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Multilocus loss of heterozygosity allelotypes identify a genetic pathway associated with progression from low to high stage disease in neuroblastoma ☆

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ABSTRACT

Neuroblastoma is a heterogeneous tumour with a variety of clinical phenotypes, ranging from a localised tumour with excellent outcome (stage 1) to a metastatic, usually fatal malignancy (stage 4). In order to investigate the genetic relationship between these tumour subtypes, a loss of heterozygosity (LOH) analysis was carried out. Composite LOH allelotypes incorporating data from 96 loci on 5 chromosomes (1p, 3p, 4p, 11q, 14q), were constructed for 62 neuroblastomas. Neuroblastomas with similar allelotypes were clustered into groups and allelotype patterns correlated with clinical features. Three distinct genetic subgroups of neuroblastoma were observed. The largest group (50% of tumours) was characterised by specific allelotype patterns indicative of a stepwise accumulation of genetic alterations (11q LOH → 1p, 4p, and/or 14q LOH → 3p LOH), associated with progression from low to high stage disease. These tumours are distinct from *MYCN* amplified neuroblastomas which have a more rapid and aggressive disease course, and also a proportion of low stage tumours, often ganglioneuromas or ganglioneuroblastomas, with restricted growth potential.

1. Introduction

Neuroblastoma, a tumour of the sympathetic nervous system, is the most frequent extracranial solid tumour in children and accounts for approximately 15% of all paediatric cancer deaths. Variability in clinical presentation is a characteristic feature of neuroblastoma, with a complete spectrum of behaviour ranging from a localised benign tumour (Stage 1) to a very aggressive, rapidly metastasizing malignancy with high mortality (Stage 4).[1]

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The relationship between these two extremes (or between these and the intermediate stages 2 and 3) is not fully understood. Screening studies have suggested that localised tumours detected in very young children (<1 year) have restricted growth potential and most do not progress to more advanced stages.[2,3] The unusual stage 4S neuroblastoma which is also found in children of this age group also appears to have limited growth potential despite metastatic spread and may, in some patients, show spontaneous regression. The very different clinical course of all of these tumour subtypes has led to the suggestion that neuroblastoma is not a single disease but includes a spectrum of related diseases.[4]

Genetic studies also provide evidence for tumour heterogeneity. Amplification of the *MYCN* oncogene for example has proved to be a very useful marker of high risk neuroblastoma, but identifies less than half of all tumours with this phenotype. A range of additional genetic alterations has been reported in a number of studies, including most frequently, loss of chromosomes 1p, 3p, 11q and 14q and gain of chromosome 17 or 17q.[1,5,6] There is some evidence to suggest that loss of chromosome 11q is inversely correlated with *MYCN* amplification, and may characterise a further group of tumours with poor outcome.[7,8] Alterations of chromosome 17 appear to be a feature of most neuroblastomas regardless of stage, although the nature of the alteration may differ – gain of the entire chromosome in low stage tumours and gain of variable portions of 17q in advanced stage tumours.

In this study we have used multilocus loss of heterozygosity (LOH) analysis to highlight key genetic features characteristic of different tumour subgroups. Our results clearly differentiate between the very aggressive *MYCN* amplified tumours which also show frequent loss of distal chromosome 1p, and a further subgroup of tumours in which a stepwise accumulation of genetic alterations appears to be associated with progression from low to high stage disease.

2. Materials and methods

2.1. Patients

A total of 62 patients, diagnosed between 1992 and 2002, was selected for study based on the availability of matched tumour and normal tissue. The distribution of tumour stage (18% stage 1, 19% stage 2, 8% stage 3, 45% stage 4 and 10% stage 4S) and the frequency of *MYCN* amplified tumours (23%) was similar to that reported for other European studies,[9,10] although a slightly increased proportion of lower stage cases in all of these studies compared to population data (Stages 1–4 and 4S: 4.9%, 11.9%, 16.7%, 60% and 5.7%, N = 1277, European Neuroblastoma Study Group (ENSG) Survey)[11] may reflect the greater availability of material from low stage tumours. Forty-seven patients were diagnosed at the Birmingham Children's Hospital and the remaining 15 at other UKCCSG (UK Children's Cancer Study Group) Centres. The tumour panel included 56 neuroblastomas, 4 ganglioneuroblastomas (tumour numbers 4, 13, 24, 34 in Fig. 2) and 2 ganglioneuromas (tumour numbers 27 and 69). The majority of samples were obtained pre-treatment, but 6 were obtained from relapse tissue. The panel included 7 early passage tumour cell lines established from bone-marrow metastases (4 obtained pre-treatment) which were used because of greater availability of material for analysis.

The study was approved by the South Birmingham Research Ethics Committee and informed consent was obtained as appropriate for the use of patient tissue.

2.2. Genetic analyses

LOH analyses and determination of *MYCN* amplification status were carried out as described previously.[8] Primer sequences for polymorphic markers listed in Fig. 2 were obtained from the Genome Database (GDB). Marker locations were obtained from the Ensembl Database, based on the NCBI July 2003 freeze of the human genome sequence.

A panel of 96 markers was used (0.5–3.0 Mb resolution), which had been chosen to include 5 chromosome regions frequently reported to show loss in neuroblastoma (1p21-p36, 3p13-p24, 4p16, 11q13-q24 and 14q32). Not all tumours were scored for all markers due to limited tissue availability, but appropriate markers were chosen from the panel to allow the extent of LOH to be determined.

Allelotype clustering was performed using 'AlleleCluster' software kindly provided by Dr. Luc Girard.[12] The identification of similar allelotypes using this package is facilitated by treating non-informative marker data as 'partially informative' based on the context: a non-informative marker or group of consecutive markers which are bordered on both sides by regions of heterozygosity are statistically more likely to be heterozygous and are coded as such; conversely non-informative markers bordered by LOH on both sides are coded as LOH. Where flanking informative markers are discordant (HET/LOH), non-informative markers are coded NI.

Cytogenetic, fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) analyses were carried out using standard methods as described previously.[13]

2.3. Statistical analyses

Kaplan–Meier estimates for event free survival (EFS) and overall survival (OS) were calculated and compared using the log-rank test. Fisher's exact test was used to test for allelotype associations, and odds ratios were calculated to measure the strength of observed associations.

3. Results

3.1. Classification of LOH in neuroblastoma

The interpretation of LOH data is highly dependent on the criteria used to define allele loss and therefore it was considered important to select appropriate criteria which would reflect the relatively frequent infiltration of neuroblastoma tissue with stromal cells, variation in ploidy, and the existence of subclones showing karyotype evolution. All of these factors may contribute to apparently incomplete allelic loss (i.e. allelic imbalance). Consequently in order to maximise the amount of information obtained, rather than apply a very stringent definition of LOH, we chose instead to classify all markers with a reduction in allele intensity (RAI) of at least

30% as showing LOH. Thus LOH was used as a qualitative term to indicate genetic alteration (loss or imbalance).

The validity of assigning LOH in tumours with RAI values of less than 60% was confirmed by comparison with cytogenetics/FISH/CGH data. Tumour 30, for example showed mean RAI values of 47%, 47% and 54% for markers on chromosomes 4, 11 and 14, respectively. G-banding (partial karyotype: add(4)(p),del(11)(q21q24),add(14)(q24)) and CGH analyses showed a diploid tumour with loss of material on 4p, 11q and 14q (Fig. 1A). These results are consistent with a reduction to hemizygosity on all 3 chromosomes, but with normal cell infiltration.

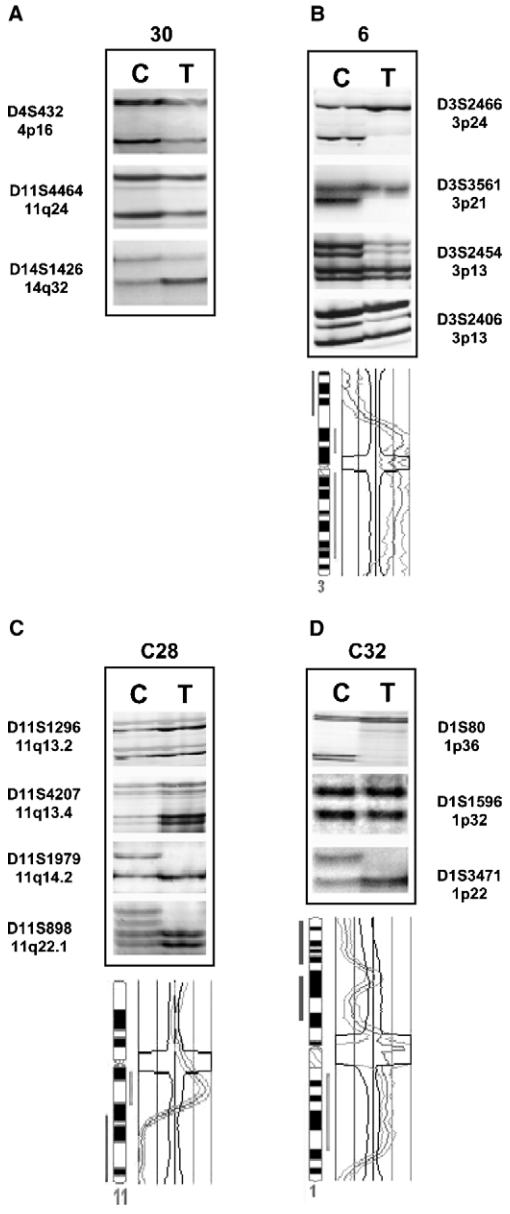

Fig. 1 – (A) Tumour 30, showing similar levels of allelic imbalance on chromosomes 4, 11 and 14, consistent with normal cell infiltration. (B–D) Variable reduction in allele intensity in tumour 6 (B) and tumour cell lines C28 (C) and C32 (D) is consistent with regions of loss and gain identified by comparative genomic hybridization (CGH). CGH profiles show regions of loss to the left of each chromosome and gain to the right. C: constitutional; T: tumour.

LOH was also successfully detected in the context of complex chromosome rearrangements, as demonstrated for example in tumour 6 (Fig. 1B) and tumour cell lines C28 and C32 (Fig. 1C and D). In these cases intrachromosomal variation in the RAI values from approximately 50–100% delimited regions of deletion and regions of imbalance/gain which were confirmed by CGH analyses.

We conclude from these results that the assessment of RAI provides an informative picture of genetic alteration in neuroblastoma tumours even in cases likely to have moderate to high levels of normal cell infiltration/clonal heterogeneity.

3.2. *Multilocus allelotyping of neuroblastoma*

Multilocus LOH allelotypes, incorporating markers from 5 chromosomal regions (1p, 3p, 4p, 11q, 14q), were constructed for a total of 55 tumours and 7 newly established neuroblastoma cell lines. All tumour stages were represented. Overall 44/62 samples (70.1%) showed LOH on one or more chromosomes. 62.6% of single chromosome allelotypes showed at least 70% RAI for most markers, 25.3% were in the 50–69% RAI range and 12.1% in the 30–49% RAI range. LOH was most frequent on chromosomes 1 and 11 (49.1% and 44.3%, respectively) followed by chromosomes 3 (32.2%), 4 (26.7%) and 14 (13.8%).

Identification of different patterns of genetic alteration among the 62 neuroblastomas was carried out by clustering tumours with similar allelotypes. Three distinct genetic groups could be identified by this analysis (Fig. 2): the largest group, allelotype group A, included 32 tumours with complex allelotypes showing multiple regions of LOH but with 11q LOH as the predominant feature; allelotype group B included 12 tumours with chromosome 1p LOH and no/minimal additional genetic loss; this group included most *MYCN* amplified tumours and also a small number of stage 1 tumours without *MYCN* amplification; allelotype group C included 18 tumours with essentially no alterations, most of which were low stage. The majority of tumours in allelotype groups B and C correspond to previously described clinical subtypes of neuroblastoma – *MYCN* amplified tumours with poor prognosis and low stage localised tumours with excellent prognosis, respectively. The remaining tumours (allelotype group A) are of particular interest since they may provide insight into the genetic factors which contribute to the variability and unpredictability of neuroblastoma.

3.3. *Clinical characteristics of allelotype group A*

Allelotype group A included tumours of all stages, but the majority (26/32, 81%) were characterised by a common genetic alteration – 11q LOH, suggesting that this is an early event in tumorigenesis. Consideration of the complete allelotype for each tumour also revealed subgroups with distinct patterns of genetic alteration which could be correlated with tumour characteristics. Among 9 low stage tumours (stages 1 and 2) for example, 11q LOH was frequently associated with 1p, 4p and/or 14q LOH (but rarely with 3p LOH). Five of these tumours recurred at the primary site subsequent to

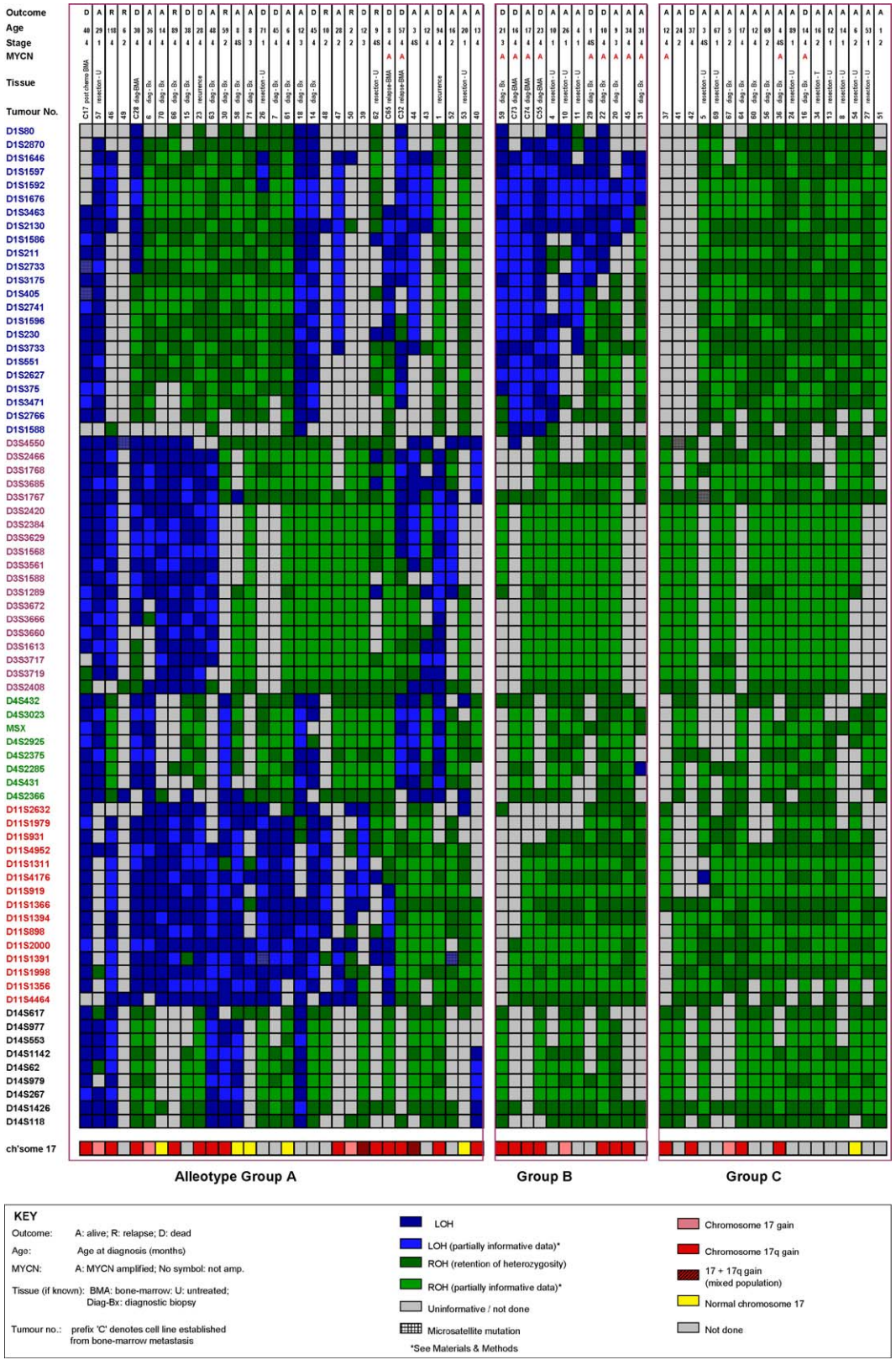

Fig. 2 – Loss of heterozygosity (LOH) allelotypes. Tumours are clustered into 3 groups on the basis of the multilocus pattern of LOH: group A – complex allelotypes characterised by multiple regions of LOH, group B – LOH restricted to chromosome 1p and group C – no significant LOH. Brief clinical details for each patient are shown above each allelotype. Data on chromosome 17 status is shown below each allelotype.

treatment, suggesting that the potential for progressive tumour growth may be a consequence of these genetic alterations. This is consistent with the absence of this allelotype pattern in 14 low stage tumours in allelotype groups B and C, which had a significantly better outcome ($P = 0.004$) (no recurrences) and which may have more restricted growth potential. It is of interest that low stage tumours in the latter 2 allelotype groups showed predominantly favourable histology and included a significantly larger number of ganglioneuroblastomas (4) and ganglioneuromas (2) ($P = 0.048$).

Stage 4 tumours showed the same range of genetic alterations as stages 1 and 2 (suggesting a genetic relationship between high and low stage tumours), but additional LOH on 3p was frequently observed (12/18 tumours) and was associated with relapse or death in many cases. 3p LOH was also observed in 3/3 Stage 4S tumours in this group suggesting that this alteration may be a significant factor in the acquisition of metastatic potential. It is of interest that the three stage 3 tumours in group A were indistinguishable from stages 1 and 2 on the basis of allelotype, consistent with the absence of widespread metastatic disease in these cases.

The association between 11q LOH and LOH of each of 3p, 4p and 14q was statistically significant ($P \leqslant 0.02$), with odds ratios of 4.1, 4.4 and 17.0, respectively, for the occurrence of each in association with 11q LOH relative to occurrence without 11q LOH.

These observations are consistent with a model in which a proportion of low stage neuroblastomas have the potential to progress to more advanced stages as a consequence of the stepwise accumulation of a defined series of genetic alterations: 11q loss → 1p, 4p, and/or 14q loss → 3p loss.

This model allows a number of predictions to be made regarding the age distribution of neuroblastoma patients, e.g. allelotype group A patients with advanced stage tumours should be older than those with low stage tumours, because of the time required for disease progression. It is likely that allelotype group A patients with stage 4 tumours will also be older than patients with *MYCN* amplified stage 4 tumours since *MYCN* amplified tumours appear to require fewer 'hits'. Patient data supported both predictions: the median age at diagnosis of group A patients with low stage tumours (stages 1 and 2) was 20 months (range 2–71 months), compared to 38 months (range 6–118 months) in patients with stage 4 tumours (Fig. 3). Furthermore, group A patients with stage 4 tumours were also older (38 months) than patients with *MYCN* amplified stage 4 tumours (17 months; range 8–57 months).

It was also observed that for patients with low stage tumours the median age at diagnosis was similar in patients in group A and in groups B/C: 19.7 months (range 2–71 months) and 15.3 months (range 1–89 months), respectively.

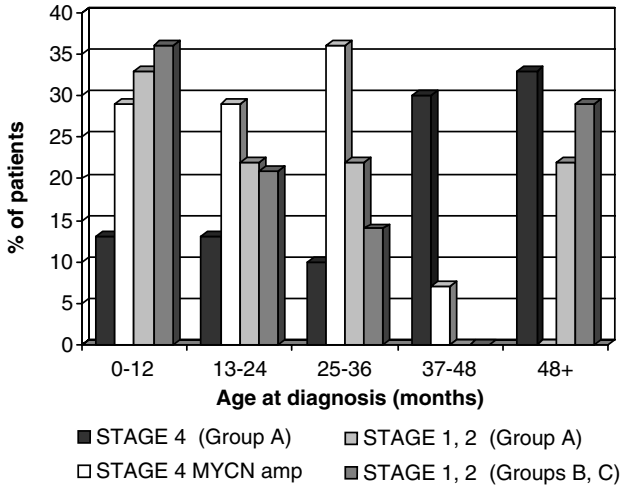

Fig. 3 – Age distribution of patients with neuroblastoma. The majority of stage 4 patients with *MYCN* amplified tumours were less than 36 months of age at diagnosis. Most stage 4 patients without *MYCN* amplification (allelotype group A) were older than 36 months. Patients with low stage tumours had a similar age distribution in all groups.

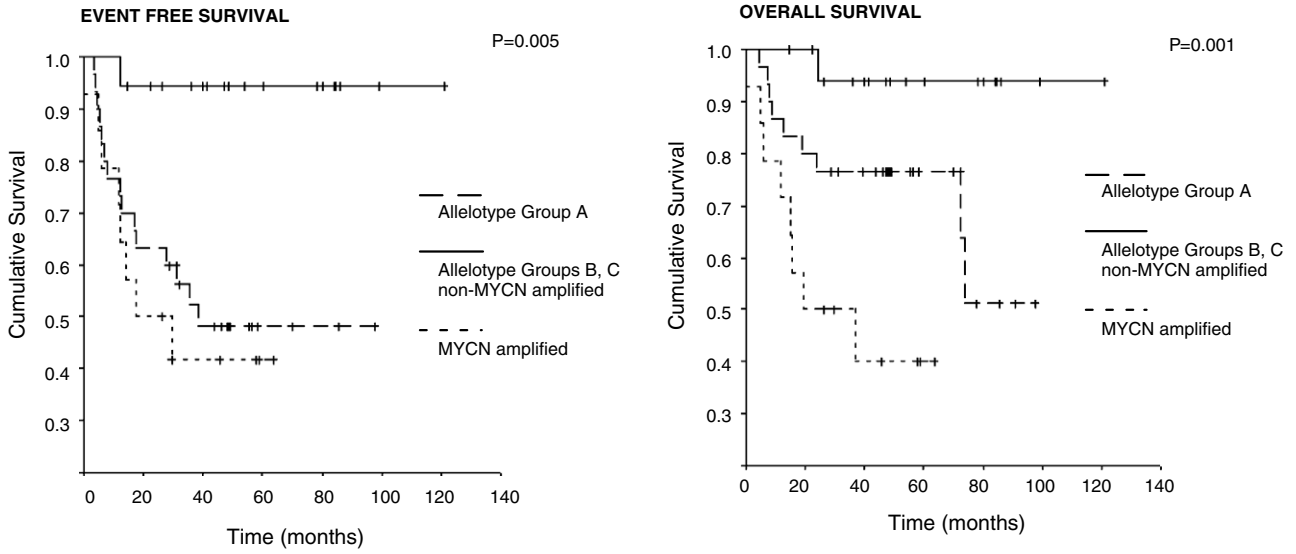

Fig. 4 – Survival of neuroblastoma patients with tumours in different genetic groups.

This is consistent with the idea that these two classes of tumour have distinct and independent etiologies, rather than representing a continuum of allelotype evolution.

Patient survival data were also consistent with a gradual disease progression in group A tumours. Although both group A and *MYCN* amplified patients showed a significant reduction in event free survival at 5 years (48.2% and 41.7% survival, respectively) (Fig. 4), a higher frequency of non-fatal events in the former group was reflected in a much greater difference in overall survival at this time point (76.7% and 40.0%, respectively). Significantly however, continued disease progression in relapsed group A patients let to a further reduction in overall survival to 51.1% by 74 months.

4. Discussion

Multilocus LOH analysis was used to identify differing patterns of genetic alteration which may be correlated with the variety of clinical phenotypes observed in neuroblastoma. Our results suggest that there are 3 distinct subtypes of neuroblastoma: rapidly progressing tumours with high mortality (characterised by *MYCN* amplification), localised benign tumours (including ganglioneuroblastomas and ganglioneuromas) with excellent prognosis (with few/no genetic abnormalities) and a further group making up approximately 50% of the total in which increasing complexity in the pattern of genetic alteration may be associated with progression from low stage to high stage disease.

In carrying out this study, it was considered important to avoid bias which might be introduced by selection of only those tumours with very clear and unambiguous LOH (e.g. >70% RAI) and therefore a broader definition of LOH was used ($\geqslant$30% RAI). Variable levels of LOH are an expected consequence of the acknowledged complexity and heterogeneity of neuroblastoma tissue. Tumours classified as 'schwannian stroma rich', for example may show an apparent reduction in the level of LOH as a consequence of dilution with stromal cells which may, in at least some cases, be non-malignant.[14,15] Clonal evolution may also contribute to intermediate RAI values.

A distinction was not made between LOH occurring as a result of complete or of partial chromosome loss. Both types of abnormality typically target the same chromosomes and thus both may contribute to tumour development. The observation of partial and complete patterns of loss in the same tumour also supports this suggestion (e.g. tumour 62: der(3)t(3;17), −4, −11; tumour 25: add(3)(p1), −4, add(11)(q2), −14).

Multilocus LOH allelotypes were considered more informative than single locus/chromosome data in the construction of a genetic classification of neuroblastoma since most neuroblastomas are characterised by multiple rather than single genetic alterations. Thus in contrast to previous studies which have proposed up to 6 different genetic subtypes of neuroblastoma,[10] in this study three allelotype groups were clearly defined: group A, characterised by a complex allelotype with 11q LOH as the major feature but with variable combinations of 1p, 3p, 4p and 14q LOH, group B: neuroblastomas also with 1p loss but no additional LOH on other chromosomes (many of which also had *MYCN* amplification), and group C, neuroblastomas, ganglioneuroblastomas and ganglioneuromas showing essentially no LOH on any of the investigated chromosomes.

The clustering of both stage 1 tumours and advanced stage *MYCN* amplified tumours in allelotype group B may be related to the fact that the analysis did not distinguish between different patterns of 1p loss, associated with different neuroblastoma subtypes. Stage 1 neuroblastomas, for example, including those detected by screening, tend to have short terminal deletions in 1p36.3, with a smallest common region of deletion (SRO) telomeric to the marker D1S214 (0–6.7 Mb from 1pter).[16–18] This same region may be targeted in stage 1 tumours in allelotype group B (which included the only tumour in the study, (#4 in Fig. 2), which was detected by screening).

There is also strong evidence for additional regions of 1p LOH in advanced stage neuroblastomas. The results of several studies are consistent with a consensus SRO in both *MYCN* amplified and non-amplified tumours in 1p36.2 between D1S214 and D1S2666 (6.7–7.8 Mb).[16,19,20] Additional more proximal regions of loss may also be located in 1p34.2–1p31.3 between D1S211 and D1S209 (43.6–61.3 Mb)[16,21] and also in 1p22 between D1S1618 and D1S2766 (85–86 Mb).[22] The majority of advanced stage tumours with 1p LOH show extensive deletion extending to at least 1p31. It is of interest however that in this study 5/5 *MYCN* amplified patients surviving >2 years had either no detectable 1p deletion or a deletion which included only the 1p36 region, suggesting that the loss of more proximal 1p locus/loci may be more closely associated with a poor outcome. 7/8 patients with *MYCN* amplified tumours and loss of both regions died from their disease.

The clustering of all of the remaining tumours (making up approximately 50% of the total) into a single group, allelotype group A, differs from classifications proposed in other studies.[10,23] However the characteristic patterns of loss observed in this group have also been reported in several other LOH, CGH and FISH investigations. A significant association between 3p and 11q loss, for example, has been reported in several studies,[24–27] as well as more complex patterns involving 11q loss together with 3p, 4p, and/or 14q.[6,10,25,28] In agreement with our results, all studies have consistently found that these patterns of alteration occur predominantly in tumours without *MYCN* amplification.

The key feature of our model, a gradual progression from low to advanced stage disease in allelotype group A patients, in parallel with the accumulation of specific genetic abnormalities, is in agreement with findings in adult tumours such as colorectal cancer.[29] It is contrary to current thinking in neuroblastoma however, where it has been suggested that low stage tumours rarely if ever progress to advanced stage.[1,30,31] We propose that the latter suggestion is in keeping with the extremes of clinical phenotype observed in allelotype groups B and C (i.e. low stage tumours, including ganglioneuromas and ganglioneuroblastomas and *MYCN* amplified tumours). Genetically and developmentally, these may represent independent disease processes. However for the remaining tumours, the results of several recent studies also support the occurrence of progression from low stage to high stage disease and are consistent with a key role for allelotype group A alterations in this process. Spitz and colleagues[32] for example noted that alterations of 1p, 3p and

11q were correlated with stage 4 disease but were also present in a subset of localised tumours and suggested a stepwise accumulation of chromosomal changes with alterations in 11q earlier than 3p. It is of interest that patients with tumours showing 11q LOH but not 3p LOH had a younger median age at diagnosis than those with both 11q LOH and 3p LOH, in both the present study (12 and 37 months, respectively), and in that reported by Spitz (30 and 47 months).[32]

Our model is not inconsistent with the differing genetic characteristics of low and high stage tumours. Many low stage tumours have a triploid chromosome complement and show predominantly numerical abnormalities, while advanced stage/poor prognosis tumours have a diploid or tetraploid karyotype with frequent structural alterations.[5,33] There is evidence however for an overlap between these two categories. The results of Tomioka and colleagues, for example, indicate that 12/39 triploid tumours (31%), investigated by CGH showed partial 11q loss (with lower levels of 3p, 4p and 14q alteration). In addition 36 advanced stage tumours (stages 3 and 4) were noted among a larger group of 109 triploid tumours.[34] Advanced stage tumours with a triploid karyotype, as well as the occurrence of both diploid and triploid clones within the same tumour have also been reported in other studies[27,35,36] and it has been suggested that clonal evolution towards diploidy is highly correlated with recurrence and lethality in locoregional neuroblastoma.[36,37]

Although the majority of neuroblastomas in this study could be unambiguously classified into one of the three allelotype groups, 2 tumours (#65 and #32) showed both *MYCN* amplification and allelotype group A alterations. Tumour 65, for example, showed 11q LOH in addition to 1p LOH and *MYCN* amplification. However this was the only tumour in which 11q loss was restricted to 11q21-qter (i.e. distal to D11S4176), unlike *MYCN* non-amplified tumours in which LOH extended to at least 11q14 in all cases. Comparison of the extent of 11q deletion in *MYCN* amplified and non-amplified tumours has not been reported but it is of interest that only 1/129 tumours with 11q LOH, in a large study carried out by Maris and colleagues,[38] did not include 11q14/D11S4176 within the deleted region. More distal breakpoints resulting in a smaller region of deletion are apparent however in several *MYCN* amplified cell lines[7,39] suggesting that a different gene may be targeted in these cases. Consistent with this suggestion, the phenotype of patient 65 was more similar to that of patients with *MYCN* amplified tumours (allelotype group B), than to allelotype group A, e.g. a younger age at diagnosis (7.5 months) and a rapidly progressing tumour with multiple sites of metastasis leading to early death of the patient. It is concluded therefore that a gene located in 11q14 is implicated in allelotype group A tumours. This conclusion is consistent with a SRO defined by Maris and colleagues (SRO1)[38] and also with the mapping of a region of constitutional deletion including 11q14 in a neuroblastoma patient.[40]

In contrast, in the case of tumour 32, the patient was diagnosed at 57 months of age with a very large abdominal neuroblastoma, continuous with a large adrenal mass and also extending into the posterior mediastinum, with low level bone-marrow involvement as the only metastatic component. This very extensive disease suggests development of the tumour over a relatively long period of time, consistent with the older age at diagnosis. The primary tumour showed loss of 1p and 4p (but not 11q) in addition to *MYCN* amplification. A subsequent bone-marrow sample taken at relapse revealed additional 3p loss. It is possible that *MYCN* amplification occurred as a relatively late event during the development of the primary tumour, substituting for 11q loss.

Other similar patients have also been reported. E.g. a 72-month-old patient diagnosed with stage 4 neuroblastoma with *MYCN* amplification and loss of 3p but not 11q.[27] Similarly, 7 patients included in the study of Plantaz and colleagues, who had stage 4 *MYCN* amplified tumours with 3p loss, with or without 11q loss, had a median age at diagnosis of 60 months in comparison with 32 months for the remaining 20 patients with *MYCN* amplified tumours without these alterations.[25] The conclusion drawn from all of these studies is that in a minority of cases, *MYCN* amplification may also participate in a stepwise disease progression, most likely as a late event. It might therefore be expected that tumour subclones with and without *MYCN* amplification may be found in the same patient. Several such cases have been reported[35,41,42] although failure to find intrapatient variation in *MYCN* amplification status in a further study,[43] suggests that this may be a relatively rare event.

Although a detailed assessment of the contribution of chromosome 17 or 17q gain in neuroblastoma tumorigenesis was outside the scope of this study, the very frequent occurrence of this alteration in neuroblastoma suggests that it is also influential. 17q gain is frequently a consequence of unbalanced translocation and the fact that the most frequent regions of chromosome loss, 1p, 11q and 3p, are also the most frequent translocation partners strongly suggests that the translocation event may augment the effects of, for example, 11q loss and 17q gain occurring independently. It is of interest that 2 stage 4 patients in allelotype group A (#6 and #70) with 'high risk' allelotypes (11q and 3p LOH), but without unbalanced 17q gain (Fig. 2) are alive at 86 and 29 months post diagnosis with no evidence of disease.

The results of this study have significant clinical implications in that they highlight the importance of early identification of tumours which have the potential to progress despite having a 'low risk' clinical phenotype. Our data, as well as the larger study of Vandesompele and colleagues[44] indicate that approximately 20% of patients with stage 1 and 2 neuroblastoma will undergo tumour progression, relapse or death. Identification of these patients at diagnosis, by screening for LOH, may justify more frequent and detailed follow-up. Further work is required however to define the best combination of chromosomal markers which should be included in any such screening and also the extent to which treatment might be modified in identified patients. It is of interest however that the results of Attiyeh and colleagues[45] demonstrating an association between both 1p36 LOH and unbalanced 11q LOH and decreased progression free survival in patients with low- and intermediate-risk disease has resulted in the Children's Oncology Group planning to use these markers to assign the number of cycles of chemotherapy in the hope of averting disease relapse.[45]

The conclusions of these studies must be reconciled however with the results of neuroblastoma screening programmes which have failed to demonstrate a correlation be-

tween early diagnosis and either reduced incidence of advanced stage disease or increased overall survival.[2,3] A possible explanation for these apparently contradictory observations is that screening may be selective for a distinct subgroup of neuroblastomas, i.e. more differentiated tumours with favourable histology (similar to the low stage tumours in allelotype groups B and C). This may be a function both of the early age at onset characteristic of these tumours as well as a typically advanced pattern of adrenergic differentiation leading to the production of high levels of the catecholamines dopamine and noradrenalin and of their respective metabolites homovanillic acid (HVA) and vanillylmandelic acid (VMA) (which are measured during screening). It has been reported that levels of catecholamines are inversely correlated with *MYCN* amplification, consistent with the fact that *MYCN* amplified tumours are rarely if ever detected by screening.[46] In our study patients in allelotype group A showed a wide range of levels of catecholamine metabolites (results not shown), suggesting that a significant proportion would not have been detected by screening. Patient 30, for example who was diagnosed with a stage 2 neuroblastoma, and who subsequently relapsed, had normal levels of both HVA and VMA.

In conclusion, neuroblastoma is a complex tumour with a variety of very different clinical phenotypes. We have for the first time, demonstrated a genetic relationship between clinically different tumours, and have shown that genes at multiple loci function cooperatively to produce aggressive forms of neuroblastoma. These findings are important in emphasizing that outcome in neuroblastoma cannot be predicted accurately using individual prognostic markers, and furthermore that both prognostic evaluation and also the search for new therapeutic targets requires a more detailed understanding of tumour biology.

Conflict of interest statement

None declared.

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