fatal in three cases, but one is presently in complete remission after intensive cytotoxic therapy. Tumour material was obtained from different centres.

Cytogenetic analysis

The tumour samples were minced and resuspended in RPMI 1640 containing antibiotics and 10% fetal calf serum for culturing. Direct and short-term culturing for chromosome preparations were undertaken. Chromosome preparations were made according to standard protocols. R-banding was performed by the chromomycin/distamycin/DAPI (CDD) staining technique [25].

Molecular biological analysis

Fresh tumour material was macroscopically separated from adhering normal tissue and mechanically disrupted. DNA from tumour tissue was extracted essentially as previously described [26]. *MYCN* copy number was analysed according to standard filter hybridisation techniques. Southern blots of *Hind*III digested genomic DNA were probed with a 800 base pair *Pst*I fragment excised from plasmid pNb-2, a generous gift of M. Schwab. PCR amplification of VNTR sequences *D1S76* and *D1S80* for determination of 1p zygosity was performed as previously described using primers 76.10 and 76.11 and 80.3 with 80.4, respectively [27].

Flow cytometric analysis

The samples were analysed with a FACStar flow cytometer (Becton Dickinson). Data were analysed using the Cellfit software (Becton Dickinson), and DNA histograms were obtained.

In situ *hybridisation*

Fluorescence *in situ* hybridisation (FISH) experiments were carried out on touch preparations, frozen sections and cytospin preparations. Touch preparations were prepared directly in the operating theatre, or by the pathologist, by gently touching one or two representative pieces of tumour on to a glass slide (usually two to three times per slide). The pieces used for the touch preparations were also used for histological examination. In addition, cytospin slides for interphase cytogenetics were prepared from disaggregated tumour cells or from bone marrow cells with a suspected tumour infiltrate [22]. The paracentromeric DNA probe pUC1-77 specific for chromosome 1 [28] and the subtelomeric DNA probe p1–79 [29], specific for the region 1p36.33 [23], were used to study the copy number and the integrity of chromosome 1. In the double target FISH assays, the digoxigenin labelled paracentromeric probe pUC1-77

(D1Z1) and the biotinylated VNTR probe p1-79 (D1Z2) were used to study the numerical and structural chromosome anomalies of the short arm of chromosome 1. Detection of the hybridised probes was performed as reported previously [22]. Briefly, labelled DNA was diluted in the presence of formamide, SSC, salmon sperm DNA and dextran sulphate to a concentration of 0.4–1 ng/μl (for repetitive sequences) or 10 ng/μl (for *MYCN*). 3–11 μl of the probe mixture were added on to the interphase nuclei. Hybridisations were performed at 37°C overnight. Post-hybridisation washing steps and immunocytochemical detection of the hybridised probe was carried out with FITC- or TRITC-conjugates as previously described [22]. Peroxidase *in situ* hybridisation (ISH) on paraffin material was performed according to methods previously described [20, 21].

Interpretation of in situ *hybridisation results*

The basis of this method lies in the detection of genomic changes within the interphase nuclei by means of adequately designed DNA probes and an appropriate experimental set-up: (i) the size of the DNA probes should be large enough to be unambiguously detected in interphase nuclei; (ii) a reference probe targeted to the same chromosome has to be used to study deletion or amplification events. The ratio of centromeric and telomeric signals in individual nuclei in normal cells is usually 2/2. In case of trisomies with intact chromosomes 1, the ratio is 3/3 per nucleus. Deviations of the numbers (e.g. 2/1 or 3/2) indicate deletions of the region in one chromosome recognised by D1Z2. Two schematic examples of different applications of interphase cytogenetics are given in Figure 1a, c. In both cases, a 1p36 deletion can be detected by a reduced number of D1Z2 signals compared with the centromeric signals. With paraffin sections, two slides must be hybridised with the appropriate probes (Figure 1a). As a certain amount of truncated nuclei are present in sectioned tissues, at least 100 nuclei must be evaluated to obtain reliable information on the somy and integrity of chromosome 1 [20, 21]. Figure 1c shows schematically an intact interphase nucleus. The intact nuclei allow the use of a double target fluoresence assay, with the centromeric probe labelled with a different hapten as compared with the subtelomeric sequences. They can be sequentially or simultaneously identified using the appropriate excitation filters. Examples of a two-colour FISH are shown in Figure 2. In Figure 2a, the nuclei clearly reveal two green signals (D1Z1) and only one red spot, indicating that one subtelomeric region, recognised by the VNTR sequence D1Z2, is lost in one homologue chromosome. In Figure 2b, an example of a triploid tumour with intact chromosomes 1 is given. A similar approach can be used for the detection of *MYCN* amplifications in interphase nuclei. Ratios of two centromeric signals (chromosome 2) and two signals of *MYCN* indicate a normal copy number of this oncogene. Amplifications of this oncogene can be seen directly in the individual cell if the numbers of signals from the *MYCN* probe exceed the number of signals of the reference probe.

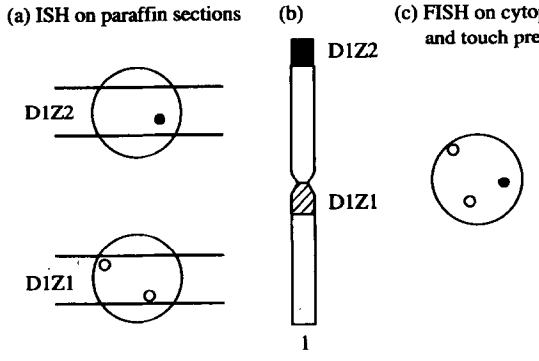

Figure 1. Demonstration of deletions at 1p36 by means of *in situ* hybridisation (ISH). Two different probes are used, one specific for the centromeric region (D1Z1) and one specific for the subtelomeric region 1p36 (D1Z2). Probes are shown on the schematic chromosome 1 in (b). In (a), an example of ISH on a paraffin section is given. Two slides are hybridised, either with D1Z1 or D1Z2, and if the number of signals with D1Z1 (○) significantly exceeds the number of signals in the slide hybridised with D1Z2 (●), a deletion is apparent. In (c), an example of a double-colour FISH is given. Both probes can be seen within the interphase nuclei, facilitating evaluation of the signals.

RESULTS

A group of localised NBs presents as morphologically undifferentiated tumours which cannot always be discriminated from aggressive tumours by means of light microscopical examination. We, therefore, separately analysed 38 tumours with localised (stages 1, 2A, 2B and 3) tumours from those with stroma-rich NBs, a well defined favourable subgroup [13].

Genetics of localised neuroblastomas not treated with cytotoxic agents

We analysed 30 radically resected tumours from patients not receiving any cytotoxic treatment (Table 1). In this group, 84% (21/25) of the tumours analysed for DNA content were in the triploid range. However, we identified three tumours with unfavourable genetic markers (two stage 1 and one stage 2B): 2 showed a deletion at 1p36.3, with one tumour being in the triploid and the other in the di/tetraploid range, the other tumour displayed amplification of the *MYCN* oncogene without deletion at 1p36.3 and showed a triploid DNA content. Despite the fact that the majority of totally resected NBs displayed favourable genetic markers, 1p deletions and/or *MYCN* amplification were noted in 10% (3/30) of cases. These patients did not receive any cytotoxic treatment, and they are alive and remain disease-free (follow-up 9–31 months).

Of the 38 local tumours, eight could not be radically operated on (Table 1). Interestingly, none of the not radically removed tumours, showing spontaneous maturation or regression, were in the diploid range, nor did they show deletions at 1p36 or amplifications of the *MYCN* proto-oncogene. These findings support the importance of the integrity of the short arm of chromosome 1, the normal number of the *MYCN* proto-oncogene, and the triploid chromosome number for the regression processes of these tumours. Therefore, in contrast to the totally resected NBs, none of the incompletely resected tumours showed any unfavourable genetic marker.

Genetics of neuroblastomas progressing into metastatic disease

We analysed five tumours from patients with localised disease that later progressed into stage 4 tumours, with bone and/or bone marrow involvement. One presented as stage I, one as stage 2B, and three as stage 3 tumours, and one had been found by screening. A deletion at the short arm of chromosome 1 was found in four tumours, of which *MYCN* was amplified in only two tumours. One tumour showed neither a del(1)(p36.3) nor a *MYCN* amplification (Table 1). These limited data indicate that 1p deletions are most frequently seen in these tumours followed by the amplification of the *MYCN* oncogene. The evaluation of the DNA content of five tumours revealed diploid and triploid tumours. In a large series of stage 4 tumours, 1p deletion was also found to be the most frequent genetic event (data not shown).

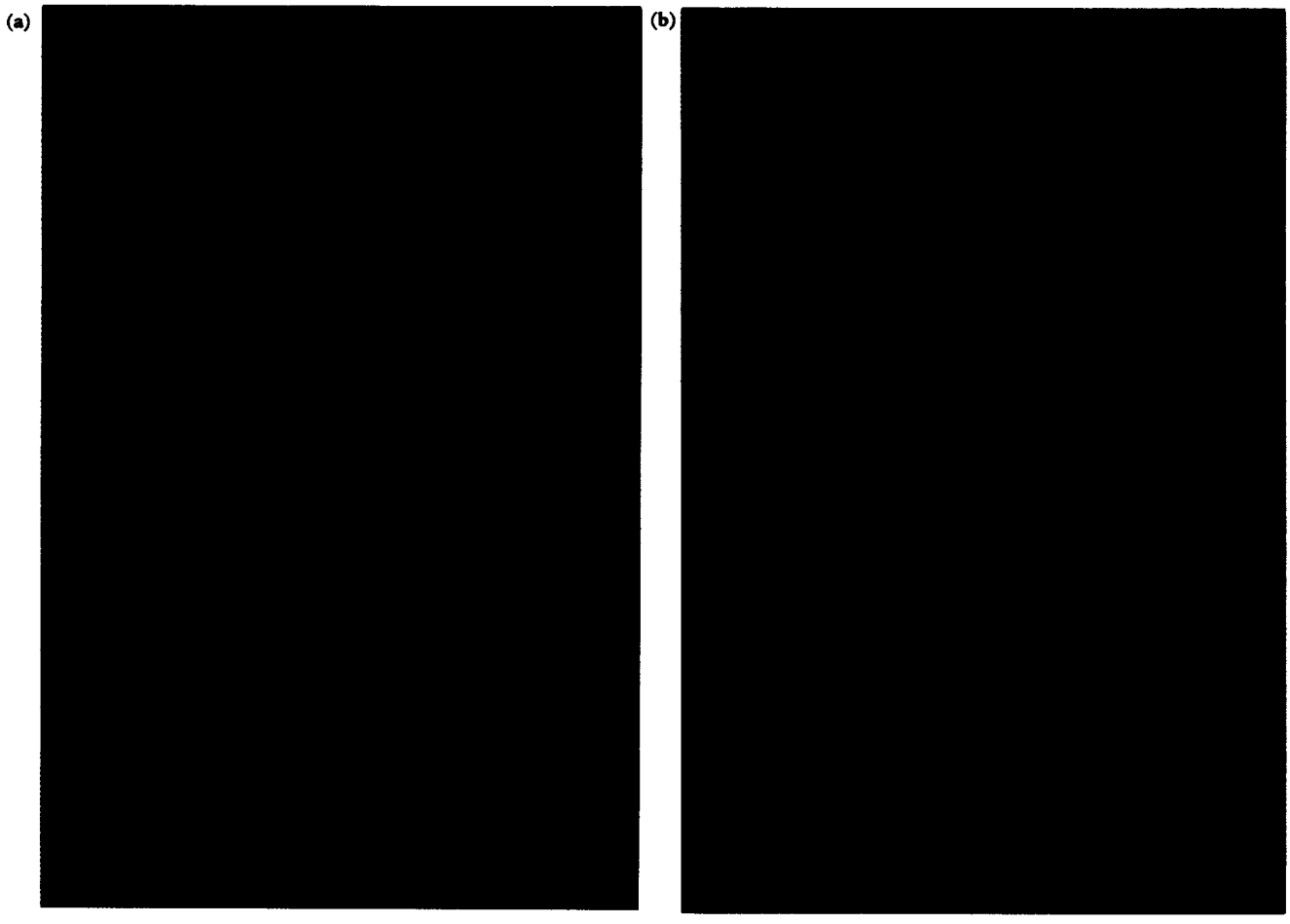

Figure 2. FISH using a centromere specific (D1Z1) and a subtelomere-specific DNA probe (D1Z2). (a) An example of interphase nuclei with a deletion at 1p36.3 is given; note that the ratio of centromeric signals (green) exceeds the number of telomeric signals (red). (b) An example of a touch preparation of a highly differentiated NB with multinucleated ganglionic cells; note that all nuclei have an equal number of centromeric (red) and telomeric signals (green) indicating an intact 1p36 region in these nuclei.

Table 1. *Genetic features of untreated and treated localised and* 4s *tumours*

	Number of tumours	2n/4n	3n	del1p	*MYCN*	Therapy	Outcome (*n*)
Local tumours							
Totally resected*	30	4	21	2	1	No	CR (30)
Not totally resected	8	None	8	None	None	No	CR (8)
Progressing tumours	5	3	2	4	2	Yes	DOD (3)
							PD (2)
4s tumours							
Regressing 4s	7	1	6	None	None	No	CR (7)
Progressing 4s–4	4	4	None	3	2	Yes	DOD (3)
							VGPR (1)

CR, complete remission; DOD, dead of disease; PD, progressing disease; VGPR, very good partial remission.
*DNA content of 5 tumours was not determined.

Genetics of stage 4s *tumours*

We analysed stage 4s tumours from 7 patients who did well without any cytotoxic treatment, and compared the genetic data of these tumours with genetic changes occurring in 4s tumours showing progression ($n = 4$). The follow-up time of the first group of patients ranges from 19 months to 79 months. Interestingly, none of the regressing 4s tumours showed amplified *MYCN* or deletion at 1p36 (Table 1). Six of the seven tumours were near-triploid, and the other was in the tetraploid chromosome range, without any other genetic aberration. Another 4s tumour from a girl with Turner's syndrome is not listed in Table 1, as this patient received some mild therapy. However, this tumour had a diploid chromosome range, a feature which is usually considered as unfavourable, but the patient has been in complete remission for 20 months. This finding indicates that diploidy/tetraploidy possibly has a different meaning in 4s

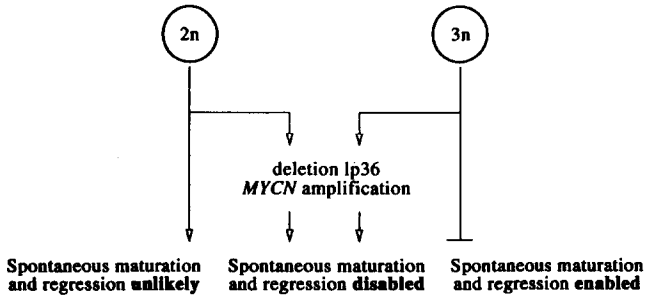

Figure 3. Genetic subtypes of NB based on ploidy. Structural genomic changes, such as deletions at 1p36 and amplifications of the *MYCN* oncogene or structural aberrations, can be present in the 2n and 3n tumours.

tumours as compared with localised tumours or tumours presenting later in childhood. The four stage 4s tumours which progressed were in the diploid or tetraploid chromosome range (one tumour had a near-tetraploid DNA index), and all showed unfavourable genetic features (Table 1). One had a deletion at 1p36, one showed *MYCN* amplification, and one displayed both.

Genetic subtypes of neuroblastoma

Generally speaking, the chromosome number (DNA content) divides the NB into two groups. This simple discrimination by ploidy is complicated by the presence of additional genetic features, such as del1p36 and/or *MYCN* amplification or other changes such as the t(1;17) [23, 30]. These aberrations can affect both genetic types (Figure 3) which makes a clear distinction based on ploidy alone impossible.

Influence of genetics on regression, maturation, and progression

The physiological processes of spontaneous maturation and regression (Figure 4a) are disabled or greatly reduced in tumours with a deletion at 1p36 and/or *MYCN* amplification (Figure 4b). Ploidy also seems to play an important role, at least in localised tumours, since regressing or maturing NBs were all in the near-triploid (or penta-hexaploid) range (Figure 4a).

DISCUSSION

New treatment strategies avoid cytotoxic treatment in localised tumours fulfilling certain clinical and biological criteria, D. Beck, this issue, pp. **. Interestingly, the majority of patients do well irrespective of residual tumour [11, 12]. In order to better understand the genetic make-up of regressing and progressing tumours, we applied classical cytogenetic and molecular genetic methods. Consistently, we found near-triploidy, lack of deletion at 1p36 and lack of *MYCN* amplification in those tumours which underwent regression or maturation [13]. Genetic criteria will help to discriminate patients needing cytotoxic treatment from those patients who can be treated by surgical intervention alone.

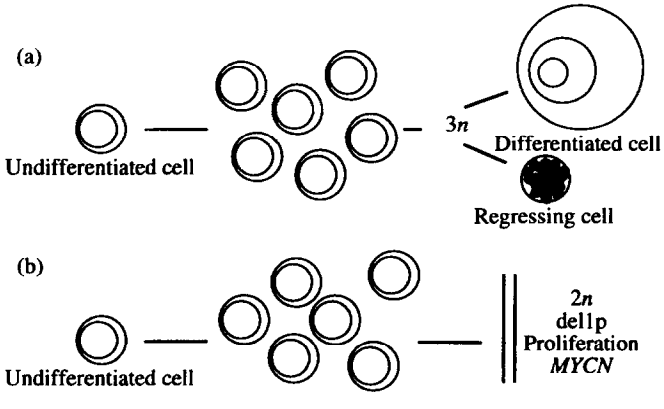

Figure 4. Differentiation and regression pathways in NB. The ability to spontaneously follow these pathways is blocked in tumours with the genetic characteristics shown in (b). NMA-*MYCN* amplification.

It is still an unresolved question whether unfavourable tumours evolve from a favourable genetic type. The data presented indicate that a localised tumour which has the capacity to metastasise into bone and/or bone marrow, already presents with unfavourable genetic markers, such as a deletion at 1p36 and/or an amplification of *MYCN*. Although the data are limited, it appears that progressing tumours can be either diploid or triploid, most often show 1p deletions and less frequently have amplification of *MYCN*. However, the acquisition of structural chromosome aberrations cannot be excluded as the cause for tumour progression since we described one such case 2 years ago [31], where the patient showed a 1p deletion in a local recurrence that was not present in the primary tumour. Absence of detectable 1p aberrations in the initial tumour may be due to tumour heterogeneity, acquisition of this genetic aberration, or due to the presence of two independent primary tumours.

Whether minimal tumour infiltration of the bone marrow at diagnosis is responsible for the metastatic process needs to be further evaluated. We studied one patient with a localised tumour who presented 2 years later with a bone infiltrate. At primary diagnosis, a low infiltration of GD2 positive cells was found, but conventional light microscopical analysis was negative. At that time, this was not a criteria for the diagnosis of stage 4 disease when light microscopical examination revealed no evidence for tumour cells.

In order to better understand the genetic make-up of regressing 4s tumours, we analysed a series of untreated tumours. This series was supplemented by the analysis of progressing 4s tumours. To date, only a limited number of reports on the genetics of these tumours are available. In the first report on chromosome aberrations of 4s tumours, the authors [32] were still undecided whether this disease should be classified as hyperplasia or neoplasia as the diploid cell population was predominant. One report indicated that these tumours are predominantly in the triploid chromosome range [18]. Other genetic aberrations in stage 4s tumours have rarely been described, but some authors [33] already discriminated three types of tumours: (i) tumours with a near triploid chrosomome number, (ii) tumours with a diploid chromosome number; and (iii) tumours with amplification of the *MYCN* proto-oncogene. However, reports on the genetics of 4s tumours, especially of patients not receiving cytotoxic treatment, are scarce and virtually no information on the deletions at the short arm of chromosome 1 are available. Therefore, it was difficult to consign the clinical heterogeneity of those tumours [34] to certain genetic subtypes.

Our data indicate that deletion at 1p36 and amplification of *MYCN* account for the majority of unfavourable 4s tumours. 3 of the 4 4s tumours displayed deletions at 1p36, one with concomitant *MYCN* amplification and another had amplification of the *MYCN* oncogene. These two genetic markers seem to identify a subcategory of stage 4s tumours which act independently from ploidy.

Genetic analysis of localised, not radically resected tumours and 4s tumours, which showed spontaneous regression or maturation, not induced by cytotoxic agents, provides clues to the prognostic impact of certain genetic aberrations. All tumours which were treated with surgery alone and which showed a benign clinical behaviour, despite residual disease, were in the near-triploid (hexaploid, pentaploid) chromosome range, with neither a deletion at 1p36 nor amplification of the *MYCN* proto-oncogene. The only exception to this "rule" was a stage 4s tumour with a tetraploid chromosome number. From these data, we can conclude that spontaneous terminal differentiation and/or regression processes in NBs are only operating in those tumours with an intact short arm of chromosome 1 and without amplification of the *MYCN* oncogene.

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