Clinical Characteristics and Outcome of Patients with Neuroblastoma Presenting Genomic Amplification of Loci Other than MYCN

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Abstract

Background: Somatically acquired genomic alterations with MYCN amplification (MNA) are key features of neuroblastoma (NB), the most common extra-cranial malignant tumour of childhood. Little is known about the frequency, clinical characteristics and outcome of NBs harbouring genomic amplification(s) distinct from MYCN.

Methods: Genomic profiles of 1100 NBs from French centres studied by array-CGH were re-examined specifically to identify regional amplifications. Patients were included if amplifications distinct from the MYCN locus were seen. A subset of NBs treated at Institut Curie and harbouring MNA as determined by array-CGH without other amplification was also studied. Clinical and histology data were retrospectively collected.

Results: In total, 56 patients were included and categorised into 3 groups. Group 1 (n = 8) presented regional amplification(s) without MNA. Locus 12q13-14 was a recurrent amplified region (4/8 cases). This group was heterogeneous in terms of INSS stages, primary localisations and histology, with atypical clinical features. Group 2 (n = 26) had MNA as well as other regional amplifications. These patients shared clinical features of those of a group of NBs MYCN amplified (Group 3, n = 22). Overall survival for group 1 was better than that of groups 2 and 3 (5 year OS: 87.5%±11% vs 34.9%±7%, log-rank p<0.05).

Conclusion: NBs harbouring regional amplification(s) without MNA are rare and seem to show atypical features in clinical presentation and genomic profile. Further high resolution genetic explorations are justified in this heterogeneous group, especially when considering these alterations as predictive markers for targeted therapy.

Introduction

Neuroblastoma (NB) is the most common extra-cranial malignant tumour of childhood, [1] and is characterised by its wide heterogeneity in clinical presentation and evolution [1–3]. Recent advances in genetic analysis of this heterogeneous tumour, using a wide panel of techniques including array Comparative Genomic Hybridization (aCGH), have revealed different recurrent genomic aberrations, most of which consist of copy number alterations. Indeed, it is now well established that the overall genomic pattern is an important prognostic marker which might be taken into account for treatment stratification [4–13]. Numerical chromosome alterations (whole chromosome gains or losses) are observed in NBs with good prognosis when exclusive. Typical segmental copy number alterations (deletions of chromosome arms 1p, 3p, 7p, 11p, 13q, and gains of chromosome arms 1q, 2q, 5q, 8q, 10q, 17q) are well-documented. MYCN amplification (MNA) is an important prognostic marker which might be taken into account for treatment stratification [4–13]. Numerical chromosome aberrations, most of which consist of copy number alterations. Indeed, it is now well established that the overall genomic pattern is an important prognostic marker which might be taken into account for treatment stratification [4–13]. Numerical chromosome aberrations, most of which consist of copy number alterations. Indeed, it is now well established that the overall genomic pattern is an important prognostic marker which might be taken into account for treatment stratification [4–13].
Comparative Genomic Hybridization and definition of genomic amplification

Tumour samples sent to the laboratory for somatic pangenomic analysis were studied by aCGH as previously described [16]. The resolution was determined by the genomic spacing of the array elements. Two types of arrays have been used: until 2009 an in-house designed array containing between 2855 and 3799 BAC-PAC clones covering the whole genome with a median probe spacing of 1 Mb [16], then a commercial array (NimbleGen) was used with an average resolution of 40 kb (72 000 probes).

Amplification was defined by at least two BAC clones (for the in-house array) or at least 3 adjacent oligonucleotide probes (for the NimbleGen array taking into account its higher resolution) with a fluorescent tumour/normal ratio $\geq$ 1.5 corresponding to a log2 ratio $\geq$ 1. Boundaries of an amplicon were described according to the genomic position of the markers located outside the amplified region (coordinates of the non amplified markers closest to the observed amplicon, according to UCSC genome draft, hg19 [http://genome.ucsc.edu/]). On chromosome band 2p24, amplicons harbouring MYCN with or without directly adjacent co-amplified genes were considered as MNA. In case of amplifications of loci distant from MYCN but still within the cytogenetic 2p24 band, we defined arbitrarily an amplicon distinct from MYCN, when the amplicon was separated from MYCN locus by at least five BAC/PAC clones with a normal fluorescence tumour/normal ratio on the in-house array, or the corresponding number of probes on the NimbleGen array.

Analysis of somatic genetic alterations with definition of losses, gains and high level amplifications was performed as previously described [16]. Precise genetic analysis of 31 of the NBs in this study has been reported previously [16] but the precise clinical data had not been analysed for these patients.

Statistical analysis

Contingency tables were analysed by the Fisher exact test. Mean values were compared by the non parametric Kruskal Wallis Test. Median follow up was calculated according to the inversed Kaplan-Meier method. Progression free survival (PFS) was defined as the time between diagnosis and the first event: relapse, progression, and death from any cause or last follow-up. Overall survival (OS) was defined as the time between diagnosis and death of any cause or last follow-up. Survival curves were analyzed according to the Kaplan-Meier method and compared using the log-rank test with a P-value of less than 0.05 considered to be significant.

Results

Genomic amplifications

Among 1100 aCGH profiles, we found a total of 12 tumours showing amplification of one or several loci distinct from MYCN without any evidence of MNA (1%). We did not get access to clinical information for two of them. For another two, the histological report was non conclusive and a definitive diagnosis of NB could not be retained. Indeed, diagnosis of malignant pheochromocytoma was suggested. These four patients were not included. Therefore eight confirmed NBs presenting regional amplification(s), without MNA, were included as group 1 (n = 8). A further 26 patients were found with NB exhibiting MNA associated with one or several amplification(s) at other loci (group 2, n = 26). Finally, 22 patients treated for NB in our institution between 1999 and 2011, for whom aCGH have been performed on tumour, were identified with amplification only located at the MYCN locus and were considered as a control group (group 3,
Figure 1. Examples of different genomic profiles with genomic amplifications obtained in neuroblastoma by array comparative genomic hybridization for each group at diagnosis. For each panel, the genome wide aCGH profile is shown with zoom on the amplified regions. Genomic profiles were obtained using the NimbleGen R platform, and images were generated using the SignalMap R software. Log2 ratio = 0 corresponds to a balanced tumour/normal DNA ratio. Amplification is indicated by plots with a log2 ratio $>1$, 5 (corresponding to a tumour/normal DNA ratio $>3$). (A) Example of group 1 profile (NB0760): NB without MYCN amplification but harbouring amplifications at loci 12q13-14 and 12q24. (B) Example of group 2 profile (NB0863): NB with MYCN amplification (2p24) and harbouring amplification at locus 12q14. (C) Example of group 3 profile (NB1244): NB with MYCN amplification and no other amplicon.

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n = 22). Examples of genomic profile for each of the 3 groups are shown in Figure 1 with zoom on the amplified regions. Altogether, the most frequent amplified regions distinct from MNA were located at chromosome band 19p12, 2p25, 2p23, 21q21, 22q11 and 12q13–14, with amplification in other chromosome regions being much rarer. Genomic findings for groups 1 and 2 cases are summarised in Table 1 and Table 2 respectively. Precise amplification boundaries are available in Table S1 and in a .BED file (File S1) enabling to export all genes possibly included in the amplicons, according to UCSC genome draft, hg19 (http://genome.ucsc.edu/).

In group 1, the amplified region 12q13–14 was recurrent, observed in 4/8 cases (NB0760, NB0830, NB0037, NB0039). For two of them, the amplification was larger and comprises chromosome bands 12q13 to 12q15. Amplification at 11q13 was observed in two cases (NB0384, NB0040). Four NBs had a single amplification and the other four (NB0037, NB0039, NB0040, NB0760) had two distinct amplicons. These amplifications arise in an overall genomic pattern of segmental aberrations (Table 1). However for 4/8 cases, aCGH showed overall atypical genomic profiles for a NB, and for two others the genomic profile was numerical.

In group 2, more than 50% of cases had at least two regions co-amplified with MNA. Among these, amplification at chromosome band 19p12 was found in 5/26 cases, amplification of ALK at band 2p23 in 5/26 cases, and ODC1 at band 2p25 in 3/26 cases (Table 2 and Table S1). The majority of tumours in group 2 had a genomic profile typical of NB, with segmental chromosome alterations including losses of 1p, 11q and gain of 17q. One had only numerical chromosome alterations and two other cases neither numerical nor segmental chromosome alterations. In this group, aCGH profiles globally showed a higher number of copy number alterations (Table 2).

In group 3, aCGH profiles showed a single amplification at the MYCN locus associated with segmental chromosomal alterations typical of NB in most cases.

Clinical characteristics

All patients from the 3 groups presented non familial and non syndromic NB. Detailed clinical characteristics and histology data for patients of groups 1 and 2 are summarised in Tables 1 and 2 respectively.

For group 1, patients were of all INSS stages, the primary site occurred in different localisations, clinical features and histology were heterogeneous (Table 1). Interestingly, three patients (NB0791, NB0830, NB0039) in this group had normal urinary catecholamines and no uptake of MIBG at scintigraphy at diagnosis. For NB0039, lung metastasis was observed at diagnosis. Of note, NB0037 had an atypical presentation with a lumbar primary site with metastatic relapse located at the spermatic cord.

Patients in groups 2 (Table 2) and 3 (data not shown) shared clinical features with NB of advanced stage. All patients in group 2, except one (data not available), had positive MIBG uptake in their primary tumour. All except one (and data were not available for six patients) presented with high level urinary catecholamines.
Table 1: Clinical characteristics, genomic findings and histology data for group 1.

| Patient | Age at diagnosis (months) | Sex | Metastasis at diagnosis | Initial treatment | Relapse | Outcome | Genomic findings | Histology |
|---------|--------------------------|-----|-------------------------|------------------|--------|---------|-----------------|----------|
| NB0384 | M 23                     | Y   | Abd                     | No               | CR(121) | CR(121) | 11p13           | Syncytial |
| NB0040 | M 54                     | Y   | Adr                     | No               | CR(127) | CR(127) | 11q13, 17q25    | Syncytial |
| NB0791 | M 18                     | Y   | Lumbar                  | No               | CT-S   | CT-S   | 12q12, 12q13    | Syncytial |
| NB0037 | M 55                     | Y   | Cervical                | No               | CT-S   | CT-S   | 13q, 17q        | Syncytial |
| NB0039 | M 67                     | Y   | Abd                     | No               | CR(145) | CR(145) | 3p, 11q         | Syncytial |

Table 1: Clinical characteristics, genomic findings and histology data for group 1.

| Genomic findings | Amplifications | Segmental alterations |
|------------------|----------------|-----------------------|
|                  | (cytogenetic grade of differentiation) | |
|                  |                 |                       |
|                  |                 |                       |
|                  |                 |                       |
|                  |                 |                       |
|                  |                 |                       |

Given the resolution of the arrays used in this study, it cannot be excluded that amplified regions smaller than the interval between the probes of the arrays might have gone undetected by our techniques. However, only few amplified regions distinct from MYCN have been observed in recent high resolution sequencing studies based on whole exome/genome sequencing [28–30], confirming that this is a rare phenomenon. Thus, our lower resolution approaches give a good overview of the majority of ampiculous in the genome. In a next step, it will be interesting to determine with accuracy genes implicated in the ampiculous using next generation sequencing.

Interestingly, patients with NBs harbouring amplifications other than MYCN, without concomitant MNA, constitute a heterogeneous group of patients with NBs arising from non-adrenal sites observed more frequently, as well as occurrence of atypical metastatic sites (lung, spermatic cord). Furthermore, an increased frequency of absence of MIBG avidity and absence of urinary catecholamine secretion was noted, when normally positive in 90–95% of NB cases [1]. In addition to atypical clinical features, the overall genomic pattern of these NBs revealed atypical segmental patterns. Although histological analysis confirmed the diagnosis of NB, novel histology characterisation using PHOX2B immunostaining might be useful in this context of atypical NB to help in the diagnosis of undifferentiated types [31]. Indeed, PHOX2B immunolabelling has been shown to improve the diagnosis of undifferentiated NB among childhood small round blue-cell tumours with high specificity and sensitivity. Considering recent publication, it would be also interesting for this atypical group of NB without MNA to further study expression of MYC protein in the tumour as it has been suggested that MYC protein expression could be a new prognostic factor indicating more aggressive clinical behaviour than MNA [32].

On the other hand, clinical features of patients whose tumours harbour regional amplifications other than MYCN together with MNA are comparable to those with MNA only.

Although limited by the small number of patients, analysis suggests that OS of patients with amplification(s) other than MYCN without MNA might be better than that of patients with MNA, whereas those harbouring both MYCN and other amplifications might have an even worse prognosis. Indeed, tumours harbouring regional ampiculous in addition to MNA showed a higher genomic instability as documented by the observation of more segmental chromosomal alterations with a tendency towards a poorer outcome, as suggested previously [16]. Furthermore, when comparing OS to a group of 170 NBs with segmental chromosomal alterations but without MYCN or other amplification (corresponding to genomic type B and D from a previous study [7]), the OS for group 1 was comparable to these NBs (type B and D) with 5 year OS of 87.6% ± 10.6% for group 1 vs 73% ± 9.85% SE for group 1 vs 73% ± 9.85% SE and this result was significantly better than OS for patients in groups 2 and 3.

The genes targeted by regional amplifications in NB have been analysed in detail in previous study [5,6,9,14,16–19]. The most frequent amplifications concern ALK amplification at band 2p23, frequently co-amplified with MYCN, accounting for 4% of NBs studied in a meta-analysis [33–35], found in five cases in our study (groups 2) and ODC1 amplification at band 2p25 always found co-amplified with MYCN (20% of cases analysed in 2 studies) [9,30], found in three cases in our study (group 2). Somatic amplification at 12q13–15 locus has also been described [6,9,12,14–16,19,36]. This amplified region contains two potential target genes: CDK4 (12q13–14) involved in cell cycle progression and MDM2 (12q15), a target gene of the transcription factor tumour protein p53 and the encoded protein can target p53 for proteasomal degradation.
Table 2. Clinical characteristics, genomic findings and histology data for group 2.

| Patient | Clinical characteristics | Genomic findings | Histology |
|---------|--------------------------|------------------|-----------|
|         | Sex | Age at dg (months) | INSS stage | Location | MIBG uptake | Urinary catechol | Relapse (months) | Outcome (FU months) | Amplifications (cytogenetic band) | Segmental alterations |         |
| NB0187  | M   | 25              | 3           | Adr      | Y            | na             | No              | CR (107)          | 19p12, 21q21              | −1p, −15q, +17q   | NB NOS   |
| NB1085  | M   | 27              | 4           | Adr      | Y            | H              | No              | CR (18)           | 1p31, 2p23 (ALK), 4q13      | −1p, +17q     | Undiff NB |
| NB0240  | F   | 26              | 1           | Adr      | Y            | H              | L(23)           | DOD (25)          | 1p36.3                    | −1p, +2p, +17q   | P. diff NB |
| NB0038  | F   | 25              | 4           | Pelvic   | Y            | H              | L(12)           | DOD (15)          | 2p23 (ALK), 8q12, 17q23, 19p12, 21q21, 22q11 | −1p         | Undiff NB |
| NB0284  | M   | 43              | 3           | Adr      | Y            | H              | L(5,5)          | DOD (6)           | 1p34.2, 1q32              | +2p, −5q       | NB NOS   |
| NB0194  | F   | 39              | 4           | Abd +mediastinum | Y          | H              | Msla(7,5)       | DOD (10)          | 2q35, 6p21                | −1p, +12q, −10p, +17q | NB NOS   |
| NB0186  | M   | 9               | 4           | Adr      | Y            | H              | No             | D tox (6)         | 2q11                      | −1p, −1q, −2p, +17q | P. diff NB |
| NB0196  | M   | 24              | 4           | Adr      | Y            | H              | Msla(3,5)       | DOD (4)           | 1q22, 17q25              | +11q13 int, −17p, +17q | GGNB     |
| NB0234  | F   | 9               | 2b          | Adr      | Y            | H              | No             | RC (156)          | 16q22.23                 | none         | NB NOS   |
| NB0185  | F   | 15              | 3           | Adr      | Y            | H              | L(15,5)         | DOD (19)          | 21q21, 22q11              | −1p, −1q, +4q, −5q, −10p, −10q, +12q, +15q, +17q, +18q, −19q | Undiff NB |
| NB0232  | M   | 17              | 4           | Adr      | Y            | H              | No             | CR (103)          | 7q22, 7q33, 3q4, 7q36     | −1p, +2q, −10q, +17q | P. diff NB |
| NB0260  | M   | 19              | 4           | Adr      | Y            | H              | Msla(23)        | DOD (25)          | 19p12                     | −1p, +3p, −5q, +7q, −16q, −17p, +17q, −19q | NB NOS   |
| NB0236  | M   | 21              | 4           | Thoraco-abd-pelvic | Y          | H              | Msas(7)         | DOD (9)           | 1p13, 6p23−24, 19p12, 21q21, 22q11 | −1p, +17q       | Undiff NB |
| NB0015  | M   | 25              | 4           | Adr      | Y            | H              | No             | CR (104)          | 19p12                     | −1p, +7q, +17q | P. diff NB |
| NB0862  | F   | 51              | 3           | Adr      | Y            | H              | No             | CR (43)           | 5p15, 5q11                | +17q         | P. diff NB |
| NB1015  | M   | 29              | 4           | Adr      | Y            | na             | Msas(8,5)       | DOD (12)          | 2p25 (ODC1)              | none         | P. diff NB |
| NB0863  | M   | 19              | 4           | Adr      | Y            | na             | Msas(14)        | DOD (15)          | 1q14                      | numerical     | P. diff NB |
| NB0250  | M   | 44              | 4           | Adr      | Y            | H              | Msas(14)        | DOD (19,5)        | 1p13, 2p23 (ALK), 2p25, 19p12 | −1p, +6p, −6q, +11q, +17q | P. diff NB |
| NB0256  | M   | 16              | 4           | Adr      | Y            | na             | Msas(58)        | DOD (65,4)        | 19p12, 21q21              | −1p, +9q, −17p, +17q | P. diff NB |
| NB0173  | M   | 6               | 45          | Adr      | na            | na             | Msas(42)        | DOD (49)          | 19q13.4                  | −1p, −17q     | P. diff NB |
| NB0248  | F   | 14              | 3           | Adr      | Y            | ni             | No             | CR (96)           | 17q23, 19p12              | −1p, −7q, −21q, +17q | P. diff NB |
| NB1173  | M   | 27              | 4           | Adr      | Y            | na             | Msas(11)        | DOD (13)          | 1q13−14, 1q4−15          | −1p, −12q, +17q | NB NOS   |
| NB0013  | F   | 13              | 4           | Adr      | Y            | na             | Msas(10)        | DOD (10)          | 2p24−25 (ODC1)           | −1p, −6q, +2p, +3q, +4q, +13q | NB NOS   |
| NB1250  | F   | 56              | 4           | Adr      | Y            | H              | Msas(6)         | DOD(10)           | 2p25.2                   | −1p, −6q, +11q, +17q | P. diff NB |
| NB1257  | F   | 149             | 4           | Adr      | Y            | H              | Msas(6.5)       | DOD (17)          | 2p23 (ALK), 2q22         | −1p, −5q, +6q, +7q, −7q, −10q, −12p, −14q, −15q, −17q, −18q | P. diff NB |
| NB0838  | M   | 8               | 4           | Adr      | Y            | H              | Msas(4)         | DOD (5,5)         | 2p25.1 (ODC1), 2p23 (ALK) | −1p         | P. diff NB |

M, male; F, female; dg, diagnosis; Adr, adrenal; Abd, abdomen; MIBG, metaiodobenzylguanidine uptake at primary tumour site; Y, yes; H, high secretion of urinary catecholamines; Nl, normal secretion of urinary catecholamines; Ms, Metastatic relapse; L, Local relapse; DOD, dead of disease; D tox, dead of toxicity; CR, complete remission; NB, neuroblastoma; GGNB, ganglioneuroblastoma; Undiff., undifferentiated; P.diff., poorly differentiated; Diff, differentiating; NOS, not otherwise specified.

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Table 3. Distribution of clinical parameters for the three groups.

| Parameters                        | Group 1 (n = 8) | Group 2 (n = 26) | Group 3 (n = 22) | Total | P   |
|-----------------------------------|----------------|-----------------|-----------------|-------|-----|
| Tumour INSS Stage                 |                |                 |                 |       |     |
| INSS Stage 4                      | 5 (62.5%)      | 18 (69%)        | 19 (86%)        | 42    | NS  |
| INSS Stage 3                      | 2 (25%)        | 5 (19%)         | 3 (14%)         | 10    |     |
| INSS Stage 1, 2, 4S               | 1 (12.5%)      | 3 (12%)         | 0 (0%)          | 4     |     |
| Median age at dg (months) [range]| 37 (17–67)     | 24.5 (6–149)    | 29 (8–73)       | NS    |     |
| Age at dg                         |                |                 |                 |       |     |
| ≤18 months                        | 2 (25%)        | 9 (35%)         | 9 (41%)         | 20    | NS  |
| >18 months                        | 6 (75%)        | 17 (65%)        | 13 (59%)        | 36    |     |
| Tumour localisation               |                |                 |                 |       |     |
| Adrenal                            | 3 (37.5%)      | 22 (85%)        | 22 (100%)       | 47    | <0.001|
| Other abdominal                   | 4 (50%)        | 3 (11%)         | 0 (0%)          | 7     |     |
| Non abdominal                     | 1 (12.5%)      | 1 (4%)          | 0 (0%)          | 2     |     |
| Relapse                           |                |                 |                 |       |     |
| Metastatic +local                 | 3 (37.5%)      | 18 (69%)        | 9 (41%)         | 30    | NS  |
| Metastatic only                   | 1 (7%)         | 6 (4%)          | 3 (11%)         | 14    |     |
| Local only                        | 1 (4%)         | 0 (0%)          | 0 (0%)          | 5     |     |

INSS, international neuroblastoma staging system; dg, diagnosis; P, Fisher’s exact test p-value; NS, not significant.
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In our study the amplicons at 12q13–14 and 12q13–15 were the most commonly amplified region in the absence of MNA with the CDK4 gene amplified constantly but MDM2 found amplified only in half of the cases. Amplifications at 12q13–14 and 12q13–15 have been reported in many other solid tumours such as malignant glioma, bladder cancer and sarcomas most often resulting in overexpression of genes in this region, with the implication of a worse prognosis in amplified cases [37–41]. CDK6 gene at 7q21 was also amplified in the absence of MNA in one case (NB0072) and CCND1 gene at 11q13 in two cases (NB0040 and NB0384).

These observations are noteworthy considering that CCND1, CDK4 and CDK6 are G1 phase-regulating genes, part of the Cyclin D/CDK4/CDK6/RB pathway found hyperactive in NB, and considering the efficacy of new small molecule inhibitor targeting CDK4/CDK6 leading to G1 arrest and cellular senescence [42].

In group 1, without MNA, seven cases among eight presented amplification containing one of these genes. Characterisation of amplicons using aCGH data combined with gene expression profiling analysis has shown that up to 25% of the genes targeted by genomic amplification are overexpressed in
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Supporting Information

**Table S1 Boundaries for amplifications found in group 1 and in group 2.** Boundaries of one amplified region are given according to the genomic position of the markers (BAC or oligonucleotide probe) located outside the amplified region (coordinates of the non amplified markers closest to the observed amplicon) and according to UCSC genome draft, hg19 (http://genome.ucsc.edu/).

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