International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee

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Neuroblastoma serves as a paradigm for utilising tumour genomic data for determining patient prognosis and treatment allocation. However, before the establishment of the International Neuroblastoma Risk Group (INRG) Task Force in 2004, international consensus on markers, methodology, and data interpretation did not exist, compromising the reliability of decisive genetic markers and inhibiting translational research efforts. The objectives of the INRG Biology Committee were to identify highly prognostic genetic aberrations to be included in the new INRG risk classification schema and to develop precise definitions, decisive biomarkers, and technique standardisation. The review of the INRG database (n = 8800 patients) by the INRG Task Force finally enabled the identification of the most significant neuroblastoma biomarkers. In addition, the Biology Committee compared the standard operating procedures of different cooperative groups to arrive at international consensus for methodology, nomenclature, and future directions. Consensus was reached to include MYCN status, 11q23 allelic status, and ploidy in the INRG classification system on the basis of an evidence-based review of the INRG database. Standardised operating procedures for analysing these genetic factors were adopted, and criteria for proper nomenclature were developed. Neuroblastoma treatment planning is highly dependant on tumour cell genomic features, and it is likely that a comprehensive panel of DNA-based biomarkers will be used in future risk assignment algorithms applying genome-wide techniques. Consensus on methodology and interpretation is essential for uniform INRG classification and will greatly facilitate international and cooperative clinical and translational research studies.

Keywords: neuroblastoma; treatment planning; genomic; translational; international consensus; INRG; genetic risk factors

Modern treatment strategies adjust the therapy of cancer patients according to the predicted biological behaviour of the individual tumour. This is especially important for neuroblastoma patients, as a subset of neuroblastic tumours will undergo spontaneous regression (in infants and in young children) or maturation (in children), whereas others will rapidly progress despite intensive multimodality therapy. This clinical heterogeneity has been known for decades (D’Angio et al, 1971; Evans et al, 1976). However, patient age and tumour stage alone cannot reliably predict tumour behaviour. Over the past two decades, tumour histology, (Shimada et al, 1999) status of the MYCN oncogene, (Brodeur et al, 1984; Seeger et al, 1985), and tumour cell DNA content (ploidy; Look et al, 1991; Ladenstein et al, 2001) have each been shown to be independently predictive of patient outcome in large retrospective and prospective studies. Besides these two genetic markers, recently 11q aberrations were also included in the INRG pretreatment risk classification (Cohn et al, 2009). However, the optimal combination of these and additional recently described prognostic biomarkers to build a treatment stratification algorithm has not been determined yet. Here, we present the consensus of the INRG Biology Committee on which markers should be used currently, on the standardised operating procedures (SOPs; partly on the basis of SIOPEN activities (Ambros et al, 2003)) for analysing neuroblastoma tumour molecular diagnostics and genetic nomenclature in this rapidly evolving field.

Genetic features of neuroblastic tumours associated with favourable clinical behaviour

Favourable clinical behaviour of neuroblastic tumours is classically characterised by a propensity to undergo spontaneous regression.
or maturation without cytotoxic therapy (Figure 1). These tumours almost always show whole chromosome gains with few, if any, segmental chromosome aberrations and without gene amplifications. The DNA content is usually in the near-triploid (penta/hexaploid) range (Ambros et al. 1995, 1996; Brodeur, 2003; George et al., 2007; Mosse et al., 2007; Schleiermacher et al., 2007). This is also the case for the neuroblastic/ganglionic cell population in Schwann cell stroma-rich tumours, whereas the Schwann cell population itself has a diploid DNA content (Ambros et al., 1996; Bourdeaut et al., 2008).

Although tumour cell hyperdiploidy (usually near-triploid DNA content) is frequently associated with spontaneous maturation (Ambros et al., 1996) and possibly also with spontaneous regression, this ploidy level alone cannot guarantee benign tumour behaviour as near-triploid tumours may also have segmental chromosomal aberrations (i.e., gains or losses of only parts of chromosomes) and MYCN amplification, which can lead to clinically aggressive behaviour.

**Genetic features of neuroblastic tumours associated with unfavourable clinical behaviour**

Neuroblastomas with an unfavourable clinical behaviour (Figure 1) have a high propensity for locally invasive growth and widespread metastatic dissemination through the lymphatic and haematogenous systems. These tumours frequently show segmental aberrations and high-level amplification of the MYCN locus is detected in a substantial subset (Schwab et al., 1983; Brodeur et al., 1984; Seeger et al., 1985) (Figure 1). MYCN amplification has been shown to be strongly associated with rapid tumour progression and poor prognosis in patients of all ages, with any stage of disease (Brodeur et al., 1984; Seeger et al., 1985; Ambros et al., 1995; Perez et al., 2000).

As expected, MYCN amplification was also highly predictive of worse outcome in the INRG cohort of patients (Cohn et al., 2009). Frequently, adjacent or more distantly located genes, such as DDX1, NAG, and ALK, are coamplified with MYCN, but amplification events in the absence of MYCN amplification are rare. Whether these coamplifications have a prognostic impact awaits clarification (Manohar et al., 1995; Squire et al., 1995; George et al., 1997; De Preter et al., 2002; Weber et al., 2004).

Although MYCN status is central to the risk stratification systems in all cooperative clinical trial groups, it is important to emphasise that the majority of metastatic neuroblastomas do not show amplification of this oncogene (see also Figure 1). Other chromosomal aberrations (and a diploid DNA content) have been assumed to predict unfavourable tumour behaviour, including deletion at the chromosomal region 1p36.3 or 11q23, (Maris et al., 1995; Caron et al., 1996b; Attiyeh et al., 2005) as well as unbalanced gain of the long arm of chromosome 17 (17q21 to 17qter; Caron, 1995; Bown et al., 1999; Spitz et al., 2003). In addition, some studies have shown that deletions on chromosome 3p, 4p, 9p, and 12p also have prognostic significance (Caron et al., 1996a; Thompson et al., 2001; Schleiermacher et al., 2007). As 11q deletions are inversely associated with MYCN amplification, this aberration has emerged as a powerful biomarker of outcome in cases without MYCN amplification (Attiyeh et al., 2005). Statistical analysis of the INRG database has confirmed this finding, and on the basis of these studies, 11q status has been included as a prognostic factor in risk classification systems in all cooperative clinical trial groups.

### Table 1

| MYCN amplification | 1p aberration | 11q aberration | 17q gain |
|-------------------|--------------|---------------|---------|
| Diploidy          | P < 0.0001   | P = 0.1242    | P = 0.3613 |
| MNA               | P < 0.0001   | P = 0.0095    |
| 1p aberration     | P = 0.0613   | P < 0.0001    |
| 11q aberration    | P < 0.0001   |
| 17q gain          | *Inversely associated. MNA MYCN amplification* |

*Figure 1* Biological pathways and genetic features in neuroblastic tumours. Tumour cell ploidy (grey columns) can be used to subdivide neuroblastoma tumours into two broad groups (separated by the long punctuated line). Although the ploidy subgroups roughly correspond to the biologic subgroups (aggressive neuroblastomas marked by a red background – either with MYCN amplification in dark red and separated by a short punctuated line from neuroblastomas without MYCN amplification vs less aggressive behaving neuroblastomas indicated by a green background), they do not totally match. Although aggressive near-triploid neuroblastomas (in red below the long punctuated line) have been observed, it is less clear if ‘benign’ diploid neuroblastomas without any structural aberrations (in green above the long punctuated line) occur. ‘Benign’ clinical behaviour refers either to spontaneous regression/maturation without any therapy or with surgery only (no cytotoxic therapy).
### Table 2a  Event-free and overall survival (EFS and OS) of genetic factors by INSS stage

| INSS stage 1 | INSS stage 2 | INSS stage 3 | INSS stage 4s | INSS Stage 1,2,3,4s | INSS Stage 4 |
|--------------|--------------|--------------|---------------|---------------------|--------------|
| n (%)        | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)        | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)        | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)        | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)        | 5-year EFS ± s.e. | 5-year OS ± s.e. |
| Hyper diploid (n = 2611) | 719 (28) | 93 ± 2 | 99 ± 1 | 415 (16) | 88 ± 3 | 96 ± 2 | 452 (17) | 82 ± 3 | 86 ± 3 | 232 (9) | 86 ± 3 | 92 ± 3 | 1818 (70) | 88 ± 1 | 94 ± 1 | 728 (28) | 47 ± 3 | 53 ± 3 |
| Diploid (n = 1086) | 196 (18) | 90 ± 4 | 96 ± 2 | 119 (11) | 84 ± 5 | 90 ± 5 | 152 (14) | 65 ± 6 | 66 ± 6 | 62 (6) | 77 ± 9 | 77 ± 9 | 529 (49) | 80 ± 3 | 84 ± 3 | 527 (50) | 31 ± 3 | 36 ± 3 |
| MYCN, not amplified (n = 5947) | 1626 (27) | 93 ± 1 | 98 ± 1 | 947 (16) | 85 ± 2 | 97 ± 1 | 1013 (17) | 81 ± 2 | 89 ± 1 | 481 (8) | 82 ± 2 | 91 ± 2 | 4067 (68) | 87 ± 1 | 95 ± 1 | 1798 (31) | 45 ± 2 | 54 ± 2 |
| MYCN, amplified (n = 1155) | 48 (4) | 50 ± 12 | 76 ± 9 | 39 (3) | 57 ± 12 | 67 ± 11 | 217 (19) | 45 ± 4 | 48 ± 4 | 47 (4) | 41 ± 9 | 45 ± 9 | 351 (30) | 46 ± 4 | 53 ± 4 | 787 (69) | 21 ± 2 | 25 ± 2 |
| Ip normal (n = 1659) | 489 (29) | 93 ± 2 | 98 ± 1 | 264 (16) | 83 ± 3 | 98 ± 1 | 313 (19) | 77 ± 3 | 88 ± 3 | 152 (9) | 86 ± 4 | 95 ± 3 | 1218 (73) | 86 ± 1 | 95 ± 1 | 430 (26) | 42 ± 3 | 50 ± 3 |
| Ip aberration (n = 493) | 48 (10) | 76 ± 9 | 100 | 29 (6) | 63 ± 11 | 81 ± 9 | 90 (18) | 52 ± 7 | 61 ± 7 | 29 (6) | 60 ± 13 | 67 ± 12 | 196 (40) | 60 ± 5 | 74 ± 4 | 280 (57) | 24 ± 4 | 32 ± 4 |
| 11q normal (n = 844) | 227 (27) | 90 ± 3 | 99 ± 1 | 141 (17) | 71 ± 7 | 95 ± 3 | 152 (18) | 77 ± 5 | 85 ± 4 | 69 (8) | 86 ± 7 | 95 ± 5 | 589 (70) | 82 ± 3 | 94 ± 2 | 233 (28) | 42 ± 5 | 50 ± 5 |
| 11q aberration (n = 220) | 11 (5) | 73 ± 2 | 91 ± 14 | 11 (5) | 45 ± 34 | 91 ± 16 | 35 (16) | 51 ± 11 | 75 ± 11 | 8 (4) | 38 ± 30 | 63 ± 38 | 65 (30) | 51 ± 10 | 79 ± 8 | 153 (70) | 28 ± 6 | 47 ± 7 |
| 17q normal (n = 187) | 36 (19) | 78 ± 9 | 97 ± 4 | 22 (12) | 77 ± 12 | 100 | 47 (25) | 74 ± 7 | 85 ± 6 | 15 (8) | 92 ± 11 | 92 ± 11 | 120 (64) | 78 ± 5 | 92 ± 3 | 60 (32) | 40 ± 7 | 46 ± 7 |
| 17q gain (n = 175) | 20 (11) | 95 ± 9 | 100 | 19 (11) | 51 ± 18 | 84 ± 14 | 16 (9) | 75 ± 13 | 94 ± 7 | 4 (2) | 100 | 100 | 59 (34) | 76 ± 8 | 93 ± 5 | 104 (59) | 24 ± 6 | 38 ± 7 |

### Table 2b  Event-free and overall survival (EFS and OS) of genetic factors, by age and primary tumour site at diagnosis

| Age <547 days | Age >547 days | Adrenal primary tumour site | Non-adrenal primary tumour site |
|---------------|--------------|-----------------------------|--------------------------------|
| n (%)         | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)         | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)         | 5-year EFS ± s.e. | 5-year OS ± s.e. | n (%)         | 5-year EFS ± s.e. | 5-year OS ± s.e. |
| Hyper diploid (n = 2611) | 1816 (70) | 88 ± 1 | 95 ± 1 | 795 (30) | 48 ± 3 | 54 ± 3 | 1023 (39) | 73 ± 2 | 78 ± 2 | 1473 (56) | 78 ± 2 | 85 ± 1 |
| Diploid (n = 1086) | 425 (39) | 70 ± 4 | 75 ± 4 | 661 (61) | 46 ± 3 | 50 ± 3 | 489 (45) | 49 ± 4 | 54 ± 3 | 556 (51) | 60 ± 3 | 64 ± 3 |
| MYCN, not amplified (n = 5947) | 3657 (61) | 88 ± 1 | 95 ± 1 | 2290 (39) | 51 ± 1 | 60 ± 1 | 2439 (41) | 69 ± 1 | 76 ± 1 | 3265 (55) | 77 ± 1 | 86 ± 1 |
| MYCN, amplified (n = 1155) | 404 (35) | 36 ± 3 | 41 ± 3 | 751 (65) | 26 ± 2 | 30 ± 2 | 732 (63) | 27 ± 2 | 33 ± 2 | 384 (33) | 34 ± 4 | 37 ± 4 |
| Ip normal (n = 1659) | 1095 (66) | 88 ± 1 | 96 ± 1 | 564 (34) | 49 ± 3 | 59 ± 3 | 751 (45) | 71 ± 2 | 78 ± 2 | 881 (53) | 77 ± 2 | 88 ± 2 |
| Ip aberration (n = 493) | 185 (38) | 57 ± 5 | 65 ± 5 | 308 (26) | 23 ± 3 | 38 ± 4 | 323 (66) | 33 ± 4 | 44 ± 4 | 157 (32) | 48 ± 5 | 58 ± 5 |
| 11q normal (n = 844) | 510 (60) | 83 ± 3 | 93 ± 2 | 334 (40) | 46 ± 4 | 57 ± 4 | 359 (43) | 64 ± 4 | 72 ± 4 | 451 (53) | 73 ± 3 | 85 ± 3 |
| 11q aberration (n = 220) | 63 (29) | 61 ± 11 | 85 ± 8 | 157 (71) | 26 ± 5 | 47 ± 6 | 126 (57) | 34 ± 6 | 52 ± 7 | 93 (42) | 35 ± 9 | 63 ± 9 |
| 17q normal (n = 187) | 104 (56) | 80 ± 5 | 92 ± 3 | 83 (44) | 44 ± 6 | 53 ± 6 | 74 (40) | 60 ± 7 | 67 ± 5 | 105 (56) | 67 ± 6 | 82 ± 5 |
| 17q gain (n = 175) | 65 (37) | 68 ± 9 | 83 ± 7 | 110 (63) | 27 ± 5 | 41 ± 6 | 116 (66) | 37 ± 7 | 51 ± 7 | 53 (30) | 53 ± 9 | 67 ± 9 |
Table 3: Consensus of genetic markers currently used for therapy stratification and proposed for future analyses

| Genetic INRG risk classification markers | Techniques recommended/accepted | DNA probes recommended and comments |
|------------------------------------------|----------------------------------|--------------------------------------|
| **Obligatory markers**                   |                                  |                                      |
| MYCN                                    | I-FISH, PCR, aCGH, MLPA          | Two colour I-FISH: BAC or other large DNA-insert clone for the MYCN gene and a clone of similar size for a gene/locus on the long arm of chromosome 2 (e.g., LAF at 2q11); commercially available probes should be used whenever possible |
| 11q23                                   | I-FISH, PCR; pan-/multigenomic techniques: aCGH (oligo, clone or SNP based); MLPA |                                      |
| Ploidy                                   | Flow or static cytometry         | Normal cells from the same patient should be used as a diploid standard |
| **Genetic markers to be analysed prospectively** |                                  |                                      |
| 1p                                       | aCGH, SNP arrays, MLPA etc.      | Commercially available platform preferred |
| 2p                                       |                                  |                                      |
| DDX1                                     |                                  |                                      |
| NAG                                      |                                  |                                      |
| ALK                                      |                                  |                                      |
| 3p                                       |                                  |                                      |
| 4p                                       |                                  |                                      |
| 7q                                       |                                  |                                      |
| 9p                                       |                                  |                                      |
| 12p                                      |                                  |                                      |
| 14q                                      |                                  |                                      |
| 17q                                      |                                  |                                      |
| Others                                    |                                  |                                      |

aCGH = array-based comparative genomic hybridisation; BAC = bacterial artificial chromosomes; I-FISH = interphase fluorescence in situ hybridisation; INRG = International Neuroblastoma Risk Group; MLPA = multiplex ligation-dependent probe amplification; PCR = polymerase chain reaction; SNP = single nucleotide polymorphism.

criterion in the INRG classification system. (Cohn et al, 2009). Recent publications clearly show the potential of comprehensive genome-wide approaches to further refine the prognostic accuracy of somatically acquired chromosomal alterations, (Vandesompele et al, 2005; Michels et al, 2007; Mosse et al, 2007; Schleiermacher et al, 2007; Tomioka et al, 2008). These studies have shown that in tumours without MYCN amplification, segmental chromosome aberrations are associated with clinically aggressive disease. These findings have been extended in a series of 493 MYCN amplified and non-amplified neuroblastomas; in tumours with only whole-chromosome copy number variations, there were no disease-related deaths. In contrast, the presence of segmental alterations (see Table 3) with or without MYCN amplification was the strongest predictor of relapse (Janoueix-Lerosey et al, 2009).

Tumour cell ploidy

The majority of tumours have a hyperdiploid (near-triploid or penta/hexaploid) DNA content, whereas less than half of neuroblastomas are diploid (Look et al, 1984; Kaneko et al, 1987). Locoregional tumours are commonly hyperdiploid; diploidy is more common in advanced-stage tumours. A number of studies have shown that in patients <18 months of age with metastatic disease, hyperdiploidy in combination with a non-amplified MYCN gene and the lack of specific segmental chromosome aberrations (such as 11q deletion) are predictive of a favourable outcome (George et al, 2005; Schleiermacher et al, 2007). However, in patients <18 months of age with metastatic disease with diploid, MYCN non-amplified tumours have a statistically significantly worse outcome.

EVALUATION OF GENOMIC MARKERS IN THE INRG ANALYTIC COHORT

Relationship of genetic aberrations

In the INRG analytic cohort (n = 8800; see Cohn et al (2009) for details), 29% were diploid, 16% were MYCN amplified, 21% had an 11q aberration, 23% had a 1p aberration, and 48% had a 17q gain. Statistically significant associations of genetic factors were identified (Table 1). 11q aberration was associated with 17q gain (P = 0.0001) and inversely associated with MYCN amplification (P = 0.0006), but 11q aberration was not associated with diploidy or 1p aberration. Diploidy and 17q gain were not associated, but all other pair-wise comparisons were highly statistically significant (P < 0.01).

Outcome by clinical and genetic subgroups

In the survival-tree regression approach of Cohn and Pearson et al (2009), patients were clustered into meaningful pretreatment groups that were homogeneous in terms of outcome and prognostic factors (clinical and biological). For the descriptive purposes of this manuscript, the INRG analytic cohort was further subdivided. Outcome by genetic factors vs INSS stage, age, and primary tumour site is shown in Tables 2a, and 2b, respectively. Within each genetic factor subgroup, the patterns of outcome differences that have been observed in the overall population prevailed: (a) EFS and overall survival (OS) decreased with increasing stage (Table 2a); (b) older patients had worse outcome than younger patients (Table 2b) and (c) patients with adrenal primary tumour site had worse outcome than those with non-adrenal tumour (Table 2b).

However, as shown in Table 2a, only a limited number of data on the prognostic impact of some segmental aberrations are available, impeding reliable interpretation of some of the data (e.g., 17q gain in stage 1 and 4s tumours). Thus, larger data sets on segmental aberrations are needed to allow final statements on their prognostic impact (Table 3).

Statistical considerations

Tests of association were carried out using a χ²-test. P-values < 0.05 were considered statistically significant. For event-free survival (EFS) analysis, time to event was defined as the time from diagnosis until the time of first occurrence of relapse, progression,
secondary malignancy, or death, or until time of last contact if no event occurred. For OS, time to event was defined as time until death, or until last contact if the patient was alive. The methods of Kaplan and Meier (1958) were used to calculate EFS and OS estimates, with s.e. according to Peto and Peto (1972).

INTERNATIONAL CONSENSUS BY THE INRG BIOLOGY COMMITTEE

Because of the relative rarity of neuroblastomas, and the uniqueness of many of the molecular diagnostic factors, the INRG Biology Committee recommended that the genetic studies required for INRG classification be carried out in experienced laboratories, typically central reference laboratories for the cooperative groups (see Table 4), to guarantee high consistency and quality of results (Ambros et al, 2003).

Consensus on the genetic markers to be currently used in the INRG risk classification system

As reported in the study by Cohn and Pearson et al (2009) survival-tree regression analyses of the INRG database confirmed the prognostic significance of MYCN amplification, 11q aberrations...
The splitting of the tumour material

Figure 2 Recommendations concerning the splitting of the tumour material for resected tumours or surgical biopsies. All specimens should be transported to the pathology laboratory as quickly as possible. From there, the snap frozen and/or OCT embedded material should then be transported immediately to the biology lab (can be used for any type of DNA, RNA or protein work). Normal reference cells (e.g., peripheral blood) should be sent to the reference laboratories.

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Table 5  MYCN terms and definitions of I-FISH results

| Terms                                      | Description                                                        | Comments                                                                 |
|--------------------------------------------|--------------------------------------------------------------------|--------------------------------------------------------------------------|
| MYCN not amplified (normal MYCN status)    | A balanced ratio between the MYCN-specific signals and the signal number of the reference probe on the chromosome 2q arm | Neuroblastic tumours have a propensity to polyploidisation, especially but not exclusively after therapy with the occurrence of giant polyploid nuclei. The number of MYCN signals must not exceed the number of reference signals on 2q (caveat: the centromeric probe is unqualified as reference probe because of frequent centromeric associations in such tumours). The number of other chromosomes or the DNA index should be checked as well. The use of a 2p specific probe in addition to 2q (optionally a centromere probe) is recommended to clarify the presence of a chromosome 2p gain or restricted MYCN gain. The discrimination could be important since a MYCN gain could indicate an ‘incipient’ MYCN amplification. Equal number of MYCN and 2p signals, exceeding 2q signals (mostly a consistent excess of one or two MYCN and 2p signals). An inconsistent MYCN excess with a varying number of excess signal numbers is much more likely in line with MYCN gain as extrachromosomally elements. The latter can also be found in tumours with heterogeneous MYCN amplification. Details on the amplification grade (a) > 4–10 times; (b) > 10 times; (c) > 30 times amplification, and also on the type of amplification either double minutes (dmin) or homogeneously staining regions (hsr) should be given in the report. Besides tumour cells with amplification defined as such, a proportion of tumour cells can also show MYCN gain. However, tumour cells without MYCN gain or amplification are extremely rare. Single cells (at least two) or cell clusters or a multitude of cells with MNA besides proven tumour cells without MNA. The terms ‘focal’ and ‘diffuse MNA’ are specifications, which however, can only be attributed after evaluation of the tissue sections. In case amplified tumour cells are found in addition to non-amplified cells, a very meticulous procedure is strongly recommended to exclude false-positive or false negative results (see Figure 3). |
| 2p24 gain:                                 | The term 2p24 gain is suggested as a descriptive generic term for MYCN signal numbers exceeding up to 4-fold the number of reference signals on chromosomal arm 2q. This pattern could reflect either: (a) a gain of short arms of chromosome 2, for example, from unbalanced translocations involving 2p; or (b) a gain of the MYCN gene either chromosomally integrated (such as duplications) or extrachromosomally |  |
| MYCN amplification, ‘homogeneous’          | More than 4-fold increase in the MYCN signal number compared with the reference probe located on the chromosome 2q | Details on the amplification grade (a) > 4–10 times; (b) > 10 times; (c) > 30 times amplification, and also on the type of amplification either double minutes (dmin) or homogeneously staining regions (hsr) should be given in the report. Besides tumour cells with amplification defined as such, a proportion of tumour cells can also show MYCN gain. However, tumour cells without MYCN gain or amplification are extremely rare. Single cells (at least two) or cell clusters or a multitude of cells with MNA besides proven tumour cells without MNA. The terms ‘focal’ and ‘diffuse MNA’ are specifications, which however, can only be attributed after evaluation of the tissue sections. In case amplified tumour cells are found in addition to non-amplified cells, a very meticulous procedure is strongly recommended to exclude false-positive or false negative results (see Figure 3). |
| MYCN amplification, ‘heterogeneous’        | Generic term for the coexistence of amplified as well as non-amplified tumour cells in the same tumour |  |
| (a) Focal heterogeneous MYCN amplification | Defined as the more or less focal (one focus or several foci – multifocal) occurrence of MYCN-amplified cells surrounded by non-amplified tumour cells. |  |
| (b) Diffuse heterogeneous MYCN amplification | Designates a tumour which contains the MYCN amplified cells in a scattered pattern besides non-MYCN amplified tumour cells. |  |
| No result                                  | Should be specified: unclear or not interpretable; inadequate tumour cell content; no tumour; not carried out |  |

I-FISH = interphase fluorescence in situ hybridisation.

Table 6  Terms and definitions for segmental chromosome aberrations of I-FISH results

| Terms                                      | Description                                                        | Comments                                                                 |
|--------------------------------------------|--------------------------------------------------------------------|--------------------------------------------------------------------------|
| Normal status                              | Balanced ratio between the signal numbers of the chromosomal region of interest and the reference signals on the opposite arm of the chromosome | In the case of a 2/2 ratio, an isodisomy with a complete LOH of all loci located on the investigated chromosome cannot be excluded. However, uniparental isodisomies are extremely rare in NB tumours. Does not necessarily correspond to an LOH, and is therefore called inconclusive. PCR should be performed to clarify this result. In NB tumours, imbalances frequently, but not always, reflect LOH. Fluorescence in situ hybridisation imbalances can also occur focally. Interphase fluorescence in situ hybridisation imbalances should only be diagnosed if hybridisation failure can be excluded. At least 200 cells should be evaluated. |
| I-FISH imbalance (inconclusive with regard to LOH) | Unbalanced ratio between the signal numbers of the chromosomal region of interest and the reference signals with more than one signals of the chromosomal region of interest | This hybridisation pattern corresponds to an LOH. Deletions can also occur focally. Interphase fluorescence in situ hybridisation deletion should only be diagnosed if hybridisation failure can be excluded. At least 200 cells should be evaluated. |
| Deletion                                   | Unbalanced ratio between the signal numbers of the chromosomal region of interest and the reference signals with only one signal of the chromosomal region of interest |  |
| Gain                                       | Up to 4-fold excess of signal numbers of the chromosomal region of interest compared with the reference signals |  |
| No result                                  | Should be specified: unclear or not interpretable; inadequate tumour cell content; no tumour; Not carried out |  |

I-FISH = interphase fluorescence in situ hybridisation.
alterations including gene amplification. To produce precise definitions, consensus was reached to introduce the term ‘segmental’ aberrations for gains and losses of chromosome parts. In neuroblastomas, most segmental chromosome aberrations are unbalanced, that is, they are associated with regional losses or gains of chromosome parts. Balanced aberrations, that is, reciprocal translocations without losses of genetic material, are thought to be relatively uncommon in neuroblastomas. Segmental aberrations (such as 11q23 and 1p36.3 deletions) can be identified by a number of techniques shown in Table 3. Currently, either I-FISH or PCR are carried out to detect segmental aberrations, but the INRG Biology Committee recommends array-based methods, multiplex ligation-dependent probe amplification (MLPA) or similar techniques in the future. The latter techniques are currently being validated (for nomenclature (I-FISH, PCR, and MLPA), see Tables 7–8; part of the nomenclature was developed by the SIOPEN Biology Group (for group members, see Table 4 and Ambros et al, 2003)).

### Tumour cell DNA content (ploidy)

Ploidy was analysed in the INRG cohort using the definitions diploid (i.e., DI ≤ 1.0) vs hyperdiploid (DI > 1.0) as published in Look et al (1991). More recent data suggest that prognostic classification may be further refined using specific ranges of DI. The INRG Biology Committee recommends recording the exact numerical DI value for each tumour, so that clinically relevant cutoffs for DI can be determined (for techniques see Table 3). Any other method can be applied as long as it allows discrimination of the diploid DNA peak. However, an unknown number of normal cells contained in the tumour specimen under investigation, including tumour-derived, but non-neoplastic diploid Schwann cells are an important source of false data interpretation. Thus, the DNA content of maturing/mature tumours, which mostly develop from near-triploid neuroblastomas is easily misdiagnosed as diploid.

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**Table 7** Terms and definitions of segmental chromosome aberrations analysed by PCR.

| Terms               | Description                                                                 | Comments                                                                 |
|---------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------|
| **Allelic loss**    | Loss of one band                                                            | One band is relatively weaker, when compared with the ratio of constitutional DNA controls. Needs further clarification by FISH (copy number of the respective chromosome has to be known) and re-evaluation of the tumour cell content (an LOH could be masked by a high number of normal cells). Alternatively, different PCR probes, located on both arms (p and q) can circumvent the need for the use of another technique. Allele disequilibrium means an imbalance between the number of maternal and paternal alleles (in the case of odd numbers of the respective chromosome), but not a physical loss of alleles |
| **No result**       | Should be specified: unclear or not interpretable; inadequate tumour cell content (<60%); constitutional homozygosity; no DNA; no tumour; not carried out | One band has completely, or almost completely disappeared |

FISH = fluorescence in situ hybridisation.

**Table 8** Terms and definitions of chromosome aberrations analysed by MLPA.

| Terms               | Description                                                                 | Comments                                                                 |
|---------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------|
| **Normal status**   | Balanced ratio between the majority of signals (signal intensity is visualised in the graphic representation of the MLPA results as height of the bars) of both chromosomal arms | In the case of a balanced ratio, a uniparental isodisomy (a uniparental isodisomy means that both chromosomes are derived from one parent. Thus, the two chromosomes are not homologus but identical. However, uniparental isodisomies are rare in NB tumours) with a complete LOH of all loci located on the investigated chromosome cannot be excluded |
| **Segmental chromosome aberration** | Loss Unbalanced ratio between the signals of the chromosomal region of interest (at least two adjacent probes) and the reference signals (at least two probes) signals | This result could correspond to a FISH deletion that reflects an LOH, or a FISH imbalance, which does not necessarily indicate an LOH. |
| Loss                | Gain Unbalanced ratio (low signal excess) between the signals of the chromosomal region of interest (at least two adjacent probes) and the reference signals (at least two) of the chromosomal region of interest | The threshold between gain and amplification needs to be determined for the experimental system with the help of other techniques, for example, I-FISH |
| **Amplification**   | Unbalanced ratio (high signal excess) between the signals of a gene and all other probes located on the same chromosome | The threshold between gain and amplification needs to be determined for the experimental system with the help of other techniques, for example, I-FISH |
| **No result**       | Should be specified: unclear or not interpretable; inadequate tumour cell content; no tumour; not carried out | |

FISH = fluorescence in situ hybridisation; MLPA = multiplex ligation-dependent probe amplification; I-FISH = interphase fluorescence in situ hybridisation.
There is clear consensus that the use of genetic data derived from diagnostic neuroblastoma tumours will remain central to patient treatment planning and gain even more power. The INRG Biology Committee addressed the issues of ongoing and future work directed at prognostic biomarker discovery and validation, as well as how these data sets can be leveraged to identify molecular targets for novel therapeutics. In Table 3, 12 genetic markers to be analysed prospectively are mentioned. Pan-genomic investigations may help to identify additional genomic areas of interest.

**DNA-based biomarkers**

A number of studies suggest that the pattern of DNA-based genomic changes is prognostic in neuroblastoma, and that whole genome analysis should be carried out rather than a series of individual locus-specific assays. Therefore, the INRG Biology Subcommittee suggests using pan- or multigenomic techniques enabling an analysis of all relevant genomic loci.

Multiplex ligation-dependent probe amplification is a PCR-based technique that detects a large variety of segmental aberrations and gene amplifications in a robust manner (for technical details, see (Schouten et al., 2002). Quantification of losses, gains, and amplifications in small amounts of DNA can be determined, and simultaneous investigation of a large number of loci, covering all currently known important aberrant regions found in neuroblastomas, can be carried out in a single assay (Villamon et al., 2008). The robust nature of the results and the relatively low cost of the MLPA kits make this technique attractive for routine neuroblastoma analysis.

Chip-based technologies have also been used to molecularly classify neuroblastoma tumours (Bilke et al., 2005; George et al., 2005; Selzer et al., 2005; Spitz et al., 2006; Stallings et al., 2006; Lastowska et al., 2007; Michels et al., 2007; Mosse et al., 2007; Tomioka et al., 2008). Comparative genomic hybridisation has reached a high coverage of the target sequences and become more widely used. The advantage of single nucleotide polymorphism (SNP)-based platforms is the simultaneous detection of physical gains and losses and the detection of copy neutral LOH (George et al., 2005). Still, these techniques cannot be considered as routine techniques but as excellent tools to identify so far undetected genomic regions of prognostic impact. The INRG Biology Committee considers it a priority to work towards a single
diagnostic tool that will reliably and accurately detect allelic deletions and gains, as well as MYCN amplification, and estimate overall DNA content (ploidy), on a single platform. This has been achieved using in-house arrays (Maris et al, 2005; Janoueix-Lerosey et al, 2009), but a commercially available platform will be needed to implement this or a similar technology (e.g., MLPA) uniformly in clinical laboratories across various groups and countries.

RNA-based biomarkers

Several groups have recently shown that genome-wide gene expression profiling can identify differentially expressed transcripts that provide reliable prognostic information (Wei et al, 2004; Ohira et al, 2005; Schramm et al, 2005; Asgharzadeh et al, 2006; Oberthuer et al, 2006; Lastowska et al, 2007; Tomioka et al, 2008). Although these studies are of outstanding quality, they each suffer from relatively small sample sizes, necessitating preliminary validation of proposed gene sets. In addition, there was very little overlap in the gene sets that were identified as prognostic by each group, so a consensus gene set, that is, predictive of outcome has not yet been identified. International collaboration will be required to unequivocally determine whether mRNA expression profiles, likely focused on a representative set of genes, are sensitive and specific enough for molecular assay to be used in the clinic, and/or if these data are synergistic with, or override the information derived from the assays of DNA alterations.

Molecular targets

Neuroblastoma treatment will continue to rely on risk grouping based on tumour genomic features. Increasing attention is now focused on utilising these data sets to discover therapeutic targets. It stands to reason that any prognostically relevant genomic aberration might also signal a molecular aberration that is critical to the maintenance of the malignant phenotype, and thus can be targeted for therapy. Regions of DNA copy number gain that result in the overexpression of a protein that is druggable in patients succumbing to the disease can theoretically be identified in carefully annotated data sets in which both high quality DNA- and RNA-based microarray data are available. Thus, a parallel focus on the discovery and validation of targets for neuroblastoma and other tumour entities will further increase the significance of genomic efforts used in the discovery, validation, or even clinical application phases of implementation.

CONCLUSION

Currently, neuroblastoma treatment planning is not possible without detailed knowledge of tumour cell genomics. International efforts were and are needed not only to identify the most significant genetic markers but also to identify and coordinate particularly suitable techniques for their evaluation in all patients. For future studies, new technologies, which provide a comprehensive picture of the tumour cell genome are recommended. Moreover, the INRG Biology Committee achieved consensus on the nomenclature of genetic aberrations and developed definitions of the terms to be used. The INRG Biology Committee is dedicated to providing the highest possible reproducibility and reliability of genetic markers enabling a uniform INRG classification and forming the basis for international clinical and translational studies. Finally, many of these recommendations apply not only to neuroblastoma tumours but also to any tumour entity for which genetic factors are essential for therapy decisions.

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